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LEVAMISOLE INFLUENCES THE IMMUNE RESPONSE OF FRESHWATER PRAWN, *MACROBRACHIUM ROSENBERGII* AND ITS RESISTANCE TO NITRITE STRESS AND *AEROMONAS HYDROPHILA* INFECTION

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The present study evaluated the effectiveness of dietary levamisole in modulation of innate immunity and reducing the percent mortality against nitrite stress or *Aeromonas hydrophila* infection in giant freshwater prawn, *Macrobrachium rosenbergii*. Haemolymph agglutinin and total protein levels, lysozyme activity, phenoloxidase (PO) activity, total and differential haemocyte counts, induced nitrite stress and mortality (%) against *Aeromonas hydrophila* challenge were measured in sub-adult *M. rosenbergii* fed with diets containing levamisole at 0, 125, 250 and 500 mg/kg feed for 7 or 14 days. *M. rosenbergii* fed with a diet containing 250 mg levamisole/kg feed for 14 days showed significant ($P<0.05$) increase in haemagglutination titre, PO activity, undifferentiated haemocyte count, resistance to nitrite stress and survival against *A. hydrophila* challenge. On the contrary, graded levels of levamisole feeding for 7 days failed to modulate most of the immune parameters or reduce the percent mortality against *A. hydrophila* challenge or nitrite stress. It is therefore concluded that administration of levamisole in the diet at 250 mg/kg feed for 14 days in sub-adult *M. rosenbergii* could enhance the immune ability and increase its resistance to *A. hydrophila* infection and nitrite stress.

INTRODUCTION

The giant freshwater prawn, *Macrobrachium rosenbergii* (de Man) of the family Palaemonidae is a migratory active species between brackish and freshwater habitat, and has a territory of Indo-Pacific region. Having improved standardized hatchery and culture technologies, the large scale farming of this species has gained momentum. Recent occurrence of nodavirus (*Mr*NV) infection in *M. rosenbergii* causing white tail disease in India, China, West Indies, Thailand and Taiwan (Cheng and Chen, 1998; Tripathy *et al*., 2006) showed indication towards poor management and quarantine system. Further, the recently occurring appendage deformity syndrome (ADS) in prawn farming causing mortality in juveniles and adults in India indicates towards poor water quality and management of culture ponds (Sahoo *et al.,* 2005). In addition, bacterial diseases are also becoming common problem in prawn farming (Cheng *et al*., 2003). Although *Aeromonas*

sp. are not generally considered to be the major threat to the commercial production of *M. rosenbergii* (Sung *et al*., 2000), they have sometimes been linked to disease outbreaks in this species (New, 1995). Nitrite also plays a major role in determining the survival of prawns in aquatic environment and its detrimental effects on immune response of freshwater prawns have been reported (Chand and Sahoo, 2006; Mallasen and Valenti, 2006). Therefore, stimulation of immune system and reduction of mortality in prawns due to infections and stress, would be of great interest for aquaculture research and the prawn farming industry.

Innate immune system of crustaceans involves various cellular mechanisms, *viz*., total and differential haemocyte counts, phenoloxidase (PO) activity, phagocytosis, encapsulation etc. and humoral mechanisms, viz., agglutinin levels and lysozyme activity. Based on the recent classification of *M. rosenbergii* haemocytes (Sierra *et al.,* 2001), large ovoid haemocytes and undifferenciated round haemocytes might be carrying out the functions of proPO system, like semigranular and granular haemocytes in other crustaceans (Johansson and Soderhall, 1989). Activation of PO from proPO is through proPO activating enzyme (ppA), a serine protease (Perazzolo and Barracco, 1997) which can be induced by several microbial polysaccharides, including β-1, 3 glucan from fungal cell walls (Vargas-Albores *et al.,* 1996). The PO activity has been well documented in *M. rosenbergii* (Kumari *et al.,* 2004; Chand *et al.,* 2006). Lectins/agglutinins play their role by enhancing the recognition interface between the invading pathogen and semigranular haemocytes by the opsonin action in the plasma (Soderhall and Cerenius, 1992). Their presence in *M. rosenbergii* haemolymph has been well documented (Vazquez *et al*., 1997; Chand *et al.,* 2006). Lysozyme, an antibacterial peptide has also been reported in *M. rosenbergii* (Kumari *et al.,* 2004).

