Vol. 57: 147–150, 2003 | DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org | Published December 3

NOTE

Vibrio alginolyticus associated with white spot disease of *Penaeus monodon*

Joseph Selvin[1,*], A. P. Lipton[2]

[1]Department of Biotechnology, Malankara Catholic College, Mariagiri, Kaliakavilai 629 153, Kanyakumari District, India
[2]Marine Biotechnology Laboratory, Central Marine Fisheries Research Institute, Vizhinjam 695 521, Thiruvananthapuram, India

ABSTRACT: In February 2000, white spot disease outbreaks occurred among cultured *Penaeus monodon* in extensive shrimp farms on the southwest coast of India. Bacteria were isolated from infected shrimp that showed reddish body coloration and white spots in the cuticle. The isolates were screened on thiosulfate citrate bile salt sucrose (TCBS) agar plates for the selection of *Vibrio* species. The primary isolate (QS7) was characterized as *V. alginolyticus* based on morphological, biochemical and physiological characteristics. Antibiotic sensitivity tests of QS7 indicated that the isolate was highly sensitive to chloramphenicol, ciprofloxacin, nalidixic acid and streptomycin. Pathogenicity tests confirmed that the isolate was virulent for *P. monodon*. Based on the lethal dose (LD_{50}) value (5×10^6 cfu per shrimp), it was inferred that shrimp weakened by white spot syndrome virus would succumb to secondary infection by QS7.

KEY WORDS: Bacterial disease · *Penaeus monodon* · Secondary pathogen · *Vibrio* · Antibiogram · Pathogenicity

Resale or republication not permitted without written consent of the publisher

INTRODUCTION

Vibriosis has been implicated as the cause of major mortality in juvenile penaeid shrimp (Lightner & Redman 1994). Disease caused by *Vibrio alginolyticus* and *V. harveyi* remains a serious problem in cultured black tiger shrimp in India, being overshadowed only by white spot syndrome virus (WSSV) infections, which cause even more serious problems throughout Asian countries. *Vibrio* species are among the normal bacterial flora of both natural and cultural populations of shrimp and the culture environment (Jiravanichpaisal et al. 1994, Otta et al. 1999), but often act as opportunistic or secondary pathogens that can cause mortality ranging from a few to 100% in affected populations under stress (Lightner 1988).

In February 2000, an outbreak of white spot disease among *Penaeus monodon* led to mortalities that culminated in the termination of grow-out activities after 60 d of culture (DOC) in extensive shrimp farms in and around the Quilon area (southwest coast of India). Infected shrimp exhibited white spots in the cuticle together with reddish patches on the shell and tail areas, indicating secondary bacterial infections (Karunasagar et al. 1997). The characteristic red patches were not observed in apparently healthy specimens. Therefore, the present study was initiated to identify secondary pathogens and to test their virulence by experimentally infecting healthy shrimp. In addition, we determined antibiotic sensitivity for possible therapeutic treatment.

MATERIALS AND METHODS

Biochemical characterization and antibiograms of isolated bacteria. The farm from which we collected samples covered an area of 0.6 ha and was stocked

*Email: selvinj@rediffmail.com

© Inter-Research 2003 · www.int-res.com

with 100 000 black tiger shrimp *Penaeus monodon*. The average body weight of the infected shrimp was 14.6 ± 1.23 g on the day of sampling (60 DOC). The culture conditions on the farm included ~25% daily water exchange and a pelleted feed provided at a rate of 3.2% of shrimp body weight d^{-1}. Shrimp (50) showing signs of white spots and red coloration were collected and transported to the laboratory in circular, 250 l volume, high-density plastic tanks provided with constant aeration. The prevalence of WSSV was confirmed with a commercial PCR-based WSSV detection kit (Genei). After recording their sizes, the shrimp were examined externally using standard methods (Austin & Austin 1989). Initial bacterial isolations were made from infected shells and hepatopancreatic tissue. The infected shell area was removed with sterile scissors and swabbed onto plates of nutrient agar supplemented with 2% NaCl (NA). Hepatopancreatic tissue was homogenized in a sterile homogenizer (Omni) using normal saline (NS). The resultant suspension was serially diluted up to 10^{-6} with phosphate-buffered saline and used for preparation of spread plates on NA. The plates were incubated at 32 ± 2°C for 18 h. Dominant colonies observed on the NA plates were further screened on thiosulfate citrate bile salts sucrose (TCBS) agar (Himedia). Thus, 7 yellow colonies observed were further screened for O/129 (Sigma) resistance using impregnated (150 µg) paper discs (8 mm diameter).

