

*Full Length Research Paper*

# ***In vitro* antimicrobial activity of shrimps haemolymph on clinical pathogens**

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Accepted 15 June, 2010

**Crustaceans, the lower invertebrates, have an immense immunological defence against pathogenic microorganisms. In such a way, this work is carried out in 3 selective shrimp namely *Penaeus indicus*, *Penaeus monodon* and *Penaeus semisulcatus*. The hemolymph were collected separately from these shrimps and tested against series of clinical pathogens as well as multi drug resistant pathogens. The results confirmed a positive test against most of the pathogens used. Previously, the work carried out in crustaceans like horse shoe crab hemolymph is remarkable. It is certain that the hemolymph of shrimp plays a vital role in modern immunology. The present work is focused on the antimicrobial property of shrimp hemolymph on human pathogens by *in-vitro* method.**

**Key words:** Hemolymph, crustacean immunology, shrimp disease, multidrug resistant pathogens.

## **INTRODUCTION**

In crustaceans, the defense system against microbes rests largely on cellular activities performed by haemocytes, such as cell adhesion, phagocytosis, encapsulation, nodule formation, and melanisation. In most of the crustacean species studied, the antimicrobial activity has been located in the haemolymph and/or in the haemocytes. However, potent antimicrobial activity has also been detected in other organs/tissues. The evolution of antibiotic-resistant pathogenic bacteria has stimulated the search for alternative antimicrobial agents from natural sources.

Penaeid shrimp are economically important aquaculture species. However, the production of shrimp was threatened by diseases and it may cause severe mortalities during larval stages of shrimps and other crustaceans. For example, septicemia by the Gram-positive bacterium *Areococcus viridans* can cause mortality in lobsters and crabs (Newman and Feng, 1982), and Gram-negative bacteria in the family Vibrionaceae, and fungi *Fusarium* spp. can cause mortality in shrimp (Song et al., 1993; Burns et al., 1979).

To overcome such problems and also avoiding the use of antibiotics, natural AMPs (Antimicrobial peptide) from shrimp have been considered. Penaeidins are AMPs that have been first isolated from the plasma and hemocytes of *Penaeus vannamei* (Shao-Yang et al., 2006). Hence the present study was carried out in order to investigate the three different shrimps hemolymph on clinical human pathogens by *in-vitro* method.

## **MATERIALS AND METHODS**

### **Collection of animals**

Three species of live Penaeid shrimps were collected from different areas along the Vellar estuarine environment (Lat 11° 29' N; 79° 46' E). They were collected using cast net.

Healthy male and female animals of different sizes were used throughout for experimental purposes and each animal was subjected to single blood collections.

### **Collection of haemolymph**

Haemolymphs were collected from the legs of the animal with a fine disposable syringe. To avoid haemocyte degranulation and coagulation, the haemolymph was collected in the presence of Sodium Citrate Buffer, pH 4.6 (2:1 v/v). Equal volume of physiological saline (0.85% NaCl, w/v) was added to it. To remove

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haemocytes from plasma haemolymph, it was centrifuged at 2000 g for 15 min at 4°C. Supernatant was collected by aspirating and stored at 4°C until use.

#### Anti-bacterial assay

The spectrum of antibacterial activity was studied using a test agent with a range of 7 different strains of human pathogenic gram-positive and gram-negative bacteria.

*In vitro* antibacterial assay was carried out using the disc diffusion technique (Bauer et al., 1996). Whatman no.1 filter paper discs with 4 mm diameter were impregnated with known amount of test samples of the shrimps haemolymph and positive control contained 250 mg of a standard antibiotic disc. Negative controls did not comprise of only sterile disc. The impregnated discs along with control (incorporated with solvent alone) were kept at the center of Agar plates, seeded with test bacterial cultures after incubation at room temperature (37°C) for 24 h. Antibacterial activity was expressed in terms of diameter of Zone (including the disc within) in mm.

#### Anti fungal assay

*In vitro* antifungal activity was determined using the technique of Bauer et al. (1996). Five different species of fungal pathogen was inoculated by spread plate method using 0.1 ml of 24 h old culture, maintained in mycological broth. Whatman no.1 filter paper (4 mm) discs impregnated with test samples of the shrimp haemolymph and positive control contained 300 mg of a standard antibiotic disc. Negative control, which did not comprise of sterile discs were placed along with control (solvent impregnated) discs on the test plates. Antifungal activity was measured in term of diameter of zone (including the disc within) in mm.

