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Recent Advances in Diagnosis of Pseudomonas Septicemia in Relation to Isopods Infestation in *Pomadasys stridense*

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ABSTRACT

Fish in the Suez Canal region, a highly diverse ecosystem where many different species of fish interact, are at risk of mixed infections caused by multiple pathogens, including parasites and bacteria. This study highlighted the naturally occurring process of parasitic bacterial co-infections, specifically involving the parasite *Livoneca redmanii* and the bacterium *Pseudomonas aeruginosa* in the fish *Pomadasys stridens*. A total of 80 *P. stridens* fish were randomly collected from the Suez Canal coasts in Egypt over a period from September to November 2022. The fish samples were tested using standard parasitological and microbiological procedures to identify the presence of *L. redmanii* and *P. aeruginosa*. To definitively identify the parasite *Livoneca redmanii*, its mitochondrial Cytochrome Oxidase subunit I gene was sequenced, and the sequence was deposited in GenBank under the accession number OR051043. Furthermore, the presence of *P. aeruginosa* was isolated from the infested fish samples with isopods, and its identification was confirmed by molecular techniques. The prevalence of *L. redmanii* was 48.75%, while *P. aeruginosa* in the infested *P. stridens* fish with isopods was 69.23%. These findings shed light on the complex dynamics of parasitic bacterial co-infections among fish species in the region.

INTRODUCTION

The striped piggy, scientifically known as *Pomadasys stridens* **(Forsskål, 1775)**, belongs to the Haemulid family and is one of its five members. This particular species of fish is relatively small in size and can be found in shallow waters. It feeds on shellfish and smaller fish species **(Froese & Pauly, 2018**). The spawning season of striped piggy is in spring and summer **(Golani *et al.*, 2006)**. The haemulid fish are scattered in the Mediterranean Sea through the Suez Canal **(Bodilis *et al.*, 2013)** and are recorded in Attaka in the Gulf of Suez **(Osman *et al.*, 2019)**.

Mixed infections often occur when multiple pathogens, such as bacteria, parasites, viruses or fungi, infect the same fish, simultaneously resulting in a secondary concurrent infection. These types of infections can have detrimental effects on fish, amplifying the

pathogenic impact of the involved pathogens, reducing the resistance to other diseases, and increasing the likelihood of fish mortality **(Abdel-Latif *et al.*, 2020)**. Infestation with ectoparasites can weaken the barrier immunity of a fish individual, making it more susceptible to infections by other pathogens such as bacteria **(Kotob *et al.*, 2017)**.

Bacterial fish diseases are one of the major life-threatening challenges facing wild and farmed fish **(Elgendy *et al.*, 2022)**. Pseudomonas infection is regarded as one of the most significant bacterial diseases affecting a variety of freshwater and marine fish, since it is identified as a fish pathogen in Egypt (**Falaise *et al.*, 2016**), causes economic losses, beside the probability of posing a potential risk of transmitting diseases from fish to humans (**El-Gohary *et al.*, 2020)**. Pseudomonas septicemia in various fish species has been linked to *Pseudomonas fluorescens, P. angulliseptica, P. aeruginosa,* and *P. putida* **(El-Barbary & Hal, 2016)**.

Pseudomonas aeruginosa is a Gram-negative, rod-shaped, obligate aerobic bacterium that can infect fish, particularly under stressful environmental conditions **(Abdullahi *et al.*, 2013)**, and it possesses a number of virulence factors related to pathogenicity, including exotoxins (A, U and S), elastase (lasB), alginate (algD), hemolytic phospholipase C (plcH), protein flagellum (fliC), and outer membrane protein (OprI). These elements are well recognized to have a substantial impact on how bacteria interact with their surroundings **(Fadhil *et al.*, 2016)**.

A family of huge, widespread, ectoparasitic crustaceans called Cymothoidae, Leach, 1818 (Crustacea: Isopoda) can be found in freshwater, brackish water, and marine environment (**Tansel, 2012)**. Cymothoidae can attach to different parts of the fish body, depending on the fish species. Some common attachment sites include the skin, fins, gills, and mouth **(Hoffman, 2019)**. Isopods in the opercular cavity seriously impair fish respiratory and cardiovascular systems **(Trilles, 1994)**. Cymothoid parasites were recovered from Haemulidae fish **(Gloria Wonodi *et al.*, 2019)**.

Livoneca redmanii is a species of parasitic isopod that commonly infests various fish species present in both freshwater and saltwater ecosystems on a global scale **(Trilles, 1994)**. In Egypt, *L. redmanii* was isolated from various fish species, such as *Dicentrarchus labrax* and *Dicentrarchus punctatus,* which were collected from the Suez Canal region indicating its wide distribution (**Eissa *et al.*, 2020)**. In addition, it has been found in *Argyrosomus regius* in northern lakes **(Fadel, 2020)**.

Molecular diagnostic techniques such as polymerase chain reactions have been useful for the rapid and accurate identification of many septicemic bacterial pathogens detectable by commercial biochemical methods **(Abdelsalam *et al.*, 2022**). In order to efficiently detect parasites in marine fish species found in the Egyptian Mediterranean waters, the use of molecular methods such as DNA barcoding is essential. Particularly the mitochondrial COI gene of *L. redmanii*, which is analyzed to achieve accurate results **(Abdallah & Hamouda, 2022)**. The purpose of this study was to investigate naturally occurring parasitic and bacterial co-infection in *P. stridense* fish in the Suez Canal region.

MATERIALS AND METHODS

Sample collection and clinical examination

A total of 80 *Pomadasys stridens* specimens were haphazardly collected from local fishermen along the Suez Canal coasts during September till November 2022. The collected samples were immediately transported to the laboratory of Animal Health Research Institute, Ismailia alive in ice boxes from the site of capture. Haemulid species (*P. stridens*) are morphologically identified following the criteria of **Fischer and Bianchi (1984)** and **Burhanuddin and Iwatsuki (2012)**. For the purpose of identifying any clinical abnormalities, a clinical examination of the living or freshly dead fish was performed both internally and externally following the method of **Noga (2010)**.

