Chitosan and levamisole induced survival and bacterial disease resistance in tiger shrimp, *Penaeus monodon* (Fabricus)

V.A.J. HUXLEY, JINO JOHN, P. SUTHAN AND A.P. LIPTON

ABSTRACT

The immunomodulating effects of a number of compounds including chitosan, and levamisole are reported. This study is intended to determine the efficacy of immunosimulants such as levamisole and chitosan in juvenile *Penaeus monodon* disease management. The study also evaluated the effective dose of immunostimulants in relation to growth and disease management. The dose 50 mg /kg levamisole (Feed C) and 50 mg /kg chitosan (Feed D) produced high AGR and SGR than other doses tested. The same doses administration gave more protection against bacterial challenge at the rate of 60% more than the control.

Key words: Shellfish, Disease management, Immunostimulants, Chitosan, Levamisole

The expression of disease is the result of a complex interaction between agent, host and the environment, although the close contact between animals in most aquaculture systems allows pathogens less time in the inhospitable marine/aquatic environment. The pathogenic stress also leads to the inhibition of growth and survival. Major problem in the control of bacterial disease is that bacteria, which are normally associated with a hatchery, may at certain times become the principal cause of mortality (Vadstein et al., 1993; Misciattelli et al., 1998). *Vibrios* is considered as one of the major causative agents among the bacterial pathogens reported in shrimp aquaculture resulting in high mortalities and severe economic losses (Lightner, 1988; Ruangpan and Kitao, 1991; Goarant et al., 1998; Rodgers and Furones, 1998). Vibriosis affects all life stages of the animals from larva to broodstock. However, it is important to realize that the presence of a potential pathogen is not equivalent to the presence of disease.

The prevention and control of diseases are now considered as the priority subjects for research and development activities in shrimp aquaculture. The developments and sustainability of this industry are very much at stake as shrimp aquaculture faces significant increase in the ecological and pathological problems on a global scale (Bachere, 2000). Immunostimulants are valuable tools for the control of diseases and may be useful in shrimp culture (Huxley, 2002; Huxley and Lipton, 2009). The immunomodulatory effects of glucan, chitin, lactoferrin, levamisole and b-glucan for shrimp have been reported (Sung et al., 1996; Selvin et al., 2004).

In this context, new efforts has been taken to determine the effective dose of immunostimulants such as levamisole and chitosan in terms of growth and disease resistance ability. This study also leads, to develop a package of management strategy in potential cultured tiger shrimp.

MATERIALS AND METHODS

Collection of experimental animal:

The tiger shrimp, *Penaeus monodon* was collected from extensive farm near Rajakamagalm area, Kanyakumari district, Tamilnadu. The collected shrimps were acclimated to laboratory conditions in one tonne fibre reinforced plastic (FRP) tanks. The tank was filled with seawater and maintained with adequate aeration and optimum water quality (salinity = 35 ± 2 ‰; temperature = 28 ± 2°C). The shrimps were fed with pelleted feed (CP Nova, Kochi).

Preparation of immunostimulated feed:

For immunostimulation of *P. monodon*, two immunostimulants *viz.*, Levamisole and Chitosan were used at different doses. The methodology of preparation of the feed and its incorporation are described below:

Levamisole feed:

Levamisole @ 300 mg/kg shrimp body wt (as Feed
A) and @50 mg/kg shrimp body wt (as Feed C) were incorporated in the shrimp grower feed (CP feeds, Cochin). The medical grade levamisole (from MBT Lab CMFRI, Vizhinjam) was finely ground in a mortar and pestle. The required quantity of fine powder was initially dissolved in a small quantity of 1% acetic acid and subsequently in 4% gelatin solution prepared in phosphate buffered saline (PBS) at pH 7.2. The resultant aliquot was sprayed on shrimp feed so as to get the required concentrations of the immunostimulants. The feed thus prepared was dried at 40°C in a hot air oven for 24 hours.

