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Vibrio alginolytigus causing shell disease in the mud crab *Scylla serrata* (Forskal 1775)

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Scylla serrata is one of the most cultured mud crab species in the aquaculture which is also susceptible to shell disease. In the present study, *Vibrio alginolyticus* MF680287.1 caused by shell disease and isolated from infected mud crab *S. serrata* grow out pond located at Mahendrapalli, Nagapattinam District, Tamil Nadu, India. Further, gross observation of infected mud crab showed shell lesion on the dorsal carapace. The histological examination of normal and diseased mud crab *S. serrata* carapace and gills was conducted. The shell lesion affected in the *S. serrata* carapace layers showed loss of membrenous layer and epithilium. The bacterial colonies were abundant in the cuticle. The gill lamellae showed cuticlar damage in the formation of haemocyte nodules and eosinophilic granular cells.

[**Keywords:** *Scylla serrata*; *Vibrio alginolytigus*; Phylogenetic tree; Histology]

Introduction

Mud crab, *Scylla serrata* (Forsskal, 1775) belongs to the genus Scylla and family Portunidae. It is one of the exotic aquaculture species and economically important crustacean species. The mud crab fattening and grow out culture has developed rapidly in the past few years worldwide. However, outbreaks of shell disease in the mud crab grow out culture led to economic losses in Pemalang district, Central Java, Indonesia, and India[1,2].

The shell disease due to mainly Vibrio species affects the culture pond with poor water quality, stress, high stocking density and environmental conditions[3]. Vibrios are gram-negative bacteria, ubiquitous in marine and estuarine as well as aquaculture farms, and comprise one of the major microbiota of these ecosystems[4,5]. Vibriosis, caused by infection of *Vibrio* spp, is one of the most prevalent diseases in the aquaculture-reared organisms and is widely responsible for mortality in aquaculture culture systems worldwide[6]. The mass mortality of shrimp and crab due to Vibrio species, such as *V. alginolyticus*, *V. damsela*, *V. parahaemolyticus*, *V. Íulnificus* and *V. penaeicida*have has been reported in Indonesia[7], Thailand[8], Philippines[9], Taiwan[10], Ecuador[11], Australia[12], and India[13]. Clinical signs of disease range from localized cuticular lesions and oral and enteric infections to septicaemia[14]. The abundance of Vibrio spp. Is due to their ubiquity, multiplication rates and ability to adapt environmental changes in shrimp and crab culture. The present study was *Vibrio alginolyticus* MF680287.1 isolated from mud crab *S. serrata* carapace causing shell disease reported in the grow out culture system.

Materials and Methods

Infected mud crab *S. serrata* samples were collected from brackish water pond of mud crab grow out culture system in Puliyandurai, Nagapattinam District, Tamil Nadu, India. The moribund samples were processed immediately in the laboratory. Infected mud crab carapace erosion was washed thoroughly with sterilized distilled water. The carapace (0.1 to 0.5 g) were dissected with sterile scissors and homogenized in physical saline (PS) under aseptic conditions. The 1 mL of a dilution of 10^{-1}, 10^{-3} and 10^{-5} CFU mL^{-1} was spread on the specific media for vibrio, TCBS (Thiosulphate citrate bile salts sucrose) and was incubated at room temperature for 24 to 48 hr[15,16]. Then, based on the morphological feature, colonies were randomly selected and purified using streak plate method. To obtain pure isolates, purification was performed and then pure isolates were stored in nutrient agar followed by gene analysis for 16S rRNA.

Identification using 16S rRNA gene sequence

Genomic DNA of bacterial isolates was extracted using the QIAamp genomic DNA kit. The 16S rRNA gene was amplified by PCR using the universal primer set (7F and 1492R)[17]. The thermal cycling

parameters used were: 5-min hot start at 95 °C; followed by 32 cycles of denaturation for 1 min at 94 °C, annealing at 55 °C for 1 min and extension at 72 °C for 1.5 min; and a final extension of 20 min at 72 °C. Approximately, 1.5-kb products were obtained. A partial fragment or full-length of the 16S rRNA gene was sequenced by big dye terminated v3.1 cycle sequencing k*it's* as per manufacturers instruction (Thermo Fisher Scientific USA).

