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Bacterial flora associated with the selected life stages and organs of farmed giant freshwater prawn *Macrobrachium rosenbergii* (de Man)

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Abstract: Bacteria associated with different life stages of giant freshwater prawn (*Macrobrachium rosenbergii* de Man) were analyzed. The gill, hepatopancreas, haemolymph of brood and juveniles as well as the egg, larvae and larval rearing water were sampled to understand the quantity and the quality of bacteria associated with the animals. A total number of 93 representative isolates were identified. The identified bacterial isolates could be distributed in to 14 genera. A mean bacterial total plate count (TPC) of $4.5x10^5$ colony forming units (cfu) g^{-1} in eggs, $6.0x10^6$ cfu g^{-1} in larvae and $4.6x10^5$ cfu ml^{-1} in water were observed. Among all the organs highest TPC of $3.5x10^7$ cfu g^{-1} were observed in juvenile's gills. The brood haemolymph was found to be devoid of any bacteria. Identification of isolates representing different colony morphotype indicated that 89.77% of the bacterial population was gram negative dominated by *Aeromonas hydrophilla* (16.74%), *Enterobacter aerogenes* (12.09%) and *Citrobacter frundii* (10.16%). Among Gram-positive bacteria *Bacillus*, *Streptococcus* and *Micrococcus* were identified. Study of quantitative and qualitative aspects of bacterial prevalence with the different life stages of *M. rosenbergii* would be helpful in identification of disease causing bacteria and therefore in better management of *M.rosenbergii* culture.

Keywords: Aquaculture, Bacteria, *Macrobrachium rosenbergii*, Total bacterial counts

INTRODUCTION

Giant freshwater prawn *(Macrobrachium rosenbergii*, de Man, 1879) has great aquaculture potential because of its large size, its tolerance to changes in water quality and for its ability to accept the unconventional feed on (El-Sayed, 1997). In India, *M. rosenbergii* is one of the important commercial crustacean as it has good demand in both domestic and export markets (MPEDA, 2010; FAO, 2013). Culture of species have been reported from Vietnam, Combodia, Malaysia, Myanmar, Bangladesh, India, Sri Lanka, Philippines, Taiwan, Japan, Israel, USA and China (New, 2003; Kennedy *et al.,* 2006). A lower survival rate of *M. rosenbergii* during its larval stages often prevents the development of the full economic potential of this crustacean. In the absence of any environmental stress, the mortality of aquatic organisms can be result of pathogenic bacteria. Some of the bacteria associated with aquatic animals may be opportunistic pathogens some others may be protective against pathogens (Hansen and Olafsen, 1999). Several studies have been done on bacterial flora associated with fish and its environment but there are not many reports regarding the normal bacterial flora associated with *M.rosenbergii* hatcheries (Prakash and Karmagam, 2013). Thus it would be important to study the number and kinds of bacteria occurring in larvae, larval rearing water and in selected organs of *M. rosenbergii*. The present study was undertaken for the quantitative and qualitative analyses of bacteria associated with the egg, larvae, water and organs (gill, hepatopancreas and haemolymph) of juveniles and brood of giant freshwater prawn, *M. rosenbergii* (de Man).

MATERIALS AND METHODS

Sample collection: Twenty four berried prawns (*M. rosenbergii)* in the same developmental stage were collected from perennial pond of 3.6 ha area with a depth ranging from 1.44 m to 1.82 m located in peri-urban area of Mumbai, Maharashtra, India. The prawns were brought in live condition to the wet laboratory of the Central Institute of Fisheries Education, ICAR (Mumbai). The brooders were maintained in three brooder tanks. For analysis, four berried prawns from each tank were randomly picked and washed three times with 0.85 % sterilized physiological saline to remove loosely bound bacteria. Before sacrificing the brooders, mature eggs from the brood pouch were collected using sterile forceps. Eggs collected from the brooders of same tank were mixed in sterile test tube containing sterile physiological saline and was considered as one sample.

