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ORIGINAL ARTICLE

Isolation and characterization of pathogenic *Vibrio alginolyticus* from sea cage cultured cobia (*Rachycentron canadum* (Linnaeus 1766)) in India

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Significance and Impact of the Study: The epizootics of vibriosis caused serious economic losses to farmers. Natural blooms of the pathogen can be prevented by sea cage management measures such as, changing the inner net of the cages, changing the location of the cages to relatively clean water (about 50 m apart) from the affected site and providing shade over the cages while the water temperature rises. Supplementation of the feed with immunostimulants and mineral mixture may be practised to improve the immune response against infection. Early diagnosis and sea cage management measures may prevent occurrences of the infection.

Keywords
cage culture, cobia, haemorrhage, sequence, *Vibrio alginolyticus*.

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Abstract

Mass mortalities of cobia, *Rachycentron canadum*, sub-adults occurred during August 2013 in cage culture in the Gulf of Mannar, Mandapam Tamil Nadu, India. The epizootic of disease was started with typical classical clinical signs followed by acute mortality. Grossly, severe haemorrhage and congestion were observed in the gastric mucosa. The abdomen was distended with peritoneal fluid. The heart revealed haemopericardium and fibrinous pericardium. Histologically, the gastric mucosa showed severe erosion and necrosis. Haemorrhagic pericarditis and an increased size of the melano macrophage centre (MMC) in the tail kidney were other histopathological changes. *Vibrio* sp. was isolated from the gastric lesions and heart blood swab of moribund fishes and it was found to be virulent to the cobia fingerlings. After the challenge, the same bacterium could be re-isolated from moribund fingerlings. The 16S ribosomal RNA of the isolate was amplified and BLAST analysis of the sequence confirmed that the pathogen was *Vibrio alginolyticus*. The confirmation was also correlated with its cultural, biochemical and pathomorphological changes. This is the second report and the first incidence of epizootics with severe pathological lesions in cultured cobia in India. The study throws light on the pathology of vibriosis. By practising cage farm management measures, occurrences of infection may be prevented.

Introduction

The cobia, *Rachycentron canadum*, is distributed worldwide in tropical and subtropical water. Cobia culture offers great possibilities in aquaculture because of its fast growth rate and commercial interest (Su *et al.* 2000).

Carli *et al.* (1993) reported that cobia farming could be an emerging aquaculture industry in India. Gopakumar *et al.* (2011) initiated the brood-stock development and achieved the first successful induced breeding, in March 2010 in India. Presumably cobia farming could become an emerging aquaculture industry in India in the near future. However, the industry faces various threats including viral, bacterial and parasitic diseases (Rajan *et al.* 2001; Lopez *et al.* 2002) in different regions where cobia aquaculture has been established. Disease caused by bacteria was an important limiting factor for the viability of fish farms (Colorni *et al.* 1981) Vibriosis, a disease caused

by numerous species of vibrio, was a primary disease of marine fish in salt and brackish waters and creates huge economic losses in the mariculture industry, affecting large numbers of fish and shellfish species, both cultured and feral (Roberts 1989). Disease outbreaks often occur when the water temperature increases in late summer in shallow and near shore waters. *Vibrio alginolyticus* was considered to be a part of the normal marine flora (Carli *et al.* 1993). However, *V. alginolyticus* has been suggested to be a pathogen of several marine fishes such as silver sea bream (Al-Sunaiher *et al.* 2010), cobia (Rajan *et al.* 2001; Rameshkumar *et al.* 2014), grouper (Lee 1995) and Asian seabass (Sharma *et al.* 2013).

