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Pathogenic changes due to inoculation of gram-negative bacteria *Pseudomonas aeruginosa* (MTCC 1688) on host tissue proteins and enzymes of the giant freshwater prawn, *Macrobrachium rosenbergii* (De Man)

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Abstract: *The freshwater giant prawn, Macrobrachium rosenbergii inoculated with Pseudomonas aeruginosa MTCC 1688 revealed pathogenic symptoms in certain tissue biochemical parameters. The tissue proteins showed proteolysis. The chitinase and phenol oxidase activity in tissues revealed marked inhibition. The culmination of these biochemical changes is attributed to the tissue pathological changes characteristic of bacterial inoculation. The chitinoclastic nature of the opportunistic bacterial pathogen inducing the above symptoms reveal that the disease outbreak in shrimps is the outcome of a pathogenic chain linkage involving environment (medium)–bacterial population (opportunistic/endemic)–shrimp population. The delinking of the above chain bywater quality maintenance, selection of shrimp species and by keeping the bacterial population below that of damage threshold would have far reaching implications in enhancing productivity in this under water agriculture.*

Key words: *Clinical signs, Chitinase, Pro-phenoloxidase, Macrobrachium rosenbergii, Pathological changes.*

Introduction

Similar to the marine species, the freshwater prawns are also prone to the attack of similar opportunistic pathogens such as viruses and bacteria. A perusal of literature has revealed that the freshwater forms have been infected with the bacterial species such as *Aeromonas sp., Pseudomonas aeruginosa* and *Edwardsiella sp.* (Hsiao and Chen, 1987; Ayala-Galvan, 1987; Anderson *et al*., 1989; Brady and Lasso, 1992; Jayashree, 2004 and Qureshi *et al*., 2000). Such studies have been made with reference to *Macrobrachium rosenbergii* in European freshwater systems. Similar to the above studies, several investigators have also carried out studies on freshwater prawns in India and revealed that they are susceptible to the diseases due to bacteria, protozoan parasites, fungal pathogens and viruses (Subrahmanyam, 1998; Kamonporn-Tonguthai, 1992; Shariff *et al*., 1992; Soundarapandian and Kannupandi, 1998). Their studies have also revealed that larval stages of *Macrobrachium sp.* are more susceptible to the pathogens, when compared to the adults.

However, some of these studies have revealed that the virulence and pathogenecity of these pathogens on *Macrobrachium rosenbergii* and *Macrobrachium malcolmsonii* are comparatively low to account for heavy mortality incidence. Hence, it is of interest to investigate the potentialities and scope of such species, which are less susceptible to infection (Sahul Hameed *et al*., 2000).

The foregoing account and comparison of data regarding the species of freshwater prawns and that of marine habitats imply the lacuna in the augmentation of farming system of the former and the shortcomings by way of human intervention. However the feasibility of culture of freshwater prawns seems to be more favourable in view of the availability of the above water resources and their non-susceptibility to the pathogenic, disease causing bacterial populations, which remain as either endemic or opportunistic species. However an understanding of their built in mechanisms of immunity as well as their biochemical and enzymatic components which afford them the stamina against infection is needed and warrants laboratory investigation.

In the culture of fishes in farms, studies have revealed that certain biochemical and enzymatic parameters help in assessing the morbidity and the resistance of species (Payne and Payne, 1985). Such parameters and studies on fishes and other organisms subjected to various stressors such as handling, transportation, overexertion, cold-heat stress, noise stress, altitudinal effects, anaesthetization, hyper muscular activity etc. have revealed that stress of any nature could affect the well being of organisms in various ways and emphasized that undue stress neds to be controlled to reduce ill health and mortality (Black, 1958; Chavin and Yond, 1970; Karuppannan and Kutty, 1978). Their studies have also pointed out that finding of good indicators, through bioassays, may reflect in short intervals, the condition of organisms in development / culture is difficult.

In view of the lacunae, in the biology and culture of freshwater prawns, the present study is attempted, to evaluate the effects of bacterial endotoxin namely, *Pseudomonas aeruginosa* over the blood and tissue chemistry. The investigation encompasses such parameters as protein content, chitinase and phenoloxidase activity.

