Natural infestation of an anchor worm, Lernaea sp. in cage culture of Asian Seabass, Lates calcarifer juveniles and its control using an anti-parasitic drug, emamectin benzoate

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Abstract

Parasitic infestations and their control programmes are one among the challenges to be considered the most significant in aquaculture. A parasitic infestation was studied elaborately in Asian Seabass, *Lates calcarifer* juveniles with clinical signs, post-mortem findings, morphological and molecular identifications. In addition, those fish were also treated with emamectin benzoate (EMB) @ 50 µg kg^{-1} of fish body weight (BW) d^{-1} for 10 consecutive days under the controlled wet lab facility by feeding through the medicated feed at 4% BW. Results showed that the parasitic prevalence, parasitic intensity (PI) and mortality were 45.5%, 8.17 ± 0.15 per fish and 40% over a period of one week in that existing cage culture. The parasite was identified as a crustacean bloodsucker, anchor worm *Lernaea* sp. and EMB was found to be 100% effective with significant reduction in PI over a period of 10 days with improved survival rate of 90% against the untreated group. Infested but treated group revealed substantial haematological improvement in parameters such as RBC, WBC, Hb, PCV, large lymphocytes, small lymphocytes and total lymphocytes ($P < 0.01$). Similarly, comparative histopathology of vital organs also revealed no discernible lesions between the healthy and treated fish juvenile as compared to that of infested untreated group. Hence, EMB can be used to control the *Lernaea* sp. infestation in Asian Seabass.

1. Introduction

The advances in demanding aquaculture have brought an equivalent critical concern for problems accompanying with infectious diseases caused by virus, bacteria, fungi and parasites whether by slow but continuous attrition or by sudden catastrophic epizootics leads to great economic loss. Parasites such as copepods (Caligidae and Lernaeidae), isopods (Cymothidae), branchiurans (Argulidae), pentastomids (Linguatulidae), and segmented leeches (Annelida: Zylanicobdella) are considered to be the most worldwide economically important parasites in aquaculture (Johnson et al. 2004; Yatabe et al. 2011; Sahoo et al. 2013; Misganaw and Getu 2016; CIBA 2019a & 2019b; Ananda Raja et al. 2020). *Lernaea* sp. (Copepoda, Lernaeidae) is considered as a significant parasite in freshwater fish with a worldwide geographical range transported to many countries by translocation of cyprinids (Piasecki et al. 2004). The economic importance of this parasite in aquaculture is increasing because of its several epizootics among the most significant culture fish species. *Lernaea* sp. which feeds on the blood, causing mechanical injuries to the host and erosions leading to augmented vulnerability of fish to super-infections, anaemia and death (Wootten et al. 1982; Pickering and Pottinger 1989; Bjorn and Finstad 1998; MacKinnon 1998; Venmathi Maran et al. 2009; Abdul Khalid and Shaharoum-Harrison 2014; Misganaw and Getu 2016; Ananda Raja et al. 2020 & 2022). Hence identification and treatment strategy for such parasitic infestation is paramount important with scientific and legal regulations. The parasite should be identified primarily based on the morphological features followed by the molecular characterization. Treatments by many compounds like organochlorines, organophosphates, carbamates, pyrethroids, and acylurea have been applied as insecticides or anti-parasiticides in aquaculture, agriculture and animal husbandry (Athanassopoulou et al. 2009). The limited climatic activity (Stone et al. 2000b), effective only against certain life stages of parasites (Horst and Walker 1996), narrow safety margin (Roy et al. 2000; Duston and Cusack 2002), and ill effects on environment and human health (Stone et al. 2000c) are limiting factors with those chemicals. Because of such restrictions, there was a necessity for new drug with advantages of in-feed administration, high tolerance in fish, effective in wide range of temperatures, capacity to destroy all the life stages of parasites, and protection for an extended period of 10 weeks after treatment with least environmental consequences. Hence, we have identified an innovative avermectin, emamectin benzoate (EMB) and studied the biosafety, withdrawal period, environmental safety and efficacy against crustacean parasites in the laboratory condition (Ananda Raja et al. 2020; Julinta et al. 2020; Kurcheti et al. 2022). There was a natural outbreak of lernaeosis in Asian Seabass, *Lates calcarifer* juvenile in freshwater cage culture, it was treated using EMB and also a wet lab experiment was designed to assess the efficacy of EMB against lernaeosis under controlled condition.

2. Materials And Methods

2.1. Cage culture and parasitic infestations

Nursery rearing of Asian Seabass juvenile in cage culture was carried out in the freshwater bore-well fed pond of the State Government of Puducherry at Thengaithittu village, Puducherry, India (Latitude – 11°54'51.3"N, Longitude – 79°48'53.6"E) for a period of 135 days post hatch (Fig. 1A). The culture was carried out in cage (length x breath x height; 3m x 1.5m x 1.5m) system with the stocking density of 80 per cubic meter. Sudden mass mortality (40%) was observed on 50th day of post hatch i.e., 15th day of nursery rearing. Clinical signs were recorded and post-mortem examination was carried out in the moribund fish juvenile. In the same pond, Asian Seabass fingerlings of different sizes between 5 and 45 g were also maintained but in separate cages.

2.2. Isolation and identification of parasite

Fish were collected and immersed in 1% sodium chloride for 30 minutes and the parasites were collected without morphological damage. Dissecting forceps with gentle pressure was used to remove the parasites that did not detach on their own. After removing the parasite, the injured area was applied with a mild disinfectant (5 parts per million [ppm] potassium permanganate). The parasites were preserved in 4% neutral buffered formalin (NBF) and 70% alcohol (Ananda Raja and Jithendran, 2015), and morphologically identified (Gnanamuthu 1951; Demaree 1967; Toksen, et al. 2014; Shivaji et al. 2016; Gervasoni et al. 2018; Hossain et al. 2018; CIBA 2019b; Hua et al. 2019). Genomic DNA was extracted by standard phenol-chloroform precipitation method, quantified by spectrophotometric method and checked in 1% agarose gel electrophoresis for quality and stored at -80 ^{o}C. Polymerase chain reaction (PCR) was performed using a gradient thermal cycler (eppendorf, HiMedia) for 28S rDNA gene (Song et al. 2008). The forward and reverse primers used were 28SF (5'–ACAACTGTGATGCCCTTAG–3') and 28SR (5'–TGGTCCGTGTTTCAAGACG–3'), respectively with the cycling condition of initial denaturation at 94 ^{o}C for 5 min. followed by 30 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 54 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min. Final extension was carried out at 72 ^{o}C for 5 min. PCR mixture contained the master mix with dNTPs and $MgCl_2$ (10 µL), forward primer (10 pmol), reverse primer (10 pmol), DNA (100 ng) and the total volume was made to 20 µL using nuclease free distilled water. After PCR, the amplified product of 696 bp was visualized under UV transilluminator, eluted, sequenced and assembled as per Ananda Raja et al. (2017).