Fish cultured in floating cages were particularly susceptible to disease when various environmental parameters such as temperature, salinity, dissolved oxygen and suspended particles fluctuated suddenly or widely, or following rough handling operations. Among the bacterial diseases, vibriosis caused severe outbreaks and serious setback to the mariculture industry (Su *et al.* 2000). Several species of vibrio from moribund farmed cobia including *V. alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* accounted for ≤45% mortalities in cage stocked juvenile cobia (Lopez *et al.* 2002). *Vibrio alginolyticus* has been commonly associated with epidemic vibriosis which leads to mass mortality in the culture of large yellow croaker resulting in considerable losses (Yan *et al.* 2007). However, it has also been suggested that this species is a pathogen of several marine animals and humans (Blake *et al.* 1980; Lee 1995; Rikelme *et al.* 1996).

Rajan *et al.* (2001) recorded a natural outbreak of vibriosis in Taiwan and re-isolated the *V. alginolyticus* infection from juvenile cobia. Further, the authors conducted experimental infection studies and found haemorrhage, ulcers on the skin, dark skin and fluid accumulation in the peritoneal cavity. Most fish died within 7 days in both cases.

High mortalities in cultured fish were observed with 100% morbidity. However, mass mortalities in cage culture of cobia occurred at the stage when the juveniles were transferred to the floating marine cages (Leong and Colorni 2002). The cobia from all stages of the production cycle might succumb to vibriosis, and the clinical signs were inappetance, exophthalmia and pale liver (McLean *et al.* 2008).

However, in the present report, an outbreak with severe inflammatory changes was recorded in cobia sub-adults. *Vibrio* species was isolated and characterized in the sub-adults of cobia weighing 2·5–4·7 kg reared in a floating cage at Mandapam, Tamil Nadu. This is the second report and the first incidence of epizootics with severe pathological lesions in cage culture of cobia in India.

Results and discussion

The severe outbreak of vibriosis in cobia sub-adults cultured in a marine floating cage was recorded in Mandapam, Tamil Nadu, India. During the August 2013, a total of 99 numbers of cobia sub-adults died. The affected fish showed the clinical signs of surfacing, off feed, anorexia, photophobia, corneal opacity, aimless or erratic swimming behaviour with frequent hitting the cage net and finally acute mortality. The total cumulative mortality in all the cages was approx. 33%. The morbidity rate was 67%. The affected fishes slowly recovered and no mortality was observed after 8 days.

Bacterial isolation and characterization

From the naturally infected and moribund fish, Gram negative, rod shaped bacteria were isolated from haemorrhagic ulcers in the stomach and in the heart blood. Greyish white, raised colonies were observed on tryptone soya agar (TSA) with 2% NaCl, whereas isolates produced yellow colonies on TCBS agar after 24 h. The colonies were showed an absence of luminescence. Based on the biochemical tests the infective bacterium was tentatively identified as *V. alginolyticus*. Koch's postulate was confirmed by the experimental infection and re-isolation from the moribund fish. Further, no bacteria could be isolated from the kidney and heart blood of the control group fishes. The LD_{50} value of *V. alginolyticus* isolate for cobia fingerlings was $10^{4·7}$ CFU per fish.

Biochemical characterization of the bacterial isolates from cobia grow out are listed in Table 1. In the antibiotic sensitivity test, the isolate was sensitive to gentamicin, ciprofloxacin, nitrofurantoin, gatifloxacin and cefotaxime. It was resistant to tetracycline, colistin, ampicillin, ceftriaxone, ceftazidime and streptomycin.

Classical clinical signs

The clinical signs started with off feed, surfacing, development of corneal opacity, abnormal swimming behaviour, hitting the cage net and swimming in a circular movement. Due to the erratic swimming behaviour lacerations was noticed over the skin from hitting the cage net or barnacles. The white corneal opacity later changed to a haemorrhagic type and showed exophthalmoses with severe congestion.

