Fishery Technology
2005, Vol. 42(2) pp : 191 - 196

Pathogenicity, Antibiogram and Biochemical Characteristics of Luminescent *Vibrio harveyi*, Associated with 'Black Shell Disease' of *Penaeus monodon*

Joseph Selvin*, A.J. Huxley and A.P. Lipton**
Department of Biotechnology, Malankara Catholic College
Mariagiri, Kaliakavilai - 629 153, Kanyakumari District, India

Bacterial isolation was made from shrimps *Penaeus monodon* showing characteristic external symptoms of 'black shell disease' from shrimp farms located in southeast coast of India. The isolates were screened on TCBS agar plates and O/129 for the selective isolation of *Vibrio* sp. Based on the morphological, biochemical and physiological characteristics, the isolate (RJM5) was characterized as luminescent *Vibrio harveyi*. Antibiogram of *V. harveyi* indicated that it was highly sensitive to chloramphenicol, ciprofloxacin, nalidixic acid and streptomycin. The pathogenicity studies confirmed that the isolate (*V. harveyi*) was moderately virulent.

Key words: Bacterial-disease, *Penaeus monodon*, *Vibrio harveyi*, opportunistic-pathogen, antibiogram, pathogenicity

Bacterial infection by *Vibrio* has been recognized as the major cause triggering mortalities up to 100% in the shrimp farming (Nash, 1990). The short generation time of *Vibrio* ensures development of rapid massive population in the infected host as well as in the environment. In Asia 11 species of *Vibrio* were isolated from shrimp culture systems (Lavilla-Pitogo, 1995). Among these, *V. harveyi* was considered as important in the grow-out ponds of *Penaeus monodon* in India (Otta *et al.*, 1999). Luminescent vibriosis by *V. harveyi* led to mass mortalities in hatchery-reared larval and juvenile *P. monodon* (Lavilla-Pitogo *et al.*, 1990).

In September 1999, prevalence of 'black shell disease' among *P. monodon* led to stunted growth and mortalities, culminated in termination of grow-out activities especially after 40 days of culture (DOC) in the extensive shrimp farms located in Kanyakumari coast. Gross examination of the diseased shrimp revealed the following symptoms: necrotised chelate legs and antenna, antenna cut, brown gills and a folded base of the tail. The objective of the present study was to identify the causative bacteria, experimentally produce infection in the apparently healthy host and determine the antibiotic sensitivity pattern for undertaking curative measures.

Materials and Methods

The chosen farm area (0.24 ha) located in Kanyakumari coast was having a stock of 40,000 black tiger shrimp. About 25% daily water exchange occurred and pellet feed was provided at a rate of 3.2% of the shrimp body weight. The shrimp, which showed characteristic external clinical symptoms, were collected (50 nos.) and transported to the laboratory. The average body weight of the infected shrimp samples was 14.6±1.23 g on the day of sampling (60 DOC). After recording the size of the specimens, they were examined for external clinical symptoms using standard methods (Austin and Austin, 1989). Diseased and moribund shrimps were dissected through the dorsal mid line using sterile scissors. The initial isolations were made from the parts such as

* Marine Biotechnology Laboratory, Central Marine Fisheries Research Institute, Vizhinjam – 695 521. Thiruvananthapuram, India
[1] Corresponding author (selvinj@rediffmail.com)

infected shell, antenna, chelate legs and hepatopancreas. The infected shell area was removed using sterile scissors and swapped on plates of nutrient agar supplemented with 2% NaCl (NA). Hepatopancreatic tissue was homogenized in a sterile homogeniser (Omni, USA) using normal saline (NS). The resultant suspension was serially diluted up to 10^{-6} dilution using phosphate buffered saline and used for preparation of spread plate on nutrient agar supplemented with 2% NaCl. The plates were incubated at 32±2°C for 18 h. Dominant colonies observed on the NA plates, were further screened on thiosulfate-citrate-biles salt-sucrose (TCBS) agar (Himedia). Five yellow colonies (RJM1 to RJM5) observed were further screened for O/129 (Sigma) resistance using impregnated (150 mg) paper discs (8mm dia).

