Occurrence of Vibriosis in Asian sea bass (*Lates calcarifer*) cultured in open sea cages

Rajaprabhu G.[1,2]*, Kirubagaran R.[1]*, Rasheeda M.K.[1], Sendhil Kumar R.[3] and Dharani G.[1]
1. Ocean Science and Technology for Islands, National Institute of Ocean Technology, Ministry of Earth Sciences, (Govt. of India), Pallikaranai, Chennai – 600100, INDIA
2. Sathyabama Institute of Science and Technology, Jeppiaar Nagar, Rajiv Gandhi Salai, Chennai – 600119, INDIA
3. Centre for Marine Living Resources and Ecology, Ministry of Earth Sciences, (Govt. of India), Puthuvype, Kochi – 682508, INDIA
*rajaprabhu@niot.res.in; kiruba@niot.res.in

Abstract
This study reports occurrence of mass mortality of Asian sea bass (Lates calcarifer) during the early stage of grow-out culture in open sea cages at Olaikuda, Rameswaram. Gross signs during the mass mortality included anorexia, stunted growth, dark pigmentation, uni/bilateral exopthalmia, distended abdomen and skin lesions with deep ulceration. Bacterial abundance in different body parts (gills, liver, spleen, muscle and kidney) of asian seabass from rearing cages confirmed an outbreak of vibriosis.

The biochemical characterization and 16s rRNA sequencing of dominant isolates confirmed that the causative agent of vibriosis was Vibrio harveyi. Diffuse haemorrhage with reduced white pulp and massive deposition of haemosiderin in the melano-macrophage centres in spleen, shrunken degenerated glomerulus and tubular necrosis in kidney and hydropic changes in liver were confirmed by histopathological examination. Water quality parameters showed significant changes during the study. The results of this study revealed that V.harveyi is the causative agent of vibriosis in L. calcarifer reared in open sea cages.

Keywords: Asian seabass, sea cage culture, mass mortality, *Vibrio harveyi*, Vibriosis.

Introduction

Asian seabass (*Lates calcarifer*) is one of the valuable cultured marine finfishes in South Asia and Australia[14]. It is also one among the three important marine fish species in India for which commercial seed production is standardized and initiated cage culture is initiated[1,10, 24]. National Institute of Ocean Technology, Chennai, initiated culture of Asian sea bass in open sea cages in three coastal states in 2011. Intensive culture has led to multiple disease outbreaks imposing a significant constraint for productive farming of seabass. Major diseases reported in Asian seabass in hatchery, land-based farm and sea-cage farming include viral nervous necrosis, iridovirus, pot belly, vibriosis, streptococcosis and nocardiosis leading to mortality ranging from 50-100%[9,12,16,19,27].

Parasite infestation has also caused significant loss in seabass during the culture period[6, 34]. Most of the disease outbreak reported from Indian waters was during the initial larval development in the hatcheries or in land-based rearing facilities[3,27,35] but very few reports exist on disease outbreak in seabass farmed in a grow-out facility[15, 34]. *Vibrio alginolyticus* infection was reported in Asian sea bass reared in open sea cage floating cages[28]. In the current study the water quality parameter during the disease outbreak and the causative agent of vibriosis outbreak was identified from disease *L. calcarifer* cultured in open sea floating cages.

Material and Methods

Sea bass culture conditions: Seabass juveniles (25000 nos., 2.5-3.5 g) procured from the Rajiv Gandhi Centre for Aquaculture (RGCA), Seabass-hatchery, Sirkhazi, Tamil Nadu, India, were reared in 2 m Ø HDPE nursery cages for 45 days at a density of 800 fishes/m^3 and later these fishes (7-8 g) were transferred to two 9 m Ø HDPE floating cages at a density of 25 fishes/m^3. During the nursery rearing and grow out phase, the juveniles were fed 6 % and 3% of their body weight twice a day with slow-sinking feed (4 mm-13 mm) procured from Lucky Star feed (Taiwan Hung Kuo Industrial Co. Ltd., Su-Ao, I-Lan, Taiwan).