The immunostimulatory effects of various substances like peptidoglycans, glucans, lipopolysaccharides (LPS), sodium alginate, polyherbal formulation, lactoferrin and other polysaccharides have been widely studied in crustaceans (Sritunyalucksana *et al*., 1999; Chang *et al*., 2003; Kumari *et al.,* 2004; Cheng *et al*., 2005; Chand *et al.,* 2006). Levamisole, a levo-isomer of tetramisole acts as potential immunostimulant in mammals and several aquatic species (Sakai, 1999). Feeding of levamisole at 125 or 250 mg/kg feed to *M. rosenbergii* could able to increase percent survival, and specific growth rate and feed conversion ratio without adversely affecting growth (Baruah and Prasad, 2005). However, its immunomodulatory effects on prawn immunity are not clear. In this study we attempted to examine various immune parameters in *M. rosenbergii* and its resistance to nitrite stress and *A. hydrophila* infection when the prawns were fed diets containing levamisole at graded levels for 7 or 14 days.

MATERIAL AND METHODS

The intermoult stage of *M. rosenbergii* weighing 15-20 g were collected from prawn farm of the Central Institute of Freshwater Aquaculture, Bhubaneswar and acclimated in the laboratory for two weeks before experimentation. Four sets of experiments were conducted to evaluate the immunomodulatory effects of dietary levamisole in prawns. For each set, prawns were divided randomly into four groups A, B, C and D (in triplicate) for two time periods (7 or 14 days) of levamisole feeding. Each set of experiment was carried out in 24 FRP tanks of 40 l capacity containing 30 l of water. Prawns were given pelleted diet for 2 weeks. Ten percent of water was renewed daily to siphone out the leftover feed and metabolites, providing better environment and maintaining optimal water quality parameters. Water quality parameters such as water temperature, pH, total alkalinity, dissolved oxygen were analyzed to maintain optimal levels (total hardness, 80-120 mg/l; total NH_3-nitrogen, < 0.1 mg/l; dissolved oxygen, 6.5-7.0 mg/l; water temperature, 23-26^oC; pH, 7.0-8.5). First set of experimental prawns was used to measure haemolymph supernatant agglutinin levels, lysozyme activity and total protein concentration after different dose and time period of levamisole feeding. Second set of experiment was conducted to see the influence of levamisole feeding on haemolymph cell counts and PO activity. Third and fourth sets were set up for determining the resistance of animals against nitrite stress and bacterial pathogen *Aeromonas hydrophila* infection, respectively. In first three sets, three prawns were kept in each tank where as in fourth set, four prawns were maintained in each tank. Pelleted feed was provided bot during acclimation and also during the experiment. The composition of the feed was as described by Chand *et al*. (2006). Experimental feed was prepared by adding levamisole hydrochloride (Sigma, St. Louis, USA) to above pelleted feed ingredient to make final concentrations of 0, 125, 250 and 500 mg levamisole/kg feed for groups A, B, C and D, respectively. Pellet feed was prepared with a hand pelletizer and the feed was air-dried and stored at 4^oC in airtight container before use. Carboxy methylcellulose was added as binder to the feed mix to avoid any possible leaching loss of levamisole. The feed was given to prawn at 4% of body weight daily in two split doses.

A known pathogenic isolate of *A. hydrophila* was grown on tryptone soy broth (TSB, Difco) for 24 h at 30^oC. The broth cultures were harvested by centrifugation at 5000 x *g* for 15 min at 4^oC. The bacterial pellet was washed by resuspension in sterile PBS (pH 7.4) and centrifugation as above and the final pellet was resuspended in the PBS at 10^9 and 2 x 10^5 cfu/ml to produce stock bacterial suspensions (as confirmed by total plate count) for the study of bacterial agglutination titre and challenge test, respectively.

Further, the bacteria to be used for bacterial agglutination assay were treated with 1% formalin and kept overnight at 4^oC. The formalin-killed cells were washed twice with

sterile PBS as described previously and suspended in PBS to its original cell count. The formalin-killed cells were stored at –4ºC until use.

