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Immunomodulatory and Growth Promoting Effect of Dietary Levamisole in *Cyprinus carpio* Fingerlings Against the Challenge of *Aeromonas hydrophila*

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Abstract

In order to determine the immunomodulatory effect of dietary intake of levamisole in the common carp, specimen were fed diets containing 0 (control), 125, 250 and 500 mg levamisole kg^{-1} of dry diet for a period of 70 days. Experimental stock was challenged intra-peritoneally with *Aeromonas hydrophila* on 30^{th} and 58^{th} day. Hematobiochemical parameters were determined on day 0, 57 and day 70. The total erythrocyte count, haemoglobin content, hematocrit value, total serum protein, albumin and globulin content were significantly ($P<0.05$) enhanced in levamisole supplemented groups particularly in 250 mg kg^{-1} group; however the TLC was significantly ($P<0.05$) higher in control (infected) group. Lysozyme activity and NBT assay were significantly stimulated in levamisole supplemented groups displaying the highest value in 250 mg kg^{-1} group on day 57. After the stock in all the groups had been challenged intra-peritoneally with *Aeromonas hydrophila* on 30^{th} and 58^{th} day, the relative percentage survival was significantly higher in 250 mg kg^{-1} group (89.6%), followed by 500 mg kg^{-1} group (81.3%) and 100 mg kg^{-1} group (47.9%). Specific growth rate (SGR) was significantly ($P<0.05$) the highest in 250 mg kg^{-1} group (2.67%) and the lowest in control group (1.82%) and feed conversion ratio (FCR) value was found to be the best in 250 mg kg^{-1} group (1.81), followed by 500 mg kg^{-1} group (1.85), 100 mg kg^{-1} group (2.02) and control group (2.32). The findings of the present investigation suggest that the incorporation of levamisole in the diet of common carp fingerlings certainly enhances the non-specific immunity, increases their resistance to infection, and reduces the mortality and enhances the growth of fish.

Keywords: hematobiochemical parameters, nitroblue tetrazolium assay, lysozyme activity, growth, *Aeromonas hydrophila; Cyprinus carpio*.

Introduction

Aquaculture is a fast developing industry. However, unmanaged fish culture practices and adverse environmental conditions affect the fish health leading to production losses. Thus, fish farmers have to carry out careful husbandry practices (Sakai, 1999). In order to achieve optimal fish production, better prophylactic, diagnostic and therapeutic measures are warranted during fish farming operations. The recent expansions of aquaculture has led to a growing interest in understanding fish disease, so that they can be treated or prevented and have at least partial success (Stoskopf, 1993). However, the emergence of antibiotic-resistant microorganisms is important obstacle to their extensive use (Anderson, 1992). Use of expensive chemotherapeutants and antibiotics for controlling disease has been widely criticized for their negative impact like residual accumulation in the tissue, development of the drug resistance and immunosuppression, thus resulting in reduced consumer preference for food fish treated with antibiotics (Anderson, 1992). Prevention of disease is much more desirable than intervention to stop and reverse the disease process once it begins. Hence, instead of chemotherapeutic agents, increasing attention is being paid to the use of immunostimulants for disease control measures in aquaculture.

Levamisole, a synthetic phenylimidazolthiazole has been extensively used in both humans and veterinary medicine as an anti-helminthic agent (Janssen, 1976). The ability of this agent to enhance the response of mammalian T-lymphocytes and macrophages of both healthy (Renoux, 1980) and immunocompromised individuals were well documented (Amery, 1978; Morimoto *et al.*, 1979; Ogunbiyi *et al.*, 1988). In fish, levamisole has been used in a few studies with the aim of enhancing the non-specific immune response (Siwicki, 1987, 1988, 1989; Kajita *et al.*, 1990; Baba *et al.*, 1993) or as adjuvant with a vaccine (Anderson and Jeney, 1992; Jeney and Anderson, 1993). The results obtained through these studies point out the potential use of levamisole in fish as an immunostimulant, although special attention must be paid to the doses administered and the timing, as the effect of levamisole are closely dose and time dependent. High dose of levamisole may suppress the immune response and much lower doses may not be effective at all (Anderson *et al.*, 1989; Siwicki *et al.*, 1990). The duration of any increased immune response in fish after the administration of levamisole as well as the duration of this induced protection remains to be determined. The immunostimulatory potential of levamisole in fish is of considerable interest in the present scenario of aquaculture in combating the

bacterial and parasitic disease of fish because the U.S Food and Drug Administration (FDA) has approved it for treatment of helminth infections in ruminants. Levamisole has been shown to have the ability to up-regulate non specific immune response in carps, rainbow trout and gilthead sea bream (Stickney, 2000).

