EFFECT OF IMMUNOSTIMULANT ON ENHANCEMENT OF THE IMMUNE RESPONSE OF KURUMA SHRIMP *Marsupenaeus japonicus* AND ITS RESISTANCE AGAINST WHITE SPOT SYNDROME VIRUS (WSSV)

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ABSTRACT

An experiment of 45 days duration to test the efficacy of the *Vibrio alginolyticus* as an immunostimulant was conducted in *Marsupenaeus japonicus* against white spot syndrome virus (WSSV) infection, considered as one of the serious pathogens of shrimps. The shrimps were fed with the experimental diets coated with different concentrations of bacteria of 17 ml / kg feed and 10 % of animal body wt. They were challenged with WSSV to assess the increase in survival rate and growth if any due to immune enhancement. Another group of shrimp was used for drawing the haemolymph to estimate the increase in the level of prophenoloxidase activity. Samplings for the above said analysis were carried out at 15 days interval for a period of 45 days. The highest prophenoloxidase activity (39.78 with sand substrate-T1) and the highest survival rate (66.66 % with sand substrate-T1) and highest growth rate (7.04 % in 17ml/kg- with sand substrate-T1) were recorded in experimental diet (17 ml / kg feed). Hence the 17 ml / kg feed of *Vibrio alginolyticus* formalin killed added to the diet could be an eco-friendly and economically viable immunostimulant for penaeid shrimps.

Key words : Kuruma Shrimp, *Marsupenaeus japonicus,* Prophenoloxidase activity, *Vibrio alginolyticus,* immunostimulant and WSSV Challenge

INTRODUCTION

Shrimp culture has undergone rapid development in most south-east Asian countries, but successful production is increasingly hampered especially by diseases in addition to environmental pollution and poor management practices. Of all the infectious diseases, bacterial infections cause serious diseases like vibriosis, ulcer disease, tail rot, necrosis etc. Vibriosis, especially luminescent disease caused by *Vibrio harveyi* leads to mass mortality in the shrimp hatcheries (De la Pena *et al.*, 1993).

At present Shrimp culture in India has high susceptibility to a plethora of diseases which resulted severe loss. Traditionally, treatment of shrimp diseases was carried out by the use of chemical and antibiotic products. Chemical treatment is intricate mainly due to the huge requirement of chemicals needed to produce the desired therapeutic effect and the after effects of hither to unknown consequences of such treatment to the ecosystem. The fear of development of plasmid mediated resistant strains of bacteria to antibiotics (Gabriel AG and Felipe AV, 2000) and accumulation in the shrimp, restricts continuous use of the antibiotics. However, this indiscriminate and unabated usage of chemicals and antibiotics has led a serious debate amongst environmentalist and governmental agencies for complete ban of these products. These reasons paved way for the development of wide array of strategies in sustaining the shrimp aquaculture. Thus, the concept of biological disease control, particularly using non-pathogenic bacterial strains for disease prevention has received wide spread attention during the last decade (Gram, *et al.*, 1999).

Effective vaccination of penaeid shrimps with a formalin – killed *Vibrio* spp. vaccine has been reported by Kou *et al.* (1989) and Itami *et al.* (1989). However, study on the development of *V.harveyi*

vaccine and its effect on vibriosis in *Fenneropenaeus indicus* was found scarce.In the present study, a more effective way of protecting cultivable penaeid shrimps against vibriosis, by using formalin - killed *V.alginolyticus* as immunostimulant was developed and the relative percent survival (RPS) of immunostimulated against control *Marsupenaeus japonicus* and growth was also investigated.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted at Muttukkadu Experimental Station of Central Institute of Brackishwater Aquaculture, CIBA, Chennai. for a period of 45 days in six distinct experimental groups namely C1, T1, T2 (with sand substrate) and C2, T3, T4 (without sand substrate) with each group having three replicates including controls. Uniform size Fiberglass Reinforced Polypropylene (FRP) tanks of 100 L capacity each were cleaned and disinfected with bleaching powder before starting the experimental trial. The tanks were filled with 70 L of filtered seawater and continuous aeration was provided throughout the experimental period. The juvenile shrimp average weight 4 ± 2 g with 2 nos/m^2 density were stocked in100 L tank capacity. The juveniles of *Marsupenaeus japonicus* from single broodstock were obtained from CIBA hatchery. PCR test was done before the shrimps were used for the experimental trials.

