



Co-infection with *Caligus clemensi* and *Vibrio Parahaemolyticus* in Egyptian Farmed Mullet: Diagnosis, Histopathology, and Therapeutic Management



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THIS manuscript detailed an investigation into parasitic and bacterial co-infections that caused significant morbidity and mortality in farmed thin-lipped grey mullet (*Liza ramada*) and keeled mullet (*Liza carinata*) populations. Examination revealed heavy infestations of the copepod parasite *Caligus clemensi* in the buccal cavity, gills, and skin. Morphological identification of *C. clemensi* was confirmed through genetic analysis using 18S rRNA sequencing and phylogenetic analysis. The postmortem examinations revealed internal signs of disease, including liver enlargement, hemorrhages, and ascites, while histopathology demonstrated severe destruction of gill tissues associated with the parasite infection. Bacterial cultures isolated *Vibrio parahaemolyticus* as the cause of secondary bacterial infections, which was further characterized phenotypically and identified through *recA* gene sequencing. Concerningly, high rates of antibiotic resistance were detected among the *V. parahaemolyticus* isolates. Therapeutic prolonged baths with Virocid® disinfectant followed by probiotic treatment with Sanolife Pro-W® effectively cleared the parasite infections, resolved secondary bacterial infections, and improved fish survival rates. In summary, heavy parasite burdens of *C. clemensi* coupled with multiple antibiotic-resistant *V. parahaemolyticus* infections, were identified as the primary causes of mullet mortalities. The integrated therapy effectively managed aquaculture outbreaks, providing an alternative to excessive chemical pesticides or antibiotics by eliminating parasitic infestations and controlling secondary vibriosis.

Keywords: Aquaculture, *Caligus clemensi*, *Vibrio parahaemolyticus*, Virocid®, Sanolife Pro-W®.

Introduction

Caligus infections are a major constraint to the sustainable development of marine aquaculture worldwide. *Caligus* species are ectoparasitic copepods that infect both wild and farmed fish

populations, causing significant economic losses to the aquaculture industry [3-4]. In Egyptian marine farms located in the Deepa triangle region of the Mediterranean Sea, three commercially important mullet species, flathead grey mullet (*Mugil cephalus*), thin-lipped grey mullet (*Liza*

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ramada), and keeled mullet (*Liza carinata*), are commonly farmed with other marine fishes such as gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*), in high stocking densities [5-6]. However, infections with the copepod sea lice *Caligus* spp. have been reported to cause severe disease outbreaks and mass mortalities among mullet farms [7-8].

Caligus infections impair the physiological functions of infected fish, leading to stress, reduced growth rates, skin lesions, and susceptibility to secondary infections [9-10]. Additionally, heavy caligid infestations, especially in juveniles, may cause osmoregulatory dysfunction, hypoxia, and anemia, frequently resulting in mortality [11]. Molecular diagnostic tools have revolutionized pathogen detection and enabled accurate identification of etiological agents compared to conventional methods [12]. For *Caligus* infections, PCR assays targeting the 18S rRNA gene have provided a specific means to identify the implicated parasite species without reliance on microscopic examination [13]. Currently, caligid control in fish farms relies predominantly on nonspecific chemical treatments with organophosphate compounds such as malathion [7-14]. However, these treatments have negative effects on the environment and may develop resistance in sea lice [15]. Therefore, there is a critical need to adopt alternative control measures against *Caligus* infections that are eco-friendly and reduce reliance on chemical pesticides.

Secondary bacterial infections by opportunistic pathogens commonly compromise the health of parasitized fish and contribute to mortality [16]. In previous studies, bacteria such as *Vibrio* spp., and *Streptococcus* spp. have been isolated from fish co-infected with *Caligus* spp. and experiencing disease outbreaks [17]. Among *Vibrio* spp., *Vibrio parahaemolyticus* has been recognized as an emerging zoonotic pathogen of global concern that causes human gastroenteritis [18, 19, 20]. Its ability to cause infection takes advantage of the compromised immunity of infected fish by *Caligus* spp. [21]. However, little is known about the role of *V. parahaemolyticus* as an opportunistic pathogen in farmed mullets co-infected with *Caligus* spp. Probiotics and disinfectants have been effectively used to control vibriosis in aquaculture.

Sanolife Pro-W® (INVE Aquaculture, Belgium) is a commercial probiotic containing *Bacillus subtilis* and *B. licheniformis* that has

shown promise in reducing *Vibrio* infections when added to the rearing water of cultured species [22]. Virocid® (Cid Lines, Ieper, Belgium) is a commercial broad-spectrum disinfectant based on a combination of quaternary ammonium compounds, glutaraldehyde, and isopropanol, shown to be effective against a wide range of fish pathogens [23]. The combined use of probiotics and disinfectants in an integrated approach can help lower the bacterial load and improve the disease resistance of cultured fish.

This study aimed to identify the etiologies contributing to mortality and morbidity in co-cultured farmed mullet species, particularly *L. ramada* and *L. carinata*, within Egyptian marine aquaculture systems. Specific objectives included employing PCR-based assays to detect and characterize *Caligus* sp. infecting mullets, isolating and identifying the incriminated *Vibrio* sp. from parasitized mullets to assess its potential opportunistic role, evaluating histopathological changes in moribund mullets, and assessing the efficacy of Sanolife Pro-W® and Virocid® against *Vibrio* and *Caligus* co-infections to develop sustainable aquaculture control strategies.

Material and Methods

Ethical Approval

This study protocol was approved by the Ethics Committee of the Faculty of Veterinary Medicine at Cairo University (VET-CU-IACUC-03162023629).

Farm visit

An extensive investigatory visit was conducted at a privately owned marine fish farm situated in the Deeba Triangle region of Egypt. This area is geographically bounded by the Mediterranean Sea to the north, the Damietta Estuary to the west, and Lake Manzalla to the south. The fish farm consists of multiple large earthen ponds each with a volume of 6,900 m³ and depth of 1.7 m used for polyculture of thin-lipped grey mullet and keeled mullet. The visit was initiated in response to morbidity and mortality events reported by farm owners among cultivated mullet stocks. Mortalities typically escalated in the spring months, prompting the need for a thorough investigation. The fish farm utilizes wild-caught thin-lipped grey mullet and keeled mullet juveniles as their seed stock. An estimated 6,000 fingerlings are stocked in each pond. Sinking feed pellets with 38% crude protein

content are used to feed the fish stocks. During farm visits, parasitic caligid copepod infestations were observed among farmed mullet.