2.3. Fish procurement and acclimatization

Seabass juvenile produced at Muttukadu Experimental Station (MES) of Indian Council of Agricultural Research-Central Institute of Brackishwater Aquaculture (ICAR-CIBA) and nursery reared in the State Government farm at Puducherry were used in this experiment. Asian Seabass juveniles (n = 90), that were grouped as healthy (n = 30) and naturally infected (n = 60) by an external crustacean parasite were collected from the nursery rearing cage culture system to the wet lab facility of ICAR-CIBA. Four days acclimatization was given in rectangular cement tanks. The fish juvenile were PCR screened for common viral infections such as irido and betanoda viruses (WOAH [OIE], 2022). Before beginning the real experiment, the juveniles were kept for three days in the indoor static experimental set up with 50% daily water exchange. Sexually immature juveniles were used in the present study (Ananda Raja et al. 2020). Water quality parameters such as pH, dissolved oxygen (DO), temperature and salinity were recorded daily with the help of Multiparameter Waterproof Meter (Hanna Instruments Inc., USA). Fish were observed daily for their general behaviour, feeding and mortality. Criteria such as position in water column (e.g., crowding at the water surface; near the outlet or inlet pipe), flashing, gasping, hyperactivity, lethargy, abnormal pigmentation, equilibrium loss, and other uncommon behaviours and/or signs were noted for assessing general fish behaviour (Misganaw and Getu 2016; Ananda Raja et al. 2020 & 2022).

2.4. Feed preparation and feeding schedule

Powdered form of EMB (0.2%) was prepared using corn starch as a base for field efficacy trial. The recommended dose was 25 g of final preparation tonne^{-1} of fish biomass day^{-1} for 10 consecutive days i.e., 50 µg of EMB kg^{-1} fish body weight (BW) day^{-1} for 10 consecutive days. For wet lab experiments at small scale, medicated pellet feed (crude protein 42.69%)

was prepared by vacuum coating (Pegasus® Vacuum coater, Netherlands) the EMB (Sigma-Aldrich; Batch: SZBF170XV) at 1.25 ppm in feed. Preliminary trials were conducted in Seabass to check the feed acceptability with good palatability. Then the experiments were carried out by feeding normal (non-medicated) and medicated pellet feeds during acclimatization and treatment, respectively. The quantity of feed was at 4% BW per day and offered thrice a day by equally dividing the total quantity with careful observation on the feeding behaviour. Five groups of feeding behaviour were categorised such as no interest and no feed consumed, little interest and approximately 25% feed consumed, moderate interest and approximately 50% feed consumed, moderate interest and approximately 75% feed consumed and aggressive feeding with 100% feed consumption. The first two groups were biologically significant and remaining three groups were categorised as biologically not significant (Ananda Raja et al. 2020).

2.5. Efficacy of EMB

The parasitic intensity per fish (PI) and parasitic prevalence (%) were calculated in the cage culture system by treating with EMB (Helna et al. 2018; Ananda Raja et al. 2020 & 2022) as mentioned below. Similarly, Asian Seabass juveniles were grouped in to three (healthy [T1], infected treated [T2] and infected untreated [T3]) of 30 fish each in triplicate for wet lab experiment. 30 L containers were used for each replicate. Fish morphometric parameters were recorded. EMB was administered to fish juvenile through medicated feed as mentioned elsewhere. The control group was also fed with EMB medicated feed to assess any adverse effect of EMB. T3 was kept with no medication for relating the results obtained from PI, haematology, histopathology and mortality. The experiment was conducted for duration of eleven days by daily recording the water quality parameters as indicate elsewhere. MS-222 was used to anaesthetise the fish following the procedure adopted by Ananda Raja et al. (2020). Parasitic distribution on fish (%), fish PI and mortality (%) were calculated for wet lab experiment (Helna et al. 2018; Ananda Raja et al. 2020) as follows:

$$Parasitic distribution on fish\,(\%) = \frac{Number of fish infested}{Number of fish observed} \times 100$$

$$Parasitic intensity per fish\,(PI) = \frac{Total number of parasites recovered}{Number of fish infested}$$

$$Mortality\,(\%) = \frac{Total number of fish dead}{Total number of fish infested} \times 100$$

2.6. Haematology

Blood was collected in heparinized vials for haematological parameters in triplicates either through tail cutting or intra-cardiac puncture after 10th day of treatment. The fish were selected randomly from each group. Red blood cells (RBC) were counted by diluting the blood with Natt-Herrick's staining solution at 1/1000 while the white blood cells (WBC) were counted with 1/100 dilutions [Natt and Herrick 1952; Sarder et al. 2001] using improved Neubauer haemocytometer. Leishman-Giemsa's stain was used to carry out the differential WBC counts (Ferguson 2006) under light microscopy (Fi3 camera attached Nikon microscope with image analysis software). Acid haematin conversion method was adopted to estimate the haemoglobin (Hb) concentration with the help of Sahli's haemoglobinometer. Packed cell volume (PCV) also called as haematocrit (HCT) values were determined using micro haematocrit capillaries in Neuation iFuge D12 (Martins et al. 2004). The following formulae were used to calculate the erythrocyte indices (Ananda Raja et al. 2020).