Fish sampling: An epizootic was recorded in farmed sea bass with daily mortality ranging from 2 to 3.2%. Twenty five affected or moribund specimens (125-180 g) were collected from each cage every week, euthanized with an overdose of MS-222 (65 mg/L, Sigma Chemical Co., St Louis, MO, USA) and dissected under sterile conditions at the laboratory to collect samples for bacteriological and histological analyses. Skin surface, scales and gills were examined for the presence of parasites under a stereo zoom dissection microscope (Nikon SMZ 1500, Tokyo, Japan). Wet mounts of mucus, lesion and portions of gill filaments were also prepared and examined for any parasite infestation. Apparently healthy fishes were also taken for analysis as control.

Water quality parameters: Water quality parameters such as temperature, salinity, dissolved oxygen (DO) and pH were recorded daily throughout the culture period using water quality monitoring devices: Thermometer, Eutech DO 6 plus, Eutech salt 6 plus, Eutech pH 5 (Vernon hills, Illinois, USA). Seawater nutrient analysis from the cage was also carried out following APHA guidelines (2012). The water parameters recorded during disease outbreak showed moderate fluctuation. The nitrite and ammonia levels varied between 0 and 0.02 ppm and 0.0 and 0.35 ppm respectively.

Histological analysis: Gill, liver, spleen, muscle and kidney samples were collected from moribund fish and fixed in neutral buffered 10% formalin solution, sectioned at 5 µm using Leica microtome RM2125 RTS (Nussloch, GmbH, Germany). The tissues were processed, stained with haematoxylin and eosin for histological analysis. Stained sections were examined at magnifications of 20-100X using a Carl Zeiss Axiophotoshop 2 microscope (Welwyn, Garden City, UK).

Bacteriological analysis: Muscle, liver, kidney, intestine and gills were aseptically removed from euthanised fish showing gross lesions or clinical symptoms. The tissues were homogenized in 100 mM sterile phosphate buffer saline, serially diluted and plated on general and selective media such as tryptic soya agar supplemented with 1.5% NaCl, zobell marine agar, thiosulphate–citrate-bile salt-sucrose agar, pseudomonas agar base and aeromonas agar base supplemented with ampicillin (Hi Media, Mumbai, India). The inoculated plates were incubated at 28ºC for 48 h. Colonies morphology was used for initial grouping of isolates for further identification and characterization.

In each plates, the colonies with similar appearance on each media type were considered as unique morphotype. At least 3 colonies of each morphotype from each plate were preserved in 15–20% (v/v) glycerol at −70°C. The isolates were sub-cultured to obtain pure cultures. Confirmation of each strain was achieved using the analytical profile index of Hi Media biochemical test strips (KB002, KB004 and KB007).

Molecular characterization: The final confirmation of the dominant strain was performed by 16S rRNA gene amplification. To achieve this, total genomic DNA was extracted from pure cultures using high salt extraction method. Subsequently 16S rRNA gene amplification was performed on an ABI 7500 thermal cycler programmed with following cycle: 95ºC -2 min, 35 cycles of 95ºC -1 min, 55ºC-1 min, 72ºC-2 min and finally 72ºC-7 min using universal primers 27F (5-AGA GTT TGA TCC TGG CTC AG-3) and 1492R (5- GGT TAC CTT GTT ACG ACT T-3) using Fermentas 2X Taq mastermix (Waltham, Massachusetts, USA).

After gel electrophoresis ~1500 bp bands were cut and eluted using Qiagen gel purification Kit according to manufacturer's instruction and squenced (Eurofins, Bangalore, India). DNA sequences obtained after amplification were compared to the RDP-II and GenBank databases using BLAST (http://www.ncbi.nih.gov/BLAST). Sequences displaying equal or more than 99% identity with their closest relative were considered to be from the same species.

Antibiotic susceptibility test: Sensitivity of bacteria against antimicrobial agents was determined by disc diffusion method on Muller-Hinton agar plate as per CLSI guidelines. Dosages of antimicrobial agents procured from Hi Media (Mumbai, India) were as follows: ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), furazolidone (100 µg), kanamycin (30 µg), norfloxcin (30 µg), oxytetracycline (30 µg), penicillin G (10 units), streptomycin (10 µg), vancomycin (30 µg), ciproflaxcin (5 µg), sulfadiazine (250 µg) and tetracycline (30 µg).

