**Journal of Fisheries**
Volume 10 Issue 1 April 2022
Peer Reviewed | Open Access

eISSN 2311-3111
pISSN 2311-729X

DOI: https://doi.org/10.17017/j.fish.300

**Original Article**

# Pathology of systemic multiple bacterial infections and peritonitis in hatchery-produced African catfish *Clarias gariepinus* (Burchell, 1822) larvae

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**Manuscript history**
Received 30 December 2020 | Accepted 14 January 2022 | Published online 20 February 2022

**Citation**
Abraham TJ, Adikesavalu H, Banerjee S (2022) Pathology of systemic multiple bacterial infections and peritonitis in hatchery-produced African catfish *Clarias gariepinus* (Burchell, 1822) larvae. Journal of Fisheries 10(1): 101204. DOI: 10.17017/j.fish.300

**Abstract**
Diseases are the major problems that have a significant impact on African catfish *Clarias gariepinus* seed production. This study reports the necropsy, microscopy, bacteriology and histopathology of diseased catfish larvae that experienced mass mortalities (>80%). The gill filaments of diseased larvae revealed no ectoparasites. The intestines had no parasitic association. About 35 – 40% of the dead larvae had ruptured abdomen. The affected larvae had abdominal haemorrhages and disintegrated intestine with marked degenerative and inflammatory changes, which indicated peritonitis. Bacteria including *Aeromonas veronii*, *Edwardsiella tarda* and *Pseudomonas putida* were isolated from the haemorrhagic exudates of diseased catfish larvae. Histopathology demonstrated dense melanomacrophage aggregates in the spleen. The intestine had extensive degeneration, basophilic margination and disintegration of the mucosal layer. The kidney section suggested a suppurative infection with necrosis of haematopoietic tissue, inflammation of the epithelial tissue, vacuolar degenerations and hypoplastic haematopoietic tissue. *Aeromonas veronii* and *E. tarda* immersion challenge at $5\times10^6$ cells $mL^{-1}$ yielded no mortalities under laboratory conditions. Nevertheless, the hatchery management measures and the laboratory analyses supported peritonitis with systemic multiple bacterial infections in the observed large-scale motilities of excessively fed larvae.

**Keywords:** *Aeromonas veronii*; African catfish; *Edwardsiella tarda*; peritonitis; ruptured abdomen

## 1 | INTRODUCTION

Catfish are produced worldwide using various production systems. *Pangasianodon hypophthalmus* and *Clarias* spp. are the major catfish species produced worldwide, which contributed 4.3% and 2.3% to the total world aquaculture production in 2018, respectively (FAO 2020). Seed production and larval rearing are the most critical steps in the aquaculture of African catfish *Clarias gariepinus* (de Graaf and Janssen 1996; Uedeme-Naa and Nwafili 2017; Basiita and Rajts 2021). The flow-through technique is employed for the mass rearing of larvae and fry in indoor facilities. This technique is based on the principles that the inflow water ensures water quality requirements, replaces the used water permanently, out-flowing water removes the accumulated metabolites and feed remnants and fish are concentrated in a relatively small easily controllable area (Janssen 1987). *Clarias gariepinus* use a wide variety of natural foods as well as formulated feeds, tolerate poor water quality, and can be grown rapidly at warm temperatures with relatively low input costs. These

attributes have made this species the most widely cultured freshwater fish in tropical and subtropical countries of Asia and Africa (de Graaf and Janssen 1996; Uedeme-Naa and Nwafili 2017; Basiita and Rajts 2021) and in the Europe and South America (Marimuthu 2019). *Clarias gariepinus* seed production also requires high protein feed, which is generally expensive (Ataguba *et al.* 2009). Bacterial diseases are recognized as one of the most severe threats to the commercial success of catfish aquaculture and have a profound impact on the production (Meyer and Bullock 1973; Bharathkumar and Abraham 2015; Paul *et al.* 2015; Melaku *et al.* 2017). In seed production, significant losses are recorded during the egg and larval stages (Melaku *et al.* 2017). High stocking densities, accumulation of organic wastes and poor water quality management are cited as the prime reasons behind the disease occurrence (Meyer and Bullock 1973; Bharathkumar and Abraham 2015; Paul *et al.* 2015). The single most important drawback of the large-scale commercial culture of *C. gariepinus* is the deficiency of quality seeds of uniform size, and free of diseases, parasites, and pests at the time of stocking in culture ponds (Marimuthu 2019).

