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Heterotrophic Bacteria Associated with Healthy and Moribund Larvae of *Penaeus monodon* H.Milne Edwards

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Abstract

Heterotrophic bacterial flora of *P.monodon* from an apparently healthy hatchery system as well as a pool with heavy mortality were isolated and studied. In the healthy systems comparatively higher generic diversity with *Pseudomonas, Acinetobacter, Bacillus, Micrococcus*, members of the family Enterobacteriaceae and coryneform group in the diminishing order of dominance was recorded. Meanwhile from the moribund larvae and rearing water *Aeromonas* and *Pseudomonas* could be isolated in almost equal proportions. Strikingly, *Aeromonas* could not be isolated from the apparently healthy larval rearing system and its exclusive occurrence in the sick culture system in comparatively higher percentage suggested its possible role in the mortality. They were found to be highly halophilic exhibiting growth at 10% NaCl. On testing their sensitivity to twenty antibiotics, four of them (Streptomycin, Gentamycin, Methamine mandelate and Cloramphenicol) were found to be effective on all the isolates of *Aeromonas* and *Pseudomonas* suggesting their possible application in the hatchery system in times of emergency. While doing so, Streptomycin would do comparatively better than the others as the minimum inhibitory dose required was comparatively lower (200ppm) within a period of 24 hours.

Introduction

Diseases due to bacterial infection in captive wild and in cultured shrimps are well known (Cook and Lofton, 1973; Delves -Broughton and Poupard,1976; Lightner and Lewis,1975; Aquacop,1977, Lightner,1977 and 1985). In every reported bacterial infection in penaeid shrimp, *Vibrio, Aeromonas* and *Pseudomonas* have been found to be involved (Lewis ,1973 ; Lightner,1977). Among the heterotrophic bacteria associated with the eggs and larvae of *Penaeus indicus* in a hatchery system, *Vibrio* has been found to be the dominant genus and their per cent increase in the system towards the later stages of the larval life turns out to be the most important factor associated with the mass mortality of mysis and the post larvae (Singh *et al.,* 1989). Singh (1990) observed *Aeromonas* as the major flora of eggs and larvae of *M. rosenbergii* during mortality and Pseudomonas during the successful completion of the larval cycle. Karunasagar *et al.,* (1994) isolated a strain of *V. harveyi* resistant to antibiotics, causing mass mortality of the larvae of *P. monodon* in hatchery. The present paper deals with the heterotrophic bacterial flora of the larvae of *Penaeus monodon* during an incident of mass mortality in a hatchery system compared to that of apparently healthy larva.

Materials and Methods

Moribund larvae (mysis) of *Penaeus monodon* from a hatchery pool which experienced heavy mortality and apparently healthy larvae from another pool where no signs of disease was reported were used as the samples. The larvae were collected and transferred to the laboratory in glass bottles at 4° C in thermocool box along with the water samples from the same pool. Moribund and healthy larvae were macerated 25 each in sterile 30ppt sea water using glass tissue homogenizer and serially diluted to 10^{-6} usiing the same diluent. The inocula thus prepared were swabbed on ZoBells agar 2216E (peptone 0.5%, yeast extract 0.1%, FePO4 0.01%, agar 2%, pH 7.5±0.2 plates and incubated at 28±04° C for 7 days. Subsequently the total heterotrophic bacteria were isolated in to ZoBells agar slants and identified to genera following Buchanan and Gibbons (1974) and Oliver (1982). The bacterial isolates obtained from moribund larvae were screened for their sensitivity to antibiotics such as Penicillin G, Streptomycin, Ampicillin, Oxytetracyclin, Tetracyclin, Gentamycin, Polymyxin B, Chloramphenicol, Neomycin, Methamine mandelate, Cefazolin, Amoxycillin, Novobiocin, ,Nalidixic acid, Chloramphenicol, Erythromycin, Kanamycin, Bacitracin, Lincomycin and Sulfadiazine using the commercially available disks (HiMedia Laboratories). The antibiotic disk plate method using ZoBells agar was used and zones of inhibition were measured and recorded. From the preliminary examination of the sensitivity of the isolates to the 20 antibiotics, Streptomycin, Gentamycin,Methamine mandelate and Chloramphenicol were selected for further studies on the grounds that all the isolates tested were uniformly sensitive to them and also because of the larger zones of inhibition formed by them. For studying the Minimum Inhibitory Concentrations (MICs) antibiotic concentrations prepared in sterile 30ppt seawater , ranging from100 to 1000 $\mu g.mL^{-1}$ were used. Filter sterlized antibiotic solutions were transferred at 10 mL aliquots to sterile test tubes and inoculated with a loopful of the 24 hour broth culture in ZoBells broth. Inhibitory action of the antibiotic was checked at 24 hours, 48hours and 72 hours after inoculation by transferring a loopful of the antibiotic + culture prepration into 10 mL ZoBells broth and observing for growth. Growth was indicated by turbidity in the broth. The concentration at which inhibition was exhibited and the duration of exposure required for the same were

