Bacteriological studies on diseased freshwater prawn Macrobrachium rosenbergii infected with vibriosis

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Abstract: During the last decade many improvements have taken place in aquaculture, especially in prawn and shrimp farming. The shift from extensive to intensive and semi-intensive farming has brought about an increase in disease outbreaks, especially by bacteria. To control these diseases, antibiotics have been used indiscriminately. To avoid the use of antibiotics and the development of resistant strains of bacteria, we studied the efficacy of levamisol Hcl, as an antibacterial and immunostimulant in Macrobrachium rosenbergii (de Man). Efficacy was evaluated in vitro by minimum lethal concentration levamisol Hcl and found to be 1.0 ppm for P. fluorescens and 1.5 ppm for E. tarda, V. alginolyticus, S. aureus and A. salmonicida. The 24 hr LD50 of levamisole Hcl for two month old M. rosenbergii was 10.0 ppm. Its immunostimulant effect was evaluated by challenging levamisole Hcl treated M rosenbergii (1.5 ppm levamisole Hcl bath for 15 days) with P. fluorescens and V. alginolyticus. The NBT (nitroblue tetrazolium) assay showed that levamisole Hcl treatment stimulated nonspecific immune response by the activation of granular cells. It also protected M. rosenbergii from disease when compared to untreated controls. A single treatment of levamisole Hcl was effective for P. 46 hours for V. alginolyticus. Bath treatment at 2.0 ppm for one hour showed successful control of bacterial infection in M. rosenbergii previously infected with the bacteria. The results showed that levamisol Hcl could be used as an effective antibacterial and immunostimulant in M. rosenbergii.

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Key words: Macrobrachium rosenbergii- V. alginolyticus- levamisole Hcl- immune response- immunostimulant.

Introduction

The ever increasing demand for prawns in the international and domestic markets has stirred tremendous interest among aquafarmers, entrepreneurs, and industrialists to experiment with new culture methods. The adoption of an intensive aquaculture system in an unplanned and unscientific manner has caused additional stress on shrimp and prawns. The indiscriminate use of antibiotic for controlling bacterial disease has led to the development of an antibiotic resistance that is transferable to nonresistant bacteria by R-plasmids (Aoki and Kitao 1985).levamisole Hcl used as antibacterials for controlling bacterial diseases in aquaculture (Anderson and Conroy 1969). It is a nonspecific antibacterial chemical used for the treatment of columnaris disease in juvenile freshwater fish and bacterial gill disease in juvenile salmonids ,it is used also for the treatment of vibriosis. It have been used for the treatment of bacterial black spot and red gill disease of juvenile and adult shrimps (Baticados et al. 1990). Jeney and Anderson (1993) developed a method to enhance the immune response in rainbow trout for protection against Aeromonas salmonicida by using a bacterin after prior immersion for 30 min in an immunostimulant solution containing levamisole. This elevated both the specific and nonspecific immune responses.

The synthetic phenylimedazolthiazole or levamisole has been extensively utilized in veterinary and human medicine as antihelminthes, antibacterial and immunomodulating effects in fish (Anderson 1992). It is reported that carp fingerlings treated with levamisole showed increased growth and reduced mortality (Siwicki and Korwin-Kossakowski 1988). This trial was conducted to ascertain the implications of antihelminthes in the culture of prawn to facilitate stimulated growth and better survival. In present study levamisole Hcl was tested for its efficacy in controlling bacterial infections (Vibrio. alginolyticus infection) and in stimulating immunity in M. rosenbergii.

Materials and Method

Bacterial species used

The bacteria utilized to induce experimental infection for minimum lethal concentration (MLC) and for challenge studies were Edwardsiella tarda, Pseudomonas fluorescens, Vibrio alginolyticus, Aeromonas salmonicida, and Staphylococcus aureus. subcultured every fifteen days and stored at 4^oC. The tests employed for the identification of the bacteria according to the method of Kreig (1991).