Parasitological examination

Pomadasys stridens fish sample was examined for the presence of isopods on different parts starting from the buccal cavity, gills, body surface, and fins. Isopods were removed from the fish by a pair of forceps. Subsequently, the prevalence of parasite and its position on the fish was recorded. The found isopod was picked up with forceps and a dissecting needle, then it was preserved in a tiny vial and cleaned with distilled water. The isopod was preserved in 70% ethanol via the instructions of **Lucky (1977)**. The isolated parasites were identified using the guidelines of **Trilles (1994, 2008)**, **Ravichandran *et al.* (2009)**, **Rameshkumar *et al.* (2011)**, **Hadfield (2013)** and **Nisreen *et al.* (2017)** based on the shape of the head, segments, pleotelson, brood pouch/marsupium and general body structure, in addition to recording the type of deformity done by the isopod on the fish sample. Leica-S6D stereo and dissection microscopes were used for the identification, according to **Pillai (1985)**.

Bacteriological examination

The fish were aseptically sampled from the site of isopod attachment, specifically the gills. The samples were then inoculated directly onto nutrient agar and incubated at 37˚C for 24hrs. Re-inoculation of cultured bacteria was performed until distinct colonies were obtained. Suspected purified colonies were collected for further identification by inoculating these bacterial isolates on different media, including MacConkey agar, blood agar and Pseudomonas F agar, following the method described by **Austin and Austin (2007)**. Identification of the isolated bacteria was performed morphologically using Gram staining, as well as biochemical tests, as indicated by **Quinn *et al.* (2002)**, **Austin and Austin (2007)** and **Markey *et al.* (2013)**. Confirmatory identification was made by PCR through the detection of 16s rDNA gene and virulence genes of the isolated bacteria following the guidelines of **Shahrokhi *et al.* (2022**) and **Noor El-Deen *et al.* (2023)**.

PCR and sequencing

I) **Polymerase chain reaction**

1) DNA extraction

A) **In case of *Pseudomonas aeruginosa***, DNA was extracted from the samples using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), with modifications made in accordance with the manufacturer's instructions. At 56°C for 10 minutes, 200µl of the sample suspension was incubated with 10µl of proteinase K and 200µl of lysis buffer. After incubation, 200µl of 100% ethanol was added to the lysate. Following a wash, the sample was centrifuged in accordance with the manufacturer's instructions. Nucleic acid was eluted using 100L of the kit's elution buffer, as shown in Table (1).

B) **In case of the Isopod,** The QIAamp DNA Mini kit (Qiagen, Germany, GmbH) was modified in accordance with the manufacturer's instructions to extract DNA from the samples. In the simplest terms, parasites were cleaned with 1% SDS before 180L of ATL buffer, 25mg of the sample, and 20L QIAGEN protease were added. Tubes were inserted into adapters that were connected to Qiagen tissueLyser clamps for sample homogenization. A rapid shaking phase that lasted for two minutes (30 Hz) was used to interrupt. Afterward, the samples were heated to lysis at 56°C. Following the lysis, 200µl of lysate was treated at 72°C for 10min with 10µl of proteinase K and 200µl of lysis buffer. 200µl of 100% ethanol was added to the lysate after incubation. Subsequently, the sample was cleaned and centrifuged in accordance with the producer's instructions. The kit's 100µl of elution buffer was used to elute the nucleic acid (Table 1).

C) **Oligonucleotide Primer:** Metabion (Germany) provided the primers used, which are indicated in Table (1).

2) PCR amplification: A 25µl reaction including 12.5µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), for assessing the primers, 1µl of each primer at 20pmol, 5.5µl of water, and 5µl of DNA template were used. *P. aeruginosa*'s reaction was performed in an Applied Biosystem 2720 thermal cycler, while *L. redmanii*'s reaction was implemented in a T3 Biometra thermal cycler.

3) Analysis of the PCR Products: PCR products were separated by electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1× TBE buffer at room temperature using gradients of 5V/ cm. For gel analysis, 20µl PCR products were loaded into each gel well for *P. aeruginosa*, while 15µl products were loaded into each gel well for *L. redmanii*. Fragment size was determined using the Generuler 100bp ladder (Fermentas, Thermo Scientific, Germany). The gel was imaged with a gel documentation system and #40; Alpha Innotech, Biometra and#41, and the data were then processed using computer software.

Table 1. Primers sequence, target genes, amplicon sizes and cycling conditions

Item	Target gene	Primers sequence	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
					Secondary denaturation	Annealing	Extension		
Pseudomonas aeruginosa	*16S rDNA*	GGGGGATCTTCGGACCTCA CCTTAGAGTGCCCACCCG	956	94˚C 5min.	94˚C 30sec.	52˚C 40sec.	72˚C 50sec.	72˚C 10min.	**Spilker *et al.* (2004)**
	toxA	GACAACGCCCTCAGCATCACCAGC CGCTGGCCCATTCGCTCCAGCGCT	396	94˚C 5min.	94˚C 30sec.	55˚C 40sec.	72˚C 40sec.	72˚C 10min.	**Matar *et al.* (2022)**
	exoS	GCGAGGTCAGCAGAGTATCG TTCGGCGTCACTGTGGATGC	118	94˚C 5min.	94˚C 30sec.	55˚C 30sec.	72˚C 30sec.	72˚C 7min.	**Winstanley *et al.* (2005)**
Isopod	COX1	GGTCAACAAATCATAAAGATATTGG TAAACTTCAGGGTGACCAAAAAATCA	703	94˚C 5min.	94˚C 30sec.	48˚C 40sec.	72˚C 45sec.	72˚C 10min.	**Geller *et al.*(2013)**

II) Sequencing of cytochrome oxidase subunit I (COI) products

QIAquick PCR Product extraction kit was used to purify PCR products (Valencia, Qiagen). The sequence reaction was conducted using the Bigdye Terminator V3.1 cycle sequencing kit from Perkin-Elmer, and it was subsequently purified using a Centrisep spin column. DNA sequences were acquired using an Applied Biosystems 3130 genetic analyzer (HITACHI, Japan). To determine the sequence's similarity to GenBank accessions, a BLAST® analysis (Basic Local Alignment Search Tool) was first carried out following the method of **Altschul *et al.* (1990)**. The MegAlign module of LasergeneDNAStar version 12.1 was used to generate the phylogenetic tree, as indicated by **Thompson *et al.* (1994)**, and MEGA6 was used to perform the phylogenetic analyses using maximum likelihood, neighbor joining, and maximum parsimony following the method of **Tamura *et al.* (2013)**.