Sample preparation: Using sterile scissors the brooders were then aseptically cut open to obtain a section of hepatopancreas and gills. The haemolymph were drawn with the help of sterilized syringes. The remaining twelve berried prawns were transferred to three spawning tanks containing brackish water of initial salinity of 13.0 ppt that was gradually reduced to 10.5 ppt. Upon hatching of eggs, larvae from spawning tanks were collected in sterile beakers. Larvae (day one old) from each three tanks were filtered to weigh one gram. Three samples of larvae from each tank were obtained. Water samples in triplicates were also collected simultaneously from larval rearing tanks in sterile bottles. Mean counts of cfu of bacteria in larvae and water samples obtained by spread plate technique were used in the statistical analysis. The eggs and larvae were washed with sterilized physiological saline 0.85% w/v NaCl, weighed and were homogenized. The remaining larvae in the tanks were reared for a period of 60 days and juveniles obtained were also similarly processed as brooders for hepatopancreas and gills and haemolymph. Samples were processed immediately after collection.

Physico-chemical parameters analysis: Temperature, dissolved oxygen (DO), pH, alkalinity, total dissolved solids (TDS), salinity, nitrite nitrogen of the brood collection pond, hatching, larval and juvenile rearing tanks were determined according to standards methods for the examination of water and wastewater (APHA, 2005).

Bacteriological analysis: All the glass wares used were sterilized in hot air oven at 180^0C for 60 minutes. Media, tips for micropipette, saline water were sterilized at 121^0C under 15 lbs pressure for 15 minutes. Hepatopancreas and gills were macerated in stomacher (Stomacher 80 Biomaster, Seward, UK) with nine volumes of physiological saline. Total *Vibrios* counts were determined by plating samples on Thiosulphate Citrate Bile salt Sucrose agar (TCBS) and incubating the agar plates at 30^0C±2^0C for 24-48 hours and examining the growth of the isolates in different concentrations of NaCl, and their sensitivity to *Vibrio* static agent *i.e* pteridine compound (0/129). Similarly, Kenner Faecal (KF) agar and Eosine Methylene Blue (EMB) agar were used for determining presumptive counts of *Streptococcaceae* and *Enterobacteriaceae* members respectively. The homogenates were suitably diluted and plated on nutrient agar (Hi-media, Mumbai, India) following spread plate technique. Purified cultures were inoculated onto Tripticase Soya Agar (TSA) slants and were kept at 4^0C for stock. Standard enumeration rules were followed to estimate bacterial population. The number of each type of colonies was noted to calculate the percentage contribution of each species to the total flora. Isolated colonies were purified and pure cultures were observed for cell shape, motility, flagellation, spores and encapsulation, and gram staining. The isolates were then subjected to a battery of biochemical tests following the criteria described in the Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994) for identification to genus or species level. In parallel, BIOLOG system (Vitec 2 Microbial identification system (Biomeriux, France) were also used.

Statistical analysis: TPC were expressed as cfu g^{-1} for hepatopancreas, gills, eggs and larvae and cfu ml^{-1} for water. The counts were transferred into log_{10} values before statistical analysis. The data was statistically analyzed using statistical software (SPSS version 16.0). One way analysis of variance (ANOVA) and Duncan's Multiple Range Test were applied to compare the significance of difference between means of microbial counts. Means were considered significantly different at $P<0.05$.