Composition of BHI agar is as follows (Table 1):

Table 1. Composition of BHI agar

Ingredients	gm·100 ml
Calf brain, Infusion from	20.00
Beef heart, Infusion from	25.00
Proteose peptone	01.00
Sodium Chloride	00.50
Disodium phosphate	00.25
Dextrose	00.20
Agar	02.00
Final pH (at 25 ºC)	7.4 ± 0.2

Minimum lethal concentration (MLC)

MLC was tested following the procedure of Alder (1970). Levamisole Hcl was mixed aseptically in sterile distilled water or in unchlorinated bore well water such that the final concentration of active ingredients ranged from 0.01% to 0.0001%. levamisole Hcl was mixed thoroughly and 25 ml/ml of 18 hour broth culture of bacteria (count 3.2 x 1011) was added. The flasks were incubated at 33ºC for 48 hours. Appropriate controls were included in the experiment. If the levamisole Hcl was effective at a particular concentration, it did not allow the multiplication of bacteria and the solution was clear. The solution became turbid in the presence of bacterial growth. Controls consisted of sterile distilled water and unchlorinated bore well water but without levmisole Hcl. To see whether the effect was bacteriostatic or bacteriocidal, 1 ml of mixture from the flask was transferred to a BHI agar plate and incubated at 33ºC for 48 hours. Presence or absence of growth was recorded. The least concentration (highest dilution) at which no bacterial growth was observed was considered as MLC of levmisole Hcl for that particular bacterium.

Lethal dose 50 (LD50)

LD50 of levamisole HCl was performed as per the procedure of Abel (1989). M. rosenbergii (20 at two months old) were reared in 25 l of water kept in a plastic tub of 0.50 meter diameter (50 liters total capacity). All experiments were conducted in triplicate. The required dose of levamisole Hcl was initially diluted in a liter of water and then added to the water in the tubs with the prawns. The different doses experimented were 0.25 ppm, 0.75 ppm, 1.0 ppm, 1.5 ppm, and 2.0 to 10.0 ppm in increments of 1.0 ppm. The prawns were observed for mortality over 96 hours. The concentration at which 50% of the experimental prawns died was considered as LD50.

\Period of activity

To check the period of activity, a series of experiments were conducted using nutrient broth. Media (300 ml) was sterilized in 500-ml conical flasks. To the sterile media, levamisole Hcl was added at either 1.0 ppm or 2.0 ppm. Hourly samples of 3 ml each were then taken in duplicate and transferred to sterile test tubes to which a loopful of bacterial cultures (V. alginolyticus) was added. These were incubated at 33°C for 24 hours before samples were streaked on nutrient agar plates, incubated at 33ºC for 24 hours to record presence or absence of growth.

Nitroblue tetrazolium (NBT) assay

To determine cellular activity, NBT assay was performed by placing a single drop of hemolymph (0.1 ml) on each of two glass cover slips. The cover slips were incubated for 30 minutes at room temperature (22ºC) on damp paper towel before being gently washed with phosphate buffered saline (PBS), pH 7.4 and the edges touched to blotting paper to drain excess solution. A drop of 0.1 ml of 0.2% NBT in PBS was taken and placed on each of two microscope slides. Cover slips were placed on top so that adherent cells could be incubated for another 30 minutes at room temperature with the NBT solution. The activated cells contained bluish granules when treated with NBT dye while nonactivated cells did not contain these bluish granules. The activated granular cells with bluish granules were counted under a microscope at 400 x. Granular cells were mostly spherical and contained large, highly refractive granules which were promptly activated when the cells were exposed to nonself materials.

Challenge studies

Challenge studies were performed to assess the immunostimulant effect and protection level of levamisoleHcl against disease causing bacteria. Twenty five prawns were treated with 1.5 ppm of levamisole Hcl for 15 days (i.e. levamisole Hcl was added to the water by 1.5 ppm at the start and no additional levamisole Hcl was added on subsequent days). Then the prawns were transferred to water free levamisole HCL and challenged by immersion with 1 x 104 organisms ml-1 with V. alginolyticus. Controls were not treated with levamisole Hcl before a similar challenge with the bacteria. The prawns were subsequently observed for gross signs of disease and mortality.

Statistical analysis

The mean and standard error of the mean (SEM) were calculated for NBT assay. Validity was determined by the Student's t test at 5% level of signifycance.

Results and Discussion

The MLC of levamisole HCL for the different

bacteria tested were found to be 1- 1.5 ppm in sterile distilled water and 1.5 ppm in unchlorinated bore well water (Table 2). P. fluorescens was highly sensitive to levamisole Hcl. Gump (1979) has reported that bacteriostatic activity of levamisole Hcl against P. aeuroginosa, V. cholera, S. aureus was 0.06 ppm, 0.51 ppm and 0.80 ppm respectively.