RESULTS

Clinical picture

In the present investigation, most of the examined *Pomadasys stridens* fish showed opened mouth, plugged operculum and the operculum protruded unilaterally in most cases; however, there was a bilateral protrusion in some cases (Fig. 1). Most of the isopod

infested fish showed signs of hemorrhage at the operculum, pelvic fins, anal fin, congested vent, and slight abdominal distention (Fig. 1). Isopods are predominantly found in the opercular cavity, in the space between the gill arches or adhering to the operculum's inner surface with its legs stuck to the gills (Fig. 2).

Most infested fish had congested and hemorrhagic gills (Plate 1C, E), with an excessive amount of mucus. Moreover, pits, ripping and sometimes large sections of gill tissues were destroyed at the sites of isopods attachment were seen (Fig. 2). Additionally, hemorrhagic spots in the liver, congested spleen and hemorrhagic kidneys were observed (Fig. 2).

Taxonomic summary of the detected isopod

***Livoneca redmanii*:** (Family: Cymothoidae): It was isolated from the branchial cavity of *P. stridens* only. The isopod's distinctive morphology is seen in its two distinct dark stripes along the body's lateral side; this is carried on till the uropod exopods. Its oval body is generally bent on one side, light brown in color with dark chromatophores. Its posterior border has three lobes, and there is only one set of eyes laterally. Pereon, consists of seven segments, with the last two appearing narrower, and the pereopods are large. Pleon: It is somewhat narrower than pereon. It has six segments, each of which becomes narrower as it gets toward the back. The length of the uropoda is identical, and it extends beyond the pleotelson border (Fig. 3).

Bacteriological examination

Results of bacteriological examination is illustrated in Table (2).

Table 2. Morphological and biochemical characters of isolated *Pseudomonas aeruginosa* from isopod infested *Pomadasys stridense* by traditional methods

Test	*Pseudomonas aeruginosa*
Gram-stain	-ve
Shape	Short rod
Motility	+
Cytochrom oxidase	+
Catalase	+
H_2S on triple sugar iron (TSI)	- K/K
Indole	-
Citrate	+
Methyl red	-
Vogaus proskauer	-
Urease production	-
Growth at 42°C	+

H_2S (TSI)= production of H_2S from triple sugar iron

K/K= alkaline/ alkaline

Fig. 1. *Pomadasys stridens* showing: **A.** An opened mouth, unilateral opercular pulging, abdominal distension and haemorrhagic pelvic fins, **B.** Haemorrhagic operculum, base of pectoral fin, pelvic fins and analfin, congested gills and haemorrhagic vent, **C.** The *L. redmanii* attached to congested gills, and **D.** Bilateral opercular protrusion with bilateral *L. redmanii*

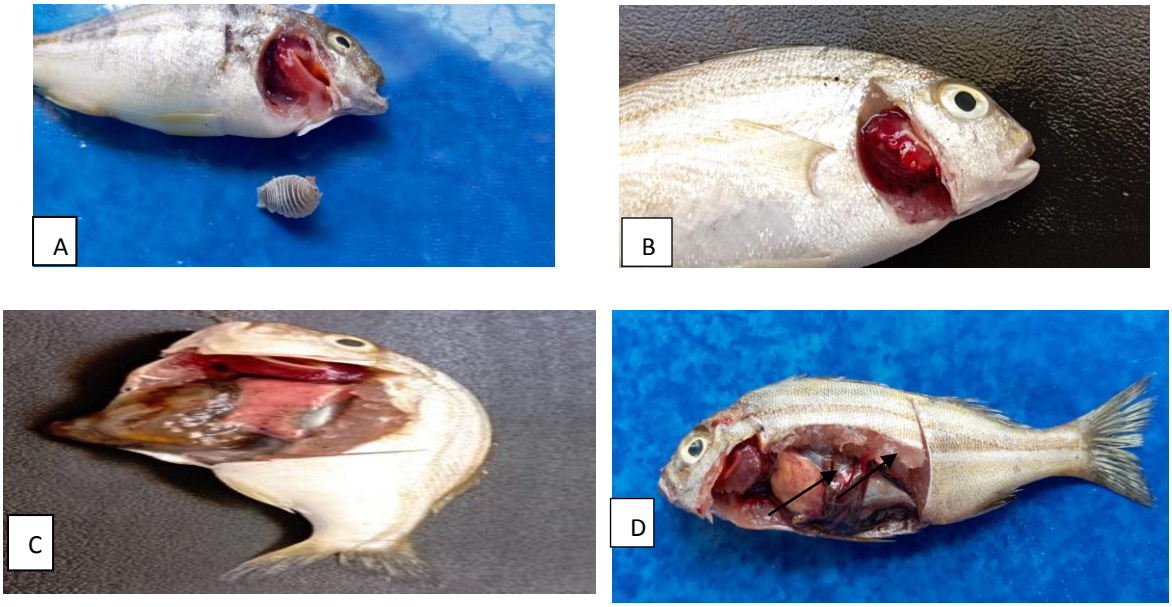

Fig. 2. *Pomadasys stridens* showing: **A.** The *L. redmanii* and distruction of a large section of gill tissue at the site of isopod attachment, **B.** Congestion, pits and ripping of gill tissues, **C.** The haemorragic spots in the liver, and **D.** The congested spleen and haemorragic kidneys

