Indian Journal of Marine Sciences
Vol. 32(1), March 2003, pp. 71-75

Pathogenicity and antibiotic susceptibility of *Vibrio* species isolated from moribund shrimps

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Received 26 April 2002, revised 11 December 2002

The population of *Vibrios* in diseased *Penaeus monodon* collected from culture ponds situated at Uran, Maharashtra (west coast of India) was studied. All animals collected were associated with more than one *Vibrio* species. Bacterial identification based on morphological and biochemical characteristics showed that four groups of Vibrios were present in shrimp hepatopancreas namely *Vibrio parahaemolyticus*, *V. alginolyticus*, *V. anguillarum* and *V. vulnificus*. The diseased shrimps displayed poor growth, lethargic movements, red discolouration and mortality. Experimental infection of healthy *P. monodon* using the isolated Vibrios produced varying degrees of mortality. Antibiotic resistance of the isolated Vibrios were investigated. All the four species were sensitive to Erythromycin, Chloramphenicol and Streptomycin. However, *V. parahaemolyticus* and *V. anguillarum* showed resistance to Oxytetracycline and Polymyxin-B respectively and *V. parahaemolyticus* and *V. vulnificus* were resistant to ampicillin. Hence, antibiotic application to control vibriosis in shrimp farms may have limited effectiveness, based on the efficacy of the drug, because of the development of resistant strains of bacteria.

[**Keywords** : Shrimp, antibiotic resistance, Vibrios, *Penaeus monodon*]

Vibrios which are members of the normal bacterial flora of shrimps, induce mass mortalities in affected populations of shrimps[1]. Bacterial diseases caused by members of the genus *Vibrio* such as *V. parahaemolyticus, V. alginolyticus, V. anguillarum* and *V. vulnificus* have often been reported among cultured penaeid shrimps[2].

In the present paper, we report an outbreak of bacterial disease in *Penaeus monodon* culture pond. The objective of this study is to explore the cause of the observed infection in grow out ponds of *P. monodon* at Uran (Maharashtra, West coast of India). The Vibrios present in the hepatopancreas of the infected shrimp were isolated in order to identify strains which could be significantly associated with diseased animals. The study also aims to determine the threshold level of Vibrios in *P. monodon* by correlating the bacterial load in surrounding water and the occurrence of mortality in shrimps. A variety of antimicrobials are used in aquaculture to control bacterial infections. Due to the indiscriminate use of antimicrobials the number of antibiotic resistant bacteria are increasing. In India, little data is available in the area of antibiotic resistance among shrimp pathogens. Hence, study of antibiotic resistance in pathogenic *Vibrio species* isolated from *P. monodon* was also carried out.

Material and Methods

Five infected shrimps of 20-25 g were manually collected from a 1 ha pond with a stocking density of 7 shrimps/m^2 and not used any antimicrobials during this culture period. Shrimps were considered diseased when they showed symptoms such as lethargy, anorexia resulting to empty digestive tracts and reddening of the body. Animals were transported live to laboratory and hepatopancreas removed aseptically for bacteriological study.

Bacteriological analysis

Shrimps were surface disinfected by swabbing with 75% alcohol. The hepatopancreases were aseptically removed and homogenised in 10 ml of autoclaved (121°C for 15 min.) peptone water. Serial dilutions were made and 0.1 ml of the sample was plated onto thiosulfate citrate bile sucrose agar (TCBS) by pour plate method. Plates were incubated at 30°C for 24 h. To analyse the composition of the *Vibrio* population of each sample, 20 bacterial colonies were chosen from TCBS plates containing 30 – 300 colonies. Selected colonies were purified and identified by standard biochemical tests[3].

Pathogenicity test

Prior to the infection experiments, healthy shrimp larvae (PL-20), without any prior antibiotic treatment,

were held in a 7 litre, glass jar, filled with 5 litres of autoclaved seawater, for 4 days for acclimation to laboratory conditions (salinity–25 ppt, temperature 24-28°C, *p*H 8.0). They were fed with commercial diet (LUX, Waterbase, crumble 1, size 0.1-0.3 mm) four times a day. The bacterial infection experiment was based on the method described by Karunasagar *et al.*[4]. Shrimp larvae were stocked in glass jars of 7 litres filled with 5 litres of autoclaved seawater at a density of 2 individual/litres. Twentyfour hour culture of *V. vulnificus, V. alginolyticus, V. parahaemolyticus* and *V. anguillarum* were separately added to glass jar to give a final concentration of 10^3 to 10^8. No bacteria were added in jars that served as control. Larvae were observed for 96 h. A record of body colour, lethargy and sluggishness was maintained.

