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Note

Antimicrobial resistance profile of *Vibrio* species isolated from the hatchery system of *Macrobrachium rosenbergii* (Deman)

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ABSTRACT

Vibrio spp. are responsible for high mortality and severe economic loss in prawn industry in all the prawn producing countries. The incidence of high antibiotic resistance is reported in hatcheries. For the present study, 23 isolates of *Vibrio* species from the larvae, larval rearing water, *Artemia* and *Artemia* rearing water from the hatchery system of *Macrobrachium rosenbergii* were used. The isolates were characterised and nine different species were identified *viz., V. harveyi, V. parahaemolyticus, V. damsela, V. splendidus, V. anguillarum, V. alginolyticus, V. fluvialis, V. vulnificus* and *V. campbelli.* Fifteen commonly used antibiotics in the hatchery system were tested against the isolated *Vibrio* species. The results showed that all the isolates (100%) were resistant to ampicillin, erythromycin, doxycycline hydrochloride and sensitive to amikacin, chloramphenicol, cefaclor, gentamycin and streptomycin.

Keywords : Antimicrobial resistance, Hatchery, *Macrobrachium rosenbergii, Vibrio*

The giant freshwater prawn, *Macrobrachium rosenbergii* is an important species cultured in many countries because of its high commercial value (New, 1982). Each step of *Macrobrachium* larval development is characterised by different type of associated microbiota, which is introduced in to the system by different ways such as seawater (in eggs and yolk-sac larvae) and live feed (*Artemia* and rotifers). Bacterial disease represents the most serious problem to the development of prawn farming industry caused by opportunistic bacteria belonging to the genera *Vibrio* sp., *Aeromonas* sp., *Alcaligens* sp., *Moraxella* sp. and *Pseudomonas* sp. High mortality and severe economic loss are caused by *Vibrio* sp. in prawn farming in all the prawn producing countries (Jayakumar and Ramasamy, 1994; Ramasamy 2000). Several species including *Vibrio harveyi*, *V. anguillarum*, *V. damsela*, *V. parahaemolyticus*, *V. vulnificus*, *V. fluvialis*, *V. alginolyticus*, *V. mimicus*, *V. furnissi*, *V. cholera* and *V. ordalli* are known to induce severe infections in shrimp aquaculture environment (Srinivasan and Ramasamy, 2009). Usual approaches to control bacterial growth in intensive rearing of larvae are based on preventive measures by way of maintaining a clean environment, by seawater treatment processes (filtration, UV-irradiation, ozonisation, disinfectants). Disinfection of eggs (Salvesen and Vadstein, 1995), treatment with hydrogen peroxide (Giménez *et al.,* 2006) or ultraviolet radiation for partial decontamination (Munro *et al.,* 1995) have been proposed but would be difficult to implement at industrial scale. Complete elimination of bacteria from the organisms and culture system is not possible. The use of antibiotics in prawn hatcheries has become widespread, although by no means universal. Earlier, studies have revealed that certain bacterial species are controlled by antibiotics (Hameed and Rao, 1994; Abraham *et al.,* 1997). The most common way to resolve the vibriosis problem is by the use of antibiotics. A wide range of antimicrobial compounds *viz.*, oxytetracycline, ciprofloxacin, nitrofurantoin, furazolidone and chloramphenicol are being used in the hatcheries and farms of freshwater prawn and marine shrimp in India to control bacterial population (Karunasagar *et al.,* 1994; Abraham *et al.,* 1997; Hameed and Balasubramanian, 2000). Continuous and improper use of antibiotics in aquaculture favours the selection of resistant isolates and the dissemination of resistance genes within bacterial populations in the environment, reflecting the pattern of drug use (Tendencia and de la Pena, 2002). Many cases of multiple antimicrobial resistances have been reported from shrimp farms in countries where the activity is well developed, such as China (Dang *et al.,* 2006), Korea (Kang *et al.,* 2005) and Chile (Miranda and Rojas, 2007). Moreover, the use of huge amount of antibiotics or chemicals is unacceptable as it is unfavourable to the environment (Park *et al.,* 2000, Nakai and Park 2002; Serrano, 2005). The situation is aggravated by the emergence of antibiotic-resistant strains (Karunasagar*et al.,* 1994) and the ability of *Vibrio* sp. to form biofilms (Karunasagar*et al.,* 1996). Hence, the present study was

initiated to assess the occurrence and antibiotic resistance profile of *Vibrio* spp. in hatchery system of the freshwater prawn *M. rosenbergii*.

