Studies on levamisole Hcl as a feed additive on the non-specific immune response and growth performance with disease resistance of *Aeromonas hydrophila* in *Clarias gariepinus*

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Abstract: Present investigation was designed for examination of levamisole Hcl on *Clarias gariepinus* as immunostimulant, growth promoter and protect fish from challenge infection with *Aeromonas hydrophila*. A total number of 150 *Clarias gariepinus* were fed diets containing 0 (control), 50, 100, 250 and 500 mg levamisole Hcl kg ^{1} dry diet for 14 days. Lysozyme and blood lymphocyte proliferation were dermined at 0, 2, 4, 6, and 8 weeks after last administration of levamisole. Fish were challenged with *Aeromonas hydrophila* 4 weeks post-treatment, and mortalities were recorded over a 40-day period. The results demonstrate that fish treated with levamisole showed significantly lysozyme elevated levels than control group ($P<0.05$). Elevated lymphocyte proliferation were recorded significantly with addition of levamisole ($P>0.05$). The levamisole treated fish were the more resistant. 50 mg levamisole kg ^{1} dry diet had no effects on the immune response of *Clarias gariepinus,* whereas 500 mg levamisole kg $^{-1}$ dry diet caused immunosuppression. The present results suggest that administration of 250 mg levamisole kg $^{-1}$ dry diet to *Clarias gariepinus* should be optimum for stimulating nonspecific defense mechanisms and the specific immune response against *Aeromonas hydrophila.*

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1. Introduction:

Water pollution and overcrowding tend to continuously cause stress of cultured fish, causing adverse effects on health. The continuous stress inhibits specific immune responses and nonspecific defense mechanisms, resulting in increasing susceptibility to infections **El-Refaey *et al*. (2016).**

Synthetic chemicals and antibiotics have been used to prevent or treat fish diseases and have achieved at least partial success. However, the emergence of antibiotic-resistant microorganisms is an important obstacle to their extensive use (**Nevien *et al*., 2008; Li *et al*., 2006)**

Immunotherapy is an approach that has been actively investigated in recent years as a method for disease prevention. It does not involve recognition of a specific antigen or targeting the immune response towards a specific pathogen, but causes an overall immune response that hastens recognition of foreign proteins (**Campos *et al*., 1993; Secombes, 1994 and Sordello *et al*., 1997**). So the use of immunostimulants for prevention of diseases in fish is considered an alternative and promising area (**Sakai, 1999**).

Levamisole, originally synthesized as an anti-helminthic, has been widely used as an immunomodulator in fish either by injection (**Siwicki, 1987**), immersion (**Siwicki and Korwin-Kosskowski, 1988**), oral administration (**Siwicki, 1989; Siwicki and Studnicka, 1994; Mulero *et al.,* 1998b Alvarez *et al*., 2006; Li *et al*., 2006**) or in vitro immunostimulation (**Siwicki *et al.,* 1992**). Levamisole as an immunostimulant has been widely studied in man and animals, including fish and shrimp. Thus the present study was designed to determine the effect of the dietary intake of levamisole HCL on the immune response, disease resistance and growth performance of *Clarias gariepinus*.

2. Materials and methods:

Fish: A total number of 150 fish *Clarias gariepinus*, obtained from a private fish farm, the same stage of growth, similar body weight and body length, normal body colour, and stable, were randomly assigned to 5 dietary treatments (A, B, C, D and E), 3 replicates of 20 fish each. The initial average weights of fish in treatment A, B, C, D and E were 125.46 g, 125.89 g, 125.12g, 125.90 g and 125.12 g, respectively. Fish were kept in aquaria (50cm×50cm ×100 cm), and were fed two times daily. Meanwhile, the full appetite of fish was recorded. Water was changed once daily about 1/4 and water temperature fluctuated from 25 to 32 °C. The experiment was started after 2 weeks.