Colony morphology was observed at 18 h, and cell shape was recorded after Gram staining followed by observation at 1000× magnification. Biochemical tests followed the methods of MacFaddin (1981). All test media were supplemented with 2% NaCl (w/v). Tests for chitinase, amylase, caesinase and gelatinase were based on those of Cowan (1974) and Austin & Lee (1992). The β-galactosidase test was done with *o*-nitrophenyl beta-D-galactopyranoside (ONPG) disks (HiMedia). Results were recorded after incubation at 30 ± 2°C, and incubation time depended on each test. The effect of NaCl concentration and temperature on growth was also tested. Bacterium (QS7) identification followed the descriptions of Baumann & Schubert (1984) and Colwell & Grimes (1984). Antibiotic sensitivity profile was determined by the Kirby-Bauer disk-diffusion method (Bauer et al. 1996) using HiMedia antibiotic discs. The isolate was considered sensitive to a particular antibiotic when the zone of inhibition equalled 8 mm.

Pathogenicity and LD_{50}. Healthy juvenile *Penaeus monodon* (40 DOC) were selected from grow-out tanks of the Marine Biotechnology Laboratory (India) aquarium and maintained at 10 shrimp per tank in 100 l glass aquaria at 30 ± 2°C. The length and weight of each shrimp were measured before starting the experiment. Prior to challenge experiments, the shrimp were sampled for the presence of bacteria to ensure that they were pathogen-free. A slant culture was activated in nutrient broth supplemented with 2% NaCl and subcultured in fresh media. An 18 h shake-culture was centrifuged at 4800 × *g* for 15 min. Cell pellets were washed twice with NS, re-suspended and serially diluted in NS, and enumerated by plating on NA plates to obtain counts of colony-forming units (cfu). Preliminary examination revealed that a challenge dose of 10^3 cfu per shrimp was insufficient to cause mortality. Therefore, concentrations of 10^5 to 10^8 cfu in 0.1 ml NS were injected intramuscularly in the ventral side of each shrimp between the second and third segment using a 1 ml tuberculin syringe. Parallel control groups received 0.1 ml of NS only; 10 shrimp were used at each challenge level. The mortality and behavior of the shrimp were observed every 15 min during the first hour post-injection (p.i.) and every 1 h thereafter until Hour 6 p.i. Subsequent monitoring was done every 12 h for a period of 7 d (Tendencia & Dureza 1997). Hepatopancreatic tissue homogenate of moribund shrimp was used for the reisolation of bacteria using Zobell marine agar (HiMedia Manual 1998) plates. The LD_{50} doses for 24 h and 7 d were calculated by the probit method, after Wardlaw (1985).

RESULTS

Biochemical characteristics of isolates

Bacteriological isolation revealed that the shrimp were infected with a single type of bacterium. Although initially the isolates were recorded as 7 different colonies, biochemical characteristics revealed their similarity. All 50 shrimp showed characteristic WSSV infection. Bacteriological isolation on the TCBS agar plates revealed that only 30 shrimp contained characteristic yellow colonies. Therefore, the prevalence of dual *Vibrio alginolyticus* and WSSV infection in the pond was assumed to be ~60%. The characteristics of the chosen isolate (QS7) are shown in Table 1. Based on this and comparison with earlier reports, the isolate was identified as *V. alginolyticus* (QS7) (Lee et al. 1996).

Antibiogram

QS7 was highly susceptible to chloramphenicol, ciprofloxacin, nalidixic acid and streptomycin with inhibition zones of 35, 30, 31 and 30 mm, respectively. Inhibition zones for gentamycin, metronidazole, rifampicin, erythromycin and ampicillin were 10 mm. The isolate was resistant to penicillin-G and bacitracin

and completely unaffected by oxytetracyline, a commonly used shrimp therapy. Sulphamethizole was grouped amongst the least active antibiotics because resistant colonies were observed inside the inhibition area. This also occurred with sulphadiazine, tetracycline, trimethaprim, cloxacillin, amoxycillin and amphotericin-B.