$$Mean Corpuscular Volume\,(MCV)\, in femtoliters\,(fl) = \frac{PCV\% \times 10}{RBC count in 10^6 cmm}$$

$$Mean Corpuscular Haemoglobin\,(MCH)\, in picogram\,(pg) = \frac{Hb ing\% \times 10}{RBC count in 10^6 cmm}$$

$$Mean Corpuscular Haemoglobin Concentration\,(MCHC)\,in gram per deciliter\left(gdL^{-1}\right)=\frac{Hbing\%\times 100}{PCV\%}$$

2.7. Histopathology

Anaesthetized fish was performed with cervical dislocation for post-mortem examination. Organs such as eye, gill, brain, muscle, skin, stomach, intestine, liver, spleen and kidney were collected in triplicates after 10th day of treatment and fixed in 10% neutral buffered formalin [NBF] as per the literature (Ananda Raja and Jithendran, 2015). Routine histopathological technique was followed with the fixed samples in Sigma-Aldrich tissue cassettes. Dehydration was done using alcohol in ascending grades. Hardening and clearing were performed in automatic Microm STP 420D tissue processor (Thermo Fisher Scientific). Tissue paraffin infiltration and embedding were carried out in HistoStar. (Thermo Fisher Scientific). The tissues were sectioned at 5 µm thickness using a semi-automated rotary microtome (Leica RM2245). Tissue sections were stained with haematoxylin and eosin [H&E] (Bancroft and Gamble, 2011). Labomed-Lx 500 Microscope with MiaCam Image HD camera and Fi3 camera attached Nikon microscope with image analysis software were used for capturing the histopathological images.

2.8. Statistical analysis

All quantitative data were expressed as means ± SE (standard error). One-way analysis of variance (ANOVA) was used to check the homogeneity of variances in the haematological parameters. Statistical analyses were completed by means of Microsoft Excel and SPSS v 18.0. Statistical significance was fixed at $p < 0.01$ and $p < 0.05$.

3. Results

3.1. Clinical signs and parasite identification

Mortality among the Asian Seabass juvenile was detected after 15 days at nursery. Clinical signs such as depression, dullness, lethargy, inappetence, flashing, hyperactivity, erratic swimming, abnormal pigmentation, loss in equilibrium, gasping, hanging at the surface, poor body condition, emaciation and, anemia were noticed. Moribund juveniles on post-mortem (PM) examination showed that there were skin ulcerations, cutaneous haemorrhages, pallor gill, anaemic and blanched visceral organs with heavy infestation of external parasites on the trunk (Fig. 1B). Deeply punctured hemorrhagic lesions, up to 2 mm in diameter, caused by this copepod parasite were noticed in the attached body surfaces (Fig. 1C & D). The parasite was interwoven by vorticellids, *Carchesium* spp. (Fig. 2A) and diatoms (Fig. 2B) and identified as anchor worm, *Lernaea* sp. based on the morphological characteristics as depicted in Fig. 2C-F. *Lernaea* sp. was identified by the morphological (Figs. 1 & 2) and molecular descriptions (Fig. 3). Mature adult female were measured about 9804.8 ± 72.0 µm in length. The egg sac length and egg diameter were about 2924.6 ± 18.5 and 104.3 ± 2.3 µm, respectively. The sequenced PCR product of 28S rDNA showed that it was 100% homology with *Lernaea cyprinacea* (OM835790.1; OM827070.1; OM827069.1; DQ107546.1; DQ107547.1; DQ107548.1). The prevalence of *Lernaea* sp. was 45.5% with the PI of 8.17 ± 0.15 per fish and mortality of 40% over a period of one week in the cage culture. Temperature, pH, salinity and DO were within the normal range of 28.93 ± 0.5 ^{o}C, 7.88 ± 0.1, 1.03 ± 0.2 parts per thousand (ppt) and 4.95 ± 0.1 ppm, respectively. Asian Seabass fingerlings sized more than 5 g were found devoid of *Lernaea* sp. infestation.

3.2. Efficacy of EMB

EMB was found to be 100% effective in controlling *Lernaea* sp. infection in cage cultured Seabass juveniles with disappearing clinical signs and improvement in survival. It was observed that there was no re-occurrence for a period of two months post medication in the cage culture system. In wet lab experiment with the same batch of fish, the average body weight (ABW) and average body length (ABL) of the juveniles in T1, T2 and T3 were 1.64 ± 0.44 g and 46.6 ± 0.39 mm, 1.62 ± 1.11 g and 43.6 ± 1.20 mm and 1.76 ± 0.90 g and 46.1 ± 0.97 mm, respectively. PI per fish was estimated to be 8.17 ± 0.15. Temperature, pH, salinity and DO were found within the normal range of 28.67 ± 0.2 ^{o}C, 7.85 ± 0.1, 1.25 ± 0.1 ppt, and 5.03 ±

0.6 ppm, respectively. PI was reduced significantly among T2 (3.68 ± 0.6) against T3 (7.91 ± 0.3) over a period of ten days and reached zero as shown in Fig. 4A. In few cases, though the parasites were firmly attached to the fish host, they were found dead and damaged on 10th day of treatment. Mortality observed in T3 was 83.3% against the same in T2 as 10% as shown in Fig. 4B. PM examination showed a significant enhancement in gross lesions in T2 as compared to that of T3. No fish mortalities and/or adverse responses were noticed in T1.

3.3. Haematology

Infested but un-treated group (T3) exhibited significant reduction in RBC, WBC, Hb, PCV, large lymphocytes, small lymphocytes and total lymphocytes ($P < 0.01$), and significant increase in juvenile neutrophils ($P < 0.01$), total neutrophils ($P < 0.05$) and monocytes ($P < 0.01$) as presented in Table 1. RBC revealed anisocytosis (unequal sized RBCs), poikilocytosis (abnormally shaped red blood cells) and dividing erythrocytes in the blood smears of T3.

Table 1
Haematology of Asian Seabass (*Lates calcarifer*) infested with anchor worm (*Lernaea* Sp.) and treated with emamectin benzoate (EMB) [Mean ± SE].

