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Note

Microbiological and histopathological investigations of *Vibrio alginolyticus* infection in cobia *Rachycentron canadum* (Linnaeus, 1766) cultured in sea cage

P. RAMESHKUMAR, C. KALIDAS, G. TAMILMANI, M. SAKTHIVEL, A. K. ABDUL NAZAR, V. ASHOK MAHARSHI, S. K. SRINIVASA RAO AND G. GOPAKUMAR

Mandapam Regional Centre of Central Marine Fisheries Research Institute, Marine Fisheries P. O. Mandapam Camp, Tamil Nadu – 623 520, India

e-mail: prkvet@gmail.com

ABSTRACT

The occurrence of disease caused by *Vibrio alginolyticus* in sea cage farming of hatchery produced cobia juveniles is reported in this paper. The affected animals showed signs of surfacing, sluggish swimming and bilateral exophthalmia followed by acute mortality. The bacterial pathogen *Vibrio alginolyticus* was isolated from systemic lesions of infected moribund cobia fingerlings which was confirmed based on biochemical characteristics. Further the 16S ribosomal RNA of the isolate was amplified and BLAST analysis of the sequence confirmed that the pathogen is *V. alginolyticus*. Histologically, the liver of affected fish showed fatty change, the eyes revealed congestion as well as infiltration of polymorphoneuclear cells in the choroid layer and acute glomerulornephritis was observed in the kidney.

Keywords: Cobia, Histopathology, *Rachycentron canadum, Vibrio alginolyticus*

The cobia, *Rachycentron canadum*, is distributed world wide in tropical and subtropical waters. Cobia is a potential candidate for aquaculture, owing to its fast growth rate and commercial interest (Su *et al.*, 1999). In India, the first sea cage farming trial of cobia with hatchery produced fingerlings was done at Mandapam Regional Centre of Cental Marine Fisheries Research Institute (CMFRI), during 2010. Fish, cultured in floating cages, become particularly susceptible to disease when various environmental parameters such as temperature, salinity, dissolved oxygen and suspended particles fluctuate suddenly or widely, or following rough handling operation. Once pathological changes develop, progress to disease in warm water environment is rapid. Early detection of behavioural changes and clinical signs in the cultured animals is critical for proper diagnosis of the disease. Vibriosis, characterised mainly by haemorrhagic septicaemia, is one of the most serious bacterial diseases in cultured marine fish worldwide (Egidius, 1987; Hjeltnes and Roberts, 1993; Ishimaru, *et al*., 1996; Austin and Austin, 1999; Lee *et al*., 2002; Alcaide, 2003). In India, Krupesh Sharma *et al*. (2011) first recorded *Vibrio alginolyticus* infection in Asian seabass reared in open sea floating cages. Further they reported that there was difference in pattern of lesions in experimental and natural infection in their study. Although no external lesions were seen in experimental infection, natural infection was characterised by haemorrhage and ulcer on body surface.

The aim of the present study was to investigate the outbreak of vibriosis in cobia juveniles cultured in floating sea cages, and describe the associated histopathological changes in different organs.

A total of 2000 numbers of cobia juveniles (100 days post-hatch) with an average weight of 44 g having average length 19 cm, were stocked at a density of 40 no. m^{-3} in three floating cages of 6 m dia each, 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) in Tamil Nadu. Mortality of juveniles was noticed from May to October 2010. Initial mortality of 25 numbers occurred during May and the length and weight of dead fish were in the range, 30 -70 g and 20 - 23 cm, respectively. After noting the gross external lesions, 10 numbers of dead/moribund fish were taken for microbiological and histopathological investigations. Gills, intestinal tract and blood smears prepared were also examined for presence of parasites if any. In freshly dead as well as moribund fishes, bacteriological investigations were carried out from tissue samples of kidney and stomach, under aseptic conditions.. The inocula were streaked on to tryptone soya agar (TSA) (Himedia, Mumbai, India) as well as thiosulfate citrate bile salt sucrose agar TCBS (Himedia) plates and incubated overnight. Motility test, and other biochemical tests (Alsina and Blanch (1994) were performed, to identify the bacterial isolates and the results were compared with that of Rajan *et al.* (2001). Molecular characterisation and confirmation of the bacterial strain CP5k1, isolated from kidney of naturally affected cobia was done in the Marine Biotechnology Division of CMFRI at Cochin. For this, total genomic DNA was extracted from bacterial cultures grown in nutrient broth by phenol–chloroform extraction. The 16S rRNA gene was amplified using universal primers; NP1F 5'-GAGTTTGATCCTGGCTCA-3' and NP1R 5'-ACGGCTACCTTGTTACGACTT-3' with Phusion Hi-fidility DNA polymerase (New England Biolabs), according to Sambrook and Russell (2001).