From the first set of prawns, 400 µl of haemolymph was collected by using 2 ml sterile plastic syringe with 26 x 0.5'' gauge needle from the ventral sinus of the animal. Haemolymph was allowed to clot in 2 ml capacity microcentrifuge tubes held at 4ºC. After one hour, clot was broken using sterile needle and kept at 4ºC for 3-4 h. The tubes were then centrifuged at 11,000 x *g* for 30 min at 4ºC. The supernatant fluid was collected and stored at –30ºC for further analysis. Lysozyme activity was studied first using part of the supernatant following Kumari *et al.* (2004) with slight modification. Supernatant fluid of 15 µl was taken in a 96 well microtitre plate in triplicate per sample. Then 150 µl of *Micrococcus lysodeikticus* solution (20 mg *M. lysodeikticus* per 100 ml 0.02 M acetate buffer, pH 5.5) was added to each well. Blank consisted of 165 µl of acetate buffer only. The optical density reading was taken immediately at 450 nm by using ELISA reader (Anthos Labtec, Austria). Then the plate was incubated for 10 minutes at 25ºC. The final optical density was read after incubation period. Reduction in optical density of 0.001 was taken as one unit of enzyme activity per 15 µl of sample. The rest of the supernatant was used to study total protein concentration following Bradford (1976), using bovine serum albumin as a standard protein, and haemagglutination and bacterial agglutination titres following Chand *et al*. (2006) using RaRBC and killed *A. hydrophila*, respectively.

From the second set of prawns, 100 µl of haemolymph was collected in 1 ml syringe with 26 x 0.5'' gauge needle containing 900 µl anticoagulant (sodium chloride 0.45 M, glucose 0.1 M, sodium citrate 30 mM, citric acid 26 mM, EDTA 20 mM, pH 4.5). Phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxy phenylalanine (L-DOPA, a product of Hi Media, Mumbai) following Hernandez-Lopez *et al*. (1996). The PO activity optical density was expressed as dopachrome formation per 50 µl haemolymph.

Using 1 ml sterile plastic syringe with 26 x 0.5'' gauge needle containing 0.45 ml anticoagulant (as described above) with fixative solution (sodium cacodylate 0.10 M and 1.5% glutaraldehyde) in 1:1 ratio, 0.05 ml of haemolymph was drawn from ventral sinus of intermoult prawn and placed on haemocytometer (Spencer, Neubauer, Germany). Total and different types of haemocytes were counted following Sierra *et al.* (2001) and Chand *et al.* (2006).

At the end of levamisole feeding for 7 or 14 days, prawns were held in 5 l of water (in each tank) containing sodium nitrite (Merck, India) at 9 mg/l concentration following Chen and Lee (1997). Nitrite water (50%) was renewed daily without providing aeration. The percent mortality was observed up to 120 h for each of the groups.

Prawns fed with levamisole diet for 7 or 14 days were injected intramuscularly between the second and third abdominal segments with 0.05 ml PBS containing 10^4 cfu of *A. hydrophila* (cultured for 24 h at 30 °C). Four prawns fed with control diet were also injected with 0.05 ml PBS and served as negative control. The mortality was observed up to five days and the cause of mortality was further confirmed by re-isolating the organism from dead prawn hepatopancreas.

The mean±standard error of each parameter for each of the experiments at each time period was calculated for all the groups. Data were analyzed using one way ANOVA. Means were compared using Duncan's multiple range tests (Duncan, 1955). The difference was considered significant when $P< 0.05$.

RESULTS

No mortality was recorded in any of the groups of prawns during the experiment. A significant ($P<0.05$) enhancement in haemagglutinin titre was marked in the levamisole-fed prawns after 14 days of levamisole feeding compared with control values. However, there was no significant difference in the level of haemagglutinin among all the groups fed levamisole for a period of 7 days (Table 1).

Table 1. Haemolymph supernatant parameters of *M. rosenbergii* fed graded levels of levamisole containing diets for 7 or 14 days.