Parameters	Control without parasite (T1)	Infested with parasite treated (T2)	Infested with parasite untreated (T3)
Red Blood Cell ($x10^6$ mm^3)**	9.6 ± 0.3^b	8.6 ± 0.3^b	4.0 ± 0.9^a
White Blood Cell ($x10^3$ mm^3)**	157.9 ± 3.2^b	149.3 ± 6.1^b	72.6 ± 2.0^a
Haemoglobin (g/dL)**	8.7 ± 0.4^b	7.9 ± 0.2^b	3.6 ± 0.6^a
Packed Cell Volume (%)**	25.8 ± 0.4^b	23.9 ± 0.5^b	11.1 ± 1.2^a
Mean Corpuscular Volume (fl)	27.1 ± 1.3	28.0 ± 0.7	29.2 ± 3.2
Mean Corpuscular Haemoglobin (pg)	9.1 ± 0.8	9.2 ± 0.2	9.3 ± 0.6
Mean Corpuscular Haemoglobin Concentration (g/dL)	33.6 ± 1.3	32.9 ± 0.2	32.2 ± 1.5
Elongate thrombocyte (%)	34.7 ± 0.9	31.7 ± 0.9	33.0 ± 1.5
Oval thrombocyte (%)	12.0 ± 1.7	8.0 ± 1.0	10.0 ± 0.6
Total thrombocyte (%)	46.7 ± 0.9	39.7 ± 0.3	43.0 ± 1.7
Large lymphocyte (%)**	9.7 ± 0.3^b	10.7 ± 0.9^b	6.7 ± 0.3^a
Small lymphocyte (%)**	22.7 ± 0.9^b	20.7 ± 0.9^{ab}	18.0 ± 0.6^a
Total lymphocyte (%)**	32.3 ± 1.2^b	31.3 ± 0.9^b	24.7 ± 0.9^a
Juvenile neutrophil (%)**	1.3 ± 0.3^a	3.3 ± 0.3^b	2.3 ± 0.3^{ab}
Mature neutrophil (%)	2.7 ± 0.3	2.3 ± 0.3	3.3 ± 0.3
Total neutrophil (%)*	4.0 ± 0.6^a	4.7 ± 0.7^a	6.7 ± 0.3^b
Myelocyte (%)	9.7 ± 0.9	9.3 ± 0.7	9.3 ± 0.9
Monocyte (%)**	7.3 ± 0.7^a	15.0 ± 0.6^b	16.3 ± 0.3^b
**P < 0.01, *P < 0.05.			
† $^{a, b}$ - Values bearing different superscripts in a row differ significantly.			

3.4. Histopathology

Comparative histopathology of gills showed that there was normal secondary lamellae (Fig. 5A), congestion in the central venous sinus (Fig. 5B) and loss of secondary lamellae (Fig. 5C) in control without parasite (T1), infested treated (T2) and infested untreated (T3) groups, respectively. Skin revealed normal epithelium (Fig. 5D), thickened epithelium with proliferated fibrous connective tissue and inflammatory mononuclear cells (MNCs) infiltration (Fig. 5E) and presence of MNCs and erythrocytes surrounding the parasite anchor in the hypodermis (Fig. 5F). Muscle tissue was normal in healthy group (Fig. 5G), separated with inflammatory MNCs (Fig. 5H) in T2 and diffuse presence of inflammatory MNCs with melanosis in T3 (Fig. 5I). Liver exhibited normal hepatopancreatic tissue (Fig. 5J & K) in T1 and T2 while T3 presented fatty change with severe congestion and hemorrhage (Fig. 5L). Kidneys showed that there were very few shrunken glomeruli with focal areas of melanomacrophage centers (MMCs) in T1 (Fig. 5M), diffuse hemorrhages with MMCs in T2 (Fig. 5N) and degenerative changes in Bowman's capsule and tubules with diffuse MMCs (Fig. 5O) in T3. There were no marked lesions in the heart, eye, stomach, intestine and brain of all the groups. Similarly, no discernible lesions were observed between the healthy and recovered fish juvenile as compared to that of infested untreated group.

4. Discussion

Asian Seabass juvenile was found infected with a crustacean parasite which was not reported so far in India. The parasite was identified as anchor worm, *Lernaea* sp. based on the morphological features as reported by earlier workers (Nagasawa et al. 2007; Toksen et al. 2014; Sayyadzadeh et al. 2016; Gervasoni et al. 2018; Hossain et al. 2018). Though the 28S rDNA sequence obtained was 100% matching with the published partial sequences of *Lernaea cyprinacea*, it could not be ascertained that the sample collected in the present study could be of the same species because of the conspecific nature between *L. cyprinacea* and *L. cruciata* (Hua et al. 2019). Morphologically, the collected parasite in the present study had single pair of branched holdfasts similar to *L. cruciata* against two pairs of holdfasts in *L. cyprinacea* (Shivaji et al. 2016; USGS 2022). Hua et al. (2019) suggested that the host-induced morphological variations in the holdfasts might lead to misidentification of *L. cruciata* and *L. cyprinacea* as different species, but both might be of the same species. Futures in depth molecular characterization studies are required to accept either both are same or different species. The anchor worms were interwoven by vorticellids and diatoms (Gnanamuthu 1951) due to their attachment on the host for long duration. This parasite is found to infect different species of fish opportunistically (Misganaw and Getu 2016; Satyanarayana and Sree Ramulu 2016). The present study showed that pH (7.88 ± 0.1), temperature (28.93 ± 0.5 ^{o}C), DO (4.95 ± 0.1 ppm) and salinity (1.03 ± 0.2 ppt) in the pond were within the adoptable range for both host and parasite. In concurrence with the present findings, prevalence and abundance of the parasite were directly proportional up to optimal range of water salinity, temperature and alkalinity (Price et al. 2010). Lernaeasis is usually observed in ponds that are characterized by slow water flow and elevated optimum water temperature of 26-36^{o}C (Berry et al. 1991; Hossain et al. 2013; Toksen et al. 2014; Sayyadzadeh et al. 2016) and reduced development below 20°C (Marcogliese, 1991; Lester and Haywood, 2006). The infected fish were stressed, exhausted and nervous with bleeding spots, dropping scales and stopped feeding. The tongue like-process ending with the holdfast organ induced congestion, skin ulcers, haemorrhages, tissue proliferation and necrosis. This parasite feeds on blood and tissue debris leading to deep ulcers, abscesses or fistulas and skin necrosis. Fish mortality was observed 40% over a period of one week with abnormal behaviors, dullness, sluggish movement, respiratory distress, emaciation and anemia in finfish hatcheries (Hossain et al. 2013) as detected in the current investigation. Lesions, up to 2 mm in diameter, caused by this copepod parasite were noticed in different areas as reported earlier. This parasite infects gills and eyes causing respiratory distress and blindness, respectively (Toksen et al. 2014). *Lernaea* sp. was also collected and identified from nostrils, fins, gills, operculum, eye, lips and body of *Capoeta saadii*, *Capoeta aculeata*, *Alburnus mossulensis* (all native cyprinids), *Carassius auratus* and *Cyprinus carpio* (exotic cyprinids) collected from the Kor River Basin (Kor River and Dorudzan Reservoir), Southwest of Iran in 2010 and 2011 (Sayyadzadeh et al. 2016). In the present study, no parasitic stages could be found neither in gills nor eyes. *Lernaea* sp. infestation can occur in skin, fins, gills, and oral cavity but in the present outbreak, it was observed only on the skin as the fish juvenile were too small. PM