Gross and histopathology

Grossly, the eye revealed keratitis and corneal opacity and after 24–48 h it became severely congested with hemorrhagic exophthalmoses. Redness or the erythematous

Table 1 Morphological and biochemical characterization of the bacterial isolates from Cobia sub-adults

Test	*Vibrio alginolyticus* (CGLP/13)	*Vibrio alginolyticus* Cobia (Liu *et al.* 2004)
Gram's stain	−	−
Motility	+	+
Swarming on TSA	+	+
Growth on TCBS	Y	Y
Growth in 0% NaCl	−	−
Growth in 3% NaCl	+	+
Growth in 6% NaCl	+	+
TSA1%	+	+
TSA8%	+	+
TSA10%	−	−
Oxidase	+	+
Catalase	+	+
Nitrate reductase	+	+
Production of H_2S	−	−
Urease	+	+
Indole	+	+
VP test	−	−
Utilization of		
Citrate	+	+
Glucose	+	+
Mannitol	+	+
Inositol	−	−
Sorbitol	−	−
Sucrose	+	+
Melibiose	−	−
Arabinose	−	−

TCBS, thiosulphate citrate bile salt sucrose agar; TSA, tryptone soya agar; +ve, positive reaction; −ve, negative strain; Y, yellow colour colonies.

patches were observed in the lower abdomen, tail and the lower mandibular region. The gills were pale with profuse mucous secretions. The abdomen was distended with 250–300 ml of clear transparent white to serosanguinous coloured gelatinous peritoneal fluid. The stomach showed severe haemorrhages and congestion in the entire gastric mucosal folds and it was empty. The heart revealed a severe haemorrhagic and sticky chalky white deposition with fibrinous adhesion was observed between the pericardium and myocardium (Fig. 1). Almost all the dead fish hearts revealed severe congestion and haemorrhage. No external or internal parasites were found in the gill, skin, abdominal cavity and intestine, respectively.

Histopathology of different organs of naturally and experimentally infected cobia was carried out. The gastric mucosa revealed haemorrhagic gastritis, engorged capillary sinuses and loss of tubular secretory glands. There was severe haemorrhage and necrosis of the tubular glands in the lamina propria (Fig. 2). The tail kidney revealed acute glomerulonephritis. The proximal convoluted tubules revealed degeneration and loss of brush

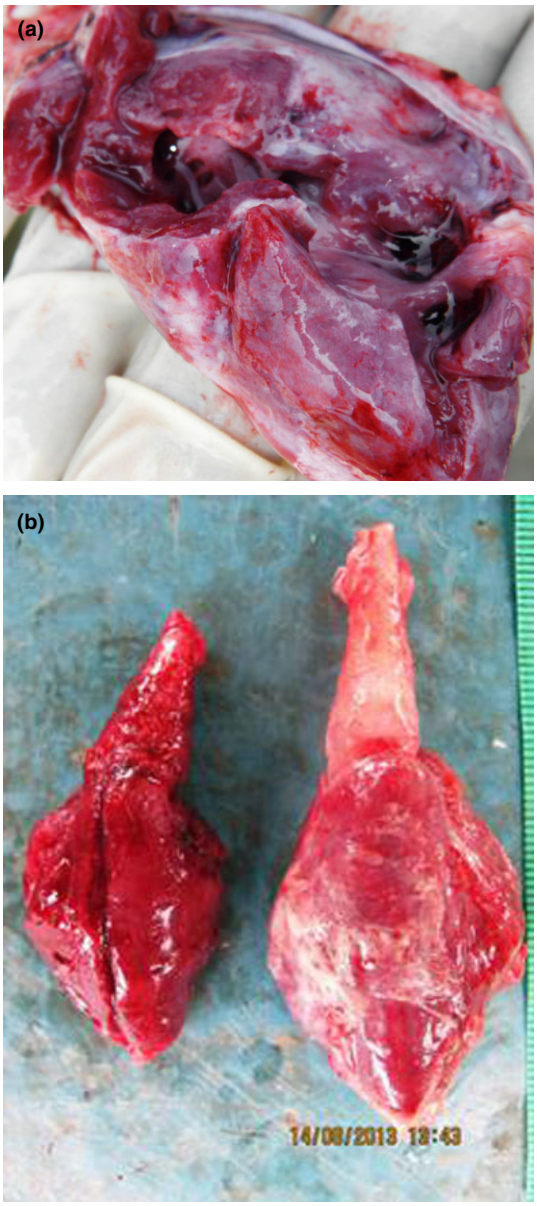

Figure 1 Heart pericardium. (a) Normal. (b) Severe haemorrhagic and sticky chalky white deposition with fibrinous adhesion. [Colour figure can be viewed at wileyonlinelibrary.com]