Table 1. *Vibrio alginolyticus.* Characteristics of bacterium (QS7) isolated from diseased *Penaeus monodon*. +: positive; –: negative; TCBS: thiosulfate citrate bile salts sucrose agar; ONPG: *o*-nitrophenyl beta-D-galactopyranoside

Test/characteristic features	QS7
Gram staining	–
Shape	Short rod
Growth on TCBS	Green
Sensitivity to vibriostatic 0/129 phosphate	+
Luminescence	–
Swarming	+
Oxidase production	+
Catalase production	+
Oxidative-fermentive test	+
Acid/gas production:	
Glucose	Acid
Sucrose	Acid
Mannitol	Acid
Maltose	Acid
Sorbitol	–
Lactose	–
Galactose	–
Arabinose	Acid
Nitrate reduction	+
Methyl red	+
Voges-Proskauer	+
Indole production	–
Hydrogen sulfide production	–
ONPG hydrolysis	–
Decarboxylase of:	
Arginine	–
Lysine	+
Ornithine	+
Growth in:	
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	–

Table 2. *Vibrio alginolyticus* (QS7). Pathogenicity to *Penaeus monodon*, based on triplicate trials

Dose (cfu per shrimp)	Length (cm)	Weight (g)	Mortality (%)	Infection (%)
10^8	6.50 ± 0.15	2.60 ± 0.52	100	0
10^7	6.26 ± 0.18	2.38 ± 0.16	80	20
10^6	6.28 ± 0.42	2.42 ± 0.23	20	80
10^5	5.96 ± 0.62	2.12 ± 0.28	0	30
10^4	6.42 ± 0.13	2.48 ± 0.18	0	0

Pathogenicity and LD_{50}

At a high dose (10^8 cfu per shrimp), all shrimp died at 6 to 24 h p.i. (Table 2). No mortality was observed within 7 d p.i. at 10^5 cfu per shrimp, although infections were noticed on Day 5. The LD_{50} value extrapolated from the graph was 5×10^6 cfu per shrimp. Signs of infection included brown or black spots on the shell, darkened or red body surface, pink or brown gills, murky whitish muscle, a lack of food in the midgut, and a folded base of the tail.

DISCUSSION

The diseased shrimp collected from the farm were under stress due to high stocking density (160 000 ha^{-1}) and subsequent deterioration of hydrological conditions from high feed inputs and accumulation of organic matter on the pond bottom. Such conditions usually develop in the middle of the shrimp culture period (de la Pena et al. 1992). Severe stress and injury to the shrimp under poor environmental conditions lowers their resistance (Liu 1990), rendering them susceptible to viral as well as bacterial infection (Sindermann 1990).

In addition, the primary WSSV infection probably weakened the shrimp and made them more susceptible to bacterial infection. The isolation of such secondary pathogens from outbreaks of WSSV in cultured tiger shrimp and consequent mass mortality has been reported in Taiwan (Lee et al. 1996). The WSSV-infected shrimp showed reddish coloration in addition to the white spots in the cuticle. According to Karunasagar et al. (1998), reddish coloration of WSSV-infected shrimp could be attributable to secondary bacterial infection.

According to an earlier report, a virulent pathogenic strain of *Vibrio alginolyticus* isolated from WSSV-infected shrimp was sensitive to chloramphenicol, ciprofloxacin, nalidixic acid, oxolinic acid, penicillin-G, and sulfonamide (Lee et al. 1996). This sensitivity pattern resembled that observed for QS7 in the pre-

sent study. Based on the QS7 results, it could be inferred that antibiotics such as ciprofloxacin, nalidixic acid and streptomycin might be useful therapy for WSSV-infected shrimp. However, because of emerging environmental and public health concerns, their usage has to be kept to a minimum.

Our pathogenicity tests confirmed that QS7 was virulent to shrimp. It has previously been reported that shrimp affected by WSSV become susceptible to invasion by environmental *Vibrio* species including *V. alginolyticus* (Karunasagar et al. 1997). *V. alginolyticus* has also been reported as a pathogen associated with shrimp diseases including 'black splinter disease' in Thailand (Ruangpan & Kitao 1991). According to Ramasamy et al. (1995), mortality in *Penaeus monodon* infected with monodon baculovirus (MBV) was hastened by secondary bacteria. The bacteria might have entered the hepatopancreas as secondary pathogens via lesions resulting from MBV (Lightner et al. 1983).

Vibrio alginolyticus was isolated as a virulent secondary bacterial pathogen from shrimp infected with WSSV and was found to be lethal for *Penaeus monodon* and kuruma prawns *P. japonicus*, with LD_{50} values of 1.13×10^5 and 2.46×10^5 cfu g^{-1} shrimp, respectively (Lee et al. 1996). These values are in the range that we found for QS7. They are also similar to LD_{50} values (1.1×10^6 to 2.2×10^7) of 6 *V. alginolyticus* strains isolated from a culture environment and used to challenge healthy *P. monodon* post-larvae (Otta et al. 1999). A virulent strain isolated from moribund kuruma prawns had an LD_{50} of 4.43×10^4 cfu g^{-1} shrimp (Lee et al 1996). The LD_{50} values in the present study indicated that the shrimp weakened by WSSV may die due to secondary bacterial infections.