## RESULTS

### Biological screening

Antibacterial activities of the haemolymph of *Penaeus monodon*, *Penaeus indicus* and *Penaeus semisulcatus* were used for the present study. Investigations against a range of 7 different bacterial strains were used: One gram positive bacteria (*Staphylococcus aureus*,) and six gram negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexineri*, *Vibrio cholerae*, and *Klebsiella sp.*). In the case of fungus, 5 different strains (*Aspergillus fumigatus*, *Rhodotorula sp.*, *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus niger*) were used. Stains of multi-drug resistant bacteria like; *Streptococcus pyogenes*, *Acinetobacter sp.* *S. typhi*, and Methicillin Resistant *S. aureus* were also used (Table1).

Plates showed a wide array of antibacterial, antifungal and multi-drug resistant bacterial activity measured as the diameter of zone of inhibition in disc diffusion assay (streak method). The result demonstrated that the penaeid shrimp haemolymph of crude sample tested against gram positive and gram negative pathogenic bacterial, fungal strains and four multi drug resistant

**Table 1.** Pathogens used for the study.

Pathogens	Isolate no.
<b>Bacteria</b>	
<i>E. coli</i>	B1
<i>P. aeruginosa</i>	B2
<i>S. aureus</i>	B3
<i>S. typhi</i>	B4
<i>S. flexineri</i>	B5
<i>Klebsiella sp.</i>	B6
<i>V. cholerae</i>	B7
<b>Fungi</b>	
<i>A. fumigatus</i>	F1
<i>Rhodotorula sp.</i>	F2
<i>C. albicans</i>	F3
<i>C. neoformans</i>	F4
<i>A. niger</i>	F5
<b>Multi-drug resistant bacteria</b>	
<i>S. pyogenes</i>	D1
<i>Acinetobacter sp.</i>	D2
<i>S. typhi</i>	D3
Methicillin Resistant <i>S. aureus</i>	D4

bacterial strains were used. In antibacterial activity, the highest zone of inhibition was observed in the haemolymph of *P. monodon* against *Klebsiella sp.* (5 mm) and there was no activity against *E. coli* and *V. cholerae*. *P. indicus* against *E. coli*, *V. cholerae* and *Klebsiella sp.* and *P. semisulcatus* against *Klebsiella sp.* The antibacterial activities also showed negative response. Thus in antifungal activity, the highest zone of inhibition was observed in the haemolymph of *P. monodon* against *Aspergillus fumigatus*, *P. indicus* against *C. neoformans*, *P. semisulcatus* against *A. fumigatus* and *C. neoformans*. Moreover, the other strains were showed negative result by these three shrimps. In the cause of multi-drug resistant bacterial activity, only *P. semisulcatus* showed the highest zone of inhibition against Methicillin Resistant *S. aureus* (2 mm) (Table 2).

## DISCUSSION

In decapods crustaceans, it is known that environmental changes may affect the immune ability to susceptibility against pathogen infection. In the present investigation, haemolymph were collected from three different shrimps such as *P. monodon*, *P. indicus* and *P. semisulcatus*, which are subjected to antibacterial and antifungal assay.

**Table 2.** Antimicrobial activity of shrimp haemolymph against various pathogenic strains.

Sample	B1	B2	B3	B4	B5	B6	B7	D1	D2	D3	D4	F1	F2	F3	F4	F5
<i>P. monodon</i>	+	+	+	4	3	-	5	-	+	-	-	+	-	-	-	-
<i>P. indicus</i>	-	+	+	1	-	-	2	-	+	-	-	-	-	-	+	-
<i>P. semisulcatus</i>	+	+	+	+	1	-	4	2	+	-	-	+	-	-	+	-

Morishima et al. (1992) tested various bacteria as elicitor of antibacterial protein synthesis in *B.mori* larvae. Some species of gram negative and gram positive bacteria were effected elicitor. In most of the invertebrates, antibacterial proteins are inducible constitutive such as Lysozyme (Powning, 1973) and Andropin (Samakovilis et al., 1991). Lysozyme occurs constitutively in invertebrates, but can also be induced by prior immunization.

In Arthropods, antimicrobial compounds were mainly studied in Chelicerates (Horseshoe crab) and insects. Their involvement in the defense reaction is quite different in these two groups. In Horseshoe crabs, they are stored after processing within their cytoplasmic granules (Iwanga and Kawabata, 1998; Ravichandran and Sudha, 2007). They are believed to be released by exocytosis upon microbial infection as reported in crustacean species of the crab: *Carcinus maenas* (Schnapp et al., 1996) and *Callinectes sapidus* (Khoo et al., 1999), but up to date there are no available data.