In West Bengal, India, the catfish culture is gaining importance due to high economic returns with low input. The majority of the fish hatcheries are involved in the breeding and production of seeds of catfish namely *C. batrachus*, *C. gariepinus*, *C. macrocephalus*, *Pangasius pangasius*, *P. sutchi*, etc. Several farmers have also taken up nursery rearing of *Clarias* spp. because of their good market value, high profitability and easy management practices (Bharathkumar and Abraham 2015; Paul *et al.* 2015). *Clarias gariepinus* farming in the majority of the derelict water bodies in and around peri-urban Kolkata, India has helped recycle wastes into wealth. It reportedly suffers from management issues and enormous economic losses (Abraham *et al.* 2018). Also, several earlier studies reported incidences of bacterial diseases such as *Aeromonas hydrophila* and *Edwarsiella tarda* infection (Sahoo *et al.* 1998), *Wohlfahrtiimonas chitiniclastica* fulminant sepsis (Reddy and Mastan 2013), motile Aeromonas septicemia (Paul *et al.* 2015), *E. tarda* infection (Abraham *et al.* 2015) and *Stenotrophomonas maltophilia* infection (Abraham *et al.* 2016) in Indian catfish culture. Antibiotics and several other chemicals are used in catfish grow-out aquaculture and larval rearing environment as a remedy for various diseases (Bharathkumar and Abraham 2015). This study reports the necropsy, microscopy, bacteriology and histopathology of diseased catfish larvae that experienced mass mortalities in the indoor facilities of a commercial hatchery due to haemorrhagic intestinal infection.

## 2 | METHODOLOGY

### 2.1 Case history and sampling

Since 2013, a large number of *C. gariepinus* hatcheries and nurseries in and around Naihati, North 24 Parganas district and Nepalgunj, South 24 Parganas district, West Bengal, India reported 80 – 100% mortalities during the summer season (authors, personal observation). In June 2015, a hatchery located in Ramchandrapur (22°53′21″N 88°28′13″E), West Bengal, India experienced mass mortalities (≥80%), where sampling was done as per standard protocol (Heil 2009). Antibiotics like oxytetracycline (2g kg.feed$^{-1}$) and enrofloxacin (1 ppm) were used by the hatchery owner to treat the larvae and water, respectively. Despite the use of antibiotics, mortalities continued to occur. The gross and clinical signs observed in the diseased larvae were lethargy, red spots on the head, dark colouration, fin and tail rot, focal cutaneous haemorrhages, abdominal haemorrhages, sanguineous fluid accumulation in the abdomen, discoloured internal organs, visceral haemorrhages, swollen intestine and internal organs and gas accumulation in the intestine (Figure 1). The affected larvae (n=100) with typical disease symptoms were brought to the laboratory in oxygen-filled polythene bags for further analysis. At the laboratory, the diseased larvae (≤1.0 g; 2.0 – 2.5 cm) with typical signs of abdominal haemorrhages ($n$ = 10) were subjected to necropsy that includes observations on external abnormalities, lesions, ulcerations, poor body condition, body discolouration, excessive mucus, exophthalmia, cloudy cornea, lens opacity, eye haemorrhage, frayed or missing fins, faecal casts as well as internal abnormalities (Heil 2009).

**FIGURE 1** *Clarias gariepinus* larva with haemorrhagic internal organs, and gas and fluid-filled abdomen.

### 2.2 Parasitologic and bacteriologic examination

Wet mount preparations of gills ($n$ = 5) on clean glass slides were observed for ectoparasites. The squash of a small section of the lower intestines ($n$ = 5) was made on glass slides and observed for the presence or absence of internal parasites (Heil 2009) under the microscope (Olympus BX51, Japan). The moribund catfish larvae ($n$ = 3) were first rinsed in sterile physiological saline, wiped with a sterile paper towel and dissected aseptically. Inocula from the haemorrhagic exudates were streaked onto the brain heart infusion agar (BHIA) and *Edwardsiella ic-*

*taluri* agar with colistin (10 µg $mL^{-1}$) supplement (EIA; Shotts and Waltman II 1990) and incubated at 30 ± 2°C for 24 h. Representative colonies based on dominance and distinct colony morphology were picked randomly from BHIA (*n* = 7) and EIA (*n* = 5) plates, purified by repeated streaking on BHIA plates and maintained on BHIA slants. A series of biochemical reactions were performed to identify the bacterial strains isolated from the haemorrhagic exudates (Collins *et al.* 2004; Austin and Austin 2012). The identity of the bacterial strains (*n* = 12) was confirmed phenotypically using GN Test kit VTK2 by Vitek-2 Compact system (bioMérieux, France).