Fish samples

400 moribund mullet specimens were randomly collected for clinical and laboratory analysis. Fish ranged 150-180 g in weight and 22-28 cm in length. Specimens were promptly transported on ice to the wet laboratory facility for clinical, postmortem, histopathological, parasitological, bacteriological, and molecular examinations [12]. This extensive farm investigation and laboratory analysis provided insights into pathogens identification, and disease management.

Parasitological examination

Following the fish examination, the skin and gills of each specimen were carefully inspected for the presence of external parasites or lesions. The gills were then meticulously dissected, scraped, and studied under a sterile microscope. Copepods were collected in phosphate-buffered saline (PBS), cleaned to remove mucus, added lactophenol as a clearing agent, and then mounted in glycerin jelly. The length-to-breadth ratio, the number of setae on each piece, the length of the egg sac, and the number of antennal and antennule segments were among the many details that were closely examined to identify the mounted Copepoda. Every measurement was taken in micrometers and recorded as (mean \pm SD). An Olympus BH2 microscope was used to photograph and inspect the mounted copepod specimens [24].

18S rRNA gene sequencing of parasite

Genomic DNA from copepods was extracted and purified using the Qiagen DNeasy blood and tissue kit (Qiagen, Inc., Valencia, CA, USA) following the manufacturer's instructions. DNA concentration and quality were measured using an ACT-Gene Nano-Drop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The 18S rRNA gene was sequenced to identify the parasitic copepods using the primer pair LCO 1490-forward (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO2198-reverse (5'TAAACTTCAGGGTGACCAAAAAATCA-3') as described previously [25]. PCR amplification of the 18S rRNA genes was performed using the MyTaq Red Mix kit (Bioline, Taunton, MA, USA) in 50- μ l reaction volumes containing 25 μ l of MyTaq Red Mix, 2 μ l for each primer, 5 μ l of DNA, and 16 μ l

of nuclease-free water. Standard PCR cycle conditions were initial denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 60 sec, and final extension at 72°C for 7 min.

Amplicons were visualized by agarose gel electrophoresis, purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), and sequenced by Macrogen Inc. (Seoul, Korea). Sequences were assembled, edited using BioEdit software [26], and deposited in GenBank after BLAST comparison to reference Caligidae sequences. The 18S rRNA sequences of current Caligidae were compared to 28 other Caligidae accessions with >87% similarity to the current *Caligus* sp. Sequence alignment and phylogenetic tree construction were performed by maximum likelihood methodology in MEGA11 [27] using the GTR+G+I model, which provided optimal accuracy based on model selection criteria. Maximum likelihood analysis was chosen as the most suitable method for aligning the current parasites with reference sequences. In this analysis, *Cyclops kolensis* (EF532820) was chosen as the outgroup.

Bacteriological examination

Moribund fish specimens were disinfected with 70% ethyl alcohol. Under aseptic conditions, tissue samples (spleen, kidney, liver) were streaked onto enriched Tryptic Soy Agar (TSA) and Thiosulfate Citrate Bile Salt Sucrose (TCBS) agar (Oxoid, USA) and incubated at 29°C for 18-24 hours [28]. From each plate, a single, distinct colony was carefully selected and sub-cultured on TCBS, and blood agar media (BA; Difco). These streaked plates were then incubated for an additional 24 hours at 29 °C. To establish stock cultures for biochemical and molecular identification, the pure colonies of bacterial isolates were stored in 15% glycerol at an ultra-low temperature of -80 °C. The phenotypic and biochemical characterization included assessments of colony morphological characteristics, sensitivity to the vibriostatic agent (O/129), Gram staining, bacterial cell shape under the microscope, and biochemical traits using the API20 E identification system (bioMérieux, Marcy l'Etoile, France) [28].

Sequencing of recA gene of Vibrio sp.

The *recA* gene, which encodes a protein involved in DNA recombination and repair, was sequenced in 8 *Vibrio* strains isolated from

diseased *L. caranita* and *L. ramada* to identify the species. The *Vibrio* strains were revived from frozen stocks and cultured on TSA supplemented with 2% NaCl at 29°C for 18 hours. Pure colonies were harvested, and genomic DNA was extracted using a commercial bacterial DNA extraction kit (Bioflux, Tokyo, Japan) per the manufacturer's instructions. The extracted DNA was stored at -20°C until use. The *recA* gene was amplified by PCR using the primer pair *recA*-1F (5'-GAAACCATTTCAACGGGTTC-3') and *recA*-1R (5'-CCATTGTAGCTGTACCAAGCACCC-3') which are specific for *Vibrio* species [29]. PCR was performed in 25 µl reactions containing 5 µl purified genomic DNA, 5 µl 10X PCR buffer, 1 µl each primer (50 pmol), 1.25 µl dNTPs (250 µM), 0.25 µl Taq DNA polymerase (5 units/µl) and sterile double-distilled water. Thermal cycling conditions were adjusted to cope with the following cycle conditions; initial denaturation at 94°C for 5 min; 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min; final extension at 72°C for 10 min. PCR products were purified using a PCR clean-up kit (BioFlux).

The *recA* amplicons were sequenced bidirectionally using Sanger sequencing (Macrogen Inc.) with the BigDye Terminator v3.1 kit (Applied Biosystems). Sequence reads were assembled, edited, and analyzed using BioEdit [26]. The assembled sequences were compared to the GenBank database using BLASTn for species identification and deposited in the GenBank database under unique accession numbers. A phylogenetic tree was constructed by the maximum likelihood method using the Kimura 2-parameter model in MEGA 11 [27]. Tree topology was tested with 1,000 bootstrap replicates. *Escherichia coli* (CP000247) was used as an outgroup.

Antibiotic susceptibility testing

The antibiotic susceptibility profiles of *V. parahaemolyticus* isolates were determined by the Kirby-Bauer disk diffusion method on Mueller-Hinton agar [30]. Briefly, pure cultures of *V. parahaemolyticus* were grown in tryptic soy broth at 30°C for 18-24 hours. The cultures were then diluted to achieve a turbidity equivalent to a 0.5 McFarland standard. The inoculum was swabbed uniformly onto Mueller-Hinton agar plates. Commercial antibiotic disks (Oxoid, Thermo Fisher Scientific) were then placed onto the inoculated plates using sterile forceps. The following antibiotic disks were tested:

oxytetracycline (30 µg), amoxicillin (30 µg), trimethoprim-sulfamethoxazole (1.25/23.75 µg), ampicillin (10 µg), Nalidixic acid (30 µg), gentamicin (10 µg), florfenicol (30 µg), ciprofloxacin (5 µg), and Erythromycin (30 µg). Plates were incubated at 30°C for 18-24 hours. After incubation, the zone of inhibition around each antibiotic disk was measured to the nearest millimeter using a caliper. Interpretation of susceptibility was based on Clinical and Laboratory Standards Institute (CLSI) breakpoints [31]. *V. parahaemolyticus* isolates were classified as susceptible, intermediate, or resistant to each antibiotic tested based on the zone diameter achieved.