Immunostimulant Preparation

Innoculum of pure *Vibrio spp., viz., V. alginolyticus,* were cultured individually in pre-sterilized alkaline peptone water broth (15g / l, pH 7.4, 1% NaCl), at room temperature for 48 hrs. After 48 hrs, the bacterial preparation was harvested by centrifugation at 8000 rpm for 5 min at 4°C. The supernatant was collected and the pellet was resuspended in sterile Phosphate Buffer Solution (PBS) .The cultures were inactivated by formalin method: Adding 0.5% Formalin to 1 L of bacterial culture (Akhlaghi, 1999). The sterility test of killed bacteria was conducted by inoculating the preparation on TCBS plates and incubating at 37°C for 24 hours. The formalin killed

bacterial cultures were diluted individually in PBS to give a series of concentration of $10^5 – 10^9$ cells / ml. The solutions were stored in glass bottles at 4°C till further use. However, during the suspension of desired concentration was prepared fresh, mixed with the shrimp feed and sun dried for 2-3 hours before feeding.

The experimental animals were fed with commercial shrimp feed (2mm size). Two experimental diets using commercial feed were mixed with T1 (17 ml $(1 \times 10^8$ cells)/kg), T2 (10% of animal body wt.) (with sand substrate) and T3, T4 (without sand substrate) and each of with formalin killed *Vibrio alginolyticus* bacterial suspension and dried for 1 hour before feeding. The control feed C1, C2 (with sand substrate) and C3, C4 (without sand substrate) were devoid of any bacterial suspension. The juveniles were fed thrice a day at 08.00 hrs, 14.00 hrs and 18.00 hrs at the rate of 8% of the body weight per day. The daily allowance was divided into three equal portions. The rate of feeding was adjusted daily depending on the feed consumptions. Complete (100%) water exchange was carried out in all the tanks everyday. Daily, before water exchange, abiotic parameters – salinity, pH and temperature were recorded. Before water exchange, samples were collected twice a week and estimated for ammonia and nitrite using standard methods.

Parameters for evaluating efficacy of immunostimulant :

Growth Rate

In order to assess the growth performance in the tank, sampling was conducted in the tank regularly with the Ricker, W. E. (1979) methodology. The final weight of the shrimps was taken at the end of the study (45 days); growth percentage and survival percentage (Sung *et al.*, 1994) were calculated using the formulae given below.

$$\text{Growth\%} = \frac{\text{(Final weight} - \text{Initial weight) of shrimps}}{\text{Initial weight of shrimps}} \times 100$$

$$\text{Survival\%} = \frac{\text{(Final number} - \text{Initial number) of shrimps}}{\text{Initial number of shrimps}} \times 100$$

Prophenoloxidase Activity Assay

Prophenolxidase activity (proPO) in haemocytes of shrimp's haemolymph was measured by the formation of dopachrome from L-Dihydroxy-phenylalanine (DOPA) (Soderhall and Unestam, 1979) on a Multiscan ELISA reader. Prophenol-oxidase activity (proPO) in the haemolymph of *M japonicus* fed with or without immunostimulant was estimated. 500µl of haemolymph was drawn from the ventral sinus (5th pleiopod) of shrimp in a 1000 µl syringe pre-chilled with 0.5 ml of cold anticoagulant solution. The haemolymph was collected in pre-cooled Eppendoff's vials (2 ml). The samples were thawed and immediately analyzed for proPO activity. The haemolymph collected and centrifuged at 5000 rpm for 5 min and the supernatant fluid i.e., haemocyte lysate supernatant (HLS) was separated from plasma protein for determination. The cell suspension was incubated with Cacodylate buffer for 30 min at room temperature. At the end of incubation, the mixture was centrifuged for 3 min at 6000 rpm. The supernatant was collected and estimated for proPO activity.

