Indian J. Fish., 61(3) : 118-121, 2014

Note

Association of *Vibrio harveyi* in mortality of mangrove red snapper (*Lutjanus argentimaculatus,* Forsskål, 1775) cultured in open sea cages

S. R. KRUPESHA SHARMA[1], M. A. PRADEEP[2], JAYASREE LOKA[1], A. K. SAHOO[3] PRAVEEN N. DUBE[1], N. SADHU[1] AND K. K. PHILIPOSE[1]

[1]*Karwar Research Centre of Central Marine Fisheries Research Institute, Karwar - 581 301, Karnataka, India*
[2]*Central Marine Fisheries Research Institute, Kochi - 682 018, Kerala, India*
[3]*Central Inland Fisheries Research Institute, Barrackpore - 700 120, Kolkata, India*
e-mail: krupeshsharma@gmail.com

ABSTRACT

A case of vibriosis caused by *Vibrio harveyi* in adult mangrove red snappers reared in floating net cages is reported for the first time from India. The pathogen was confirmed using biochemical characterisation, by nested PCR and 16S rRNA gene sequencing. Histopathology revealed chronic nature of the disease. The outbreak of mortality was consequent to elevated water temperature and handling. Importance of stress factors as stimulus to outbreak of mortality is discussed.

Keywords: Cage culture, Red snapper, Stress, *Vibrio harveyi*

Cage culture of important marine food fish like cobia, snapper, pompano, sea bream and Asian seabass is becoming more popular in India in recent years. The mangrove red snapper, *Lutjanus argentimaculatus* is a highly priced food fish in many countries (Liao *et al*., 1995). Bacterial infections have been one of the limiting factors for the sustainability of aquaculture (Colorni *et al*., 1981). Several reports on the occurrence of mortalities due to vibriosis in cage cultured finfish have been documented (Tedencia, 2002; Sharma *et al.*, 2013). *Vibrio harveyi* is reported to be a serious pathogen in marine fish (Zhang *et al*., 2001). Earlier researchers have isolated this pathogen from the common snook (Kraxberger-Beatty and Mc Gare, 1990), cobia (Liu *et al.*, 2004) and snappers (Stephens *et al*., 2006; Gomez-Gil *et al*., 2007). In this paper, a case of mortality in red snapper caused by *V. harveyi* in open sea net cages is presented.

Mortality in broodstock of mangrove red snappers (mean weight: 850±150 g; mean total length: 22±4 cm) with a stocking density of 280 nos. in a 6 m diameter cage installed at the marine fish farm of the Karwar Research Centre of Central Marine Fisheries Research Institute, India was recorded. Water temperature, salinity, pH and dissolved oxygen (DO) of the marine farm site (lat. 14° 48′ N; long. 74° 06′ E) were monitored using portable instruments. Freshly dead and moribund fish with gross lesions were brought to the laboratory for further investigations. The fish were also examined for the presence of external and internal parasites.

After recording the gross lesions, attempts were made for bacterial isolation under aseptic conditions from blood, liver and kidney of five moribund fish on trypticase soya agar (TSA; supplemented with 2% NaCl). The samples were incubated for 24 to 48 h at room temperature to obtain visible bacterial growth. Four colonies were randomly picked from each plate and purified in fresh TSA plates and incubated for 24 to 48 h at room temperature to obtain pure colonies. A pure colony from each TSA plate was transferred to thiosulphate citrate bile salts sucrose (TCBS) agar and trypticase soya broth (TSB) and incubated for 24 h at room temperature. Samples from TSB were again sub-cultured on TSA plates and colonies thus obtained were subjected to study the morphology and biochemical characteristics (Baumann and Schubert, 1984). For performing the antibiotic sensitivity, the isolate was cultured in TSB, washed thrice in phosphate buffered saline (PBS, pH 7.2) and then diluted to an absorbance of 0.6 at 610 nm with PBS. Bacterial suspension (0.1 ml) was spread onto triplicate TSA plates, antibiotic discs were placed and plates were incubated for 24 h at room temperature. Zones of inhibition of the bacteria by the antibiotics were scored according to the method described by Barry *et al.* (1979).