Histopathology

Normal and infected mud crab carapace and gill filaments were preserved in Davidson`s fixative for 48 hr after they were transferred in 70% ethanol. The biopsies were processed as per standard procedure like embedding in paraffin wax and preparing the thin sections of 4 μm with rotary microtome (Yorco YSI-115). The sections were stained with haematoxylin and eosin (H&E) and evaluated for infection under the light microscope (Magniis).

Results

Gross morphologic finding

In male *Scylla serrata* infection, shell disease was in the left side dorsal carapace (crab weight 230 g; carapace length 9.2 cm; width 7.3 cm; shell lesion length 2.8 cm). The shell erosion was characterized by oval shape, outer surface carapace lesion in pale colour. The severe lesion of the carapace varied from hard to soft tissue in texture (Figs 1A and B).

Fig.1 — (A) Shell disease infected mud crab *S. serrata* (circle), (B) Close up view of shell disease (arrow).

Histopathology

The results of histological investigation indicated that normal *S. serrata* carapace was present in the epicuIticle, exocuIticle, endocuticle, epithilium and membrenous layer. In case of severe erosion on the *S. serrata,* carapace was degenerated and the membrenous layer and epithilium entirely interrupted. The highly vacuolated cuticular from underlaying epithilium was observed. The erosion of the carapace was due to bacterial colonies found abundant in the surface of the cuticle (Fig. 2).

The result of histological investigation of normal *S. serrata* gill lamelae present in the end of cuitcle is shown in Fig. 3A. The infected gill was characterized by swollen and club-shaped lamellae (Fig. 3B). The severe lesion of the gill stem infected in the haemocyte nodules and eosinophilic granular cells were accumulation of epithilial cells (Figs 3C and D).

Phylogenetic analysis

The identification of deep rooted bacteria *V. alginolyticus* was through 16S rRNA gene sequencing

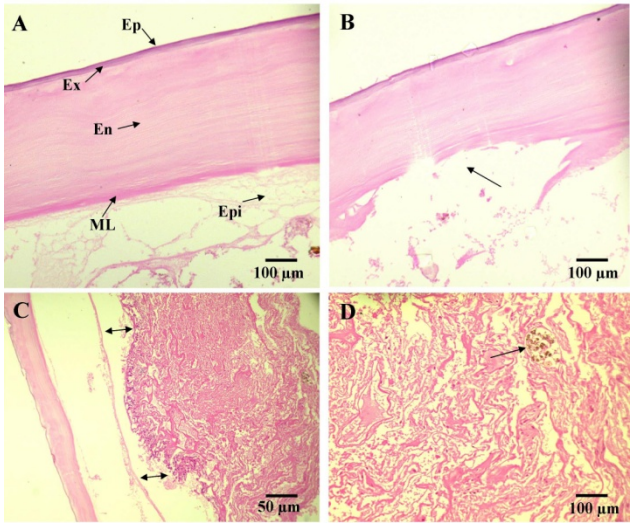

Fig. 2 — Histological section of exoskeleton of mud crab *S. serrata* infected by bacteria, (A) Normal *S. serrata* carapace, (B) Severe erosion of membranous layer, (C) A space separates the cuticle from the epithelium (double arrow), (D) Bacteria invading in the cuticle (arrow). Key: Ep-epicuticle; Ex-exocuticle; En-endocuticle; ML-membranous layer; epi-epithelium; eg-eosinophilic granular haemocytes.

and phylogenetic analysis. The homology analysis by BLAST concluded that the isolate ggss2 (MF680287.1) closely resembled *V. alginolyticus* MF062663.1 (100%). In addition, phylogenetic tree analysis showed that the monophyly of isolate with respect to reference taxa and extremely short tree branches indicate minimal difference among the analyzed sequence (Fig. 4). In addition, the phylogenetic tree indicated close relationship between vibrio.

