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Immune responses of *Paratelphusa jacquemontii* (Rathbun) against artificial infection of *Staphylococcus aureus* and *Vibrio parahaemolyticus*

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Abstract

The edible crab *Paratelphusa jacquemontii* (Rathbun) supports as an important fishery in fresh water sources in many Indian states including Tamilnadu. The present study is on immune responses of the crab which were collected from the rice fields and irrigation canals of Orathanadu, Pattukotai District, Tamilnadu. Though diseases are natural component of crustaceans and mortalities observed in few outbreaks, their immune responses are largely unknown. As a well developed immune system are not reported in shellfishes and as immunological memory is lacking the mechanisms behind, their defense against invading pathogens to be studied in different species as a common mechanism in these organisms cannot be expected. Hence the present study on *P. jacquemontii* (Rathbun). The present attempt included evaluation of certain immune responses like enhanced total hemocyte count, phenol oxidase activity, agglutination activity against various human and fish pathogens which revealed the presence of various defense mechanisms.

Keywords: immune responses, *Paratelphusa jacquemontii*, *Staphylococcus aureus* and *Vibrio parahaemolyticus*

Introduction

Crustacean aquaculture represents a major industry in tropical developing countries. As a result of high culture densities and increasing extension of aquaculture farms, the presence of diseases has also increased, inducing economic losses. Invertebrates, which lack adaptive immune systems, have developed defense systems that respond against antigens on the surface of potential pathogens. The defense mechanisms of crustaceans depend completely on the innate immune system that is activated when pathogen-associated molecular patterns are recognized by soluble or by cell surface host proteins, such as lectins, antimicrobial, clotting, and pattern recognition proteins, etc., which, in turn, activate cellular or humoral effector mechanisms to destroy invading pathogens.

In invertebrates, there is little conclusive evidence to confirm whether any of these factors are antigen-specific. However, some cellular and cell-free hemolymph factors show high specificity for non-self or damaged cells, similarly as demonstrated for antibodies (Vasta *et al*., 1999). Crustaceans possess several defense mechanisms that become activated depending on the pathogen's characteristics. When foreign material is recognized, different effector

mechanisms are activated, such as the prophenoloxidase (proPO) system, phagocytosis, and encapsulation.

Based on the aforementioned, it can be implied that crustaceans possess efficient defense mechanisms in spite of lacking immunological memory and antibodies, which are found in vertebrates (Vazquez *et al*., 2009). It has been considered that certain physiological factors are importantly involved in the immunity of these species. However mechanisms against pathogens seem to be different for different crustacean species. Hence the mechanism should be studied in individual species. Hence the present study on *Paratelphusa jacquemontii*.

Materials and Methods

Experimental animals and laboratory maintenance

Freshwater adult male crabs, with a body weight of 30±3 g (carapace width of 34±3 mm) were collected from the rice fields and irrigation canals around Oratha Nadu, Pattukottai Dist, Tamilnadu, India. Animals were housed 4-6 per plastic tank (length/width/ height=30:40:30 cm) with soil and fresh water and transferred to fresh medium daily. They were acclimatized to the laboratory conditions (Temperature - 27±1°C and relative humidity - 75%) for 7 days. Crabs were fed with goat meat throughout the period of maintenance in the laboratory. Only male crabs were used in the present study.

Bacterial challenge/Injection of bacteria

Each group (contains 3 Nos.) of crabs were injected with 100 µl of a suspension containing about 10^6 CFU/ml bacteria (*Staph.aureus* and *V.parahaemolyticus*) in phosphate buffered saline (PBS). Control crabs (3 Nos.) received 100 µl of PBS only. Injections were made inserting the needle of 1-ml sterile syringe into the third walking leg. After injection, control and injected animals were immediately transferred to three different plastic tanks with fresh water and soil.
Note: *Staphylococcus aureus* and *V. parahaemolyticus* were isolated from infected crabs.

Bacterial density estimation

After inoculation of pathogens on various time periods after (1hr, 1day and 10days) haemolymph from challenged animals were collected at the rate of 100µl and density of *S. aureus* and *V.parahaemolyticus* were estimated using respectively Baird- Parker (Hi-crome) medium (g/L) (Casein enzymic hydrolysate -12.0g; Pancreatic digest of gelatin - 3.0g; Beef extract - 6.0g; Yeast extract- 5.0g; Sodium pyruvate -10.0g; Lithium choride -5.0g; Chromogenic mixture- 2.1g; Agar-20.0g; pH- 7± 0.2) and TCBS agar medium (g/L) (Peptone - 10g; Yeast extract -5g; Sodium thiosulphate-10g; Sodium citrate -10g;Oxgall - 8g; Sucrose -20g;Sodium chloride -10g; Ferric citrate -1g; Bromothymol blue- 0.04g; Thymol blue -0.04 Agar - 15g; Distilled water- 1000ml) adopting spread plate technique. Density was calculated and expressed as CFU/ml.
Note: *S. aureus* develops brown black colonies with a clear zone around in Baird- Parker medium.