Histopathological examination

Gill and liver tissues from each fish were fixed in 10% neutral buffered formalin. Standard histological processing, including dehydration, xylene clearing, and paraffin embedding, was followed by sectioning (5 µm thickness) using a rotary microtome. Slides were stained with Hematoxylin and Eosin per established protocols [32] and analyzed under a light microscope.

Field treatment trial

A comprehensive therapeutic regime was implemented to control the infectious outbreak in farmed mullets, consisting of immersion baths in Virocid disinfectant followed by supplementation with the probiotic Sanolife Pro-W®. Prolonged immersion baths were performed over 3 successive days using a potent Virocid formula at a concentration of 300 ml per acre. The goal was to thoroughly disinfect surfaces and contain disease spread within the affected mullet populations. Several precautions were instituted during the Virocid® baths. The pond water drainage was closed for 24 hours during treatment. Water quality parameters including dissolved oxygen and pH levels were carefully monitored. Aerators were operated continuously to maintain oxygenation and optimize Virocid® efficacy [33]. After the 3-day Virocid® regimen, the probiotic Sanolife Pro-W® was applied for an additional 3 successive days. The probiotic was prepared by first adding 100 g to 500 liters of water in a holding tank and allowing 4 hours of activation before introduction to the ponds [34-35]. The overall therapeutic efficacy was evaluated through post-treatment monitoring of mortality rates and comprehensive parasitological and bacteriological testing. This provided important insights into the success of

the Virocid® disinfectant and Sanolife Pro-W® probiotic interventions for safeguarding the health and productivity of the farmed mullet populations.

Results

Farm visit

Farm visits revealed high morbidity, with 75% of fish displaying clinical signs and 30% mortality during outbreaks. Infected mullets exhibited lethargy, sluggish swimming, skin irritation, respiratory distress, and brown copepod clusters on the head, buccal cavity, and skin. Severely affected fish showed abundant mucus, gill hemorrhaging, loss of appetite, rubbing behaviors, and scale detachment. Late-stage infections developed hemorrhagic gill patches, exterior ulcers, head hemorrhaging, ascites fluid, and ocular opacity. Internal examination revealed progressive pathology from early hepatomegaly to extensive late-stage congestion and hemorrhage of various organs (Fig. 1.).

Parasitological analysis

Parasitological examinations of the buccal cavities of mullets revealed the presence of parasitic copepods in 75% of fish specimens. The copepods were identified as *Caligus clemensi* based on morphological features. The most distinguishing feature was the presence of 9-16 genital segments on the parasites, indicative of the species *C. clemensi*. The male *C. clemensi* parasites had 4 legs and measured 1.8-2.5 mm (average 2.2 mm) in total body length. The females were larger, measuring 2.3-3.9 mm (average 2.85 mm) in length. Both sexes had a segmented body consisting of a cephalothorax and abdomen. The segmented bodies and specialized cephalothoracic appendages are characteristic of copepods in the genus *Caligus*. Key identifiable features of the cephalothorax included the lateral zones, thoracic zone, cephalic zone with frontal plate, and the connecting apron. Posteriorly, the abdomen contained a caudal ramus. The cephalothorax was comprised of distinct but partially fused cephalic, lateral, and thoracic sections. The cephalic area contained a well-developed frontal plate with disk-shaped lunules. A posterior apron on the cephalothorax connected the third legs and the genital complex segment, which was attached to the thorax (Fig. 2.).

Genetic Characterization of Parasite

In this study, a DNA fragment of the 18S rRNA gene, spanning 1020 base pairs, was

sequenced from the identified *Caligus* sp. Sequence analysis revealed a robust association of the obtained *Caligus* sp. with the family Caligidae Burmeister (1835), within the genus *Caligus* Müller (1785). The sequences were unequivocally confirmed as belonging to *C. clemensi*. Subsequently, the 18S rRNA gene sequence was deposited in GenBank under the accession numbers OR563781 and OR563782. Intriguingly, the two sequences derived from distinct mullet species exhibited identical 18S rDNA regions, indicating the conservation of this gene segment across *C. clemensi* populations. Comparative analysis unveiled a high similarity between the obtained sequence (OR563781) and known sequences, particularly with *C. clemensi* (DQ123833; MT151385), showing a similarity range of 99.80-99.71%. This firmly establishes the species designation. Further comparisons indicated decreasing identities 98.34% with *C. gurnardi* (EF088410), 97.95% with *C. elongatus* (MF077736), 97.85% with *C. belones* (EF088405), 97.75% with *C. centrodonti* (EF088406), 97.74% with *C. tetradontis* (MW925124), 97.07% with *C. quadratus* (KR048776), and 96.46% with *C. dakari* (MW925120). Additionally, a similarity range of 96.39-96.12% was observed when comparing the sequence with *Lepeophtheirus* spp. isolates, including *L. salmonis*, *L. longicauda*, *L. nordmannii*, and *L. chilensis* (OY804523, EF088414, EU929084, LC512444, and JX896398). This pattern of sequential decrease in similarity from congeneric to confamilial genera confirms the taxonomic classification.

Upon examination of the phylogenetic tree in Fig. 3., it is evident that the 18S rRNA sequence obtained from the current *C. clemensi* specimen clusters closely with known *C. clemensi* isolates, forming a monophyletic branch supported by a robust bootstrap value of 100%. This provides strong molecular evidence confirming the morphological identification of the parasite as *C. clemensi*. Furthermore, the clustering pattern illustrates close evolutionary relationships between the current *C. clemensi* and other *Caligus* spp. such as *C. quadratus*, *C. elongatus*, *C. belones*, *C. tetradontis*, and *C. gurnardi*. More distantly related genera like *Lepeophtheirus* form outgroups in separate major clades. Further sampling of 18S rRNA from diverse sea lice species, along with multi-gene phylogenies, would provide better resolution of interrelationships within the family Caligidae. Overall, the phylogenetic analysis

supplements the morphological and molecular characterization, while providing insights into the taxonomic classifications and evolutionary history of the sea louse species.