As less attention has been paid to the culture of freshwater prawns, compared to the marine species, the giant freshwater prawn, *Macrobrachium rosenbergii* is selected for the present investigation. As the species *Macrobrachium rosenbergii*

represents a hardy and resistant one to infection compared to other species (as revealed by the perusal of literature), an understanding of its resistant potential through the selected parameters of tissue and blood chemistry may be useful for its culture techniques of either intensive or semi-intensive nature.

Materials and Methods

Specimens of *Macrobrachium rosenbergii* were collected from the commercial farm of Kanathur, along the coastal area of Chennai. After being brought to the laboratory, the giant prawns were acclimated to the laboratory conditions in stocking glass tanks (Salinity 2ppt, temperature 28 $\pm$ 2ºC). The prawns were kept for a minimum of 15 days prior to the experimentation as suggested by Drach (1939) for aquatic crustaceans. *Pseudomonas aeruginosa* is an ubiquitous microbe in the environment and is found in the water, soil and on plants (Sabath, 1980). The bacterial strain, *Pseudomonas aeruginosa* was selected as the biotoxin for the study, because of its pathogenic effects on *Macrobrachium rosenbergii*. The bacterial strain *Pseudomonas aeruginosa* was brought from the institute of microbial type culture collection and gene bank, Chandigarh, India. Culture was done as prescribed by the above institute. The bacterial inoculum was prepared by the procedure adopted by Lightner and Lewis (1975). The live bacteria were harvested which was made with different dilutions viz., 10^8, 10^7, 10^6 and 10^5. About 0.05 ml of the inoculum of the different dilutions was taken in 1 ml tuberculin syringe and injected in between 5th and 6th abdominal segment of *Macrobrachium rosenbergii*. The bacterial count or the colony forming units in the inoculum of LD_{50} was determined by the procedure followed by Brown and Poxton (1997). The LD_{50} for 96 hrs concentration of inoculum was determined to be 10^7. The bacterial count for LD_{50} was taken for the time course study with intervals of 24, 48, 72 and 96 hr after injecting the inoculum for biochemical studies. The tissues body muscle, hepatopancreas and haemolymph were taken for the above analysis.

Total protein was estimated following Lowry *et al.* (1951) method with Folin-Phenol reagent. Chitinase was determined by the method of Reissig *et al.,* (1995), using Bausch and Lomb Spectronic 21 Spectrophotometer (585 nm). Phenoloxidase was determined by the procedure followed by Nelliappan and Ramalingam (1980), using Bausch and Lomb Spectronic 21 Spectrophotometer (480 nm). The data obtained for different intervals of inoculation were compared with the control using ANOVA as given by Zar (1974).

Results and Discussion

Clinical signs: In the present study, *Macrobrachium rosenbergii* inoculated with *Pseudomonas aeruginosa* MTCC 1688, exhibited clinical signs such as translucent appearance of the abdominal musculature and whitish opaque colouration; slightly darkening of the dorsal portion of the integument; eroded localized lesions in the cuticle which subsequently melanized and showed brown to slight greenish colour, presence of black spots on the uropod; pale and atrophied hepatopancreas and empty guts etc (Fig. 1). Similar clinical observations have been reported in both freshwater and marine speciesdue to vibriosis. Mukherjee and Chandra (1991) revealed that *Pseudomonas* infection in the freshwater prawn *Macrobrachium rosenbergii* caused thickening of the joints of the uropods and pleopods which were covered with greenish yellow filamentous deposits seen through out the body surface, eyestalk, antennae and carapace. Gill lamellae were pale and grayish black in colour and clogged with filamentous growths.

Bechtler and Holler (1996) revealed the clinical signs of vibriosis in shrimps viz., loss of appetite, halt growth, broken antennae, necrosis, lesions and black spots in the muscle tissue. Bower *et al.* (1996) reported the clinical signs of black discolouration of the cuticle especially around the edges of the body segments and black stippling on the surface of the hepatopancreas in the 'Stained prawns disease' of *Pandalus platycerus* caused by rickettsial infection.

The differential effects of the pathological clinical symptoms such as the translucent musculature, cuticular erosion and atrophy of the tissues may be deduced to the various enzymatic actions of the bacterial species. The inoculum containing the bacteria might have secreted the extracellular enzymes such as proteases, chitinase, oxidase, catalase and gelatinase etc. And these extracellular enzymes might have acted independent of one another and thus brought the above differential symptoms. The darkening of the dorsal tegument and the black spot on the uropods might be attributed to the action of the phenoloxidase enzyme of the prawns, which would have produced melanin formation in the infected cuticle.