Bacteria (presumptive *Vibrio* spp.) were isolated from the larvae of *Macrobrachium rosenbergii,* hatchery tank water (seawater and mixedwater), *Artemia* nauplii and *Artemia* rearing water from freshwater prawn hatcheries, located at Kovalam, Bay of Bengal, India and at Vandalur, Chengelpet near Chennai, India. The bacteria were isolated on the selective medium Thiosulphate Citrate Bile Salt Sucrose Agar (TCBS Agar). Presumptive *Vibrio* isolates were picked from TCBS agar plates according to the colour of the colony (indicating sucrose fermentation) as well as colony morphology and size. The isolated colonies were then purified by streaking on Nutrient Agar plates (with 2% NaCl) and the purified colonies were transferred to NB broth (with 2% NaCl), cultured at 32 °C for 18 h and then stored at –20 °C, after the addition of 30% (v/v) glycerol until further studies. They were characterised and identified based on standard biochemical and morphological tests (Alsina and Blanch, 1994).

Commercially available antibiotic discs (Hi-media, Mumbai, India) were used to test the susceptibility patterns. The antibiotic discs tested include amikacin (Ak) 30µg, ampicillin (A) 10µg, azithromycin (At) 15µg, cefaclor (Cj) 30µg, cephotaxime (Ce) 30µg, chloramphenicol (C) 30µg, ciprofloxacin (Cf) 30µg, doxycycline hydrochloride (Do) 30µg, erythromycin (E) 15µg, furazolidone (F)50µg, gentamicin (G) 10µg, kanamycin (K) 30µg, nalidixic acid (Na) 30µg, neomycin (N) 30µg and streptomycin (S) 10µg. The assay was carried out as per Molitoris *et al.* (1985). Pure isolated colonies of bacteria were suspended in 0.85% sterile saline and the turbidity was adjusted to match with 0.5 McFarland standard. The inoculum was spread over Mueller-Hinton agar (Himedia, Mumbai) medium and the antibiotic discs were placed on the surface of the agar plates. The plates were incubated at 32ºC and the zones of inhibition were recorded after 24 and 48 h. The susceptibility of the bacterial isolates to various antibiotics were determined based on the reference range. Multiple antibiotic resistance (MAR) index of the isolates against the tested antibiotics was calculated according to Sarter *et al.* (2007) as: MAR index = X/(Y×Z), where X = total number of antibiotic resistance case; Y = total number of antibiotics used in the study and Z = total number of isolates tested. MAR index greater than 0.2 implies that the strain of such bacteria originate from an environment where several antibiotics are used (high risk) and MAR index values less than or equal to 0.2 indicates that those isolates are from environments where these antibiotics are seldom used (low risk).

A total of 23 isolates of *Vibrio* spp. were collected from the larvae of *Macrobrachium,* hatchery tank water, *Artemia* nauplii and *Artemia* rearing water. The present study revealed the occurrence of nine different species of vibrios *viz., V. parahaemolyticus* (4 isolates), *V. splendidus* (4 isolates), *V. fluvialis* (3 isolates), *V. alginolyticus* (3 isolates), *V. anguillarum* (3 isolates), *V. harveyi* (2 isolates), *V. damsela* (2 isolates), *V. vulnificus* (1 isolate) and *V. campbelli* (1 isolate). All the isolates were rod shaped, motile, Gram negative, halophilic and oxidase as well as catalase positive. All the 23 isolates fermented D-glucose without gas production and reduced nitrate. Results of carbohydrate fermentation showed that *V. anguillarum* was lactose negative and positive to carbohydrates such as maltose, galactose, fructose, and sucrose. *V. parahaemolyticus* isolates were positive to maltose and negative to sucrose as well as galactose. None of the *Vibrio* isolates produced acid from inositol, inulin, raffinose, melibiose, xylose, lactose and rhamnose. Similar observations were reported by Abraham and Palaniappan (2004).

