Effect of *Pseudomonas aeruginosa* on the giant freshwater prawn, *Macrobrachium rosenbergii* - histopathological and electron microscopic study

K. Ramalingam¹ and S. Ramarani²

¹PG (Aquaculture) and Research Department of Advanced Zoology and Biotechnology, Government Arts College, Nandanam, Chennai-600 035, India
²Chellamal College for Women, Guindy, Chennai-600 035, India

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**Abstract:** The giant freshwater prawn *Macrobrachium rosenbergii* was injected with an inoculum containing LD₅₀ 96 hr dose of 10⁷ *Pseudomonas aeruginosa* (MTCC 1688) to determine the histopathological effects in vivo. The comparison of tissues of both the control and the bacterial endotoxin treated prawns after 96 hr revealed significant degenerative changes in treated prawns. Both light microscopic and electron microscopic observations revealed the infiltration of the tissues of *Pseudomonas* sp in the muscular and hepatopancreatic tissues of prawn. The muscular tissue changes in the myofibrillar arrangement with blockage at the gap junctions and necrotic lesions were observed. The hepatopancreatic cells were vacuolated with hypertrophied nucleus. Atrophy of hepatopancreatic tubules was conspicuous. The pathogenicity of *Pseudomonas aeruginosa* is attributed to its infiltration and multiplication inside the tissues and the consequent release of extra-cellular enzymes for its metabolism. The degeneration of host tissues is also attributed to the latter.

**Key words:** *Macrobrachium rosenbergii*, *Pseudomonas aeruginosa*, Extracellular proteases, Endotoxin

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**Introduction**

In shrimp culture, the disease outbreaks in growout ponds have been a perennial threat to their growth and productivity. Such diseases also bring heavy economic loss for the culturists and investors due to mass mortality. Both bacterial and viral diseases have been established in prawn/shrimp culture (Sugita et al., 1987; Anderson, 1989; Mukherjee and Chandra, 1991 and Ra-anan et al., 1991; Ramalingam and Ramarani, 2006). The literature has revealed that both pathogenic and non pathogenic bacterial population exist in natural freshwater and marine water sources. The complex nature of these disease outbreaks in crustaceans have necessitated the need for an understanding of the causative pathogens and their metabolism and the progress of their infection *in vivo* in order to apply control measures and managerial strategies.

Several investigations have been carried out to understand the different aspects of commercially important prawns in India. However, the cases documented at present are not comprehensive, and considerable research efforts are required to survey the diseases that occur in growout ponds and to understand the host-pathogen relationship, pathogenesis, disease diagnosis, biochemical profile of the host and on specialized aspects like virology, bacteriology, mycology and immunology.

The gross and histopathological findings, both at macro (gross) and micro level, are of diagnostic value in the identification of the nature of infection. Such identification/prognostic analysis of symptoms in shrimp culture farms would go a long way in minimizing the risks involved or in eliminating the pathogenic population from water.

Towards this end, a time bound analysis of tissues of prawns, after the inoculation of the pathogen would be of value in assessing the pathological effects over a specific period. Since mortalities have been reported to begin towards the end of the grow out period when the shrimps approach the market size, the diagnosis of pathogenic infection is crucial and prudent at this stage. In the present study, the giant freshwater prawn inoculated with an LD₅₀ 96 hr dose of *Pseudomonas aeruginosa* MTCC 1688 was subjected to histopathological analysis. The period of observation was after 96 hours.

**Materials and Methods**

Specimens of *Macrobrachium rosenbergii* were collected from the commercial shrimp farm at Kanathur, along the coastal area of Chennai. After being brought to the laboratory, the giant prawns were acclimated to the laboratory conditions in the stocking glass tanks (salinity 2 ppt, temperature 28 ± 2°C). 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 ubiquitous in the environment and is found in the water, soil and on plants (Sabath, 1980). *Pseudomonas aeruginosa* was selected as the biotoxin for the study, in view of its pathogenicity on *Macrobrachium rosenbergii*. The bacterial strain 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 from 24 hr culture using sterile bacterial loop and mixed with double distilled water. This was then diluted to two-fold serial
dilutions of the bacterial suspension, which was made into different dilutions viz., 10^6, 10^7, 10^8, and 10^9. 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 LD_{50} concentration of inoculum was 10^7. The bacterial count for LD_{50} value of inoculum was calculated as 2.41 x 10^7 CFU/0.05 ml. The above LD_{50} dose of 10^7 was taken for the study and injected into the prawns. The muscular, hepatopancreatic tissues were taken for both light microscopic and electron microscopic observations.

The muscular and hepatopancreatic tissues of control and test groups were selected for histological studies. Tissues were separated and immediately fixed in aqueous Bouin’s fluid (saturated solution of picric acid -75 ml, Formaldehyde -20 ml and Acetic acid 5 ml) for a period of 24 hr. After fixing, they were washed overnight in running water to remove the fixative. To remove the water content present in the tissues, dehydration was carried out by transferring them to a series of gradually increasing concentrations of alcohol in water. The materials were then cleared in xylol and embedded in wax (melting point 52°C). Sections were cut at 7µ thickness and stained with haematoxylin and eosin (Culling, 1957; Humason, 1972).

