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# Modulation of selected hemolymph factors in the Indian edible oyster *Crassostrea madrasensis* (Preston) upon challenge by *Vibrio alginolyticus*

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## ABSTRACT

Juveniles of *Crassostrea madrasensis* (mean weight 85.5 ± 2.3 g) were exposed to live cells of *Vibrio alginolyticus* (1.2 x $10^6$ cells $g^{-1}$) by intramuscular injection. Hemolymph samples were collected at different time intervals to study the modulations in the cellular and humoral factors. There was an increase in total hemocyte count, percentage granulocytes, serum protein, serum acid phosphatase, serum phenol oxidase and serum lysozyme in response to bacterial challenge upto three to five days post-injection. A decrease in the ability of hemocytes to phagocytose yeast cells was also noted. The hemolymph parameters of the test group became similar to that of control animals within two weeks of exposure to live bacterial cells.

Keywords: *Crassostrea madrasensis*, Hemolymph factors, Indian edible oysters, *Vibrio alginolyticus*

## Introduction

Molluscs frequently encounter diseases due to microbial infections. The quality of response of the internal defense system during the period following microbial challenge is a major factor in the success or failure of infective process. Molluscs become vulnerable to infection when they are physiologically weak and the attacking organisms acquire virulence. The defense system in molluscs could be generally divided into two *viz.*, cellular and humoral (Oubella *et al.,* 1996). Very few studies have been conducted in order to understand the changes that occur in these factors subsequent to a bacterial challenge, especially in the Indian edible oyster, *Crassostrea madrasensis* (Preston) which is an important species used for culture.

One of the important bacterial pathogen which causes mass mortality in cultured molluscs is *Vibrio alginolyticus* (Anguiano-Beltran, 1998; Luna-González *et al*., 2002; Gómez-León *et al.,* 2005). The present study was carried out in order to elucidate the defense reactions in *C. madrasensis*, subsequent to challenge by a strain of *V. alginolyticus.*

## Materials and methods

### *Experimental design*

Edible oysters, *C. madrasensis* (mean weight 85.5 ± 2.3 g) collected from the backwaters of Kochi, Kerala, were used for the study. Sixty animals each were maintained in three replications in tanks having capacity for holding 1000 l of seawater at a salinity of 12 ppt. The animals were fed *Chaetoceros* sp. *ad libitum*. The tanks were cleaned and 50% water exchange was done every day. A strain of *V. alginolyticus* obtained from the Central Institute of Fisheries Technology, Kochi, India was used for the experiment. It was a non-pathogenic strain isolated from marine sediments. Overnight cultures of bacterial strain on nutrient agar slants were harvested into 10 ml of sterile seawater of 2% salinity (Suresh and Mohandas, 1990). The cell concentration was assessed by measuring optical density at 600 nm. The test animals were challenged by intramuscular injection (in the adductor muscle) of bacterial suspension at a dose of 1.2 x $10^6$ cells $g^{-1}$ weight of the animals (0.02 ml) under asceptic conditions using a sterile hypodermic syringe. Control animals were injected (i/m) with 0.02 ml of sterile seawater of 2% salinity. Hemolymph was collected at 2 h, 24 h, 3 d, 5 d, 7 d and 14 d post-injection, from the adductor muscle under aseptic conditions. For hemolymph sampling, 10 animals each were bled from each replication of control and test groups. The samples of each replication were pooled. In order to avoid repeated bleeding, the animals once bled were discarded. About 1 ml of hemolymph was collected from each animal.

### *Total and differential hemocyte count*

The total and differential hemocyte counts were estimated following the method of Nakayama *et al.* (1997) using hemocytometer (Hausser Scientific, PA, USA).

