



## Short Communication

## First report of isolation and characterization of *Photobacterium damsela* subsp. *damsela* from cage-farmed cobia (*Rachycentron canadum*)

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Cobia (*Rachycentron canadum*) has been considered as a potential species for aquaculture because of its fast growth and commercial value. As a consequence of successful spawning and seed production, there has been a significant demand for cage farming of cobia in India (Philipose *et al.* 2013). Not many reports are available on diseases of cage-farmed cobia. Globally, mortality due to pasteurellosis in cage-cultured cobia caused by *Photobacterium damsela* subsp. *piscicida* has been reported (Liu, Lin & Lee 2003). Vibriosis caused by *Vibrio alginolyticus*, *V. harveyi*, *V. parahaemolyticus* and *V. vulnificus* has also been responsible for mortalities in cage-cultured cobia (Rajan *et al.* 2001; Liu *et al.* 2004).

*Photobacterium damsela* subsp. *damsela* was originally isolated from the skin lesions of damselfish, *Chromis punctipinnis* (Love *et al.* 1981). Subsequently, diseases caused by this pathogen have been reported in several cultivable fish species like seabream (Vera, Navas & Fouz 1991), eel (Ketterer & Eaves 1992), turbot (Fouz *et al.* 1992),

ovate pompano (Zhao *et al.* 2009) and Asian seabass (Kanchanopas-Barnette *et al.* 2009). Infection caused by *P. damsela* subsp. *damsela* has also been recorded in marine mammals like dolphins (Fujioka *et al.* 1988) and several wild fish species (Han *et al.* 2009). Pathogenicity of this bacterium in several marine fish species and humans has been reviewed (Rivas, Lemos & Osorio 2013a). There have been no reports on mortality caused by this pathogen in cobia. This study was envisaged with the objectives to describe the disease epizootic caused by *P. damsela* subsp. *damsela* in cage-farmed cobia and characterize the pathogen.

Mortality was reported in cobia (mean length: 26 cm; mean weight: 550 g) maintained in 6-m-diameter steel cages (5 cubic m<sup>-1</sup>) during May 2013 in the marine farm (latitude: 14° 48'N; longitude 74° 06' E) of the Central Marine Fisheries Research Institute, Karwar, India. Fish were fed with fresh sardine (*Sardinella longiceps*) at 5% of biomass thrice daily. pH, salinity and temperature of the cage water were measured at weekly intervals using portable instruments, and the values were statistically analysed using SPSS software. Mortality with clinical signs lasted for 2 weeks with a total mortality of 40%. Temperature of the water during the period of outbreak was 32 ± 0.6 °C which was significantly ( $P < 0.05$ ) higher when compared with previous months (30 ± 0.8 °C). There was no significant

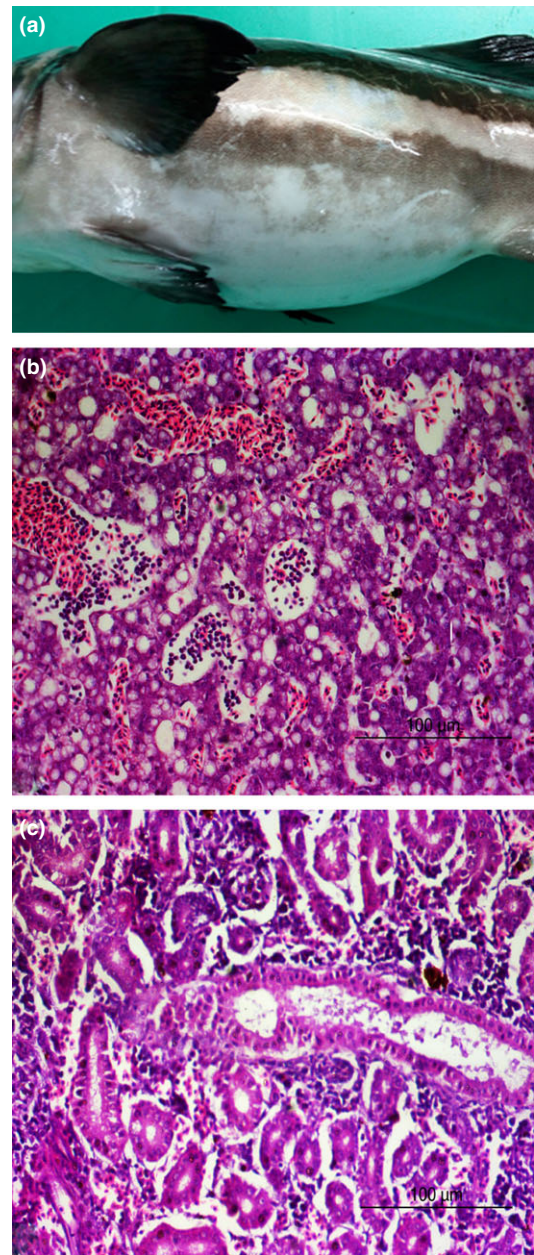
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difference in pH (8.0–8.2) and salinity (34–35‰) of the water during the culture period.