recorded. The isolates from moribund larvae were tested for their ability to grow at varying concentrations of NaCl *viz,* 0, 1.5, 3, 6, 8 and 10 % w/v in Nutrient broth (peptone 1.0%, beef extract 0.5%, yeast extract 0.1%, pH 7.5±0.2). The test broth were inoculated from overnight slant culture, incubated for 72 hours and observed for growth.

Results and Discussion

Generic composition of the heterotrophic bacteria isolated from moribund larvae and healthy larvae and the corresponding rearing water is summarized in Table-1. From the moribund larvae and rearing water, *Aeromonas* and *Pseudomonas* could be isolated in equal proportions. On the other hand from the apparently healthy larvae genera such as

Table - 1. Generic composition (%) of heterotrophic bacteria isolated fro the 'sick' and the 'healthy' culture system

Genera	Sick culture pool		Healthy culture pool	
	larvae	water	larvae	water
Total number of isolates	**13**	**15**	**24**	**38**
Aeromonas	46.15	46.67	-	-
Pseudomonas	53.85	53.33	-	39.47
Acinetobacter	-	-	12.50	28.97
Enterobacteriacese	-	-	8.33	18.42
Micrococcus	-	-	41.67	-
Bacillus	-	-	37.50	10.52
Coryneform group	-	-	-	2.63

Micrococcus, Bacillus, Acinetobacter and members of the family Enterobacteriaceae and from the rearing water *Pseudomonas, Acinetobacter*, members of the family Enterobacteriaceae, *Bacillus* and coryneform group were isolated in the diminishing order of dominance. *Aeromonas* were conspicuous by their absence in the healthy system. Comparison of the heterotrophic bacteria isolated from the larvae and the rearing waters of both the healthy and the sick pool revealed a marked reduction in the generic diversity in the culture pool with high mortality, culminating in only two genera, such as *Aeromonas* and *Pseudomonas*. All the Gram positive forms such as *Micrococcus, Bacillus* and Coryneform group which were seen in the 'healthy' larval rearing system had disappeared from the 'sick' pool. Similar patterns of reduction in the generic diversity in the larval rearing systems during times of mortality had been reported by Singh *et al.*, (1989). *Pseudomonas* seen in considerable proportions in the water of the 'healthy' pool were also present in the 'sick' culture system suggesting that the strains of *Pseudomonas* encountered in this instance may not be with any pathogenicity. The exclusive occurrence of *Aeromonas* in the sick culture pool strongly suggest the profound role these organisms have played in causing mortality of the larvae. Yasuda and Kitao (1980) noted that cultured and wild penaeids had abundant *Pseudomonas* population in the gut and when the *Aeromonas* species were dominant, the prawns showed poor growth. Similarly Singh *et al.*, (1989) demonstrated that in a penaeid hatchery system, the per cent increase of *Vibrio* towards the later stages of the larval life is one of the most important detectable factors associated with the mass mortality of larvae of *Penaeus indicus.* Later while working on the larval rearing system of the giant fresh water prawn *Macrobrachium rosenbergii*, Singh (1990) observed that *Aeromonas* formed the major flora of the sick culture system and *Pseudomonas* those of the healthy ones.

Sensitivity of *Aeromonas* and *Pseudomonas* isolated from the moribund larvae and rearing water to twenty antibiotics are summarized in Table-2. All the isolates tested were found to be inhibited by four of the 20 tested antibiotics such as Streptomycin, Gentamycin, Methamine mandelate and Chloramphenicol suggesting their usefulness in the hatchery system. All the isolates of *Pseudomonas* were resistant to 13 of the 20 antibiotics tested while the *Aeromonas* isolates were resistant to 9 of them. Thus the isolates tested showed multiple drug resistance which could prove disasterous in hatcheries. The occurrence of drug resistant strains in the aquaculture environment has increased over the years and may be attributed to the intensive use of antimicrobials. For instance from 1992 to 1996 the percentage of bacteria resistant to oxytetracycline were reported to increase from 14 % to 45% (Raungpan,1996; Raungpan and Kitao, 1992).

