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Vibrios associated with *Macrobrachium rosenbergii* (De Man, 1879) larvae from three hatcheries on the Indian southwest coast

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Abstract

Surveys for bacteriological analysis of larval samples to isolate the associated vibrios were carried out during 1985–1992, 2001 and 2002 in three different hatcheries located on the southwest coast of India. *Vibrio* isolates were examined for their species diversity, virulence based on haemolysis in prawn blood agar, lipolysis, proteolysis and chitinolysis and antibiotic sensitivity. *Vibrio cholerae* was the predominant species in the apparently healthy larval samples, whereas *V. alginolyticus* and *V. vulnificus* dominated during disease and morbidity. No correlation was found between the hydrolytic properties and haemolytic activity of the vibrios associated with the larvae. All isolates were resistant to erythromycin and resistance to oxytetracycline, ampicillin and streptomycin sulphate was prevalent among the larger section of the *Vibrio* population. This suggested that antibiotic application may not be of much use to protect the larvae from vibriosis. This is the first report on the diversity of *Vibrio* species associated with *Macrobrachium rosenbergii* larvae and their virulence characteristics based on haemolysis in prawn blood agar.

Keywords: vibrios, *Macrobrachium*, haemolytic, pathogenicity

Introduction

Giant freshwater prawn (*Macrobrachium rosenbergii* de Man, 1879) or scampi is an important commercial candidate species with considerable export value, ideal for cultivation both under low saline conditions and in freshwater zones (Kurup 1994). Augmentation of grow-out systems of scampi during the past decade demanded hatchery production of seed, which, in turn, resulted in the expansion of scampi hatcheries in India. Presently, 71 hatcheries are under operation in different states, having a cumulative capacity to supply 1.83 billion scampi seed annually (Bojan 2003). Ironically, in spite of two and a half decades of research in production systems and production processes, several issues still remain unresolved. One among them is vibriosis, which has hampered scampi seed production, culminating in low yield because of mass mortality. Vibriosis has become the most important disease in penaeid and non-penaeid larvae, and is often reported as a limiting factor in hatcheries (Felix & Nanjaiyan 1992; Abraham, Shanmugam & Sundararaj 1993; Lightner 1996; Nayak & Mukherjee 1997).

Vibrios are ubiquitous in marine and estuarine environments and are associated with fish and other poikilothermic animals, existing as part of the normal microbiota and as primary or secondary pathogens as well (Cahill 1990; Austin & Austin 1993). On several occasions, mortalities in finfish and shellfish have been associated with an increase in the *Vibrio* populations (Sung, Li, Tsai, Ting & Chao 2001). Several species of *Vibrio* are associated with surfaces and internal organs of marine invertebrates and vertebrates (Huq, Small, West, Huq, Rahman & Colwell 1983; Colwell & Grimes 1984; Ortigosa, Garay &

Pujalte 1994), and have been isolated from lesions or haemolymph in most of the reported bacterial infections in shrimps.

Vibrio alginolyticus (Felix & Devaraj 1993), *V. anguillarum* (Nammalwer & Thangaraj 1980), *V. cholerae* (Prem Anand, Patterson Edward & Ayyakannu 1996), *V. fluvialis* (Ponnuraj, Murugesan, Sukumaran & Palaniappan 1995), *V. parahaemolyticus* (Abraham *et al.* 1993), *V. mimicus* (Karunasagar, Susheela, Malathi & Karunasagar 1990), *V. vulnificus* (Karunasagar, Ismail, Amarnath & Karunasagar 1992), *V. damsela* (Aravindan & Kalavati 1997), *V. harveyi* (Abraham & Manley 1995) and *V. proteolyticus* CW8T2 (Verschuere, Heang, Criel, Sorgeloos & Verstraete 2000) have been isolated so far from larval shrimps. Among the different species of vibrios, *V. alginolyticus* has been isolated frequently from diseased shrimp as the aetiology of vibriosis, and has been described as the principal pathogen in both penaeids and non-penaeids (Lightner 1988; Lee, Yu, Chen, Yang & Liu 1996). Moreover, *Vibrio* spp. appears to be more virulent in the larval stages either because of their ability to produce exotoxins/exoenzymes (Nottage & Birkbeck 1987; Santos, Bandin, Núñez, Montero, Silva & Toranzo 1992; Riquelme, Hayashida, Toranzo, Vilches & Chavez 1995) or because of their invasiveness or both.

