Journal of Fish Diseases 2006, **29,** 187–191

Short communication

Isolation of a pathogenic strain of *Vibrio alginolyticus* from necrotic larvae of *Macrobrachium rosenbergii* (de Man)

N S Jayaprakash[1], S S Pai[1], R Philip[2] and I S B Singh[1]

1 Centre for Fish Disease Diagnosis and Management, School of Environmental Studies, Cochin University of Science and Technology, Cochin, Kerala, India

2 Department of Marine Biology, Microbiology and Biochemistry, Cochin University of Science and Technology, Cochin, Kerala, India

Keywords: haemolysis, *Macrobrachium rosenbergii*, necrosis, pathogenicity, prawn larvae, *Vibrio alginolyticus*.

Giant freshwater prawn, *Macrobrachium rosenbergii* (de Man), is an important commercial species with considerable export value, ideal for cultivation under low saline conditions and in freshwater zones (Kurup 1994). However, despite more than a decade of research on its larval production systems, vibriosis still hampers seed production resulting in high mortality rates. Among the different species of vibrios, *Vibrio alginolyticus* has been isolated frequently from diseased shrimp as the aetiological agent of vibriosis and has been described as a principal pathogen of both penaeids and non-penaeids (Lightner 1988; Baticados, Cruz-Lacierda, de la Cruz, Duremdez-Fernandez, Gacutan, Lavilla-Pitogo & Lio-Po 1990; Mohney, Lightner & Bell 1994; Lee, Yu, Chen, Yang & Liu 1996). *Vibrio fluvialis*, *V. alginolyticus*, *V. cholerae* non-O1 (Fujioka & Greco 1984), *Aeromonas liquifaciens* and *V. anguillarum* (Colorni 1985) have been isolated from the larvae of *M. rosenbergii*. A profound relationship between the abundance of members of the family Vibrionaceae and larval mortality (Singh 1990) and the predominance of *Vibrio* in eggs, larvae and post-larvae of *M. rosenbergii* (Hameed, Rahaman, Alagan & Yoganandhan 2003) was reported. The present paper reports the isolation, characterization, pathogenicity and antibiotic sensitivity of *V. alginolyticus* associated with *M. rosenbergii* larvae during an occurrence of severe mass mortality at the ninth larval stage.

Correspondence *I S B Singh, Centre for Fish Disease Diagnosis and Management, School of Environmental Studies, Cochin University of Science & Technology, Lake Side Campus, Fine Arts Avenue, Cochin 682 016, Kerala, India (e-mail: bsingh@md3.vsnl.net.in)*

Moribund larvae (about 500) were collected from M/s Rosen Fisheries (Trichur, Kerala). The larvae were reared in 15‰ sea water, fed on freshly hatched *Artemia* nauplii until stage 6, and supplemented with egg custard subsequently. Water quality parameters monitored were pH (7.5–8.0), temperature (25–28 °C), total ammonia (< 0.1 ppm) and nitrite (< 1.0 ppm). Partial water exchange (30%) was provided daily. The diseased samples were obtained from a tank (5-tonne capacity with 500 000 mysis as the initial stocking density) where the majority of the larvae at stage 9 had displayed anorexia, inactivity, poor growth, morbidity and necrotic appendages. Collection of moribund larvae and the rearing water was made in sterile polypropylene bottles (autoclaved at 121 °C for 15 min) with sterile sea water (15‰) as the transport medium. The samples were transported in an ice-chest at 4 °C and analysed in the laboratory within 2 h of collection.