In the present study the darkening of the dorsal portion of the integument might be due to the bacterial action of *pseudomonas aeruginosa* (MTCC 1688), which possesses chitinase, oxidase and catalase enzymes.

Total protein: In the present study the prawns inoculated with *Pseudomonas aeruginosa* revealed profound changes in the total protein content of body muscle and hepatopancreas (Table 1). The consistent decrease of the total protein after the inoculation at all intervals reveals the proteolysis in tissues. The above proteolysis may be attributed to the action of the extra cellular secretion of the proteases by the bacterial strain. In toxicological studies the processes of proteolysis and the consequent mobilization of tissue proteins have been attributed for the derivation of the energy (Ramalingam, 2003). The changes in the protein profile of the tissue intoxicated with contaminants and the increase in the amino acid pool have been attributed to the hydrolytic action of enzymes such as proteolytic enzymes and transaminases of the organisms subjected to toxicity stress.

These studies have also revealed the sparing role of proteins next to carbohydrates in meeting the energy demand. However, the same inference could not be attributed in the case of prawns subjected to *Pseudomonas aeruginosa* infection of their endotoxin toxicity. Recent studies have revealed that bacterial species can synthesis extra cellular proteases (Chen

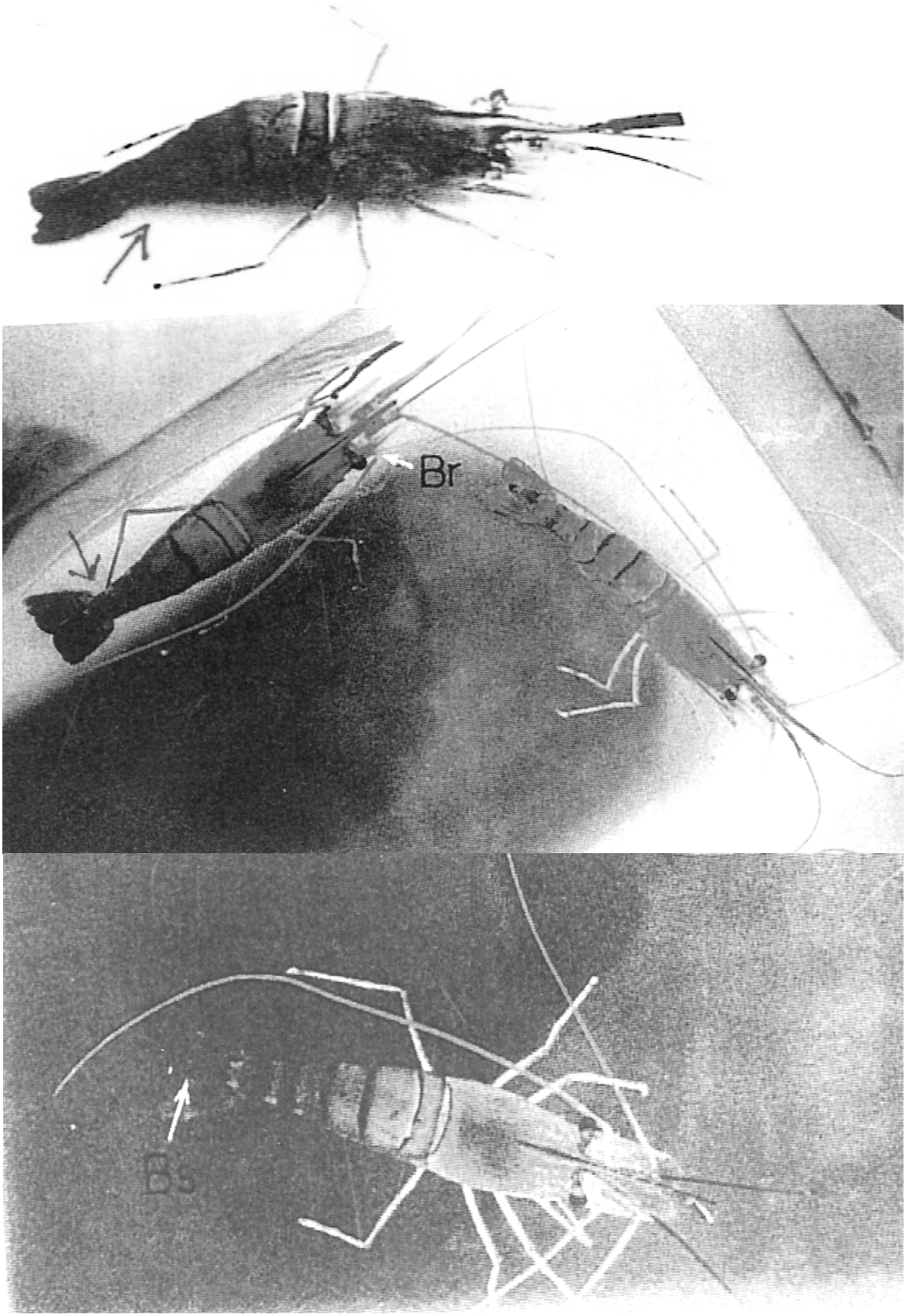

Fig. 1: Clinical signs of *Macrobrachium rosenbergii* inoculated with *Pseudomonas aeruginosa MTCC 1688.*

Table – 1: Total protein levels in the tissues (body muscle, hepatopancreas (units/mg) and haemolymph (units/ml) of *Macrobrachium rosenbergii* control (0 hr) Vs test group inoculated with *Pseudomonas aeruginosa* MTCC 1688.

Times of interval	Body muscle units/100mg wet wt.	Hepatopancreas univets/100mg wet wt.	Haemolymph units/ml
0 hr control	1.673 ± 0.091	1.188 ± 0.071	2.498 ± 0.686
24 hr	1.387 ± 0.102	0.957 ± 0.083	2.665 ± 0.479
48 hr	1.057 ± 0.124	0.884 ± 0.048	2.851 ± 0.254
72 hr	0.756 ± 0.116	0.650 ± 0.081	2.672 ± 0.621
96 hr	0.348 ± 0.088	0.223 ± 0.089	2.832 ± 0.627