The bacterial strain CP5k1 identified as *Vibrio alginolyticus* was used for experimental challenge study in healthy cobia fingerlings. A dose range of 10^3 to 10^7 CFU per fish was used to enumerate the dose required for causing 50% mortality of challenged fish (Reed and Muench, 1938). For the challenge experiment, six numbers of cobia fingerlings of 30-35 g weight in duplicate for each dosage was maintained. The fishes 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. The mortality pattern was observed for a period of one week post-challenge. For histopathology, representative samples of tissues from skin, kidney and stomach, showing gross lesions were fixed in 10% neutral buffered formalin. The tissues were processed, embedded in paraffin wax and sections cut at 5µ thickness, stained with haematoxylin and eosin (Lillie and Fulmer, 1976) and photomicrographs were taken.

The total cumulative mortality in all three cages during the period of five months was approximately 60.7%. Initial clinical signs included anorexia, sluggish swimming and frequent surfacing. In acute cases, bilateral exophthalmia with extensive congestion of both the eyes were noted followed by mass mortality. Gills were pale with profuse mucous secretions. Petechial haemorrhages were observed in the base of the dorsal fin and tail region. Liver was often pale (Fig.1) with petechiae. Hepatic lesions included congestion, haemorrhages and swollen hepatocytes with honey comb vacuolation (Fig. 2). Abdomen was distended with peritoneal fluid accumulation. The gastric mucosa showed reddish appearance with congestion, no ingesta was found within the gastric lumen. These findings were similar to that of the *Vibrio alginolyticus* infection in cobia cultured in Taiwan (Rajan *et al.,* 2001) and in seabass (Azad *et al*., 2011; Sharma *et al*., 2012).

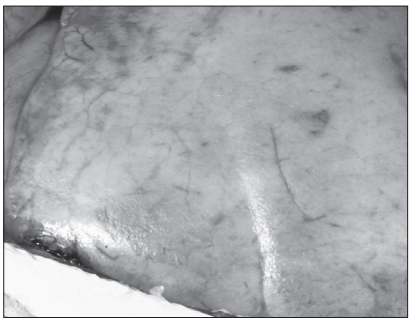

Fig. 1. Pale liver from *Vibrio alginolyticus* infected cobia showing fatty degeneration

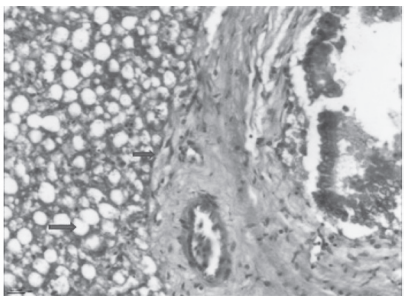

Fig. 2. Histological section of liver from *Vibrio alginolyticus* infected cobia showing fatty degeneration, honey comb vacuolation and bile duct hypertrophy (arrow). H&E; X400

Bacteria isolated from the kidney and haemorrhagic ulcers in stomach from naturally infected fish were found to grow on TCBS agar with the formation of yellow colonies. Colonies were circular in shape with Gram negative rod shaped bacteria. The morphological and biochemical characteristics of the bacterial isolates are listed in Table 1 and the isolates were identified as *Vibio alginolyticus*. Further the bacterial isolate (CP5k1) from kidney was identified as *V. alginolyticus* by BLAST search in GenBank (http://blast.ncbi.nlm.nih.gov/), and the 16S rRNA sequence (GenBank Acc. No. KC734518) showed 100% similarity to that of *V. alginolyticus*.