RESULTS AND DISCUSSION

The physico-chemical parameters recorded were found to be within the optimum range for larval rearing of *M. rosenbergii*. We recorded water temperature vary between from 28.13-29.17 ^{o}C in different tanks. Kanaujia and Mohanty (1998) reported that temperature range of 28-30^{o}C enhanced molting and reduced larval development duration. In our study pH range was observed to vary between 7.43- 7.73. Cheng *et al.* (2000) mentioned that pH ranges of 7.5-7.7 are good for freshwater prawns. Uddin *et al.* (2005) also reported improved survival of *M .rosenbergii* in water pH ranging from 7.2-7.9. In the present study alkalinity values varied between 116.0-133.33 mg l^{-1}. Brown *et al.* (1991) reported maximum growth of freshwater prawn at water hardness ($CaCO_3$) of more than 53 mg l^{-1}. In the present experiment temperature, pH, alkalinity, dissolved oxygen, nitrite nitrogen and salinity were consistently observed to be within the acceptable range (Table 1).

Giant freshwater prawn (*Macrobrachium rosenbergii* de Man*,* 1879) (or scampi) is an important commercial species due to property as food supply as well as a valuable export product (Deen *et al.,* 2013). The literature has little information on the micro-floral associated with freshwater prawn culture system, larvae and brood organs of *M.rosenbergii.* Most of the researcher focused on pathogens of marine shrimps (Yasuda *et al.,* 1980; Hameed, 1993; Gomez-Gil *et al.,*1998) but there are not many reports regarding the normal bacterial flora associated with *M.rosenbergii* hatcheries (Prakash and Karmagam *et al.,* 2013). Biological contaminants such as viruses, bacteria, fungi, protozoa and helminthes constitute the major cause for *M. rosenbergii* (Lalitha *et al*., 2010).In the present study bacterial flora associated with different stages of prawn and selected body organs were identified. TPC and specific counts of bacteria recorded have been presented in Table 2. Among egg, larvae and water significantly ($P<0.5\%$) higher TPC were observed in larvae 6.77±0.05 log10 cfu g^{-1}. Lower TPC in eggs of

Table 1. Physico-chemical (Mean± SD) parameters of brood pond, hatching, larval rearing and juveniles tanks of three replicates observed during sampling July 2012.

Sample	Temperature (^{0}C)	pH	DO* (mg^{-1})	Alkalnity(mg^{-1})	TDS*(mg^{-1})	Salinity(‰)	NO_2-N(mg^{-1})
Brood pond	28.13±0.12	7.73±0.50	5.33±0.46	116.0±2.0	1.35±0.04	0.20±0.00	0.02±0.01
Hatching tank	28.67±0.58	7.43±0.06	6.07±0.06	133.33±2.31	1.42±0.04	6.07±0.42	0.02±0.01
Larval rearing tank	28.93±0.60	7.46±0.25	6.20±0.40	130.00±6.93	1.50±0.04	12.10±1.39	0.03±0.01
Juveniles tank	29.17±0.29	7.43±0.06	6.00±0.35	119.33±8.08	1.36±0.14	0.23±0.25	0.03±0.01

* DO= Dissolved oxygen, TDS=total dissolved solids.

Table 2. Mean microbial counts (log 10 cfug^{-1}±SD†) of different samples of *M. rosenbergii* (n=3).

Bacterial groups	Egg*	Larvae*	Water**	Haemolymph** (Juvenile)	Hepatopan-creas* (juveniles)	Gills* (Juveniles)	Hepatopan-creas* (Brood)	Gill* (Brood)
TPC	5.65±0.06^{d}	6.77±0.05^{e}	5.66±0.05^{d}	3.23±0.07^{a}	4.55±0.08^{c}	7.54±0.01^{g}	4.45±0.02^{b}	6.35±0.04^{f}
Enterobacteriaceae	4.68±0.02^{f}	4.53±0.16^{e}	4.33±0.15^{dc}	3.01±0.02^{a}	3.95±0.05^{b}	4.13±0.04^{c}	4.21±0.00^{dc}	5.02±0.010^{g}
Vibrionaceae	3.29±0.11^{e}	4.59±0.23^{f}	4.45±0.06^{f}	0.00^{a}	0.00^{a}	2.79±0.1^{d}	1.11±0.19^{b}	1.68±0.12^{c}
Streptococcaceae	2.08±0.04^{ab}	2.06±0.22^{ab}	1.90±0.12^{a}	2.12±0.10^{b}	2.26±0.06^{bc}	2.34±0.12^{c}	3.05±0.04^{d}	3.26±0.01^{e}

* cfu g$^{-1,}$ ** cfu mL-^{1}, SD†- Standard Déviation, Different superscript in a row indicate significant difference.