The test inocula were prepared by streaking 18-24 h bacterial culture on TSA with 1.5% (w/v) NaCl. The bacteria were harvested after 24 h using sterile wire loop, placed in pre-weighed sterile test tube and suspended in sterile normal saline solution (0.85% NaCl). Dilutions were made to produce different doses of inocula.

Bacterial re-isolation was performed by homogenising the gut and exoskeleton, consisting mainly of pleopods and the cephalothorax of the experimental shrimps and plating on TSA. Identification was done as earlier described. Mortalities were attributed to an isolate only if it was recovered in virtually pure culture from the dead animals. Aseptic techniques were used throughout the processing of samples. The LD_{50} was calculated by probit method[5].

Antibiotic sensitivity test

Purified cultures of the test organisms were picked with a wire loop and introduced into a test tube containing 4 ml of trypticase soy broth. These tubes were then incubated for 20-24 h to produce a bacterial suspension of moderate cloudiness. For the sensitivity tests, large (15 cm) Petri plates were used with Mueller-Hinton agar (5 to 6 mm depth). Plates were dried before use. The bacterial broth suspention was streaked evenly in 3 planes onto the surface of the medium with a cotton swab. After the inoculam dried the antibiotic discs (Pastaur pharmacuticals) were placed on the agar with flamed foreceps and gently pressed down to ensure contact. The discs used were Oxytetracycline (30µg), Erythromycin (15µg), Ampicillin (10µg), Chloramphenicol (30µg), Streptomycin (10µg) and Polymyxin-B (300 units). After overnight incubation at 30°C, the zone diameter (including the disc) were measured with a ruler. The end point was taken as complete inhibition of growth as observed visually. The results were interpreted according to Bauer *et al* [6]. The significance of mortalities in pathogenicity test was assessed statistically by employing chi-square test.

Results

A total of 100 colonies were chosen from hepatopancreas of 5 diseased shrimps. Four major groups of isolates were identified based on their cultural, morphological and biochemical characteristics. Characteristics of the four groups and their identification[3] is given in Table 1. The microbiological and histopathological studies of hepatopancreas indicated absence of viral occlusion bodies in the cells. It confirms that the disease infecting the cultivated prawn is not a viral disease. Experimentally infected prawns exhibited a reddish body colouration, lethargy, and sluggishness in swimming. A bacterium was isolated in pure culture from the gut and exoskeleton of moribund or recently dead shrimps and it was confirmed by biochemical test to be the initial inoculated bacterium.

Table 2 shows the cumulative mortality from the pathogenicity tests of *V. parahaemolyticus*, *V. alginolyticus, V. anguillarum* and *V. alginolyticus*. *Vibrio vulnificus* isolates were the most virulent with an LD_{50} of 6.54×10^3. The LD_{50} of *V. alginolyticus* was 2.54×10^6, *V. parahaemolyticus* was 5.99×10^5 and *V. anguillarum* was 6.13×10^5, indicating low virulence. The mortality is not significantly different at 10^3 as compared to 10^4-10^8. Only test with *V. anguillarum* showed significant increase in mortality at 10^6 than 10^5, for other three isolates the increase was not significant.

Comparative study of various antibiotics based on the zone of inhibition of bacteria is given in Table 3. All the four species were sensitive to erythromycin, streptomycin and chloramphenicol. *Vibrio parahaemolyticus* was resistant to ampicillin and oxytetracycline and showed intermediate sensitivity to polymyxin-B. *Vibrio alginolyticus* was sensitive to all the six antibiotics tested while *V. anguillarum* and *V. vulnificus* were resistant to polymyxin-B and ampicillin respectively.

Discussion

Experimental infection of *Penaeus monodon* with Vibrios produced varying degrees of mortality (Table 2). Pure isolates were re-isolated from the gut and exoskeleton of dead and moribund shrimps indicating

Table 1 — Characteristics of the four isolates of *Vibrio* spp. from diseased *Penaeus monodon*.