Table 1. Morphological and biochemical characteristics of *Vibrio alginolyticus* isolated from cobia juveniles

Test	*V. alginolyticus* (CP5k1).	*V. alginolyticus* from cobia (Rajan *et al.,* 2001)
Gram's staining	–	–
Motility	+	+
Swarming on TSA	+	+
Growth on TCBS	Y	Y
Growth in 3% NaCl	+	+
Growth in 6% NaCl	+	+
Oxidase	+	+
Catalase	+	+
Nitrate reductase	+	+
Production of H_2S	–	–
Urease	+	+
Indole	+	+
VP test	–	–

TCBS : Thiosufate citrate bile salt sucrose agar, TSA : Triptic soy agar
+ : Positive reaction, - : Negative reaction, Y - Yellow colonies

The LD_{50} value of *V. alginolyticus* isolate (CP5k1) for cobia fingerlings was found to be $10^{4.5}$CFU per fish. In the experimental infection studies, *V. alginolyticus* was re-isolated from heart blood and kidney of moribund cobia fingerlings, while no bacteria could be isolated from the kidney and heart blood of the control group fishes.

Microscopically, in naturally infected fish, hepatic parenchyma revealed extensive fatty changes. In few places small vacuoles coalesced to form large fatty cysts displacing the nucleus to the periphery. Some of the area showed mild to moderate congestion and hypertrophy of the bile duct. *Azad et al*. (2011) reported necrotic changes in the hepatic tissues resulting in 'honeycomb' vacuolation in seabass juveniles naturally infected with *V. alginolyticus*. In myocardium, loss of cross striations in the fibers and infiltration of polymorphoneuclear cells in to the endocardium (Fig. 3) was noted. Eyes revealed congestion and infiltration of polymorphoneuclear cells in the choroid layer. Acute glomerulonephritis was observed in kidney. Increased expression of melano-macrophage centres (MMC) (Fig. 4) was observed throughout the kidney parenchyma. The proximal convoluted tubules revealed degeneration and loss of brush borders with the degeneration of the entire tubular epithelium. The kidney parenchyma showed brownish-yellow round metallic sheen, as

haemochromatosis deposits in the entire glomeruli indicating the intravascular haemolysis. Gastric mucosa showed engorged capillaries and loss of tubular glands in the gastric pit. No parasite could be detected or observed in the gills, intestine and in the impression smears.

The outbreak of vibriosis reported in cobia fingerlings in marine floating cage culture is the first description from India. Only a few pathological studies have been reported in cobia (Chen *et al*., 2010). Susceptibility of fish to vibriosis is enhanced when the animals are subjected to stressful conditions, such as handling, transport, overcrowding and low dissolved oxygen (Austin and Austin, 1993). Horizontal transmission is the most probable route in vibriosis, with bacteria being shed from open lesions (Leong Tak Seng and Angelo Colorni, 2002). In the present study, the natural outbreak of vibriosis in cobia was caused by *V. alginolyticus* which was confirmed through biochemical and molecular methods. The *V. alginolyticus* (CP5k1) strain isolated was found to be virulent to juvenile cobia and the bacterium is therefore confirmed to be a pathogen of the fish. The source of the pathogen can be attributed to the carrier seabass fishes cultured nearby, in which vibriosis outbreak was reported two months prior to the present outbreak. Good husbandry practices and adequate nutrition are essential to prevent the development of vibriosis in sea cages.

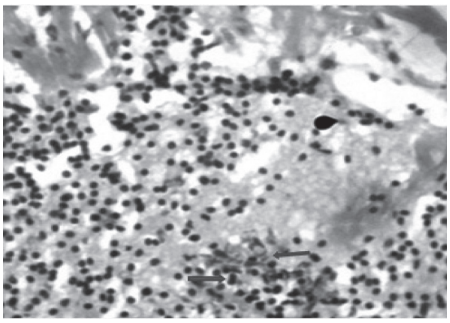

Fig. 3. Myocarditis and polymorphoneuclear cells infiltration (arrow) with presence of rod shaped bacteria (arrow) in *Vibrio alginolyticus* infected cobia. H&E; X1000