For molecular diagnosis, pure cultures of the phenotypic isolate in log phase in Luria-Bertani (LB) broth were used for DNA extraction using bacterial genomic DNA extraction kit (Sigma, USA). A gene specific for *V. harveyi*, a unique consensus region

within the 16S rRNA gene sequences, was amplified by nested PCR. In the first step of the nested PCR, bacterial 16S rRNA gene was amplified using the combination of universal primers 27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ GGTTACCTTGTTACGACTT 3′). In the second step, a gene specific for *V. harveyi* was amplified using the primers VAHF1 (5′-ATCATGAATAAAAC TATTACGTTACT-3′) and VAHR1 (5′-AAAGGATGGT TTGACAAT-3′) following Fukui and Sawabe (2007) in a thermal cycler (Eppendorf, Germany). The 16S rRNA PCR product was purified using the nucleic acid purification kit (Axygen Biosciences, USA) and sequenced. Bacterial identity was deduced by searching GenBank database using BLAST algorithm to ascertain its closest related sequences. Percent identity of the isolate with other *Vibrio* spp. was calculated with MEGALIGN program of DNASTAR. Phylogenetic tree was constructed with MEGA 5 using neighbour-joining method. The evolutionary distances were computed using the p-distance method and tree topologies were evaluated by bootstrap analysis of 1,000 data sets (Felsenstein, 1985; Saitou and Nei, 1987). The nucleotide sequence of 16S rRNA gene was submitted to GenBank database under the accession number KC345010.

Representative samples of kidney, liver, spleen and stomach were also preserved in 10% buffered formalin for histopathology. Tissues were processed by a routine method and embedded in paraffin wax. Sections cut at 5 µ thickness were stained with Harri's hematoxylin and eosin and photomicrographs were taken.

Mean values of water temperature, pH, DO and salinity recorded from the marine farm site were statistically analysed by one-way ANOVA using SPSS 13.0 for Windows.

The mean water temperature (32 ± 0.8˚C) during the week in which mortality occurred was significantly ($p<0.05$) higher than the preceding weeks (29 ± 0.6 to 30 ± 0.4 ˚C). No significant differences were found with respect to mean weekly values of salinity (31 to 33‰), pH (7.8 ± 0.3) and DO (4.5 to 5.5 mg l^{-1}).

Mortality (40%) occurred for two days. Dead and moribund fish showed haemorrhage and ulceration on the body surface and exophthalmia (Fig. 1). No external or internal parasites were found. The liver was pale and kidney was congested. The mucosa of the stomach was thickened with excessive mucus in the lumen. Based on the morphological and biochemical characteristics, only one phenotypic isolate (0012KARWAR Table 1) was identified (out of 60 isolates). The isolate was sensitive

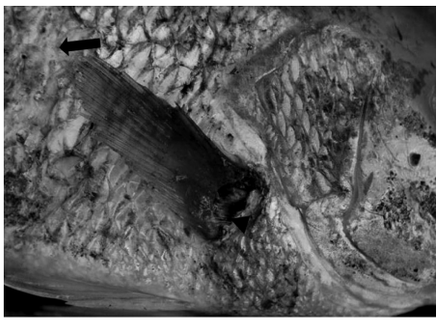


Fig. 1. Cage reared red snapper showing haemorrhage at base of the fin (arrow head) and erosion on the body (arrow).

Table 1. Characteristics of the isolate 0012KARWAR compared with Baumann and Schubert (1984)