Larvae ($n = 30$) were washed in a 20-mL volume of sterile (autoclaved at 121 °C for 15 min) sea water (15‰) three times for a duration of 1 min each, and macerated to a fine paste in a sterile glass

tissue homogenizer. The homogenized sample was diluted serially to 10^{-6} in autoclaved sea water (15‰). Volumes (0.2 mL) were spread over ZoBell's marine 2216E agar plates prepared in 15‰ sea water (peptone 0.5%, yeast extract 0.1%, ferric phosphate 0.01%, pH 7.5) in duplicate and incubated at 28 ± 1 °C for 24 h. Colonies were grouped based on their morphology following Cappuccino & Sherman (1996) (Table 1) and from each group 10% of the colonies were isolated into ZoBell's marine agar slants. The colonies were repeatedly streaked on ZoBell's agar plates until purity was attained. All Gram-negative isolates were identified to genera following Oliver (1982), and Gram-positive cultures following McFaddin (1980). Among seven morphological types, type A was dominant comprising 57.7% of the isolates and was identified as *V. alginolyticus* following Alsina & Blanch (1994). All isolates of this species were uniformly rod shaped, able to swarm on ZoBell's marine agar plates, fermentative without gas production from glucose, catalase and oxidase-positive, motile, produced yellow colonies on thiosulphate citrate bile-salts sucrose agar plates, sensitive to O/129 (150 μg), negative for arginine dihydrolase but positive for ornithine and lysine decarboxylases, positive in the Voges–Proskaeur reaction, able to utilize citrate, produce indole, and utilize D-glucosamine as sole carbon source. The isolates produced acid from sucrose, but not from salicin, arabinose and inositol, hydrolysed gelatin, tributyrin, DNA and chitin, did not grow without NaCl, but grew at 10% NaCl, and showed growth at 30, 35 and 40 °C but not at 4 °C. The organisms were maintained in ZoBell's marine agar and antibiotic sensitivity tests were carried out by the disc diffusion method in Müeller–Hinton agar supplemented with 1.5% NaCl, using the following antimicrobial agents (μg/disc): ampicillin (10), streptomycin (10), rifampicin (2), neomycin (30), erythromycin (10), kanamycin (30), chloramphenicol (10), ciprofloxacin (5), oxytetracycline (30), novobiocin (30), furazolidone (50), nitrofurantoin (100), trimethoprim (5) and tetracycline (30). On examining the level of sensitivity of *V. alginolyticus* isolates to antibiotics, the organisms were resistant to ampicillin, erythromycin, chloramphenicol, oxytetracycline and tetracycline but sensitive to others (12–15 mm clearing zone).

Table 1 Generic composition of heterotrophic bacteria from necrotic larvae of *Macrobrachium rosenbergii* and the rearing water

Morphological types	Number of colonies in larvae (10^{-3} dilution)	Genera	% Composition (in larvae)
A	75	*Vibrio*	57.7
B	20	*Aeromonas*	15.4
C	3	*Pseudomonas*	2.3
D	3	*Alcaligenes*	2.3
E	22	*Cytophaga*	16.9
F	6	*Alteromonas*	4.6
G	1	*Bacillus*	0.8

A – Small, circular, convex, entire, translucent colony; B – moderate, circular, umbonate, entire, translucent colony; C – moderate, circular, convex, entire, brown colony; D – small, circular, convex, entire, pale white colony; E – small, circular, convex, entire, yellow colony; F – small, irregular, raised, undulate, white colony; G – moderate, irregular, flat, rhizoidal, white colony.