For electron microscopic study, the cuticle, muscular and hepatopancreatic tissues of both control and test groups of Macrobrachium rosenbergii were fixed for 24 hr at 4°C in glutaraldehyde buffered to pH 7.2 with 0.1 M sodium cacodylate-HCl containing 3% sucrose and 0.55 sodium chloride (Ramasamy, 1995). They were washed and stored at 4°C in 4% sodium cacodylate buffer until required further. The tissues were post fixed for one hour with 15% aqueous osmium tetroxide, dehydrated through ethanol to propylene oxide and embedded in Epoxy resin. The section of 60-90 nm were cut using a Richter E Ultramicrotome with diamond/glass knives. Sections were collected on copper grids stained with uranyl acetate and lead citrate and examined using a Philips Transmission Electron Microscopy operating at 100 KV.

Results and Discussion

In the uninfected control (0 hr) forms of shrimps, the muscular tissue showed normal longitudinal arrangement of myofibrillar filaments without any pathogenic infection. After 96 hr, muscle tissues revealed the presence of melanized haemocytic granulomas in the connective tissue around the haemal sinuses together with haemocytic aggregation in necrotic musculature and broken myofibrillar arrangement (Fig. 1).

The hepatopancreas revealed the presence of normal hepatopancreatic tubules with F and B cells and normal brush border in the control group of Macrobrachium rosenbergii. After 96 hr, multiple haemocytic nodules in the hepatocytes and increased number of melanised haemocytes markedly affecting the tissue was noticed (Fig. 2).

In the electron microscopic sections, the body muscle of Macrobrachium rosenbergii showed the regular arrangement of myofibrillar filament of the muscle tissue with the presence of sarcoplasm and interstitial matrix in between the muscle bundles in the control group (Fig. 3).

After 96 hr of inoculation with Pseudomonas aeruginosa, MTCC 1688, the significant changes observed were, i) localization of bacteria invading the muscle; ii) longitudinal shape of the muscle being constricted, iii) loosened myofibrils with gap regions, iv) disappearance of nuclear materials, and v) large necrotic lesions with bacterial colonies incorporated in them (Fig. 5).

The hepatopancreas of uninfected shrimps showed the presence of hepatopancreatic cells with intact nuclear membrane, less number of nuclear pores and vacuoles, eccentric positions of nucleolus and more number of microvilli (Fig. 3, 4).

After 96 hr of inoculation with Pseudomonas aeruginosa, MTCC 1688, the changes observed were more number of vacuoles and nuclear pores; hypertrophied nucleus; disappearance of nuclear membrane and loss of microvilli at the apical surface of the tubules (Fig. 6).

The structural morphology of cuticle in both uninfected (control) and infected shrimps at 96 hr revealed marked difference with reference to the intactness of the layer and also the permeability as envisaged by the translucent appearance in the infected cuticle (Fig. 4, 6).

The results of the histopathological study in the Pseudomonas aeruginosa inoculated prawns revealed the characteristic degenerative changes in both body muscle and hepatopancreas. The abnormalities were greatly discernible after 96 hr of inoculation. In the muscle the changes included, i) muscular lesions containing bacteria, ii) changes in the myofibrillar arrangement from longitudinal disposition to diagonal direction, iii) necrotic changes, iv) melanised haemocytic granulomas around the haemal sinuses, v) haemocytic aggregation in the necrotic musculature and vi) broken myofibrillar arrangements, etc. Likewise in the hepatopancreas, the changes included i) melanised hepatopancreas tubules, ii) haemocytic nodules and iii) irregular luminal cavity of the tissues. Earlier workers have reported similar destructive changes in the different tissues of shrimps due to both bacterial and viral infections. Lightner (1984) reported the presence of a large number of lesions in the tissues of hepatopancreas, antennal gland and mandibular organs in the penaeid prawns infected with bacteria. Leone et al. (1994) have reported haemocytes infiltration alongside with nodule and granuloma formation in the epidermis and connective tissues of Penaeus vannamei infected with vibriois. Jiravanichpaisal et al. (1994) reported the occurrence of bacteria phagocytes in the tissues of prawns infected with Vibrio harveyi. Cai and Cai (1996)
reported multi-organ necrosis affecting hepatopancreas, gills, heart and gonads in their histopathological findings in *Macrobrachium rosenbergii* infected with Tortulosis. Esteve and Herrera (1997), also revealed the tissues destruction and accumulation of bacteria in the hepatopancreatic tubular lumen of *Penaeus vannamei* infected with *Vibrio anguillarum*. Cheng et al. (1998), reported the presence of melanized haemocytic granulomas in the connective tissue around haemal sinuses together with haemocytic aggregation in necrotic musculature in *Macrobrachium rosenbergii* infected with Enterococcus bacterium. Similar to bacterial infection, Arcier et al. (1999) reported various histopathological changes due to viral infection in the post larvae of the giant freshwater prawn *Macrobrachium rosenbergii* which were similar to bacterial infection.

