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The haemolymph response of *Penaeus indicus* to the extracellular products of *Aeromonas hydrophila*

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Abstract

The effects of Extracellular products (ECP) of *Aeromonas hydrophila* on *Penaeus indicus* were assessed by haemolymph study. ECP was obtained by growing the pathogenic bacterium on sterile cellophane sheets over TSA plates followed by suspension in normal saline, centrifugation and filtration. Protein profile of ECP was analysed by SDS-PAGE. Five groups of *P. indicus* and a control group were maintained to study the effect of five different concentrations of the ECP of *A. hydrophila* on haemolymph factors. There were rapid behavioural changes and gross pathological alterations like loss of appetite, abnormal swimming behaviour, melanisation on abdomen and soft shell syndrome. The mortality rate for each concentration of ECP was determined. The total haemocyte count was assayed in all groups at different hours. There was reduction in the number of haemocytes with each higher concentration of the ECP. These changes are attributed to the virulent factors in the ECP of *Aeromonas hydrophila.*

Introduction

Among the bacterial diseases of shrimps, the diseases caused by bacteria belonging to the family Vibrionaceae occupies paramount position (Sindermann, 1971; Lightner, 1977, 1983). *Aeromonas hydrophila* is an important member of Vibrionaceae which cause disease condition like shell disease, haemocytic enteritis, juvenile septicaemia and wound infections (Lightner, 1977). Reports of occurrence of *Aeromonas* sp. were mostly from *Penaeus japonicus, P. indicus and P. stylirostris* (Yasuda and Kiyao., 1980; Lewis *et al.,* 1982; Singh *et al.,* 1985). *Aeromonas* sp. were isolated from a number of conditions in *P. indicus*. They have also been associated with soft shelled prawns (Baticadas *et al.,* 1986). *Aeromonas* occupied a dominant position in 10-20 ppt in rearing tanks of shrimps (Huang *et al.,* 1994) and frequently isolated from *P. monodon* and *P. indicus* (Chang *et al.,* 1996).

These bacteria produce certain extracellular toxins which has got pathogenic effect on fishes. The ECP of *Aeromonas hydrophila* are important in causing the disease manifestations. The pathogenesis of most of the members of the family Vibrionaceae were attributed to the extracellular products secreted by them. Beta haemolysin (Berheimer *et al.,* 1974), enterotoxins (Wadstrom *et al.,* 1976) and leucocytolytic factor (Fuller *et al.,* 1977) have been observed in the ECP of *A. hydrophila.* A multienzyme complex with acyltransferase and phospholipase activity has been reported in *A. hydrophila* (MacIntyre and Buckley, 1978).

Though a lot of studies has been done on the ECP isolated from *A. hydrophila,*

these studies were mostly confined to characterisation of various factors and their activity *invitro*. Not much work has been done on the effect of these toxins *invivo*. There were no reports on the effect of ECP on shrimps. Hence a study was planned to assess the effect of ECP of *A. hydrophila* on the haemolymph factors of *P. indicus*.

The haemolymph is an important component involved in the respiration, digestion and the defence mechanism of crustaceans. Hence any effect on the haemolymph has a bearing on the immunity of the animal. The attempt was made to understand the effect of toxins on the cellular defence factors of the prawn.

Material and methods.

The effect of ECP of *Aeromonas hydrophila* was studied in *P. indicus* of mean body weight 5-7 gm. The experimental set up consisted of maintaining 6 numbers of 50 litre plastic tubs stocked with 8 shrimps each. Salinity was maintained between 15 and 20 ppt and mean temperature being 28 ± 1°C. The shrimps were fed with pelleted feed 2-3% of body weight twice daily. Triplicates were maintained for the experiment. Bacterial strains for the experiment was isolated from diseased fish sample. The pathogenic strain of *A. hydrophila* was inoculated in TSB and routinely cultured in TSA at 28°C for 24-48 hours. The strain was also stored on TSA slants for further use at 4°C.