### *Phagocytosis*

Phagocytic and endocytic indices were determined as per the method of Bayne *et al.* (1979), with slight modifications using formalin inactivated yeast cells as the

target. Hemocyte monolayers were prepared on glass slides, rinsed with sterile seawater of 2% salinity and incubated with yeast suspension in sterile seawater of 2% salinity for 60 min at 25 °C. The slides were rinsed with sterile seawater of 2% salinity, fixed in 10% methanol for 15 min, air dried and stained with Giemsa (SRL, India) for 20 min, differentiated in acetone and mounted in DPX. The slides were then observed using a compound microscope under oil immersion objective (Leica DMLS, Munich, Germany)

*Total serum protein*

The total protein concentration of serum was estimated following the method of Lowry *et al.* (1951) with bovine serum albumin (Himedia) as standard. The results were expressed in µg $ml^{-1}$ of serum.

*Serum acid phosphatase*

The method followed by Varley (1980) was used for the estimation of serum acid phosphatase. The amount of phenol released per 100 ml serum was determined from a standard curve constructed using known amount of phenol. The results were expressed in KA units (mg phenol released per 100 ml serum per h).

*Serum phenol oxidase*

The method of Preston and Taylor (1970) was modified for the purpose. The test serum (0.3 ml) was added to 2.7 ml of 0.01 M L-dopa in 0.05 M tris - HCl buffer at pH 7.5. Sodium dodecyl sulphate (SRL, India) was added to the mixture at a concentration of 1mg $ml^{-1}$. The increase in O.D of the sample for 1 min was noted at 420 nm using spectrophotometer (Varian Inc., USA). The phenol oxidase activity was calculated as increase in O.D per min per mg protein of serum.

*Serum lysozyme*

The method of Parry *et al.* (1965) was modified for the estimation of serum lysozyme. The decrease in the absorbance of a suspension of *Micrococcus lysodeikticus* (Sigma, USA) with and without serum sample was read between 0.5 min and 4.5 min at 530 nm. The unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001 per min (Lysozyme unit).

## Results

*Total hemocyte count*

The total hemocyte count at different time intervals such as 2 h, 24 h, 3 d, 5 d, 7 d and 14 d post-injection are given in Fig.1. The hemocyte count in the test group was significantly high ($p<0.05$) compared to control at 2 h post-injection (hpi). The count dropped significantly in both control and test animals at 24 hpi. The reduction in the total hemocyte count observed at 24 hpi in the test sample was more than that in the control group. Since 5th day post-injection (dpi), there was no significant difference between the values obtained for the control and test animals at different intervals.

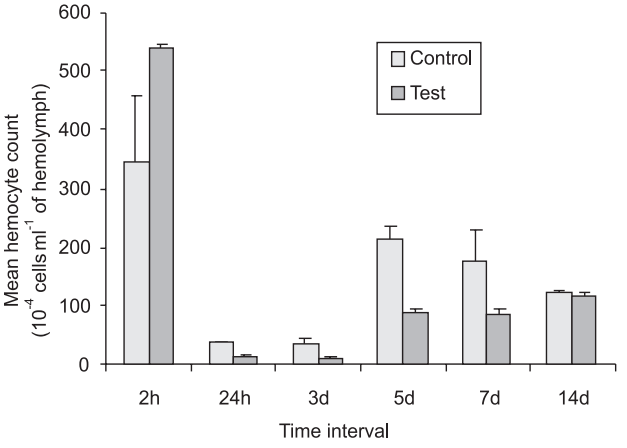


Fig. 1. Mean hemocyte count of *C. madrasensis* injected with saline and *V. alginolyticus* at different time intervals

*Differential hemocyte count*

The mean percentage of granulocytes at different time intervals for the test group was significantly ($p< 0.05$) higher than the control at all sampling intervals except at 14 d, where the result obtained for control and test groups were not significantly different (Fig. 2). Upto 3 dpi, the mean percentage of semigranulocytes was significantly ($p<0.05$) lower in test animals compared to control group (Fig. 3). However there was no significant difference between mean percentage of semigranulocytes in the control and test animals by 14 dpi. The mean percentage of hyalinocytes in test group was less compared to control upto 24 hpi. At 3 dpi, the mean percentage of hyalinocytes in the test group