Bacteriological examination

A total of 8 presumptive *Vibrio* isolates were recovered from the infected fish. Subculturing isolates on TCBS agar yielded characteristic *Vibrio* colonies, measuring 2-3 mm in diameter and exhibiting a round, green morphology with a distinct dark blue center. Microscopic examination of Gram-stained preparations revealed Gram-negative, slightly curved rod-shaped bacteria. Biochemically, the isolates were oxidase and catalase positive. Growth was inhibited by novobiocin (30 µg) and the vibriostatic compound O/129(2,4-diamino-6,7-di-isopropylpteridine phosphate), indicating susceptibility. Analytical profile indexing using API 20E test strips enabled phenotypic identification of the isolates as *V. parahaemolyticus* (n=8), with profile codes 4146107 and 4146105.

Molecular identification of Vibrio sp.

PCR successfully amplified the housekeeping gene *recA* from all 8 *Vibrio* isolates, generating ~692 bp amplicons. Sanger sequencing of these amplicons followed by nucleotide BLAST analysis confirmed all isolates as *V. parahaemolyticus*. Notably, the 4 *recA* sequences from *L. ramada* isolates were 100% identical and deposited in GenBank under accession numbers PP101570-PP101573. Similarly, the 4 sequences from *L. caranita* isolates showed complete identity and were assigned accession numbers PP101574-PP101577. Intraspecies sequence similarity between *V. parahaemolyticus recA* sequences from the two mullet species was 99.13%, with only 6 bp differences. Comparative sequence analysis of the *V. parahaemolyticus recA* sequences against *V. parahaemolyticus* reference strains in GenBank revealed percent sequence similarities ranging from 99.71% to 96.65% for strains including (ATCC17802T, ON248832, LMG2850T, DSM10027T, LR861019, KC543007, KJ656688, CP099934, and JQ959395). Lower similarity levels (90.80% to 88.29%) were observed with *V. alginolyticus* strains (FM204833T, AP022861, AP022862, and AP022863).

Phylogenetic analysis of V. parahaemolyticus

The phylogenetic reconstruction based on *recA* gene sequences demonstrated two primary lineages (Fig. 4.). The first major lineage further

diverged into two subclades. The first subclade subsequently split into two distinct branches. The 8 *V. parahaemolyticus* isolates from this study formed a well-supported monophyletic clade with a bootstrap value of 100%, clustering together with other reference *V. parahaemolyticus* strains. Based on this clear clustering, our isolates can be definitively classified within the *V. parahaemolyticus* species. It was differentiated from its sister branch containing *V. alginolyticus* and other more distantly related *Vibrio* species like *V. campbellii*, and *V. harveyi*. The second subclade of the first major lineage contained a diversity of other *Vibrio* sp including *V. cholerae*, *V. mimicus*, and *V. vulnificus*. The second major lineage contained the well-defined species of *V. anguillarum*.

Antimicrobial Susceptibility Testing

The antibiotic susceptibility profiles of 8 *V. parahaemolyticus* isolates were ascertained against a panel of 9 antimicrobial agents encompassing 5 drug classes using a standardized disk diffusion methodology. All isolates (8/8, 100%) exhibited resistant phenotypes to the β-lactam antibiotics ampicillin (10 µg) and amoxicillin (30 µg). High rates of resistance were also observed for the aminoglycoside gentamicin and the folate pathway inhibitor combination trimethoprim-sulfamethoxazole, with resistance rates of 62.5% (5/8) and 50% (4/8), respectively. Additionally, 3/8 isolates (40%) displayed resistance to the fluoroquinolone ciprofloxacin and the macrolide erythromycin. More moderate resistance frequencies of 25% (2/8 isolates) were seen against the tetracycline antibiotic oxytetracycline and quinolone nalidixic acid. In contrast, low resistance rates were detected against the phenicol florfenicol (30 µg). Antibiotic resistance patterns in these bacteria highlight the crucial need for continuous monitoring in aquaculture. This will guide the proper use of antibiotics against persistent *Vibrio* infections.

Histopathological findings

The histopathological findings of gills showed the presence of parasites between gills and gill lamellae with the presence of severe tissue destruction (Fig. 5 a-b), there was congestion of primary gill lamellae blood vessels (Fig. 5c) and congestion of gill arch blood vessels (Fig. 5d). Sections of parasites are present embedded in gills with various degrees of tissue destruction (Fig. 5e). On the other hand, hepatic tissues showed severe vacuolar degeneration of hepatocytes (Fig.

5f). Some cases showed hepatocellular steatosis of hepatocytes (Fig. 5g).

Field treatment trial

The application of prolonged immersion baths with Virocid disinfectant followed by in-pond supplementation with Sanolife Pro-W probiotics proved effective for controlling the parasitic and bacterial infections in the afflicted mullet populations. Following treatment, noticeable improvements were observed in fish health and survival. Treated fish displayed normal swimming behavior, appetite, and feed intake compared to infected cohorts. By conclusion of the 6-day Virocid and Sanolife Pro-W regimen, examinations showed complete elimination of *C. clemensi* parasitism. No parasites were detectable on skin or gills. Additionally, no further disease outbreaks or mortality occurred in treated fish groups, in contrast to pretreatment mortalities approaching 30%. There were no discernible adverse effects from the Virocid baths or probiotic water supplementation. On the contrary, fish activity and feeding patterns improved after therapy. Signs of secondary *V. parahaemolyticus* infections also resolved post-treatment, with bacteriological sampling verifying eradication of *Vibrio* bacteria. These results demonstrate that this combined parasiticide bath and in-pond probiotic protocol successfully controlled *C. clemensi* infestations and bacterial co-infections, restoring fish health and preventing further production losses from disease.

Discussion

This comprehensive investigation has provided novel insights into the parasitic sea lice *C. clemensi* and secondary bacterial infections affecting mullet aquaculture in Egypt. Our study represented the first definitive confirmation and genetic characterization of *C. clemensi* infestations in farmed thin-lipped grey mullet (*L. ramada*) and keeled mullet (*L. carinata*). This was achieved through 18S rRNA gene sequencing and phylogenetic analysis, as no previous records have documented *C. clemensi* occurrences in Egyptian aquaculture settings. More recently, *C. clemensi* was recorded parasitizing wild striped seabass (*Morone labrax*) from Damietta Governorate, Egypt [13]. Earlier Egyptian surveys have reported diverse *Caligus* spp parasitizing both mariculture and wild fish stocks, including *C. curtus*, *C. carangis*, *C. kuwaitnesis*, *C. minimus*, *C. elongatus*, and *C. longipedis* infesting European seabass, gilthead

sea bream, grey mullet, and other farmed species [36-37]. Collectively these underscore a rich diversity of caligid sea lice present in Egyptian marine and brackish waters exerting impacts on regional aquaculture [38].

