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Bacterial Load and Diversity in *Portunus pelagicus* (Crustacea, Decapoda, Portunidae) Larvae and Rearing Media

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Abstract: A study was conducted to estimate the bacterial load as well as diversity in the larvae and rearing system of the marine blue swimmer crab *Portunus pelagicus*. Berried females of mean carapace width 159.86±3.04 mm were collected from the wild and the zoeae from a single brood were used for the investigation. The bacterial load in the filtered seawater, rearing seawater, live and dead larval tissue were estimated and the bacterial species diversity was identified following standard identification keys based on the morphological, phenotypic as well as biochemical characteristics of the colonies isolated on Trypticase Soy Agar (TSA) and Thiosulphate Citrate Bilesalt Sucrose agar (TCBS). The bacterial loads recorded were 1.2×10^4, 2.9×10^5 CFU mL^{-1}, 2.3×10^5 and 4.8×10^7 CFU g^{-1} for filtered seawater, rearing seawater, live larvae and dead larvae, respectively. The dominant bacterial strains identified were *Vibrio vulnificus* and *Vibrio alginolyticus* in rearing seawater; *Pseudomonas alcaligenes*, *Pseudomonas acidovorans*, *Bacillus marinus* and *Alcaligenes* sp. in live larval tissue homogenate and *Vibrio splendidus*, *V. vulnificus*, *V. alginolyticus* and *Vibrio parahaemolyticus* in dead larval tissue homogenate. No growth was observed on TSA and TCBS plates inoculated with filtered seawater.

Key words: Bacterial load, bacterial profile, crab larval rearing, *Portunus pelagicus*, TSA and TCBS plates, India

INTRODUCTION

The marine blue swimmer crab, *Portunus pelagicus* is an important candidate species for aquaculture in India. There is no organized culture practice of the species chiefly due to the unavailability of seeds. Difficulty in obtaining seed from the wild has been one of the factors that promoted research for developing a methodology for hatchery production of seed. One of the major constraints faced in developing hatchery technology for the blue swimmer crab is the heavy mortality during the larval stages.

Laboratory cultures of crab larvae often suffer severe mortality from diseases, particularly from epibiotic bacteria and larval mycosis (Hamasaki and Hatai, 1993). In the larvae of brachyuran crabs such as *Erimacrus isenbekii*, *Chionoecetes opilio*, *Portunus trituberculatus*, *Scylla serrata* and *Scylla paramamosain*, the pathogenic bacteria increase due to decaying organic matter and infect injured and settled, weak larvae, causing mass mortality (Hamasaki *et al*., 2002). They have also opined that among bacteria, *Vibrio* sp., account for the maximum destruction during the larval phase of crustaceans. In shrimp and crab hatcheries, bacterial infections, especially those caused by luminescent *Vibrio* sp., result in serious diseases that affect animal growth and total production (Garcia *et al*., 1994). Different species of Vibrio, commonly found in shrimp hatcheries and crab larval rearing systems are *Vibrio anguillarum*, *V. vulnificus*, *V. damsela*, *V. alginolyticus*, *V. harveyii*, *V. parahaemolyticus*, *V. splendidus*, *V. campbelli*, *V. pelagius* and *V. orientalis* (West and Colwell, 1984). Waters near shore is one of the major sources of infection; the midgut contents of shrimp and crab broodstocks are suspected to be the main source of luminous vibriosis pathogens (Lavilla-Pitogo *et al*., 1992). Probiotics have been effective in improving seed production of many portunid crabs (Nogami and Maeda, 1992). The present study was undertaken with the objectives of estimating the total bacterial load in the larvae and rearing medium of *P. pelagicus* as well as to isolate and identify the associated bacterial species.

MATERIALS AND METHODS

Animal collection, maintenance and hatching: Three healthy females of mean carapace width 159.86±3.04 mm with yellow berry were collected from the Gulf of Mannar, off Mandapam (9°09′N, 78°44′E). The animals were brought to the wet laboratory in battery-aerated

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containers and stocked in black, oval FRP (Fibreglass Reinforced Plastic) tanks of 1 ton capacity at the rate of one animal per tank. Feeding was done with raw clam (*Meritrix meritrix*) and squid (*Sepia pharaonis*) meat at a ratio 1:1 *ad libitum*. The animals were examined daily and at dark grey berry stage, they were transferred to cylindro-conical FRP (Fibreglass Reinforced Plastic) hatching tanks of 500 L capacity.