Aeromonas hydrophila has been reported to cause fin rot disease in hatchery reared *Cyprinus carpio* fingerlings under temperate climatic conditions of Kashmir valley (Hussain *et al.*, 2005). The authors recommended the use of multiple antibiotics as a bath for controlling fin rot disease. However, as mentioned above, controlling diseases with antibiotics is not a safe procedure in aquaculture practices. Hence, the present study is proposed to undertake with following objectives:

1. To determine the effect of levamisole on the non-specific immune parameters of common carp.
2. To determine the efficacy of the levamisole against the challenge of *A. hydrophila*.
3. To evaluate the effect of levamisole on the growth and survival of common carp fingerlings after the challenge of *A. hydrophila.*

Materials and Methods

Fish and Rearing Conditions

Healthy and disease free advanced fingerlings of common carp (*Cyprinus carpio*) having an average weight of 16±2 g and total length of 11±2 cm were procured from fish farm of Faculty of Fisheries, SKUAST-K. The experimental stock was acclimatized for a period of 2 weeks in concrete ponds containing same source of water which was used for conducting the experimental trial and the stock was fed on control diet (D_1). Four experimental concrete ponds with proper inlets and outlets and measuring 6.0 x 6.0 x 1.5 m were used for conducting the experiment. These were thoroughly treated with quick lime and disinfected with $KMnO_4$. The ponds were then filled with the spring water and a uniform water column of 1.2 m was maintained through out the experimental period. Pond water replenishment was carried out every week by replacing 40-50% of the pond water. After acclimatization, the fish were divided into four treatments of 60 specimens each and each treatment was further divided into three replication with 20 specimens each and stocked in the respective experimental ponds. Water quality parameters like temperature, dissolved oxygen, pH and free CO_2 were recorded on weekly basis. Water and air temperature were recorded by a standard quality thermometer. Dissolved oxygen (DO) and pH were recorded with digital DO and pH meter respectively. Free CO_2 was determined titrematically by following the standard procedures (A.P.H.A. 1998). The source of water used for conducting the experimental trial was natural spring water.

The dissolved oxygen content of water throughout the experimental period ranged between 7-10 mg L^{-1}, pH ranged between 7.0–8.5, temperature of the water in the ponds ranged between 31°C during August and 19°C during the end of October. The free CO_2 content of the pond water ranges between 0 to 5 mg L^{-1}.

Experimental Feed

Feed ingredients viz, groundnut oil cake (32.00%), rice bran (26.10%), soybean meal (15.90%), fish meal (3.95%) and wheat flour (20.05%) were procured, screened and subjected to proximate analysis following standard procedure (A.O.A.C. 2006). All the ingredients were properly weighed as per their inclusion rates in the four experimental diets. Diet D_1 served as control diet as it was not supplemented with levamisole, where as diets D_2, D_3 and D_4 comprised of same ingredients as that of D_1 but these were supplemented with Levamisole hydrochloride (Sigma, USA) to give 125, 250 and 500 mg levamisole kg^{-1} of dry diet respectively. The dried pelleted diets were packed in airtight polythene bags and stored at -20°C.

Experimental stock in all the treatments was fed twice daily for a period of 70 days. The feeding rate was set to be 5% of their body weight. The feed was offered in the feeding trays, which were immersed in the ponds at a depth of 0.7 m and inspected to monitor the consumption of feed, which was always found to be consumed in full with in an hour.

Bacterial Strain and Challenge Study

A virulent strain of *Aeromonas hydrophila* received in Tryptose Soya Agar Slants (TSA) from IMTECH (Institute of Microbial Technology, Chandigarh, India) was maintained at 4°C in the Division of Veterinary Microbiology and Immunology, SKUAST-K from this slant culture, sub-cultures were maintained on Tryptose Soya Agar (TSA) slants (Hi-media, Mumbai) at 5°C. A stock culture in Tryptose Soya Broth (TSB) (Hi-media, Mumbai, India) was maintained at -40°C with 0.85% NaCl (w/v) and 20% (v/v) glycerol to provide stable inocula throughout the study period as followed by previous reports (Chabot and Thunne, 1991; Yadav *et al.*, 1992).