The colony morphology was observed after 18 h followed by Gram staining and observation under 1000x magnification. Luminescence was observed under dark and confirmed over UV illumination. Biochemical tests were followed after MacFaddin, (1981). All the tested media were supplemented with 2% NaCl. Tests for chitinase, amylase, protease and gelatinase were based on Cowan (1974) and Austin and Lee (1992). The b-galactosidase test was done with ONPG disks (Himedia). Results were recorded after incubation at 30±2°C. The effect of NaCl concentration and temperature on the growth of the pathogens was also tested. Classification followed as per Baumann and Schubert (1984) and Colwell and Grimes (1984).

Antibiotic sensitivity profile was determined by the Kirby-Bauer disk diffusion method (Bauer *et al.*, 1996) using Himedia antibiotic discs. Bacterial suspensions of 10 fold dilutions were prepared using 18 h fresh shake culture was inoculated onto the Mueller-Hinton agar plates to get the lawn concentration of about 1.5×10^6 cfu/cm^2. Six discs were dispensed on the seeded lawn at 60° apart to each other. The diameter of inhibition zone around the discs was measured after incubation at 30±2°C for 24 h. The colonies observed inside the inhibition zone was considered as specific resistant strain.

For challenge studies, healthy juvenile black tiger shrimp *Penaeus monodon* (30 DOC) were segregated from the grow-out tanks of Marine Biotechnology laboratory aquarium and maintained at a rate of 10 shrimps/tank in 100 l glass aquaria. The length and weight of each shrimp was measured before starting the experiment. Prior to the infection experiments, random sampling of shrimp was made for the bacterial isolation to ensure the shrimp were pathogen free. Isolations were made from the parts such as shell, body tissue, hepatopancreas and haemolymph on TCBS agar plates to ensure the shrimps were free from characteristic yellow colonies. The 18 h fresh shake culture was centrifuged at 4000 rpm for 15 min and washed twice in normal saline (NS). The purified pellets were serially diluted in NS and enumerated in a haemocytometer. This was also plated on NA plates to get the colony forming units (cfu). Preliminary examinations revealed that challenge dose of 10^3 cfu per shrimp could not kill the injected shrimp. Therefore the concentrations of 10^5 to 10^8 cfu per shrimp were taken in 0.1 ml saline and inoculated intramuscularly using a 1 ml tuberculin syringe at ventral side between the second and third segment of healthy shrimps. Parallel control groups received 0.1 ml of NS only. Ten shrimps were used for each inoculation level. The mortality and reflexes of the shrimps were observed for every 15 min in the first hour of post-inoculation and every 1 h until the 6th h. Subsequent monitoring was done every 12 h for a period of seven days (Tendencia & Dureza, 1997). Moribund shrimps were sampled for the bacterial re-isolation in Zobell Marine Agar (ZMA) plates. LD_{50} dose for 24 h and 7 days were calculated by the probit method, after Wardlaw (1985).

Results

Results of the morphological, biochemical and physiological characterization of the bacterial isolates are presented in Table 1. The chosen isolate (RJM5) was swarming, luminescent, Gram negative and actively motile short rods. It grew on TCBS agar, required NaCl for growth, sensitive to the vibriostatic

Table 1. Characteristics of the isolate of *Vibrio harveyi* from the infected *P. monodon*