Table 2. Minimum lethal concentration of levamisole for different bacteria. Bacterial species MLC

	in water (ppm)	unchlorinated bore well water (ppm)
P. fluorescens	1.0	1.5
E. tarda	1.5	1.5
V.	1.5	1.5
A.salmonicida	1.5	1.5
S. aureus	1.5	1.5

The LD50 of levamisole HCL for M. rosenbergii (PL20) was 3.0 ppm in 96 hours and 10 ppm in 24 hours. The period of activity of levamisole HCL was tested

For V. alginolyticus (Table 3) in nutrient broth. It was found to be 46 hours at 1.0 ppm and 49 hours at 2 .0 ppm.

Table 3. Period of activity of levamisole HCl on V. alginolyticus in nutrient broth.
Serial no. Time rowth in hours

		1 ppm	2 ppm
1.	Before adding levamisole		
2.	Immediately after adding	+	+
3.	1 hour	-	-
4.	6 hours	-	-
5.	12 hours	-	-
6.	18 hours	-	-
7.	24 hours	-	-
8.	30 hours	-	-
9.	36 hours	-	-
10.	42 hours	-	-
11.	48 hours	+	+
12.	49 hours	+	+
13.	50 hours	+	+

Table 3. Number of activated granulocytes (± SEM) from M. rosenbergii challenged with bacteria in combination with levamisole Hcl.

Group	Activated cells/field
Negative control	2.01 ± 1.01
Positive control	5.05 ± 1.03
levamisole Hcl and bacteria	13.04 ± 2.45

The positive control (bacteria only) and levamisole HCL (1.5 ppm) treated groups were significantly different from the negative control group ($P < 0.05$). The levamisole Hcl treated group was significantly different from the positive control group ($P < 0.05$).

The NBT assay (Table 3) showed that untreated M. rosenbergii (negative control) had a baseline of 2.01 ± 1.01 activated granular cells/field while prawns challengedwithout immunostimulant had 6.05 ± 1.03. Prawns treated with 1.5 ppm levamisole Hcl and then challenged showed 12.04 ± 2.45 activated cells/field. The activated cells contain bluish granules when treated with NBT dye while nonactivated cells do not contain these bluish granules. Certain cells like neutrophils ingest and reduce nitroblue tetrazolium (NBT) dye. The yellow dye is taken up by the activated cells and reduced to blue derivative. The blue particles containing cells are counted directly under a microscope. The dye is only reduced in activated cells, therefore the number of cells reducing the dye gives an idea of the proportion of activated cells in vivo (Hudson and Hay 1989). The NBT assay was tried to assess the activation of cells, for the first time in crustaceans.

M. rosenbergii treated with levamisole Hcl and challenged with pathogenic bacteria showed no gross signs of disease and there was no mortality. By contrast, prawns not treated with levamisole Hcl and challenged with pathogenic bacteria showed signs of disease. Prawns infected with V. alginolyticus showed degeneration of tissues of the telson. A precise knowledge of the relationship among levamisole, feed requirements and body weight of cultured animals is essential to attain the desired growth. Deterioration of water quality with the dumping of excess feed and ensuing excreta might have worsened the living conditions of the animals. Moreover, the duration of moulting period, growth inhibiting hormones and cannibalism affect normal growth and survival invariably. Nevertheless, application of levamisole would be helpful to effectively minimize the natural mortality of the farmed prawn. This experiment evinces a positive tilt towards the dietary application of levamisole in Macrbrachium rosenbergii culture but further comprehensive and field trials are highly war-ranted to establish its efficacy as a growth facilitator. Future studies that employ similar methodologies, however, may shed more light on whether dietary ingestion of levamisole really increases, decreases or does not affect the growth and survival of scampi at lower, higher or differing doses.

Mulero et al. 1998 reported that bathing rainbow trout for 30 minutes in immunostimulant solution before a 2-minute bath in A. salmonicida antigen bacteria elevated both the specific and nonspecific defense mechanisms. Bath treatment of prawns challenged with V. alginolyticus with 2.0 ppm

levamisole for one hour was found to be successful and the prawns were clinically healthy for 7-10 days thereafter.

Each group of prawns treated with levamisole HCl followed by a challenge with V. alginolyticus showed increased nonspecific defenses when compared to prawns given the bacterin alone. The mode of action of levamisole in increasing the protection was not known. There is no clear explanation why levamisole is so dose sensitive since it is widely assumed as a growth enhancer (Mulero et al. 1998) therefore, further research would be forthcoming.

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