Table 3. Percentage distribution of the main bacterial groups and genera associated with *M.rosenbergii* organs of juveniles and brood.

Name of bacteria	Egg		Larvae		Haemo.*		Hepat.*		Gills*		Hepat.**		Gill**		Total
	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	
Bacillus sp.	-	-	5.88	1	-	-	-	-	-	-	-	-	7.69	1	2
Stenotrophomonas maltophilia	7.14	1	-	-	-	-	-	-	-	-	-	-	-	-	1
Vibrio sp.	14.29	2	17.64	3		-	-	-	9.09	1	-	-	7.69	1	7
Pseudomonas aeruginosa	7.14	1	17.64	3	13.33	2	-	-	9.09	1	-	-	15.38	2	9
Citrobacter freundii	21.43	3	5.88	1	20	3	7.69	1	18.18	2	-	-	15.38	2	12
Pantoea	14.29	2	-	-	6.67	1	-	-	-		-	-	-	-	3
Acinetobacter haemolyticus	-	-	23.52	4	-	-	-	-	-	-	-	-	-	-	4
Aeromonas hydrophila	14.29	2	17.64	3	-	-	38.46	5	36.36	4	28.57	2	30.77	4	20
Proteus mirabilus	-	-	5.88	1	-	-	-	-	9.09	1	-	-	-	-	2
Enterobacter aerogenes	14.29	2	5.88	1	-	-	30.77	4	18.18	2	28.57	2	15.38	2	13
Plesiomonas sp.	-	-	-	-	-	-	7.69	1	-	-	14.29	1	-	-	2
Streptococcus	7.14	1	5.88	1	6.67	1	15.38	2	-	-	28.57	2	-	-	7
Sphingomonas paucimobilis	-	-	-	-	40	6	-	-	-	-	-	-	-	-	6
Micrococcus	-	-	-	-	13.33	2	-	-	-	-	-	-	-	-	2
Unidentified	-	-	-	-	-		7.69	1	-	-	14.29	1	7.69	1	3
Total	100.00	14	99.96	18	100	15	99.99	14	99.99	11	100	8	99.98	13	93

*=Haemolymph, hepatopancreas and gills of juvenile, **= Hepatopancreas and gills of brood.

M.rosenbergii has been reported by Phatarpekar *et al.* (2002). Phatarpekar *et al.* (2002) further mentioned that lower counts of TPC in eggs may be attributed to the presence of membrane, which prevents the attachment and subsequent proliferation of bacteria from surrounding water and the absence of protective membrane in larvae unlike that in the egg might be responsible for the increase in bacterial population. Similar to our observation Hameed (1993) also reported increased TPC in *P. monodon* larvae. In another study Hameed *et al*. (2003) reported numbers of aerobic heterotrophic bacteria from *M. rosenbergii* eggs, larvae and post larvae to vary from 7.9×10^4 to 8.2×10^6, 0.8×10^5 to 81.1×10^5 and 38.3×10^5 to 10.9×10^6 cfu/g respectively. Hameed (1993) suggested that interaction between the larval surface and increased adhesion sites are responsible for association of greater number of bacteria in larvae. In our study we observed high number of *Enterobacteriaceae* compared to water and larvae in eggs, this may be due to the proximity of egg sac to the excretion ports. Larvae were harboring significantly ($P<0.5\%$) higher *Vibrionaceae* counts than water and egg. This could be because of salinity in larval rearing tank which favors the growth of *Vibrio* spp. Among brood organs higher ($P<0.05$) counts of *Enterobacterriaceae* and *Streptococcaceae* were observed in gills. In juveniles organs also gills were harboring significantly ($P<0.05$) higher TPC and specific counts of *Enterobacterriaceae, Vibrionaceae* and *Streptococcaceae.* This may be because of gills direct contacts with water increasing the possibility of dominance of existing bacteria.