Characteristics	0012KARWAR	Baumann and Schubert (1984) (VH41)
Gram reaction	-	-
Cell morphology	r	r
Luminescence	-	d
Swarming	-	-
Growth in % NaCl		
0	-	-
3	+	+
6	+	d
8	+	d
10	-	d
Decarboxylase test		
Arginine	-	-
Lysine	+	+
Ornithine	+	+
Catalase	+	+
Oxidase	+	+
Acid from		
Sucrose	-	-
Arabinose	-	-
Mannose	+	+
0/129 sensitivity		
10 µg	+	d
150 µg	+	+
Growth at ˚C		
40	-	-
35	+	+
30	+	+

r- rod; d - doubtful

to oxacillin, ampicillin, enrofloxacin, ciprofloxacin, norfloxacin, furozolidone, tetracycline, chloramphenicol, penicillin, vancomycin, amoxycillin, streptomycin and gentamycin. It was resistant to trimethoprim. Nested PCR confirmed the isolate as *V. harveyi* (Fig. 2). The 16S rRNA sequencing showed 100% identity with *V. harveyi* strain GU262992 and 99.9% identity with *V. harveyi* strains KC455398 and JX861207. The

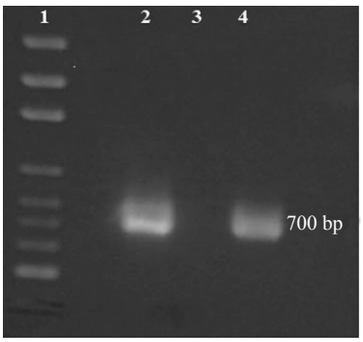


Fig. 2. Detection of *V. harveyi* using nested PCR. Lane 1: marker Lane 2: 0012KARWAR; Lane 3: negative; Lane 4: positive

phylogenetic tree based on the above 16S rRNA sequences is shown in Fig. 3. Microscopic lesions (Fig. 4) revealed extensive granuloma in sub-mucosa of the stomach characterised by proliferation of connective tissue and new blood vessels. The muscularis mucosa was replaced by the granulation tissue in some places. Hyperplastic epithelium and proliferative fibroblasts were seen in the hepatic bile ducts. In some sections, necrotic hepatic parenchyma was replaced by erythrocytes. Enlargement of sinusoids and sub-capsular haemorrhage were also evident in sections of the liver. Kidney sections showed presence of necrosis, haemorrhage, inflammatory cells and increased expression of melanomacrophage centres (MMCs). The MMCs were discrete, pigmented and randomly distributed. Depletion of cells in the spleen was also observed.

The above results revealed a case of vibriosis in cage reared red snappers caused by *V. harveyi*. This bacterium has been reported to be a primary pathogen infecting fish and shellfish species over a wide geographical area (Zhang and Austin, 2000). Most of the pathogenic *Vibrios* are opportunistic in nature causing disease when fish are subjected to stress (Lightner and Redman, 1998). Histopathology suggested a chronic course of the disease. In the present case, mortality of snappers which were in a phase of chronic vibriosis was associated with elevated water temperature and handling stress. Grimes *et al*. (1985) reported that *V. harveyi* was a serious pathogen in immunocompromised hosts. Rise in water temperature and stress due to handling as factors for vibriosis was also reported in turbot (Horne *et al*., 1977) and cage reared Asian seabass (Sharma *et al*., 2013). Tedencia (2002) observed heavy rain fall as a reason for stress that predisposed cage cultured Asian seabass to *V. harveyi* infection.