ELISA Reading

Triplicates of 50µl of haemolymph sample were taken and to which 50µl of trypsin was added and incubated at room temperature. After 20 min of incubation, 50µl of L-DOPA was added and the optical density was measured at 490 nm on an ELISA reader every one minute interval for 5 minutes. Protein concentrations of haemolymph were determined by Lowry *et al.,* (1951), method. To 1 ml of diluted protein standard (BSA) and haemolymph sample, 5 ml of alkaline copper sulphate was added and mixed thoroughly. The solution was put under incubation. After 30 minutes, the blue colour so developed was measured at 640 nm using UV-Spectrophotometer (Shimadzu). Using the standard curve, protein concentration of unknown samples were determined by plotting concentration (mg / ml) of BSA against

standard 640 nm . One enzyme activity unit was equivalent to an increase of 0.001 in absorbance. Prophenoloxidase (unit/min/mg of protein) was calculated using the formula.

$$\text{Change in Absorbance} = \frac{2^{nd} - 1^{st}}{1} + \frac{3^{rd} - 2^{nd}}{1} + \frac{4^{th} - 3^{rd}}{1} + \frac{5^{th} - 4^{th}}{1} \times 2$$

$$\text{Prophenoloxidase level} = \frac{\text{Change in absorbance}}{\text{Protein value of plasma}}$$

Challenge studies

The tank cultured shrimps from both treatment and control tanks were challenged with *V.alginolyticus* as well as White Spot Syndrome Virus (WSSV). The harvested live shrimps from both treatment and control were maintained in troughs (15 l) and acclimatized for 24 hours prior to the challenge test. The infected WSSV shrimp were cut into small pieces and fed at the rate of 3% of the body weight of the shrimps to both groups-treatment and control groups which were maintained separately. After 4 hours of feeding the residual feed and water was removed and replaced with fresh sea water. The shrimps were fed with commercial feed till the conclusion of the experiment. On forty-five days of post vaccination, the Juveniles were challenged with *V. harveyi* (6.1×10^5 cfu/ml- challenge dose) and the mortality of the shrimps was recorded for a period of 5 days (Itami *et al.,* 1989). Mortality number and time of death was monitored for 7 days and clinical signs were verified by re-isolation of WSSV from freshly challenged dead shrimp or survivors through PCR tests. Cumulative mortality was registered and percent survival was calculated.

Polymerase chain reaction (PCR) screening for WSSV :

WSSV Isolation and Preparation

Virus used in this study originated from an infected shrimp farm located near Muthukadu, Chennai. The presence of WSSV in infected shrimp was confirmed by PCR analysis of DNA isolated. The WSSV stock

solution was prepared following the method of Tsai *et al.* (2000). Infected shrimp were homogenized in TNE and then centrifuged at 1700xg for 10 min at 4°C. The supernatant was filtered through a 0.45 ìm membrane and was stored at -80°C until used for challenging.

Detection of WSSV

Different parts of shrimp body such as pleopods, gills and tail were cut using a sterilized scissor. The parts were immediately digested using the solubilising buffer (NaOH -100 µl of 0.05 N, Sodium dodecylsulphate solution-0.025%). The contents were thoroughly homogenized with a Teflon homogenizer and the same was placed in water bath at 100°C for 5 min and then centrifuged at 6000 rpm for 5 min. 5 µl of the collected supernatant were transferred to PCR tubes. PCR cocktail reagents were added and the contents of the PCR vials were mixed gently by tapping with the fingers. The amplification of DNA molecules was performed using the PCR program specific to the kit (WSSV detection kit, Genei, Bangalore and CIBA, Chennai) was used.

Statistical Analysis :

The data collected on the different parameters of the experimental study was subjected to statistical analysis by one way analysis of variance ANOVA (Snedecor and Cochran, 1968), the statistical level of significance was tested at 1% and 5% levels.

RESULTS AND DISCUSSION

Effect of immunostimulant on growth percentage

An insignificantly higher ($P \leq 0.05$) growth percentage was observed irrespective of the treatments compared to control groups. The highest growth percentage was recorded in treatment group (T1) compared to other groups over the period of 45 days with the sand substrate (Table 1). There is no significant difference between the control and treatment groups (C1, T1 and C1, T2). Similarly there

is no significant difference ($P \geq 0.05$) between the control and treatment groups (C2, T3 and C2, T4) in without sand substrate during the experimental period. The maximum average mean value of growth percentage was found to be 7.033 ± 0.274 in with sand substrate treatment group (T1) compared to other groups.