Larval rearing: Once the eggs hatched, the most active larvae from the same brooder were collected after removing the animal and stocked at a density of 25 no L^{-1} (as this stocking density gave better results during earlier trials) in 200 L rectangular FRP tanks. The temperature, salinity, pH and photoperiod maintained were 28±1.0°C, 35 ppt, 8.1±0.2 and 12 hl:12 hd (l/d = light/dark), respectively.

The temperature was maintained using titanium electronic aquarium heaters with thermostats (AZOO, USA) and the pH was adjusted by adding 1 N sodium carbonate or 1 N hydrochloric acid as required. Zoeae 1 and 2 were fed twice daily with *Skeletonema costatum* and *Brachionus plicatilis* (cell densities maintained at 50,000 and 25 no mL^{-1}, respectively).

The larvae were also fed with formulated prawn feed (Frippak 2 CD-INVE, Belgium) at a rate of 0.5 g ton^{-1} (4 times daily). Zoeae 3 and 4 were also fed with *S. costatum*, *B. plicatilis* and formulated prawn feed (at cell densities 50,000 cells mL^{-1}, 30 no mL^{-1} and at the rate 0.5 g ton^{-1}, respectively). The frequency of feeding was the same as that in the case of zoeae 1 and 2. Megalopae were fed daily once with *Moina macrura* and newly hatched *Artemia* nauplii (cell densities maintained were 3 and 5 no mL^{-1}, respectively). They were also fed twice daily with freshly prepared egg custard at the rate of 0.5 g ton^{-1}. Rotifers and *M. macrura* were collected from the wild and mass cultured in 1 ton FRP tanks on microalgae.

Estimation of bacterial load: Since, the mortality rates were comparatively higher during zoea 4 stage in previous trials conducted in the laboratory, the rearing seawater and larval homogenates at zoea 4 stage were taken for microbiological analyses. Filtered seawater, larval rearing seawater and larvae (live and dead) were analyzed for bacterial load by Total Plate Count (TPC) method.

Six larvae were collected, weighed, washed with sterile seawater, macerated and homogenised in 1 mL sterile seawater. Since, earlier trials indicated that the dilutions 10^{-2} and 10^{-3} were sufficient for getting separate countable colonies, serial dilutions up to 10^{-3} were prepared with sterile seawater for all the samples. The water samples and larval homogenates were inoculated onto nutrient agar (supplemented with 2.5% NaCl) plates and incubated at room temperature (29±2.0°C). Care was taken to do the entire procedure under aseptic conditions. The bacterial colonies in each plate were counted after 48 h and the results were recorded as number of Colony Forming Units (CFU) per mL (CFU mL^{-1}) of undiluted sample or as CFU (CFU g^{-1}) of the material. Inoculation was done in triplicate with control for each set.

Bacterial diversity: For identification of the bacterial species present in each sample, undiluted samples of filtered seawater, rearing seawater, live larvae and dead larvae (larval tissue homogenates prepared as mentioned earlier) were inoculated into Trypticase Soy Agar (TSA) and Thiosulfate Citrate Bile-salt Sucrose (TCBS) agar plates (both supplemented with 2.5% NaCl) under aseptic conditions. Triplicate sets were inoculated with control to minimize error.

The plates were incubated at room temperatures (29±2.0°C) for 48 h and were examined for bacterial growth. The morphological characteristics such as colour, shape, size, etc. of various colonies in both TSA and TCBS plates were recorded and the dominant colonies were transferred to nutrient agar slants. They were further purified by streaking on nutrient agar plates and stored for further studies.

The bacterial isolates were then characterised based on their phenotypic characteristics like growth on selective media, colony characteristics, gram staining, motility and a series of biochemical tests such as amino acid decarboxylase test, sugar fermentation test, growth in NaCl, growth at 42°C, cytochrome oxidase test, nitrate reduction test, catalase test, Methyl Red and Voges Proskaeur tests (MRVP), indole production, Triple Sugar Iron agar (TSI) and Kligler Iron Agar (KIA) reactions, Oxidation/Fermentation (O/F) tests, urease test, gelatinase test, citrate utilisation test, O-Nitrophenyl β-D Galactopyranosidase test (ONPG) and sensitivity to 0/129 discs (Holding and Collee, 1971; Paik, 1980; West and Colwell, 1984). All the media used for the tests were supplemented with 2.5% NaCl. The isolates were identified to species level as per the standard identification keys based on the morphological, phenotypic as well as biochemical characteristics of the colonies (Alsina and Blanch, 1994; Brenner *et al*., 2005; Noguerola and Blanch, 2008).