et al., 1999). The above proteases of bacterial origin may be said to degrade the tissue proteins in the prawns. The marked changes noticed in both muscle, hepatopancreas and the histopathological symptoms may also be attributed to the above bacterial proteases. When proteases of such bacterial origin attack the tissues, the histamine bound to the cell protein could have been released. In the microbial infections histamines are of importance, because they are attributed to bring deleterious effects of tissue inflammation.

The deleterious action of bacteria over the host tissues have been unequivocally proved long back in animals. Histamine producing bacteria have been attributed to the spoilage of the host tissues such as fishes. Though *Pseudomonas* species may not by synthesizing histamine, being gram negative forms, their endotoxin might have strongly activated the tissue systems such as muscle and hepatopancreas and the histidine carboxylase activity in them and resulted in the inflammatory changes. In vertebrates, various bacterial spcies and Freund's adjuvant have been reported to activate the histidine decarboxylase activity in the tissues rich in reticulo-endothelial system and the consequent conversion of histidine to histamine (Schayer, 1960).

Unlike the above the higher vertebrates wherein the tissues are endowed with anastomizing reticulo endothelial system, the crustaceans lack such blood supply through vessels as the haemolymph bathes the tissues. However, the pathological manifestations of the gram-negative *Pseudomonas aeruginosa* seem to be similar in the phylogenetically simpler and archaic organisms like prawns also.

Chitinase activity: In the present study, the chitinase enzyme in *Macrobrachium rosenbergii* inoculated with *Pseudomonas aeruginosa*, resulted in a decrease in all the three tissues viz., body muscle, hepatopancreas and haemolymph at all intervals, except at 48 hr in the hepatopancreas with respect to the control group (Table 2).

Similar decrease of chitinase enzyme activity has also been observed by Bayoumi *et al.* (1997) in the leaf worm *Spodoptera littoralis*, due to treatment with pesticides like chloroflauzon and fluefenzuron and by Donachie *et al*. (1995) in *Meganyctiphaenes norvegicus* infected with chitinoclastic bacteria.