ECP preparation

The ECP was prepared according to Nieto and Ellis (1986). In order to obtain ECP free of the cultured medium, the culture of *Aeromonas hydrophila* in Tryptic Soya Broth (TSB) were inoculated into sterilized cellophane sheets overlying Tryptic Soya Agar (TSA) plates and incubated at 28°C for 24 hours. The log phase culture was harvested and cells were suspended in about 5-7 ml of normal saline and centrifuged at 6000 rpm for 20 min. The supernatant (ECP) was sterilized by filtration by passing through 0.22µ millipore filter. For each experiment, the ECP was freshly prepared.

Total Protein Assay

Total protein content in the ECP of *Aeromonas hydrophila* was estimated by the Folin-Ciocalteau phenol method of Lowry *et al.*, (1951).

Protein Profile of ECP.

The protein profile of bacterial ECP was studied by SDS-PAGE as per the methods of Laemmeli (1970).

Standardisation

The shrimps reared were exposed to minimum concentration of the ECP for standardising the experiment. The ECP was injected between the abdominal segments on the ventral side of the animal.

Study of mortality pattern

The levels of administration of ECP was divided into five concentrations according to the protein content in the ECP. Group I - Control, II - 0.5 µg ECP/ml, III - 1 µg ECP/ml, IV - 1.5µg ECP/ml, V - 2 µg ECP/ml, VI - 2.5µg ECP/ml.

Haemolymph study

The haemolymph was assayed as per

the method of Nakayama *et al.* (1997). The total haemocytes of *P. indicus* of the six groups reared in triplicate was determined. Sampling of the animals were done from each group at 24 hours, 48 hours and 72 hours respectively. Haemolymph was taken from the heart with a heparinised plastic syringe. The haemoilymph was extracted along with filtered May-Gruenwald's - Giemsa stain and taken on the haemocytometer. The haemocytes were counted under the light microscope after 20 min observation.

Results

The shrimps were injected with different concentrations of *Aeromonas hydrophila* ECP to study the haemolymph response. Behavioural changes included abnormal swimming and loss of appetite. The control animals were injected with normal saline. In control animals there was neither abnormal swimming behaviour nor loss of appetite. In Group II, that were administered with 0.5µg ECP, the animals showed normal behaviour but consumed feed at a reduced rate (1% of body weight). In Group III (1µg) and Group IV (1.5µg) the shrimps consumed less feed and showed erosions on the appendages and telson. In Group V (2µg) and Group VI (2.5µg) that received highest concentration of ECP, complete loss of appetite was observed. Melanisation and erosion was noted in Group IV and Group V animals (Fig 1). In Group VI (2.5µg) soft shelling was observed in the moribund animals.

The percentage of mortality was assayed after the administration of ECP in different groups. Group II (0.5µg) and

Fig. 1.

Group III (1µg) animals showed 50% mortality in 24 hours. 80% mortality was observed in Group IV animals. Group V (2µg) showed 50% mortality in 24 hours and remaining died in about 72 hours. High mortality was observed in Group VI with complete death in 48 hours.

The protein profile of ECP of *A. hydrophila* revealed three bands in lane l, lane 2 showed 15 bands and lane 3 revealed 5 bands (Fig. 2)

For haemotological study, the animals were sampled at 24 hours, 48 hours and 72 hours. In Group I normal saline was injected. The total haemocyte count ranged

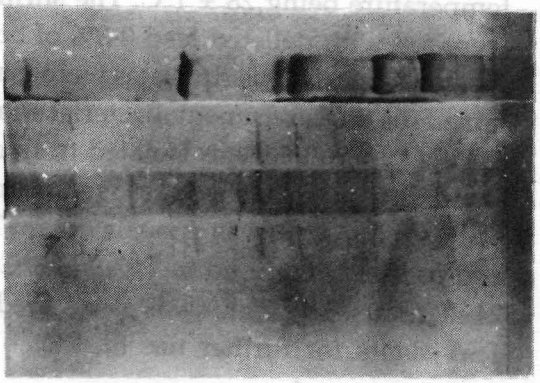

Fig. 2.