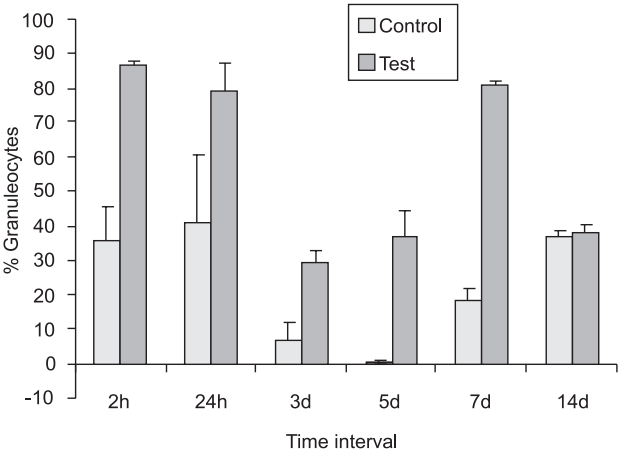


Fig. 2. Mean percentage of granulocytes of *C. madrasensis* injected with *V. alginolyticus* at different time intervals

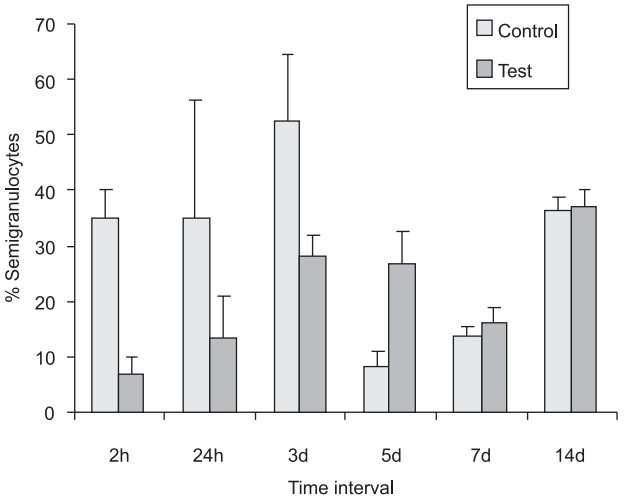


Fig. 3. Mean percentage of semigranulocytes of *C. madrasensis* injected with *V. alginolyticus* at different time intervals

increased and both control and test samples gave almost similar values. At 7 dpi, the percentage of hyalinocytes decreased to a significantly ($p< 0.05$) low value in the test compared to control. By 14 dpi, the control and test groups gave almost similar values (Fig. 4).

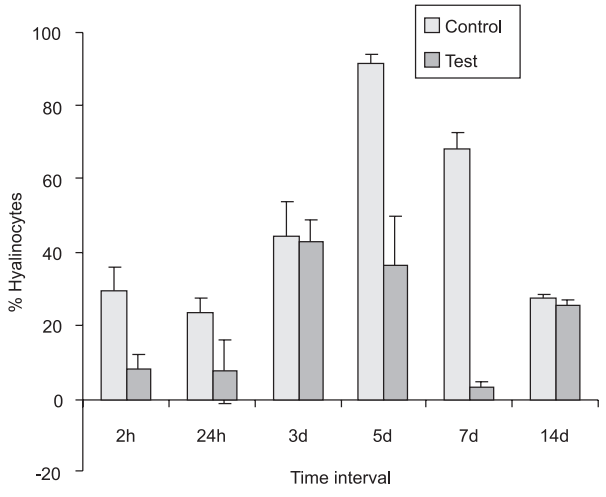


Fig. 4. Mean percentage of hyalinocytes of *C. madrasensis* injected with *V. alginolyticus* at different time intervals

*Phagocytosis*

The mean phagocytic index of the hemocytes for the control and the test groups did not show significant difference ($p>0.05$) at any of the different time intervals. The mean endocytic indices of hemocytes at different time intervals are given in Fig. 5. Up to 3 dpi, both the control and test animals gave very low values for endocytic index. At 5 d the control value increased significantly ($p<0.05$) compared to the test. By 14 dpi, value for the test group also increased and there was no significant difference between control and test samples.