**Chitosan feed:**
Chitosan @300 mg/kg shrimp body wt (as Feed B) and @50 mg/kg shrimp body wt (as Feed D) were incorporated in the shrimp grower feed (CP feeds, Cochin). The chitosan (manufactured by: CIFT, Kochi) powder was initially dissolved in a small quantity of 1% acetic acid and subsequently in 4% gelatin solution prepared in phosphate buffered saline (PBS) at pH 7.2. The resultant aliquot was sprayed on shrimp feed so as to get the required concentrations of the immunostimulants. The feed thus prepared was dried at 40°C in a hot air oven for 24 hours.

**Experimental design:**
The collected juvenile of *Penaeus monodon* were divided into five groups (40 each). They were fed with different doses of immunostimulants (Levamisole and Chitosan) at a rate of 5% of body wt and the other one group was fed with control feed without IS. Before starting the experiment, the wt and survival were noted. During the experimental period, periodic water exchange and unfed removal were done.

**Growth parameters:**
The initial (Day 0) and Final (Day 30) live wet weight of all the experimental group of shrimps was recorded. The growth rate was calculated from the results obtained.

**Challenge experiments:**
For challenge experiments, *Vibrio harveyi* strain obtained from MBT Laboratory, VRC of CMFRI, Vizhinjam was used. On the 30th day, three groups each of healthy and immunostimulated shrimps (10 individuals per group) were challenged with the median lethal dose (LD50) of *Vibrio harveyi*. After the administration, the shrimps were transferred to 60-liter glass aquaria. They were observed for a period of 4 days for mortality and infections. Parallel controls (10 no’s) received 0.2 ml normal saline (0.85%) only. The chosen concentration was inoculated intra-muscularly at the ventral part of second segment. The mortality/infecitivity percentages were estimated by the following formula:

\[
\text{% of mortality} = \frac{\text{No. of dead/infective shrimp}}{\text{Total no. of injected shrimp}} \times 100
\]

**Determination of PRP**
The PRP was determined by the following expression:

\[
\text{PRP} = 1 - \frac{\% \text{ Mortality in the treated group}}{\% \text{ Mortality in control group}} \times 100
\]

**RESULTS AND DISCUSSION**
The results obtained from the present investigation are presented below:

**Growth of shrimps fed with immunostimulants:**
The entire immunostimulated group showed the higher absolute growth rate (AGR) over the control. However, Feed C (@50 mg/kg shrimp body wt levamisole, fed shrimp showed high AGR value 0.106 g/body wt/day followed by Feed D (@50 mg/kg shrimp body wt chitosan) showed 0.076-g/body wt/day. The relative growth rate (RGR) also had the same trend. Feed C fed shrimp showed high RGR value 0.96% followed by @50 mg/kg shrimp body wt chitosan, which gave 0.70%. The detailed results are given in Table 1 and the percentage over the control are depicted in Fig. 1.

**Table 1: Absolute growth and relative growth rate of *P. monodon***

<table>
<thead>
<tr>
<th>Experiments</th>
<th>Absolute growth rate (g/body wt/day)</th>
<th>Relative growth rate (RGR) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0086</td>
<td>0.077</td>
</tr>
<tr>
<td>Feed A</td>
<td>0.0090</td>
<td>0.079</td>
</tr>
<tr>
<td>Feed B</td>
<td>0.0320</td>
<td>0.286</td>
</tr>
<tr>
<td>Feed C</td>
<td>0.1060</td>
<td>0.961</td>
</tr>
<tr>
<td>Feed D</td>
<td>0.0760</td>
<td>0.701</td>
</tr>
</tbody>
</table>

Challenge experiments:

The survival rate of \textit{P. monodon} after challenging with \textit{V. fischeri} \((5.5 \times 10^6 \text{ cells/ml})\) is shown in Table 2. Feed C \((@50 \text{ mg/kg shrimp body wt levamisole showed survival rate of 60\% and was recorded as maximum. The percent relative protection given by the immunostimulated feeds are also given in (Table 2). Feed C \((@50 \text{ mg/kg shrimp body wt levamisole and Feed A \((@300 \text{ mg/kg shrimp body wt levamisole gave the protection to the tune of 60.0\% and 50.0\%, respectively.}}\)

Most of the earlier studies on immunostimulation of shrimps have involved injection or immersion. Since oral administration would be more practical in aquaculture situations, the possibility of improving the disease resistance of black tiger shrimp, \textit{P. monodon}, by oral administration of different concentration of immunostimulants was evaluated.