from 206 x 10^4 cells/ml to 300 x 10^4 cells/ml. In Group II (0.5µg) there was gradual reduction in the number of haemocytes from 24 to 72 hours of observation. The mean value was 217 x 10^4 cells/ml in 24 hours, 173 x 10^4 cells/ml in 48 hours and 155 x 10^4 cells/ml in 72 hours respectively. In Group III (1µg) the haemocyte counts varied from 177 x 10^4 cells/ml to 120 x 10^4 cells/ml. The mean value in the group was 164 x 10^4 cells/ml in 24 hours, 150 x 10^4 cells/ml in 48 hours and 124 x 10^4 cells/ml in 72 hours. In Group IV (1.5 µg) the total haemocyte count ranged from 146 x 10^4 cells/ml to 56 x 10^4 cells/ml. The mean value was 135 x 10^4 cells/ml in 24 hours, 123 x 10^4 cells/ml in 48 hours and 77 x 10^4 cells/ml in 72 hours. In Group V (2µg) the total haemocyte count ranged from 109 x 10^4 cells/ml to 125 x 10^4 cells/ml. The mean count was 117 x 10^4 cells/ml. In Group VI, the haemocyte count ranged from 80 x 10^4 cells/ml to 105 x 10^4 cells/ml. The mean count was 97 x 10^4 cells/ml. Most of the shrimps in Group V and Group VI died within 24 hours. The results in the different groups indicated that there is a time and concentration dependent reduction in the haemocyte number of toxin treated groups. Analysis of variance showed significant difference in the total haemocyte count number of treatment groups when compared to the control.

Table 1 . *Study of mortality pattern in different groups at different time intervals*

Groups	24 hours	48 hours	72 hours
GI	1	-	-
GII	3	-	-
G III	3	-	-
G IV	1	3	1
G V	3	2	1
G VI	5	1	-

Discussion

The approach of this study was to elucidate the morphological, behavioural and haemotological alterations in *P. indicus* at different concentrations of extracellular products of *A. hydrophila*. The occurrence of *Aeromonas sp* has been observed by Lakshamanaperumalsamy *et al.*, (1982) who isolated a few *Aeromonas* strains from the blackened lesions of *P. indicus* caught from Cochin backwaters. *Aeromonas* sp have also been reported by several workers in penaeid species (Yasuda and Kiyao, 1980; Owens *et al.*, 1992; Chang *et al.*, 1996). In our conditions a drop in salinity is a common feature during monsoon that favours the occurrence of *A. hydrophila.*, which is mostly found in freshwater habitat. However, the pathogenecity studies regarding their effects on shrimps were limited, hence the present study was taken up.

Table 2. *Mean haemocyte count in the control and experimental groups at different intervals*

Groups	24 Hours	48 Hours	72 Hours
GI	300 x10^4	-	-
G II	217 x 10^4	173 x 10^4	155 x 10^4
GIII	164 x 10^4	150 x 10^4	124 x 10^4
G IV	135 x 10^4	123 x 10^4	77 x 10^4
G V	117 x 10^4	-	-
G VI	97 x 10^4	-	-

The protein profile of ECP of *hydrophila* was analysed by SDS-PAGE. Thune *et al.*, (1982) reported three extracellular proteases which had molecular weights of 56 KDa, 34-35 KDa and 19.5 KDa respectively. In the present study, bands corresponding to 50-55 KDa, 32-36 KDa and 29-14.3 KDa were seen in three different lanes. Since we have not confirmed the protease activity of these bands, further studies are necessary in this direction.

An attempt was made in the present study to describe the virulent factors involved in the *Aeromonas* infection of *P. indicus*. The ECP of *A. hydrophila* was injected into the shrimps at various doses. The changes observed were abnormal swimming behavior and loss of appetite. Sis *et al.*, (1980) had reported similar changes in *Vibrio* infections of penaeid prawns. Our studies also indicate that the extracellular products of *Aeromonas* are responsible for the behavioural changes observed in *P. indicus*. The gross pathological changes in the present study was melanisation and erosion on appendages, exoskeleton and telson in Group IV and Group V. Melanin has a bacteriostatic, clotting and localising function and melanin spots were observed in areas where injury to skeleton has occurred (Rosen, 1970; Unestam and Weiss, 1970). Probably, they may be the reason for presence of melanin in Group IV and Group VI shrimps. In Group VI (2.5 µg) the treated shrimps showed soft shelling phenomenon. *A. hydrophila* had been isolated from moribund *P. indicus* showing symptoms of soft shell syndrome (Anon, 1992., Baticadas *et al.*, 1986). The behavioural changes were in agreement with the observation of Remesh (1988) in soft shelled prawns. The role of *A. hydrophila* toxin in the pathogenesis of soft shell syndrome which is a common disease problem need to be investigated.