Experimental diet:

The basal diet was commercial diet composed of fish meal, soybean meal, wheat bran, peanut meal,, rice bran, calcium phosphate, corn oil, vitamin premix, mineral mixture, capsulated vitamin C, and chloride. The experimental pellet feed of treatments A, B, C, D and E was supplemented with levamisole

hydrochloride to give 0, 50, 100, 250 and 500 mg levamisole kg^{-1} dry weight. After 2 weeks administration of levamisole, fish were fed with the levamisole-free basal pellet diet.

Levamisole® Hcl

It is a commercial product available in the market manufactured by Memphis Pharmaceutical, Cairo, Egypt. It is used as anti helminthic and immunostimulant for large animals and fish in farms. Each ml contains 0.1g levamisole Hcl. The dose was calculated to be 0, 50,100, 250 and 500 mg /kg diet then mixed with the basal diet and pellets were made. The pellets were prepared biweekly, air dried at room temperature and stored in a refrigerator (-4 °C) for daily use.

Determination of immunological competence

Blood samples were collected from the caudal vein of 8 fish of each group and were divided into two parts. One part of the blood samples were allowed to clot at 4 °C in a refrigerator for 4 h. Following centrifugation, the serum was removed and frozen at -20 °C until use for the examination lysozyme at 0, 2, 4, 6, and 8 weeks after last administration of levamisole. The other part of blood samples were anti-coagulated by sodium citrate for the examination of lymphocyte proliferation. Lysozyme was obtained as described by **Gordon *et al.* (1974)** and lymphocyte proliferation was obtained through the MTT colorimetry as described by **Zhu and Chen (2000).**

Determination of growth performance:

Fish samples were collected from each treatment and control groups at 1st and 28th days of the experiment, then weighted for determining (Average body weight, Body weight gain, Average daily gain (ADG), condition factor (CF) and specific growth rate according to **Ricker, (1979)** using the following equations:

(a) Total gain (g / fish) = Wt – Wo

(b) Average daily gain (g /fish /day) = Wt – Wo/n

(c) Condition factor (CF) = weight (gm) / length (cm) x 100

(d) Specific growth rate.

SGR= (Lin Wt – Lin Wo) 100 / n

Wo: Is the initial fish weight (gm) at the start of the experiment.

Wt: Is the final fish weight (gm) at the end of the experiment.

n: Is the duration period of the experiment in day.

Lin: Is the natural logarithm.

Experimental challenge infection:

10 fish for each group (held together) were experimentally infected with the virulent strain *Aeromonas hydrophila* (**Li Guifeng *et al.*, 2001**) by intramuscularly injected on the base of the dorsal fin at a dose of 0.1 ml with 1×10^8 bacteria/ml 30 days after the last administration of levamisole. Mortalities were noted over a 40 day period.

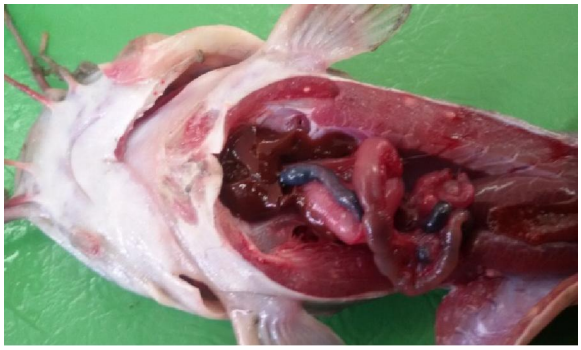
Statistical analysis

Data were analyzed by the Statistic software SPSS11.0 for analysis of variance and the data were expressed as mean+standard error (S.E.). Differences were considered statistically significant when $P<0.05$.

3. Results:

Clinical signs and postmortem lesions of challenge *C. gariepinus*:

The most clinical signs noticed on the examined fishes infected with *Aeromonas hydrophila* suffered from increased mucous secretion. Respiratory failure, some fish suffered from abnormal coloration with abrasions of skin, eroded fins and wounds particularly at the base of the dorsal and caudal fins. In advanced infections, fish were laying on the bottom of aquaria, with dullness and become off food and loss of escape reflex**.** The internal examination of infected fish were recorded as congestion of liver, kidneys with enlargement and congestion of spleen and distended gall bladder **Fig. 1.**



Fig, 1: Showing *C. gariepi*nus infected by *Aeromonas hydrophila* displayed congestion of internal organs (liver and kidney) and distended gall bladder.