Discussion

The outbreak of bacterial and fungal infection results in huge loss to aquaculture industry throughout the world[18]. The pathogenic bacteria play a negative role as they compete with shrimps and crabs causing stress and shell disease (Moriarty). Generally gram-negative bacteria are found to be the dominant forms in the shrimp and crab culture ponds. The bacteria belonging to the genus Vibrio, such as *V. harveyi, V. alginolyticus and V. parahaemolyticus* were frequently reported to be involved in shell disease outbreaks in *L. vannamei* shrimp and mud crab *S. serrata*[19,20,21]. Vibrio species are the major disease-causing bacteria normally found in the polluted environment[22,23,24]. In previous studies, *V. anguillarum* was found to be one of the most virulent pathogenic strains in marine environment[25,26]. However, the pathogenic bacteria *V. alginolytigus* MF680287.1 causes high mortality of mud crab culture in the farm. *V. alginolytigus* MF680287.1 were isolated from infected *S. serrata* from grow out pond. This strain was the dominate bacterium recovered from the dorsal carapace of infected crab.

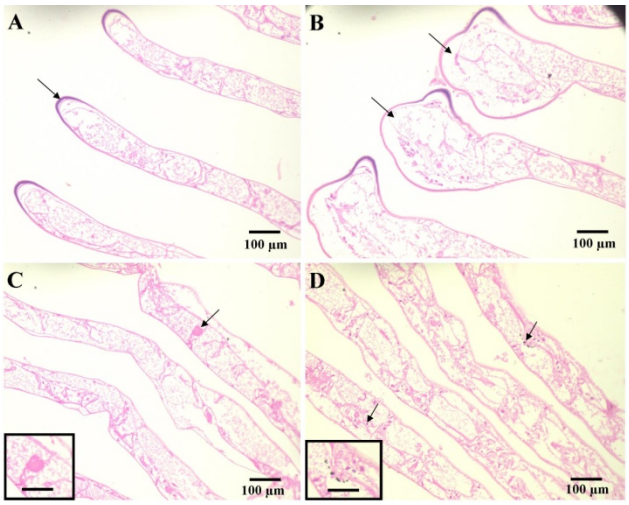

Fig. 3 — (A) Histological section of normal *S. serrata* gill lamellae, (B) Infected mud crab *S. serrata,* swollen and club shape of gill lamellae (arrow), (C) Severe lesion of gill lamellae infected in haemocytic nodules (arrow), insets (bar: 200 µm), (D) Accumulation of eosinophilic granular haemocytes (arrow), insets (bar: 200 µm).

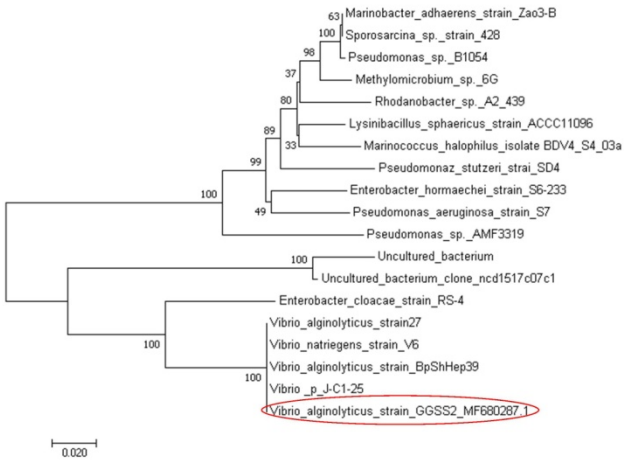

Fig. 4 — Phylogram showing the relationship between vibrio alginolyticus strain GGSS2 MF680287.1 and other related species.

The studies observing shell disease have relied entirely upon gross evaluation[27,28]. Our study suggest that infected mud crab diagnosis based on gross observation might be used as a crude indicator of shell disease infecting the dorsal carapace and it is oval in shape and pale in colour.