Table 1 : Growth percentages of Kuruma shrimp *(M. japonicus)* fed with immunostimulant reared with and without sand substrate in 45 days of experimental duration.

Serial Number	Treatment	Tank Nos.	Growth Percentage (Mean $\pm$ S.E.)
With sand substrate			
1.	Control	C1	6.149 ± 0.269^a
2.	17 ml / kg feed	T1	7.033 ± 0.274^a
3.	10 % of animal body wt.	T2	6.676 ± 0.271^a
Without sand substrate			
4.	Control	C2	6.472 ± 0.244^a
5.	17 ml / kg feed	T3	6.494 ± 0.234^a
6.	10 % of animal body wt.	T4	6.665 ± 0.169^a

Means not sharing a common superscript letter between column values for each indexes differ significantly ($P < 0.05$).

Effect of immunostimulant on Prophenoloxidase activity

Prophenoloxidase activity observed significant difference ($P \leq 0.05$) between control and treatment groups in with sand substrate (Table 2). The highest Prophenoloxidase activity was recorded in treatment group T1, 39.78 ± 0.365 compared to other groups over a period of 45 days with sand substrate. There is no significant difference ($P \geq 0.05$) between the treatment groups (T3 and T4) but there were significant difference between control and treatment groups (C2, T3 and C2, T4) in without sand substrate during the experimental period.

Table 2 : Prophenoloxidase -Activity in haemolymph of kuruma shrimp *M. japonicus* fed with immunostimulant reared with and without Sand substrate in 45 days period.

Serial Number	Treatment	Tank Nos.	Prophenoloxidase Activity(units / min / mg / protein) (Mean ± S.E.)
With sand substrate			
1.	Control	C1	29.98 ± 0.215^a
2.	17 ml / kg feed	T1	39.78 ± 0.365^b
3.	10 % of animal body wt.	T2	38.81 ± 0.317^c
Without sand substrate			
4.	Control	C2	33.14 ± 0.384^a
5.	17 ml / kg feed	T3	39.01 ± 1.163^b
6.	10 % of animal body wt.	T4	39.62 ± 0.342^b

Means not sharing a common superscript letter between column values for each indexes differ significantly (P < 0.05).

Effect of immunostimulant on survival percentage

Survivay percentage showed that shrimps reared with and without sand substrate at the end of 45 days of feeding with immunostimulant had complete mortality (100%) in the 4th day of post challenge in both control groups (C1 and C2) (Table 3). But in with sand substrate treatment groups (T1 and T2) were recorded in the survival of shrimps in the 7th day of post challenge The highest survival was found to be 66.66 ± 2.842 in with sand substrate treatment group (T1) compared to other treatment and control groups. A PCR test was conducted randomly to check whether shrimps have positive or negative WSSV in all the experimental tanks. It was found that all treatment groups showed negative results except without sand substrate control group which showed positive result of WSSV (Fig. 1 and 2).

Table 3 : Survival percentages of Kuruma shrimp *(M. japonicus)* fed with immunostimulant reared with and without sand substrate for a period of 45 days.

Serial Number	Treatment	Tank Nos.	Survival Percentage (Mean ± S.E.)
With sand substrate			
1.	Control	C1	38.33 ± 1.123^a
2.	17 ml / kg feed	T1	66.66 ± 2.842^b
3.	10% of animal body wt.	T2	50 ± 3.015^c
Without sand substrate			
4.	Control	C2	41.66 ± 4.578^a
5.	17 ml / kg feed	T3	58.33 ± 3.859^b
6.	10% of animal body wt.	T4	56.33 ± 3.859^b