The fingerlings in all the groups were challenged with 100 µl of *Aeromonas hydrophila* at a concentration of 1.5±0.3 x 106 CFU ml^{-1} in PBS as a medium. The bacterial suspension in PBS was inoculated intra-peritoneally in all specimens of all the groups by 1 ml insulin syringe on 30^{th} day and the specimens were re-challenged on 58^{th} day. Due care was taken to avoid any injury while challenging the experimental stock with *A. hydrophila*. All the challenged specimens were released back into their respective ponds and were observed for their response

against the injected bacterial strain.

Experimental Regime

The stock was released of 60 specimens each in experimental ponds P1, P2, P3 and P4 and fed on diets D_1, D_2, D_3 and D_4, respectively.

Sampling Schedule:

0 day - 1st blood sample collection.

1st to 70th day - feeding with levamisole supplemented diet (only for treatment groups)

30th day - 1st infectious challenge with *A. hydrophila* (in all the groups).

57th day - 2nd Blood sample collection.

58th day - 2nd infectious challenge with *A. hydrophila* (in all the groups)

70th day - 3rd Blood sample collection.

Length and weight of nine randomly selected specimens, tree from each replication of every treatment were recorded at fortnight intervals and ration was adjusted accordingly on the basis of fish biomass. The fortnight recorded data was used for calculating the feed conversion ratio (FCR) and specific growth rate (SGR).

Six specimens were sampled at random and blood samples were collected on day 0, 57 and 70 from the caudal vein. Collected blood was subjected to different haematological and serum biochemical studies.

Mortality was recorded through out the study period and the relative percentage survival (RPS) was calculated as per the Baulny *et al.* (1996).

Blood and Serum Collection

The blood sampling was carried out for the analysis of the blood parameters, neutrophil activity and serum lysozyme activity. Three fish from each treatment were anaesthetized with MS222 (100 ppm, Sigma, USA). Blood was collected from the caudal vein using a syringe, which was previously rinsed with 2.7% EDTA solution. The blood was then transferred immediately to an Eppendroff tube containing a pinch of EDTA powder, shaken gently and kept at 4°C. The blood was used for determination of haemoglobin content, hematocrit value, total erythrocyte and leucocyte count and for NBT assay. For serum, another three fish from each treatment were anaesthetized and blood was collected in 5 ml test tube without anticoagulant and allowed to clot for 2 h at room temperature in a slanting position. The tubes were centrifuged at 2500 x g for 15 min and the supernatant serum was separated and collected in screw cap Eppendroff tubes and stored at -40°C for further serum biochemical analysis.

Hemato-immunological Parameters

The haemoglobin level of blood was analysed by the cyanmethaemoglobin method using Drabkins fluid (Qualigens Diagnostics, division of Glaxo Smithkline Pharmaceutical Ltd, India) and a haemoglobin standard concentration of 0.06 g dl^{-1} (Oser and Hawk, 1965). The haemoglobin concentration was then calculated by using following formula:

Haemoglobin(g dl^{-1})=[OD(T)/OD/(S)x25 1/1000]x60

where; OD (T) = Absorbance of test; OD (S) = Absorbance of standard.

Erythrocyte count and leucocyte count were determined using a haemocytometer with Neubauer counting chamber as described by Blaxhall and Daisley (1973).

The following formula was used to calculate the number of erythrocytes and leucocytes per ml of the blood sample:

Number of cells = (Number of cells counted x dilution) (ml^{-1})/(Area counted x depth of fluid)

For estimation of hematocrit value, the blood was drawn into heparinized hematocrit pipette up to the graduation mark. The lower opening of the pipette was closed up to 2 cm depth using sealant and heating it carefully over the spirit lamp which closes the upper opening. The pipettes were centrifuged for three minutes with a speed of 3000 rpm and placed on the reading device and read-off. The hematocrit value was expressed as % blood cells in total volume of blood.

Total Serum Protein, Albumin, Globulin and AG Ratio: Serum protein was estimated by Biuret and BCG dye binding method of Reinhold (1953) using the total protein (Qualigens Diagnostics). Albumin was estimated by the bromocresol green binding method of Doumas *et al.* (1971) using albumin kit (Qualigens Diagnostics). Globulin was calculated by subtracting albumin values from total plasma protein. A/G ratio was calculated by dividing albumin values by globulin values.

Serum Lysozyme Activity: Serum lysozyme activity was measured using an ion exchange chromatography kit (Bangalore Genei, India). Serum samples were diluted with phosphate buffer (pH 7.4) to a final concentration of 0.33 mg ml^{-1} protein. In a suitable cuvette, 3 ml of *Micrococcus luteus* (Bangalore Genei, India) suspension in phosphate buffer (A450 ¼ 0.5e0.7) was taken, to which 50 ml of diluted serum sample was added. The content of the cuvette was mixed well for 15 s and reading was taken in a spectrophotometer at 450 nm exactly 60 s after the addition of the serum sample. This absorbance was compared with standard lysozyme of known activity following the same procedure as above. The lysozyme activity was expressed as IU ml^{-1} per mg protein of serum.