examination of the moribund juveniles shown that there were skin ulcerations, cutaneous haemorrhages, muscle necrosis, intense inflammatory response, anaemic, pallor gill, blanched abdominal viscera and death as reported by the earlier workers (Khalifa and Post 1976; Berry et al. 1991; Daskalov et al. 1999; Hemaprasanth et al. 2008). *Lernaea* sp. infestation was reported in 3.33% of wild sea bass (*Dicentrarchus labrax* L.) in the northern Adriatic Sea (Coz-Rakovac et al. 2002). Hossain et al. (2018) recorded highest prevalence 72% with *L. cyprinacea* in January to March, while the lowest prevalence (8%) was recorded during April-June. Infestations with *Lernaea* sp. are most prevalent in the summer months and occur more commonly in stagnant or slow-moving water bodies (Marcogliese 1991; Hossain et al. 2013). Similarly, the present study revealed the prevalence of *Lernaea* sp. was 45.5% with the PI of 8.17 ± 0.15 per fish and mortality of 40% over a period of one week. The prevalence, parasite range and mean abundance of *L. cyprinacea* in Rainbow Trout (*Oncorhynchus mykiss*) [ABW, 80–100 g] farmed in Turkey were 100%, 4–9 and 6.2, respectively (Toksen et al. 2014). It is a common parasite all over the world affecting almost 46 species of Cyprinidae (Toksen et al. 2014; Sayyadzadeh et al. 2016). Fish can survive with *Lernaea* sp. infestation, but chronic infestations lead to lowered productivity in terms of growth retardation with behavioural changes and fish become more vulnerable to secondary bacterial and fungal infections which ultimately kill the fish (Robinson and Avenat-Oldewage 1996). It is a highly adapted crustacean that penetrates the host's skin to form an extremely strong and damaging attachment. The adult females are usually attached to the surface of the fish as observed in the present study (Gervasoni et al. 2018). The male parasite dies after copulation. The female bores into the host tissue, eventually by means of a large anchor on her anterior ("head") end to permanently embed into the skin and muscle of the fish. The female matures within 24 hours as a very prolific adult, may begin to release eggs from a pair of sacs on its posterior ("back") end. In the present study, no developmental stages such as free living nauplii and/or parasitic copepodid could be recovered since treatment was done with EMB.

Lernaea sp. is an immense threat to aquaculture due to the fact that it can infect all freshwater fish and even frog tadpoles and salamanders. This parasite does not have any host specificity (Hossain et al. 2013 & 2018). It can easily get adapted in any water quality conditions and any hosts to complete its life cycle as evidenced from extensive morphological adaptations of the majority of lernaeids (Piasecki et al. 2004). But rarely, reported in Asian Seabass belonging to species of Latidae. Increased human population with enhanced transport facility and globalization have hastened the biological invasions and proportion of introductions of any alien species throughout the world, which is of major environmental issue of public concern (Vitousek et al. 1997; Sakai et al. 2001; Gozlan et al. 2010; Lymbery et al. 2014). Aquatic invasive species (AIS) and bio-invasions (BI) are global issues in marine, brackish and freshwater ecosystems with special reference to alien and invader parasites. Fingerlings become moribund if they are infected by more than six adult female parasites ultimately leading to epizootics and high mortalities in cultured fish (Daskalov et al. 1999; Lester and Haywood 2006; Hossain et al. 2018). In the present study, the PI was 8.17 ± 0.15 per fish with mortality of 40%. In the same pond, Asian Seabass fingerlings of different sizes between 5 and 45 g were maintained in separate cages and found no *Lernaea* sp. infestation. This may be due to the fact that the scales are not well developed in fish juvenile compared to that of fingerlings and above life stages. A small rigid plate like structures grows out of the skin in fish is called as scale. Though the scales from the bony fish are originated from different tissue, but are considered similar to the structure to teeth. The skin of Asian Seabass is shielded with these defensive scales, which can also afford active camouflage by reflection and coloration. In addition, fingerlings are covered with a layer of mucus (slime) which can protect against pathogenic organisms such as parasites, bacteria, viruses and fungi. The mucus layer eventually increases the swimming speed with reduced surface resistance. Since the scales are typically formed late during the developmental stages of fish, it makes the fish more vulnerable to parasites and the parasitized fish become more susceptible to super infections as well (Bandilla et al. 2006).