Means not sharing a common superscript letter between column values for each indexes differ significantly (P <0.05)
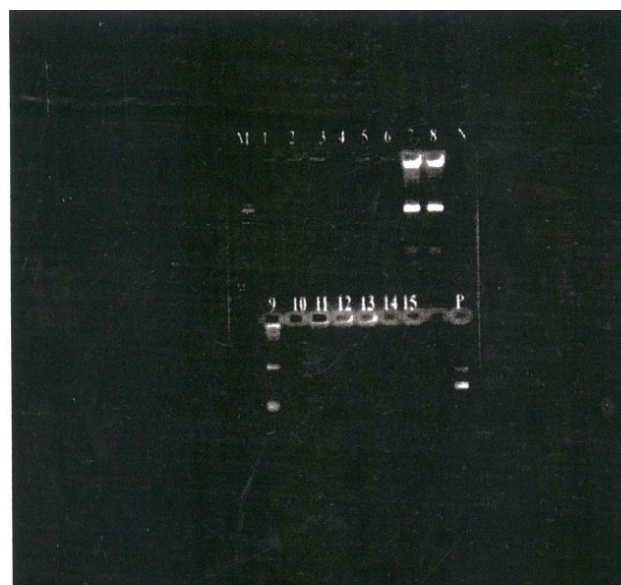

Fig. 1 : Agarose Gel before the Challenge Test from shrimp *Marsupenaeus japonicus* fed with immunostimulant for the period of 45 days.

Lane M-Marker, Lane 1 to 2 -C1, C2, Lane 3 to 6 -T1, T2, T3, T4, Lane 7 to 8-C3, C4, Lane 9 to 11-T5, T6, T7, T8, Lane N-Negative, Lane P-Positive
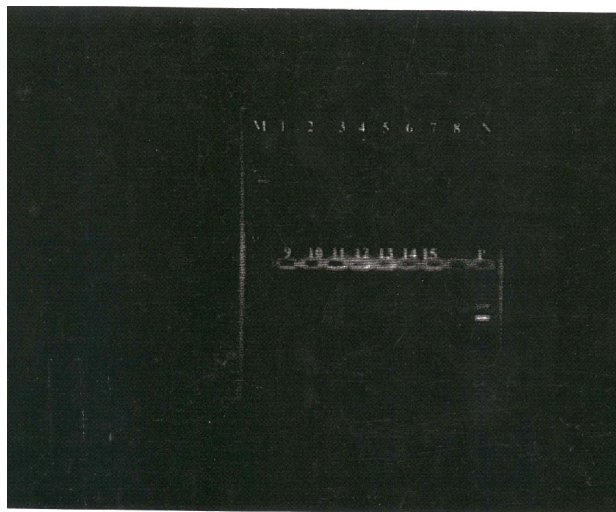

Fig. 2 : Agarose Gel after the Challenge Test from shrimp *Marsupenaeus japonicus* fed with commercial feed for the period of 45 days.

Lane M-Marker, Lane 1 to 2-C1& C2, Lane 3 to 6-T1, T2, T3, T4, Lane 7 to 8-C3, C4

Lane 9 to 11-T5, T6, T7, T8, Lane N-Negative, Lane P-Positive

Different approaches to prevent shrimp diseases have normally involved antibiotics, vaccination against specific pathogens and even different immunostimulants that protect shrimps nonspecifically. Till some years ago scientists believed that shrimps do not possess acquired immunity but recent studies showed that shrimps have acquired immunity and even memory cells that make the vaccinations efficient in these groups of animals (Van Hulten, *et al.*, 2009). In present study we compared two immunostimulants i.e. (a) 17ml / kg feed (b) 10% of animal body wt. in two types of substrates (a) with sand substrate, (b) without sand substrate in Kuruma shrimp, *Marsupenaeus japonicus* looking at its commercial importance. Physiological concentration and the efficacy of immunostimulants were also tested.

The growth percentage was found to be higher in all the treatment groups than the control group. The highest growth percentage was observed in 17ml/

kg feed treatment group T1 (7.033 $\pm$ 0.274) and the lowest growth percentage was recorded in 10% of animal body wt. treatment groups T2 (6.676 $\pm$ 0.271) fed with immunostimulants. In without sand substrate experimental groups, percentage of growth was found to be higher in all the treatment groups than the control group, C2 was 6.472 $\pm$ 0.244 for a period of 45 days. This result is in accordance with study by Montero-Rocha *et al.* (2006); that showed an increase in body weight during 15 days of using Ergosan in intermoult stage shrimp. Similarly, (Sang HM and Fotedar R, 2004) recorded the highest growth of western king prawn, *Penaeus latisulcatus* reared at 34 ppt followed by 22 ppt and 46 ppt. The result obtained in the present study is concurrent with the results obtained by (Muthu MS, 1980) and (Liao IC, 1988). The enhanced survival and growth with immunostimulation suggests that immuno-stimulant elevates the physiological condition and possibly it has an indirect role in the osmoregulation process.