Neutrophil Activity: For the nitroblue tetrazolium assay, Secombes (1990) method was followed with modification described by Stasiak and Baumann (1996). The heparinized blood was collected in silica coated Eppendroff tubes and puffy coat was separated by centrifuging at 500 rpm for 10 min. Fifty microlitres of the puffy coat was placed into each well of a 96 well U bottomed microtitre tube plate (Tarson, India) and incubated at 37°C for 1 h to facilitate adhesion of cells. Then the supernatant was removed and 50 µl of 0.3% NBT was added. After incubation for 1 h, the NBT was removed. The cells were then fixed with 100% methanol and washed thrice with 70% methanol and theplate was air dried. Sixty microlitres of 2 N potassium hydroxide and 70 µl dimethylmsulphoxide were added into each well to dissolve the formazan blue precipitate formed. The turquoise-blue colored solution was then read in a microplate reader at 655 nm.

Growth Parameters

The recorded data on weight was used for calculation of the feed conversion ratio (FCR) and specific growth rate (SGR). On each sampling day, the SGR or percent body weight increase per day and FCR for all the experimental groups was calculated according to Ricker (1979) as follows:

SGR =[(Ln of final weight – Ln of initial weight) / t (time interval in days)] x 100.

FCR = [Feed given (dry weight)/Weight gain (wet weight)]

Mortality

Recorded mortality data was used for calculating the relative percentage survival (RPS) following Amend (1981)

RPS=1- [(Mortality (%) in treated group) x 100] (Mortality (%) in control group)

Statistical Analysis of the Experimental Data

The experimental data was subjected to the statistical analysis following the (Completely Randomized Design) CRD and the statistical difference between the treatment means and within the treatment means was assessed by two way analysis of variance (ANOVA) techniques followed by Duncan's multiple range test using statistical package (SPSS) to find out the significant difference at 5% level ($P<0.05$) of significance.

Results and Discussion

Haemato-biochemical Assay

The results related with hematological and serum biochemical parameters obtained in present investigation after challenging *Cyprinus carpio* with *Aeromonas hydrophila* are presented in Table 1, 2 and 3 respectively. The TEC, HCT and Hb content in the control (infected) group showed a significant ($P<0.05$) decrease when compared with the levamisole supplemented groups which may be attributed to any of the following reasons; (1) activation of the alternative complement pathway by lipopolysaccharide (Bradley, 1979), (2) increased phagocytosis of endotoxins-coated red blood cells (Kabir *et al.*, 1978); and/or (3) direct lysis by the bacterial toxins or enzymes (Rigney *et al.*, 1978). Another possible reason may be that, once *A. hydrophila* enters the body of fish, it digests the material such as gelatin and hemoglobin. Our findings are in agreement with that of Bruno and Munro (1986) who reported that the experimental infection of rainbow trout and Atlantic salmon with *Renibacterium salmoninarum* and the subsequent progression of Bacterial Kidney Disease (BKD) resulted in the significant decline in TEC, hematocrit and hemoglobin levels. Similar findings were also reported by (Wedemeyer and Ross, 1973; Suzumoto *et al.*, 1977; Aldrin *et al.*, 1972; Kimura, 1978). During the experimental *A. hydrophila* infection in gold fish, *Carassius auratus*, the RBC count, hemoglobin and HCT content decreased following intra-muscular injection (Brenden and Huizinga, 1986). The highest value of TEC and hemoglobin was recorded in D_3 group indicating the immunomodulatory effect of levamisole. Similar findings were reported by Dina Rairakhwada *et al.* (2007) who concluded that TEC and hemoglobin content were significantly enhanced in the 0.2% levan fed group after the fish were challenged with *Aeromonas hydrophila*.

Yoo *et al.* (2007) also reported that the immunostimulant supplemented group (ß-1,3 glucan) had higher HCT value than the control. Similar findings were reported by Findlay and Munday (2000) while working with levamisole. Present findings differ from Mulero *et al.* (1998) who reported that the levamisole treated group showed significantly ($P>0.05$) lower HCT value than the control. Siwicki and Anderson (1993), also demonstrated that levamisole has less effect on hematocrit levels, there was no significant difference in the hematocrit levels between control and levamisole treated group.