In-depth phylogenetic analysis in our study unequivocally established the identity of the parasite as *C. clemensi*, based on its close evolutionary relationship with other *C. clemensi* isolates in comparison to other *Caligus* spp. This molecular evidence aligned with and bolstered our initial morphological identification, enabling definitive designation of *C. clemensi* as the causative species. Our results are consistent with previous reports from the Mediterranean basin documenting *C. clemensi* as a common ectoparasite of wild fish hosts [13]. However, our investigation newly reveals *C. clemensi* poses an escalating threat for Mediterranean mariculture, having expanded its distribution range from native tropical Indo-Pacific waters via human translocation [25]. Significant disease outbreaks were triggered in farmed *Liza* spp. by heavy *C. clemensi* infestations, with mortality rates reaching 30% - comparable to epizootics induced by other *Caligus* spp infecting susceptible aquaculture stocks globally [39]. Extreme proliferation with loads exceeding 50 lice per fish emphasizes *C. clemensi* possesses highly invasive attributes that could profoundly impact regional aquaculture sustainability if left unchecked [40].

A concerning finding was the consistent isolation of *V. parahaemolyticus* strains from moribund mullets concurrently infected with *C. clemensi*. Molecular sequencing of *recA* housekeeping gene enabled definitive *V. parahaemolyticus* identification and phylogenetic analysis. As an emerging zoonotic pathogen of global public health significance [41-42], its presence in parasitized fish attributes a possible opportunistic role. *Caligus* attachments cause hemorrhagic skin erosions, scale loss, and mucus production [43]. This compromises skin barriers, enables bacterial invasion, and weakens immunity against opportunists like *Vibrio* spp. [44-45]. Secondary infections exacerbate pathology and mortality risks [46]. Mixed *Vibrio* infections are common in *Caligus*-infested fish [11]. Shared *V. parahaemolyticus* strains between mullet species imply inter-species transmission facilitated by high host densities. Antibiotic susceptibility profiling indicated appreciable rates of resistance

among the *V. parahaemolyticus* isolates to frontline drugs for vibriosis, including ampicillin, gentamicin, and trimethoprim-sulfamethoxazole. Wise use of antimicrobial agents in aquaculture is vital to contain antimicrobial resistance proliferation [47]. Antibiotic susceptibility testing indicated appreciable resistance rates, necessitating alternatives like probiotics [28]. Histopathology revealed severe gill damage induced by the embedded *C. clemensi* parasites, including lamellar fusion, congestion, and hemorrhage. Similar pathology has been described in other fish species due to *Caligus* infestations [48]. Hepatic lesions were also evident, including steatosis, vacuolization and necrosis. These pathological changes likely contribute to the morbidity and mortality seen in heavy infestations. They emphasize the need for prompt therapeutic interventions.

Encouragingly, this investigation provides the first evidence that the integrated therapeutic protocol incorporating prolonged immersion in Virocid® disinfectant followed by in-pond Bacillus probiotic supplementation effectively eliminated *C. clemensi* infestations and controlled secondary *V. parahaemolyticus* infections in afflicted farmed *Liza* spp. Post-treatment, *C. clemensi* infestations were eliminated and associated mortalities ceased, in contrast to substantial pretreatment losses near 30%. Fish appetite and activities markedly improved as well. These positive outcomes validate the safety and efficacy of this sustainable integrated strategy for controlling *Caligus* epidemics and bacterial co-infections to safeguard aquaculture production. Virocid® is an aquaculture-registered disinfectant containing a synergistic blend of aldehydes, quaternary ammonium compounds, and alcohols [49]. Its multi-pronged mode of action likely disrupted *C. clemensi* attachment and lifecycle progression through membrane permeabilization [23]. Prolonged immersion baths ensured adequate Virocid® penetration of the parasite's enclosed surfaces. When used as directed, Virocid® has demonstrated safety for fish even at efficacious levels, with any residues easily rinsed away after treatment [50]. Its broad-spectrum activity against fungi, viruses, and bacteria allowed simultaneous targeting of *C. clemensi* and *Vibrio* pathogens [23]. Supplementation with Sanolife Pro-W®, a commercial probiotic containing diverse *B. subtilis* and *B. licheniformis* strains, likely aided disease control through competitive exclusion of *V. parahaemolyticus* made possible

by rapid growth rates of the *Bacillus* strains and production of inhibitory metabolites [51-52]. In particular, the *Bacillus* strains in Sanolife PRO-W have been documented to improve growth rates and survival in Pacific white shrimp postlarvae through enhanced digestive enzyme activity and inhibition of pathogenic *Vibrio* bacteria [53-54]. *Bacillus* probiotics have also enhanced growth and immune parameters in Nile tilapia when used as a dietary supplement or water additive [55]. Therefore, the *Bacillus* formulation in Sanolife PRO-W demonstrated potential as a defined probiotic for aquaculture systems. The probiotic's introduction into pond water complemented the disinfectant bath effects of Virocid®. Sanolife Pro-W® Bacillus probiotics are generally recognized as safe status and have a long track record of vibriosis control efficacy in aquaculture without adverse impacts [56]. The sustainability of Egyptian aquaculture is under significant threat due to the prevalence of parasitic and bacterial infections in fish farms [57-59]. These persistent challenges not only harm fish health and production but also hinder the industry's long-term viability. Therefore, implementing new, eco-friendly control measures is crucial to safeguard the future of aquaculture in Egypt and protect the environment from potential harm caused by excessive chemical use.

Conclusions

Extensive analysis revealed parasitic sea lice (*C. clemensi*) and bacterial (*V. parahaemolyticus*) co-infections causing mortality in farmed mullets. Gene sequencing confirmed the identities of *C. clemensi* and *V. parahaemolyticus*. Our findings suggested complex interactions between these pathogens in moribund fish. An integrated approach using Virocid® and Sanolife Pro-W® successfully controlled both infections without resorting to excessive pesticides or antibiotics, highlighting its potential for sustainable sea lice management. Further studies are needed to optimize this strategy.

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Conflict of interest

There is no conflict of interest.