borders. An increased size of melano macrophage centres (MMC) was also observed in the entire parenchyma. The heart showed severe haemorrhagic and fibrinous pericarditis with infiltration of inflammatory cells (Fig. 3). The liver parenchyma revealed fatty degeneration. All the small vacuoles coalesced to form a large fatty vacuole and displaced the nucleus to the periphery. Some of the area showed mild to moderate congestion and hypertrophy of the bile duct.

In the experimentally infected fish, severe congestion and redness was observed at the base of the dorsal fin and eye.

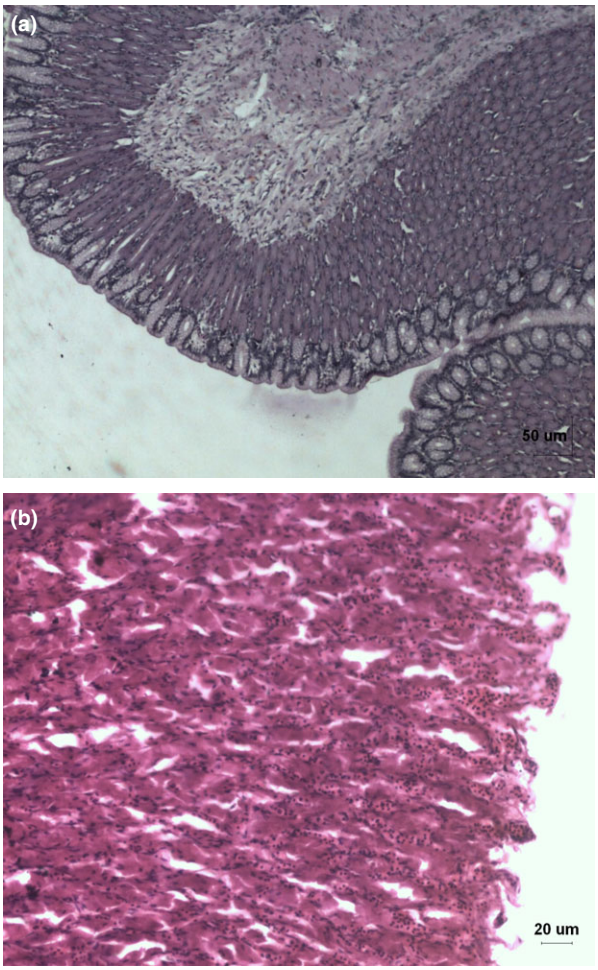

Figure 2 Gastric mucosa. (a) Normal histological structure H&E × Bar = 50μm. (b) Haemorrhagic gastritis, engorged capillary sinuses and loss of tubular secretory glands H&E Bar = 20μm. [Colour figure can be viewed at wileyonlinelibrary.com]

Internal organs showed generalized congestion. No external or internal parasites were observed in the experimental fish. Histopathologically there were no significant changes found except engorged sinuses in the liver and spleen.