Average TPC load in juvenile and brood hepatopancreas were $3.65x10^4$ to $2.88x10^4$ cfu g^{-1}. Uddin *et al.* (1998) reported $1.9x10^3$ to $2.9x10^4$ cfu g^{-1} of total bacterial load in hepatopancreas of *M. rosenbergii.* Contrary to our observation of absence of bacteria in haemolymph Uddin *et al.* (1998) reported *Vibrio* and *Staphylococcus* as dominant genera in haemolymph of farmed *M.rosenbergii*. Similar to our study the presence of *Vibrio* in larvae agrees with the earlier reports of Yasuda and Kitao, (1980) and Hameed, (1993) but do not agree with Phatarpekar *et al.* (2002). The higher bacterial counts recorded in the larvae may be attributed to the larval rearing techniques employed as many of the works were carried out in green water system (Anderson *et al.,* 1989; Miyamoto *et al.,* 1983). In green water system the addition of phytoplankton might suppress bacteria in water and larvae (Kogure *et al.,* 1979).

Bacterial composition: On the basis of phenotypic traits and biochemical tests, the selected isolates could be grouped into 14 genera (Table 3). Most dominant general were *Aeromonas, Enterobacter* and *Citrobacter*. Diversity of isolated bacterial genera was found to be highest nine genera in larvae followed by eight from eggs, six each from haemolymph and gills of juveniles and five from hepatopancreas of juveniles. The gills of brood harboured six genera while hepatopancreas harboured four. Ogbondeminu and. Okoye (1992) mentioned that the bacterial composition in all the fish species appeared to be a reflection of that their respective environments. The bacterial flora of fish also reflects the quality of the aquatic environment (Shewan and Hobbs, 1967). Out of 14 bacterial genera isolated in the present study majority were Gram-negative. Presence of Gram-negative bacteria such as *Aeromonas, Enterobacter, Vibrio* and *Micrococcus* on carapace and pond sediment of *M.rosenbergii* has been also previously reported (Uddin and Al-Herbi, 2005). In eggs *Citrobacter* sp*., Enterobacter* sp*., Pantoea* sp., hitherto are unreported genera in earlier studies conducted by Phatarpekar *et al.* (2002) and Kennedy *et al.* (2006). Moreover, the faecal coliforms as *Escherichia coli* are generally considered as indisputable indicators of faecal contamination from warm blooded animals (Ward and Hackney, 1991).

Out of nine genera isolated from larvae, only six were common to that of egg. In contrast Phatarpekar *et al*. (2002) reported only one genera *Pseudomonas* common in egg and larvae. In our study *Pseudomonas* were observed in eggs, larvae, gills and haemolymph of *M.rosenbergii.* This is one of the most diverse bacterial genera, containing over 60 validly described species (Jensins *et al*., 2004). Recently, *P.aeruginosa* was identified as harmful to *M.rosenbergii* (Ramalingam and Ramarani, 2007). Gomez-Gil *et al*. (1998) isolated *Vibrios* from hepatopancreas and haemolymph of *P.vannamei*. Among bacterial isolates of hepatopancreas and haemolymph of juveniles *Citrobacter* and *Streptococcus* were common genera observed. Out of 11 genera from juveniles haemolymph and hepatopancreas *Citrobacterm, Pantoe, Plesiomonas* and *Enteronbacters* were common to Uddin *et al.* (1998) observation. Presence of bacteria in haemolymph is indicative of septicemia and a common sequel to stress (Lightner, 1988). We do not observe any bacteria in the haemolymph of brood which may be indicative of healthy brood stock. Hopkins and Nott (1980) have claimed that bacteria are not commonly found in hepatopancreas because they are prevented from entering the gastric sieve which excludes particles larger than 0.1 mm. It has been suggested that the sieve may combine with digestive enzymes to prevent bacteria gaining access to or colonizing the hepatopancreas and therefore the presence of bacteria in hepatopancreas may represent a failure to these mechanisms (Alday-Sanz, 1994). However, the possibility of gastric sieve damage as a result of feeding commercial pellets has also been explained, providing chance of entry of bacterial flora into hepatopancreas (Gomez-Gil *et al.,* 1998). In the present study, the hepatopancreas were found to carry a good load of bacteria. Role of some of bacteria observed such as *Pseudomonas* and *Aeromonas* in spoilage of prawn (Reilly and Dangla, 1986) and in