In prawns (non-penaeids), vibrios are known to be pathogenic (Anderson, Shamsudin & Nash 1989) and systemic infections and necrotic appendages caused by *Vibrio* have been reported in hatcheries (New 1995). In 1984, Fujioka and Greco isolated *V. fluvialis*, *V. alginolyticus* and *V. cholerae* non-01 from *M. rosenbergii* larvae. Meanwhile, Singh (1990) observed a profound relationship between the abundance of the members of family Vibrionaceae and the mortality of larvae during the midlarval cycle. The same observation was later made by Hameed, Rahaman, Alagan and Yoganandhan (2003), who observed that *Vibrio* species comprised the dominant taxon in eggs, larvae and post larvae of *M. rosenbergii*. Lombardi and Labao (1991) documented the association of vibrios with necrosis (black spot) and gill obstruction.

Information on *Vibrio* species related to prawn hatcheries is essential to find out appropriate control measures. All the earlier studies on the bacteriology of *M. rosenbergii* larval culture systems were related to total bacterial count and data on *Vibrio* species are scarce. Moreover, information on the species diversity of vibrios and its pathogenicity based on haemolytic activity in prawn blood agar is not available. In this paper, we report for the first time on the isolation and haemolytic activity of different *Vibrio* species associated with *M. rosenbergii* larvae sampled during different periods from three different hatcheries located on the southwest coast of India. We examined the relation between the virulence characteristics of the isolates based on haemolytic activity in prawn blood agar and also hydrolytic properties such as lipase, protease and chitinase. The antibiotic sensitivity of the *Vibrio* isolates to selected antibiotics was also assessed.

Materials and methods

Sample sources

Repetitive samplings of larvae for bacteriological analysis took place in 1985–1992 in an *M. rosenbergii* larval rearing system of the Regional Shrimp Hatchery located in Azhikode, Kerala, and the bacterial isolates were identified to be family Vibrionaceae (Singh 1990) and subjected to numerical taxonomy (Bhat 1998; Bhat & Singh 1998). The isolates are maintained at the Centre for Fish Disease Diagnosis and Management, Cochin University of Science and Technology.

In 2001, necrotic moribund larvae were collected from the *M. rosenberbergii* larval production facility of M/S Rosen Fisheries (Trichur, Kerala). The larval samples were obtained from a tank (5-tonne capacity with about 500 000 mysis as the initial stocking density) where majority of the larvae (stage nine) had displayed anorexia, inactivity, poor growth, necrotic appendages and morbidity.

In 2002, moribund larval samples were obtained from Matsyafed Prawn Hatchery (Quilon, Kerala), where a batch of *M. rosenbergii* larvae had suffered heavy mortality.

In all cases, larval samples were collected in sterile glass containers and transported to the laboratory in insulated coolers with frozen gel packs to maintain a temperature of around 4 °C. All specimens were plated within 2 h of collection.

Bacterial isolation and storage

Larvae (10–15 specimens) were washed with sterile seawater, macerated in sterile glass homogenizers and diluted serially using seawater of 15 g L^{-1} salinity. Aliquots (0.2 mL) of the dilutions were plated on ZoBell's marine agar 2216E (ZoBell's agar) plates and incubated at 28 ± 1 °C for 3–5 days. In 1985–1992, 20 colonies were isolated randomly from each sample at a time. During the period between 2001

and 2002, instead of random isolation, a pattern of differential selective isolation based on colony morphology was adopted. Accordingly, colonies were grouped based on their morphology following Cappuccino and Sherman (1999). Subsequently, from each group, 10% of the colonies were isolated into ZoBell's agar slants. The colonies were repeatedly streaked onto ZoBell's agar plates until purity was attained.