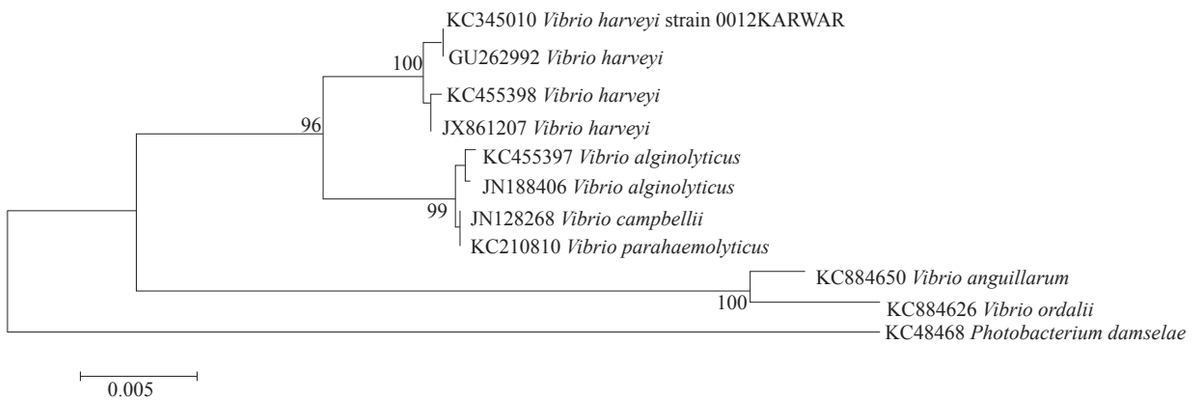
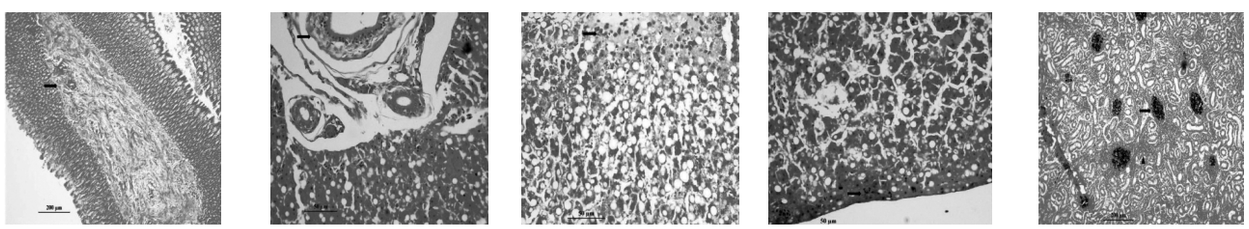


Fig. 3. Phylogenetic relationships of KC345010 *Vibrio harveyi* strain 0012KARWAR with selected *Vibrio* spp. Bootstrap values (expressed as percentages of 1000 replication) are shown at the branch points; Values greater than 95% were considered significant. *Photobacterium damselae* was used as an out-group for rooting trees



(a) (b) (c) (d) (e)

Fig. 4. Histopathology of red snapper infected with *V. harveyi* (H & E): (a) Section of stomach showing granuloma (arrow). (b) Section of liver showing hyperplastic epithelium and proliferative fibroblasts in the hepatic bile ducts (arrow). (c) Section of liver showing necrotic hepatic tissue occupied by erythrocytes (arrow). (d) Section of liver showing sub-capsular haemorrhage (arrow). (e) Section of kidney showing haemorrhage (arrow head) and increased expression of MMCs (arrow)

Gross lesions recorded were similar to previous reported cases of vibriosis in different fish species (Rasheed, 1989; Sharma *et al*., 2013). Mohi *et al*. (2010) observed granulomatous lesions in different organs of tiger puffer infected with *V. harveyi*. However, in the present study, granulomas were limited only to stomach. Increased MMCs in liver and kidney of snapper infected with *V. harveyi* was also reported by Stephens *et al*. (2006). It is reported that MMCs develop during late stages of chronic inflammatory response to severe tissue damage (Agius and Roberts, 2003). Even though pathogenicty trails were not conducted in the present study, amplification of a specific gene by nested PCR and 16S rRNA sequencing confirmed the causative agent as *V. harveyi* which was repeatedly isolated from the infected snapper.

The present investigation highlighted the importance of *V. harveyi* causing mortality in cage reared snappers. Although *Vibrio* induced mortality of marine fish associated with stress in culture conditions is not uncommon, the present study gains distinction in view of the growing demand for culture and broodstock maintenance of red snapper in marine cages. Hence, alleviating stress factors in marine cage farming should merit importance to prevent fish mortality since *Vibrio* species are ubiquitous in the marine environment.

References

Agius, C. and Roberts, R. J. 2003. Melano-macrophage centres and their role in fish pathology. *J. Fish Dis*., 26: 499-509.

Barry, A. L., Amsterdam D., Coyle M. B., Gerlach, E. H., Thornsberry, C. and Hawkinson, R.W. 1979. Simple inoculum standardising system for antimicrobial disk susceptibility tests. *J. Clin. Microbiol*., 10: 910-918.