Growth:

The organism’s different array of feeding behavior normally reflects the growth rate \cite{Cho1985}. From the results of the dissertation, it is inferred that the orally administered immunostimulants helped the experimental groups of \textit{P. monodon}, to attain increased growth rate over the control group. \textit{Shi-Yen-Shiau and Yi-Ping-Yu} \cite{Shi-Yen-Shiau1998} found that chitin supplementation enhanced the growth of \textit{P. monodon}. Experimental results of \textit{Itami et al.} \cite{Itami1991} indicated that the quality of larvae of \textit{P. monodon} improved after the administration of microencapsulated killed \textit{Vibrio} cells. Observations of \textit{Song and Hung}, \cite{Song1993} confirmed the enhancement of growth in shrimp, \textit{P. monodon} by immunostimulation with glucan. However, \textit{Boonyaratpalin et al.} \cite{Boonyaratpalin1993} have reported that higher levels of peptidoglycan in feed had adverse effects in tiger prawn, \textit{P. monodon} and were reflected in the growth and survival. In the present experiments, the higher growth in shrimps fed with 50mg/ kg body wt of levamisole showed safety index than the other groups. The mechanism of better growth might have been possible due to the dose dependent immunostimulation of levamisole. However, in the chitosan fed groups @50mg/kg body wt also had better growth increment.

Challenge experiments:

The beneficial effects of 50mg/kg body wt of levamisole, which manifested the high PRP as well as survival value \((60\%)\) against \textit{V harveyi} over control was recorded. The findings of \textit{Takahashi et al.} \cite{Takahashi1995} after oral administration of immunopotentiators, such as beta -1,3-glucan and peptidoglycan, to shrimp also indicated increased resistance against bacterial infection. Previous data from \textit{Robertson et al.} \cite{Robertson1990} indicated that the use of beta-glucan into Atlantic salmon resulted in a marked increase in resistance to both vibriosis and enteric red mouth disease. Based on their results and the present observations on the duration of enhanced protection in shrimp, levamisole apparently had the potential to be used prophylactically as a short-term immunostimulant for shrimp. According to \textit{Huxley} \cite{Huxley2002}, the immense increase in PRP as well as survival of \textit{P. monodon} when fed with different doses of immunostimulants (levamisole 150 mg/kg body wt shrimp) gave survival up to 70\%. According to \textit{Chang et al.} \cite{Chang1999} shrimp fed with experimental diets of beta -1,3-glucan for post larvae and juvenile and when challenged with WSSV showed less mortality \((p < 0.005)\) in all the glucan-fed groups than in the respective non-glucan control groups. \textit{Liao et al.} \cite{Liao1996} performed challenge experiments in grass prawn fed with four doses of beta-1, 3-glucan by intra muscular injection with \textit{V. damsela}. Their results showed that the beta-1, 3-glucan @0.5-1.0 \text{ g/kg diet significantly (p<0.05) increased the resistance of the prawn against vibriosis. Thus, the protection of the host against invading microbial pathogens can be achieved by using the required quantity of immunostimulants.}

Acknowledgements:

The authors are thankful to the Dept. of
Biotechnology for providing financial assessments in the form of sponsor project. Thanks are extended to Dr. Mohan Joseph Modayil, Former Director and Dr. R. Paul Raj, Former Head PNP Division, CMFRI for the encouragement express my gratitude to Shri K. Silvadasan, Field Assist. DBT project for specimen collection.

Authors’ affiliations:

JINO JOHN, Biotech Research Laboratory, Department of Zoology, Thiru. Vi. Ka. Govt. Arts College, TIRUVARARU (T.N.) INDIA

P. SUTHAN AND A.P. LIPTON, Marine Biotechnology Laboratory, Central Marine Fisheries Research Institute, Vizhinjam Research Centre, VIZHINJAM (THIRUVANANTHAPURAM) INDIA

LITERATURE CITED