All Gram-negative bacterial isolates were identified to genera following Oliver (1982), and subsequently characterized for presumptive identification (Baumann & Schubert 1984). Isolates of *Vibrio* were further identified to species level following Alsina and Blanch (1994).

All the isolates were maintained at room temperature on ZoBell's agar vials by overlaying with sterile liquid paraffin as well as by sealing with paraffin wax. Working cultures were maintained on ZoBell's agar slants at room temperature and also at 4 °C.

Haemolytic assay in prawn blood agar

Haemolytic assays were carried out following the basic protocol of Chang, Liu and Shyu (2000) with slight modifications. Haemolymph required for the assay was drawn from wild-caught adult (30 ± 10 g) *M. rosenbergii* quarantined for a week in the laboratory. During quarantine, they were fed a scampi diet (Higashimaru , Cochin, Kerala, India) and provided 100% water exchange. To facilitate aseptic collection of blood from the rostral sinus, the area beneath the rostral spine was disinfected with sodium hypochlorite (200 mg L^{-1}) by allowing the solution to flow through the area. This was followed by administration of 70% ethanol in the same pattern. The area was washed with sterile distilled water repeatedly and wiped dry with sterile absorbent cotton swabs. Haemolymph was collected using sterile capillary tubes.

To prevent clotting of haemolymph, the capillary tubes were rinsed with citrate-EDTA buffer (glucose 0.1 M, trisodium citrate 30 mM, citric acid 26 mM and EDTA 10 mM in double-distilled water). The pH was adjusted to 4.6 and osmolality to 350 mOsm (by adding sodium chloride), and sterilized at 115 °C for 10 min. The capillary tubes were rinsed with citrate-EDTA buffer before blood collection. One millilitre of collected haemolymph was transferred to a sterile 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. Aseptically, 1 mL of the stained haemolymph preparation was added to 15 mL of the prepared basal agar medium, followed by gentle mixing and pouring into petri plates. The basal agar medium comprised 10 g Bacto peptone, 5 g sodium chloride and 15 g Bacto agar dissolved in 1000 mL distilled water, adjusted to pH 6.8, autoclaved for 15 min at 121 °C and cooled to 45–50 °C in a water bath. The bacterial isolates were streaked onto the prawn blood agar plates and incubated at 28 ± 1 °C for 3–7 days. The plates were observed for clearing zone around the growth and lysis of haemocytes under a microscope with a × 10 objective lens.

Hydrolytic properties

Proteolytic activity was assessed in nutrient gelatin agar (Frazier 1926). A medium that contained per litre peptone 5 g; yeast extract 1 g; beef extract 5 g; NaCl 15 g; gelatin 20 g and agar 20 g, at a pH of 7.3 ± 0.2, was autoclaved at 121 °C for 15 min and poured into plates. The test organisms were spot inoculated, and the plates were incubated at 28 ± 1 °C for 3–5 days. Gelatinase production was indicated by zone of clearing around the colonies after flooding the plates with mercuric chloride solution ($HgCl_2$ 15 g; concentrated HCl 20 mL; distilled water 80 mL).

Lipolytic activity was assessed in tributyrin agar (Rhodes 1959) having per litre: peptone 5 g; beef extract 5 g; yeast extract 1 g; NaCl 15 g; tributyrin 10 mL; agar 20 g and a pH of 7.3 ± 0.2. The medium was autoclaved at 121 °C for 15 min, and plates were poured while mixing well each time. Test organisms were spot inoculated, and the plates were incubated at 28 ± 1 °C for 3–5 days. Zones of clearing around the colonies indicated hydrolysis of tributyrin.