The mortality pattern was observed after the administration of *Aeromonas hydrophila*. The maximum mortality was observed in Group VI that received the highest concentration of ECP (2.5µg). The mortality pattern was in proportion to the doses of ECP administered to the different groups of shrimps. Leong and Hanrahan (1980) reported high mortality when ECP of *Vibrio parahaemolyticus* and *V. alginolyticus* were injected into penaeid shrimps. Probably the mortality observed in the experiment may be due to the exotoxins present in the ECP of *Aeromonas hydrophila*.

The haemocyte studies with regard to diseases in crustaceans are limited though the haemocytes are the major effector cells in the crustacean defence mechanism. In Gaffkemia of lobsters, the haemocytes were reported to undergo reduction in number (Stewart and Rabin, 1970). ECP was reported to contain lytic factors which may be responsible for the reduction of haemocytes (Bernheimer *et al.*, 1974). Though this is a first report about the reduction of haemocytes in a disease condition, further studies are required to draw a conclusion about haemocyte dynamics.

In the present investigation, it was shown that the pathogenic effect of

A. hydrophila was probably due to the production of exotoxins by the virulent bacterial strain.

References

Anon, 1992. Disease investigation and disease control in culture system. *CMFRI Annual report. pp. 37-38*

Baticadas, M. C. L., R. M. Coloso., R. C. Duremdez. 1986. Studies on the chronic soft shell syndrome in the tiger prawn, *Penaeus monodon* Fabricus, from brackish water ponds, *Aquaculture,* **56**(3-4) : *271-285.*

Bernheimer, A. W., and L. S. Avigad. 1974. Partial purification of Aerolysin, a lytic exotoxin from *Aeromonas hydrophila. Infect. Immun.,* **9** : 1016-1021.

Chang, Chan-I, H. Chung., G. Kou. 1996. Numerical taxonomy of bacteria isolated from the hepatopancreas of giant tiger prawn, *Penaeus monodon;* fleshy prawn, *P. chinensis* and their culture water. *Journal of the fisheries Society of Taiwan,* **23**(2) : 117-136.

Fuller, D. W., K. S. Pilcher and J. I. Fryer. 1977. A leukocytolitic factor isolated from cultures of *Aeromonas salmonicida. J. Fish. Res. Board Can,* **3** : 1118-1125.

Huang, Mei-Ying and Liao, I. Chiu. 1994. A study on microflora found in grass prawn culture tanks at different salinities in a circulating system. *J. Taiwan Fish Res,* **2**(2) : 31-40.

Lakshamanaperumalsamy, P., I. Bright Singh., I. Thomas., M. Chandramohan. 1982. Brownspot disease in *Penaeus indicus. Symp. Dis. finfish and Shellfish, 1-3 March 1982, Univ. Agri. Sci., College of Fish., Mangalore, India (Abstract)*

Laemmeli, Ű. K. 1970. Cleavage of structural protein during the assembly of the head of the bacteriophage T4. *Nature,* **227** : *680-685.*

Lewis, D. H., T. K. Leung and C. Mock. 1982. Aggregation of Penaeid shrimp larvae due to microbial epibionts. *Aquaculture,* **27**(2) : 149-154

Leong, J. K and D. S. Hanrhan. 1980. Effects of vibrio toxins on survival and haemolymph coagulation in white shrimp. In. *Aquaculture* : Public Health regulatory and Management Aspects. The sixth FDA Symposium. Texas A & M. University Press.