Group	Dose (mg levamisole /kg feed)	Haemagglutination titre (log_2)		Bacterial agglutination titre (log_2)		Total protein (g/dl)		Lysozyme activity (OD/15 µl supernatant)		PO activity (OD/50 µl haemolymph)	
		7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days	7 days	14 days
A	0	5.11± 0.24	5.00± 0.69^{a}	2.64± 0.10^{ab}	2.78± 0.11^{a}	16.87± 0.43	17.35± 0.58	0.002± 0.0006	0.002± 0.0006	0.20± 0.04	0.16± 0.02^{a}
B	125	5.17± 0.94	7.22± 0.22^{b}	2.44± 0.29^{a}	3.00± 0.69^{a}	17.02± 0.66	15.47± 1.38	0.003± 0.0014	0.003± 0.0009	0.35± 0.08	0.38± 0.04^{b}
C	250	6.56± 0.72	7.66± 0.66^{b}	3.11± 0.19^{b}	2.83± 0.44^{a}	18.41± 1.28	17.96± 0.64	0.004± 0.001	0.003± 0.0004	0.31± 0.05	0.37± 0.01^{b}
D	500	4.56± 0.61	7.50± 0.50^{b}	3.11± 0.19^{b}	1.16± 0.44^{b}	17.85± 0.48	17.50± 0.20	0.004± 0.0004	0.001± 0.0003	0.33± 0.05	0.63± 0.07^{c}

Data represent mean ± S.E of 9 (three prawns of replicate groups) prawns. Means bearing different superscript(s) are significantly ($P<0.05$) different.

The bacterial agglutinin level was not influenced significantly in both the upper dose group prawns as compared to its control in case of 7 days of levamisole feeding.

However, prawns fed levamisole at 125 mg/kg feed showed significantly lower agglutinin level compared to prawns fed with levamisole at 250 and 500 mg/kg feed. After 14 days of levamisole feeding, a decline in bacterial agglutinin level in group D prawns was noticed compared to other group prawns (Table 1).

Feeding of levamisole at any of the dose levels for 7 or 14 days could not influence total protein level and lysozyme activity in prawns compared to control group prawns (Table 1).

The PO activity was significantly increased in groups B, C and D prawns compared with control values after 14 days of levamisole feeding. The highest PO activity was noticed in group D prawns fed levamisole at 500 mg/kg fed for 14 days. On the contrary, no significant change in PO activity was noticed in prawns compared with control values after 7 days of levamisole feeding (Table 1).

Total haemocyte count (THC) with mean values ranging from 0.83 x 10^7 to 2.04 x 10^7 cells/ml of haemolymph was obtained in various groups of prawns. THC was not significantly (P>0.05) influenced by levamisole feeding compared to control prawns after 7 days of exposure. However, significant decrease in THC was noticed in groups B and C compared to control group prawns after 14 days of levamisole feeding (Fig. 1a).

All the three types of haemocytes *viz*., fusiform cells, large granular cells and undifferentiated round cells were observed under microscope. The mean of fusiform cells varied from 59.71 to 68.38% which were the predominant cell types in this species. There were no significant differences in the population of large granular and undifferentiated round cells among graded levels of levamisole feeding for 7 days compared to control groups prawns. Group C prawns showed significantly higher numbers of fusiform cells as compared to group B prawns after 7 days of levamisole feeding. Similarly, no changes in fusiform and large granular cell populations were marked by levamisole feeding after 14 days. The mean number of large granular cells varied from 22.15 to 30.19%. The mean number of undifferentiated cells ranged from 9.46 to 14.45%. However, a significant increase in undifferentiated round cells was marked after 14 days levamisole feeding in levamisole fed prawns compared with control values (Figs. 1b,c,d).

Exposure of prawns to 9 mg/l of sodium nitrite caused mortality on the second day onwards and the mortality was recorded up to the end of the fifth day after 7 and 14 days of levamisole feeding. After 120 h, the mean mortality was varied from 33.33 to 66.66% in the 7 days levamisole fed groups. Groups A and B showed similar mortality rate i.e. 66.66% compared to 33.33% and 50% in groups C and D, respectively. However, there was significant reduction in percent mortality (P<0.05) in levamisole fed prawns in case of 14 days levamisole fed prawns compared to control. The control group prawns

showed 100% mortality compared to 33.33% in both groups B and C, and 49.99% in group D prawns (Table 2).