Results

Clinical observations: The 4 fishes (90 ± 6g mean weight: 16 ± 2 cm mean length) reared in sea cage exhibited gross clinical signs such as sluggish movement, scaling superficial/ deep skin ulceration; abdominal distension, tail and fin rot with exophthalmia, stunted growth, pigmentation and liver with numerous yellow lesions (Fig.1).

Mass mortalities were recorded over a period of 4 weeks with a peak occurring in the first two weeks. Postmortem examinations were conducted on moribund and healthy fishes. Macroscopic examination of gills and body surface revealed no sign of cyst or external parasites. The outbreak caused daily mortality of 2.0 - 3.2% and considerable economic losses.

Water Quality parameters: Temperature of the sea water during the culture period was 30 ± 0.5° C and during the period of infection, the temperature was 32 ± 0.06 ° C. Amongst the other factors, salinity and pH showed no significant changes during the cultivation period. The pH ranged from 6.98 to 8.7, elevated pH was found at 33 day of culture before the onset of mass mortality. Then after an average of 7.64 pH was measured throughout the experiment.

There was significant different in the DO value during the culture period. Total DO range was between 6.12 to 7.01mg/l and low DO level of 4.5 mg / l was recorded at 31st day. It was observed that the change of the pH and DO elucidates high level relationship between these factors. The water temperature and salinity were within optimal ranges for culturing fishes. Results of physico - chemical parameters of water sample from the cage culture site are present in fig. 3.

Histopathology: Several gross pathological changes were observed after carrying out histopathological studies on spleen, liver, gills kidney and muscle tissue of moribund fishes (Fig. 2). The worst affected internal tissues were the liver, spleen muscle and kidney. Spleen showed melanomacrophage centres with lytic areas and signs of congestion with reduced white pulp (Fig. 2a, 2b, 2c). Liver sections displayed multifocal hepatocellular necrosis with degenerative vacuoles (Fig. 2d, 2e, 2f). Fusions of secondary lamellae with hyperplasia and hypertrophy of epithelial cells were observed in the gills (2g, 2h and 2i). Kidney sections displayed shrunken glomerulus with diffuse necrosis and vacuolation of renal tissue (2j, 2k, 2l). Muscle tissue showed degenerated muscle fibers (2m, 2n, 2o).