Preparation of serum

Hemolymph samples of 0.2 to 0.5ml were collected from the walking leg of the crabs adapting aseptic procedure. The serum and hemocytes were prepared according to the methods described by Chen *et al.*, 2010. The serum was prepared from hemolymph by centrifugation which separated hemocytes and care was taken to use serum for challenging experiments without any delay.

Evaluation of Immune response

Immune responses of crab towards bacterial challenges were evaluated using total hemocyte count, prophenol oxidase estimation, agglutination assay, etc., Control values were compared with the values obtained from challenged animals.

Total Hemocyte Count

Appropriately diluted hemolymph samples were used to count the total hemocytes using Neubauer hemocytometer, which was used under Olympus compound microscope under 40x magnifications.

Phenoloxidase (Po) Activity

PO activity was assayed as described by Sung *et al*., (1994) using L-3, 4-dihydroxyphenyl-alanine (L-dopa) (Sigma) as a substrate. To obtain lysates, frozen haemocytes were thawed and frozen several times. Haemocyte lysate supernatants (HLS) and plasma were assayed for PO activity using a spectrophotometer (Hitachi U-2000) to measure the OD at 490 nm. The concentration of total serum protein was determined by the Lowry *et al*., (1951) using bovine serum albumin (Bio-Rad Protein assay

Kit II) as a standard. One unit of PO activity was defined as an increase in absorbance of 0·001 min/mg/protein. Control and challenged haemolymph were used for the study.

Agglutination activity of haemolymph

Agglutination activity of serum obtained from control as well as *Staphylococcus aureus, Vibrio parahaemolyticus* challenged crabs were done to assess the immune response. Human pathogens like *Staphylococcus aureus, Vibrio cholerae, Salmonella paratyphi, Klebsiella pneumoniae, Salmonella typhi, Proteus mirabilis, Klebsiella oxytoca, E.coli* and *Pseudomonas aeruginosa* were used. The pathogenic strains were obtained from CMC, Vellore. Fish pathogens such as *V.harveyi, V.parahaemolyticus, V.vulnificus, V.anguillarum, V.mimicus, V.alginolyticus, A.hydrophila* and *Yersinia ruckeri* were obtained from CIFE (Mumbai) and Fisheries collage (Tuticorin). The titter values of control and challenged serum were compared.

Results and Discussion

In control crabs during the period of observation the total hemocyte count was found to in the range of $1.8x10^7$ to $2.1x10^7$ /ml. In the control (saline alone injected) the observed THC was $1.9x10^7$, $1.8x10^7$ and $2.1x10^7$ /ml at 1hr, 1day and 10^{th} day respectively. When *S.aureus* was injected maximum reduction to the level of $1.8x10^4$/ml was observed in the 1^{st} 1hr period, which was gradually increased to reach the maximum on the 1day ($2.0x10^4$/ml) and $8.6x10^6$/ml on 10^{th} day. Compared to *S.aureus* the injection of *V.parahaemolyticus* resulted in more reduction in hemocyte count of $2.0x10^3$/ml 1^{st} 1hr period, which increased at a faster rate reaching maximum at the 1day ($4.0x10^3$/ml) and $2.24x10^6$/ml on 10^{th} day. Though the trend seems to be the same the changes were more with *V.parahaemolyticus* strain. However the reduction in the 1^{st} 1hr period observed in the both the pathogens might be due to the aggregation process. The vertebrate coagulation system acts as a 1^{st} line of defense mechanism at wound site. The haemocyte segregation resulted in removal of haemocytes in circulation for a short period. This reaction is important as it indicated the response of recognition of self and non-self foreign particles (Figs. 1-2).