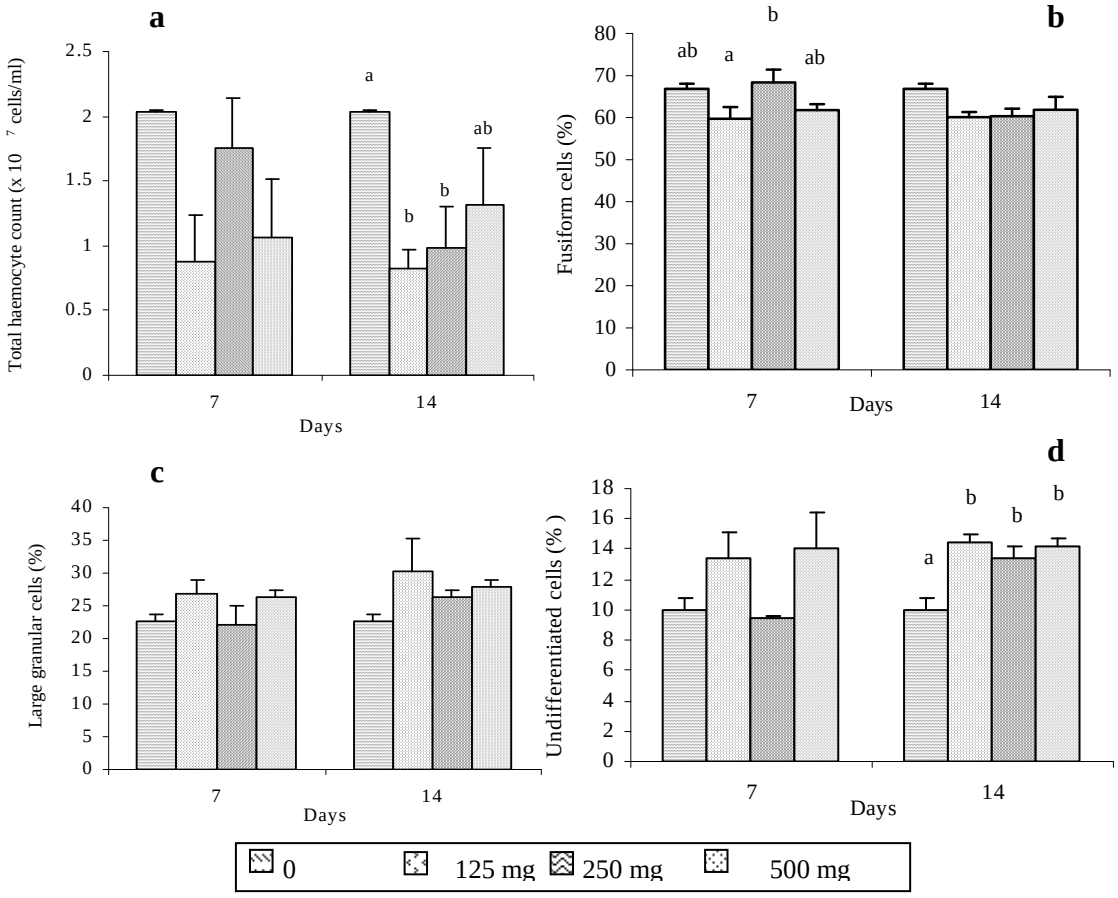

Fig. 1. Total and differential haemocyte counts of *M. rosenbergii* given levamisole containing diets for 7 or 14 days. Bars represent mean ± S. E of 9 determinations. Bars for same time with different letter(s) at a particular period are significantly (P<0.05) different.

Table 2. Mortality levels (%) after challenge of prawns exposed to nitrite or *A. hydrophila* challenge after 120 h.

Group	Dose (mg of levamisole/kg feed	Exposure to nitrite		*A. hydrophila* challenge	
		7 days	14 days	7 days	14 days
A	0	66.66±19.24	100±0.00^{a}	72.22±2.77	72.22±2.77^{a}
B	125	66.66±19.24	33.33±0.00^{b}	66.66±8.33	41.66±8.33^{bc}
C	250	33.33±19.24	33.33±19.24^{b}	83.33±8.33	33.33±8.33^{b}
D	500	50.00±28.86	49.99±9.62^{b}	91.66±8.33	58.33±8.33^{ac}

Data represent mean±S.E of 9 prawns (three prawns of each replicate) for nitrite exposure and 12 prawns (four prawns of each replicate) for *A. hydrophila* challenge. Means with different superscript(s) are significantly (P<0.05) different.