Prophenoloxidase is the key enzyme in the syntheses of melanin. It occurs in haemolymph as an inactive pro-enzyme prophenoloxidase (proPO) and is activated when it reacts with zymosan (carbohydrates for yeast cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ions, trypsin, or heat and converts to active form as proPO. This active proPO is one of the terminal components of a cascade of proteolytic reactions that accompany the recognition of foreign materials in numerous crustaceans and insects (Soderhall K *et.al.,* 1990), (Johansson M W and Soderhall K, 1992) (Nappi A and Vass E, 1993a) and (Nappi AJ and Sugumaran M, 1993b). Results from several experiments of the putative proPO activating system, stimulate several cellular defence reactions, including phagocytosis, module formation, encapsulation and haemocyte locomotion (Soderhall K and Smith VJ, 1986).

At the end of 45 days the prophenoloxidase activity was estimated from the haemolymph in the treatment and control group. All the treatment groups showed higher prophenoloxidase activity than the control groups with sand substrate and without sand substrate. With sand substrate group, highest mean prophenoloxidase activity was recorded in 17 ml / kg feed treatment group T1 (39.78 $\pm$ 0.365) than 10

% of animal body wt. treatment group T2 (38.81 + 0.317). But the case of without sand substrate experimental groups showed highest prophenol-oxidase activity in 17 ml / kg feed treatment group T3 (39.01 + 1.163) than 10% of animal body wt. treatment group T4 (39.62 + 0.342) and followed by the control group C2 (33.14 + 0.384).

The prophenoloxidase factor as a measure of immune enhancement in penaeid shrimps had been demonstrated in number of studies (Soderhall K, Cerenius L and Johansson MW, 1996), (Vargas-Albores F, Jimenez-Vega F and Soderhall K, 1996), (Perazzolo LM and Barracco MA, 1997), (Devaraja *et al.*, 1998) ;(Karunasagar I and Karunasagar I, 1999a) and (Karunasagar I and Karunasagar I, 1999b), (Felix *et al.*, 2004) (Citarasu *et al.*, 2006) observed that prophenoloxidase value increased significantly in the shrimps fed with immunostimulant, while in immunostimulant deficient control groups, it decreased. In the present study the prophenal-oxidase was high in with sand substrate treatment tanks compared to without sand substrate treatment groups, in 17 ml/ kg feed immunostimulant. But it was noticed that prophenoloxidase activity was less in both the control groups. The prophenoloxidase (proPO) activity of haemolymph, an important enzyme linked mediator of crustacean immunity has been demonstrated to be an effective tool that can be used independently or along with challenge studies for confirmation of immune enhancement pattern in penaeid shrimps. The effectiveness of the Vibrio vaccine as an immunostimulant in penaeid shrimps is comparable to those of other immunostimulants (LPS, chitin and â-Glucan) tested with proPO effects (Felix S and Sivakumar, 2003).

The survival percentage was found to be higher in all the treatment groups than the control group, C1 was 38.33 + 1.123. The highest survival was recorded in 17 ml/ kg feed treatment group T1 was 66.66 + 2.842. The lowest survival was recorded in 10% of animal body wt. treatment groups T2 was 50 + 3.015. The highest survival was observed in 17 ml / kg feed of immunostimulant concentration compared to 10 % of animal body wt. of immuno-stimulant concentration.

Without sand substrate experimental group's survival percentage found to be higher in all the treatment groups than the control group, C2 was 41.66 + 4.578. The highest survival in 17ml/kg feed treatment group recorded in T3 was 58.33 + 3.859 and followed by lowest survival in the 10% of animal body wt. treatment group T4 was 56.33 + 3.859. Compared to with sand substrate the survival was low in without sand substrate. The different parameters of the present work revealed that the immunostimunats are very effective against WSSV infection and the outcome of the results will serve long way towards the sustainable shrimp culture which at present struggling under the impact of disease threat.

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