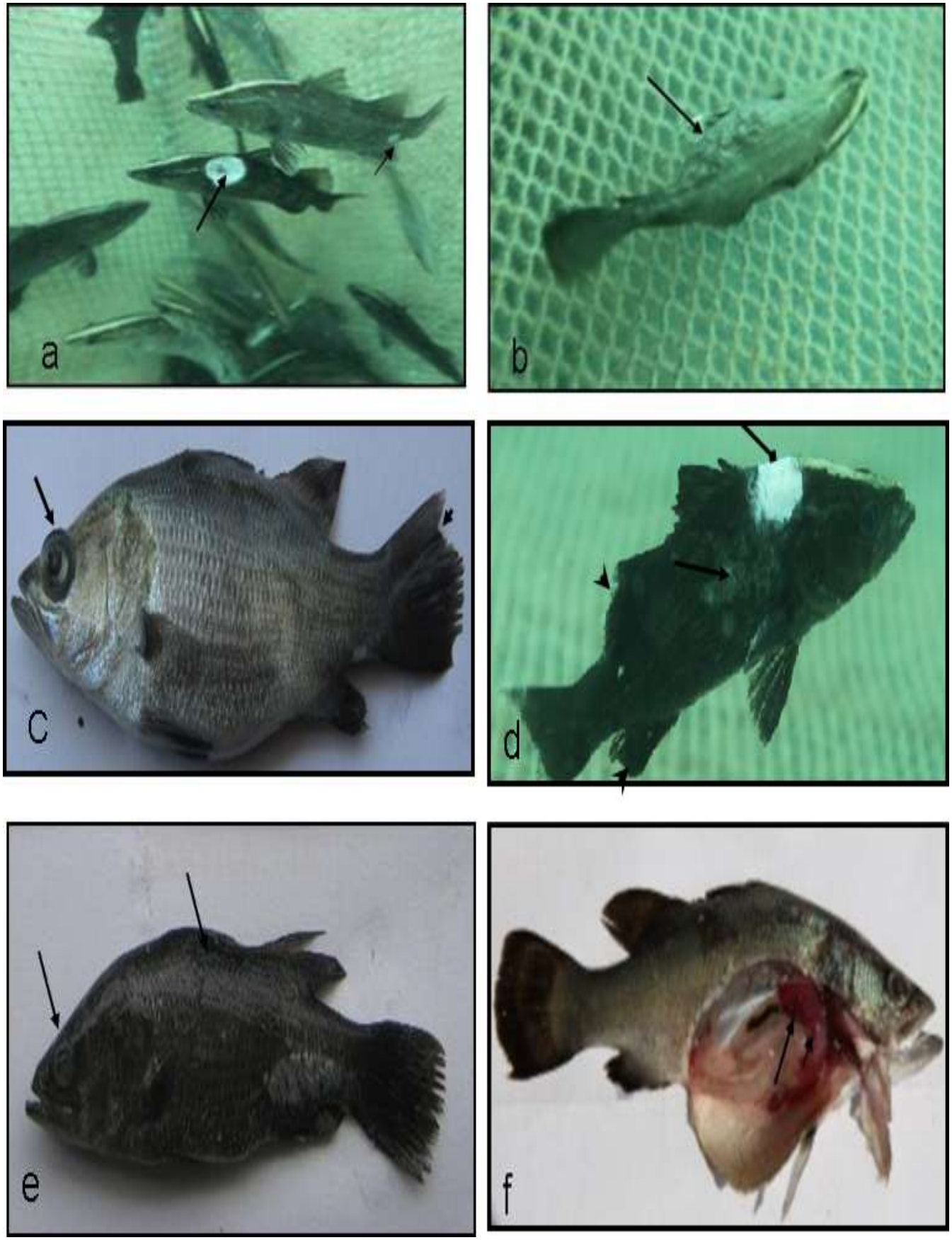
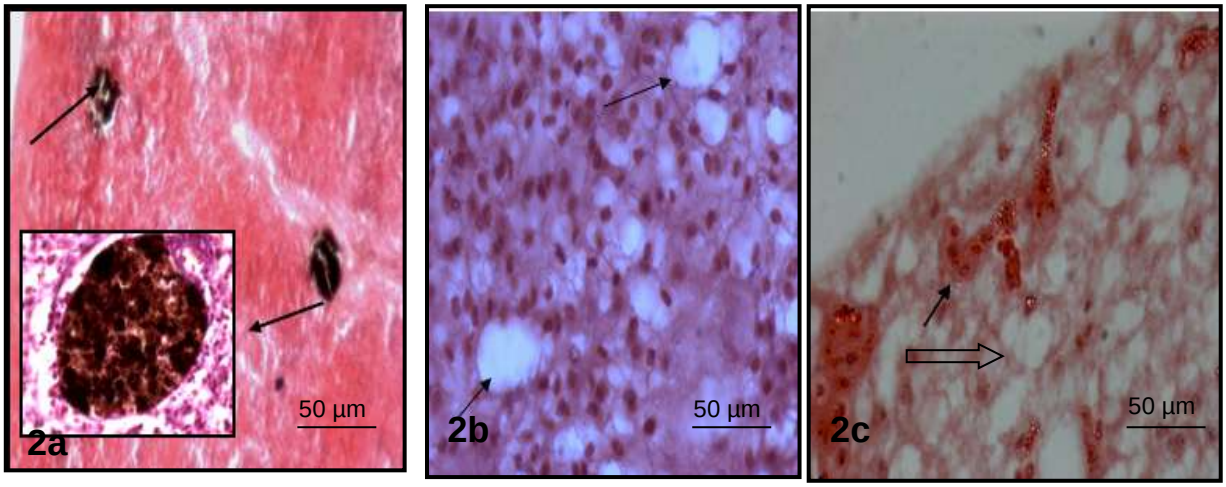
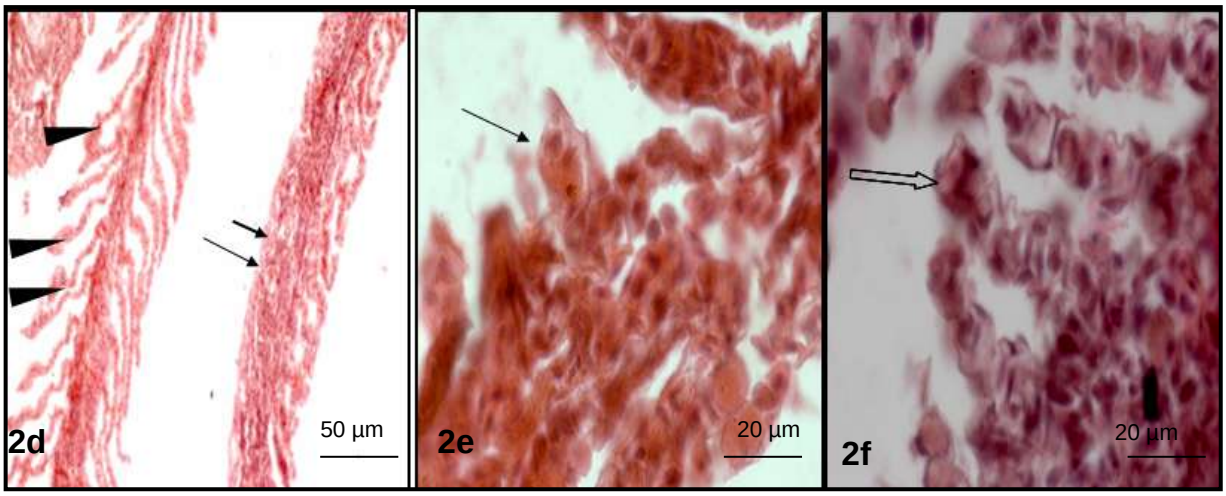