No mortality was observed in prawns injected only with PBS. The percent mortality reached its peak after 24 h in the animals. Feeding of levamisole at any of the dose levels for a period of 7 days could not bring the reduction in mortality of prawns. However, prawns challenged after 14 days of levamisole feeding showed significant decrease (P<0.05) in mortality in groups B, C and D compared to control group prawns. Group C prawns showed significantly lower mortality compared to group D prawns (Table 2).

DISCUSSION

To combat disease problems, knowledge of prawn defence mechanism is very essential. Innate immune system of prawns and its induction mechanisms for disease resistance have been explored, but not so sufficient to give perfect solution for disease problems. Thus, for short-term protection from disease in culture system, use of immunostimulants is widely accepted. As these substances are biocompatible, biodegradable, safe for the environment and human health along with having certain nutritional values, they are slowly replacing antibiotics and vaccines for prevention of diseases. Different immunomodulatory effects of levamisole have been established in higher vertebrates including fish (Sahoo and Mukherjee, 2002; Li *et al*., 2004; Kumari and Sahoo, 2006). Levamisole is not a constituent in prawn feed and it can be easily incorporated in feed at known concentrations.

Levamisole is a synthetic compound, which has widespread effects like enhancement of serum lysozyme activity, serum antibody titres after immunization, the expression of cytokines by macrophages, lymphocyte proliferation and antitumor responses (Sakai, 1999). However, immunosuppressive effects of levamisole have already been reported (Mulero *et al*., 1998; Li *et al*., 2004). Previous studies regarding the possible

effects of levamisole on fish immune system have enriched our knowledge to use it as an immunostimulant in fish farming. However, no studies focus on the optimal dose, time period of feeding and efficacy of levamisole in the sub-adult giant freshwater prawn *M. rosenbergii.* Therefore, the present study aims towards gaining information regarding levamisole action on the innate immune system of *M. rosenbergii.*

In this study, for reasons of economy and efficiency, we looked for the minimum dose that would induce immunostimulation in prawn in the shortest time possible. Since time and/or dose-dependent effects are to be expected, we tested three dose levels over two different time periods, parameters which are fundamental in any immunomodulatory strategy (Sakai, 1999; Chand *et al*., 2006). Levamisole is water-soluble and gets easily absorbed in digestive tract, which is a requirement for triggering protective responses in the prawn. It is stable at room temperature and is also relatively resistant to proteolytic degradation, as it is not a protein. Thus, it can overcome the problem of being attacked by proteolytic enzymes in the gut. To overcome the problem of handling related stress, we planned oral administration of dietary levamisole to prawns. Taking the above points into consideration, we tried to evaluate incorporation levels of 125, 250 and 500 mg levamisole/kg feed on immunity and disease resistance of prawn after 7 or 14 days feeding. Feeding of levamisole at 500 mg/kg feed did not cause even any mortality during 7 or 14 days trial, thus indicated non-toxic nature up to this dose and time period of feeding to this species as also being observed by earlier workers (Baruah and Prasad, 2005).

Nitrite, a byproduct of ammonia excretion is a major threat to intensified aquaculture as it is highly toxic to aquatic animals at high concentration. It has been reported that the concentration of nitrite increased along with culture period, and reached as high as 20 mg/l in grow-out ponds (Tacon *et al.,* 2002). Elevated nitrite has been reported to cause growth suppression, increased rate of moulting, oxygen consumption, ammonia excretion and in extreme cases, death of decapod crustaceans (Chen and Chen, 1992). The effect of nitrite and nitrate on prawn growth has already been studied with *Penaeus indicus* and *P. monodon* as well as in *M. rosenbergii*, where reduction of growth occurred up to 50% in *P. indicus* when 6.4 mg NO_2-N/l was applied to it. Nitrite was found to be more toxic to *M. rosenbergii* than marine shrimp (Wickins, 1976). Among prawn species, *M. malcolmsonii* is more susceptible to nitrite than *M. rosenbergii* (Chand and Sahoo, 2006). Chen and Lee (1997) observed 96 h LC_{50} value of NO_2-N to be 8.5 to 12.9 mg/l in *M. rosenbergii.* Exposure of prawns after 7 days or 14 days of levamisole feeding to 9 ppm of sodium nitrite showed variation of mortality from 33.33% to 66.66% after 120 h of observation in 7 days levamisole-fed groups. The least mortality was found in group C prawns fed with levamisole of 250 mg/kg feed compared to control group prawns which showed 66.66% mortality. After 14 days of levamisole feeding, a significant reduction in mortality was observed in different groups of levamisole feeding compared

