Research Article

Antibacterial activity of the hemolymph of *Uca triangularis* and its bacterial load

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Abstract

Background: Crustacean is very important phylum, whose commercial outputs have not been understood clearly. In the present study the innate immune response among the male and female species of *Uca triangularis* was studied. **Objective:** To understand the immune response generated in *Uca triangularis* and to check if there is any difference in immune response among the male and female speciemen. **Material and methods:** Four different types of bacteria were isolated and identified from the hemolymph of both male and female healthy *Uca triangular is,* collected from the coast of Marina beach, Chennai. The bacteria were identified as *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus vulgaris* based upon their biochemical characterization and their growth pattern upon the selective media. The enzymatic biomarkers like glutathione-S transferase, glutathione reductase, super oxidase dismutase and non-enzymatic stress parameter such as reduced glutathione were estimated. The total hemocyte cell count in both male and female sample were estimated. Further the crude hemolymph preparation was subjected to the analyses of antibacterial activity by well diffusion technique. **Results and conclusion:** The maximum sensitivity pattern was observed against *Staphylococcus aureus* (24mm ± 0.0025- male hemolymph and 21mm ± 0.0037- female hemolymph) and the least inhibition was observed for *Proteus vulgaricus* (9mm ± 0.0015- male hemolymph and 5mm ± 0.0017- female hemolymph) was observed. It is evident from the present study that *Uca triangularis* harbors bacteria as commensal and its hemolymph contains antibacterial peptides, which prevents the bacteria becoming pathogen to the animal and the male hemolymph generates maximum inhibitory activity than female sample.

***Keywords*:** *Uca triangularis*, bacteria, hemolymph, antibacterial activity

Introduction

Crustaceans are the largest habitants of the both freshwater and marine habitats. Crustaceans are good examples to study the immune responses, as they survive in variety of habitats (Smith and Chisholm, 1992; Soderhall and Cerenius, 1992; Swetha et al., 2015). There are increased number of research and their respective publications pertaining to immune responses of other animals and not on Crustaceans (Lorena et al., 2009). In these primitive animals the immune responses are innate and are well developed (Chirs, 2012; Bernard and Sekhar, 2015). The brachyuran Crabs, like *Uca triangularis* plays an important role in the balancing of coastal ecosystem, specially the mangrove ecosystem (Subhasish and Susanta, 2014). *Uca triangularis* is one the important brachyuran crabs, and quite often found in the Eastern coastal area. There are reports related to the feeding, behavioral and toxicologically studies, but there are no reports available in regarding with the *Uca triangularis* immune responses.

The respiratory burst due to NADPH-oxidase is one of the most vital characteristic feature in invertebrate phagocytic immune responses (Arumugam et al., 2000; Lee and Söderhäll, 2002). These mode of immune responses are highly dependent on production of superoxide molecules. According to Singaram et al. (2011), ROS production was linked with generation of antioxidant responses also. Thus due to the presence of bacteria in the hemolymph, immune

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responses are generated, to combat these bacterial infection and also the antioxidants like enzymes such as Glutathione-S-transferases (GSTs) (Zhao et al., 2010). Glutathione reductase, reduces to Glutathione (Singaram et al., 2013). Thus animal does not face any deleterious effects. There increases its survival status in natural or captivity breeding.

Materials and methods

Collection of hemolymph from *Uca triangularis*

Uca triangularis was collected from Marina coast, Tamil Nadu; India. In this present study both male and female specimens of approximate weight of 5 g was used. The animals were segregated into two groups; Group-A male animals and group-B female animals and triplicates of each group was maintained. The surface of the animal was wiped with sterile pad on all sides completely. The hemolymph from both the group-A and group-B were collected from the third walking leg (Sumalatha et al., 2018) and was immediately treated with 1 unit of heparin as the anticoagulant.

Estimation of total protein

The total protein in the hemolymph of the both groups were estimated by Lowry et al. (1951). The Bovine serum albumin (1mg/ml), was used as the standard.

Estimation of biomarker enzymes

(a.) Glutathione S Transferase

Glutathione-S-transferase was assayed by the method of Habig et al. (1974) with slight modification.

$$GST = \frac{\Delta \text{ O. D/min x Vol. of assay x } 100}{9.6 \text{ x Vol. of conjugate enzyme x protein (mg)}}$$

(b.) Glutathione Reductase

The GR activity was measured by spectrophotometric method described by Staal et al. (1969) with slight modifications.

$$GR = \frac{\Delta \text{ O. D x Vol. of assay x } 1000}{6.22 \text{ x Vol. of enzyme x protein (mg)}}$$

(c.) Superoxide Dismutase

The enzyme was estimated by the method of Marklund and Marklund (1974).