A haemolytic assay in prawn blood agar was done according to the protocol of Chang, Liu & Shyu (2000), with modifications. Prior to blood collection, adult *M. rosenbergii* (30 ± 10 g) were maintained in fresh water for a week on a scampi diet (Higashimaru, India), with 100% daily water exchange. The area beneath the rostral spine was disinfected with sodium hypochlorite (200 mg L^{-1}) for 5 min followed by administration of 70% ethanol. The area was washed with autoclaved (121 °C for 15 min) distilled water repeatedly and wiped dry with autoclaved absorbent cotton swabs. Haemolymph was drawn from wild caught adult (30 ± 10 g) *M. rosenbergii* using sterile capillary tubes. To prevent clotting of the haemolymph, citrate–EDTA buffer (0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA dissolved in double distilled water) was used to rinse the capillary tubes prior to blood collection. The pH of the buffer was adjusted to 4.6 and osmolality to 350 mOsm (by adding sodium chloride) and the solution sterilized at 115 °C for 10 min. One millilitre of haemolymph was transferred to an autoclaved tube containing 0.2 mL citrate–EDTA buffer and stained by addition of 140 μL of 2% (w/v) Rose Bengal (dissolved in citrate–EDTA buffer) with gentle rotation to achieve complete mixing. The basal agar medium (10 g bacto peptone, 5 g sodium chloride and 15 g bacto agar dissolved in 1000 mL distilled water) was adjusted to pH 6.8, autoclaved and cooled to 45–50 °C in a water-bath. Aseptically, 1 mL of the stained haemolymph preparation was added to 15 mL of the prepared basal medium followed by gentle mixing and pouring into Petri dishes. The isolates of *V. alginolyticus* were streaked on to prawn blood

agar and incubated at 28 ± 1 °C for 3–7 days. The plates were observed for clearing around the growth and lysis of haemocytes using a microscope with a 10× objective. All eight isolates of *V. alginolyticus* were haemolytic on prawn blood agar. Ruangpan & Kitao (1991), Chang, Lee, Shyu & Liao (1996) and Chang *et al.* (2000) suggested bacterial haemolysin as an important virulence factor of pathogenic vibrios and correlated it with their pathogenicity more than factors such as adhesion and invasion (Ruangpan, Tabkaew & Sangrungruang 1994). Zhang & Austin (2000) highlighted the importance of haemolysin as a virulence factor of pathogenic vibrios in fish. Additionally, the haemolytic assay has been recognized to be useful in differentiating the virulent strains among suspected pathogens by Ullah & Arai (1983), Chen, Lai & Huang (1995) and Lee, Chen & Liu (1995). Chang *et al.* (2000) used prawn blood agar haemolysis to screen bacteria pathogenic to cultured tiger prawn, *Penaeus monodon*. The present report is the first demonstration of haemolytic activity of *V. alginolyticus* associated with the larvae of *M. rosenbergii* in prawn blood agar.

Hydrolytic properties such as proteolysis, lipolysis, chitinolysis and DNase production were carried out following the techniques described by Frazier (1926), Rhodes (1959), Holding & Collee (1971) and Jeffries, Holtman & Guse (1957), respectively. The results indicated that all the isolates of *V. alginolyticus* in this study produced gelatinase, lipase, DNase and chitinase, all probable virulence factors, which might help the organisms to invade the larvae and proliferate. Toranzo, Barja, Colwell & Hetrick (1983) and Ellis (1991) suggested this as a general feature of expression of pathogenicity of vibrios. A comprehensive search for virulence factors among vibrios by earlier workers has unequivocally revealed the role of proteases, lipases, DNase, chitinase and plasmids coding for iron chelators, apart from haemolysins, in initiating infectious mortality (Reid, Woods & Robb 1980; Horne 1982; Toranzo *et al.* 1983; Moustafa, Kodama, Ishiguro, Mikami & Izawa 1984; Nottage & Birkbeck 1987; Janda, Powers, Bryant & Abbott 1988; Wong, Ting & Shieh 1992; Austin, Stobie, Robertson, Glass & Stark 1993). Cipriani, Wheller & Sizemore (1980) reported that chitinolytic activity is fundamental to lesion progression; microbial proteases and lipases may be involved in exoskeleton breakdown, particularly in the initial stages of shell disease. Vogan, Costa-Ramos & Rowley (2002) considered chitinolytic bacteria to be involved in shell disease syndrome in the edible crab, *Cancer pagurus*. It seems reasonable to suggest that the hydrolytic enzymes of *V. alginolyticus* described here may play a role in pathogenesis in the larvae of *M. rosenbergii*.