Molecular characterization

The bacterial isolate was determined to be that of *Vibrio alginolyticus* using BLAST search on GenBank (http://blast.ncbi.nlm.nih.gov/), our 16S rRNA sequence gave a 100% match to *Vibrio alginolyticus*. The percent identity calculated using MEGALIGN (DNASTAR) showed 100% identity with *V. alginolyticus* strains KC455397 and JN188406, the isolate also showed a high percent identity with 16S rRNA sequence of other *Vibrio* spp, such as *Vibrio parahaemolyticus* – KC210810 (99·9%), *Vibrio campbellii* – JN128268 (99·8%), *Vibrio harveyi* –HQ161740 (99·8), *Vibrio anguillarum* –KC884650 (95·6), *Vibrio ordalii* – KC884626 (95·6%). The phylogenetic tree constructed based on the above 16SrRNA sequences is shown in Fig. 4. Phylogenetic relationships of *Vibrio alginolyticus* – KM985650 (strain Mandapam) with selected *Vibrio* spp. Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. *Photobacterium damselae* was used as an out-group for rooting trees.

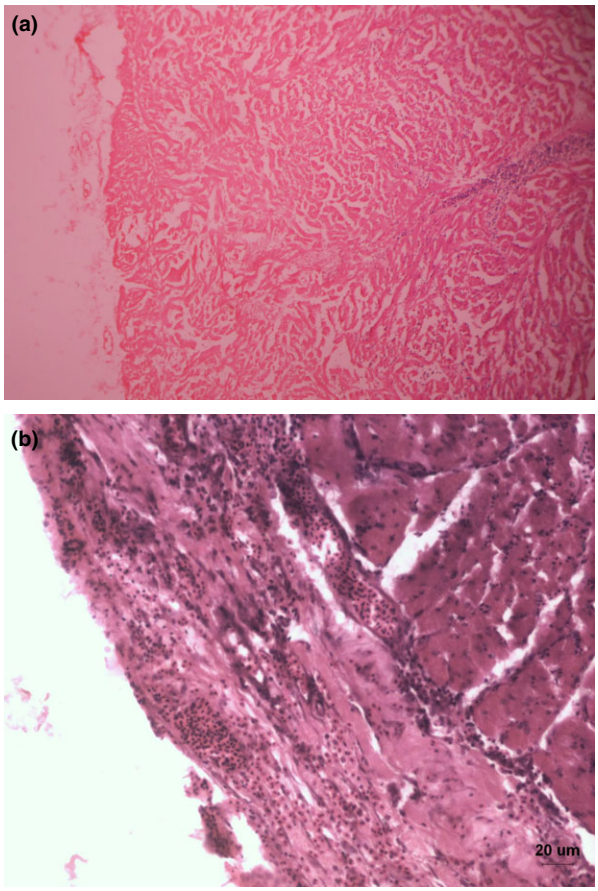

Figure 3 Heart pericardium. (a) Normal histology. H&E × 400×. (b) Severe haemorrhagic with engorged sinuses and infiltration of inflammatory cells. H&E Bar = 20μm. [Colour figure can be viewed at wileyonlinelibrary.com]

All the samples preserved at −20°C were analysed with the VNN kit (Thermo Fisher Scientific, Pune, Maharashtra, India) method and the results were negative for *βetanoda* virus.

Cobia is a newly farmed fish in mariculture, only a few pathological studies have been conducted in that species (Chen *et al.* 2001). The majority of marine fishes cultured in cages are susceptible to vibriosis, with some fish species more sensitive to the infection than others. Vibriosis occurs frequently during periods of fluctuations in salinity, increased organic load, or stress brought about by net changing and grading of fish. Horizontal transmission is the most probable route, with bacteria being shed from

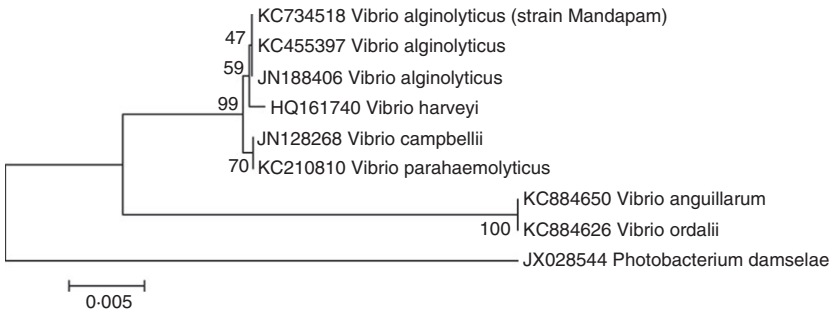

Figure 4 Phylogenetic relationships of *Vibrio alginolyticus* – KM985650 (strain Mandapam) with selected *Vibrio* spp.