The marked decrease in the chitinase enzyme activity in the tissues of *Pseudomonas aeruginosa* inoculated prawns in the present study suggests its inhibition as well as the failure of chitin synthesis in the host prawns. Studies have also demonstrated in the invertebrate predators, fishes and even in humans the chitinase formed from the glycol-hydrolases are used for defense against pathogenic fungi, parasites and other microbes. However, the clinical symptoms of damage in the exoskeletal structure of *Macrobrachium rosenbergii*, revealing the lesions and also the softening of the cuticle suggest that the enzyme becomes non-functional and ineffective in the defensive function. It remains to be understood how the host enzyme chitinase being inhibited while that of the bacterial enzyme is enhanced to enable its penetration of the exoskeletal and peritrophic membranes of the arthropod organisms like prawns.

The inhibition of the host chitinase in prawns is not uncommon. Fox (1993) revealed that increased levels of dietary chitin decreased the chitinase activity in the hepatopancreas of *Penaeus monodon*. Similarly, chitino plastic/chitinolytic bacterial infection in prawns has been observed to reduce the chitinase activity in the host shrimps, belonging to penaeid species. Watanabe and Kondo (1997) isolated cDNA encoding the chitinase protein from the cuticular tissues of *P. japonicus*. Wortman *et al.* (1986) have demonstrated chitinase determinants of *Vibrio vulnificus*.

Previous studies have suggested that the extracellular chitinase domains of chitinoplastic species might have exhibited its catalytic function by combining with non-catalytic domains in the *in vivo* condition to increase their host-chitin binding capacity and their consequent digestion of the chitin. As long as the intricacies involved in the bacterial genome expression and the genomic functions of the host cells are not properly understood, the contaminant of such pathogenic infections by the characteristic chitinoclastic forms is far from comprehension and alleviation. Probably the genetic engineering methodology may be in a promising position to probe the above host versus pathogen infection and interaction. It may be concluded however that the bacterial enzymes are activated *in vivo* in the manifestation of the clinical symptoms of Vibriosis.

Phenoloxidase activity: In the present study, the phenoloxidase activity decreased significantly after 24, 48, 72 and 96 hr in the tissues of body muscle and hepatopancreas, whereas in the haemolymph of *Macrobrachium rosenbergii* inoculated with *Pseudomonas aeruginosa*, there was a significant increase at 96 hr of inoculation (Table 3). In crustaceans and insects (arthropods)

Table – 2: Activity of chitinase enzyme in the tissues (body muscle, hepatopanreas (units/mg) and haemolymph (units/ml) of *Macrobrachium rosenbergii*. Control (0 hr) Vs test groups inoculated with *Pseudomonas aeruginosa* MTCC 1688.

Times of interval	Body muscle units/100mg wet wt.	Hepatopancreas units/100mg wet wt.	Haemolymph units/ml
0 hr control	4.866 ± 0.216	37.033 ± 2.007	163.25 ± 2.923
24 hr	4.666 ± 1.080	35.850 ± 1.013	160.016 ± 2.957
48 hr	3.683 ± 1.068	39.333 ± 1.570	139.683 ± 3.546
72 hr	2.750 ± 0.882	35.583 ± 2.354	130.516 ± 5.286
96 hr	2.258 ± 0.347	32.083 ± 1.463	92.75 ± 6.469

Table – 3: Activity of henoloxidase enzyme in body muscle, hepatopancreas (units/mg) and haemolymph (units/ml) of *Macrobrachium rosenbergii*. Control (0 hr) Vs test group inoculated with *Pseudomonas aeruginosa* MTCC 1688. (Units/min/mg of rotein/100mg of wet tissue)

Times of interval	Body muscle units/100mg wet wt.	Hepatopancreas units/100 mg wet wt.	Haemolymph units/ml
0 hr control	0.163 ± 0.062	0.181 ± 0.065	0.413 ± 0.038
24 hr	0.105 ± 0.062	0.108 ± 0.058	0.163 ± 0.065
48 hr	0.015 ± 0.005	0.015 ± 0.008	0.186 ± 0.100
72 hr	0.015 ± 0.01	0.065 ± 0.013	0.113 ± 0.027
96 hr	0.018 ± 0.007	0.063 ± 0.029	0.648 ± 0.047

the phenoloxidase is not merely considered an enzyme of cuticle tanning process but also considered to play an important role in the immune functions (Brehlin *et al*., 1989; Gregoria and Ratcliffe, 1991; Lanz *et al*., 1993).