Chitinase production was assessed in chitin agar according to Holding and Collee (1971) by incorporating colloidal chitin (20 g L^{-1}) in the basal medium composed per litre of: peptone 5 g; beef extract 5 g; NaCl 15 g; agar 20 g and a pH of 7.5. The medium was autoclaved at 121 °C for 15 min and poured into plates. The test cultures were spot inoculated and incubated at 28 ± 1 °C for 3–5 days. Hydrolysis of chitin was detected by clearing zones around the colonies.

Antibiotic sensitivity

The standard disc diffusion method (Bauer, Kirby, Sherris & Turck 1966) was followed for testing the

sensitivity of the *Vibrio* isolates to 12 antibiotics. Nutrient agar plates supplemented with 1.5% sodium chloride were swabbed with overnight-grown bacterial suspension cultures using sterile cotton swabs and readymade antibiotic discs (Himedia Laboratories, Bombay, India) were placed on the agar and incubated at 28 ± 1 °C for 24 h. The zone of inhibition was recorded at the end of the incubation period. The antibiotic discs tested were ampicillin 10 mcg, streptomycin 10 mcg, rifampicin 2 mcg, neomycin 30 mcg, erythromycin 10 mcg, kanamycin 30 mcg, chloramphenicol 10 mcg, ciprofloxacin 5 mcg, oxytetracycline 30 mcg, novobiocin 30 mcg, furazolidone 50 mcg and nitrofurantoin 100 mcg.

Results and discussion

From among the bacterial isolates recovered during 1985–1992, 2001 and 2002 from three scampi hatcheries, 55 isolates of *Vibrio* could be identified (Table 1). The genus was composed of nine species such as *V. cholerae*, *V. nereis*, *V. vulnificus*, *V. alginolyticus*, *V. mediterranei*, *V. parahaemolyticus*, *V. splendidus* II, *V. proteolyticus* and *V. fluvialis*. The association of *V. cholerae*, *V. alginolyticus* and *V. fluvialis* with the larvae of *M. rosenbergii* has been reported earlier (Fujioka & Greco 1984). Meanwhile, *V. alginolyticus* has been described as the principal pathogen of non-penaeids (Lightner 1988; N. S. Jayaprakash, pers. comm.).

A haemolytic assay of the vibrios in prawn blood agar revealed that all isolates of *V. alginolyticus* and *V. parahaemolyticus* were haemolytic. Meanwhile, among *V. nereis*, *V. vulnificus* and *V. mediterranei,* both haemolytic and non-haemolytic isolates were observed. However, all isolates of *V. cholerae*, *V. splendidus* II, *V. proteolyticus* and *V. fluvialis* from normal larval rearing systems investigated during the period from 1985 to 1992 were non-haemolytic (Table 2). Bacterial haemolysin has been suggested as an important virulent factor of pathogenic vibrios (Chang, Lee, Shyu & Liao 1996). The haemolytic assay has been used to differentiate the virulent strains among suspected pathogens as well (Chang *et al.* 2000). The importance of haemolysin as a virulent factor of pathogenic vibrios in fishes has been suggested by Zhang and Austin (2000). According to them, *V. harveyi*, which is most pathogenic to salmonids, produced extracellular products with the highest titre of haemolytic activity against Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) erythrocytes. In crustaceans, Chang *et al.* (2000) were able to use prawn blood agar haemolysis effectively to screen out bacteria pathogenic to cultured tiger prawn, *Penaeus monodon*.

All isolates of *Vibrio* were lipolytic and all but two isolates of *V. splendidus* II were proteolytic. However, the chitinolytic property was not widespread among the isolates (Table 2). Toranzo, Barja, Colwell and Hetrick (1983) and Ellis (1991) suggested that the production of gelatinase, lipase and chitinase might be considered as potential virulence factors in vibrios. A comprehensive examination of virulence factors among vibrios revealed unequivocally the role of proteases, lipases, chitinase and plasmids coding for iron chelators apart from haemolysins as being capable of initiating an infectious death (Reid, Woods & Robb 1980; Moustafa, Kodama, Ishiguro, Mikami & Izawa 1984; Nottage & Birkbeck 1986; Wong, Ting & Shieh 1992). Among these hydrolytic enzymes, the importance of protease has been demonstrated in *V. alginolyticus*, (Lee, Yu & Liu 1997) and *V. parahaemolyticus* (Sudheesh & Xu 2001). Among crustaceans living in aquaculture systems, elevated levels of shell disease associated with chitinolytic bacteria have been reported (Prince, Bayer, Gallagher & Subramanyam