Author contributions

All authors contributed in creating this article and approved the final manuscript

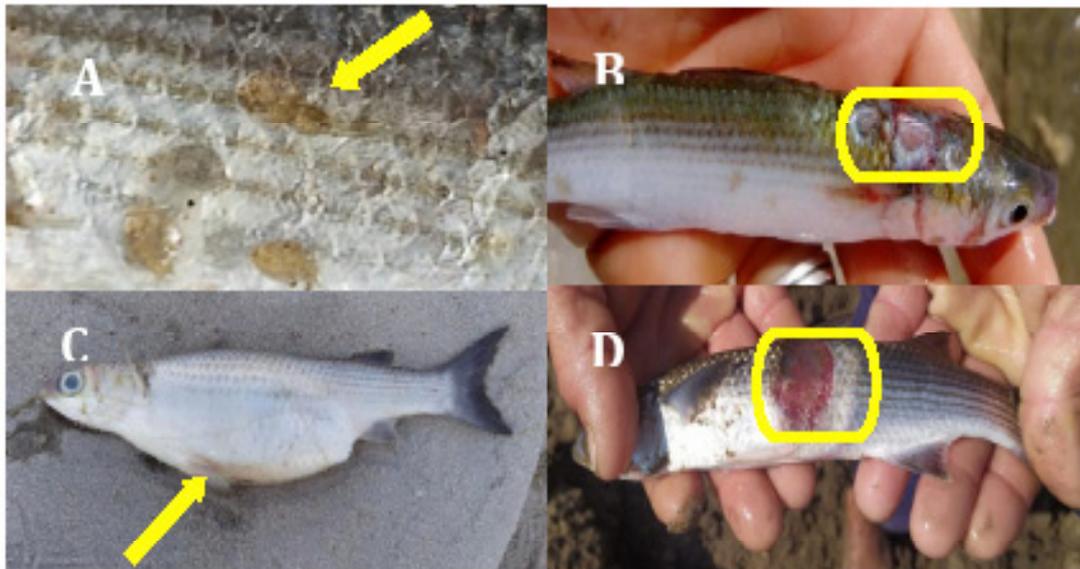


Fig. 1. Clinical signs manifested by moribund mullets.

(A) Heavy copepod infections on farmed mullets, with parasites visible on the body surface (arrows).

(B) Skin ulceration on the head (circles) of a naturally infected fish.

(C) Ascites (arrow).

(D) Skin ulceration on the body (circles) of naturally infected fish.

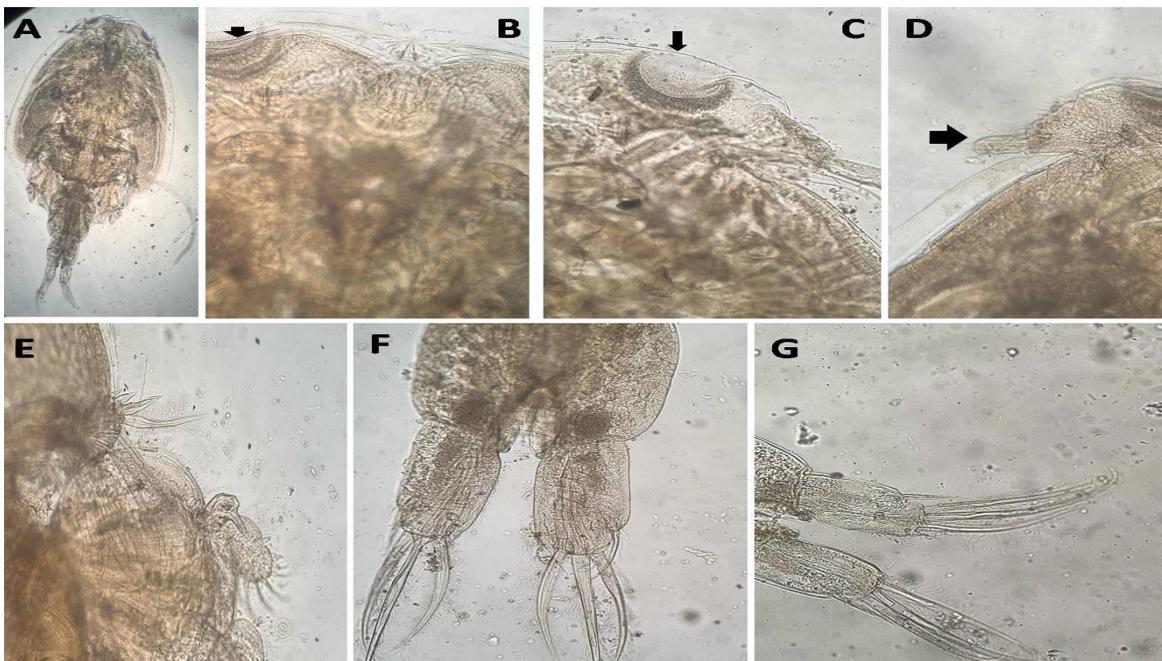


Fig. 2. Light microscopic micrograph of adult *C. clemensi*.

A: Whole adult which is oval-shaped cephalic, lateral, and thoracic sections comprise the cephalothoracic shield.

B-C: The cephalic area contained a well-developed frontal plate with disk-shaped lunules (arrows).

D: showing anterior antennule.

E: showing the legs and their hair.

F-G: The posterior part of the abdomen contains caudal ramus.