Studies have demonstrated that various elicitors of phenoloxidase or prophenoloxidase include (Nelliappan and Ramalingam, 1980), I) B-1,3 glucan from fungal cell wall or lipo-polsaccharides; ii) Bacterial peptidoglycans; iii) Temperature; iv) Calcium concentrations; v) Vibiro cells; vi) Divalent cations Ca^{2+} and Mg^{2+}; vii) Trypsin activates.

Sung *et al.* (1998) have reported that both calcium and magnesium are required to enhance the phenoloxidase activity, both in tiger prawns and giant freshwater prawns. Cheng *et al*. (2000) have opined that the quality of water is critical in maintaining proper concentrations of calcium or magnesium in order to enhance the pro-phenoloxidase activity to strngthen the defense mechanism of the shrimps. Sung *et al.* (1998) have also observed that phenoloxidase activity in the giant freshwater prawn is significantly greater than that of tiger prawn and attributed it to higher levels or large number of granular haemocytes. Soderhall and Smith (1983) have revealed that semi-granular and granular haemocytes participate in the pro-phenoloxidase system and are involved in the antibacterial activity.

With the above inference from the reports, the decreased phenoloxidase activity in the present study may be attributed to the inhibitors of the above enzyme system *in vivo* as well as to the circulatory haemocytes, their nature or types and the specific decease of those cells involved in immune function.

The fact that *Macrobcrachiums roserbergii* survives in freshwater for longer periods with greater hardiness (Ra' anan *et al*., 1983) suggests that in the experimentally inoculated prawns the microbial metabolism may be interfering with its hardiness by affecting the phenoloxidase activity which has been said to play a role in the phagocytic and encapsulation processes of the non- self or foreign antigen (Hose *et al*., 1990).

The conspicuous increase in the phenoloxidase activity in the haemolymph at 96 hr in the bacterial inoculated prawns is of interest to suggest that the suppression or inhibition of phenolxidase activity is not of a permanent nature and that it could also be revived. However, the intricate mechanisms in enhancing the phenoloxidase activity remains to be explained in the *in vivo* condition.

Towards the above, the reports of a few investigators with regard to the function of phenoloxidase are of interest to mention. Chisholm and Smith (1992) observed no correlation between antibacterial activities and phenoloxidase in the shore crab *carcinus maenus*. However, Du *et al.* (1997) revealed that phenoloxidase activity increased with the bacteriolytic activity in *Penaeus chinensis* against *Vibrio alginolyticus* after immunopotentiation with the incorporation of garlic oil in the feed.

In the present investigation, the sample population of *Macrobrachium rosenbergii*, subjected to *Pseudomonas aeruginosa* infection survived up to ten or more days. However, the increase of dosage caused their mortality within 72 hr. The above observation also suggests that the circulatory blood cells specifically the granular haemocytes and the phenoloxidase system alongside with the haemagglutination principles may be rendering the prawns survival and the potentiation of their defense mechanism.

Hence, the bacterial (*Pseudomonas aeruginosa*) inoculum bringing about the disease symptoms such as whitish

opaque appearance, slight darkening of the tegument, black spot on the uropods and tissue damage are attributed to the success of infection of *Pseudomonas* in the intermoult adult prawns, *Macrobrachium rosenbergii*.

From these studies, it is of interest to note that *Pseudomonas* infection is not specific to definite stage of growth of larval or adult stage *Macrobrachium rosenbergii*. Hence, the contaminant of the infectivity and pathogenicity of *Macrobrachium rosenbergii* by the bacterial pathogens (opportunistic) depends upon conducting time course studies to establish the development of pathogen in different stages of host development. Such time course studies in the experimental infection would throw light about infection of pathogen with reference to the host age; susceptibility of larval, early post larval, late post larval and adult stages of the host.

The laboratory results of the present investigation, using the inoculum *Pseudomonas aeruginosa* MTCC 1688 revealed that these endemic bacterial populations might become opportunistic pathogens, when hydroecological conditions are altered in the farm ponds and could bring the fatal episodes of mass mortality.

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