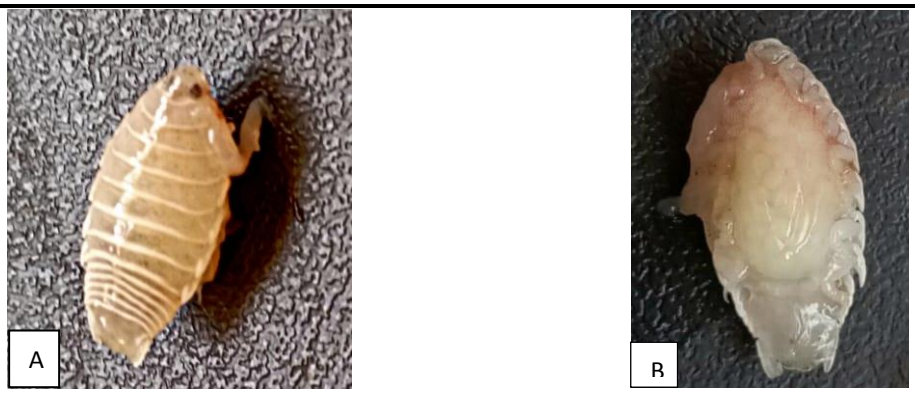

Fig. 3. *Livoneca redmanii* showing: **A.** Dorsal aspect and **B.** The ventral aspect showing marsupium

Polymerase chain reaction

A) Result of molecular identification of 16S rDNA and two virulence genes (toxA and exoS) of pathogenic *Pseudomonas aeruginosa*

Fig. (4) shows the presence of the 16s rDNA gene and two virulence genes, toxA (exotoxin A) and exoS (exotoxin S), in four selected isolates of *Pseudomonas aeruginosa*. The 16s rDNA gene at 956 bp, the toxA gene at 396 bp and the exoS gene at 118bp were all present in the four *Pseudomonas aeruginosa* isolates**.**

B) Results of *Livoneca redmanii's* molecular identification and phylogenetic analysis

The mitochondrial COI gene was utilized to identify species using an amplicon of approximately 703 base pairs, Under the accession codes OR051043 *Livoneca redmanii* RhShEg, these Cytochrome Oxidase subunit I sequences were submitted to GenBank on the NCBI website. Putting the acquired sequences (OR051043 *Livoneca redmanii* RhShEg) in a blast search, which resulted in 100% identity with the USA isolate (KX360234 *L. redmanii* 1409697, KT959417 *L. redmanii* 1286837, KT959449 *L. redmanii* 1286856) and Egypt isolate (MZ208985 *L. redmanii* ESHA/Isopoda2), the current investigation isopod isolates, and the earlier isolated isopod Egyptian isolates with close identity to the result of this study (OP430942 *L. redmanii* Bitter_Lakes_1. Identity (99.6%), Op430945 L. redmanii Burullus_Isopod1. (99.6%), OP430931 *L. redmanii* Qarun_Isopod4. (99.4%) and OP430950 *L. redmanii* Manzala_Isopod2. (97.5%). In the maximum likelihood analysis using the neighbor joining and maximum parsimony in MEGA6 **(Tamura *et al*., 2013)**, the phylogenetic analysis showed that the isopod isolates from this study and other strains of *L. redmanii* shared a strong evolutionary connection found in the United States and in previous studies conducted in Egypt. These isolates formed a monophyletic group, which suggests that they are all descended from a common ancestor (Figs. 5, 6 & Table 3).

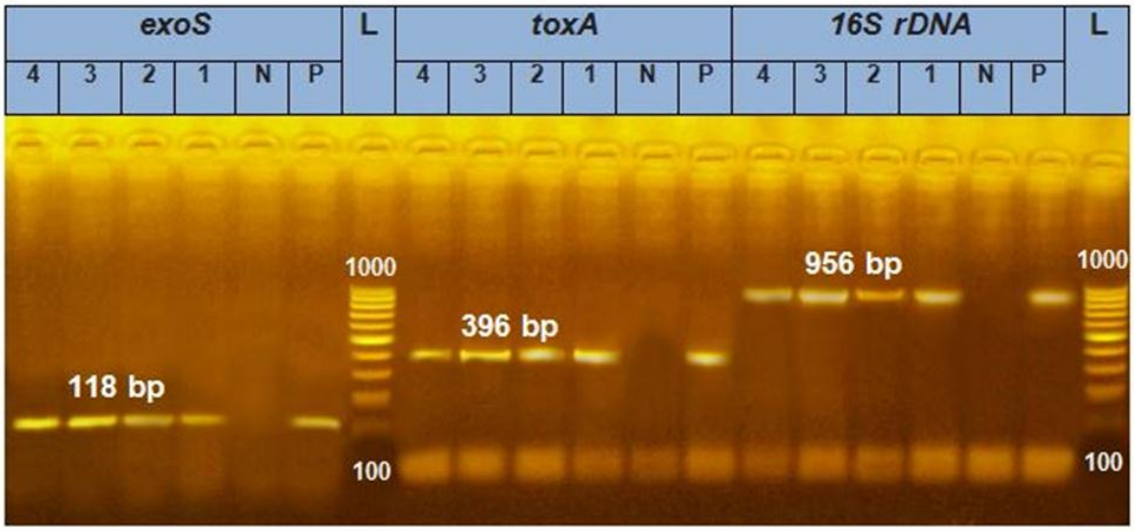

Fig. 4. Detection of *Pseudomonas aeruginosa* 16s rDNA (956bp) gene and two virulence genes toxA (396bp) and exoS (118bp) by PCR. Lanes 0: Negative control, lanes 1- 4: *P. aeruginosa* positive strains for 16S rDNA genes, lanes 1- 4: Positive strains for toxA genes, and lanes 1-4: positive strains for exoS genes

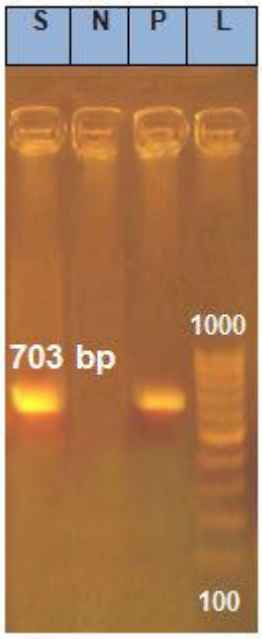

Fig. 5. Prestained 1.5% agarose gel for the PCR products for mitochondrial cytochrome oxidase subunitI gene regions for detection of *Livoneca redmanii* giving band at (703bp)