After recording the gross lesions, wet scrapings of gills and skin were observed under the microscope. Ailing fish ( $n = 18$ ) with gross lesions were immediately transported to the laboratory. Representative samples of spleen, liver, kidney, gills and brain from freshly dead or ailing cobia were preserved in 10% neutral buffered formalin for histopathology. Tissues were routinely processed, and sections cut at 5  $\mu$  thickness were stained with haematoxylin and eosin. Grossly, swollen abdomen with haemorrhagic patches was consistently observed (Fig. 1a). Internally, abdominal cavity was filled with yellow gelatinous fluid with haemorrhagic liver and kidney. No external parasites were observed on skin and gills. Microscopically, cellular degeneration, haemorrhage and infiltration by inflammatory cells in liver and kidney were observed (Fig. 1b,c).

Bacterial isolations were made aseptically from blood, liver, kidney, spleen, brain and ascitic fluid on thiosulfate–citrate–bile salt–sucrose (TCBS; Himedia) agar plates and tryptone soya broth (TSB; Himedia) supplemented with 2% NaCl and incubated at 30 °C for 24 to 48 h. Cultures from the broth were transferred to Tryptone Soya Agar (TSA; Himedia) supplemented with 2% NaCl and incubated for 24 h at 30 °C to obtain pure cultures. Five pure cultures of the isolate were subjected to various biochemical tests following Krieg & Holt (1984). The biochemical characteristics were compared with that of Kanchanopas-Barnette *et al.* (2009) and Labella *et al.* (2011). Raised, small, creamy colonies were observed on TSA. Morphological and biochemical characteristics of the isolate are given in Table 1. All the isolates showed similar morphological and biochemical characteristics.

Bacterial extracellular products (ECP) were harvested, and the activities of caseinase, gelatinase, phospholipase and lipase were assayed following Lee & Ellis (1990). Haemolytic activity was determined on blood agar containing 5% cobia blood and incubated at 30 °C for 24 h. Clear zone around the colony indicated haemolysis. API ZYM kit (BioMérieux) was also employed for enzymatic profiling of the isolate and ECP. To ascertain heat stability, the ECP was incubated at 100 °C for 10 min and enzymatic activities were tested. ECP exhibited phospholipase, lipase and



**Figure 1** Gross and microscopic lesions (H&E) in cobia naturally infected with *P. damsela* subsp. *damsela*. (a) Abdominal swelling in the infected fish; (b) haemorrhage, accumulation of inflammatory cells and degenerative changes in the hepatic parenchyma; (c) tubular degeneration, haemorrhage and infiltration of inflammatory cells in the kidney.

haemolytic activity. Enzyme profiling using of API ZYM assay is shown in Table 2.

For molecular characterization, pure cultures of the phenotypic isolate in log phase in Luria-Bertani broth were used for DNA extraction

**Table 1** Biochemical and phenotypic characteristics of *P. damsela* subsp. *damsela* (0029KARWAR) compared with Asian seabass isolate

Characteristics	0029KARWAR	Kanchanopas-Barnette <i>et al.</i> (2009)	Labella <i>et al.</i> (2011)
Gram stain	–	–	–
Growth on TCBS	G	G	G
Swarming	–	–	–
Motility	+	+	+
Nitrate reduction	+	+	+
Gas from glucose	+	+	+
Indole production	–	–	–
Urease	+	+	V
Lysine decarboxylase	+	+	V
Arginine dihydrolase	+	+	+
Ornithine decarboxylase	–	–	–
O/129 sensitivity			
10 µg	+	+	NT
150 µg	+	+	NT
Growth in % NaCl			
0	–	–	–
3	+	+	NT
6	+	+	+
8	–	–	–

+, positive; –, negative; G, green; NT, not tested; V, variable.