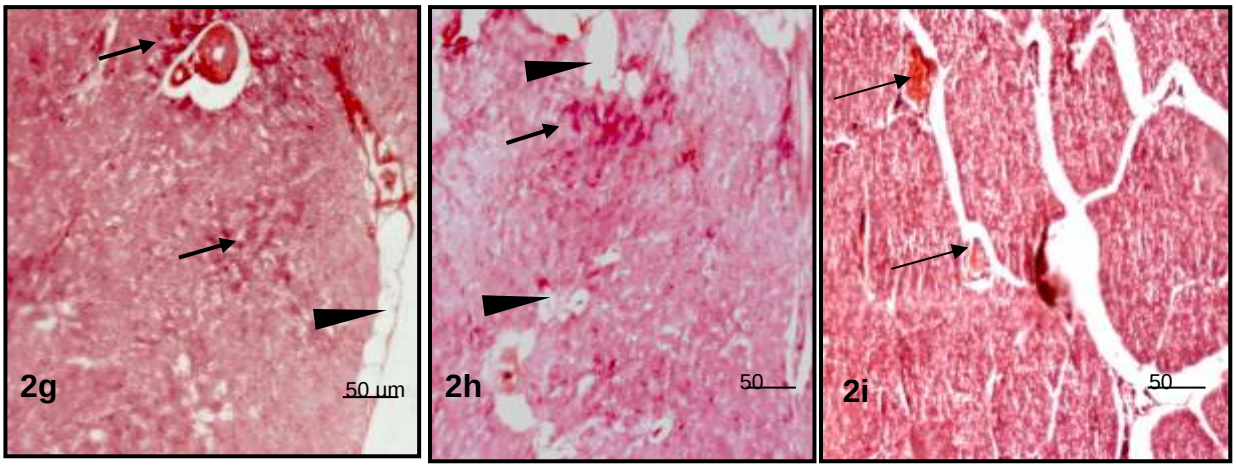
Fig. 1: Main external symptoms and pathological signs observed in affected *Lates calcarifer* reared in sea cages: arrows show the clinical symptoms (a) deep skin ulceration (arrow) (b) abdominal distension (arrow) (c) tail rot (arrow head) with exophthalmia (arrow) (d) scaling (arrow), superficial ulceration (diamond arrow) with fin rot (arrow head) (e) stunted growth and deep pigmentation (arrow) and (f) liver with numerous yellow lesions (arrow)