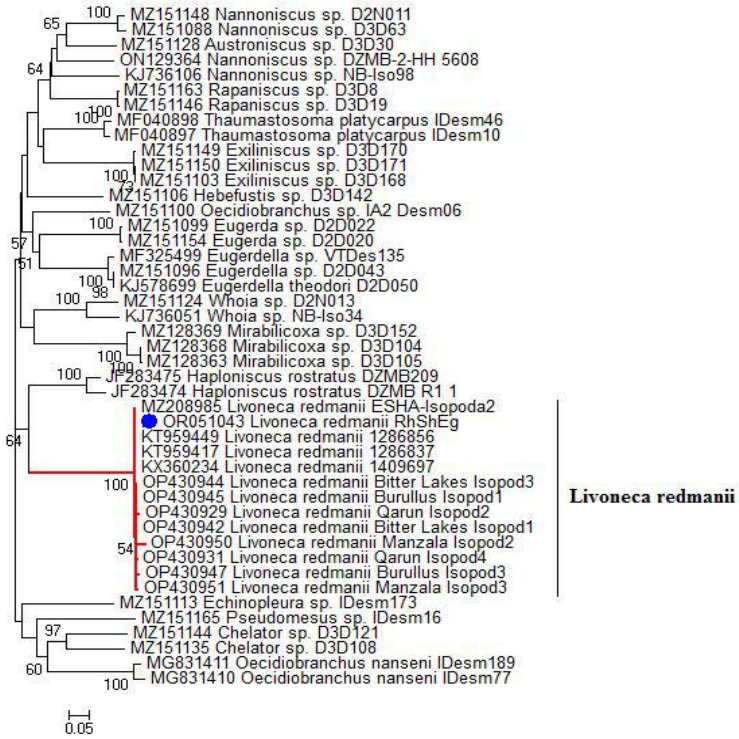

Fig. 6. The isolates of *Livoneca redmanii* (OR051043) from this study, which was isolated from *Pomadasys stridense*, were among the cymothoid isopods whose phylogenetic relationships were inferred using the maximum likelihood technique based on the mitochondrial cytochrome c oxidase subunit I sequence. The phylogenetic tree was created by the MegAlign module of LasergeneDNAStar version 12.1 and phylogenetic analyses were done using maximum likelihood, neighbor joining and maximum parsimony in MEGA6

Table 3. Estimates of pairwise distance between Cymothoid species sequences

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
1		100.0	100.0	99.6	99.4	99.6	99.6	98.9	99.1	99.2	97.5	100.0	100.0	63.9	67.9	61.6	61.8	64.1	63.7	64.1	62.4	62.8	64.8	65.0	64.8	63.1	1
2	0.0		100.0	99.6	99.4	99.6	99.6	98.9	99.1	99.2	97.5	100.0	100.0	63.9	67.9	61.6	61.8	64.1	63.7	64.1	62.4	62.8	64.8	65.0	64.8	63.1	2
3	0.0	0.0		99.6	99.4	99.6	99.6	98.9	99.1	99.2	97.5	100.0	100.0	63.9	67.9	61.6	61.8	64.1	63.7	64.1	62.4	62.8	64.8	65.0	64.8	63.1	3
4	0.4	0.4	0.4		99.8	100.0	100.0	99.2	99.4	99.6	97.9	99.6	99.6	63.9	67.9	62.0	61.8	64.5	63.7	63.7	62.4	62.8	64.5	65.0	64.8	63.1	4
5	0.6	0.6	0.6	0.2		99.8	99.8	99.1	99.2	99.4	97.7	99.4	99.4	64.1	67.7	61.8	61.6	64.3	63.5	63.5	62.2	62.6	64.3	64.8	64.7	62.9	5
6	0.4	0.4	0.4	0.0	0.2		100.0	99.2	99.4	99.6	97.9	99.6	99.6	63.9	67.9	62.0	61.8	64.5	63.7	63.7	62.4	62.8	64.5	65.0	64.8	63.1	6
7	0.4	0.4	0.4	0.0	0.2	0.0		99.2	99.4	99.6	97.9	99.6	99.6	63.9	67.9	62.0	61.8	64.5	63.7	63.7	62.4	62.8	64.5	65.0	64.8	63.1	7
8	1.1	1.1	1.1	0.8	1.0	0.8	0.8		98.7	98.9	97.2	98.9	98.9	63.3	67.7	61.4	61.6	64.3	63.3	63.1	61.8	62.2	63.9	64.5	64.1	62.6	8
9	1.0	1.0	1.0	0.6	0.8	0.6	0.6	1.3		99.1	97.4	99.1	99.1	63.7	67.3	61.4	61.2	63.9	63.1	63.1	61.8	62.2	63.9	64.5	64.3	62.6	9
10	0.8	0.8	0.8	0.4	0.6	0.4	0.4	1.1	1.0		97.5	99.2	99.2	63.5	67.5	61.6	61.4	64.1	63.3	63.3	62.0	62.4	64.1	64.7	64.5	62.8	10
11	2.5	2.5	2.5	2.1	2.3	2.1	2.1	2.9	2.7	2.5		97.5	97.5	63.5	66.9	60.9	61.1	63.9	62.6	62.9	61.8	62.0	63.7	64.1	64.3	62.2	11
12	0.0	0.0	0.0	0.4	0.6	0.4	0.4	1.1	1.0	0.8	2.5		100.0	63.9	67.9	61.6	61.8	64.1	63.7	64.1	62.4	62.8	64.8	65.0	64.8	63.1	12
13	0.0	0.0	0.0	0.4	0.6	0.4	0.4	1.1	1.0	0.8	2.5	0.0		63.9	67.9	61.6	61.8	64.1	63.7	64.1	62.4	62.8	64.8	65.0	64.8	63.1	13
14	49.3	49.3	49.3	49.3	49.0	49.3	49.3	50.4	49.7	50.0	50.1	49.3	49.3		61.6	59.5	67.5	64.1	65.4	66.2	68.2	69.6	69.9	68.1	63.3	66.5	14
15	39.6	39.6	39.6	39.6	39.9	39.6	39.6	39.9	40.6	40.2	41.2	39.6	39.6	50.3		64.5	66.4	65.6	64.3	68.8	66.0	67.5	65.6	67.3	65.8	67.5	15
16	53.4	53.4	53.4	52.6	52.9	52.6	52.6	53.8	53.7	53.3	55.1	53.4	53.4	59.8	44.5		63.3	65.4	65.0	66.7	65.2	66.7	63.1	66.0	64.1	63.7	16
17	53.5	53.5	53.5	53.5	53.9	53.5	53.5	53.9	54.7	54.2	55.2	53.5	53.5	43.9	41.5	51.2		65.4	66.9	68.4	64.7	67.3	66.5	66.9	66.4	67.7	17
18	49.1	49.1	49.1	48.2	48.6	48.2	48.2	48.6	49.3	48.9	49.3	49.1	49.1	50.7	42.5	46.3	47.3		67.1	66.9	68.2	70.5	63.1	69.0	72.4	71.3	18
19	50.2	50.2	50.2	50.2	50.6	50.2	50.2	51.0	51.3	50.9	52.4	50.2	50.2	47.9	45.5	47.4	45.0	43.8		69.9	72.4	75.4	63.9	71.5	69.9	69.2	19
20	48.6	48.6	48.6	49.4	49.8	49.4	49.4	50.5	50.5	50.1	50.8	48.6	48.6	45.9	37.1	44.4	42.0	43.9	39.4		71.3	75.4	66.5	67.9	68.1	68.6	20
21	52.7	52.7	52.7	52.7	53.1	52.7	52.7	54.0	53.9	53.5	54.0	52.7	52.7	42.5	41.9	46.8	49.9	41.8	35.1	36.8		79.0	65.0	71.1	72.6	68.8	21
22	52.0	52.0	52.0	52.0	52.4	52.0	52.0	53.2	53.2	52.7	53.5	52.0	52.0	40.3	39.4	44.7	44.6	37.7	30.6	30.3	24.9		66.4	72.0	70.1	68.8	22
23	47.9	47.9	47.9	48.7	49.0	48.7	48.7	49.7	49.8	49.4	50.0	47.9	47.9	39.2	43.1	51.3	45.1	53.5	51.6	45.7	48.4	46.3		64.8	66.4	66.9	23
24	47.8	47.8	47.8	47.8	48.2	47.8	47.8	48.9	48.9	48.5	49.6	47.8	47.8	43.0	39.6	45.9	44.4	40.2	36.8	42.8	37.3	35.9	49.0		69.9	67.9	24
25	46.9	46.9	46.9	46.9	47.3	46.9	46.9	48.4	48.0	47.6	48.0	46.9	46.9	52.2	41.9	48.5	45.3	34.5	38.6	42.0	34.1	38.3	45.9	38.8		69.4	25
26	50.9	50.9	50.9	50.9	51.2	50.9	50.9	52.0	52.0	51.6	52.8	50.9	50.9	45.8	39.1	50.3	43.2	36.3	40.1	41.4	40.8	40.9	44.9	42.6	39.7		26
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	