Test/characteristic features	RJM5	Standard* reactions
Gram staining	-	-
Shape	short rod	short rod
Growth on TCBS agar	Yellow	Y/G
Sensitivity to 0/129 phosphate	+	+
Luminescence	+	+/-
Swarming	-	+
Oxidase production	+	+
Catalse production	+	+
Oxidative-fermentive test	F	F
Acid/gas production:		
Glucose	-	-
Sucrose	Acid	Acid
Mannitol	Acid	Acid
Maltose	Acid	Acid
Sorbitol	-	-
Lactose	-	-
Galactose	Acid	Acid
Arabinose	Acid	Acid
Nitrate reduction	+	+
Methyl red	+	+
Voges-Proskauer	-	-
Indole production	+	+
Hydrogen sulfide production	-	-
ONPG hydrolysis	+	+
Decarboxylase of:		
Arginine	-	-
Lysine	+	+
Ornithine	+	+
Growth at 4°C	-	-
40°C	+	+
Growth in peptone with NaCl		
0%	-	-
0.5%	+	+
1%	+	+
3%	+	+
6%	+	+
8%	+	+
10%	-	-
Production of exo-cellular enzymes:		
Amylase	+	+
Caesinase	+	+
Gelatinase	+	+
Chitinase	+	+
Urease	-	-

*Baumann and Schubert (1984)

agent O/129 and produced cytochrome oxidase, catalase, gelatinase, caesinase, amylase and chitinase. Nitrate was reduced and fermentation and acid production from glucose, arabinose, lactose, mannitol, maltose and sucrose was noted. Growth occurred at 40°C. Decarboxylase of lysine and ornithine were positive whereas arginine was negative. Based on the morphological, biochemical and physiological characteristics of the isolate and comparison with the earlier reports, it was identified as *Vibrio harveyi* (RJM5).

The antibiotic sensitivity pattern of shrimp bacterial isolates is given in Table 2. The antibiogram indicated that *V. harveyi* was highly susceptible to chloramphenicol (CAP), ciprofloxacin, nalidixic acid and streptomycin. CAP and nalidixic acid produced a higher bactericidal activity to the extent of 28 mm of inhibition. In the case of ciprofloxacin and nalidixic acid, the bacterium was inhibited to the extent of 24 mm inhibition respectively. The antibiotics amoxycillin, gentamycin, metronidazole, rifampicin, erythromycin and ampicillin gave intermediate sensitivity pattern to the range of 10 to 20 mm. Notably, the bacterium was resistant to b amphotericin, tetracycline, bacitracin, Co-trimoxazole, nystatin, pencillin-G and trimethoprim.

Table 2. Antibiotic sensitivity pattern of *Vibrio harveyi*

Antibiotics	Concentration mcg/disc	Sensitivity pattern*
Amoxycillin	30	Intermediate
Amphotericin-B	100 U	Resistant
Ampicillin	25	Intermediate
Bacitracin	10 U	Resistant
Chloramphenicol	25	Sensitive
Ciprofloxacin	10	Sensitive
Cloxacillin	10	Intermediate
Co-Trimoxazole	25	Resistant
Erythromycin	15	Intermediate
Furazolidone	50	Intermediate
Gentamicin	10	Intermediate
Metronidazole	5	Intermediate
Nalidixic acid	30	Sensitive
Neomycin	30	Intermediate
Nitrofurazone	100	Sensitive
Nystatin	100 U	Resistant
Oxytetracycline	30	Intermediate
Pencillin-G	5 U	Resistant
Rifampicin	15	Intermediate
Streptomycin	10	Sensitive
Sulphadiazine	100	Intermediate
Sulphamethizole	300	Sensitive
Tetracycline	10	Resistant
Trimethoprim	5	Resistant

* Sensitive = > 20 mm dia inhibition zone; Intermediate = 10 to 20 mm; Resistant = < 10 mm
**Resistant colonies observed inside the inhibition zone

Table 3. Pathogenicity of *Vibrio harveyi* (RJM5) to *P. monodon*

Dose (cfu/ shrimp)	Length (cm)	Weight (g)	Mortality (%)*	Infection (%)*
10^8	8.6±0.26	3.8±0.39	100	0
10^7	8.25±0.32	3.6±0.27	60	40
10^6	7.89±0.32	3.28±0.24	30	70
10^5	8.68±0.18	3.83±0.62	40	20
10^4	8.52±0.08	3.71±0.08	0	0

* Based on triplicate trials

Although sulphamethizole showed activity against the isolate, it could be grouped as the least active antibiotic because resistant colonies were observed inside the inhibition area.