RESULTS AND DISCUSSION

The bacterial load was comparatively higher in the live and dead larval samples whereas it was lowest in the

filtered seawater (Fig. 1). Different types of colonies were observed on TSA and TCBS plates inoculated with larval rearing seawater, live larval tissue and dead larval tissue

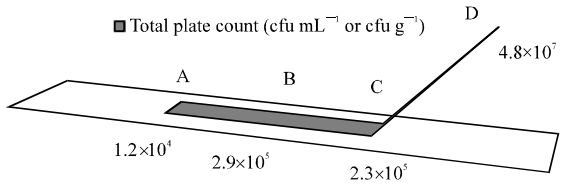

Fig. 1: A) Total plate count of filtered seawater; B) Rearing seawater (CFU mL^{-1}); C) Live larval tissue and D) Dead larval tissue (CFU g^{-1})

samples. However, no growth was observed on plates inoculated with filtered seawater. The dominant bacterial strains identified based on the phenotypic characteristics and biochemical tests were *Vibrio vulnificus* and *Vibrio alginolyticus* in rearing seawater; *Pseudomonas alcaligenes*, *Pseudomonas acidovorans*, *Bacillus marinus* and *Alcaligenes* sp., in larval tissue sample and *V. splendidus*, *V. vulnificus*, *V. alginolyticus* and *Vibrio parahaemolyticus* in dead larval tissue sample (Table 1-3). The bacterial load in the larval culture tank is significant in determining the survival rate of the larvae. According to Phatarpekar *et al.* (2003), as long as the physico-chemical parameters are maintained within

Table 1: Phenotypic and biochemical characteristics of *Vibrio* sp.

	Isolates							
Biochemical tests	RS	RS	RS	RS	DL	DL	DL	DL
Gram reaction	-	-	-	-	-	-	-	-
Cell morphology	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Growth on TCBS	Y	Y	G	G	G	G	Y	G
Luminescence	-	-	-	-	-	-	-	-
Swarming on solid media	+	+	-	-	-	-	+	-
Motility	+	+	+	+	+	+	+	+
0/129 sensitivity								
10 µg	R	R	S	S	S	S	R	R
150 µg	S	S	S	S	S	S	S	S
Decarboxylase tests								
Arginine	-	-	-	-	-	-	-	-
Lysine	+	+	+	+	-	+	+	+
Ornithine	+	+	+	+	-	+	+	+
Growth NaCl (%)								
0	-	-	-	-	-	-	-	-
1	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+
8	+	+	-	-	-	-	+	+
10	-	-	-	-	-	-	+	-
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+
ONPG	-	-	-	+	-	-	-	-
Voges proskauer	+	+	-	-	-	-	+	-
Methyl red	+	+	+	+	-	+	+	+
O/F test	F	F	F	F	F	F	F	F
Growth at 42°C	+	+	+	+	-	+	+	+
Acid from								
Dextrose	+	+	+	+	+	+	+	+
Sucrose	+	+	-	-	-	-	+	-
Arabinose	+	+	-	-	-	-	+	+
Cellobiose	-	-	+	+	-	+	-	-
Inositol	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-
Lactose	-	-	-	+	-	-	-	-
Mannitol	+	+	+	+	+	+	-	+
Mannose	+	+	+	+	-	+	+	+
Trehalose	+	+	+	+	+	+	+	+
Rhamnose	+	+	-	-	-	-	-	-
Fructose	+	+	+	+	+	+	+	+
Esculin	-	-	-	-	-	-	-	-
Gas production	-	-	-	-	-	-	-	-
Gelatinase	+	+	+	+	+	+	+	+
Urease	+	+	+	+	-	+	+	+

Table 1: Continued

	Isolates							
Biochemical tests	RS	RS	RS	RS	DL	DL	DL	DL
Nitrate reduction	+	+	+	+	+	+	+	+
Simmon citrate	+	+	+	+	+	+	+	+
KIA	K/A	Y/A	K/A	A/A	K/A	K/A	K/A	K/A
TSI	A/A	K/A	K/A	K/A	K/A	K/A	A/A	K/A
Indole production	+	+	-	-	-	+	+	+
H_2S production	-	-	-	-	-	-	-	-
Identification	*V. alginolyticus*	*V. alginolyticus*	*V. vulnificus*	*V. vulnificus*	*V. splendidus*	*V. vulnificus*	*V. alginol--yticus*	*V.parahae--molyticus*

+: Positive reaction; -: Negative reaction; G: Green colony; Y: Yellow colony; S: Sensitive; R: Resistant; K: Alkaline; A: Acidic; O: Oxidative; F: Fermentative; Y: Yellow; RS: Rearing Seawater; DL: Deadlarvae

Table 2: Phenotypic and biochemical characteristics of *Bacillus* sp.