to control after 120 h of exposure. Mortality was the lowest i.e. 33.33% in groups B and C fed at 125 and 250 mg/kg feed respectively whereas control group prawns showed 100% mortality. Chand *et al*. (2006) found the lowest mortality of 16.67% compared to 75% in control group after 120 h of nitrite treatment after 14 days of lactoferrin feeding to prawns.

The role of immunostimulants of different origin in protecting shrimps from wide range of pathogens is well known. In shrimp, oral administration of β-1, 3- glucan was found to be effective against bacterial infections (Takahashi *et al*., 1995; Liao *et al*., 1996). Chand *et al.* (2006) found a significant reduction in mortality in lactoferrin fed *M. rosenbergii* after 7 or 14 days when challenged with virulent *A. hydrophila*. In this study, a significant reduction in mortality was observed in groups B and C prawns compared to control after 14 days of levamisole feeding. Thus, levamisole has a positive effect on the disease resistance against pathogens. The enhanced survival due to bacterial challenge and nitrite stress is well correlated with the enhanced immune response marked in those prawns.

Several crustacean lectins have been characterized with a heat labile and calcium dependent substances called agglutinins (Acharya *et al*., 2004). Feeding of levamisole at any of the dose levels for 7 days did not bring any change in the serum haemagglutinating activity against rabbit RBC. Similarly, feeding with lactoferrin at different dose levels to *M. rosenbergii* was not able to raise haemagglutinin level against rabbit RBC (Chand *et al*., 2006). On the other hand, considerable enhancement in haemagglutinin level was marked in this experiment after 14 days of levamisole feeding compared to control. Sritunyalucksana *et al*. (1999) and Chand *et al*. (2006) also observed similar result in haemagglutinating titre in *P. monodon* and *M. rosenbergii*, respectively. Immuplus (AquaImmu), a herbal immunomodulator (Indian Herbs, Saharanpur, India), was also found to enhance haemagglutination titre level in *M. rosenbergii* after incorporating 1g/kg feed for 3 weeks (Kumari *et al*., 2004). The highest haemagglutination titre was found in group C prawns fed levamisole at 250 mg/kg diet for 14 days.

Previous studies have shown that prawn and shrimp have agglutinins in their sera that can agglutinate Gram-negative bacteria (Acharya *et al*., 2004). Bacterial agglutinins against *Bacillus cereus* and *Aeromonas* sp. in *M. rosenbergii* (Vazquez *et al*., 1996), *Vibrio* sp. and *Pseudomonas* sp. in *P. indicus* (Jayasree, 2001) have also been reported. Lectin recognizes different polysaccharide components like bacterial O-keto O-methyl containing sugars, the N-acetyl sugars residue and teichoic acid from the polysaccharide cell wall. Bacterial agglutinin level did not differ significantly among different groups compared to control group after 7 days of levamisole feeding. However, raised agglutinin levels were marked in groups C and D compared to group B. Further, after 14 days of levamisole feeding, a significant decrease in titre was obtained in group D prawns

compared to other groups. Earlier studies also indicated no significant enhancement in agglutinin levels after 14 days of either lactoferrin feeding to prawns (Chand *et al*., 2006) or feeding with 0.4% peptidoglycan or 0.002% lipopolysaccharide in *P. monodon* (Sritunyalucksana *et al*., 1999).

