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Antibacterial effectiveness of hemolymph serum of *Scylla tranquebarica* inoculated by *Pseudomonas aeruginosa* ATCC 27853

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Abstract
The present investigation was aimed to determine the antibacterial effectiveness of the serum of *Scylla tranquebarica* challenged with *Pseudomonas aeruginosa* ATCC 27853 in two forms viz., heat killed form and live form. The antibacterial activity was discernible only in the heat killed group exhibited the antibacterial activity after 48 hours. After 96 hours the sham control showed again a significant antibacterial activity. Throughout the test period of 96 hours, the live group serum failed to show the antibacterial activity. In order to study the secondary response, the three groups were inoculated with a secondary dose of saline, heat killed (10^{-7}) and live (10^{-7}). The results revealed that no significant response was discernible in all the three groups. After 72 hours the immunoreactivity of these peptides became undetectable in tissue like gills as well as in the cuticle infiltrating hemocytes. From these studies, it is understood that antimicrobial peptides is a local protection system to prevent invasive infectious pathogens such as bacteria, fungi and also play role in wound healing and during injury.

Keywords: Mud crabs, Pseudomonas, Primary immune response, Secondary immune response, Exotoxin.

1. Introduction
Pharmaceutical market is growing rapidly and continuously in worldwide but, still the demand for new drug discovery is encouraged. The reason behind this motivation is the growth of numbers drug resistant infectious disease and more upcoming disorders to humans and animals. The terrestrial resources have been greatly explored. Nowadays, the researchers are expecting the lead molecules and compounds from the new resources especially from the marine environments. The oceans cover more than 70% of the Earth's surface and contain more than 3,00,000 species of plants and animals and it will represent an important resource for the discovery of novel bioactive compounds (Donia *et al*, 2003). The marine environment comprises of complex ecosystem with a plethora of organisms and many of these organisms are known to possess bioactive compounds. Almost every class of marine organisms contains variety of molecules with single structural features due to the physical and chemical conditions of the marine environment (Chin *et al*, 2006) [12]. The bioactive compounds are involved in biological functions of marine organisms such as communication, infection, reproduction and self-defense. More than 12,000 natural products have been isolated from marine algae, sponges, coelenterates, ascidians, echinoderms and bryozoans (Matthee *et al*, 1999; Costantino *et al*, 2004) [13, 14] and many of these organisms are known to possess bioactive compounds but still now most of them are unexplored especially from the molluscs family.
Marine invertebrates are known to depend on innate immune mechanisms by interacting cellular and humoral components to protect against pathogens for their safe (Tincu and Taylo, 2004) [15]. Furthermore their small size makes then easy to synthesize without cells or tissues and they rapidly diffuse to the point of infection (Rameshkumar *et al*., 2009) [16]. Recently, brachyuran crabs have shown pronounced antibacterial activities and may be useful in the biomedical area. The potential of marine crabs as a source of biologically active products is largely unexplored. A broad, based screening of marine crabs for bioactive compounds is necessary. The present investigation was aimed to determine the antibacterial effectiveness of the serum of *Scylla tranquebarica* challenged with *Pseudomonas aeruginosa* ATCC 27853 in two forms viz., heat killed form and live form.

2. Materials and Methods

2.1 Species selection and identification

Scylla tranquebarica being a commercially important species was selected as the test animal for the study. Sexually immature crabs (carapace width: 8 to 9 cm) were selected for the experiments. All the animals were in intermoult stage. The species were identified by according to the basis of the taxonomic criteria suggested by Drach (1939) [1].

2.2 Procurement and culture of *Pseudomonas aeruginosa* MTCC 1688

The bacterial strain *Pseudomonas aeruginosa* ATCC 27853 used in this study was procured from the ATCC Corporation, Manassas, Va, USA.

2.3 Preparation of *Pseudomonas aeruginosa* MTCC 1688 inoculum

The bacterial inoculum was prepared by the procedure adopted by Lightner and Lewis (1975) [2]. 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., 10^8, 10^7, 10^6, and 10^5.