The 23 different *Vibrio* spp. used in the present study were isolated from the larvae of *Macrobrachium* (8 isolates), seawater (2 isolates), hatchery tank water (2 isolates), *Artemia* nauplii (3 isolates) and *Artemia* rearing water (7 isolates). From *M. rosenbergii* larvae, 5 species of vibrios *viz*., *V. parahaemolyticus, V. alginolyticus, V. damsela, V. anguillarum* and *V. vulnificus* were isolated with *V. parahaemolyticus* being the predominant species. Abraham *et al.* (2001) reported *V. harveyi* to be the dominant flora invariably in penaeid shrimp hatchery components. Lavilla-pitago *et al.* (1990) reported the presence of luminous *V. splendidus* biotype I in shrimp hatcheries.

In the present study, *V. splendidus* and *V. harveyi* were isolated from the larval rearing hatchery tank water, where as *V. parahaemolyticus* and *V. fluvialis* were recorded from source seawater. Lavilla-Pitogo *et al.* (1990) reported that *V. harveyi* has been recovered from nearshore seawater. *V. parahaemolyticus* is abundant in the seawater column and sediments during summer (Kumazawa *et al.,* 1991; Ruangpan and Kitao, 1991). The untreated effluents from shrimp farms/hatcheries are drained off into the coastal areas and these may be one of the primary sources contributing to the increased concentration of *Vibrio* sp. in the coastal area of Bay of Bengal, India.

In the present study, *V. parahaemolyticus*, *V. damsela* and *V. splendidus* were isolated and identified from the nauplii of *Artemia. V. splendidus*, *V. anguillarum, V. fluvialis* and *V. campbelli* were identified in the *Artemia* rearing water. *Pseudomonas* sp. (34%), *Moraxella* sp. (2%), *Cytophaga* sp. (6%) besides *Vibrio* sp. (43%) were

reported in the *Artemia* (Tanasomwang and Muroga 1990; Verdonck*et al.,* 1991; Hameed*et al.,* 2000; Torres and Partida 2001). Lavilla-Pitogo *et al.* (1992) recorded luminous bacteria upto 0.005% and 0.17% of the total vibrio count (TVC) in *Artemia* nauplii and rearing water. Abraham and Palaniappan (2004) reported the occurrence of luminescent bacterium *V. harveyi* in *Artemia* rearing water and *Artemia* nauplii.

In the present study, all the isolates of *Vibrio* were sensitive to amikacin, chloramphenicol, gentamicin and streptomycin whereas they showed resistance to ampicillin (100%), erythromycin (100%), doxycycline hydrochloride (100%), cefaclor (100%), cephotaxime (91%), kanamycin (47%), furazolidone (30%), ciprofloxacin (26%), nalidixic acid (21%), neomycin (21%) and azithromycin (8%) (Table 1 and 2). Similar observation was reported by Hameed and Balasubramanian (2000) that 90% of the bacterial isolates from the larvae and post-larvae of freshwater prawn showed resistance to erythromycin, oxytetracycline and furazolidone. *V. harveyi* isolated from different shrimp farming facilities and from unfarmed waters in Asia were resistant to ampicillin (Teo *et al.,* 1999). Roque *et al.* (2001) reported that 100% of the 11 isolates from seawater were resistant to ampicillin. Oxytetracycline is a commonly used antibiotics in aquaculture but only 57% of the *Vibrio* isolates showed any sensitivity to this antibiotic and only 29% of them were highly sensitive (Roque *et al.,* 2001). Karunasagar *et al.* (1994) observed that luminous *V. harveyi* developed resistance to drugs such as streptomycin, chloramphenicol and cotrimazole. Baticodos *et al.* (1990) also reported the occurrence of antibiotic resistance to erythromycin, kanamycin, penicillin and streptomycin in luminous strains of *V. harveyi* and *V. splendidus* isolated from shrimp larvae. The continuous use of antibiotics and their persistence tends to lead to the development of antibiotic

Table 1. Resistance profile of *Vibrio* spp. isolated from freshwater prawn hatchery