Divergence

1 KX360234 L. redmanii 1409697
2 KT959417 L. redmanii 1286837
3 KT959449 L. redmanii 1286856
4 OP430942 L. redmanii Bitter_Lakes_1
5 OP430931 L. redmanii Qarun_Isopod4
6 OP430945 L. redmanii Burullus_Isopod1
7 OP430944 L. redmanii Bitter_Lakes_3
8 OP430929 L. redmanii Qarun_Isopod2
9 OP430947 L. redmanii Burullus_Isopod3
10 OP430951 L. redmanii Manzala_Isopod3
11 OP430950 L. redmanii Manzala_Isopod2
12 MZ208985 L. redmanii ESHA-Isopoda2
13 OR051043 L. redmanii RhShEg
14 MZ151144 Chelator sp. D3D121
15 JF283475 H. rostratus DZMB209
16 MZ128369 Mirabilicoxa sp. D3D152
17 MZ151165 Pseudomesus sp. IDesm16
18 MZ151096 Eugerdella sp. D2D043
19 MZ151163 Rapaniscus sp. D3D8
20 MZ151124 Whoia sp. D2N013
21 MZ151148 Nannoniscus sp. D2N011Z151148
22 MZ151128 Austroniscus sp. D3D30
23 MZ151113 Echinopleura sp. IDesm173
24 MZ151106 Hebefustis sp. D3D142
25 MZ151100 Oecidiobranchus sp. IA2 Desm06
26 MZ151099 Eugerda sp. D2D022

Prevalence of isopod infestation and Pseudomonas concurrent infection

The overall prevalence of *Livoneca redmanii* infestation in the examined *Pomadasys stridens* fish from September to November 2022 was 48.75%, while the prevalence of naturally occuring *P. aeruginosa* from isopod infested fish was 69.23%.

Table 4. Prevalence of isopod infestation and bacterial infection in isopod infested *Pomadasys stridens*

	Number	**Prevalence (%)**
Examined fish	**80**	**----**
Isopod infested fish	**39**	**(39/80) 48.75%**
Isopod infested fish with *Pseudomonas aeruginosa*	**27**	**(27/39) 69.23%**