The present study indicates that there was an increase in TLC in the control (infected) group after the fingerlings were intra-peritoneally challenged with *Aeromonas hydrophila* while the levamisole supplemented groups showed depressed level of leucocyte count. This is mainly due to the immune response of the fish immune system against the bacterial invasion. The gradual reversion of the leucocyte count back to the normal in the immunostimulant supplemented groups may be indicative of recovery from systemic damage. These results are similar to those reported by Siwicki (1987)

Table 1. Mean±SE values of feed conversion ratio and specific growth rate values at the end of the experiment

Treatments	Mean FCR	Mean SGR
Control D1	2.32±0.083[a]	1.82±0.104[a]
D2	2.02±0.072 [b]	2.20±0.119 [b]
D3	1.81±0.092 [c]	2.67±0.086[c]
D4	1.85±0.127[c]	2.38±0.093[d]

Values with same superscript in a column do not differ significantly (P>0.05)

Table 2. The value of relative percentage survival (RPS)

Treatment	Total no. of fishes	No. of Mortalities	Survival (%)	RPS (%)
Control D1	60	48	20.00	---------
D2	60	25	58.3	47.90
D3	60	5	91.7	89.60
D4	60	9	85.00	81.30

Table 3. Hematobiochemical parameters of *Cyprinus carpio* intra-peritoneally challenged with *Aeromonas hydrophila* (Mean±SE)

Parameters	Treatments/days	Control D1	D2	D3	D4
TLC ($1x10^3$/ml)	57	42.3±1.0[a]	30.4±0.4[c]	28.7±0.4[c]	34.7±0.5[c]
	70	48.5±0.6[a]	38.2± 0.5[c]	35.2± 0.5[c]	40.2± 0.5[c]
TEC($1x10^6$/ml)	57	1.28±0.01[a]	1.46±0.08 [b]	1.52±0.02[c]	1.50±0.01[d]
	70	1.19±0.02[a]	1.22±0.01[a]	1.31±0.02[b]	1.27±0.02[b]
Hb (g/dl)	57	5.8±0.1[a]	7.5±0.07[ba]	9.0±0.2[c]	8.2±0.2[c]
	70	3.8±0.1[a]	5.7±0.1[b]	7.8±0.2[c]	7.0±0.2[c]
HCT (%)	57	28.2±0.3[a]	33.9±0.4[b]	36.4±0.6[c]	34.6±0.5[c]
	70	22.2±0.5[a]	25.4±0.6[b]	30.4±0.77[c]	28.7±0.8[c]
Total serum protein (g/dl)	57	2.02±0.04[b]	1.89±0.02[a]	2.81±0.02[c]	2.4±0.02[c]
	70	0.99±0.05[a]	1.73±0.03[b]	2.39±0.02[c]	2.15±0.01[c]
Albumin content (g/dl)	57	0.67±0.01[a]	0.81±0.02[b]	0.90±0.03[b]	0.85±0.02[b]
	70	0.37±0.02[a]	0.52±0.03[c]	0.80±0.02[b]	0.72±0.01[b]
Globulin content (g/dl)	57	1.35±0.04[a]	1.08±0.02[c]	1.91±0.04[b]	1.55±0.02[b]
	70	0.62±0.04[a]	1.21±0.04[c]	1.59±0.03[b]	1.43±0.02[b]
Albumin: Globulin	50	0.49±0.02[ac]	0.75±0.03[b]	0.47±0.02[ac]	0.55±0.01[a]
	70	0.59±0.03[a]	0.43±0.04[bc]	0.50±0.02[b]	0.50±0.01[bc]

and Siwicki (1989). TLC increased in yellow tail, *Seriola quinquiradiata* infected with *Nocardia kampachi* (Ikeda *et al*., 1976). Studies on levamisole reported a significant decrease in leucocyte levels in levamisole treated group (Unal and Dorucu, 2005).

The results of the present study revealed that the total serum protein content in the control (infected) group decreased significantly (P>0.05) when fish were intra-peritoneally challenged with *Aeromonas hydrophila*. Total serum protein content at the end of experimental trial was lowest in control (infected) group and highest in D_3 group. Dina Rairakhwada *et al.* (2007) also found that total serum protein content was significantly enhanced in levan fed common carp fingerlings against the infection of *Aeromonas hydrophila* while the depressed values were found in control (infected) group. Similar results were obtained by Bruno and Munro (1986) in rainbow trout and Atlantic salmon experimentally infected with *Renibacterium salmonarium*.