The isolate (RJM5), luminescent *V. harveyi*, produced more or less same level of infection and mortalities as observed in the field condition. The severity of the infection varied with dose range. At higher dose of inoculation (10^8 cfu/shrimp), all the experimental shrimps were died at 6 h of post-inoculation. However at lower doses, the infection started on the 4th day of post-inoculation and culminated to mortality on the 7th day of post-inoculation (Table 3). The LD_{50} value extrapolated from the graph was accounted for $6x10^6$ cfu/shrimp. The external clinical symptoms were recorded as necrosis in the chelate legs and antenna, loss of legs, antenna cut, black spots on the shell, murky whitish muscle, anorexia, lethargy and a folded base of the tail. The hepatopancreas was pale and soft.

Discussion

The symptoms of the infected shrimp confirmed the typical 'black shell disease' condition. All the infected shrimps had external symptoms such as broken antenna and leg loss in addition to the melanized fissures on the carapace and shells. The field observations indicated that the shrimps were under stress due to the high stocking density (1.6 lakh/ha). The same isolate was observed in the farm water (Selvin, 2002). Therefore the isolate became opportunistic pathogen due to the mid-culture stress. Deterioration of hydrological conditions arise as a result of high stocking densities and increased inputs leading to accumulation of organic matter in the pond bottom. Such situation developed mostly in the middle of the culture period (de la Pena *et al.*, 1992). These conditions, which cause severe stress and injury to the cultured animals, were rendering them susceptible to bacterial infection (Sindermann, 1990).

The colony morphology of RJM5, which was characterized as *V. harveyi* was also earlier reported as cream coloured, occasionally translucent, raised and shiny colonies (Alvarez *et al.*, 1998). The specific characteristic of indole production and negative VP reaction were also observed for the present *V. harveyi* strain (RJM5). According to Lightner *et al.*, (1992), identification of *Vibrio* bacteria required complete biochemical characterization otherwise, *V. harveyi* would be misidentified as *V. parahaemolyticus* or *V. alginolyticus*. However in the present study, the biochemical tests carried out were found to be adequate to identify the isolate.

According to Jiravanichpaisal *et al.*, (1994), the characteristics of a strain of *V. harveyi* isolated from *P. monodon* was chitinase negative, grew in 8% NaCl and H_2S positive. It was contrary to the report of Baumann and Shubert (1984) in which they stated that only the chitinase positive bacteria could be pathogenic to shrimp. In the present study, the isolate was found to be a chitinoclastic bacterium. Therefore this bacterium had the capablility to produce chitinase, with which chitin in the shrimp's exoskeleton can be eroded.

The antibiogram of the isolates indicated the sensitivity pattern of commonly used and other conventional antibiotics and which in turn may be useful in determining the therapeutants. The commonly used antibiotic, chloramphenicol and oxytetracycline were inhibitory to the isolate. Based on the present findings, it could be inferred that the antibiotics such as ciprofloxacin, nalidixic acid and streptomycin may form additional therapeutics for shrimp. However due to the emerging environmental and public health consequences, their usage has to be minimized (Selvin & Lipton, 2003).

In the present experiment, the infection or mortality started after two to five days of post-challenge. The mechanism of such delayed responses may be due to the initial bacterial clearance by the haemolymph and growth rate as well as generation time to attain minimum infectivity dose in the host. However, higher dose, the mortality started within 6 h of post-inoculation. At this dose, the bacteria may have overwhelmed the shrimp's defense system during the first two days resulting in mortalities. Nash *et al.*, (1992) reported that shrimp injected with bacteria were weak in the first two days but could recover within 3 to 5 days.