DISCUSSION

Co-infection typically occurs when two or more distinct pathogens infect the same host at the same time or as a result of a subsequent competitive infection. The severity of the negative effects on the exposed host enhances their pathogenic effects **(Abdel-Latif *et al.*, 2020)**. Most of the examined *Pomadasys stridens* fish showed opened mouth and plugged operculum in the current study, which has been explained by **Helal and Yousef (2018)** and **Mahi Gobashy (2000)**, who stated that the characteristic signs of *Livoneca redmanii*-infested fish, include hypoxia, flaring opercula, and gasping mouths. These signs are caused by the pressure of the isopod on the gill lamellae, which shortens the lamellae, ruptures the epithelial layer, releases blood content, increases mucus production, and impairs gas exchange. Mostly, the operculum protruded unilaterally in most cases, but there were bilateral protrusion in some examined *P. stridense*; this result leans to agree with **Maather El-Lamie and Heba Abdel-Mawla (2015)**, **Nisreen *et al.* (2017)**, **Ali and Aboyadak (2018)** and **Abdallah and Hamouda (2022)**. Additionally, the majority of *Pseudomonas aeruginosa*-infected *P. stridens* exhibited signs of septicemia, including hemorrhages at the operculum, pelvic fins, anal fins, a clogged vent, slight abdominal distention, hemorrhagic spots in the liver, congested spleen and hemorrhagic kidneys. These present results are almost identical to those of **Ezzat *et al.* (2021)**, **Duman *et al.* (2021)**, **Rashed *et al.* (2021)**, **Emam *et al.* (2022)** and **Nada *et al.* (2023)**. Moreover, the results depend on the fact that *Pseudomonas aeruginosa* is part of the normal microflora of fish; however, in stressful conditions, such as malnutrition, overcrowding and parasitic infestation, this bacterium becomes highly opportunistic, pathogenic forms that can lead to serious diseases such as hemorrhagic septicemia **(Ardura *et al.*, 2013)**. As a result of the release of potent bacterial proteolytic enzymes, there is a loss of electrolytes and proteins, leading to the disruption of blood circulation **(Mortia, 1975)**.

Based on previously documented morphological characteristics, the isolated isopod was recognized and identified as *Livoneca redmanii* according to **Bruce (1990)**, **Trilles (2008)** and **Hadfield (2013)**. Comparable isopod isolated from other the Egyptian

fish hosts had similar morphological features to *L. redmanii* isolated in this study, as reported by **Helal and Yousef (2018)**, **Khalaf-Allah and Yousef (2019)**, **Mohammed-Geba *et al.* (2019)** and **Fadel (2020)**.

Depending on the type of fish, different cymothoidae attachment sites exist, it can attack the skin, fins, gills, and mouth. Some species have been entangled in fish musculature **(Hoffman *et al.*, 2019)**. Mainly, *L. redmanii* was found in the branchial cavity of the examined *Pomadyases stridense*, aligning with the results of **Rameshkumar and Ravichandran (2014)** and **Abdallah and Hamouda (2022)**, who identified *L. redmanii* in the opercular cavity between the gill arches, where it attaches to the inner surface of the operculum. On the other hand, **Ugbomeh and Nwosu (2016)** and **Gloria Wonodi *et al.* (2019)** documented that the isopods were more prevalent in the mouth and on the fins of the *Pomadasysidae*.

Chronic parasites as cymothoids don't detach from their hosts; they stick there permanently **(Bunkley-Williams & Williams, 1998)**. The current study exposed pits, ripping and sometimes large section of gill tissues were destroyed at the sites of isopods attachment, these lesions could be a consequence of feeding habits. Presence of mucus acting as a means of defense to fend off an infestation and diminish irritation. These results are corroborated by studies conducted by **Rameshkumar and Ravichandran (2014)**, **Rania and Rehab (2015)**, **Thamban *et al.* (2015)**, **Ali and Aboyadak (2018)** and **Helal and Yousef (2018)**. Additionally, the pressure of large parasites frequently results in mechanical harm and lamellar structure atrophy, which impairs opercular respiratory motions. This result coincides with that of **Elgendy *et al.* (2018)** who explained that isopod feed on the blood of the infested fish. Isopods are harmful parasites that can seriously endanger the survival of fish by sucking and biting, leading to deformities and stunted growth. All these factors make the fish unfit for human consumption. According to **Nisreen *et al.* (2017)**, cymothoid isopods are chronic ectoparasites of freshwater and marine fish that provide major health risks to their fish hosts, either directly by inflicting harm on fish tissues or inadvertently by serving as a point of entry for further fish infections.

This study revealed that all isolates were not lactose fermenters and thus formed bright colonies on MacConkey agar. In addition, the production of pyocinin and pyoverdin pigments formed greenish blue colonies on the nutrient agar medium with a distinctive fruity grape-like odor (Sweetish odor) of aminoacetophenone (an aromatic molecule), and Pseudomonas F agar also produced bluish- green colonies. On blood agar, beta hemolysis was seen as a clear zone surrounding the colonies **(Abd El-Tawab *et al.*, 2019)**. These results are consistent with those of **Mai *et al.* (2020)**, **Ezzat *et al.* (2021)**, **Nair *et al.* (2021)**, **Emam *et al.* (2022)**, **Shahrokhi *et al.* (2022)**, **Nada *et al.* (2023)** and **Noor El-Deen *et al.* (2023)**.

On the other hand, isolates were gram-negative, rod-shaped, motile, positive for oxidase test, catalase test, and citrate utilization test. They were negative negative for

indole test, urease production, hydrogen sulfide (H2S) production, Vogues-Proskauer test and methyl red test. Growth at 42°C is considered an important differential test to distinguish *P. aeruginosa* from other fluorescent *pseudomonas* species. *P. aeruginosa* displayed an alkaline reaction on TSI agar without producing H_2S and without fermenting sucrose and lactose. These results match the findings of **Abd El-Tawab *et al.*(2019)**, **Mai *et al.* (2020)**, **Ezzat *et al.*(2021)**, **Nair *et al.* (2021)**, **Emam *et al.* (2022)**, **Shahrokhi *et al.* (2022)**, **Nada *et al.* (2023)** and **Noor El-Deen *et al*. (2023)**.