Effects of levamisole on levels of lysozyme in serum:

Levels of lysozyme at different stages were indicated below. Treatment B and E had higher levels of lysozyme than the control throughout the experiment but not significant. Treatment D had a significantly higher level of lysozyme than the control immediately after last administration of levamisole, changing smoothly after 2 weeks. However, the level of lysozyme of treatment C was significantly higher than the control until 2 weeks, but for up to 4 weeks (table 1).

Table 1. Showing concentration of lysozyme of *Clarias gariepinus* group specimens 0, 2, 4, 6 and 8 weeks after the last administration of levamisole Hcl.

group / Weeks	Group A	Group B	Group C	Group D	Group E
O	0.30±004	0.33±00.1	0.35±00.3	0.46±000.0	0.23±00.2
2	0.31±00.4	0.39±02.0	0.35±00.3	0.45±00.1*	0.48±00.3*
4	0.32±00.5	0.34±00.4	0.41±00.2*	0.38±00.3	0.33±007
6	0.29±00.6	0.34±00.6	0.52±00.4*	0.37±00.4	0.31±00.8
8	0.33±00.9	0.36±007	0.43±00.1*	0.37±00.0	0.32±00.2

*Data represent the mean+S.E. denote statistically significant differences (P<0.05) between control and levamisole-treated groups.

Effects of levamisole on lymphocyte proliferation in vitro:

Lymphocyte proliferation was obtained through the MTT colorimetry. The results indicated with exception of treatment B with 50 mg levamisole kg^{-1} diet at 2 weeks post-treatment, levamisole-treated groups had higher lymphocyte proliferation compared to that observed in control, but not significant (table 2).

Table 2: Showing lymphocytes proliferation in *Clarias gariepinus* 0,2,4 and 8 weeks after the last administration of levamisole Hcl.

group / Weeks	Group A	Group B	Group C	Group D	Group E
2	0.035±000.4*	0.03±0007	0.04±000.3	0.04±000.6	0.05±000.4
4	0.034±000.6*	0.042±0007*	0.045±000.3*	0.044±000.6*	0.048±000.2*
8	0.025±000.9	0.030±000.3	0.043±000.2*	0.051±000.4*	0.053±000.7*

* Data represent the mean+S.E. denote statistically significant differences (P<0.05) between control and levamisole-treated groups.

Effects of levamisole on the disease resistance:

Levamisole-treated groups had a lower cumulative mortality than that of the control, and the protection rates of treatment B, C, D and E were 28%, 42%, 71% and 42%, respectively (Table 1). Thus, levamisole can enhance protection of *Clarias gariepinus* against *Aeromonas hydrophila*.

Table 1: Mortality rate *Clarias gariepinus* after challenge with *Aeromonas hydrophila*

groups	Infected fish	Dose of levamisole Hcl (mg)	Dead fish	Mortality rate (%)	Protection rate %
A	10	0	7*	70*	-----
B	10	50	5*	50	28
C	10	100	4*	40	42*
D	10	250	2	20	71*
E	10	500	4*	40	42*

* Data represent the mean+S.E. denote statistically significant differences (P<0.05) between control and levamisole-treated groups.

Table 2: Growth performance by the end of experiment of *Clarias gariepinus* fed on Levamisole for 2 weeks

groups	Infected fish	Dose of levamisole Hcl (mg)	Wight gain	ADG	SGR (% / day)	CF (%)
A	10	0	1.30± 0.12*	0.047±0.01	0.126 ± 0.05	0.089 ± 0.03
B	10	50	2.42 ±0.44*	0.086±0.02	0.326 ± 0.09	0.213 ± 0.08
C	10	100	3.23±0.22*	1.22±0.4	1.222±0.06*	0.567±0.03
D	10	250	3.95±0.56*	2.56±0.7*	2.234±0.11*	1.213±0.11*
E	10	500	1.45±0.34*	0.067±0.02	0.452±0.05	0.146±0.23

* Data represent the mean+S.E. denote statistically significant differences (P<0.05) control and levamisole-treated groups.