Characteristics	*Vibrio parahaemo-lyticus*	*Vibrio alginolyticus*	*Vibrio anguillarum*	*Vibrio vulnificus*
Gram staining	-	-	-	-
Rods	Straight	Straight	Curved	Curved
Cytochrome Oxide Test	+	+	+	+
Oxidative/ fermentaive	F	F	F	F
Sensitivity to 0/129	+	+	+	+
Motility	+	+	+	+
Catalase	+	+	+	+
Growth at 40°C	+	+	+	-
Gas from glucose	-	-	-	-
Nitrate reduction	+	+	+	+
ONPG	-	-	+	+
Oxidase	+	+	+	+
Voges-Proskauer	-	+	+	-
Ornithine de-carboxylase	+	+	-	+
Arginine di-hydrolase	-	-	-	-
Lysine de-carboxylase	+	+	-	+
Citrate utiliza-tion	+	+	+	+
Amylase	+	+	+	+
Gelatinase	+	+	+	+
Utilization of				
L-Arabinose	+	-	+	-
Cellobiose	-	-	+	-
Galactose	+	+	-	+
Lactose	-	-	-	-
D-mannitol	+	+	+	+
D-mannose	+	+	+	+
Melobiose	-	-	-	-
L-rhamnose	-	-	-	-
Sucrose	-	+	+	-
Growth on 8% NaCl	+	+	-	-

virulence of the isolates. The first 24 h did not show any mortality in the case of *V. anguillarum* and *V. alginolyticus* at the tested concentrations. Nash *et al.*[7], reported shrimps injected with bacteria are weak in the first two days but can recover within 3-4 days. In this study also shrimps exposed to *V. anguillarum* and *V. alginolyticus* at 10^5 cfu/ml were weak for about 48 h, but recovered within 96 h. It could therefore, be stated that 10^5 cfu/ml of with *V. anguillarum* and *V. alginolyticus*, would cause an

Table 2 — Cumulative mortality observed from the pathogenicity test of Vibrios on *Peneaus monodon* postlarvae

Isolates used for challenge test	Challenge dose (Bacteria/ml)	Percent mortality (10)* 24 h	48 h	72 h	96 h
V. anguillarum	10^3	0^a	0^a	0^a	0^a
	10^4	0^a	0^a	0^a	0^a
	10^5	0^a	0^a	0^a	10^{ab}
	10^6	0^a	20^{abc}	50^{bcdef}	60^{cdefg}
	10^7	0^a	40^{bcde}	80^{efg}	100^g
	10^8	0^a	80^{efg}	100^g	100^g
V.alginolyticus	10^3	0^a	0^a	0^a	0^a
	10^4	0^a	0^a	0^a	0^a
	10^5	0^a	0^a	0^a	0^a
	10^6	0^a	0^a	10^{ab}	30^{abcd}
	10^7	0^a	10^{ab}	50^{bcdef}	80^{efg}
	10^8	0^a	20^{abc}	80^{efg}	100^g
V. parahaemolyticus	10^3	0^a	0^a	0^a	0^a
	10^4	0^a	0^a	0^a	0^a
	10^5	0^a	0^a	10^{ab}	20^{abc}
	10^6	0^a	0^a	30^{abcd}	50^{bcdef}
	10^7	10^a	20^{abc}	70^{defg}	100^g
	10^8	10^a	40^{bcde}	100^g	100^g
V. vulnificus	10^3	0^a	0^a	0^a	30^{abcd}
	10^4	0^a	0^a	10^{ac}	50^{bcdef}
	10^5	0^a	20^{abc}	30^{abcd}	80^{efg}
	10^6	20^{abc}	50^{bcdef}	80^{efg}	100^e
	10^7	50^{bcdef}	70^{cde}	90^{fg}	100^e
	10^8	80^{efg}	100^g	100^g	100^e

*Number of larvae tested
Values with the same letter are not significantly different ($P > 0.05$)

infection, but may not result in disease. The pathogenicity study shows that a higher dose of inocula is needed to elicit disease caused by the four *Vibrio* spp tested[8]. This agrees with Lightner's[9] observation that isolates of *Vibrio* spp. from diseased shrimps may not always produce experimental infection, except with massive doses. With *V. vulnificus* chances of infection turning into disease at a lower concentrations seems higher. The infection with *V. vulnificus* at 10^5,10^6 and 10^7 cells/ml resulted in 0, 20 and 50% mortality respectively within 24 h. Exposure to 10^6 and 10^7 cells/ml of *V. vulnificus* for 96 h resulted in 100% mortality.