causing gastroenteritis (Kirov, 1997) has been established. Human pathogenic bacteria and viruses in aquaculture ponds have been reported to be potential threat to human health (Ogbondeminu and Okoye, 1992). Black spot, brown spot and shell disease are caused by *Vibrio* spp., *Pseudomonas* spp*.,* and *Aeromonas* spp., (FAO, 2014). Prakash and Karmagam (2013) pointed that *P. aeruginosa* was found to be 18.3% and 30% from farm cultured *M. rosenbergii*.

The presence of *Aeromonas* and *Vibrio* genera in *M. rosenbergii* indicates a potential risk to human and prawn (Lalitha *et al.,* 2004). Biological contaminants such as bacteria, viruses, fungi and helminthes constitute the major cause of food borne disease such as cholera, *E. coli* gastroenteritis, salmonellosis, shigellosis, amoebiasis, brucellosis with varying degree of severity varying from mild indisposition to chronic or life threatening illness (Phyllis, 2007). Food security is a complex issue, where fish and fishery products are generally regarded as high risk commodity in respect of pathogen contents, natural toxins and other possible contaminants and adulterants (*Yousuf et al.,* 2008*)*. The prevalence of these bacteria reinforces the opinion that although member of these genera are potentially pathogenic and exist in the natural pond environment, they may not be necessarily causing diseases unless the environment stress weakens the defense system of the animals. It is also clear that aquatic organism such as the one described in this study can contain bacteria that are known to be pathogenic for human being. Some of the bacteria may cause threat to public health, needs precaution in relation to handling and consumption. There was no clear succession observed among the bacterial genera in different life stages of the *M.rosenbergii*. Some of the species of *Bacillus, Pseudomonas and Streptococcus* have been reported to have antagonistic effects against pathogens.

Conclusion

This investigation evaluate the quantitative and qualitative of bacterial load in *M.rosenbeergii*. Gills of *M.rosenbergii* juveniles carry highest mean bacterial total plate count (TPC) of $3.5x10^7$ cfu g^{-1} whereas lowest of $4.5x10^5$ colony forming units (cfu) g^{-1} were observed in eggs. Among the isolates *Aeromonas hydrophilla* (16.74%), *Enterobacter aerogenes* (12.09%) and *Citrobacter frundii* (10.16%) were the major species indicating the dominance of gram negative bacterial flora. In eggs *Citrobacter frundi* (21.43%), in larvae *Acinetobacter haemolytycus* (23.57%), in haemolymph *Pseudomonas* and *Micrococcus* (13.33%) were dominating. *A.hydrophilla* were more prevalent in hepatopancreas (38.46%), and in gills (36.36%) of juveniles. Brood hepatopancreas (28.57%) and gills (30.77%) were also dominated by *A. hydorphilla*. The variations in the bacterial species composition in different life stages as seen in this study may be attributed to the differences in culture environment, rearing systems where organic manure and feed are the main input used. Some of the microflora like *Vibrio* and *Enterobacter* are known to be pathogenic for human beings. Therefore, the present work provides potential approaches for improving the quality assurance and to create awareness among consumers.

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