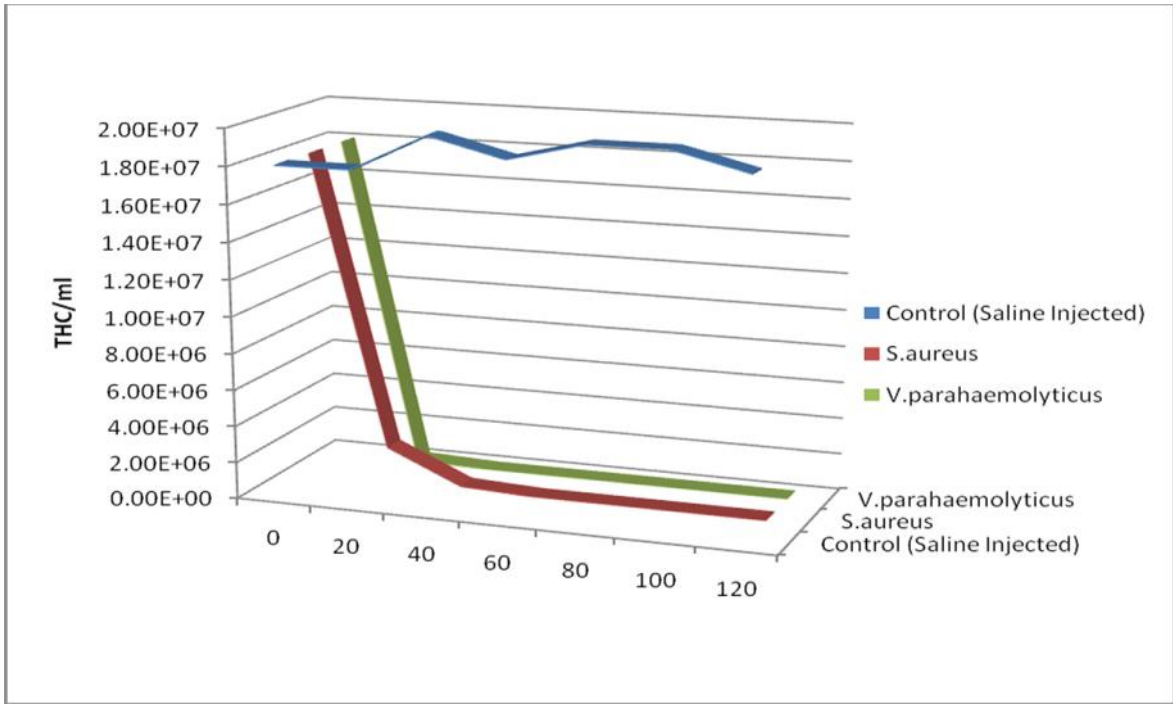

Fig.1: THC up to 1hr

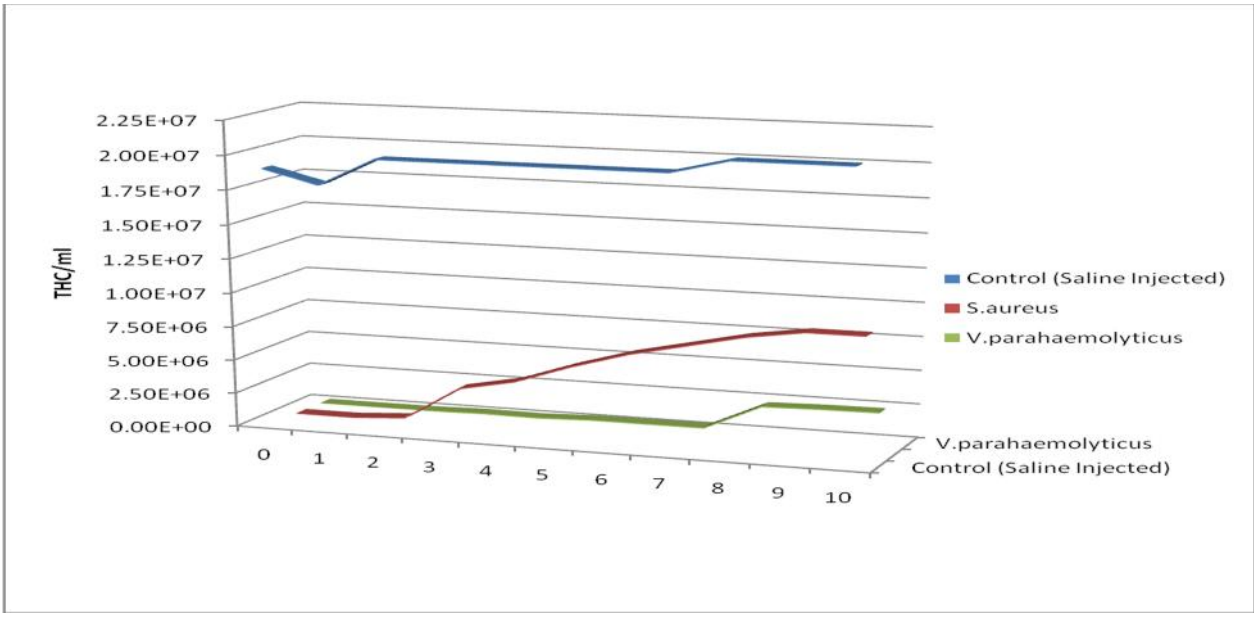

Fig.2: THC up to 10days

The circulating haemocyte number is a stress indicator (Le Moullac *et al.*, 2000) and haemocyte counts may be a valuable tool in monitoring the health status of crustacean species (Mix *et al.*, 1980). The increased hemocyte number indicated the response to the entry of pathogens.

The formation of haemocyte aggregations can be seen histologically in arteries and haemal spaces of various tissues, including the heart; as the infection progresses, nodules can be seen in gills, heart, antennal gland, and other organs (Johnson 1976). Apparently healthy crabs may exhibit a haemocytic response seen histologically which may be the result of recovery from a previous light bacterial infections**.**

Bacterial density

Regarding bacterial density in control crabs no *V.parahaemolyticus* was found in haemolymph. But *S.aureus* was found at the range 0 to 1.0X 10^1 CFU/ml. When inoculated with *S.aureus* containing 1.0x10^6 CFU/ml, the pathogen in haemolymph of crab was found to be 