Table 1 *Vibrio* isolates from *Macrobrachium rosenbergii* larval rearing systems

Location	*Vibrio* species (nos)	Code
Regional Shrimp Hatchery, Azhikode, Kerala	*V. cholerae* (12)	Vc 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39
	V. nereis (6)	Vn 24, 28, 29, 30, 31, 33
	V. mediterranei (6)	Vm 15, 18, 22, 32, 34, 36
	V. parahaemolyticus (4)	Vpa 1, 6, 8, 9
	V. splendidus II (2)	Vs 3, 5
	V. proteolyticus (2)	Vpr 4, 10
	V. fluvialis (1)	Vf 26
M/S Rosen Fisheries, Trichur, Kerala Matsyafed Prawn Hatchery, Quilon, Kerala	*V. alginolyticus* (8)	Va 1, 2, 3, 4, 5, 6, 7, 8
	V. nereis (5)	Vn 13, 29, 32, 34, 35
	V. vulnificus (8)	Vv 5, 9, 10, 19, 20, 23, 33, 36
	V. mediterranei (1)	Vm 27

Table 2 Lipolytic, proteolytic, chitinolytic and haemolytic activities of *Vibrio* isolates

Code	Lipase	Gelatinase	Chitinase	Haemolysis	Isolate
Vc 11, 12, 13, 16, 17, 19, 20, 21, 23, 35, 37, 39	+	+	+	–	*V. cholerae*
Vm 15	+	+	–	+	*V. mediterranei*
Vm 22, 32, 34, 36	+	+	+	+	*V. mediterranei*
Vm 27	+	+	–	–	*V. mediterranei*
Vm 18	+	+	+	–	*V. mediterranei*
Vv 5, 33	+	+	+	+	*V. vulnificus*
Vv 10	+	+	–	–	*V. vulnificus*
Vv 9, 19, 20, 23, 36	+	+	+	–	*V. vulnificus*
Vn 31, 33	+	+	+	+	*V. nereis*
Vn 24 , 35	+	+	–	+	*V. nereis*
Vn 29, 30	+	+	+	–	*V. nereis*
Vn 28, 13, 29, 32, 34	+	+	–	–	*V. nereis*
Vpa 1, 6, 8, 9	+	+	+	+	*V. parahaemolyticus*
Vs 3, 5	+	–	–	–	*V. splendidus* II
Vpr 4, 10	+	+	+	–	*V. proteolyticus*
Vf 26	+	+	+	–	*V. fluvialis*
Va 1, 2, 3, 4, 5, 6, 7, 8	+	+	+	+	*V. alginolyticus*