open lesions (Leong and Colorni 2002). Liu *et al.* (2004) reported two outbreaks of vibriosis in cobia juveniles during the summer season due to *V. alginolyticus*. The present severe outbreak was recorded during the pre-monsoon season, when the temperature, humidity and salinity were unstable. The species of vibriosis involved in diseases reflect regional differences. In Southeast Asia, *V. parahaemolyticus* was prevalent (Wong and Leong 1990), whereas *V. alginolyticus* was the main species noticed in the present report.

Liu *et al.* (2004) observed that cobia was susceptible to all three groups of pathogens. The authors isolated *V. anginolyticus* in cobia 100–120 g, as well as in cobia 8–12 g, from two different outbreaks in Taiwan and recorded over 80% serious mortality among cultured juvenile cobia. Lin *et al.* (2006) recorded that fish less than 4 months old, <500 g, appeared to be the most susceptible with the highest mortalities to these bacterial pathogens and also observed outbreaks associated with *Vibrio* spp. in early grow-out of cobia, under 4 months of age and below 500 g. In our observation, the outbreak of vibriosis in cobia sub-adults with the average length and weight of 91·4 cm and 13·5 kg, respectively, were recorded and a total of 99 fishes died with a cumulative mortality of 33%.

Rajan *et al.* (2001) recorded skin ulcers due to *V. alginolyticus* infection in natural cases. In the present observations, there were no skin ulcers in the natural outbreak nor in the experimentally infected fishes. The stomach showed severe haemorrhagic gastritis and ulcer in the natural outbreak, and only generalized congestion was observed in the experimental infection. Azad *et al.* (2004) reported that the necrotic changes in hepatic tissues resulted in honeycomb vacuolation in naturally infected seabass juveniles. Our findings were also similar to those of earlier workers.

Al-Sunaiher *et al.* (2010) reported that some fish represented skin darkness and scale detachment, small and large areas of haemorrhage distributed over many parts of the body, particularly at base of the fin, mouth region and abdominal area, which varied in its severity from fish to fish. In India, Sharma *et al.* (2013) first recorded *V. alginolyticus* infection in Asian seabass reared in sea floating cages and the natural infection was characterized by haemorrhage and ulcers on the body surfaces. Rameshkumar *et al.* (2014) recorded a vibriosis outbreak first in cobia fingerlings in floating cages. Our findings or observations also corroborated earlier workers. But we recorded epizootics of vibriosis in sub-adults with severe pathological lesions.

The extracellular products of *V. alginolyticus* were very hydrolytic and these hydrolytic activities have been considered as virulence factors since they allow the bacteria to survive, proliferate and invade the host tissues (Campbell *et al.* 1990; Ellis 1991). Vibriosis produces a wide variety of proteases and extracellular enzymes that were responsible for the extensive tissue damage (Thune *et al.* 1993).

In the present cases, all the fish showed typical septicaemic changes, such as ascites, haemo and fibrinous pericardium, erythematous and petechial haemorrhages in the external surfaces mainly in the lower abdomen and jaw regions. The *V. alginolyticus* was found to be virulent to cobia fingerlings and was further confirmed by challenge studies. During the experimental study the fingerlings also exhibited the same initial infective clinical signs such as corneal opacity, surfacing and off feed as observed in the natural infection. But the severity of the infection such as haemorrhagic pericarditis, haemorrhagic gastritis was not observed in the experimental fish as in the natural infection. Moderate congestion of all the internal organs was observed due to the septicaemic conditions. It was observed that *V. alginolyticus* can cause acute septicaemic infection in cobia sub-adults leading to mass mortality. The vibrio organism is ubiquitous in that aquatic environment. So sea water, waste fish used as feed to the sub-adults and the fouling of cage nets could be the main sources of the bacterium.