In the present study, the serum biochemical parameters like total serum protein, albumin and globulin were significantly (P<0.05) enhanced in the levamisole supplemented groups particularly in 250 mg levamisole kg^{-1} of diet. The highest values were recorded in levamisole supplemented groups which was at par with chitosan supplemented group. Similar results were obtained by Anderson and Siwicki (1994) who found that total serum protein was elevated by the dietary immunostimulants. Misra *et al.* (2005) also reported that total serum protein content increased significantly (P<0.05) in the fish fed with dietary doses of ß-glucan when compared with control group. Siwicki *et al.* (1994) also found the elevation of total serum protein content after feeding glucans. Popov and Popova (1997) also suggested alternative complement pathway is significantly enhanced by the use of molecule with repeating subunits like levamisole, glucan and levan to facilitate a better aggregation of immunoglobulin, cells and viruses

which are subsequently removed by the phagocytic cells.

In the present study, the mean albumin content in the levamisole supplemented groups was significantly (P<0.05) higher as compared to the control (infected) group when challenged with *A. hydrophila*. The findings of the present study are similar to those of Sahoo and Mukherjee (2002) who reported that the albumin content was higher in α-tocopherol fed group of fish when compared with the control. Results demonstrated by Misra *et al.* (2005) contradict with our findings who reported that albumin content of the ß-glucan fed group do not differ significantly (P>0.05) when compared with control. Dina Rairakhwada *et al.* (2007) also reported that the albumin content do not differ significantly between levan fed group and control group.

Globulin content in the present study was significantly higher (P<0.05) in levamisole supplemented group when compared to control. Levamisole showed the tendency to maintain the globulin level in the serum, which is main source of immunoglobulin production, thus proving its immunostimulatory potential. Similar findings were reported by Misra *et al.* (2005) with ß-glucan as immunostimulant which maintained globulin level significantly higher as compared to control. Anderson and Siwicki (1994a) also reported the similar findings. Dina Rairakhwada *et al.* (2007) also found that the globulin content was significantly enhanced in levan fed common carp fingerlings when challenged with *Aeromonas hydrophila*.

Albumin globulin ratio in the present study does not show a regular trend. In all the groups, A:G ratio does not differ significantly (P>0.05) between pre and post infection sampling days through out the period of experimental study. Similar results were reported by Dina Rairakhwada *et al.* (2007). Misra *et al.* (2005) also reported that A:G ratio in different glucan fed groups was not significantly different from the control group. Sahoo and Mukherjee (2002), reported that reduced A:G ratio was marked in high α-tocopherol fed groups which contradicts with the findings of the present investigation. Increase in A:G ratio after glucan administration has been reported by Mondal *et al.* (2004). The levamisole particularly at the concentration of 250 and 500 mg levamisole kg^{-1} of diet was effective in maintaining the blood and serum parameters of common carp in the normal range as compared to control group in which the hematobiochemical parameters were showing negative trend after being infected with virulent strain of *Aeromonas hydrophila*.

NBT Assay and Lysozyme Activity

Studies on neutrophil activity presented in Figure 1 clearly showed the enhancing effect of dietary levamisole supplement on neutrophil respiratory burst activity, which is evident from the increased NBT reduction. The neutrophil activity was enhanced in all levamisole supplemented groups but the highest significant NBT reduction was achieved in D_3 group on 57^{th} day (0.83±0.07) followed by D_4 group (0.60±0.04), D_2 group (0.48±0.05) and control (D_1) group (0.41±0.04). The production of superoxide radicals as examined by NBT reduction was significantly (P<0.05) influenced by supplementation of levamisole in the diet. Maximum increase in the NBT reduction value was observed in the group fed on diet D_3, which was similar (P>0.05) to the D_4.

The NBT assay is a quick inexpensive test focusing on the ability of phagocytes to reduce the dye by the production of oxygen radicals. In animals, the oxygen radicals are focused at the destruction of bacterial invaders. The ability of macrophages to kill pathogenic microbes is probably one of the most mechanisms of protection against disease among fishes. The higher optical density in the NBT assay was observed in all the treatments. Similar results were obtained by Gopalakannan and Venkatesan