Lightner, D. V. 1977. Shrimp Disease. In : Sindermann, C. J., (Ed.) *Disease diagnosis and control in North American Marine Aquaculture,* **6** : *10-21, Elsevier, New York.*

---- 1983. Diseases of Penaeid Shrimp. In : Sindermann, C. J. and Lightner, D. V., (Eds.) *Disease diagnosis and control in North American Marine Aquaculture II Edition, Elsevier, Amsterdam, pp. 18*

Lowry, O. H., N. I. Rosebrough., A. L. Farr., R. J. Randall. 1951. Protein measurement with Folin-Phenol reagent. *J. Biol. Chem.* **193** : *265-275.*

MacIntyre, S and J. T. Buckley 1978. Presence of glycerophospholipid cholesterol acyltrans-ferase and phospholipase in culture supernatant of *Aeromonas hydrophila J. Bacteriol.,* **135** : *402-407.*

Nakayama, K., A. M. Nomoto., M. Nizhijima., T. Maruyama. 1997. Morphological and functional characterisation of haemocytes in the Giant clam, *Tridacna crocea. Journal of Invertebrate Pathology.,* **69**(2), pp : 105-111.

Nieto, T. P. and A. E. Ellis (1986). Characterisation of extracellular metallo and serine proteases of *Aeromonas hydrophila. Journal of General Microbiology,* **132** : 1975-1979.

Owens, L., P. Muir., D. Sutton., and M. Wingfield. 1992. The pathology of microbial diseases in Tropical Australian Crustacea. Diseases in Asian Aquaculture. I *Proceedings of The First Symposium on Diseases In Asian Aquaculture, 26-29 Nov 1990 Bali Indonesia. Fish Health Section, Asian Fisheries Society, Manila* : **165**-172.

Remesh, P. R. 1988. Histopathological studies of soft prawns. *Dissertation sumitted in partial fulfillment of M. Sc programme.* Cochin University of Science and Technology. pp : 25-26.

Rosen, B. 1970. Shell disease of aquatic crustaceans. In : Sneisko, S. F., (Ed.). *A. Symposium on Diseases of fishes and shellfishes. Spec. Publ. 5, American Fisheries Society, Washington D. C., pp 409.*

Sindermann, C. J. 1971. Diseases caused mortalites in Mariculture, status and predictions. *Proc. World. Maricult. Soc., 2 : 69-74.*

Singh, I., I. Lakshmanaperumalsamy and D. Chandramohan. 1985. Heterotrophic bacteria associated with eggs and larvae of *Penaeus indicus* in a hatchery system. *Proceedings of the First Intl. conference on the culture of Penaeid Prawns/shrimps, Iloilo City, Philippines,* 4-7 Dec, 1984, 1985 p. 169. (Eds) Taki, Y., J. H. Primavera, J. A Llobera.

Sis, R. F., J. Neff., V. L. Jacobs., H. Armstrong., C. C. Corkern., R R. Tarpley and G. G. Stott. 1980. Normal historlogy and histopathology of benthic invertebrate and Demersal platform associated pelagic fishes. pp. 393 - 527. In : *C. A. Bedinger (ed). Ecological investigations of petroleum production platforms in the Central Gulf of Mexico, Vol 1 part 5., New Orleans.*

Stewart, J. E., and H. Rabin. 1970. Gaffkemia, a bacterial disease of lobsters (Genus *Homarus*) In : *A Symp. Dis. Fishes and Shellfishs. S. F. Sneiszko (ed.) Spec. publ. No. 5, 431-439. Am. Fish soc. Washington, D. C.*

Thune, R. L. T. E. Graham., L. M. Riddle and R. L. Amborski. (1982). Extracellular proteases from *Aeromonas hydrophila* : partial purification and effects age - O channel catfish. *Transactions of the American Fisheries Society . 111, 749-754.*

Unestam, T and D. W. Weiss. 1970. The host parasite relationship between freshwater crayfish and the crayfish disease Fungus, *Aphanomyces astaci* : Responses to infectivity by a susceptible and a resistant speicies. *J. Gen. Microbiol., 60 : 77-90.*

Wadstorm, T., A. Ljungh and B. Wretlind. 1976. Enterotoxin, Haemolysin and Cytotoxic protein in *Aeromonas hydrophila* from Human infections. *Acta Pathol. Microbiol. Scand. Sect. B, 84 : 112-114.*

Yasuda, K and T. Kiyao. 1980. Bacterial flora in the digestive tract of prawns, *Penaeus japonicus* Bate. *Aquaculture,* 193(3) : 229-234.