employing bacterial genomic DNA extraction kit (Sigma). Bacterial 16S rRNA gene was amplified using the combination of universal primers 27F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTACGACTT 3'). PCR product was purified using nucleic acid purification kit (Axygen Biosciences) and Sanger sequenced. Bacterial identity was deduced by searching GenBank database using BLAST algorithm. The nucleotide sequence of 16S rRNA gene of our isolate (0029KARWAR) was submitted to GenBank database under the accession number KC466554. To differentiate *P. damsela* subsp. *damsela* from *P. damsela* subsp. *piscicida*, we also employed a multiplex PCR that combines specific primers for 16S rRNA and urease genes as previously described (Osorio *et al.* 2000b). For the detection of damselysin encoding *dly* gene, primer sets were used to amplify a fragment flanking the 567-bp internal fragment of *dly* gene as previously described (Osorio *et al.* 2000a). The 16S rRNA sequencing showed 100% identity with *P. damsela* subsp. *damsela* (KC884630), *P. damsela* subsp. *piscicida* (KF95638) and *P. histaminum* (AB032014). Using multiplex PCR, two amplification products of 267 and 448 bp (Fig. 2)

**Table 2** Enzymatic profile of *Photobacterium damsela* subsp. *damsela* and its extracellular products using API ZYM system

Enzyme	Activity (nmol of hydrolysed substrate)		
	Bacteria	ECP	Heat inactivated ECP
Alkaline phosphatase	>40	>40	–
Esterase	30–40	20–30	20–30
Esterase lipase	20–30	20–30	20–30
Lipase	–	–	–
Leucine arylamidase	>40	30–40	–
Valine arylamidase	–	–	–
Cystine arylamidase	–	–	–
Trypsin	–	–	–
α-chymotrypsin	<5	–	–
acid phosphatase	>40	>40	>40
Naphthol-AS-BI-phosphohydrolase	20–30	20–30	20–30
α-galactosidase	–	–	–
β-galactosidase	–	–	–
β-glucuronidase	–	–	–
α-glucosidase	20–30	–	–
β-glucosidase	–	–	–
α-mannosidase	+	–	–
α-fucosidase	–	–	–

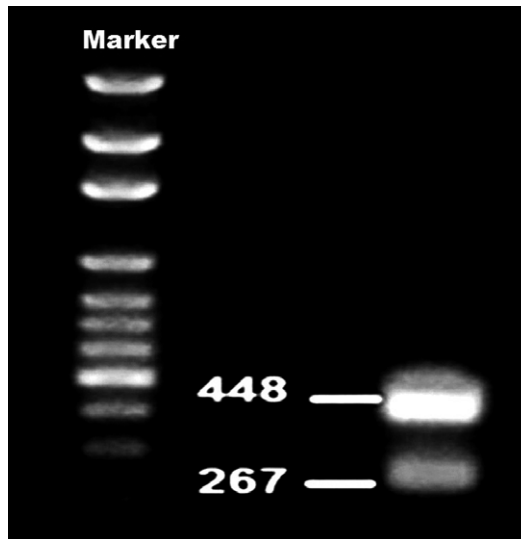
–, absent.

were obtained on 1% agarose gel. All the five phenotypic isolates showed the specific 567-bp PCR product corresponding to the phospholipase-D gene on agarose gel (Fig. 3).

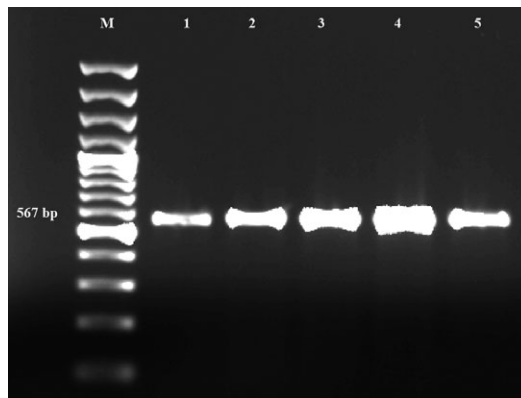
For studying the virulence, cobia (mean length: 13 cm; mean weight: 20 g) were maintained in six groups in glass tanks containing 200 l filtered sea water (salinity: 34‰; DO: 5 mg L<sup>-1</sup>; pH: 8.2; temperature: 29 °C) with 12 fish in each tank in duplicates. Fish in tanks one to five were intraperitoneally injected with a 24-h old bacterial suspension (in PBS; pH: 7.4) at a concentration of 10<sup>3</sup>–10<sup>7</sup> CFU g<sup>-1</sup> fish. Sterile PBS was injected into the fish in the sixth tank. Mortalities were recorded daily for 2 weeks. Bacteria were re-isolated from the blood of freshly dead fish. LD<sub>50</sub> values were calculated following Reed & Muench (1938). The virulence studies indicated LD<sub>50</sub> values of 4.1 × 10<sup>5</sup> CFU g fish<sup>-1</sup> for juvenile cobia. *Photobacterium damsela* subsp. *damsela* was re-isolated in pure culture from blood and liver of all freshly dead fish post-challenge.