The pathogenicity of *V. alginolyticus* for larvae of *M. rosenbergii* was evaluated under laboratory conditions. Larvae of *M. rosenbergii* at stage 9 were brought to the laboratory from the hatchery of M/s Rosen Fisheries, Trichur, Kerala in oxygen filled polythene bags. They were maintained in 15‰ sea water and were fed with freshly hatched *Artemia* nauplii. The experiment was conducted as follows: sea water (15‰) was autoclaved (121 °C/15 min) and transferred (1 L) into 2-L capacity round plastic containers and aerated at a rate of 1 L min^{-1}. Apparently healthy larvae, characterized by their photosensitivity, were removed from the top of the holding tank and distributed in groups of 30 to 12 experimental containers, nine as tests and three as controls. Larvae in both sets of containers were fed uniformly with freshly hatched *Artemia* nauplii at a rate of 300 nauplii per larva of *M. rosenbergii* daily. An overnight culture of *V. alginolyticus* (MRNL-3) was harvested from ZoBell's marine agar plates and the bacterial cells were transferred to autoclaved (121 °C/15 min) sea water (15‰) and diluted to a concentration equivalent to optical density 1.0 at $A_{600\ nm}$. This suspension of bacteria contained approximately 1×10^{10} CFU mL^{-1}, as determined by serial dilution and plating on ZoBell's marine agar. The containers with larvae demarcated as 'tests' were challenged by inoculating the rearing water to a final bacterial number of 10^6, 10^7 and 10^8 CFU mL^{-1}. All challenge concentrations were done in triplicate. Three control tanks were maintained without challenge. The larvae were observed for 4 days and mortality assessed daily to determine the cumulative mortality. During this period no water exchange was provided and the water quality parameters such as temperature, pH, ammonia and nitrite recorded were within the ranges recommended by New & Singholka (1982) and Correia, Suwannatous & New (2000), i.e. 28–29 °C, pH 7.5–8, ammonia and nitrite < 0.1 ppm. On challenging the larvae with *V. alginolyticus* (MRNL-3) at 10^6, 10^7 and 10^8 CFU mL^{-1} in triplicate, percentage mortalities of 80 (±10), 87 (±6) and 100 (±0) were observed, respectively, within 96 h. In the control group, the corresponding

mortality was 37 (±10)%. The chi-squared test showed that the mortality was significantly higher ($P < 0.01$) in the test groups than in the control. Signs preceding larval death included anorexia, settling to the bottom and weak swimming. The significantly higher mortality rate observed in the challenged set of larvae demonstrated the pathogenicity of *V. alginolyticus* to the larvae of *M. rosenbergii* in a confined system. However, this does not necessarily indicate that the strain of *V. alginolyticus* tested here (MRNL-3) is the putative pathogen responsible for the observed necrosis in the hatchery. Even though significant mortality was observed on challenging the larvae with the isolate, no sign of necrosis was seen. Moreover, the role of other associated flora in the necrosis was not determined.

Acknowledgements

This study was carried out under a project supported by the Department of Science and Technology (Project Code: SP/SO/C-40/99), Government of India. One of the authors (N.S. Jayaprakash) is grateful to the Department for providing a fellowship during the study. The authors are grateful to Mr C.D. Sebastian, Rosen Fisheries, Trichur, Kerala, for providing the larvae.

References

Alsina M. & Blanch A.R. (1994) A set of keys for biochemical identification of environmental *Vibrio* species. *Journal of Applied Bacteriology* **76,** 79–85.

Austin B., Stobie M., Robertson P.A.W., Glass H.G. & Stark J.R. (1993) *Vibrio alginolyticus*: the cause of gill disease leading to progressive low-level mortalities among juvenile turbot, *Scophthalmus maximus* (L.), in a Scottish aquarium. *Journal of Fish Diseases* **16,** 277–280.

Baticados M.C.L., Cruz-Lacierda E.R., de la Cruz M.C., Duremdez-Fernandez R.C., Gacutan R.Q., Lavilla-Pitogo C.R. & Lio-Po G.D. (1990) *Diseases of Penaeid Shrimps in the Philippines*. Aquaculture Department, SEAFDEC, Tigbauan, Iloilo, Philippines.