Genotypic characterization of one each of the bacterial strains ($CGB_4$, $CGE_2$ and $CGH_3$) with distinctly different phenotypic characteristics was done by 16S rRNA gene amplification and sequencing. Genomic DNA extraction using genomic DNA isolation kit (Macherey-Nagel, Germany), amplification of 16S rRNA gene using universal primers (8F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-ACGGCT ACCTTGTTACGACTT-3′) of amplification size 1400 bp (Eden *et al.* 1991), quantification and purification of amplicons, sequencing, electrophoresis and data analysis were as described in Abraham *et al.* (2016). The reaction mixture (25 µL) comprised of 12.5 µL 2 × PCR TaqMixture, 1.0 µL forward primer 8F (10 pMol $\mu L^{-1}$), 1.0 µL reverse primer 1492R (10 pMol $\mu L^{-1}$), 1.0 µL DNA template and 9.5 µL molecular biology grade water. Preliminary phylogenetic analysis was carried out by the basic local alignment search tool (BLAST) of the National Center for Biotechnology Information (NCBI) for confirmation of bacterial strains.

**2.3 Experimental fish and bacterial strains**

Healthy *C. gariepinus* larvae of weight ≤1.0 g were procured from a reputed hatchery in Ramchandrapur, West Bengal, India and brought to the laboratory in oxygen-filled polythene bags. On reaching the laboratory, the larvae (*n* = 400) were disinfected with 5 ppm potassium permanganate for 10 min and maintained in fibreglass reinforced plastic (FRP) tanks of 500-L capacity at 200 numbers per tank. All larvae were maintained in FRP tanks with 300-L water under the optimal conditions for 12 days and fed daily with commercial pellet feed crumbles with 30% protein twice daily at 4% body weight. The wastes and faecal matter were siphoned out on alternate days with 40 – 50% water exchange. Before the challenge, the larvae were examined for gross and external signs of disease (Heil 2009). Besides, two healthy larvae were euthanized using clove oil (25 µL $L^{-1}$), dissected aseptically and inocula from the kidneys were streaked onto the Rimler-Shotts agar (RSA) supplemented with novobiocin (10 µg $mL^{-1}$) and EIA. The absence of gross and external signs of diseases and the bacterial growth on RSA and EIA confirmed that the stocks were healthy and devoid of *Aeromonas* spp. and *E. tarda* infection. One each of the bacterial strains, viz., *A. veronii* $CGB_4$ and *E. tarda* $CGE_2$ were used in the immersion challenge experiments. The bacterial cell suspensions were prepared as described in our earlier work (Abraham *et al.* 2016). Before being used for the challenge, a portion of the cell suspension was suitably diluted up to $10^{-9}$ in sterile saline. The number of bacterial cells $mL^{-1}$ of suspension was determined by spread plating on BHIA after incubation at 30°C for 24 h.

**2.4 Immersion challenge**

Healthy *C. gariepinus* larvae were stocked at 20 larvae per glass aquaria (60 cm × 40 cm × 30 cm) and acclimatized for three days with continuous aeration. The larvae were grouped into five groups namely group A – E, in duplicate. After acclimatization, challenge by immersion for the groups' A and B was achieved by immersing 20 catfish larvae for 30 min in 1000 mL of *A. veronii* $CGB_4$ suspensions containing ≈$5\times10^6$ and ≈$5\times10^5$ cells $mL^{-1}$, respectively. Groups C and D were immersed in *E. tarda* $CGE_2$ suspensions containing ≈$5\times10^6$ and ≈$5\times10^5$ cells $mL^{-1}$, respectively. Similarly, the larvae of group E were given a 30 min immersion in 0.85% saline and served as control. All the larval groups, after the immersion, were transferred to the respective aquaria with 30-L water and fed with pellet feed crumbles twice daily at 4% body weight. The larvae were monitored daily for the external signs of infection, behavioural abnormalities and mortality for 28 days. The wastes and faecal matter were siphoned out on alternate days with 40 – 50% water exchange.