4. Discussion:

During the last decade there was an increasing interest in the modulation of the non specific immune response of fish to elevate the general defense barriers and hence increase resistance against diseases through use of immunostimulants (**Sahoo and Mukherjee, 2002; Li *et al*., 2006 and Nevin *et al*. 2008**). Concerning the clinical sings and post mortem lesions of infected C.gariepinus challenged with Aeromonas hydrophila post administrated levamisole. The present results agree with the results get by **Ahmed (2001)** and **Kldchakan (2005).**

A variety of immunomodulatory effects of levamisole has been established in a large number of studies (**Cuesta *et al*., 2004; Sahoo and Mukherjee, 2002; Cuesta *et al*., 2002; Masahiro, 1999; Mulero *et al*., 1998**; **Li *et al*., 2006)**. Levamisole as an immunostimulant can promote recovery from immunosuppression states (**Mulero *et al.,* 1998; Masahiro, 1999**) and also can enhance both the innate and specific humoral and cellular immune responses.

Both 100 and 250 mg levamisole kg^{-1} diet enhanced lysozyme levels of *Clarias gariepinus* significantly as **Siwicki (1987, 1989**) suggested. **Siwicki (1987, 1989)** also reported that levamisole enhanced neutrophil. **Mulero *et al.* (1998**) pointed out that fish fed with 250 mg levamisole kg^{-1} diet for 10 days enhanced lymphokine production by head-kidney lymphocytes 5 weeks post-treatment. The experiment demonstrated that levamisole-treated groups had higher lymphocyte proliferation induced by concanavalin A (Con A) than the control but not significant.

Clarias gariepinus were experimentally infected with the virulent strain *Aeromonas hydrophila*, and the protection rates of treatment B, C, D and E were 28%, 42%, 57% and 57%, respectively, as suggested previously in other studies. Olivier *et al.* (1985) pointed out that Coho salmon (Oncorhynchus kisutch) and Chum salmon (Oncorhynchus keta) injected with levamisole mixed with Freund's complete adjuvant (FCA) showed increased resistance to Escherichia coli. **Kajita *et al*. (1990)** reported that rainbow trout injected with levamisole showed increased protection against *Vibrio anguillarum*, caused by the enhancement of nonspecific immune responses such as phagocytic activity, chemiluminescance responses of leucocytes and NK cell activities. **Baba *et al*. (1993)** reported that carp immersed in a levamisole bath (10 mg/ml, 24 h) showed enhanced resistance against Aeromonas hydrophila. **Mulero *et al.* (1998)** also reported that gilthead seabream fed with levamisole enhanced resistance against *Vibrio anguillarum*. **Baruah** and **Prasad (2001)** described that Macrobrachium rosenbergii were fed diets containing 0 (control), 125 and 250 mg levamisole kg^{-1} dry diet for 115 days, and then were experimentally infected with the virulent strain Pseudomonas fluorescent, and showed that the death of shrimp was delayed compared to that observed in control.

In conclusion, such findings suggest that levamisole can be incorporated in feed in order to increase immune function and protection against Aeromonas hydrophila in *Clarias gariepinus*. There were no differences between treatment B (50 mg levamisole kg^{-1} dry diet) and the control on nonspecific immune responses throughout the experiment. This demonstrates that low doses of levamisole have no effect on the immune system. Both 100 and 250 mg levamisole kg^{-1} dry diet enhance the immune system slowly over a longer period. Although 500 mg levamisole kg^{-1} dry diet can stimulate the immune system rapidly, this effect decreases later some immune factors can indicate that the dose of 500 mg levamisole kg^{-1} dry diet suppresses some of the immune responses.

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