$$SOD = \frac{\Delta \text{ O. D sample x O. D blank x } 100}{\Delta \text{ O. D sample x } 50 \text{ x Vol. of sample mg protein}}$$

(d.) Reduced Glutathione

Total reduced glutathione was determined by the method of Moron et al. (1979) with slight modifications.

$$GSH = \frac{\Delta \text{ O. D/min x Vol. of assay x } 100}{1.36 \text{ of mole GSH conjugate/gm tissue}}$$

Estimation of Hemocyte count

1ml of hemolymph was diluted with 4ml of 1M PBS (p H-7.2 ±0.2). 50µl of each of the hemolymph samples was taken in a sterile microfuge tube and 10 µl of methylene blue stain was added and 10 µl of the suspended sample was placed over the hemocytometer and were counted (Mats et al., 2000; Parrinello et al., 2015; Nithya et al., 2017).

Culture of bacteria from the hemolymph

1ml of hemolymph from groups-A & B were transferred to 9ml of nutrient broth and were incubated in room temperature for 24 hrs (Rivera et al., 1999). After the incubation, 100 µl of the broth sample was plated onto the plate count agar and the bacterial colony forming units were recorded. The individual bacterial colonies were subjected to various biochemical characterization like the Gram's staining, hanging drop technique to determine the motility, catalase, oxidase, indole, methyl red, VP tests, culture on MacConkey agar, Blood agar, TSI medium, Urease detection medium, Nitrate reductase test, Citrate utilization test, oxidate- fermentative test for glucose by standard protocol as per Bergey's manual of systematic bacteriology (2005).

Antibacterial activity

The hemolymph of both groups was treated with heparin and was diluted (1:5) with sterile PBS (p H 7.2 ±0.2) and was then subjected to syringe sterilization, to remove the hemocytes and the other contaminants. This was used as the sample for antibacterial activity. The antibacterial activity was performed by the well diffusion method and the bacteria which were previously isolated from the hemolymph were lawn cultured on the Muller Hinton agar and the wells were punched to add the sterile hemolymph. The antibacterial activity was determined after 24 hrs. of incubation at room temperature (Edward et al., 1994). The zone of sensitivity was measured using the standard antibiotic scale (HiMedia) and were tabulated.

Statistical analysis

The data obtained in the present investigation were statistically analyzed and expressed as mean, SEM (Standard Error of Mean). The value of probability was obtained from degree of freedom by using the standard table. The following levels of significance were used $P<0.001$ and $P<0.05$ for significant data and $P>0.05$ for insignificant data. The level of significance (P-value) was calculated for these groups using two tailed paired T-test using the online tool Graphpad-Quickcalcs.

Results

The heparin treated hemolymph was collected from the

both the groups and stored at refrigerator (20°C) until further use. Immediately the total protein was estimated from the collected hemolymph. It is evident that in both the groups the protein concentration in male as 6.57 ± 0.93 and in female as 6.04 ± 0.34 and was found to be similar. The oxidative stress markers such as glutathione-S Transferase, Glutathione reductase, superoxide dismutase and reduced glutathione were estimated in both the groups. In male samples the oxidative stress markers were found to be slightly increased than the female samples (Table 1). The total hemocytes from the crab samples were counted using hemocytometer and in male the cells were found to be higher than the female (Table 2).

The bacterial cell count was determined as the colony forming units (CFU) on the plate count agar and tabulated (Table.2). The bacterial colony counts were found to be higher in case of female than male samples. The biochemical results for the bacterial identification in this present study was performed by comparing the results obtained with the standard growth patterns of bacteria as described in Bergey's manual of systematic bacteriology (2005), (Tables.3a& b). From the hemolymph of both male and female samples *E. coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus vulgaris* were isolated and confirmed.

The antibacterial activity of both male and female hemolymph was performed by well diffusion method and the male hemolymph exhibited better antibacterial activity than the female sample (Table.4). Among the four bacteria studied, *Staphylococcus aureus* exhibited better sensitivity pattern and *Proteus vulgaris* exhibited the least pattern. The sensitivity pattern of *Staphylococcus aureus* against male hemolymph was found to be 24mm ± 0.0025 and 21mm ± 0.0037 for female hemolymph. The sensitivity pattern of *Proteus vulgaris* against male hemolymph 9mm ± 0.0015 and 5mm ± 0.0017 for female hemolymph and this generated the least zone of inhibition. The P value $p<0.005$ was considered to be the level of significance in this present research.

Table 1. Results of different biomarker enzymes level

Experimental Groups	Glutathione-S-Transferase (µmol/ml//min/mg of protein)	Glutathione reductase (µmoles of NADPH oxidized/min/mg of protein)	Superoxide dismutase (% inhibition of pyrogallol autoxidation)	Reduced glutathione (NADPH oxidized/min/mg protein)
Group-A	302.2 ± 0.6	1.62 ± 0.2	1.68 ± 0.023	4.96 ± 0.86
Group-B	278.9 ± 0.43	0.96 ± 0.18	1.04 ± 0.054	4.02 ± 0.06

Each value represents mean ± SEM of three individual observations. Group-A versus B *p<0.005, Significant.