Table 3 Antibiotic resistance of isolated *Vibrio* species

Isolate	Total number of isolate	Antibiotics to which resistance recorded
V. chlolerae	12	Erythromycin (10 mcg) – all isolates neomycin (30 mcg) – Vc 19 streptomycin (10 mcg), neomycin (30 mcg) and kanamycin (30 mcg) – Vc 20 streptomycin (10 mcg) Vc 35
V. nereis	11	Erythromycin (10 mcg) – All isolates rifampicin (2 mcg), neomycin (30 mcg), ciprofloxacin (5 mcg) – Vn 28 chloramphenicol (10 mcg) and oxytetracycline (30 mcg) – Vn 13,29,32,34,35
V. vulnificus	8	Erythromycin (10 mcg) – All isolates oxytetracycline (30 mcg) – vv 9
V. alginolyticus	8	Erythromycin (10 mcg), ampicillin (10 mcg), Streptomycin (10 mcg) and oxytetracycline (30 mcg) – all isolates
V. mediterranei	7	Erythromycin (10 mcg) – all isolates streptomycin (10 mcg) – Vm 22,32 chloramphenicol (10 mcg) and oxytetracycline (30 mcg)-Vm 27
V. parahaemolyticus	4	Erythromycin (10 mcg), ampicillin (10 mcg) – all isolates oxytetracycline (30 mcg) – Vpa 1,9
V. splendidus II	2	Erythromycin (10 mcg), rifampicin (2 mcg), oxytetracycline (30 mcg), novobiocin (30 mcg) and nitrofurantoin (100 mcg) – all isolates ampicillin (10 mcg) – Vs 3 chloramphenicol (10 mcg) and Furazolidone (50 mcg)- Vs 5
V. proteolyticus	2	Erythromycin (10 mcg) and oxytetracycline (30 mcg) – all isolates
V. fluvialis	1	Erythromycin (10 mcg) and oxytetracycline (30 mcg)

1995). Vogan, Costa-ramos and Rowley (2002) reported that chitinolytic activity is fundamental to lesion progression and microbial proteases and lipases may be supporting the process of exoskeletal breakdown, particularly in the initial stages of shell disease. However, if haemolysis can be considered as a virulent determinant, the proteolytic, lipolytic and chitinolytic properties may serve as the supplementary factors only helping the organisms to invade the host. Further studies at the molecular level on the importance of haemolytic activity and the hydrolytic properties of *Vibrio* may help in elucidating the possible virulence factors.

All the 55 *Vibrio* isolates from *M. rosenbergii* larvae were examined for their sensitivity to 12 antibiotics (Table 3). Although variations in the resistance patterns could be observed among the bacterial isolates, all of them proved to be practically resistant to erythromycin used in hatcheries as a prophylaxis. The incidence of bacterial resistance to oxytetracycline

was high (40%) followed by ampicillin (24%) and streptomycin (22%). The prevalence of resistance to kanamycin, ciprofloxacin and furazolidone was the least observed among the individual antibiotics tested. It is known that the prophylactic use of antibiotics during larval rearing results in an increase in the frequency of antibiotic-resistant bacteria in aquaculture systems. This is true in the present study, and our observations agree with the previous work on the antibiotic-resistant bacteria isolated from *M. rosenbergii* larvae (Hameed *et al*. 2003). According to them, 95% of the bacterial isolates were resistant to erythromycin and oxytetracycline. All the isolates of *V. alginolyticus,* obtained from the diseased necrotic larvae, were found to be resistant to ampicillin, streptomycin, erythromycin and oxytetracycline, which indicated that their use in larval culture may have no effect. Moreover, if these resistance factors are carried in mobile genetic elements, they can spread rapidly within the bacterial population in the hatchery (Kerry, Hidney, Coyne, Cazabon, NicGbhainn & Smith 1994). Moreover, *Vibrio* spp. present in aquaculture settings may be transmitted to humans who come in contact with this ecosystem (Blake, Merson, Weaver, Hollis & Heublein 1979). For example, *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* have been associated with infections among persons working in aquaculture ecosystems (Bisharat & Raz 1996). With respect to the potential significance of these results for the environment and for human health, antibiotic usage in prawn hatcheries has to be discouraged either as therapy or for prophylaxis. Instead, biological control methods such as application of antagonistic probiotics and bacteriophages must be used for sustainable larval production.

To summarize, an attempt was made in the present study to determine the species diversity of vibrios associated with *M. rosenbergii* larvae and their pathogenicity based on haemolytic activity in prawn blood agar. Haemolytic activity was widespread among the *Vibrio* species tested, and all the isolates of *V. alginolyticus* and *V. parahaemolyticus* were found to be haemolytic. This study provides an avenue for further investigations on virulence and in designing biological methods for their control.

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