Electron microscopic study in control and inoculum injected groups of prawns revealed that in control group muscles, regular arrangement of myofibrillar filaments of the muscle tissue was clear. By contrast the muscles of prawns inoculated with *Pseudomonas aeruginosa*, showed the following changes; viz., i) localization of bacteria invading the muscle; ii) longitudinal shape of the muscle being constricted, iii) loosened myofibrils, with gap regions; iv) disappearance of nuclear materials; v) large necrotic lesions with bacterial colonies infiltrated in them. Similarly in control group, the hepatopancreas of *Macrobrachium rosenbergii* showed a normal arrangement with intact nuclear membrane, less number of nuclear pores and vacuoles, eccentric position of nucleolus and more number of microvilli etc. By contrast, the hepatopancreas of prawns inoculated with *Pseudomonas aeruginosa* showed the following

**Fig. 1:** Photomicrographs of body muscle of *Macrobrachium rosenbergii*  
(MF = Myofibrillar filament, S = Sarcoplasm, G = Granuloma, B = Broken filament, Ne = Necrosis)
particles seem to suggest that the complete immune mechanism of multiplication into colonies, which are discernible as occluded of the bacterium the cytoplasm. The ultrastructural changes revealing the invasion hepatopancreatic tubules and vi) more number of free ribosomes in the cytoplasm. The ultrastructural changes revealing the invasion of the bacterium Pseudomonas aeruginosa and the infiltration and multiplication into colonies, which are discernible as occluded particles seem to suggest that the complete immune mechanism of host failed to resist their multiplication in vivo. Moreover, this bacterial form Pseudomonas aeruginosa endowed with their own intrinsic enzyme systems would have facilitated the degeneration of the tissues and further extractions of the nutrient elements of the host tissues for their own growth and multiplication. In this context, the disassemblage of myofibrils in the Pseudomonas aeruginosa infected muscle may be attributed to the extra cellular proteases characteristic of the chitinoclastic bacteria and the consequent protein disintegration.

Fig. 2: Photomicrographs of hepatopancreas of Macrobrachium rosenbergii (Lum = Lumen, Brb = Brush border, HPC = Hepatopancreatic cell, V = Vacuole, WL = Widened lumen, M = Melanized hemocytes, Ht = Hepatopancreatic tubule, S = Sinus)
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Fig. 3: Transmission electron micrographs of body muscle and hepatopancreas
(INM = Interstitial matrix, MF = Myofibrilar filament, S = Sarcoplasm, Hc = Heterochromatin, NM = Nuclear membrane, M = Mitochondria, ER = Endoplasmic reticulum, V = Vacuole)
Fig. 4: Transmission electron micrographs of cuticle and hepatopancreas
(MV = Microvilli, Control - Cuticle - Intactness of the layers)

Similar views were extended by Liu et al. (1997), Singh et al. (1998), Herrera et al. (1999) and Gooday et al. (1999). Singh et al. (1998), revealed that the growth and multiplication of vibrio colonies in the muscles of *Penaeus indicus* occurs due to action of extracellular enzymes and degradation of the metabolic nutrients in the muscle tissue and the consequent uptake by the bacteria. Gooday et al. (1999) have reported the destruction in the shape of the longitudinal myofibrillar filament and the granulation of the myofibrils inoculated with vibrios leading to muscle degradation. Liu et al. (1997) have reported that the inoculated vibrios producing extra cellular enzymes are the causative factor of cell lysis and their components. Lamas et al. (1994), have reported that the host
immune response was not able to resist the bacterial multiplication, which results in the proliferation of bacterial colonies in the host tissues. In this context it is of interest to note that in shrimps infected with pathogens experimentally, phenoloxidase activity and the total haemocyte count were drastically reduced. Increased number of vacuoles noticed in the present study may represent the degeneration of cells due to lack of lipid reserves, which could have been used for bacterial metabolism. Similar reports of tissue and cellular damage with reference to giant freshwater prawn species are found mostly meager except a few (Owens et al., 1992). The tissue degenerative changes in both muscle and hepatopancreas as elucidated by both light microscopic and TEM study in *Macrobrachium rosenbergii* inoculated with *Pseudomonas aeruginosa* reveal the pathogenicity of the bacterial population *in vivo* after 96 hr. The significant changes as noticed in the permeability of the cuticle by the translucent appearance in the inoculated prawns also suggest that the exoskeletal barrier layer of the cuticle is being
Fig. 6: Transmission electron micrographs of hepatopancreas and cuticle
(NPI = Nucleoplasm, DNM = Disappeared nuclear membrane, NP = Nuclear pore
V = Vesicle, M = Mitochondria, Cuticle – translucent appearance)
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disrupted by the extracellular proteases of the bacteria thus enabling their in vivo infiltration.

References