Table 2. Total hemocytes and Bacterial colony count

S.No.	Experimental Groups	Total hemocytes, cells/ml	CFU/ml
1.	Group-A	3910± 0.0097	234± 0.003
2.	Group-B	3213± 0.0083	314±0.026

Each value represents mean ± SEM of three individual observations. Group-A versus B *p<0.005, Significant.

Table 3a. Bacterial biochemical characterization

Isolates	G. Rx	H.D.	Ca	Ox	In	MR	VP	MacConkey agar growth	TSI	Blood agar growth	Ni	Ur	Ci	OF-test, with Glucose
Isolate-A	+	-	+		-	+	+	No growth	Acid slant and butt with no H_2S	Hot-cold hemolyses	+	+	+	O(+)/F(-)
Isolate-B	-	+	+	-	+	+	-	Pink pin-point colonies	Acid slant and butt with no H_2S	Non-hemolytic colonies	+	-	-	O/F (+)
Isolate-C	-	+	+	+	-	-	-	Translucent non- lactose fermentive colonies	Alkali butt and slantsNo H_2S	Hemolytic, grey coloured colonies	+	-	+	O(+)/F(-)
Isolate-D	-	+	+	-	V	+	+	Non-lactose fermentive colonies	Alkali slant and Acid Butt, with H_2S	No hemolyses, colonies with swarming motility	+	+	+	O(+)/F(-)

*G.Rx-Gram's reaction, HD-Hanging drop, Cat-Catalase, Ox-Oxidase, In-Indole, MR-Methyl red, VP-Voges P, TSI-Triple Iron sugar medium, Nit-Nitrate, Ur-Urease, Ci-Citrate, OF-Oxidative-fermentative test, G-Glucose.

Table 3b. Growth on Confirmatory Medium

Isolates	EMB agar	King's B medium	Mannitol salt agar	Nutrient agar
Isolate-A	-	-	+	+
Isolate-B	+	-	-	+
Isolate-C	-	+	-	+
Isolate-D	-	-	-	+ with swarming colony appearance

Table 4. Antibacterial activity by well diffusion test

S.No.	Experimental Groups	*E. coli*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Proteus vulgaris*
1.	Group-A	15mm ± 0.0032	24mm ± 0.0025	12mm ± 0.0045	9mm ± 0.0015
2.	Group-B	16mm ± 0.0026	21mm ± 0.0037	8mm ± 0.0038	5mm ± 0.0017

Each value represents mean ± SEM of three individual observations. Group-A versus B *p<0.005, Significant

Discussion

In present scenario reports states that there is a growing concern about the microbial diseases among human population and increased cases of antibiotic resistance. Hence there is quest for the alternative therapies to curtail these infections, like plant based therapies. Crustacean aquaculture has been increased during the recent years and hence there is always a necessities for antimicrobial studies. In this study hemolymph was used as the sample and total protein concentration was found to be similar in both the genders as reported by Rekha et al. (2014) as the muscle protein concentration as 173.6 mg/g and in female sample it was 129.1 mg/g.

The biomarkers such as Glutathione –S transferase, Glutathione reductase, Glutathione peroxidase, Superoxidase dismutase and reduced glutathione also plays a vital role in combating the ill effects (Wan et al., 2017). Varadharajan et al. (2012) and Lipshi et al. (2015) have reported the presence of bacteria present in the hemolymph of *Oziotelphusa senex senex*. The antibacterial effect of crustacean hemolymph has been studied throughout the world. Kumaran et al. (2013) reported the total hemocyte count as 5720 ± 28.67. There are many crustacean species which has antimicrobial peptides in their hemolymph (Anbuchezhian et al., 2009). The present study indicates that hemolymph of male sample contains increased level of antibacterial peptides, than female hemolymph sample, similar results was also reported by Sumalatha et al. (2016).

In this present study it can be concluded that male crab hemolymph has increased antibacterial molecules when compared to the female hemolymph. From this study it is evident that even in the presence of bacteria in the hemolymph of *Uca triangularis*, the antimicrobial molecules are present in the hemolymph, to have microcidal effect and thus the animal is able to survive. At the same time the animal also produces biomarkers to neutralize the effect of the innate immune responses, which is reactive species. The anti-microbial peptides from marine crab like *Scylla serrate* has been well characterized (Hoq et al., 2003). Similarly the antimicrobial peptides from *Uca triangularis* has to be characterized to confirm their pharmacological significance.

Conflicts of interest

The author would like to state that there is not conflict of interest.

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