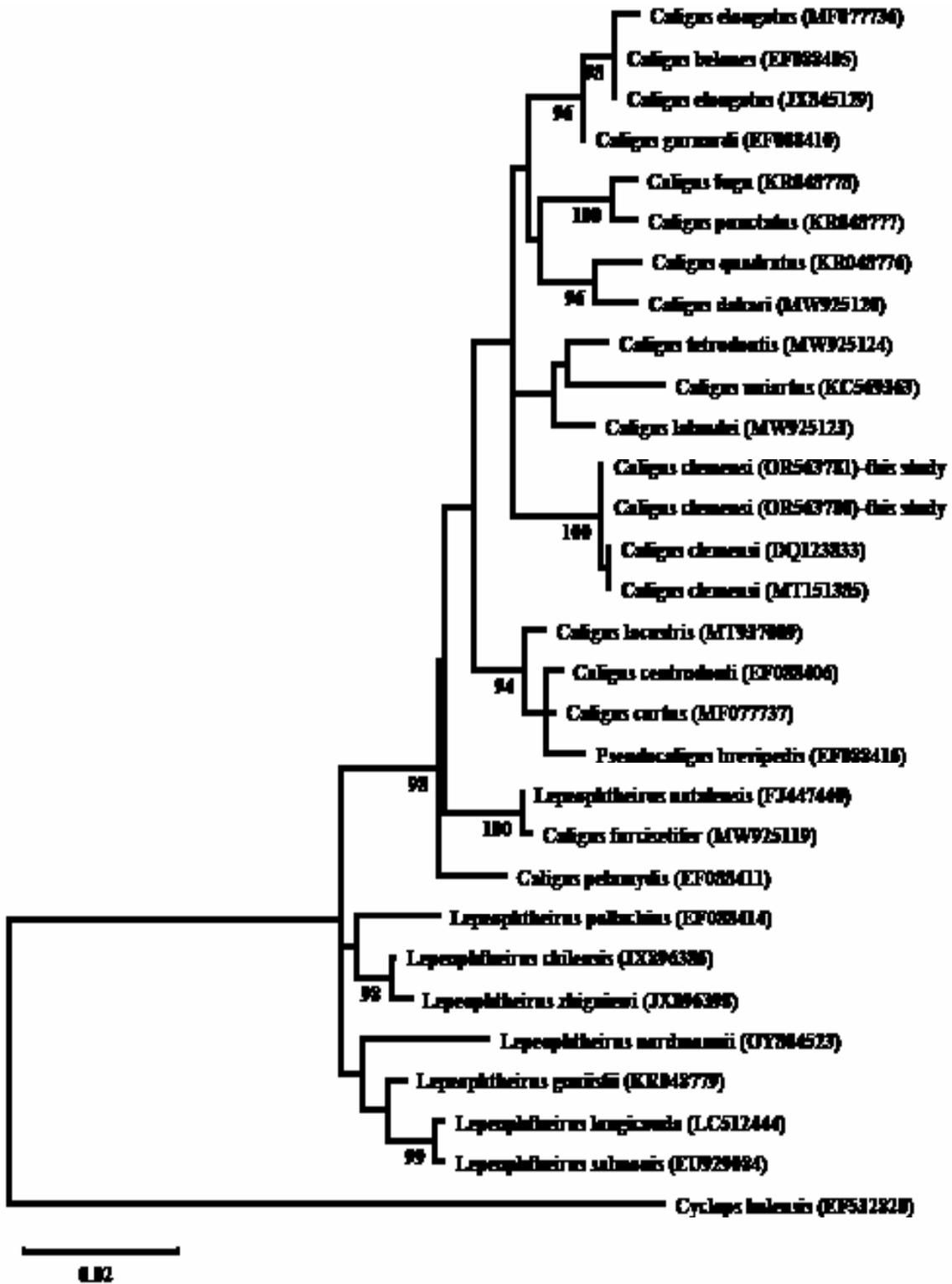


Fig. 3. Phylogenetic tree constructed using the maximum composite likelihood model and the neighbor-joining method, based on 18S rDNA sequences. The analysis includes the present *C. clemensi* specimen and other closely related members of the Caligidae family retrieved from GenBank.

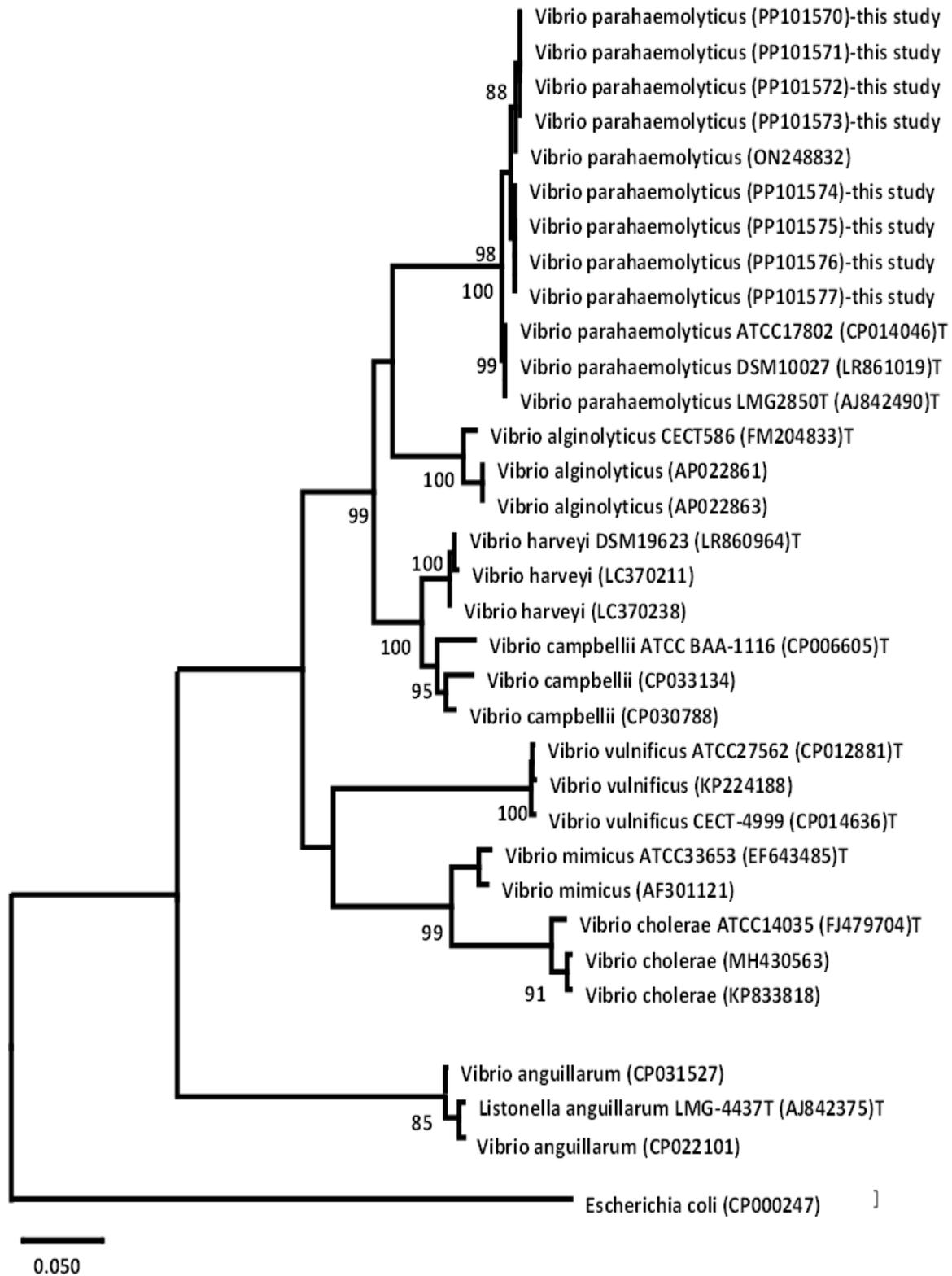


Fig. 4. A phylogenetic tree constructed using partial *recA* gene sequences from eight *V. parahaemolyticus* isolates (four from *L. ramada* and four from *L. carinata*) revealed distinct clusters. Related *Vibrio* spp. were also included for comparison.