Cappuccino J.G. & Sherman N. (1996) *Microbiology – A Laboratory Manual*. Addison Wesley, Harlow, UK.

Chang C.I., Lee W.C., Shyu C.Z. & Liao I.C. (1996) The development of shrimp blood agar for testing the hemolysis of shrimp's haemocyte by bacteria. *Chinese Journal of Microbiology and Immunology* **29,** 232–239.

Chang C.I., Liu W.Y. & Shyu C.Z. (2000) Use of prawn blood agar hemolysis to screen for bacteria pathogenic to cultured tiger prawns *Penaeus monodon*. *Diseases of Aquatic Organisms* **43,** 153–157.

Chen J.D., Lai S.Y. & Huang S.L. (1995) Cloning of the hemolysin gene from *Edwardseilla tarda*. *Journal of the Fisheries Society of Taiwan* **22,** 267–277.

Cipriani G.R., Wheller R.S. & Sizemore R.K. (1980) Characterization of brown spot disease of Gulf Coast shrimp. *Journal of Invertebrate Pathology* **36,** 255–263.

Colorni A. (1985) A study on the bacterial flora of the giant prawn *Macrobrachium rosenbergii* larvae fed with *Artemia* nauplii. *Aquaculture* **49,** 1–10.

Correia E.S., Suwannatous S. & New M.B. (2000) Flow-through hatchery systems and management. In: *Freshwater Prawn Culture* (ed. by M.B. New & W.C. Valenti), pp. 52–68. Blackwell, Oxford.

Ellis A.E. (1991) An appraisal of the extracellular toxins of *Aeromonas salmonicida* ssp. *salmonicida*. *Journal of Fish Diseases* **14,** 265–277.

Frazier W.C. (1926) A method for detection of changes in gelatin due to bacteria. *Journal of Infectious Diseases* **39,** 302.

Fujioka R. & Greco S. (1984) Enumeration of *Vibrio* sp. in marine, brackish and freshwater systems used in aquaculture. In: *Abstracts of the Annual Meeting of the American Society for Microbiology*, p. 180. ASM, Washington.

Hameed A.S.S., Rahaman K.H., Alagan A. & Yoganandhan K. (2003) Antibiotic resistance in bacteria isolated from hatchery-reared larvae and post-larvae of *Macrobrachium rosenbergii*. *Aquaculture* **217,** 39–48.

Holding A.J. & Collee J.G. (1971) Routine biochemical tests. In: *Methods in Microbiology* **6A** (ed. by J.R. Norris & D.W. Ribbons), pp. 1–32. Academic Press, London.

Horne M.T. (1982) The pathogenicity of *Vibrio anguillarum* (Bergman). In: *Microbial Diseases of Fish. Special Publication of the Society for General Microbiology* (ed. by R.J. Roberts), pp. 171–187. Academic Press, London.

Janda J.M., Powers C., Bryant R.G. & Abbott S.I. (1988) Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clinical Microbiology Reviews* **1,** 245–267.

Jeffries C.D., Holtman D.F. & Guse D.G. (1957) Rapid method for determining the activity of microorganisms on nucleic acid. *Journal of Bacteriology* **73,** 590–591.

Kurup M. (1994) Status of fishery, seed production, and farming of *Macrobrachium rosenbergii* in Kerala. In: *Freshwater Prawn Farming in India* (ed. by N.K. Thakur, R. Tewari & M. Joseph), p. 113. Proceedings of the Workshop on Freshwater Prawn Farming in India, 17–18 March, 1994. Special Publication No. 10. Asian Fisheries Society Indian Branch, Mangalore, India.

Lee K.K., Chen F.R. & Liu P.C. (1995) A haemocytolytic assay for tiger prawn, *Penaeus monodon*. *Fish & Shellfish Immunology* **5,** 385–387.