The MICs of Streptomycin, Gentamycin , Methamine mandelate and Chloramphenicol were 200, 600, 400, and 1000ppm respectively against the *Aeromonas* isolates studied and 500, 400, 600 and 1000ppm respectively against the *Pseudomonas* isolates . Thus at 24 hours, for the *Aeromonas* isolates the activity was in the order of Streptomycin > Methamine mandelate > Gentamycin > Chloramphenicol, while for *Pseudomonas* it was in the order of Gentamycin > Streptomycin > Methamine mandelate > Chloramphenicol. Thus among the antibiotics tested Streptomycin sulphate which turned out ot be most effective, may be recommended for restricted application at times of emergency. However the dosage has to be fixed only after assessing its lethal and sublethal toxicity on larvae.

On testing the halophilism of *Aeromonas* and *Pseudomonas* isolates from the larvae and rearing water of the sick pool, strikingly all the isolates of *Aeromonas* were found to be highly halophilic as they could grow even at 10 % w/v NaCl with no growth in medium without NaCl. On the other hand, *Pseudomonas* could grow only in medium containing 1.5% w/v NaCl and failed to grow in medium without NaCl and in any of the other concentrations of NaCl tested. The higher degree of halophilism exhibited by the isolates of *Aeromonas* clearly indicate that they are autocthonous flora of the sea and can adapt very well to the hatchery system.

Table 2. Antibiotic sensitivity of *Aeromonas* and *Pseudomona* isolated from the sick larval rearing pool.

Antibiotics	Conc./disk	Pseudomonas isolates (15 nos.)		Aeromonas isolates (13 nos.)	
		Sensitivity	Zone diameter (mm)*	Sensitivity	Zone diameter (mm)*
Penicillin G	10 units	R	0	R	0
Streptomycin**	30 mg	S	27	S	32
Ampicillin	10 µg	R	0	R	0
Oxytetracyclin	10 µg	R	0	R	0
Tetracyclin	10 µg	R	0	R	0
Gentamycin **	30 µg	S	28	S	24
Polymyxin B	10 µg	R	8	MS	18
Chlortetracyclin	300 units	R	0	R	0
Neomycin	30 µg	S	24	MS	18
Methamine mandelate **	30 µg	S	38	S	22
Cefozolin	3 mg	R	0	R	0
Amoxycillin	10 µg	R	0	R	0
Novobiocin	30 µg	R	0	MS	20
Nalidixic acid	30 µg	R	0	S	28
Chloramphenicol **	30 µg	S	32	S	30
Erythromycin	15 µg	MS	14	MS	30
Kanamycin	30 µg	MS	14	MS	14
Bacitracin	10 units	R	0	R	0
Lincomycin	2 µg	R	0	R	0
Sulphadiazine	300 µg	R	0	S	32

R (Resistant) : halozone < 11mm; S (sensitive) : halozone> 20 mm
MS (Moderately sensitive) : halozone 11-20 mm
** All the isolates tested positive
* The values are an average of the number of isolates tested.

However, the data generated by this study and also in earlier similar investigations (Singh *et al.,* 1989 and Singh, 1990) strongly suggest the possibility of exploiting the high generic /species diversity seen in the healthy larval rearing system to exclude the pathogenic *Vibrio* and *Aeromonas*. Gram positive bacteria such as *Micrococcus* and *Bacillus* and non pathogenic strains of *Pseudomonas* and *Acinetobacter* can serve as components of such healthy flora to prevent growth of Vibrios and Aeromonads. An important consideration to be made in such species exclusion method for protecting the larvae is to employ the strains whose generation time matches with those of the pathogens in culture conditions. *Pseudomonas* forms the major flora of the healthy culture system (Singh, 1990) , and as also confirmed by this study, non pathogenic strains of *Pseudomonas* sp. may be incorporated in the larval culture systems in numbers sufficient to prevent proliferation of other pathogenic forms, after ascertaining their generation which should either surpass that of the pathogenic forms or at least match with.

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