In this study mortality (Table 2) was not seen after 24 h exposure to *V. parahaemolyticus* at 10^5 and 10^6 cells/ml, but a mortality of 20 and 50% was noted at the end of 96 h. At the concentration of 10^7 cells/ml, it killed all the animals within 96 h. *Vibrio parahaemolyticus* causing up to 100% mortality within 24 hr by injecting a dose of 10^5-10^7 cfu/ml was reported earlier[8].

In penaeid shrimps, differences in the pathogenicity of bacteria could depend on the species tested[10] and on

Table 3 — Antibiotic sensitivity of *Vibrio species*

Antibiotics	Sensitivity of isolates			
	Vibrio alginolyticus	*Vibrio anguillarum*	*Vibrio parahaemolyticus*	*Vibrio vulnificus*
Oxytetracycline (30µg)	S	S	R	S
Erythromycin (15µg)	S	S	S	S
Ampicillin (10µg)	S	S	R	R
Chloramphenicol (30µg)	S	S	S	S
Streptomycin (10µg)	S	S	S	S
Polymyxin-B (300units)	S	R	I	I

S - sensitive, R – resistant, I - intermediate

the strain characteristics[11]. This study supports this view as it was observed that mortality resulting from challenge test depended on the dose and species. Infection with *V. vulnificus* resulted in 100% mortality after 96 h at 10^6 cfu/ml, whereas infection with *V. alginolyticus* showed 30% mortality. These results show direct relation between chances of disease occurrence and number of pathogens present in the rearing water.

The data (Table 2) in this study indicate that the four Vibrios isolated from diseased shrimps can cause disease when present in sufficient numbers, of which *V. vulnificus* was most virulent followed by *V. anguillarum, V. parahaemolyticus* and *V. alginolyticus*. Post-infected shrimps exhibited reduced feeding, low rate of survival, reddish colouration, and sluggishness in swimming and lying at the bottom of the tank. Difficulty in moulting was seen in some of the infected shrimps.

In the present study, *Vibrio* spp. were isolated from diseased shrimp, symptoms of which were reproduced by experimentally infecting with *V. parahaemolyticus, V. vulnificus, V. anguillarum* and *V. alginolyticus*. The disease may be a syndrome with more than one causes and based on the results of this study, *Vibrio* spp. are one of them.

Many shrimp farms and hatcheries in India and outside use antibiotics to control bacterial infection in shrimps[4,12]. The excessive use at sub-therapeutic dosage may result in the development of resistant strains. Numerous studies suggest a correlation between findings of increased bacterial resistance levels in and around culture farms and the antimicrobial used at the farms[13]. In this investigation antibiotic resistance was studied in ponds with no antibiotic use for 6 months prior to sampling, nor was any other farm present in the vicinity. Hence, the chances of developing antibiotic resistant bacteria due to previous exposure to antibiotic seem meagre.

Present study revealed that all the four isolates were sensitive to chloramphenicol. In earlier reports, chloramphenicol showed marked effect at low dosages on the zone of inhibition of chitin degrading Vibrios[14]. In some cases increased resistance of *V. anguillarum, V. parahaemolyticus* and *V. vulnificus* to chloramphenicol is attributed to the indiscriminate use of antimicrobial drugs[2].

All the isolates were sensitive to chloramphenicol, streptomycin and erythromycin. In addition *V. alginolyticus* was sensitive to all tested antimicrobials. *Vibrio parahaemolyticus* was resistant to oxytetracycline and ampicillin. *Vibrio vulnificus* was also resistant to the later, *V. anguillarum* to Polymyxin B, *V. parahaemolyticus* and *V. vulnificus* showed intermediate zone of inhibition to Polymyxin B.

Among the individual pathogens tested in this study, *V. parahaemolyticus* was resistant to oxytetracycline, but *V.alginolyticus*, *V. anguillarum* and *V. vulnificus* were susceptible. Oxytetracycline is most widely used antibiotic in aquaculture. This seems to be the only antibiotic successful in controlling vibrosis in shrimp culture, even though laboratory results sometimes show that the bacteria are resistant to oxytetracycline[15].

The present isolates were susceptible to most of the antibiotics tested. But proper attention is to be given before the use of antibiotics in aquaculture. The indiscriminate use may not only have an adverse effect on shrimp growth but may cause health hazards in humans. It is therefore necessary to ensure the chemotherapeutic agents used are efficacious to the target species and safe for the user of aquaculture products.

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