**2.5 Histopathology**

For histopathology, the whole diseased larvae were first fixed in Bouin's fixative for 48 h and then preserved overnight in 70% ethyl alcohol. The fixed samples were processed, sectioned and stained using standard techniques (Roberts 2012).

**3 | RESULTS**

In the affected hatchery, *C. gariepinus* larvae were stocked at high densities (≥200 larvae $L^{-1}$) in indoor cemented tanks (L: 5 m × B: 4 m) with a water depth of about 15 – 17 cm. The water circulation, aeration, feeding and health management measures were inadequate. Many a time, the larvae were fed excessively due to their avid feeding behaviour. The debris, feed remnants and other wastes were found accumulated in one corner of the tank. The mortality was about above 80% within a week and about 35 – 40% of the dead larvae had ruptured abdomen. No ecto- and endo-parasites were observed, respectively in the gills and intestines of diseased catfish larvae.

Phenotypically, the bacterial flora of the haemorrhagic exudates of diseased catfish larvae on BHIA were identified as members of the genera *Aeromonas* and *Pseudomonas* with the dominance of motile aeromonads.

The strains were identified by the Vitek-2 Compact system as *A. veronii* and *P. putida*. The association of *E. tarda* was also confirmed by its growth on EIA and phenotypic characterization. All *A. veronii* strains were β-haemolytic; while *E. tarda* and *P. putida* strains were α-haemolytic. Minor phenotypic variations in *A. veronii* (L-histidine assimilation, O/129-resistance and β-N-acetyl-galactosaminidase), *E. tarda* (L-lactate alkalinisation) and *P. putida* (malonate) were observed (Table 1). Genotypically, the strains $CGB_4$, $CGE_2$ and $CGH_3$ were identified and confirmed as *A. veronii*, *E. tarda* and *P. putida*, respectively, with more than 99% DNA homogeneity of the NCBI GenBank database.

The behavioural and feeding activities of the immersion challenged catfish larvae were normal. No mortality was observed at the two challenge doses ($5\times10^6$ and $5\times10^5$ cells $mL^{-1}$) for both bacterial strains. The sections of the diseased larval internal organs had degenerative changes (Figure 2A), extensive degeneration, basophilic margination and disintegration of the intestinal mucosal layer (Figure 2B). The spleen showed loosely packed red and white pulp, and dense melanomacrophage aggregation (Figure 2C). The kidney had necrosis of haematopoietic tissue, vacuolar degenerations and hypoplastic haematopoietic tissue (Figure 2D).