The total viable counts of the sea water were higher during the pre-monsoon season ($55{\cdot}77 \pm 1{\cdot}41 \times 10^5$ per ml) compared with the monsoon ($7{\cdot}4 \pm 0{\cdot}37 \times 10^5$ per ml) and in the summer season ($4{\cdot}88 \pm 0{\cdot}33 \times 10^5$

per ml). So the higher and sudden increment of sea water temperature (33°C) during the pre-monsoon season could be one of the predisposing factors for the higher bacterial cell count in the water. Variation in the salinity and temperature occurs during the monsoon season when the salinity and temperature drop to 32 ppt and 28°C, respectively. However, during the monsoon, no such mortality was observed.

The sudden fluctuation in the environmental parameters such as temperature, salinity, dissolved oxygen and suspended particles during the pre-monsoon season could have contributed to the stress factors that resulted in the activation of the normal non-pathogenic form of *V. alginolyticus,* to the pathogenic form and caused the outbreak of the disease and mass mortality.

In conclusion, the natural outbreak of vibriosis was caused by *V. alginolyticus* in cobia sub-adults and was confirmed through cultural, biochemical and molecular methods. The 16S ribosomal RNA of the isolate was amplified and BLAST analysis of the sequence confirmed that the pathogen is *V. alginolyticus*. The confirmation was also correlated with its cultural, biochemical and pathomorphological changes. Since *V. alginolyticus* CGLP/13 was found to be virulent to the juvenile cobia, the bacterium is therefore confirmed to be a pathogen of the fish. The natural blooms of the pathogen could be prevented by sea cage management measures, such as changing the inner net of the cage and its location and providing shade, while the water temperature rises. This is the second report and the first incidence of epizootics with severe pathological lesions in cage culture of cobia in India.

Materials and methods

Natural outbreak

A total of 300 cobia sub-adults were maintained in two HDPE circular cages of 6 m diameter and 3·5 m depth. The average length and weight of the cobia was 83·27 cm and 4·2 kg, respectively, they were stocked at a density of 50 no per m^3 in three floating cages in the Gulf of Mannar region (lat 9°16′8·9″ N to 9°16′12·6″ N; long 79°7′87·8″ E to 79°7′98·1″ E), Mandapam in Tamil Nadu, India. Sudden mass mortality of cobia grow out was observed during August 2013 in the cage culture before the north-east monsoon.

Bacterial isolation and characterization

An attempt for bacterial isolation from the natural outbreak of moribund fish was made from the haemorrhagic ulcers of stomach, kidney and heart blood swab on TSA (HiMedia, Mumbai, India) supplemented with 2% NaCl and in TCBS (HiMedia) Agar. Morphological and other biochemical characters were identified based on Bergey's Manual of Systematic Bacteriology (Baumann and Schubert 1984). From the experimental moribund fish, kidney and heart blood swabs were taken aseptically and subjected to re-isolation and identification of the bacterial isolates.

Histopathology

Organs showing gross lesions were fixed in 10% neutral buffered formalin (NBF) for histopathological examination. The formalin fixed tissues were cut into pieces of 2–3 mm thick and washed thoroughly with water for several hours before placing in ascending grades of alcohol for dehydration. The dehydrated tissues were cleared in turpentine oil and embedded in paraffin wax. Sections 4–5 μm thick were made from paraffin blocks and stained with haematoxylin and eosin (Lillie and Fulmer 1976).