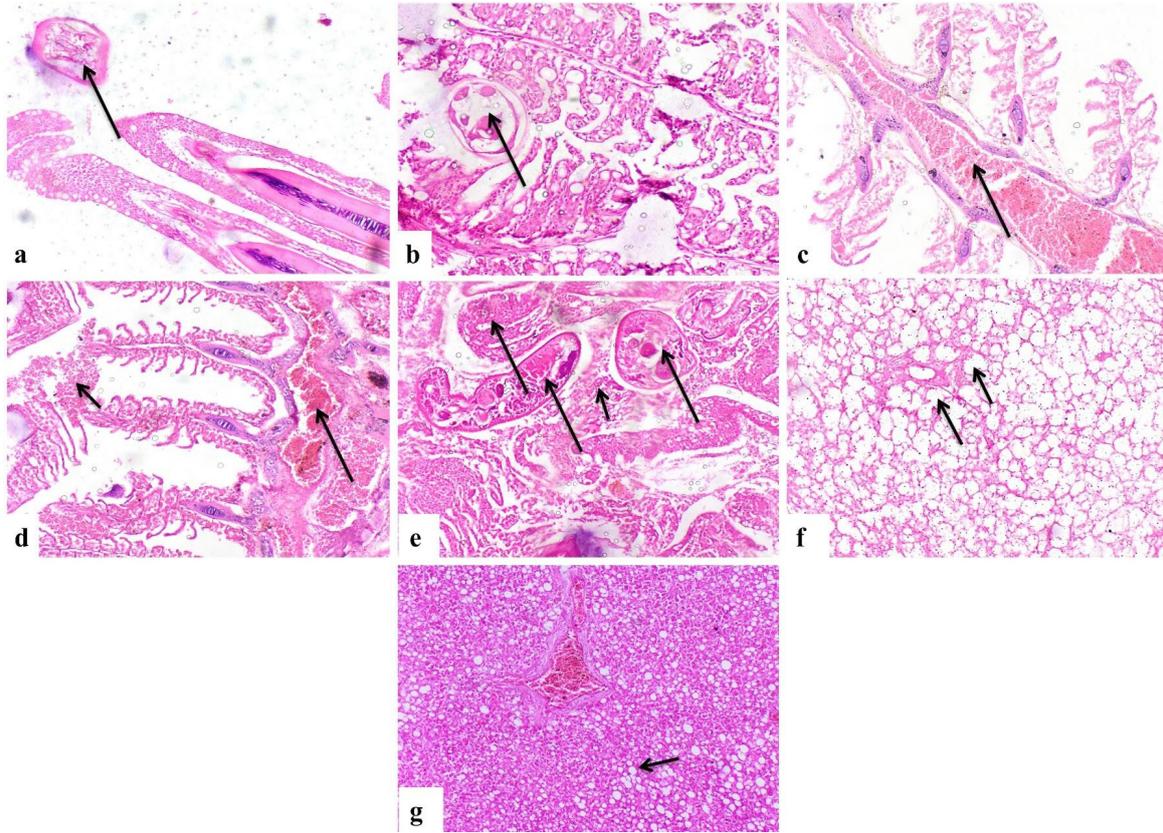


Fig. 5. Photomicrograph of mullet tissue sections showing pathological changes associated with *Caligus* sp. parasitism.

(a) and (b) gills showing localization of *Caligus* sp. between gills (arrow).

(c) gills showing congestion of primary gill lamellae blood vessels (arrow).

(d) gills showing vascular congestion of the gill arch (long arrow) and hemorrhage between gills (short arrow).

(e) gills showing sections of parasites between gill lamellae (long arrows) and destruction of gill filament (short arrow).

(f) liver showing severe vacuolar degeneration of hepatocytes (arrows).

(g) liver showing hepatocellular steatosis (arrow). (H&EX 200).

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العدوى المشتركة بقمل البحر (كاليجوس كليمنسي) والبكتيريا (فيبريو باراهيموليتيكوس) في أسماك البوري المصرية المستزرعة: التشخيص، والهستوباثولوجيا، والعلاج

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تُعدّ تربية أسماك البوري من أهم الأنشطة الاقتصادية في العديد من الدول العربية، ولكن تواجه هذه الصناعة تحديات متعددة، من أهمها العدوى المشتركة بين "قمل البحر" (كاليجوس كليمنسي) و"البكتيريا" (فيبريو باراهيموليتيكوس). تُسبب هذه العدوى معدلات عالية من الإصابة والنفوق في أسماك البوري مُرَقَط الشفاه (ليزا رامادا) والبوري ذو الزعنفة الظهرية (ليزا كاريناتا) المُستزرعة، مما يُهدد استدامة هذه الصناعة. تُقدم هذه الدراسة استراتيجية علاجية جديدة لمكافحة هذه العدوى المُزدوجة. فمن خلال فحص أسماك البوري المُصابة، تم الكشف عن انتشار كثيف لـ "قمل البحر" في فمها وخياشيمها وجلدها، بينما أظهرت الفحوصات بعد الوفاة علامات مرضية داخلية، مثل تضخم الكبد، ونزيف داخلي، وتجمع السوائل في البطن. أظهر الفحص النسيجي تدميرًا شديدًا لأنسجة الخياشيم بسبب إصابة "قمل البحر"، بينما حددت المزارع البكتيرية "فيبريو باراهيموليتيكوس" كمسبب للعدوى البكتيرية الثانوية، مع مقاومة عالية للمضادات الحيوية. أثبتت حمات مطولة من "مُطهر فيروسيد" تليها معالجة "بروبيوتيكية ب سانولايف برو-دبليو" فعاليتها في القضاء على إصابات "قمل البحر"، وعلاج العدوى البكتيرية الثانوية، وتحسين معدلات بقاء أسماك البوري. تُشير نتائج الدراسة إلى أن انتشار "قمل البحر" ووجود عدوى بكتيرية ثانية مقاومة للمضادات الحيوية من نوع "فيبريو باراهيموليتيكوس" هما السببان الرئيسيان لنفوق أسماك البوري. تُقدم هذه الدراسة استراتيجية علاجية متكاملة باستخدام "مُطهر فيروسيد" و معالجة "بروبيوتيكية ب سانولايف برو-دبليو" لإدارة نقشي الأمراض في الاستزراع المائي بفعالية، وتُقدم بديلاً عن الاستخدام المفرط للمبيدات الحشرية الكيميائية أو المضادات الحيوية، مما يُساهم في حماية البيئة وضمان استدامة تربية أسماك البوري. وتُعدّ هذه الاستراتيجية خطوة هامة نحو تطوير ممارسات تربية أسماك البوري بشكل أكثر كفاءة وأماناً..

الكلمات الدالة: فيبريو باراهيموليتيكوس، كاليجوس كليمنسي، الاستزراع المائي، فيروسيد.