Under wet lab EMB experimental condition, intense neutrophilia and lymphocytopenia were noticed as reported in the *Lernaea* sp. affected fish (Silva-Souza et al. 1999). These conditions are also shown in relationship to the spawning period (Pickering, 1986) or stress due to low water temperature (Bennet and Neville, 1975). Similar deviations can also occur in bacterial, viral (Alvarez-Pellitero and Pinto, 1987) and parasitic infections and treatments (Mahajan et al. 1979; Silva-Souza et al. 1999; Das et al. 2022) as observed in present study. A possible influence of the spawning and/or low temperature, which was shown to cause neutrophilia and lymphocytopenia by other authors, cannot be applied in this present study

because the fish infested were neither spawning nor maintained in low temperature. Monocytosis and the high levels of immature neutrophil counts were recorded from the affected groups. Lymphocytes are considered to be the most abundant leucocytes found in the peripheral blood of healthy fish. On the other hand, neutrophils are scarce, and basophils and eosinophils only occasionally seen (Ananda Raja et al. 2020). RBCs collected from the infested fish showed poikilocytosis, anisocytosis and dividing erythrocytes, which are indicative of parasitic anaemia (Martins et al. 2004) as detected in the current investigation. Anchor worm is incriminated as a voracious blood sucker, causing heavy blood loss leading to anaemia in fish as noticed in the present study. Comparative histopathology of gills, skin, muscle, liver and kidney showed that there were marked lesions in the infested untreated groups as compared to that of healthy and recovered fish juvenile. But no specific difference was observed in heart, eye, stomach, intestine and brain tissues of all the groups. Conspicuous lesions such as epidermis degeneration, presence of inflammatory MNCs and hemorrhagic lesion surrounding the parasite anchor in the hypodermis, fatty change with severe congestion and extensive haemorrhage in liver and Bowman's capsule degeneration were observed in T3 as reported by the earlier workers (Khalifa and Post, 1976; Coz-Rakovac et al. 2002; Hossain et al. 2013).

This study proved that the EMB was 100% effective after 10th day of treatment against a crustacean parasite, *Lernaea* sp. in Asian Seabass juvenile. The symptoms observed before, disappeared after treatment as noticed by Toksen (2006) against *Argulus foliaceus* on Oscar, *Astronotus ocellatus* (Cuvier 1829) and Ananda Raja et al. (2020) against *Caligus minimus* on Asian Seabass fingerlings. EMB treatment was not attributed to any fish mortalities and/or adverse reactions since the control group was also fed with EMB treated feed (Stone et al. 1999; Ananda Raja et al. 2020). EMB has also been presented effective for controlling many parasitic infestations such as *Lepeophtheirus salmonis* (Kréyer) in *Salmo salar* L. (Stone et al. 1999, 2000a, 2000b, 2000c, 2002; Armstrong et al. 2000), *Salmincola edwardsii* in *Salvelinus fontinalis* (Duston and Cusack, 2002), *Argulus coregoni* (Hakalahti et al. 2004) and *Salmincola californiensis* (Bowker et al. 2012; Gunn et al. 2012) in *Oncorhynchus mykiss*, *Lernanthropus kroyeri* in *Morone* [*Dicentrarchus*] *labrax* (Toksen et al. 2006), A. *foliaceus* in *Cyprinus carpio domestica* (Braun et al. 2008), *Caligus curtus* in *Gadus morhua* (Hamre et al. 2011), *Argulus* spp. in *Carassius auratus* (Hanson et al. 2011), *Acolpenteron ureteroecetes* in *Micropterus salmoides* (Reimschuessel et al. 2011), and *Anguillicoloides crassus* in *Anguilla rostrata* (Larrat et al. 2012). But, reports using EMB against *Lernaea* sp. was scanty. But there is a similar report in controlling *Lernaea* sp. using doramectin by incorporating in feed at 1 mg kg^{-1} BW of fish for 10 days in *Labeo fimbriatus* fingerlings and *Catla catla* yearlings without causing any kind of adverse responses or toxicities to the host fish (Hemaprasanth et al. 2008). However, detailed investigations on the environmental toxicity, pharmacodynamics and pharmacokinetics of doramectin upon their dosing to the aquatic organisms are essentially to be completed before final recommendation for the safe use of this drug to control lernaeosis in Asian Seabass. Since such studies are already reported for EMB (Ananda Raja et al. 2020), which can be scientifically recommended with 100% efficacy and safety to use against lernaeosis in the aquaculture ponds.

5. Conclusion

Outcomes of the present investigation determined that the therapeutic dosing of EMB against *Lernaea* sp. in *L. calcarifer* juvenile could be 100% successful in controlling this parasite. However, the future consequences and influences of EMB on continuous usage for controlling the parasitic infestations in aquaculture under tropical climatic conditions needs to be elaborately studied as there are many publications on its reduced effectiveness in temperate countries (Bravo et al. 2008, 2010, 2013; Jones et al. 2013; Saksida et al. 2013; Ljungfeldt et al. 2014). In addition, integrated pest management (IPM) program is necessary with all scientific knowledge on parasites, hosts and their life cycle (Jithendran et al. 2008).

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

Ananda Raja, R: Conceptualization; Investigation; Methodology; Formal analysis; Data curation; Validation; Roles/Writing - original draft.

Patil, P.K., Avunje, S and Jithendran, K.P: Methodology; Resources; Supervision; Validation; Visualization.

Ambasankar, K., De, D., Kumaran, M and Anand, P.R: Investigation; Methodology; Resources.

Alavandi, S.V and Vijayan, K.K: Funding acquisition; Project administration; Resources; Supervision; Validation.

Animal welfare statement

Fish in the present study were used with approval from Institutional Animal Ethics Committee (IAEC) [O/o the Chairman, IAEC F.No. CIBA/IAEC/2019-05].

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Statement and Declaration

The authors declare that they have no conflict of interest.