7.5x10^3 CFU/ml at 1hr. After 1day the density was 6.4x10^5 CFU/ml and it was 3.2x10^3CFU/ml at the end of 10^{th} days. Regarding *V.parahaemolyticus* cells were inoculated at the range of 1.0x10^6 CFU/ml. At the end of 1hr, 1day and 10^{th} day the observed bacterial densities were 1.28x10^4 CFU/ml, 9.3x10^3 CFU/ml and 7.6x10^3 CFU/ml respectively (Fig. 3).

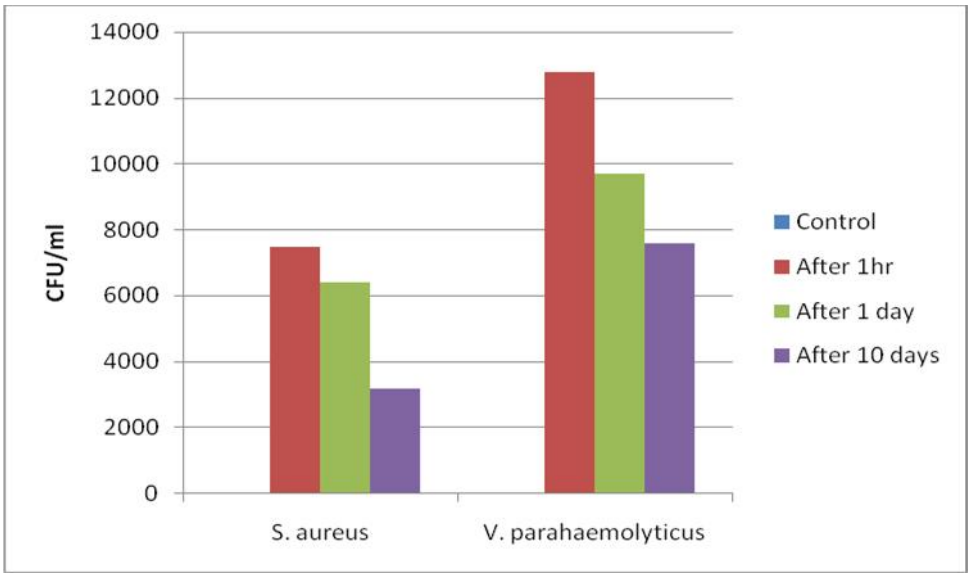

Fig. 3: Bacterial density

Estimation of prophenol oxidase

Prophenol oxidase in control crabs were in the range of 0.8 - 1.0 units/ mg/min. In *S. aureus* injected crabs it was increased to 1.38 -1.78 units/mg/min with in 1 hr. Where as in *V.parahaemolyticus* infected crabs, the range was 1.6 -1.77 units/mg/min. This enzyme level was maintained for 10days and it was slowly decreased afterwards. At the end of 10 days time it was found to be 0.82units/mg/min and 1.52 units/mg/min respectively in *S. aureus* and *V.parahaemolyticus* injected crabs. In crab survived after 10 days the level was comparable to that of control crabs. The entry of pathogens resulted in increased in prophenol oxidase activity.