Biochemical tests	Isolates RS	LL
Gram reaction	+	+
Cell morphology	Rod	Rod
Production of endospores	+ (round spores)	+ (round spores)
Motility	+	+
Decarboxylase tests		
Arginine	-	-
Lysine	-	-
Ornithine	-	-
Growth NaCl (%)		
1	+	+
3	+	+
6	+	+
8	-	+
10	-	-
Catalase	+	+
Oxidase	+	+
ONPG	+	-
Voges proskauer	-	-
Methyl red	-	+
O/F test	F	F
Growth at 42°C	+	-
Acid from		
Dextrose	+	+
Sucrose	+	+
Arabinose	+	-
Cellobiose	-	-
Inositol	-	-
Sorbitol	-	-
Mannitol	+	-
Mannose	-	+
Trehalose	+	+
Rhamnose	-	-
Fructose	+	+
Esculin	+	+
Gas production	-	-
Urease	-	-
Gelatinase	+	+
Nitrate reduction	+	+
Simmon citrate	+	-
Indole production	-	-
Identification	*Bacillus* sp.	*Bacillus marinus*

+: Positive reaction; -: Negative reaction; F: Fermentative; RS: Rearing Seawater; LL: Live Larvae

Table 3: Phenotypic and biochemical characteristics of *Pseudomonas* and *Alcaligenes* sp.

	Isolates		
Biochemical tests	LL	LL	LL
Gram reaction	-	-	-
Cell morphology	Rod	Rod	Rod
Growth on pseudomonas isolation agar	+	+	+
Motility	+	+	+
Decarboxylase tests			
Arginine	+	-	+
Lysine	-	+	+
Ornithine	-	+	+
Catalase	+	+	+
Oxidase	+	+	+
Voges proskauer	-	-	+
Methyl red	-	+	+
O/F test	O	O	O
Growth at 42°C	+	-	+
Acid from			
Dextrose	-	-	+
Sucrose	-	-	-
Arabinose	-	-	-
Cellobiose	-	+	-
Inositol	-	-	-
Sorbitol	-	-	-
Lactose	-	-	-
Mannitol	-	+	+
Mannose	-	+	-
Trehalose	-	-	+
Rhamnose	-	+	-
Fructose	-	+	-
Esculin	-	-	-
Gas production	-	-	-
Urease	-	+	-
Gelatinase	+	-	-
Nitrate reduction	+	-	-
Simmon citrate	+	+	-
Indole production	-	+	-
Identification	*P. alcaligenes*	*P. acido--vorans*	*Alcaligenus* sp.

+: Positive reaction; -: Negative reaction; O: Oxidative; LL: Live Larvae

the normal range and healthy rearing techniques are practiced, high bacterial populations could be a result of factors such as intensive feeding. He also opined that the pathogenic bacteria could enter hatchery systems from three principal routes: rearing water, broodstock and feeds. In the present study on *P. pelagicus*, the bacterial load was comparatively low in filtered seawater, rearing seawater and live larvae but in the dead larval sample it was high. Comparable TPC range of 10^{-2}-10^{4} and 10^{-4}-10^{-6} CFU mL^{-1} was recorded in the raw seawater and rearing seawater during larval rearing of *Penaeus monodon* (Baticados *et al*., 1990). Similarly, a TPC range of 1.1×10^{-4} to 9.8×10^{-6} CFU mL^{-1} and 2.5×10^{-4} to

1.6×10^{-8} CFU g^{-1}, respectively for rearing seawater and larval tissue homogenate was recorded in *Macrobrachium rosenbergii* (Phatarpekar *et al*., 2003). Mass mortality of *P. monodon* larvae was reported at a total bacterial count of 5.7×10^{7} CFU mL^{-1} in rearing seawater (Le Groumellec *et al*., 1995). In shrimp and crab hatcheries, bacterial diseases are considered to be among the most devastating ones which can destroy hatchery production completely (Haryanti and Nishijima, 2003).

The bacterial strains identified in the present study were *V. vulnificus* and *V. alginolyticus* (rearing seawater), *P. alcaligenes*, *P. acidovorans*, *B. marinus* and *Alcaligenes* sp. (live larvae) and *V. splendidus*, *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus* (dead larvae). Though, bacterial growth was not observed in TSA and TCBS plates inoculated with filtered seawater, the total plate count confirmed the presence of other groups of bacteria in the same. *V. alginolyticus* was isolated from larval tissue of *L. vannamei* (Vandenberghe *et al*., 1999). In *Scylla* sp., vibriosis is caused by *V. alginolyticus* and *V. harveyi* (Haryanti and Nishijima, 2003). The former species was detected in the rearing media and dead larval sample tissue but the latter was not detected in any of the samples under the present study. Among aerobic bacterial assemblages, species belonging to the genera Pseudomonas, Vibrio, Acinetobacter, Alteromonas and Flavobacterium are common in seawater (Maeda *et al*., 1997).