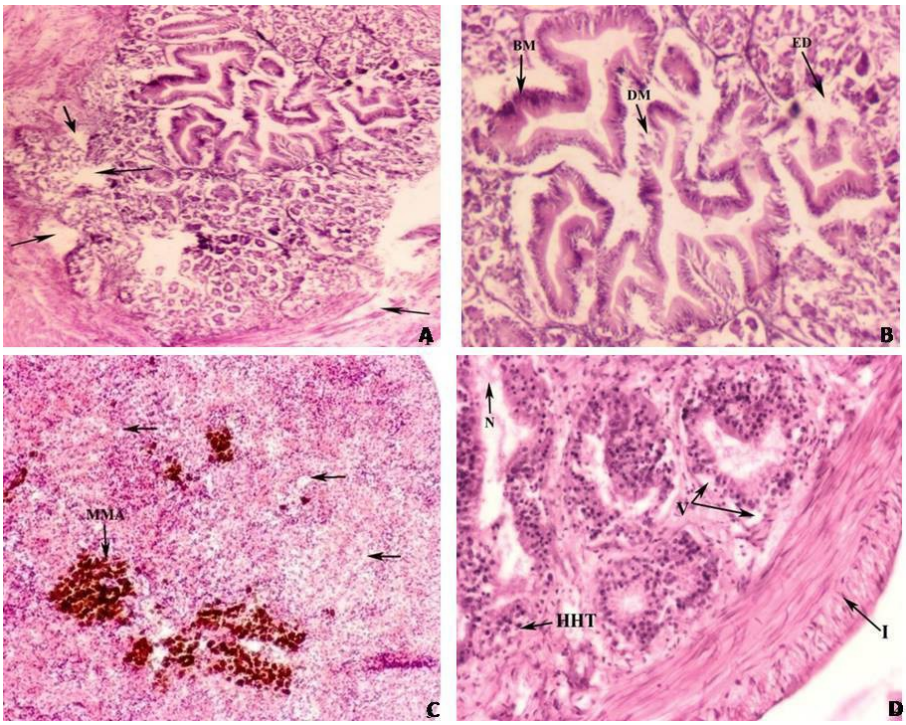


**FIGURE 2** Histopathological sections of the **[A]** internal organs of diseased *Clarias gariepinus* larva showing degenerative changes (→), X200 H&E staining, **[B]** extensive degeneration (ED), basophilic margination (BM) and disintegration of the mucosal layer of the intestine (DM), X400 H&E staining, **[C]** loosely packed red and white pulp (→), and dense melanomacrophage aggregates (MMA) within the spleen parenchyma, X100 H&E staining, and **[D]** necrosis of haematopoietic tissue (N), inflammation of the epithelial tissue (I), vacuolar degenerations (V) and hypoplastic haematopoietic tissue (HHT) in the kidney, X200 H&E staining.

## 4 | DISCUSSION

Bacterial diseases are recognized as one of the most severe threats to the commercial success of *Clarias* spp. larval production (Bharathkumar and Abraham 2015; Paul *et al.* 2015). The interview with many of the *C. gariepinus* hatchery seed producers and suppliers revealed the incidence of similar conditions in the hatchery production cycles particularly during the summer months (May and June) for years, although with variable success. The hatcheries continued to produce *C. gariepinus* seeds with minimal management practices due to their high demand. A similar diseased condition was also noted in the years 2016 and 2017. The larvae require a highly oxygenated environment, i.e., ≥5 mg $L^{-1}$ and this can generally be obtained with a water flow rate of about 3 – 5-L $min^{-1}$ (Janssen 1987). Possibly, poor and improper management measures led to stress and disease outbreaks. The antibiotic-therapy regimen was also inadequate and within a week, 80% of the catfish larvae succumbed to the disease. In catfish hatcheries, abuse of antibiotics and the incidence of multiple antibiotic-resistant (MAR) bacterial flora (Bharathkumar and Abraham 2013, 2015) and mortalities due to MAR bacteria were also recorded (Paul *et al.* 2015). In this investigation, about 35 – 40% of the dead larvae had ruptured abdomen, a condition similar to ruptured intestine syndrome (RIS) or open belly syndrome (Boon *et al.* 1987). The RIS starts as a local inflammation of the ileum or the rectum resulting in a perforation of the intestine. High feeding levels resulted in a higher percentage of RIS affected fish as the excessive consumption may result in autodigestion of the gut mucosa (Boon *et al.* 1987). Histopathological observations of the affected larval catfish confirmed the disintegration of the intestinal mucosal layer as well as the intestinal wall with marked degenerative as well as inflammatory changes in the internal organs. The disintegration of the

intestine and abdominal organs is an indication of peritonitis, which possibly indicated the involvement of virulent bacterial pathogens that originated from the gut of heavily fed larvae. The spread of inflammation in the gut and abdominal muscles prompted haemorrhagic exudates production in the abdominal cavity, thereby contributing to a dropsy-like condition. The degeneration and finally maceration of the abdominal wall might have developed a ruptured abdomen with the fluid leaving the abdominal cavity.