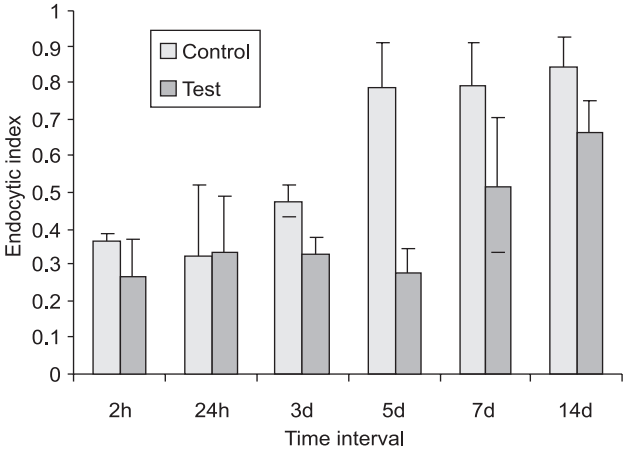


Fig. 5. Mean endocytic index of *C. madrasensis* injected with saline and *V. alginolyticus* at different time intervals

*Total serum protein*

The serum protein values at different time intervals are depicted in Fig. 6. At 3 dpi, the control animals had significantly high value compared to test group. The test value significantly increased ($p< 0.05$) compared to the control value at 5 dpi. By 14 dpi, both control and test animals gave almost similar values.

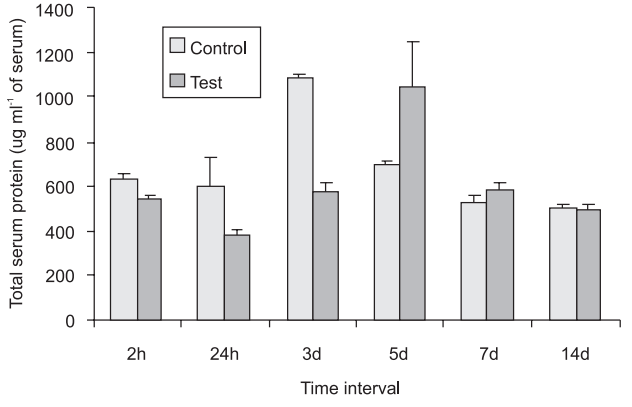


Fig. 6. Mean serum protein of *C. madrasensis* injected with saline and *V. alginolyticus* at different time intervals

*Serum acid phosphatase*

The serum acid phosphatase values obtained for the test group were significantly high compared to control group at 2 hpi and 24 hpi ($p<0.05$). There was no significant difference between the control and test values at all other sampling times (Fig. 7).

*Serum phenol oxidase*

The test value of serum phenol oxidase was significantly higher ($p<0.05$) at 24 hpi and 3 dpi compared to the control animals (Fig. 8). At all other sampling

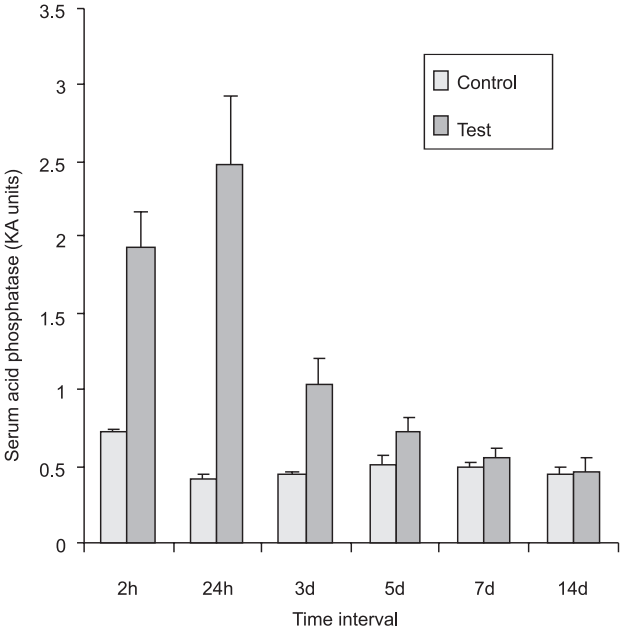


Fig. 7. Mean serum acid phosphatase of *C. madrasensis* injected with saline and *V. alginolyticus* at different time intervals