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Figures

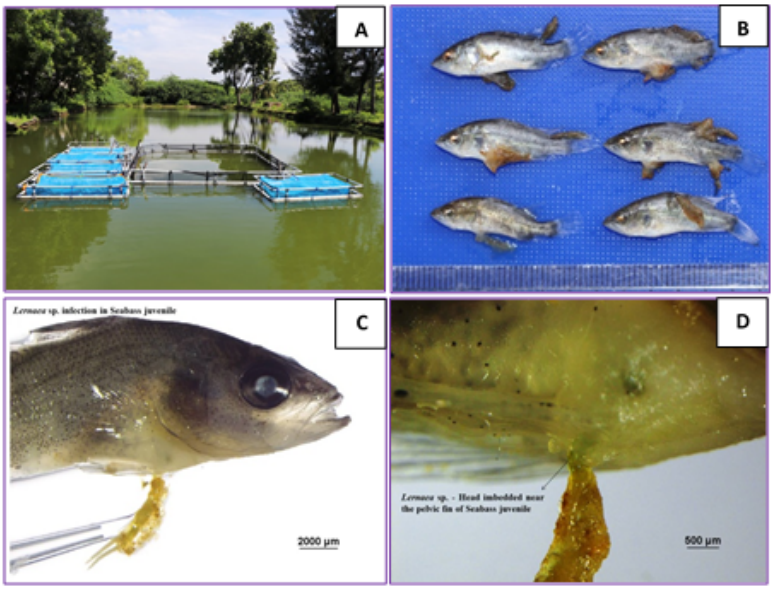

Figure 1

Asian Seabass juvenile with *Lernaea* sp. (A) Nursery rearing of Asian Seabass juvenile in freshwater cages. (B) Asian Seabass, *Lates calcarifer* juvenile infested with crustacean ecto-parasite, *Lernaea* sp. (C) Mature female *Lernaea* sp. infesting Asian Seabass juvenile. (D) Head of a mature female *Lernaea* sp. imbedded near the pelvic fin of Asian Seabass juvenile.

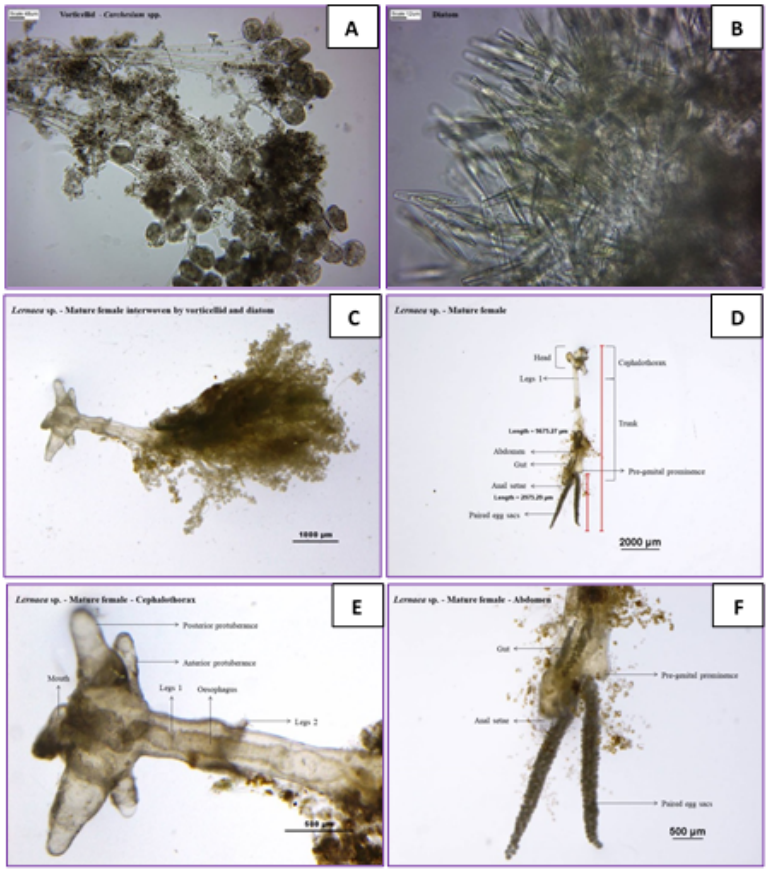

Figure 2

Female crustacean ecto-parasite, *Lernaea* sp. (A) Vorticellid collected from external surface of *Lernaea* sp. Scale bar : 48 µm. (B) Diatom collected from external surface of *Lernaea* sp. Scale bar : 12 µm. (C) Mature female *Lernaea* sp. interwoven by vorticellid and diatom. Scale bar : 1000 µm. (D) Morphology of a mature female *Lernaea* sp. Scale bar : 2000 µm. (E) Mature female *Lernaea* sp. - Cephalothorax. Scale bar : 500 µm (F) Mature female *Lernaea* sp. - Abdomen. Scale bar : 500 µm.

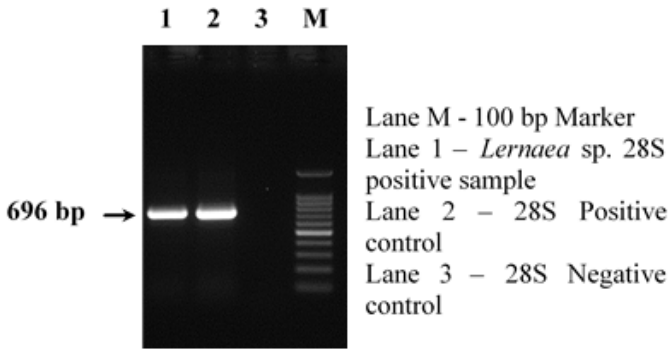

Figure 3

PCR gel picture showing the amplified product of 28S rDNA gene from *Lernaea* sp.