Baumann, P. S. and Schubert, R. H. W. 1984. The family II Vibrionaceae Veron. In: Krieg, N. R. (Ed.), *Bergey's manual of systematic bacteriology*. Wiliams and Wilkins, Baltimore, p. 515-538.

Colorni, A., Paperna, I. and Gordin, H. 1981. Bacterial infections in gilthead seabream *Sparus aurata* cultured at Elat. *Aquaculture*, 23: 257–267.

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783-791.

Fukui, Y. and Sawabe, T. 2007. Improved one-step colony PCR detection of *Vibrio harveyi*. *Microbes Environ*., 22: 1-10.

Gomez-Gil, B., Fajer-Avila, E. and Garcı´a-Vargas, F. 2007. Vibrios of the spotted rose snapper *Lutjanus guttatus* Steindachner, 1869 from north-western Mexico. *J. Appl. Microbiol*., 102: 1518–1526.

Grimes, D. J., Gruber, S. H. and May, E. B. 1985. Experimental infection of lemon sharks, *Negaprion brevirostris* (Poey), with *Vibrio* species. *J. Fish Dis*., 8: 173–180.

Horne, M. T., Richards, R. H., Roberts, R. J and Smith, P. C. 1977. Per-acute vibriosis in juvenile turbot *Scophthalmus maximus*. *J. Fish Biol*., 11: 355–361.

Kraxberger-Beatty, T., Mc Gare, D. J. 1990. *Vibrio harveyi,* an opportunistic pathogen of common snook, *Centropomus undecimalis* (Bloch) held in captivity. *J. Fish Dis*., 13: 557-560.

Liao, I. C., Su, M. S. and Chang, S. L. 1995. A review of the nursery and grow-out techniques of high-value marine finfishes in Taiwan. In: Main, K. L. and Rosenfeld, C. (Eds.), *Culture of high-value marine fishes*. Oceanic institute, Hawaii, p. 121–137.

Lightner, D. V. and Redman, R. M. 1998. Shrimp diseases and current diagnostic methods. *Aquaculture*, 164: 201–220.

Liu, P. C., Lin, J. Y., Chuang, W. H. and Lee, K. K. 2004. Isolation and characterisation of pathogenic *Vibrio harveyi* (*V. carchariae*) from the farmed marine cobia Fish *Rachycentron canadum* L. with gastroenteritis syndrome. *World J. Microbiol. Biotechnol*., 20: 495-499.

Mohi, M. M., Kuratani, M., Miyazaki, T. and Yoshida, T. 2010. Histopathological studies on *Vibrio harveyi* infected tiger puffer, *Takifugu rubripes* (Temminck et Schlegel), cultured in Japan. *J. Fish Dis*., 33: 833–840.

Rasheed, V. 1989. Vibriosis outbreak among cultured seabream (*Acanthopagrus cuvieri*) broodstock in Kuwait. *Aquaculture*, 76: 189–197.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol*., 4: 406-425.

Sharma, S. R. K., Rathore, G., Verma, D. K., Sadhu, N. and Philipose, K. K. 2013. *Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in open sea floating cages in India. *Aquacult. Res*., 44: 86–92.

Stephens, F. J., Raidal, S. R., Buller, N. and Jones, B. 2006. Infection with *Photobacterium damselae* subspecies *damselae* and *Vibrio harveyi* in snapper, *Pagrus auratus* with bloat. *Aust. Vet. J*., 84: 173-177.

Tedencia, E. A. 2002. *Vibrio harveyi* isolated from cage-cultured seabass *Lates calcarifer* Bloch in the Philippines. *Aquacult. Res*., 33: 455-458.

Zhang, X. H. and Austin, B. 2000. Pathogenicity of *Vibrio harveyi* to salmonids. *J. Fish Dis*., 23: 93–102.

Zhang, X. H., Meaden, P. G. and Austin, B. 2001. Duplication of hemolysin genes in a virulent isolate of *Vibrio harveyi*. *Appl. Environ. Microbiol*., 67: 3161-3167.

Date of Receipt : 21.11.2013
Date of Acceptance : 27.01.2014