Lee K.K., Yu S.R., Chen F.R., Yang T.I. & Liu P.C. (1996) Virulence of *Vibrio alginolyticus* isolated from diseased tiger prawn, *Penaeus monodon*. *Current Microbiology* **32,** 229–231.

Lightner D.V. (1988) *Vibrio* disease of penaeid shrimp. In: *Disease Diagnosis and Control in North American Marine Aquaculture. Developments in Aquaculture and Fisheries Science*,

Vol. 6 (ed. by C.J. Sindermann & D.V. Lightner), pp. 42–47. Elsevier, New York.

McFaddin J.F. (1980) *Biochemical Tests for Identification of Medical Bacteria*, 2nd edn. Williams and Wilkins, Baltimore, MD.

Mohney L.L., Lightner D.V. & Bell T.A. (1994) An epizootic of vibriosis in Ecuadorian pond reared *Penaeus vannamei* Boone (Crustacea: Decapoda). *Journal of the World Aquaculture Society* **25,** 116–125.

Moustafa M., Kodama H., Ishiguro S., Mikami T. & Izawa H. (1984) Partial purification of extracellular toxic material of fish vibrios. *American Journal of Veterinary Research* **45,** 2208–2210.

New M.B. & Singholka S. (1982) Freshwater prawn farming. A manual for the culture of *Macrobrachium rosenbergii*. *FAO Fishery Technical Paper* **225,** Rev. 1, 118.

Nottage A.S. & Birkbeck T.H. (1987) Production of proteinase during experimental infection of *Ostrea edulis* L. larvae with *Vibrio alginolyticus* NCMB 1339 and the antigenic relationship between proteinase produced by marine vibrios pathogenic for fish and shellfish. *Journal of Fish Diseases* **10,** 265–273.

Oliver J.D. (1982) Taxonomic scheme for the identification of marine bacteria. *Deep-Sea Research* **29,** 795–798.

Reid G.C., Woods D.R. & Robb F.T. (1980) Peptone induction and rifampin-insensitive collaginase production by *Vibrio alginolyticus*. *Journal of Bacteriology* **142,** 447–454.

Rhodes M.E. (1959) The characterization of *Pseudomonas fluorescens*. *Journal of General Microbiology* **21,** 221–263.

Ruangpan L. & Kitao T. (1991) *Vibrio* bacteria isolated from black tiger shrimp, *Penaeus monodon* Fabricius. *Journal of Fish Diseases* **14,** 383–388.

Ruangpan L., Tabkaew R. & Sangrungruang K. (1994) Bacterial flora of intensive cultured black tiger shrimp, *Penaeus monodon*. *Suisanzoshoku* **42,** 485–490.

Singh I.S.B. (1990) Bacterial flora of larvae and larval rearing system of the giant fresh water prawn *Macrobrachium rosenbergii*. In: *Proceedings of the Second Kerala Science Congress* (ed. by N.B. Nair), pp. 190–194. Trivandrum, India.

Toranzo A.E., Barja J.L., Colwell R.R. & Hetrick F.M. (1983) Characterization of plasmids in bacterial fish pathogens. *Infection and Immunity* **39,** 184–192.

Ullah A. & Arai T. (1983) Pathological activities of the naturally occurring strains of *Edwardsiella tarda*. *Fish Pathology* **18,** 65–70.

Vogan C.L., Costa-Ramos C. & Rowley A.F. (2002) Shell disease syndrome in the edible crab, *Cancer pagurus* – isolation, characterization and pathogenicity of chitinolytic bacteria. *Microbiology* **148,** 743–754.

Wong H.C., Ting S.H. & Shieh W.R. (1992) Incidence of toxigenic vibrios in foods available in Taiwan. *Journal of Applied Bacteriology* **73,** 197–202.

Zhang X.H. & Austin B. (2000) Pathogenicity of *Vibrio harveyi* to salmonids. *Journal of Fish Diseases* **23,** 93–102.

Received: 22 November 2004
Revision received: 5 October 2005
Accepted: 5 October 2005