The enhanced activity of prophenol oxidase is important as when trigged it was reported to generate immunologically activity substances such as opsonins, cytotoxic molecules (Zhang *et al.*, 2003).

ProPO is naturally activated by bacterial and fungal cell wall components (Söderhäll and Cerenius, 1998). Quantification of PO/ProPO activity offers the potential for assaying 'health', as increased activity correlates with higher levels of pathogen resistance in several study systems (*Tenebrio molitor* (Barnes and Siva- Jothy, 2000; Armitage and Siva-Jothy, 2005), *Drosophila* (Braun *et al.*, 1998), *Glossina* sp. (Nigam *et al*., 1997) and *Manduca sexta* (Eleftherianos *et al*., 2006).

Fagutao *et al*., 2009 showed that knocking down the activity of inactive precursor of phenoloxidase by RNA interference in a significant increase in the bacterial load of kuruma shrimp *Marsupenaeus japonicas* even in the absence of a bacterial or viral challenge. Silencing of proPO also let to a sharp increase in shrimp mortality.

Activation of proPO in insects by a cascade of proteolytic cleavages results in the generation of a phenoloxidase (PO). PO oxidizes phenolic com-pounds to produce quinones that polymerize to form melanin (González-Santoyo and Córdoba-Aguilar, 2011). Insects used melanin to encapsulate non- self organisms and seal wounds. The same thing might be have in caustaced also.

The ProPOAS is a series of complex interactions between enzymes and their zymogens, leading to the production the enzyme phenoloxidase (PO; monophenol, L-dopa:oxygen oxidoreductase, EC 1.14.18.1). PO activity ultimately results in the formation of melanin, which is subsequently involved in cuticle sclerotisation, wound healing and cellular defence responses (Cerenius *et al.*, 2008). Due to the cytotoxic nature of the by-products of this process, PO is commonly stored as its inactive precursor prophenoloxidase (ProPO), and activated as part of the cascade following the recognition of foreign compounds (Söderhäll and Cerenius, 1998).

Agglutination

The serum obtained from control samples of *P.jacquemontii* showed lower agglutination titter values against both human and fish pathogens tested. However, varying results were obtained with the serum of *S.aureus* and *V.parahaemolyticus* challenged crabs.

Regarding control serum the titter values against human pathogens were in the range of 2 to 16 were the minimum was observed against *P. aeruginosa* where as the maximum was against *S.aureus* and *S.typhi*. The serum obtained from *S. aureus* challenged animals the agglutination activity was considerably increased and it was in the range of 8 to 32. Similarly the *V.parahaemolyticus* challenged serum also showed enhanced titter values i.e 8 to 64. Likewise same trend was observed with fish pathogens also (Tables 1and 2). The study clearly indicated the enhanced agglutination activity through exposure to selected pathogens which were artificially inoculated into the crab *P.jacquemontii*. In conclusion, the present study confirmed the presence of various innate immune responses and humoral defenses to fight against the pathogens.

Table 1: Agglutination activity of serum against human pathogens

Pathogens tested	Control	Against *Staphylococcus* challenged serum	Against *Vibrio* challenged serum
Vibrio cholerae	8	16	64
Proteus mirabilis	4	8	8
Pseudomonas aeruginosa	2	16	32
Escherichia coli	8	16	16
Staphylococcus aureus	16	32	32
Labtobscillus bulgaricus	8	16	32
Klebsiella oxytoca	8	8	8
Klebsiella pneumoniae	8	16	16
Salmonella typhi	16	32	32
Salmonella paratyphi	8	8	32

Table 2: Agglutination activity of serum against fish pathogens

Pathogens tested	Control	Against *Staphylococcus* challenged serum	Against *Vibrio* challenged serum
Vibrio vulnificus	8	8	16
V. parahaemolyticus	8	16	64
V. mimicus	2	16	32
V. alginolyticus	4	8	8
V. harveyi	16	16	32
V. anguillarum	4	4	64
A.hydrophila	16	16	32
Yersinia ruckeri	4	4	8

Coagulation and inflammation are the interdependent process attributed to the host defense responses to injuries. Crosstalk between coagulation and inflammation is considered to inherit from primitive coagulation systems to invertebrates.

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