In the present study, polymerase chain reaction (PCR) was used for the detection of 16s rDNA gene and two virulence genes *toxA* (exotoxin A) and *exoS* (exotoxin *S*) in the selected 4 isolates giving bands at 956, 396, and 118bp. respectively. These results agree with those of **Noor El-Deen *et al. (*2023)** who reported that, PCR could be used for the detection of 16s rDNA gene in the selected isolates of *P. aeruginosa* from marine fish giving bands at 958bp. Moreover, **Shahrokhi *et al.* (2022)** mentioned that the PCR technique was used to detect virulence genes of *P. aeruginosa* and revealed that the most abundant virulence genes were algD, algU, lasB, toxA, exoS, exoT, and apr. Additionally, **Algammal *et al.*(2020)** found that the PCR results showed that all of the *P. aeruginosa* under examination contained virulence genes (oprL and toxA) that produced bands of 504 and 396bp, while only 22.2% of them had the phzM gene. Furthermore, **Mai *et al.* (2020)** detected 16S rDNA gene and Virulent ToxA gene in all *P.aeruginosa* isolates at 956 and 352bp., respectively. Therefore, based on the previously mentioned morphology, biochemical characters, and PCR analysis, the bacterium under discussion is identified as *Pseudomonas aeruginosa*.

The taxonomy of the Cymothoidae should be detected by molecular methods in addition to the morphological investigations. The mitochondrial COI gene was utilized to identify species using an amplicon of approximately 703 base pairs in the current study. The sequences acquired in this study's phylogenetic analysis showed a tight relationship and 100% identity with *L. redmanii* isolates from the United States which were recorded in the GenBank (KX360234 *L. redmanii* 140969 and KT959417 *L. redmanii* 1286837). Furthermore, *L. redmanii* isolated from *D. labrax* from the Egyptian Mediterranean waters with 100% identity was (MZ208985 L. redmanii ESHA/Isopoda2) recorded by **Abdallah and Hamouda (2022)** in the GenBank. The current isolates, the Egyptian isolates mentioned previously, and the USA isolates all form a monophyletic group within the Cymothoidae family, which is distinct from the other groups of cymothoid isopods, hence the parasite under investigation is the *L. redmanii.*

Environmental elements, such water salinity, temperature, the intensity of light and food supply have an impact on isopod numbers and spread **(Dufour, 2007)**. In this study, the total prevalence of *Livoneca redmanii* in the sampled *Pomadasys stridens* was 48.75%. This high prevalence may be due to the truncated tail of *P. stridens*, which reduces its swimming speed and makes it easier for isopods to attack. This result is higher than those reported by **Maather El-Lamie and Heba Abdel-Mawla (2015)** (22.6%), **Ali**

and Aboyadak (2018) (38%), and **Nisreen *et al.* (2019)** (19%), who found lower prevalence rates of cymothoid isopods in different marine fish species. However, this result is similar to that of **Helal and Yousef (2018)** who found that 46.7% of *Mugil cephalus* in Lake Qarun, Egypt was infested with isopods. Furthermore, this result is lower than those reported by **Gloria Wonodi *et al.* (2017)**, who found a prevalence of 69.1% for Pomadasyidae (96.7% for *Pomadasys perotetei*, and 57.1% for *Pomadasys jubelini*), and **Mohammed-Geba *et al.* (2019)** reported prevalence rates of 77.1%, 77.9%, and 78.9% for *Argyrosomus regius* in different locations in the Egyptian northern lakes. This discrepancy in prevalence rates may be due to differences in host species, sample location, water quality, and temperature **(Nisreen *et al.*, 2019)**.

This study demonstrated that prevalence of *Pseudomonas aeruginosa* isolated from *Pomadasys stridens* fish infested with isopod was about 69.23%. *P. aeruginosa* was found at the isopod attachment site in the gills. This result may be due to the effects caused by parasites, which reduce fish immunity and lead to increased mortality rate in fish co-infected with parasites and bacteria **(Salama & Yousef, 2020)**. Additionally, this fact is confirmed by **Eissa *et al.* (2015)**, who reported that bacterium is pathogenic and when entrained or carried by isopods as a secondary intruder, it causes high morbidity, affecting fish health and ultimately leading to death. Therefore, *P. aeruginosa* was isolated from the site of attachment at the gill chamber. Moreover, **Rajkumar *et al.* (2007)** demonstrated that *Nerocila phaiopleura*, a parasitic isopod, was taken from *Stolephorus commersonii* fish and caused secondary bacterial infections with Vibrio, Salmonella, and Pseudomonas. The counts of Salmonella and Pseudomonas were higher in the branchial region of the infested host than in the body surface since the gills were exposed to the outer environment directly and the lesions there were more severe. In addition, **Rameshkumar (2013)** recorded that isolated strains, such as *A. hydrophila, P. fluorescens, P.putida*, Photobacterium, Bacillus, Mycobacterium, Flexibacter, *A. salmonicida* were found on the parasitic attachment area of the host fish. The results found in this study were higher than those reported by **Nada *et al.* (2023)**, who described that, at least one isopod was detected during the examination of marine water fish, and isolated Vibrio, Pseudomonas, and Aeromonas from these infested fish with prevalence of 64.0, 26.0 and 52.0%, respectively. On the other hand, **Mai *et al.* (2020)** concluded that the prevalence of *P. aeruginosa* from naturally infected pangasius fish was 71.42%. This difference in results can be attributed to the fish species, the survey time, the total number of fish surveyed, and the type and age of the fish surveyed.

CONCLUSION

This study aimed to definitively identify *Livoneca redmanii* and *Pseudomonas aeruginosa*, both of which were isolated from *Pomadasys stridens* fish in the coasts of the Suez Canal in Egypt. To achieve this, a combination of morphological and molecular techniques was employed. The use of genetic barcoding proved to be a highly effective

tool in confirming the identity of *L. redmanii*, an invasive cymothoid isopod species. The presence of this isopod in the branchial region of the fish hosts has resulted in significant problems. The isopod either directly damages the gill tissues or serves as an entry point for other fish pathogens such as bacteria. As a consequence, the quality of fish is reduced due to these harmful effects.

Further investigations are strongly recommended to improve our understanding of the various infections and their effects on the *Pomadasys stridens* fish in the Suez Canal region of Egypt. It is crucial to acquire more knowledge in this area as the current understanding is limited. This will enable researchers and experts to develop effective strategies for disease prevention and management ultimately benefitting the health and conservation efforts of these fish populations.

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