Source of isolation	Isolate code	Species identified	Resistant	Susceptible
Larvae	LAR1	*V. parahaemolyticus*	A:Do:E:Ce:F	Ak:C:G:S:Cj:At: Cf: K:Na:N
Larvae	LAR2	*V. alginolyticus*	A:Do:E:Ce:K:Na:N	Ak:C:G:S:Cj:At: Cf:F
Larvae	LAR3	*V. alginolyticus*	A:Do:E:Ce:K:Na:Cf	Ak:C:G:S:Cj:At: F:N
Larvae	LAR4	*V. damsela*	A:Do:E:Ce:K:Na:N:Cf	Ak:C:G:S:Cj:At: F
Larvae	LAR5	*V. vulnificus*	A:Do:E:Ce:K:N:Cf	Ak:C:G:S:Cj:At: F:Na
Larvae	LAR6	*V. anguillarum*	A:Do:E:Ce:K:F:N	Ak:C:G:S:Cj:At: Cf:Na
Larvae	LAR7	*V. alginolyticus*	A:Do:E:Ce	Ak:C:G:S:Cj:At: Cf:F:K:Na:N
Larvae	LAR8	*V. parahaemolyticus*	A:Do:E:Ce:F	Ak:C:G:S:Cj:At: Cf:K:Na:N
Sea water	SWA1	*V. parahaemolyticus*	A:Do:E:Ce:K:F	Ak:C:G:S:Cj:At: Cf:Na:N
Sea water	SWA2	*V. fluvialis*	A:Do:E:Ce:F:Cf:At:Cj	Ak:C:G:S: K:Na:N
Mixed water	MWA1	*V. splendidus*	A:Do:E:Ce:F	Ak:C:G:S:Cj:At: Cf:K:Na:N
Mixed water	MWA2	*V. harveyi*	A:Do:E:Ce	Ak:C:G:S:Cj:At: Cf:F:K:Na:N
Mixed water	MWA3	*V. harveyi*	A:Do:E:Ce	Ak:C:G:S:Cj:At: Cf:F:K:Na:N
Artemia	ART1	*V. parahaemolyticus*	A:Do:E:Ce:K:Cf	Ak:C:G:S:Cj:At: F:Na:N
Artemia	ART2	*V. damsela*	A:Do:E:Ce	Ak:C:G:S:Cj:At: Cf:F:K:Na:N
Artemia	*ART3*	*V. splendidus*	A:Do:E:Ce:K	Ak:C:G:S:Cj:At: Cf:F:Na:N
Artemia rearing water	ARW1	*V. splendidus*	A:Do:E:Ce:K	Ak:C:G:S:Cj:At: Cf:F:Na:N
Artemia rearing water	*ARW2*	*V. anguillarum*	A:Do:E:Ce:Cf	Ak:C:G:S:Cj:At:F:K:Na:N
Artemia rearing water	*ARW3*	*V. anguillarum*	A:Do:E:F:Na:N	Ak:C:G:S:Cj:At: Ce:Cf:K
Artemia rearing water	*ARW4*	*V. fluvialis*	A:Do:E:Ce	Ak:C:G:S:Cj:At: Cf:F:K:Na:N
Artemia rearing water	*ARW5*	*V. splendidus*	A: Do:E :Ce:	Ak:C:G:S:Cj:At: Cf:F:K:Na:N
Artemia rearing water	*ARW6*	*V .fluvialis*	A: Do:E: Ce:K	Ak:C:G:S:Cj:At: Cf:F:Na:N
Artemia rearing water	*ARW7*	*V. campbelli*	A: Do:E:At:K:Na	Ak:C:G:S:Cj: Cf:Ce:F:N

Amikacin (Ak) 30 µg, Ampicillin (A) 10 µg, Azithromycin (At) 15 µg, Cefaclor (Cj) 30 µg, Cephotaxime (Ce) 30 µg, Chloramphenicol (C) 30 µg, Ciprofloxacin (Cf) 30 µg, Doxycycline hydrochloride (Do) 30 µg, Erythromycin (E) 15 µg, Furazolidone (F) 50 µg, Gentamicin (G) 10 µg, Kanamycin (K) 30 µg, Nalidixic acid (Na) 30 µg, Neomycin (N) 30 µg and Streptomycin (S) 10 µg