Experimental challenge studies

The natural outbreak of vibrio isolate was grown on TSB supplemented with 2% NaCl for 24 h at room temperature (33–34°C). The bacterial cells were harvested by spinning at 10,000 rev min^{-1} for 10 min and the pellet was washed in sterile PBS (pH 7·2). The pellet was re-suspended and washing was repeated and a final bacterial suspension of 10^6 CFU per ml was made in the PBS buffer. Each bacterial suspension (0·1 ml having 10^5 CFU) was injected intramuscularly to six (n = 6) cobia fingerlings (30–35 g). Another six cobia fingerlings were injected 0·1 ml of sterile PBS as a control. Both sets of fish were kept in separate tanks under observation for 7 days. The fish were fed *ad libitum* with floating pellet feed (Lucky Star, Taiwan) and a daily water exchange of 30% water was made. From the moribund fishes, kidney and heart blood swabs were aseptically collected and plated on TCBS agar. A single colony on TCBS was confirmed to be pure culture and it was used for further investigations.

Determination of LD_{50}

A dose range of 10^3–10^7 CFU per fish was used to enumerate the dose required for causing 50% mortality of challenged fish. For the challenge experiment, six (n = 6) cobia fingerlings of 30–35 g weight in duplicate for each dosage was maintained. The fish were injected intramuscularly with 0·1 ml of the bacterial suspension containing 10^3, 10^4, 10^5, 10^6 and 10^7 CFU, respectively, at the base of the dorsal fin. The control fishes were injected with 0·1 ml of sterile PBS. Challenged fishes were kept under observation for 7–10 days. The fishes were fed *ad libitum* with floating pellet feed (Lucky Star) and a daily water

exchange of 30% water was made. The mortality of the fish was recorded from each tank. During the experimental study the clinical signs showed by the fingerlings were recorded. From the moribund animals, kidney or heart blood swab samples were taken for bacteriological examination and fresh tissues were preserved in 10% NBF for histopathology. Control fish were euthanized with AQUI-S-20E (Aquatic Anaesthetic AQUI-S® 20E New Zealand Ltd, USA). The LD_{50} values were then calculated by adopting the methodology of Reed and Muench (1938).

Molecular characterization

DNA extraction, 16S rRNA amplification and sequencing
Total genomic DNA was extracted from bacterial cultures grown in nutrient broth using phenol–chloroform extraction method (Sambrook and Russel 2001) and quantified using a UV spectrophotometer. The 16S rRNA gene from the genomic DNA was amplified using universal primers; NP1F 5′-GAGTTTGATCCTGGCTCA-3′ and NP1R 5′-ACGGCTACCTTGTTACGACTT-3′ (Pai *et al.* 2010) with Phusion Hi-fidelity DNA polymerase (New England Biolabs, MA, USA) according to the manufacturer's instruction. The amplified products were purified (HiPurA PCR product purification kit, HiMedia) and sequenced. The nucleotide sequence of 16S rRNA gene was submitted to Gen-Bank database (accession number KM985650). The bacterial identity was determined by searching GenBank database using BLAST n algorithm (Altschul *et al.* 1997). The multiple alignments of the sequences were performed with CLUSTALW (Thompson *et al.* 1994).The percent identity of our sequence with other *Vibrio* spp. were deduced using the MEGALIGN program of DNASTAR. A phylogenetic tree was constructed using MEGA 5, the evolutionary history was inferred using the neighbour-joining method, the evolutionary distances were computed using the p-distance method and tree topologies were evaluated by bootstrap analysis of 1000 data sets using MEGA 5 (Felsenstein 1985; Saitou and Nei 1987; Nei and Kumar 2000; Tamura *et al.* 2013).

Brains from dead or moribund fish and part of the dorsal fin from live fish were also collected and preserved in RNA later and stored at −20°C for further analysis. These samples were sent to the Marine Bio-technology Division, Cochin for the analysis of viral nervous necrosis (VNN) or *βetanoda* virus diagnosis to assess the sub-adults carrier status.

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Conflict of Interest

Each co-author contributed their respective work part to the fulfilment of this article. It is hereby declared that there was no conflict of interest among the co-authors in this article publications.

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