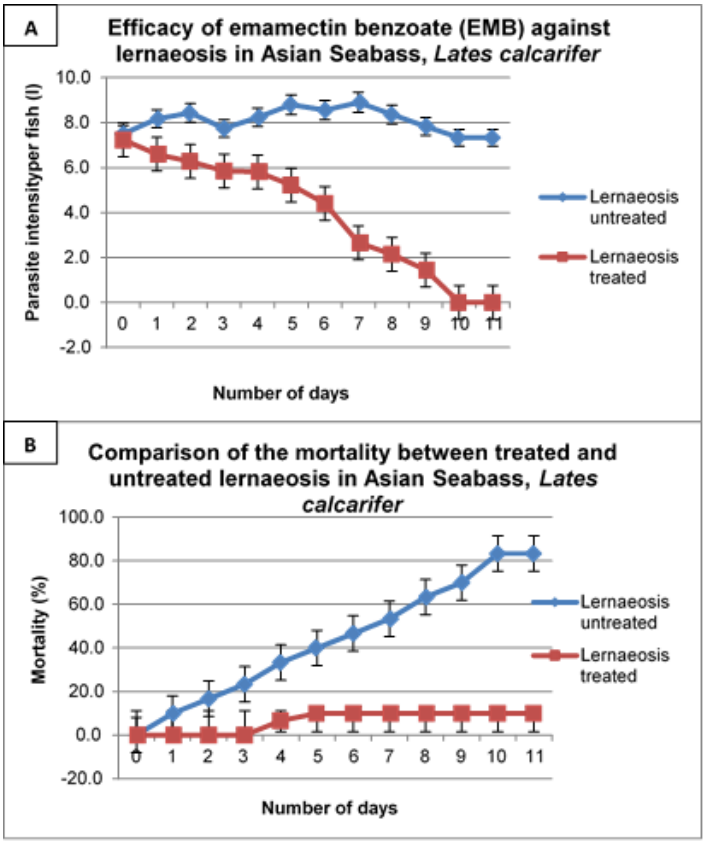

Figure 4

Efficacy of emamectin benzoate (EMB) against lernaeosis in Asian Seabass, *L. calcarifer* juvenile. (A) Parasitic intensity per fish [I] and (B) Mortality [%].

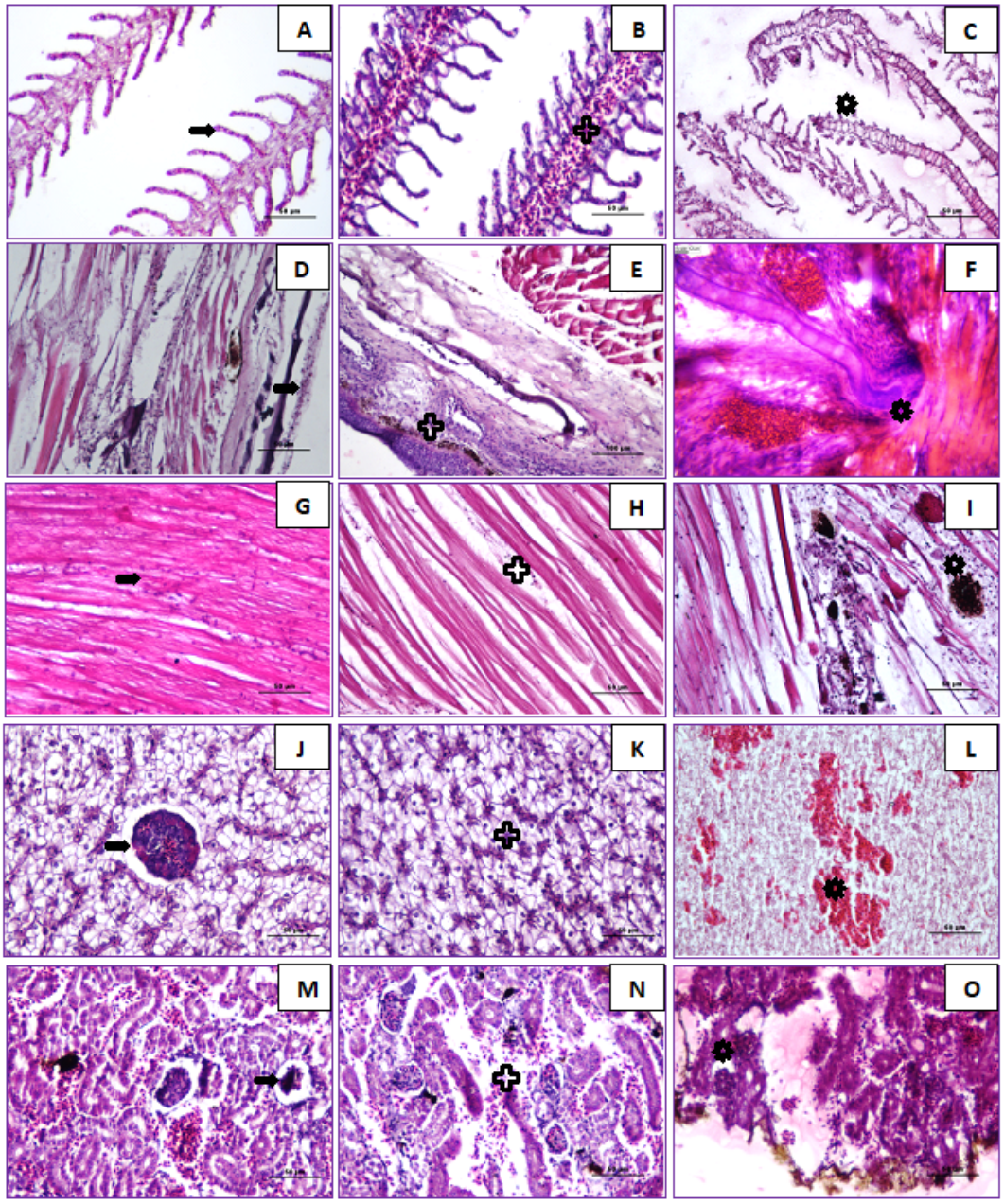

Figure 5

Comparative histopathology of healthy control, infested treated and infested untreated groups. (A) Gills with normal secondary lamellae in control without parasite (T1) group. (B) Gill showing congestion in the central venous sinus in infested treated (T2) group. (C) Loss of secondary lamellae in infested untreated (T3) group. (D) Skin with normal epithelium in T1. (E) Skin showing thickened epithelium with proliferated fibrous connective tissue and inflammatory mononuclear cells (MNCs) infiltration in T2. (F) Presence of inflammatory MNCs and erythrocytes surrounding the anchor of the parasite in the hypodermis in T3. (G) Normal muscle tissue in healthy group. (H) Muscle fibres separated with inflammatory MNCs in T2. (I) Muscle fibres revealing diffused presence of inflammatory MNCs with melanosis in T3. (J & K) Liver tissue with normal hepatopancreatic tissue. (L) Fatty degeneration with severe congestion and extensive haemorrhage in liver of T3. (M) Kidneys with very few shrunken glomeruli and focal areas of melanomacrophage centers (MMCs) in T1. (N) Kidney with diffuse hemorrhages and MMCs in T2. (O) Degenerative changes in Bowman's capsule and tubules with diffuse MMCs in T3.H&E. Scale bar as depicted in figure.