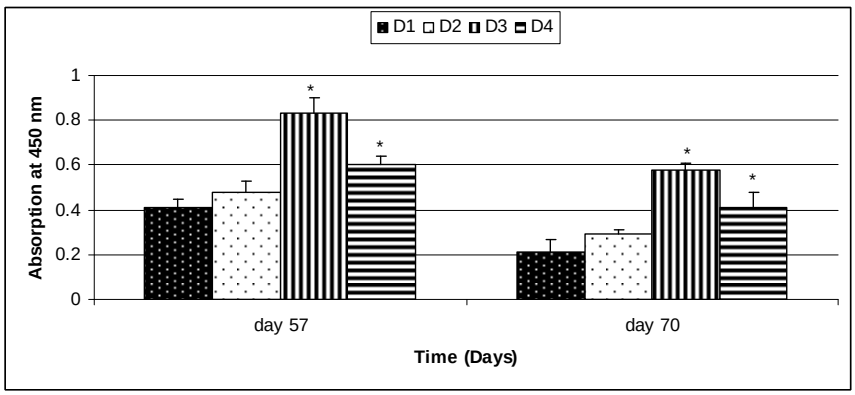

Figure 1. NBT reduction of common carp fed levamisole (100, 250 and 500 mg kg^{-1} diet) and a non-supplemented control diet for 70 days and weighed every 15 days. Asterisks denote statistically significant differences (P<0.05) between control and levamisole treated groups. Data represented as means±SE.

(2006). Kumari and Sahoo (2006) also demonstrated that respiratory burst (NBT) activity in levamisole fed group was found significantly higher as compared to the CYP-treated control group. Similarly, in another study the injection of Ocimum sanctum (20 µg) extract into *Tilapia massambicus* produced a higher neutrophil activity (Logambal *et al*., 2000). In the present study, the respiratory burst activity of phagocytes measured by reduction of NBT by intracellular superoxide radicals produced by leucocytes showed enhanced activity with increasing concentration of levan, wherein the highest was registered in the diet supplemented with 0.5% of levan. Dina Rairakhwada *et al.* (2007) demonstrated that the NBT values for levan fed fish were higher when compared with the control fish. Sakai *et al.* (2001) also found that the NBT values of *Cyprinus carpio* fed with dietary nucleotide from yeast RNA were higher than the control group. The NBT values of *Penaeus mondon* fed with ß-glucan gave the highest OD of 0.08 at 630 nm at the concentration of 0.2% glucan (Chang-Fang *et al.*, 2003).

A gradual increasing trend of lysozyme activity as shown in Figure 2 was found with dietary levamisole supplementation. Maximum activity was found in the D_3 group on the 57th day (3257±176 IU) followed by D_4 group (2894±193 IU), D2 group (1938±265 IU) and control (D_1) group (1296±129 IU). Gopalakannan and Venkatesan (2006) also demonstrated that the lysozyme activity of the fish fed on 250 mg levamisole kg^{-1} diet (D_3 group) was significantly higher when compared with control group. The serum lysozyme concentration was significantly increased by lower nisin dose (0.0025 µg/fish) in turbot *Scophthalmus maximus* (Villamil *et al.*, 2003). Similarly, many authors reported that administration of ß-glucans enhances lysozyme activity in Atlantic salmon (*Salmon salar*) and turbot (*S. maximus*) (Engstad *et al.*, 1997; Jorgensen *et al.*, 1993; Santarem *et al.* 1997; Paulsen *et al.* 2001; Baulny *et al.* 1996). Robertsen *et al.* (1994) showed an increased protection against the fish bacterial infection correlated to an increment in the serum lysozyme levels, phagocytic activity and bacterial activity of the head kidney leukocyte. Siwicki (1987, 1989) described the immunostimulatory activity of levamisole in the carp spawners, with treated fish displaying elevated leucocyte and neutrophil numbers and also increase in the lysozyme levels and natural antibody titres. Increased lysozyme levels by intraperitoneal injection of glucan has been reported by various authors (Engstad *et al.* 1992; Jorgensen *et al.* 1993a, 1993b; LaPatra *et al.* 1998)

Increased respiratory burst activity can be correlated with increased bacterial pathogen killing activity of phagocytes (Sharp and Secombes, 1993). Although the number of leucocytes did not show a significant difference when fed with levan supplemented diet, the enzymatic potential of these cells to mount an innate response through the oxidative pathway was detected.

Growth Parameters

Specific Growth Rate (SGR) and Feed Conversion Ratio (FCR): The data of SGR and FCR presented in Table 1 shows that dietary levamisole supplementation (250 mg levamisole kg^{-1} of diet) significantly enhances the SGR of *Cyprinus carpio* when compared with the fish fed on control diet. Similar findings were reported by Gopalakannan and Venkatesan, (2006). Siwicki and Korwin-Kossakowski (1988) proved that levamisole stimulates the growth of *Cyprinus carpio* larvae without affecting the survival and rate of development. The fishes treated with levamisole were larger and heavier by the end of experimental study when compared to the control (Mulero *et al.*, 1998). Precedent exists for growth enhancing effect of levamisole as observed in carp fingerlings (Siwicki and Korwin-Kossakowski, 1998).