The severe shell disease revealed histopathological changes of several internal organs and tissues[29,30,31,32]. The increased nephrocytes number and dimension in *S. serrata*, along with necrotic and degenerative changes in the gills and hepatopancreas, respectively, have been frequently observed as a consequence of constant exposure to polluted environment[33]. However, the severe lesion effect on the crab carapace was the loss of cavity, including membranous layers and epithelium; The bacterial colonies were abundant in the cuticle. The severity of shell disease was found in *Liocarcinus puber, L. corrugatus* and *L. depurator* with gill damage of nephrocytes[34]. The present results show gill lamellae damage observed in *S. serrata*. The eosinophilic granular haemocytic infiltration and nodule formation in the gill lamellae of *S. serrata* has been observed in the nephrocytic changes of shell disease, which might be directly due to *V. alginolytigus* MF680287.1 In the present study *V. alginolytigus* was isolated from *S. serrata* carapace found responsible for organisms becoming vulnerable to other diseases and poor marketability thereby, due to unfavourable conditions such as polluted water.

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References

1 Lavilla-Pitogo, C.R., & de la Peña, L.D., Diseases in farmed mud crabs *Scylla spp.:* Diagnosis, prevention, and control. Aquaculture Dept., Southeast Asian Fisheries Development Center (2004).

2 Austin, B., & Allen-Austin, D., A review. *Journal of Applied Microbiology*, 58(5) (1985) 483-506.

3 Austin, B., Austin, D.A., Vibriosis, Bacterial fish pathogens and disease in farmed wild fish. Chapter 13, Ellis Harwood Ltd. England (1993) 263-287.

4 Hjeltnes, B., Roberts, R.J., Vibriosis, In Bacterial Diseases of Fish, Edited by Inglis V Roberts, RJ, Bromage NR. Oxford: Blackwell Scientific (1993) 109-121.

5 Chen, F.R., Liu, P.C., Lee, K.K., Lethal attribute of serine protease secreted by *Vibrio alginolyticus* strains in Kurama Prawn *Penaeus japonicus*. Zool Naturforsch 55 (2000) 94-99.

6 Karunasagar, I., Otta, S.K., & Karunasagar, I., Biofilm formation by Vibrio harveyi on surfaces. *Aquaculture*, 140(3) (1996) 241-245.

7 Sunaryanto, A., & Mariam, A., Occurrence of a pathogenic bacteria causing luminescence in penaeid larvae in Indonesian hatcheries. *Bull Brackishwater Aquac Dev Cent*, 8 (1986) 105-112.

8 Jiravanichpaisal, P., Miyazaki, T., & Limsuwan, C., Histopathology, biochemistry, and pathogenicity of Vibrio harveyi infecting black tiger prawn *Penaeus monodon*. *Journal of Aquatic Animal Health*, 6(1) (1994) 27-35.

9 Lavilla-Pitogo, C.R., Baticados, M.C.L., Cruz-Lacierda, E.R., & Leobert, D., Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. *Aquaculture*, 91(1) (1990) 1-13.

10 Liu, P.C., Lee, K.K., Yii, K.C., Kou, G.H., & Chen, S.N., News & Notes: Isolation of *Vibrio harveyi* from Diseased Kuruma Prawns *Penaeus japonicus*. *Current Microbiology*, 33(2) (1996) 129-132.

11 Robertson, B.R., Button, D.K., & Koch, A.L., Determination of the biomasses of small bacteria at low concentrations in a mixture of species with forward light scatter measurements by flow cytometry. *Applied and environmental microbiology*, 64(10) (1998) 3900-3909.

12 Pizzutto, M., & Hirst, R.G., Classification of isolates of Vibrio harveyi virulent to *Penaeus monodon* larvae by protein profile analysis and M13 DNA fingerprinting. *Diseases of Aquatic Organisms*, 21(1) (1995) 61-68.

13 Karunasagar, I., Pai, R., Malathi, G.R., & Karunasagar, I., Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*, 128(3) (1994) 203-209.

14 Lightner, D.V., A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp (1996).

15 Brock, T., Madigan, M., Martinko, J.M., & Parker, J., Biology of microorganisms. 6th (1991).

16 Sarjito, Aplikasi Biomolekuler untuk Deteksi Agensia Penyebab Vibriosis pada Ikan Kerapu dan Potensi Bakteri Sponge sebagai Anti Vibriosis. [Disertasi]. Program Pasca Sarjana, Universitas Diponegoro, Semarang 2010.

17 Liu, C., & Shao, Z., Alcanivorax dieselolei sp. nov., a novel alkane-degrading bacterium isolated from sea water and deep-sea sediment. *International Journal of Systematic and Evolutionary Microbiology*, 55(3) (2005) 1181-1186.