The LD_{50} value of *V. harveyi* isolated from 'shell disease' shrimp was 10^6 cfu/shrimp. A strain of *V. harveyi* isolated from the shrimp *P. monodon* with 'red disease syndrome' could be reproduced by injection with 10^7 cfu/shrimp (Tendencia & Dureza, 1997). The LD_{50} values of *V. harveyi* were reported to range from $1.4x10^6$ to $2.8x10^7$ cfu/shrimp, which indicated low virulence (Otta *et al.*, 1999). Lavilla-Pitogo *et al.*, (1990) concluded that virulent isolates of *V. harveyi* were lethal at 100 cells/ml in seawater while non-luminescent isolates did not cause mortality even at a challenge dose of 10^6 cells/ml (Pizzautto & Hirst, 1995). This report in turn suggests that luminescence may be one of the indicators of virulence. Considering this, the present luminescent *V. harveyi* can be considered as moderately virulent (10^6 cfu/shrimp). The pathogenic characteristics of RJM5 was similar to *V. alginolyticus*, one of the common shrimp pathogen of India (Selvin & Lipton, 2003).

The significance of *V. harveyi* as a shrimp pathogen is further reinforced by reports from tropical countries where the bacteria caused mortalities up to 100% in shrimp hatcheries. It was commonly isolated only from diseased but not healthy larvae as well as from the rearing water (Jiravanichpaisal *et al.*, 1994; Karunasagar *et al.*, 1994). In the adults, Jiravanichpaisal *et al.*, (1994) isolated *V. harveyi* as a minor component from the exoskeleton of female black tiger shrimp in Thailand. Nevertheless, the proof for the pathogenic role of *V. harveyi* was rare in infectivity experiments. In the present study, the isolate obtained from the diseased shrimp demonstrated certain degree of virulence. In agreement with these results, others have also reported the high LD_{50} values ($4.9x10^7$ and $1.56x10^9$ cfu) for fish (Saeed, 1995). But lower doses of 10^2 to 10^3 cells/ml were determined as lethal for larval shrimp (Lavilla-Pitogo, *et al.*, 1990). Hepatopancreas was reported as the target tissue for the reisolation of *V. harveyi* from diseased shrimp (Alvarez *et al.*, 1998). The present isolate also was easily reisolated from the hepatopancreas of infected shrimp. Based on the present findings, the strain could be inferred as a moderate pathogen, which act as opportunistic one in the culture system.

Authors are thankful to Dr. M.J. Modayil, Director and Dr. R. Paul Raj, Head, PNP Division for the facilities and encouragement.

References

Alvarez, J.D., Austin, B., Alvarez, A.M. and Reyes, H. (1998). *Vibrio harveyi*: a pathogen of penaeid shrimps and fish in Venezuela. *J. Fish. Dis.* **21**, pp313-316.

Austin, B. and Lee J. V. (1992). Aeromonadaceae and Vibionaceae. In: *Identification Methods in Applied and Environmental Microbiology*. (R.G. Board. D. Jones and F.A. Skinner, Eds). Technical Series of the Society for Applied Bacteriology. Blackwells. Oxford. pp 163-182

Austin, B. and Austin, D.A. (1989). *Methods for the Microbiological Examination of Fish and Shellfish.* Ellis Horwood, Chichester 298p

Bauer, A.W., Kirby, W.M.M., Shenis, J.C. and Turck, M. (1996). Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* **45**, pp 493-498

Baumann, P. and Schubert, R.H.W. (1984). Family U. Vibrionaceae. In: *Bergey's manual of systematic bacteriology*. Vol 1, (N.R. Krieg and J.G. Holt, Eds). Williams and Wilkins, London. pp 516-538

Colwell, R.R. and J.A. Grimes. (1984). *Vibrio* diseases of marine fish populations. *Helgolander Wissen Sehaftliche Meeresuntersuchungen*, **37**, pp 265-287