Out of the different species of bacteria isolated, some are harmful to the total health of the system whereas some other could act beneficially. The use of beneficial bacteria which control pathogens through a variety of mechanisms is increasingly viewed as an alternative to antibiotic treatment. Many strains of bacteria like *Bacillus* sp. (*B. subtilis*, *B. licheniformis*), *Acidophilus* sp., *Nitrobacter* sp., *Aerobacter* sp., *Saccharomyces cerevisiae*, etc., can be used as probiotics for treating larval rearing water. *Bacillus* sp. is one of the most widely used putative probiotic group of bacteria due to their ability to secrete many exoenzymes (Moriarty, 1999).

The bacterial profile of the dead larvae is of relevance as the possibility of harmful bacteria entering into the culture medium or being accidentally taken in through mouth by the live larvae is high. In this study along with *V. alginolyticus* and *V. vulnificus* which were also present in the rearing medium, *V. splendidus* and *V. parahaemolyticus* were detected in the dead larval tissue. Though, the live larvae of *P. pelagicus* in the present investigation was found to be free from *Vibrio* sp., stress of any kind would make them vulnerable to vibriosis as the rearing medium and dead larval sample were detected positive for a few *Vibrio* sp. This notion is strengthened by the suggestion that vibriosis is caused by one or more *Vibrio* sp. which normally occurs in seawater and invades marine animals as opportunistic pathogens under stressful conditions (Olsen *et al*., 1995).

Since, many *Vibrio* sp. have been isolated from healthy penaeid shrimps, hypothesis on the opportunistic nature of vibrios associated with penaeid shrimp has become widely accepted (Vandenberghe *et al*., 1998). Continuous exposure to heavy load of *Vibrio* sp. in the medium resulted in vibriosis in *P. monodon* (Lavilla-Pitogo *et al*., 1990). Apart from the rearing media, live feeds also might act as agents causing large scale destruction of the larvae (Olsen *et al*., 1995). Several species of bacteria are isolated from crustacean larvae and their rearing media.

As per Nash *et al*. (1992), *V. alginolyticus* is a pathogen of *P. monodon* larvae. Nevertheless, Austin *et al*. (1995) showed that *V. alginolyticus* could be used as a probiotic for limiting the development of pathogens such as *Aeromonas salmonicida*, *V. anguillarum* and *V. ordalli*. Sriwongpuk *et al*. (2006) isolated *Aeromonas hydrophila*, *V. vulnificus*, *V. alginolyticus*, *V. cholera* and *V. mimicus* from the giant freshwater *M. rosenbergii* larval rearing water. The water in which rotifers are cultured contains a high concentration of dissolved organic matter derived from the food and faeces of rotifers which is an ideal growth media for *Vibrio* sp. and therefore, the gut of rotifers is abundant in *Vibrio* sp. (Maeda *et al*., 1997). Quinitio *et al*. (2001) reported that *Vibrio* sp. apparently dominated the bacterial population in the larvae of *S. serrata* as shown by high presumptive Vibrio counts as early as day 1 or 2. The researchers also observed that when Vibrio count reached 10^{-2} CFU mL^{-1} in the rearing media, infection occurred through oral route. Though, the live larvae was free of *Vibrio* sp. in the present study, possibility of the same entering the healthy larvae from the rearing seawater or dead larvae causing vibriosis appears to be high.

CONCLUSION

Various measures are taken in hatcheries to minimise or prevent bacterial disease outbreaks. The methods adopted include disinfection of rearing water by treating with chlorine/UVrays/ozone, provision of efficient filtering system, washing of eggs to remove adhering dirt and sand, washing of live feeds prior to feeding and

maintenance of hatchery hygiene (Garriques and Arevalo, 1992). According to Suwanto *et al*. (1998), the diversity of species in the genus Vibrio is causing difficulty in developing vaccines against specific luminescent strains of *Vibrio* sp. which are dangerous. So, it is imperative to develop and practice biological control techniques like probiotics or bio-conditioners to prevent diseases and to stabilise production in crustacean hatcheries.

According to Skjermo and Vadstein (1999), microbial maturation (settling of the water for a number of days resulting in a diverse bacterial flora dominated by non-opportunists that act as a stable buffering system restricting the growth of opportunistic and potentially pathogenic bacteria) is another simple process which if followed in hatcheries could minimise the problems from disease outbreaks effectively.

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