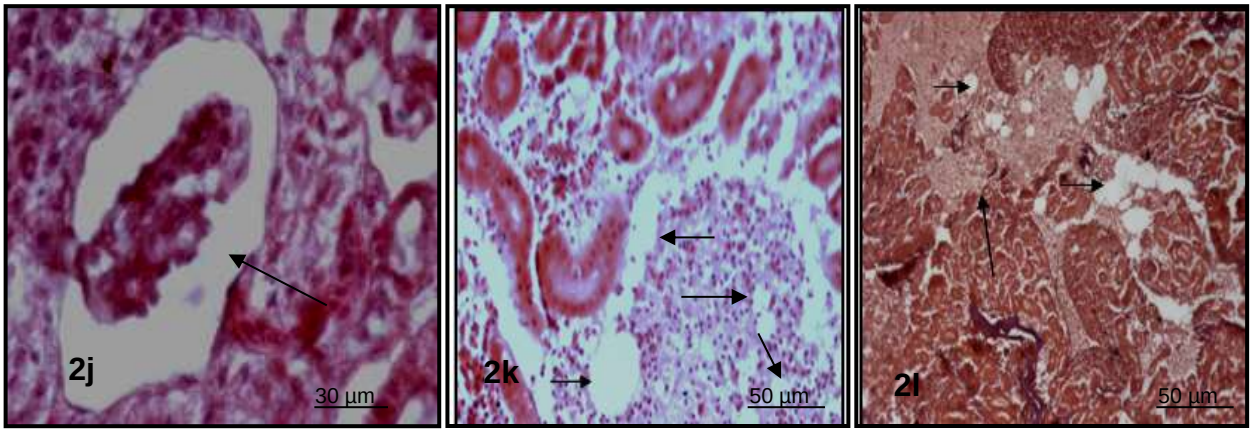
Spleen histopathology: (2a) spleen with melanomacrophage centre (arrows), inlet shows massive deposition of haemosiderin in melanomacrophage centres, (2b) lytic area (arrows) (2C) sections displays reduced white pulp (block arrow) and congestion (arrow)



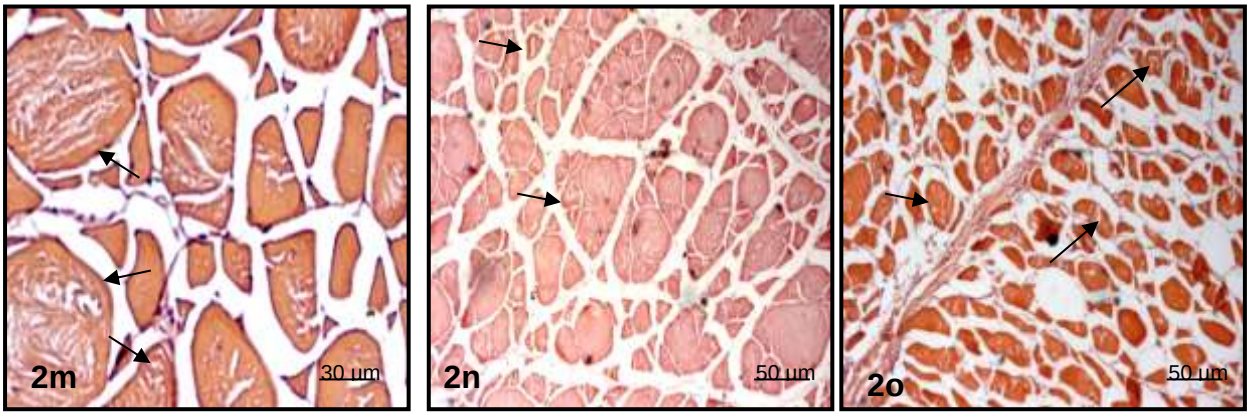
Gill histopathology: (2d) Clubbed secondary lamella tip (arrow heads), (2d, 2e) fused secondary lamella (arrows) and (2f) epithelial cells hypertrophy (block arrows)