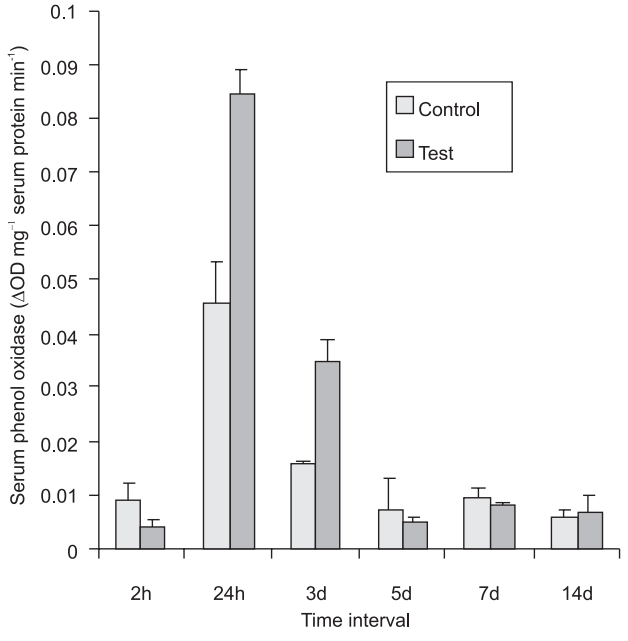


Fig. 8. Mean serum phenol oxidase of *C. madrasensis* injected with saline and *V. alginolyticus* at different time intervals

intervals, there were no significant difference ($p<0.05$) between the control and the test groups.

*Serum lysozyme*

The mean serum lysozyme levels in the test group was significantly high ($p<0.05$) compared to the control value at 2 hpi, 24 hpi and 3 dpi. At all the other time intervals, the control and test groups gave similar values (Fig. 9).

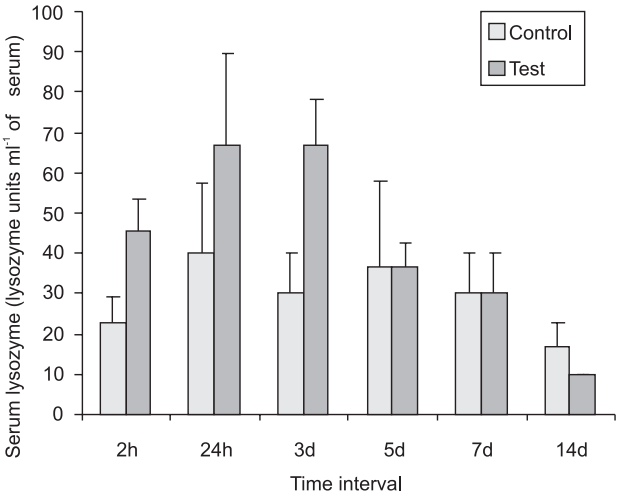


Fig. 9. Mean serum lysozyme of *C. madrasensis* injected with saline and *V. alginolyticus* at different time intervals

## Discussion

Both sham injection and injection with *V. alginolyticus* resulted in significant increase in the hemocyte count in *C. madrasensis* at 2 hpi, followed by a significant decrease at 24 hpi. Suresh and Mohandas (1990) studied modulations in hemocyte count in *Sunetta scripta* and *Villorita cyprinoides* var. *cochinensis* in response to *V. alginolyticus.* Apart from sham and bacteria injected group, they also maintained an untampered control. They got significantly high total hemocyte count in sham-injected and bacteria-injected group, compared to the untampered control. This indicated that the handling of animals itself caused increase in hemocyte count as observed in the control group in the present study. The increase in the total hemocyte count subsequent to pathogenic challenge is also reported elsewhere (Anderson *et al.*, 1992; Oubella *et al.*, 1993; 1996). It is proposed that the increase in the hemocyte count is the result of mobilization and migration of hemocytes from the tissues to hemolymph compartments in response to pathogen (Oubella *et al.,* 1996). Granath and Yoshino (1983) reported a decrease in the number of circulating hemocytes after pathogenic challenge. According to them, the decrease in hemocyte count is because of encapsulation and phagocytic reactions. It is proposed that phagocytes, after phagocytosis might migrate across epithelial borders leading to their elimination. In the present experiment, increase in the total hemocyte count soon after the injection might be because of the mobilization of host response against pathogen, and subsequent decrease in hemocyte count might be the result of elimination of the hemocytes with the phagocytosed bacteria through epithelial borders. By 14 d, both control and test gave normal values of healthy animals (Ittoop, 2005)