In the present investigation, levamisole show positive effect on growth of common carp, depicting significantly higher SGR values and lower FCR

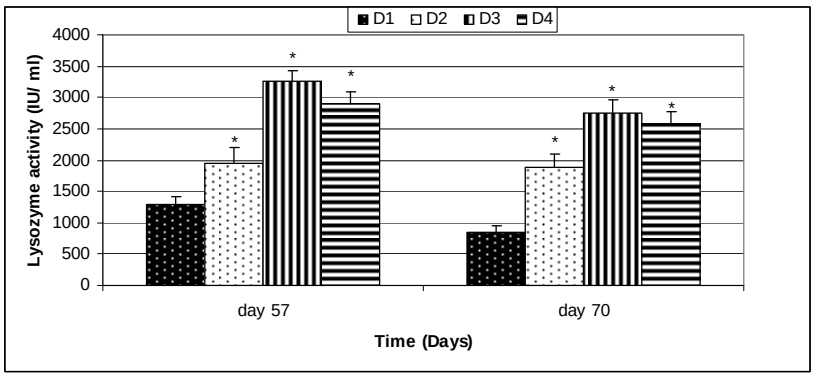

Figure 2. Lysozyme activity of common carp fed levamisole (100, 250 and 500 mg kg^{-1} diet) and a non-supplemented control diet for 70 days and weighed every 15 days. Asterisks denote statistically significant differences (P<0,05) between control and levamisole treated groups. Data represented as means±SE.

values when compared to the control (infected) group. The highest growth rate was observed in 250 mg levamisole kg^{-1} of diet group followed by 500 mg levamisole kg^{-1} of diet and 100 mg levamisole kg^{-1} of diet. Alvarez Pellitero *et al.* (2006) also reported that specific growth rate was higher in the levamisole 500 mg Kg^{-1} treated groups than in the control (C). Niki *et al.* (1991) also found that immunostimulant (levamisole) significantly increases the SGR in fish when compared with the control group. Misra *et al.* (2005) reported that feeding rohu with 250 and 500 mg of ß-glucan kg^{-1} diet resulted in significantly ($P<0.05$) higher SGR and lower FCR values than shown by control fish.

Relative Percentage Survival (RPS)

The result of relative percentage survival (RPS) was shown in Table 2 and Figure 2. The mortality percentage was found highest (68.3%) in the control (infected) group and lowest (5.0%) in D_3 group. The relative percentage survival was found highest (92.7%) in D_3 group and lowest (65.9%) in D_2 group. In the present study, when the fish were intra-peritoneally challenged with *Aeromonas hydrophila* on 30th and 58th day, the RPS was significantly higher ($P<0.05$) in D_3 group followed by D_4 and D_2 group. This might be due to the enhancement of the non specific immune system of the fish by levamisole. Similar findings were reported by Gopalakannan and Venkatesan (2006) while challenging *Cyprinus carpio* with *Aeromonas hydrophila*. Baba *et al*. (1993) also reported that survival rate after challenging the fish with *Aeromonas hydrophila* was enhanced in common carp treated with levamisole. Levamisole has also been found to be a possible modulator of the immune response of *Cyprinus carpio* (Siwicki, 1987, 1989; Baba *et al*., 1993) and rainbow trout, *Oncohrynchus mykiss* (Kajita *et al*., 1990). After treatment with levamisole, both the fish species showed enhanced non-specific immune response activities and resistance to experimental challenge with pathogenic bacteria. Mortality percentage in the levamisole supplemented group was only 31% and 88% in the control (infected) group when all the groups were challenged with *Vibrio anguillarum.*

Dina Rairakhwada *et al*. (2007) reported highest RPS (100%) was recorded in 0.5% levan fed and the least RPS was recorded in 1% levan fed fish.

From the results of the present investigation, it can be concluded that levamisole can be incorporated in diet of common carp fingerlings in order to increase immune function and protection against infection of *Aeromonas hydrophila*. A dose of 250 mg and 500 mg levamisole kg^{-1} diet is optimum to stimulate the immune function of common carp and confer a high degree of protection against the invading bacterial pathogen. Same doses of levamisole were equally effective in stimulating the growth of common carp under temperate climatic condition of Kashmir valley. It is hoped that this base line information will of immense importance to the fish farmers. It is the first study of such kind conducted under temperate climatic condition of Kashmir valley.

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