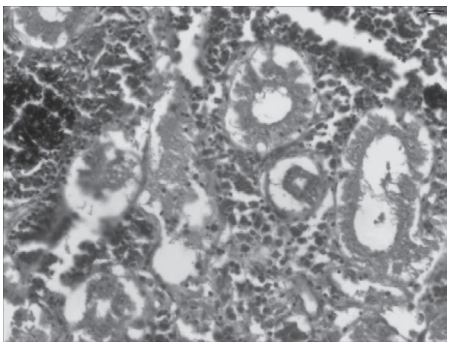

Fig. 4. Acute glomerulonephritis and necrosis of convoluted tubules in kidney of *Vibrio alginolyticus* infected cobia. H&E; X400

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References

Alsina, M. and Blanch A. R. 1994. A set of keys for biochemical identification of environmental *Vibrio* species. *J. Appl. Bacteriol.,* 76: 79–85.

Austin, B. and Austin, D. A. 1993. *Bacterial fish pathogens,* 2nd edn. Ellis Horwood, Chichester, U. K.

Azad, I. S., Thirunavukkarasu, A. R., Kailasam, M. and Rajan, J. J. S. 2004. Virulence and histopathology of *Vibrio anguillarum* like (VAL) bacterium isolated from hatchery produced juveniles of *Lates calcarifer* (Bloch). *Asian Fish. Sci.,* 17 : 101-110.

Chen, S. C., Kou, R. J., Wu, C. T., Wang, P. C. and Su, F. Z. 2001. Mass mortality associated with *Sphaerospora* - like myxosporidean infestation in juvenile cobia, *Rachycentron canadum* (L), marine cages cultured in Taiwan. *J. Fish. Dis.,* 24: 189-195.

Egidius, E. 1987. Vibriosis: Pathogenecity and pathology - A review. *Aquaculture,* 67: 15-28.

Hjeltnes, B. and Roberts, R. J. 1993. Vibriosis. In: Inglis, V., Roberts, R. J. and Bromage, N. R. (Eds.), *Bacterial diseases of fish,* Blackwell scientific Publications, Oxford, p. 109-121.

Ishimaru, K., Akagawa-Matsushita, M. and Muroga, K. 1996. *Vibrio ichthyoenteri* sp. nov., a pathogen of japanese flounder (*Paralichthys olivaceus*)larvae. *Int. J. Syst. Bacteriol.,*46: 155-159.

Krupesha Sharma, S. R., Gaurav Rathore, Dev, K. Verma, Narasimhulu Sadhu and Kuttickal Kuriakose Philipose 2011. *Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in open sea floating cages in India. *Aquacult. Res.*, p. 1–7.

Leong Tak Seng and Angelo Colorni 2002. Infectious diseases of warm water fish in marine and brackish waters. In: Woo, P. T. K., Bruno, D. W. and Lim, L. H. S. (Eds.), *Diseases and disorders of finfish in cage culture.* CAB publishing, p. 193-230.

Lee, K. K. 1995. Pathogenesis studies on *Vibrio alginolyticus* in the grouper, *Epinephelus malabaricus*, Bloch and Schneider. *Microb. Pathog.,* 19 : 39-48.

Lillie, R. D. and Fulmer, H. M. 1976. *Histopathologic technique in practical histochemistry*, 4th edn., McGrawHill, New York.

Rajan, P. R., Carmen Lopez, John Han-You Lin and Huey Lang Yang 2001. *Vibrio alginolyticus* infection in cobia

(*Rachycentron canadum*) cultured in Taiwan. *Bull. Eur. Ass. Fish. Pathol.,* 21(6): 228.

Reed, M. J. and Muench, H. 1938. A simple method for estimating fifty percent end point. *Am. J. Hyg.,* 27: 493-497.

Roberts, R. J. 1989. The bacteriology of teleosts. In: Roberts, R. J. (Ed.), *Fish. Pathol.,* Bailliere Tindall, London, p. 289-319.

Sambrook, J. and Russel, D. W. 2001. Rapid isolation of yeast DNA. In: Sambrook, J. and Russel, D. W. (Eds.), *Molecular cloning, a laboratory manual.* Cold Spring Harbor Laboratory, New York, p. 631-632.

Su, M. S., Chien,Y. H. and Lio, I. C. 1999. Potential of marine cage aquaculture in Taiwan. *Book of Abstracts, The first international symposium on cage Aquaculture in Asia*, p. 24.

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