Table 2. Percentage of resistance/sensitivity of *Vibrio* spp. isolated from *Macrobrachium rosenbergii* hatchery against various antibiotics tested

Antibiotics (µg)	Resistant (%)	Sensitive (%)
Amikacin (30)	0	100
Ampicillin (10)	100	0
Azithromycin (15)	8	92
Cefaclor (30)	4	96
Cephotaxime (30)	92	8
Chloramphenicol (30)	0	100
Ciprofloxacin (30)	26	74
Doxycycline hydrochloride (30)	100	0
Erythromycin (15)	100	0
Furazolidone (50)	30	60
Gentamicin (10)	0	100
Kanamycin (30)	47	53
Nalidixic acid (30)	21	79
Neomycin (30)	21	79
Streptomycin (10)	0	100

resistant strains, which may complicate disease treatment. Antibiotic use reduces natural microbial activity, which leads to waste accumulation and reduced degradation and nutrient recycling (Kautsky *et al.,* 2000). Terrestrial bacteria entering into the seawater with antibiotic resistant plasmids may also contribute to the prevalence of resistance genes in the marine environment (Chandrasekaran *et al.,* 1998).

Multiple antibiotic resistance (MAR index) values recorded for *V. anguillarum* (0.46), *V. alginolyticus* (0.4), *V. damsela* (0.4), *V. campbelli* (0.24), *V. fluvialis* (0.37), *V.vulnificus* (0.46), *V.parahaemolyticus* (0.36), *V.splendidus* (0.31) and *V. harveyi* (0.26) were higher than 0.2 (Table 3). MAR index values reported by Lee *et al*. (2009) also indicated that the hatchery water source, *M. rosenbergii* post-larvae and sediment tanks were at high-risk exposure to the 15 tested antibiotics. Use of oxytetracycline has caused increased bacterial resistance in shrimp farms (Nash *et al.,* 1992). Increased resistance to chloramphenicol has emerged through misuse of antibiotics in shrimp hatcheries in Ecuador and Philippines (Baticados and Paclibare 1992). Leano *et al.* (1999) reported that *Vibrio* sp. and *Aeromonas* sp. from shrimps were resistant to streptomycin but sensitive to oxolinic acid. Similarly, in Thai shrimp farms, the use of oxytetracycline as a prophylactic antibiotic rather than for treatment resulted in reduced sensitivity of Gram-negative bacteria especially *Vibrio* sp. (Nash *et al.,* 1992). In Mexico, for instance, the most commonly used antibiotics in shrimp farms are oxytetracycline, florfenicol, trimethoprim- sulfamethoxazole and more recently, sarafloxacin and enrofloxacin administered by mixing through balanced feed or applied directly in water (Roque *et al.,* 2001). Luminous bacteria developed resistance to broad-spectrum antibiotics in certain shrimp culture systems of West Bengal (Sengupta *et al.,* 2003). Tendenica and de la Pena (2001) have compared antibiotic resistance in bacterial isolates from pond water, pond sediment and cultured shrimp and reported that the incidence of resistance to oxytetracycline was the highest followed by furazolidone, oxolinic acid and chloramphenicol. The isolates of *Vibrio* spp. were resistant to most of the antibiotics tested, which may be due to the source water that is contaminated with antibiotic residues. This was supported by the MAR values obtained in this study, which showed that *Vibrio* spp. is widely exposed to the tested antibiotics. In order to minimise the use of antibiotics, application of probiotics is suggested in both grow-out culture ponds and hatcheries (Garrieques and Areval 1995; Rengpipat *et al.,* 1998; Moriarty, 1998). Application of probiotics and prebiotics as immunostimulants is an interesting prospect for replacement of antibiotics in the aquaculture industry and could be a useful tool in the rearing of delicate early stages of certain marine species (Daniels *et al.,* 2010).

Table 3. Multiple antibiotic resistance (MAR) values of *Vibrio* spp. isolated from *Macrobrachium rosenbergii* hatchery

Vibrio sp. isolates	MAR Index
V. harveyi	0.26
V. parahaemolyticus	0.36
V. damsela	0.4
V. splendidus	0.31
V. anguillarum	0.4
V. aiginolyticus	0.4
V. fluvialis	0.37
V. vulnificus	0.46
V. campbelli	0.24

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