Haemocyanin, a respiratory protein, is the most abundant molecule of the haemolymph (60-90% of total protein) (Djangmah, 1970) followed by clotting protein and other humoral components. In this study, there was no significant alteration in the total protein level after 7 or 14 days of levamisole feeding to prawns indicated negligible effect of levamisole on total protein level.

Lysozyme is an enzyme, which has bacteriolytic effect. It hydrolyses β-1, 4 glycosidic bond of bacterial cell wall peptidoglycan. In Indian river prawn *M. malcolmsonii*, lysozyme level of 0.04 units/ml of haemolymph has been found by Acharya *et al.* (2004). Kumari *et al.* (2004) found a significant high level of lysozyme activity after 3 weeks of treatment of 1 g/kg feed Immuplus (AquaImmu) in *M. rosenbergii.* On the contrary, we did not find any significant difference in the lysozyme activity at any of the dose levels of levamisole feeding and time periods among different groups of levamisole fed prawns.

Experimenting on different substances for their immunostimulatory effects, activation of prophenoloxidase system is an important criterion for the judgement of effectiveness of that substance. In the present study, no alternation in the PO activity among different groups after 7 days of levamisole feeding to prawns was noticed. Chand et al. (2006) found enhanced PO activity after 7 days of lactoferrin feeding to prawns. However, after 14 days of levamisole feeding, a significant enhancement in the PO activity in the prawns fed diets containing levamisole at all the graded levels compared to control was noticed. Kumari *et al*. (2004) obtained similar result by feeding Immuplus at 1 g/kg feed up to 3 weeks to the same species. In *P. monodon*, the highest level of PO activity was marked after 9 days by dietary feeding of β-1, 3-glucan at 2, 10 or 20 g/kg concentration (Chang *et al*., 2003). Thus, levamisole can be considered along with LPS, glucan, sodium alginate, lactoferrin and herbal products for triggering PO activity in prawns indicating an increase in immune ability.

In this study, there was no significant change in the total haemocyte number among different groups of prawns fed levamisole diet for 7 days. However, a marked decline of THC in the groups B and C prawns fed levamisole diet at 125 and 250 mg/kg was noticed compared to control prawns after 14 days. Chand *et al.* (2006) observed that incorporation of lactoferrin at 50 mg/kg diet for a period of 7 days increased the THC compared to other groups and there was no significant difference in different groups of lactoferrin fed prawns after 14 days. The reduction in haemocyte count has also been

marked in other crustaceans after exposure to immunostimulant (β-1,3-glucan) as a consequence of cell clumping in vivo implying a specific cellular recognition of and reaction to soluble non-self molecules in crustaceans (Smith *et al*., 1984; Johansson and Soderhall, 1985). This study indicates that levamisole do not stimulate more production of haemocytes and the resistance observed in terms of increased survival in levamisole fed prawns to nitrite or bacterial stress may be routed through mostly humoral factors viz., PO activity and haemagglutinin levels.

We measured the count of three different haemocytes in different groups of experimental prawns and the ranges showed similarity with the finding of Vazquez *et al.* (1997) who suggested the presence of 70% fusiform or hyaline haemocytes, 20% large granular cells and 10% undifferentiated cells in the intermoult *M. rosenbergii*. Except a significant difference in fusiform cells between groups B and C prawns fed at 125 and 250 mg of levamisole/kg feed, there was no observable alternation in fusiform cells, large granular cells and undifferentiated round cells among graded levels of levamisole fed prawns after 7 days. Similarly, fusiform cells and large granular cells showed no significant differences after 14 days of levamisole feeding. However, a marked increase in undifferentiated cells was observed in prawns fed at 125, 250 and 500 mg/kg of levamisole feed compared to control prawns. It has been proposed that undifferentiated cells are nothing but immature form of granular cells (Vazquez *et al*., 1997) or granular cells releasing their granules that contain the proPO system. Thus, the increase in percentage of undifferentiated cell might be partly responsible for increase in PO activity observed in levamisole-fed prawns for 14 days.

Thus, it may be concluded that levamisole can be incorporated in feed of grow-out *M. rosenbergii* at 250 mg/kg feed for a continuous feeding of 14 days, to enhance their immune status by increasing haemagglutination titre, phenoloxidase activity, undifferentiated cell count and increase in resistance to bacterial infection and nitrite stress.

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