In this study, a pathogenic strain of *P. damsela* subsp. *damsela* was isolated for the first time from an epizootic of mortality in cage-farmed cobia. This bacterium is reported to be an emerging pathogen due to its frequent isolation from newly cultured fish species with diverse geographical distribution (Labella *et al.* 2010). The gross lesions were analogous to those reported in Asian





**Figure 2** Agarose gel electrophoresis of the multiplex PCR products of *P. damsela* subsp. *damsela*. Lane 1: 100-bp ladder; Lane 2: amplification products corresponding to the 448- and 267-bp internal fragments of the *ureC* and 16s rRNA genes, respectively.



**Figure 3** PCR amplification product of the 567-bp *dly* gene fragment of *P. damsela* subsp. *damsela* strains. M: 100-bp ladder; one to five: five phenotypic isolates of the pathogen.

seabass infected with *P. damsela* subsp. *damsela* (Kanchanopas-Barnette *et al.* 2009).

Bacterial ECP exhibited several enzymatic activities. Not much is known about the role of ECP in the pathogenesis of *P. damsela* subsp. *damsela* in fish. The components of the ECP are chief antigenic proteins in many vaccine preparations (Collado *et al.* 2000). Labella *et al.* (2010) observed phospholipase, lipase, amylase and protease activity without the presence of caseinase and gelatinase activities in the ECP of *P. damsela* subsp. *damsela* isolated from several cultured

marine fish species. Haemolytic activity was present in only one strain, and phospholipase activity was lost post-heat treatment. Fouz *et al.* (1993) demonstrated phospholipase and haemolytic activities in the ECP. However, bacterial isolates in this study exhibited caseinase, phospholipase and haemolytic activity with the absence of gelatinase activity. Reports on the enzyme activity of *P. damsela* subsp. *damsela* using API ZYM system are scanty. The enzymatic activities of our isolate as recorded by the API ZYM system were similar to those reported by Labella *et al.* (2010) with the exception of  $\alpha$ -chymotrypsin activity, which was lowest in their strains. Further, in our study, the activities of  $\alpha$ -chymotrypsin and  $\alpha$ -glucosidase were absent in the ECP and  $\alpha$ -chymotrypsin activity was lowest in live cells. Absence of activity of many enzymes in the ECP was attributed to internalization of some substrates and association of enzymes with the cell envelop (Magarinos *et al.* 1992).

Pedersen *et al.* (2009) attributed the haemorrhagic nature of the infection to the presence of a potent haemolysin known as damselysin, encoded by *dly* gene. Damselysin is a best studied virulence factor in *P. damsela* subsp. *damsela* (Fouz *et al.* 1993). In this study, all the five isolates showed the presence of *dly* gene suggesting that the pathogen was strongly haemolytic. According to Labella *et al.* (2010), the virulence and haemolytic activity were directly related in *P. damsela* subsp. *damsela* isolated from different fish species. However, *dly* gene was not present in all the strains. The authors further observed that the strains without *dly* gene were non-haemolytic or with weak haemolytic activities which could be due to the presence of other haemolytic proteins. Recently, it was reported that the haemolytic activity of plasmidless strains of *P. damsela* subsp. *damsela* was due to the chromosome-encoded *hlyA* gene which was present in all the strains of *P. damsela* subsp. *damsela* (Rivas *et al.* 2013b). A synergistic effect between *dly* and *hlyA* was responsible for the contribution of haemolysis for the virulence.

The experimental studies indicated that the *P. damsela* subsp. *damsela* strain was virulent to cobia.

Increased water temperature observed during the period of disease outbreak in the present case could be a predisposing factor for the outbreak of mortality in cobia confined to cages. As *P. damsela* subsp. *damsela* can survive in sea water and

sediment for a long period of time by maintaining infectivity and pathogenic properties (Fouz *et al.* 1998), new host species can be infected through water when temperature and salinity are favourable (Fouz *et al.* 2000). Further, Pedersen *et al.* (2009) suggested that the occurrence of disease caused by this pathogen is likely to increase due to anticipated increase in seawater temperatures consequent to global climate changes because this bacterium prefers warm water.

To conclude, a pathogenic strain of *P. damsela* subsp. *damsela* isolated from an epizootic outbreak of mortality in cobia cultured in sea cages was characterized. The occurrence of this pathogen in cage-reared cobia should merit research interest due to two reasons: the pathogen being strongly haemolytic, would be responsible for mortality in cobia leading to economic losses and the pathogen is able to cause ailments in humans.

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