Oubella *et al.* (1996) observed a significant decrease in the number of hyalinocytes and a concomitant increase in the granulocytes after bacterial challenge. The formation

of granuloma by the granulocytes subsequent to pathogenic challenge has been reported by Uzhazy *et al.* (1988). The involvement of granulocytes in the formation of focal abscesses is reported by Balquet and Poder (1985). In the present experiment, the increase in percentage of granulocytes and the corresponding decrease in percentage of hyalinocytes may be because of the involvement of granulocytes in such reactions subsequent to bacterial challenge.

The phagocytic index using yeast as the target was not significantly different at any of the sampling times in the test and control groups. The endocytic index was significantly less up to 3 dpi in the test group compared to control group. This showed a reduced ability of the hemocytes of *C. madrasensis* to phagocytose yeast. Probably the lectins/receptors of the hemocyte surface were engaged in binding bacteria and therefore were not available for binding yeast particles. So the ability of the hemocytes to recognize yeast particles for phagocytosis might have decreased. The reduced ability of the hemocytes to adhere and ingest yeast cells is also reported in diseased *Mya arenaria* (Beckmann *et al.,* 1992). These studies indicate that multiple infections at the same time increase the susceptibility of *C. madrasensis* to diseases.

The reduction of the total protein in response to infection is reported by Ford (1986) and Loker and Hertel (1987). However, increase of the same in response to infection, due to production of lectins and antibacterial proteins is also reported (Hubert *et al.*, 1996). In the present study, there was an initial reduction in the serum protein concentration soon after the injection and the value increased at 3 dpi. The increased value at 3 dpi could be attributed to the production of antibacterial proteins.

The amount of acid phosphatase in the serum of test animals increased significantly followed by bacterial challenge. An increase in acid phosphatase in response to pathogen is also reported by Granath and Yoshino (1983), Cheng and Mohandas (1985) and Jyothirmayi and Rao (1988). Acid phosphatase may be playing a significant role in bacterial clearance. In the present study, the amount of serum lysozyme in test group was high compared to control up to 3 dpi. In *Crassostrea virginica,* infection with *Minchinia velsoni* led to decrease in serum lysozyme levels, whereas the infection with *Bucephalus* sp. increases the lysozyme levels (Feng and Canzonier, 1970). Lysozyme is unaffected by the infection of *Perkinsus marinus* in the same species (Chu and Peyre, 1989). Thus the response of the lysozyme towards an infection varies with species and also with pathogen. The present observations indicated that in bacterial infections, lysozyme exert its influence at the earlier stages of infection. There are reports that these enzymes *viz.,* acid phosphatase, phenol oxidase and lysozyme have their origin from hemocytes. The granulocytes secrete these enzymes into the hemolymph when there is an infection in *Mercenaria mercenaria* (Moore and Gelder, 1985) and *C. virginica* (Cheng, 1990). In the present study, there was an increase in the granulocytes in the hemolymph and a corresponding increase of enzymes in the serum. Thus, there is an indication that these enzymes are released from granulocytes in this species also.

The result of the present study indicates the role played by the granulocytes in fighting disease by phagocytosis as well as by enzyme production as revealed by the increase in percentage of granulocytes, serum acid phosphatase, phenol oxidase and lysozyme in response to *V. alginolyticus* challenge.

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