Liver histopathology: (2g, 2h) Multifocal hepatocellular necrosis with hydropic changes (arrow head) (2i) lobulated liver with perivasculitis



Kidney histopathology: (2j) shrunken glomerulus (arrow) (2k, 2l) kidney vacuolated with disperse necrotic tissue



Muscle histopathology: (2m, 2n, 2o) degenerated muscle fibres shown by arrows

Fig. 2: Histopathology of moribund seabass (*Lates calcarifer*) affected with disease in sea-cages

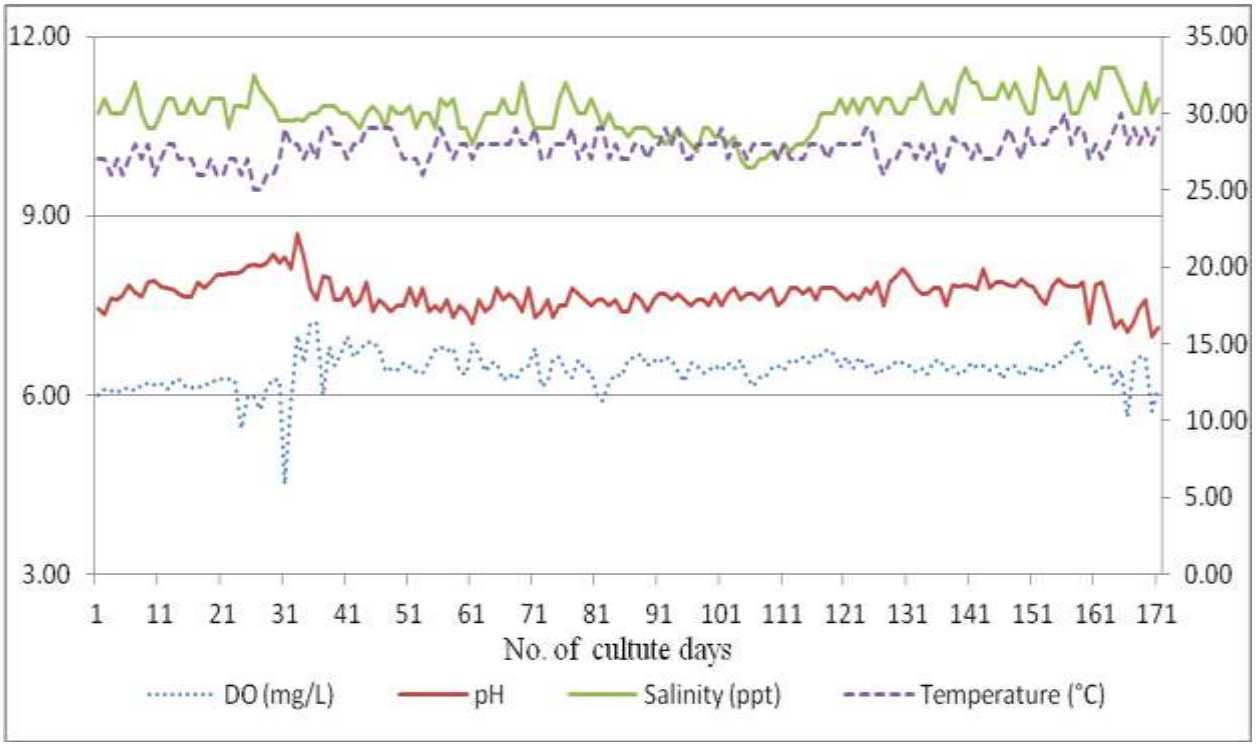


Fig. 3: Sea water quality parameters during the culture period

Isolation of pathogen and bacterial pattern: Culture reports from deep dermal lesion and visceral organs of infected fishes showed high prevalence of gram negative bacteria. Biochemical characteristics of the isolated bacteria showed positive results for oxidase, catalase, urease, methyl-red, lysine decarboxylase, indole production, nitrate reductase and haemolytic test, however it was negative for Voges-Proskauer, citrate utilization, arginine dihydrolase and phenylalanine deamination tests. The isolate was able to produce acid aerobically and anaerobically from fructose, cellobiose, mannose, dextrose, sucrose, maltose, mannitol and galactose. In contrast, no acid was produced aerobically or anaerobically from lactose, raffinose, rhamnose, arabinose or xylitol. Interestingly, no dominant communities bacteria could be isolated from internal organs of healthy fishes.