Cowan, S.T. (1974). *Cowan and Steel's manual for the identification of medical bacteria*, 2nd edition. Cambridge University Press, London.

de la Pena, L.D., Momoyama, K., Nakai, T. and Muruga, K. (1992). Detection of the causative bacterium of vibriosis in kuruma prawn, *Penaeus japonicus. Fish Pathol.* **27**, pp 223-228

Jiravanichpaisal P., Miyasaki T. and Limsuwan C. (1994). Histopathology, biochemistry, and pathogenicity of *Vibrio harveyi* infecting black tiger prawn *Penaeus monodon. J. Aqua. Anim. Health*, **6**, pp 27-35

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

Lavilla–Pitogo, C.R. (1995). Bacterial diseases of penaeid shrimps an Asian view. In: *Diseases in Asian Aquaculture II.* (Shariff, M, Arthur, J.R. and Subasinghe, R.P, Eds). Fish health section. Asian Fisheries Society, Manila, Philippines. pp. 107-121

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

Lightner, D.V., Bell, T.A., Redman, R.M., Mohney, L.L., Natividad, J.M., Rukyani, A. and Poernomo, A. (1992). A review of some major diseases of economic significance in penaeid prawns/shrimps of the Americas and Indopacific. In: *Diseases in Asian Aquaculture I.* (Shariff, I.M., Subasinghe, R.P. and Arthur, J.R, Eds). Fish health section, Asian Fisheries Society, Manila, Philippnies. pp 57-80

MacFaddin, J. F. (1981). Biochemical tests for identification of medical bacteria, 2nd edition. Williams and Wilkins, Baltimore. pp 527

Nash, G. (1990). *Penaeus monodon* grow-out diseases. In: *Proceedings of Aquatech 90*, Putra World Trade Centre, Kuala Lumpur. 11-14, June 1990. pp 172-187

Nash, G., Charetana, N. Cholada, T., Anutra, A., Phusit, P and Pongcham, R. (1992). Vibriosis and its control in pond-reared *Penaeus monodon* in Thailand. In: *Diseases in Asian Aquaculture I.* (Shariff M., Subasinghe, R.P. and Arthur J.R, Eds). Asian Fisheries Society. Philippines pp143 – 155

Otta, S.K., Karunasagar, I. and Karunasagar, I. (1999). Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon* in India. *J. Aqua. Trop.* **14**, pp 309-318

Pizzutto, M. and Hirst, R.G. (1995). Classification of isolates of *Vibrio harveyi* virulent to *Penaeus monodon* larvae by protein profile analysis and m13 DNA fingerprinting. *Dis. Aqua. Org.* **21**, pp 61-68

Saaed M.O. (1995). Association of *Vibrio harveyi* with mortalities in cultured marine fish in Kuwait. *Aquaculture*, **11**, pp 21-29

Selvin, J. (2002). *Shrimp disease management using secondary metabolites isolated from marine organisms.* Ph.D thesis submitted to M.S. University, Tirunelveli, India. pp 204

Selvin, J. and Lipton, A.P. (2003). Leaching and Residual Kinetics of Chloramphenicol Incorporated Medicated Feed Treated to Juvenile Black Tiger Shrimp *Penaeus monodon* Fabricious. *Fish. Technol.* **40**, pp 13-17

Selvin, J. and Lipton, A.P. (2003). *Vibrio alginolyticus* associated with white spot disease of *Penaeus monodon. Dis. Aqua. Org.* **57**, pp 147-150

Sindermann, C.J. (1990). Bacteria. In: *Principal Diseases of Marine Fish and Shellfish.* Vol. 2 Academic Press, San Diego, CA, pp 41-71

Tendencia, E.V.A. and Dureza, L.A. (1997). Isolation of *Vibrio* sp. from *Penaeus monodon* (Fabricius) with red disease syndrome. *Aquaculture* **154**, pp107-114

Wardlaw A.C. (1985). Practical Statistics for Experimental Biologists. John Wiley and Sons, Chichester.