Antimicrobial peptides have been established as key players in animal defense systems. An antimicrobial peptide, which is isolated from a decapod crustacean (a crab named *Thalamita crenata*), possess an immense antibacterial activity (Rameshkumar et al., 2009a). In addition to these crustaceans, crabs have an immense antimicrobial property in their hemolymph. One of such Antimicrobial protein from the crab hemolymph (*Charybdis lucifera*) has been extensively studied against *E.coli* and *P. aeuroginosa* type bacteria's (Rameshkumar et al., 2009b). Some antimicrobial peptides from the crustacean have chitin-binding activity because of the cysteine-rich region in their COOH-terminal domain. This property is considered essential not only for antimicrobial activity, but also for chitin assembly in wound healing. Thus, it may also play an important role in preventing invasive infections (Destoumieux et al., 1997). Penaeidin-5 displayed a similar motif in its COOH-terminal domain. Thus, we suppose that the penaeidin-5 also has chitin binding properties that are involved in resistance to infections. A structural feature of the penaeidin family is that the mature peptides possess an N-terminal cyclisation (Bachere et al., 2000). Circular dichroism having the lower wave length of 190 nm implies a coil or loop structure. Furthermore, in comparing the results of circular dichroism with those of 3D structural modeling, it can expect that an  $\alpha$ -helix structure existed in the C-terminus of penaeidin-5, it contains six cysteine residues

that are probably engaged in the formation of three intra molecular disulfide bridges that contribute to the formation of the  $\alpha$ -helix structure. The helix structure was similar to that of CS $\alpha\beta$ -type antimicrobial peptides such as drosomycin. It is noteworthy that the CS $\alpha\beta$  motif antimicrobial peptides are effective against a wide variety of microbes such as Gram negative bacteria and yeasts.

In the present study, the shrimp haemolymph showed antimicrobial activity against a range of different pathogenic strains of both gram positive and gram negative bacteria and pathogenic fungal strains including few antibiotic resistant strains. Similar observation was made by Noga et al. (1996) in *P. setiferus*. The result suggests that shrimp can produce antimicrobial substances instantly to combat bacterial infection. A comparative antimicrobial effect of six brachyuran crabs revealed that the maximum antibacterial effect of crude haemolymph is shown by *Dromia abrolhensis* against *E.coli* and the minimum is shown by *S. serrata* crab against *Klebsiella oxytoca* (Ravichandran et al., 2009). Induction of antibacterial compounds was also observed on the basis of sarcotoxin I (Okada and Natori, 1985), sapecition (Matsuyama and Natori, 1988), lebocin (Chowdhury et al., 1995) and Scropin-B (Taniai et al., 1995) in *Bombyx mori*, as the haemolymph showed that the antibacterial compounds were secreted in response to immunization. Similarly, observations were also found by Nakamura et al. (1988) in *Tachypleus tridentatus* and Morishima et al. (1992) in *B.mori*. The influence of crab hemolymph against wide range of clinical pathogens proves that crustaceans are very good source of antimicrobial potency (Anbuchezhian et al., 2009).

As described for *Limulus* (Toh et al., 1991), and to some extent for mammalian antimicrobial peptides, some of the penaeidins stored in the blood cells appear to be released into haemolymph upon stimulation. Actually microbial stimulation is known to trigger haemocyte degranulation as one of the most immediate haemocytic reactions in the crustaceans (Smith and Soderhall, 1983; Johansson and Soderhall, 1985) and in freshwater cray fish (*Pasificastacus leniusculus*), degranulation was shown to be associated with a rapid decrease in RNA and protein synthesis in granular cells.

Results reported in the assays of growth of *E. coli* incubated in naive plasma reveals that *P.vannamei* plasma constitutes a rich nutritive medium for bacterial growth *in-vitro*. This contrasts with the results obtained by

Noga et al. (1996), who obtained antibacterial activity in the haemolymph of apparently unstimulated *P. setiferus*. This discrepancy may indicate a difference in the inherent toxicities of naive *P. vannamei* and *P. setiferus* plasma to bacteria. It could also be due to procedural differences. The previous researchers used whole haemolymph, which was allowed to clot before crushing, centrifugation and subsequently used in antibacterial assays. Clotting in crustaceans involves plasma gelation by cell bound coagulogen (Durliat and Vranck, 1981). The exocytosis provides mechanisms for the simultaneous release of other bioactive molecules and the triggering of the proPO activating system (Smith and Chisholm, 1992; Soderhall and Cerenius, 1998). In addition, homogenization of the clot would break the haemocytes, releasing the cellular antibacterial factors that may have led to the observed antibacterial activity, although it can not be measured. But in the other cause, no zone of inhibition was detected. It is interesting to know from this finding that shrimps, being marine animal have the ability to dispose bacteria upon infection. As the bacterium is a human pathogen, it is important that sea water should be free from this type of bacteria. In conclusion, the present study indicates that the haemolymph of shrimps would be a good source of antimicrobial agents.

## ACKNOWLEDGEMENT

We gratefully acknowledge the Director of the Centre of Advanced Study in Marine Biology for rendering encouragement to this research.

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