2.4 Acute toxicity test

Acute toxicity bioassay to determine the LD_{50} of the inoculums was carried out by the method described by Reed and Muench (1938) [3]. About 4 groups of crabs, each consisting of 10 crabs was selected for acute toxicity studies. The crab in each group was inoculated with bacterial suspension (*Pseudomonas aeruginosa* ATCC 27853) at varying concentrations viz., 10^8, 10^7, 10^6, and 10^5. The bacterial suspension (about 0.5 ml) was injected into the infrabranchial sinus present at the base of the walking leg. This was taken as the test groups for the determination of the lethal dosage of *Pseudomonas aeruginosa* ATCC 27853 in *Scylla tranquebarica.* The cumulative percentage of mortality at the intervals of 24, 48, 72 and 96 hrs was noted. From the data on mortality, the LD_{50} for 96 hours (Probit method) was calculated (Itami *et al*., 1989) [4]. The death of the crabs was determined by observing the crabs for the following changes: blackening of the carapace, avoiding the feed (loss of appetite), lethargic movement, formation of black spots on the exoskeleton. Before death the crabs become moribund and fail to respond to physical disturbance (prodding).

2.5 Antibacterial activity

The antibacterial activity of the crab serum was carried out using the procedure adopted by Hoq *et al*., (2003). The hemolymph of four groups viz., control, saline injected sham control, heat killed and live groups were collected at four different intervals viz., 24, 48, 72 and 96 hr. the hemolymph was collected from the arthrodial membrane at the base of the walking legs. The hemolymph was collected in a syringe containing sodium citrate buffer, pH 4.6 (2:1 v/v). equal volume of physiological saline was added (0.85 Nacl, w/v) to it. To remove hemocytes from the plasma, the hemolymph was centrifuged at 800 g for 10 min at 4^oC, supernatant collected by aspiration and stored at 4^0C. The disc diffusion technique (Bauer *et al.*, 1966) with the modification was applied for the in vitro investigation of antibacterial activity. All bacterial suspensions were calibrated and routinely standardized to a concentration of approximately 10^6 cfu/ml. antibacterial activity was expressed in terms of diameter of zone of inhibition in mm.

3. Results and Discussion

In the present study the results of antibacterial activity determined by disc diffusion method revealed that among the four groups, after 24 hours only the sham control showed the symptoms of antibacterial activity. After 48 hours the antibacterial activity was discernible only in the heat killed group exhibited the antibacterial activity against *Pseudomonas aeruginosa* ATCC 27853. After 96 hours the sham control showed again a significant antibacterial activity against *Pseudomonas aeruginosa* ATCC 27853. Throughout the test period of 96 hours, the live group serum failed to show the antibacterial activity against *Pseudomonas aeruginosa* ATCC 27853. In order to study the secondary response, the three groups were inoculated with a secondary dose of saline, heat killed (10^{-7}) and live (10^{-7}) after 96 hours. The results revealed that no significant response was discernible in all the three groups. The results infer that in the *Scylla tranquebarica,* the synthesis of antibacterial peptide is existing. However in the live bacterium inoculated group the animals failed to elicit the synthesis of the antipeptides. The heat killed groups elicited the response and this may be due to the action of the lysed products of the bacterium. In the case of sham control, the saline would have induced the synthesis of the above antipeptides nonspecifically. The failure of both primary and secondary response in the live inoculums injected group suggests that the bacterial population may have some to inhibit the above antipeptide synthesis. In this context, it is of interest to note that the Pseudomonas exotoxins inhibited the protein synthesis when they are injected as immunoconjulates to the target cells (Iglewski and Kabat, 1975) [7]. Studies have also revealed that the exotoxins of Pseudomonas are a single peptide toxin with molecular weight of 72 kDa. The toxin once inside the cytoplasm of eukaryotic cells can interfere with protein synthesis by inactivating the elongation factor - 2 (EF-2) by ADP-ribosylation ie., by the addition of ADP-ribose from NaD^+ to a unique amino acid. Dipthamide which is formed by the post-translational modification of specific histidine residues.