Nucleotide sequence analysis: To identify the dominant strains (n=10) having similar phenotypic and biochemical properties, 16S rRNA analysis was carried out using primers 27F and 1492R and later sequenced. The results obtained were compared with published sequences in the GenBank database by using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast). The closest match observed was obtained with *Vibrio harveyi* GIT-2-Talpur - 10 (GenBank accession no. JX861207.1) isolated from intestine. The partial sequence of 16S rDNA was deposited in the GenBank with accession numbers – KF607055).

Antibiotic susceptibility test: *In vitro* studies on the susceptibility of *V. harveyi* to various antibiotic agents indicated that it was susceptibile to tetracyclines, chloramphenicol, sulfonamides and levofloxcin and resistant to aminoglycosides and β-lactam family drugs. Since oxytetracycleine was effective and is an approved drug for farmed fishes, it was chosen to treat the diseased fish through immersion. Immersion treatment was given for 1 h daily for 5 consecutive days and proved effective with clear sign of wound healing and scars on the skin.

Discussion

The virulent strain was identified as *V. harveyi* by 16s rRNA gene sequencing and biochemical analysis. Vibrios, Aeromonads, Pseudomonads, Photobacteria, Streptococci and Staphylococci are the reported bacterial pathogens for disease outbreak in fingerlings, juveniles, adults and brood stocks of most marine fish species[23,25,26,33]. In the present study, the culture results demonstrated that 84 fishes were found to be infected with gram negative bacteria and only 16 fishes were infected with gram positive bacteria out of 100 fish samples tested. Comprehensive epidemiological information on vibriosis and associated risk factors are found to the limited in Asian countries[21].

The isolation of *V. harveyi* from diseased *L.calcarifer* in the present study as bacterial pathogen associated with deep skin lesions, hemorrhagia of fin base, tail and fin rot and exopthalmia was in concurrence with a numerous reports from marine fishes that include silvery black porgy, groupers, red drum, cultured flounder, cobia and Asian seabass[8,17,18,30-32].

Further histopathology results obtained from liver and spleen of moribund fish showed signs of congestion, hemorrhage with multifocal necrosis and distended gut filled with clear fluid confirming vibriosis outbreak. Researches have proved that fishes are prone to vibriosis when they are in stressed conditions. The variations in DO and pH may be influential factors which enhanced the susceptibility of *L. calcarifer* to vibriosis[29]. In sea cage forming, fishes were exposed to seasonal variation of water temperature, DO and pH. Moreover, a minor change in water temperature influenced the growth of the fishes[13] and may also lower DO level in water[5]. Similarly significant pathological changes were observed in the internal organs vibriosis infected fishes[7, 20, 28].

The DO level is generally influenced by environment parameters, microbial metabolism and fish respiration. Our result showed normal DO level before the mass mortality. These findings contradict the finding of early studies showing impact of DO on cobia culture in submerged cages[4]. The water pH varied from 7.35 to 8.7 during mass mortality. Microscopical investigation of infected fishes showed significant changes in liver and spleen. Numerous granulomas were observed through the spleen, liver and kidney[15].

Results from the antibiotic susceptibility tests showed *V. harveyi* to be resistant to ampicillin, penicillin G, erythromycin, novobiocin and kanamycin. However, they were sensitive to chloramphenicol, ciprofloxacin, furazolidone and oxytetracycline. Since treatment with chloramphenicol, nitrofurans and fluoroquinolones is not approved for use in aquaculture; the infected fish were treated with oxytetracycline by immersion. Immersion treatment proved effective with healings of skin wounds. Similarly, treatments with oxytetracycline in Salmonids and furazolidone in turbot also helped in curtailing the disease[2,11].

The above study based on the results of gram staining, morphological observation, physiological and biochemical test, histopathological examination along with 16S rDNA confirms *Vibrio harveyi* as the etiological agent for disease outbreak in cage cultured *L. calcarifer*.

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