18 Karthikeyan, V., Selvakumar, P., & Gopalakrishnan, A., A novel report of fungal pathogen *Aspergillus awamori* causing black gill infection on *Litopenaeus vannamei* (pacific white shrimp). *Aquaculture*, 444 (2015) 36-40.

19 Esteve, M., & Herrera, F.C., Hepatopancreatic alterations in *Litopenaeus vannamei* (Boone, 1939)(Crustacea: Decapoda: Penaeidae) experimentally infected with a *Vibrio alginolyticus* strain. *Journal of invertebrate pathology*, 76(1) (2000) 1-5.

20 Soto-Rodriguez, S.A., Gomez-Gil, B., & Lozano, R., 'Bright-red'syndrome in Pacific white shrimp *Litopenaeus vannamei* is caused by *Vibrio harveyi*. *Diseases of aquatic organisms*, 92(1) (2010) 11-19.

21 Soto-Rodriguez, S.A., Gomez-Gil, B., Lozano, R., del Rio-Rodríguez, R., Diéguez, A. L., & Romalde, J.L., Virulence of *Vibrio harveyi* responsible for the "Bright-red" Syndrome in the Pacific white shrimp *Litopenaeus vannamei*. *Journal of invertebrate pathology*, 109(3) (2012) 307-317.

22 Yasuda, K., & Kitao, T., Bacterial flora in the digestive tract of prawns, *Penaeus japonicus* Bate. *Aquaculture*, 19(3) (1980) 229-234.

23 Sharmila, R., Jawahar Abraham, T. and Sundararaj, V., Bacterial flora of semi-intensive pond reared *Penaeus indicus* (H.Milne Edwards) and the environment. *Journal of Aquaculture in the Tropics* 11 (1996) 193–203.

24 Sindermann, C.J., Pollution-associated diseases and abnormalities of fish and shellfish: a review. *Fishery bulletin*, 76(4) (1979) 717-749.

25 Olsen, G.J., Larsen, N., & Woese, C.R., The ribosomal RNA database project. *Nucleic Acids Research*, 19 (1991) 2017.

26 Chythanya, R., Karunasagar, I., & Karunasagar, I., Inhibition of shrimp pathogenic vibrios by a marine Pseudomonas I-2 strain. *Aquaculture*, 208(1) (2002) 1-10.

27 Sandifer, P.A., & Eldridge, P.J., Observations on the incidence of shell disease in South Carolina blue crabs, *Callinectes sapidus*. In *Proceedings of Gulf Coast Regional Symposium on Diseases of Atlantic Animals, Baton Rouge, La,* 16 (1974) 161-184.

28 Sindermann, C.J., The shell disease syndrome in marine crustaceans (1989).

29 Vogan, C.L., Costa-Ramos, C., & Rowley, A.F., A histological study of shell disease syndrome in the edible crab *Cancer pagurus*. *Diseases of aquatic organisms*, 47(3) (2001) 209-217.

30 Smolowitz, R.M., Bullis, R.A., & Abt, D.A., Pathologic cuticular changes of winter impoundment shell disease preceding and during intermolt in the American lobster,

Homarus americanus. *The Biological Bulletin*, 183(1) (1992) 99-112.

31 Ayres, P.A., & Edwards, E., Notes on the distribution of "black spot" shell disease in crustacean fisheries. *Chemistry in Ecology*, 1(2) (1982) 125-130.

32 Ryazanova, T.V., Histopathological changes associated with shell disease in the red king crab *Paralithodes camtschaticus* (Tilesius, 1815). *Russian Journal of Marine Biology*, 31(6) (2005) 359-366.

33 Stentiford, G.D., Evans, M., Bateman, K., & Feist, S.W., Co-infection by a yeast-like organism in Hematodinium-infected European edible crabs Cancer pagurus and velvet swimming crabs Necora puber from the English Channel. *Diseases of aquatic organisms*, 54(3) (2003) 195-202.

34 Comely, C.A., & Ansell, A.D., The occurrence of black necrotic disease in crab species from the west of Scotland. *Ophelia*, 30(2) (1989) 95-112.