Table 1: Antibacterial activity of hemolymph serum of *Scylla tranquebarica* challenged with *Pseudomonas aeruginosa* ATCC 27853

Groups	Zone of inhibition procured by induced hemolymph (mm in diameter)			
	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**
Control	-	-	-	-
Sham control	9 (-)	-	-	13
Heat killed	- (-)			111527
Live	-(-)	-	-	-

3.1 Secondary response given in brackets

Such Pseudomonas exotoxins bind to the receptor moieties on the cell surface and are internalized. Immunoconjugates of Pseudomonas exotoxin have been successfully employed as molecular probes and also for the cytotoxic killing of cancer target cells such as breast cancer cells and HCLA cells. Considering this, it may be suggested that the specific action of Pseudomonas exotoxin should have occurred non-specificity in the crustacean host tissue cells. Considering these points and also the observations of failure of secondary response of antipeptide synthesis consequent to the challenge of secondary antigenic dosage, it can be construed that the synthesis of antipeptides and the animals capacity to synthesize the above in different organs seem to be limited. Moreover, if hemocytes were to synthesize the peptides

continuously, the secondary response would have been observed. On the country, the secondary response is a failure in this species as revealed by the present study. In this context, the hemocytes also show similarity to the vertebrates primary response of B cells, which synthesize a considerable repertoire of immunoglobins (Igs) and remain in shut off condition, at a later period. However in vertebrates, the sub populations of B cell clones maintain distinct categories of B cells and plasma cells for Ig synthesis and memory cells which keep an anamnaestic memory of the antigen. At this point only the crustacean phagocytic cells divulge from that of vertebrate phagocytic cells and once again establish that their immune system is primitive and archaic.

A recent study on the antimicrobial peptides in *Penaeus vannamei* experimentally challenged with bacterial strain such as *Serococcus viriders, Vibrio alginolyticus* and by the fungus *Fusarium oxysporum* has revealed that in these shrimps the antimicrobial peptides are constitutively synthesized and stored in the hemocytes. They are specifically localized in the granulocyte cytoplasmic granules. Following microbial stimulation the antipeptide mRNA levels decrease in the circulating hemocytes in the first 3 hours and the concentration of antipeptides increased in the plasma. Their study also revealed the immune reactivity of these antipeptides in cuticular tissue and their chitin binding activity. Time course analysis of antipeptides expression (Penaedin) in hemocytes revealed that the relative expression in challenged prawns compared to unchallenged forms, is enhanced to 175% after 24 hours.

Similar to shrimps, the expression and secretion of antimicrobial peptides and storage has been shown in horse shoe crabs and their release by exocytose into the blood. Iwanaga and awabata, 1998 have speculated that that the chitin binding activity of the antimicrobial peptides, in the cuticular surfaces could prevent the shrimps from invasive infections by a systemic protection. After 72 hours the immunoreactivity of these peptides became undetectable in tissue like gills as well as in the cuticle infiltrating hemocytes. From these studies, it is understood that antimicrobial peptides is a local protection system to prevent invasive infectious pathogens such as bacteria, fungi and also play role in wound healing and during injury. Despite these results some of the tissues in the establishment of disease control on the basis of gene expression for these antibacterial peptides have to be addressed (Cordwell and Nouwens, 2004; Kavanagh and Reeves, 2004) [9, 10]. They are (I) how far these antimicrobial peptides afford protection and against pathogenic viruses, (II) whether such synthesis in the tissues other than hemocytes occur (III) if so how these originated peptides reach the granules of hemocytes (IV) what would be the immunoreactivity of antipeptides with the chitin synthesizing organs other than the exoskeleton etc. All these clarifications will delineate more information regarding these antipeptides and their modulation to increase the resistance of crustacean species.

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