Acknowledgements. The authors thank Dr. M. J. Modaiyl, Director and Dr. R. Paul Raj, Head, PNP Division, CMFRI, Cochin, for encouragement and use of facilities. This paper was part of the PhD thesis work of J.S.

LITERATURE CITED

Austin B, Austin DA (1989) Methods for the microbiological examination of fish and shellfish. Ellis Horwood, Chichester

Austin B, Lee JV (1992) Aeromonadaceae and Vibionaceae. In: Board RG, Jones D, Skinner FA (eds) Identification methods in applied and environmental microbiology. technical series of the society for applied bacteriology. Blackwell, Oxford, p 163–182

Bauer AW, Kirby WMM, Shenis JC, Turck M (1996) Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol 45:493

Baumann P, Schubert RHW (1984) Family U. Vibrionaceae. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, Vol 1. Williams & Wilkins, London, p 516–538

Colwell RR, Grimes JA (1984) *Vibrio* diseases of marine fish populations. Helgol Wiss Meeresunters 37:265–287

Cowan ST (1974) Cowan and Steel's manual for the identification of medical bacteria, 2nd edn. Cambridge University Press, London

de la Pena LD, Momoyama K, Nakai T, Muruga K (1992) Detection of the causative bacterium of vibriosis in kuruma prawn, *Penaeus japonicus.* Fish Pathol 27:223–228

HiMedia Manual (1988) HiMedia Manual for microbiology laboratory practice. HiMedia Laboratories, Mumbai

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

Karunasagar I, Otta SK, Karunasagar I (1997) Histopathological and bacteriological study of white spot syndrome of *Penaeus monodon* along the west coast of India. Aquaculture 153(1–2):9–13

Karunasagar I, Otta SK, Karunasagar J (1998) Monodon baculovirus (MBV) and bacterial septicemia associated with mass mortality of cultivated shrimp (*Penaeus monodon*) from the east coast of India. Indian J Virol 14(1):27–30

Lee KK, Yu SR, Yand TI, Liu PC, Chen FR (1996) Isolation and characterization of *Vibrio alginolyticus* from diseased kuruma prawn, *Penaeus japonicus*. Lett Appl Microbiol 22:111–114

Lightner DV (1988) Diseases of cultured penaeid shrimp and prawns. In: Sinderman CJ, Lightner DV (eds) Disease diagnosis and control in North American marine aquaculture, 2nd edn. Elsevier, Amsterdam, p 8–127

Lightner DV, Redman RM (1994) New and emerging infectious and non-infectious diseases in commercial shrimp culture. In: International Symposium on Aquatic and Animal Health. Programme and Abstracts, University of California, Davis, p V–3

Lightner DV, Redman RM, Bell TA (1983) Observation on the geographic distribution, pathogenesis and morphology of the baculovirus from *Penaeus monodon* Fabricus. Aquaculture 32:209–233

Liu CI (1990) The diseases of cultured *Penaeus monodon* with emphasis on recent discoveries in Taiwan In: Kou GH, Wakabayashi H, Liao IC, Chen SN, Lo CF (eds) Fish diseases. Proc R Jpn Symp. Taipei, Taiwan. NSC Symp Ser 16:180–201

MacFaddin JF (1981) Biochemical tests for identification of medical bacteria, 2nd edn. Williams & Wilkins, Baltimore

Otta SK, Karunasagar I, Karunasagar I (1999 Bacterial flora associated with shrimp culture ponds growing *Penaeus monodon* in India. J Aquacult Trop 14(4):309–318

Ramasamy P, Breman GP, Jayakumar R (1995) A record and prevalence of *Monodon* baculovirus from postlarval *Penaeus monodon* in Madras, India. Aquaculture 130: 129–135

Ruangpan L, Kitao T (1991) *Vibrio* bacteria isolated from black tiger shrimp, *Penaeus monodon* Fabricious. J Fish Dis 14:383–388

Sindermann CJ (1990) Bacteria. In: Principal diseases of marine fish and shellfish, Vol 2. Academic Press, San Diego, p 41–74

Tendencia EVA, Dureza LA (1997) Isolation of *Vibrio* sp. from *Penaeus monodon* (Fabricius) with red disease syndrome. Aquaculture 154:107–114

Wardlaw AC (1985) Practical statistics for experimental biologists. John Wiley & Sons, Chichester

Editorial responsibility: Timothy Flegel, Bangkok, Thailand

Submitted: April 4, 2003; Accepted: September 6, 2003
Proofs received from author(s): October 27, 2003