No ectoparasites were observed in the gill filaments. The squash of the lower intestines of diseased catfish larvae did not reveal any internal parasites. Nevertheless, the association of *A. veronii*, *E. tarda* and *P. putida* in the haemorrhagic exudates of diseased catfish larvae were noted. This, however, contradicts the earlier findings (Boon *et al.* 1987), which indicated a significant influence on feeding level. According to them, the flow rate and the number of bacteria and parasites did not influence the incidence of RIS. Also, the motile aeromonads, pseudomonads and *E. tarda* are considered opportunistic pathogens (Austin and Austin 2012). *Aeromonas veronii* $CGB_4$ and *E. tarda* $CGE_2$ challenge by immersion neither affected the behavioural and feeding activities of the catfish larvae nor resulted in mortalities even at the highest challenge dose ($5\times10^6$ cells $mL^{-1}$). Nevertheless, both strains were found to harbour a variety of virulent genes (data not shown). The challenged larvae were normal throughout the observation period as they were healthy and devoid of physical damages. These results ruled out the bacterial association as a sole reason for the observed mortalities. Perhaps, co-habitation of weak larvae with physical damages and the proliferation of virulent bacteria in the improperly managed larval rearing system paved the spread of disease among the healthy larval population. We speculate that the association of virulent motile aeromonads and *E. tarda* together with excess feeding, accumulation of debris, feed remnants, faeces and other wastes in the tank, adoption of inappropriate management measures and some unknown biotic or abiotic stressors might have triggered the mass mortalities. It is noteworthy that several earlier studies reported an association of motile aeromonads and *E. tarda* in disease conditions with peritonitis. For example, aeromonads have been implicated as the causative agent of clinical infections in humans, including septicaemia and peritonitis (Janda and Abbott 2010). Equally, *E. tarda* was also associated with extraintestinal infection and peritonitis in humans (Hirai *et al.* 2015). Further, *E. tarda* was isolated from the peritoneal exudates of a northern sea lion *Eumetopias jubata* with peritonitis (Coles *et al.* 1978). The accumulation of sanguineous fluid in the abdomen of tilapia hybrids, which is accompanied by edwardsiellosis, peritonitis and pancreatitis was also documented (Garcia *et al.* 2012). In fish, peritonitis is a characteristic feature of infections by *Mycobacterium neoaurum* and *Vagococcus salmoninarum* (Austin and Austin 2012). *Pseudomonas putida* and other *Pseudomonas* spp. are often isolated from internal organs of diseased fish or haemorrhagic ascites (Austin and Austin 2012); however, these species are mostly accompanying bacterial flora (El-Barbary and Hal 2017; Pękala-Safińska 2018).

The findings of the present study supported the earlier observations (Paul *et al.* 2015), which documented a systemic infection with the loss of typical tubular epithelial lining, epithelial inflammation, necrosis, cellular and nuclear hypertrophy, pycnotic nuclei, karyolysis and hypoplastic haematopoietic tissue due to *Aeromonas* invasion in the kidney of *C. batrachus* larva. In contrast, cloudy swelling, hydropic degeneration, necrosis in the renal tubules and degenerative changes in the glomerular epithelium and inflammatory cells of the kidney were noted in *A. hydrophila* infected *C. gariepinus* (Laith and Najiah 2013). The inflammatory responses observed in the kidney of *C. gariepinus* larvae were suggestive of suppurative infection as *E. tarda* infection in some fish was suppurative (Miyazaki and Kaige 1985). The spleen of diseased catfish had dense melanomacrophage aggregates within the parenchyma, which usually contain a variety of pigments, including melanins. These aggregates increase in the presence of cachectic disease (Agius and Roberts 2003). Likewise, the presence of melanomacrophage aggregates in *P. hypophthalmus* (Faruk *et al.* 2012) and estuarine catfish Arius maculates (Alagappan *et al.* 2009) was documented during *A. hydrophila* infection.

The histopathological alterations quite similar to bacterial infections with degenerative as well as inflammatory changes in the vital internal organs, and the isolation of *A. veronii*, *E. tarda* and *P. putida* in the haemorrhagic exudates evinced systemic multiple bacterial infections in the observed mass mortalities of excessively fed *C. gariepinus* larvae in a poorly managed hatchery. Better management measures such as improved water circulation, aeration, optimal feeding, removal of wastes, prudent use of antibiotics and proper drying of the larval rearing units after each operation would help reduce the incidence and spread of such infectious diseases in catfish hatcheries.

**ETHICAL STATEMENT**

All of the methods, animal care, and experimental protocols used in this study followed the relevant guidelines and regulations of the Government of India.

**ACKNOWLEDGEMENTS**

This research was supported by the Indian Council of Agricultural Research, New Delhi under the Niche Area of Excellence program vide Grant F. 10(12)/ 2012-EPD dated 23.03.2012. The authors thank the Vice-Chancellor, West Bengal University of Animal and Fishery Sciences, Kolkata

for providing the necessary infrastructure facilities to carry out the work.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

**AUTHORS' CONTRIBUTION**

**TJA** designed the experiment, supervised the research work, interpreted the experimental data, drafted and finalized the manuscript. **HA** and **SB** collected the field samples and executed the parasitological, bacteriological, molecular biology, challenge and histopathological examinations.

**DATA AVAILABILITY STATEMENT**

Sharing of data does not apply to this article.

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