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Research Article

Serum lysozyme activity of *Clarias batrachus* subjected to attenuated antigens of *Pseudomonas fluorescens* along with levamisole, vitamin-E and selenium

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ABSTRACT

Influence of immunomodulators (levamisole, vitamin-E and selenium) on the lysozyme level has been evaluated in *Clarias batrachus* challenging with levamisole injection (@ 100 µg & 500 µg ml^{-1} $fish^{-1}$), immersion (@ 1.25 & 2.50 ppm), feed supplementation (@ 5 – 10% of body weight of fish) with pellated feed of vitamin – E (@ 800 mg & 1600 mg Kg^{-1} feed), selenium (@ 1.8 mg & 3.6 mg Kg^{-1} feed) and the mixture of levamisole (@75 & 150), vitamin-E (@800 & 1600) and selenium (@1.8 & 3.6) mg Kg^{-1} feed. Determination of serum lysozyme concentration of controlled and challenged CB has been accomplished by lysate plate method in which wells were developed in agarose plate, loaded with serum samples, incubated at 37°C in humid chamber and diameters of lysed zones were measured and concentration of serum lysozyme was determine following regression equation. Lysozyme concentration has been found to be elevated in HLD1, HLD2; HED1, HED2, MD1, MD2, HMD1, HMD2, OMD1 and OMD2 treated CB while in other groups this level declined.

KEY WORDS: Immunomodulators(levamisole, vitamin-E and selenium), *Pseudomonas fluorescens*, Lysozyme and *Clarias batrachus*

Introduction

Fishes are prone to various diseases because they live in a potentially hostile world filled with a bewildering array of infectious agents which would very happily use fish as aliment and render them to a variety of ailments. To cope up with such problems caused by bacterial fish pathogens an array of chemo-therapeutants (Inglis *et al.*, 1996) and antibiotics (Prasad *et al.*, 2005) have been used *in vitro* as well as *in vivo*. A number of chemicals (Formaline, $KMnO_4$, $CuSO_4$ and H_2O_2), antibiotics (Ofloxacin, Tetracycline etc.) probiotics and adjuvants have been reported to be used in European countries (Adams *et al.*, 1988; Wakabayashi, 1994; Hawke & Thune, 1992; Chen & Ainsworth, 1992; Ruiz *et al.*, 1998; Madsen & Dalsgaard, 1999; Thomas-Jinu & Goodwin, 2004 and Farmer, 2004)

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A number of chemicals (Formaline, $KMnO_4$, $CuSO_4$ and H_2O_2), antibiotics (Ofloxacin, Tetracycline etc.) probiotics and adjuvants have been reported to be used in European countries (Adams *et al.*, 1988; Wakabayashi, 1994; Hawke & Thune, 1992; Chen & Ainsworth, 1992; Ruiz *et al.*, 1998; Madsen & Dalsgaard, 1999; Thomas-Jinu & Goodwin, 2004 and Farmer, 2004) and very few have been tried in Indian aquaculture (Sahoo and Mukherjee, 2002 & 2005 and Prasad *et al.*, 2005). A number of immunostimulents including levamisole, vitamin C & E, chitin, selenium, glucagons etc. have been proved to be good immunomodulators and modulate the non-specific immune response in mammals and other animals including fish (Anderson, 1992). In last few decades there has been increasing interest in the modulation of non-specific immune system of fish as prophylactic measure against various bacterial diseases (Findlay & Munday, 2000). In fact adjuvants promote the proliferation of leucocytes which is also evident from the present findings resulting in to the enhancement of lysozyme

which alleviate the bacterial population below threshold level. There is another advantage in the use of these compounds they augment resistance against the infectious diseases not by enhancing the non-specific defense mechanism but also modulating specific immune response as evident from the present study.

Materials and Methods

Experimental Fish

Indian catfish, *Clarias batrachus* (CB) measuring not less than 15-25 cm in length and 25-50 g in weight was selected for this study, collected from Nakatia River and Zher fishpond of Bareilly and Puranpur fish pond of Pilibhit districts, transported to the laboratory in plastic bags, kept in cemented tanks (2 x 2 x 5m) for acclimatization for 8-10 d and fed with artificial feed. Thereafter, they were transferred to the glass aquaria (12x12x36') filled with water in which temperature was maintained at 20 to 25^{0}C. They were divided into different groups containing 10 fishes (n=10) in each group. One group was maintained as control (considered as negative control) and another group was immunized (considered as positive control) with either 'H' antigen or 'O' antigen of *Pseudomonas fluorescens* (PF) and rest groups were subjected to adjuvants (levamisole, vitamin-E and selenium) through injection, immersion and feed.

Preparation of Attenuated antigen (Bacterian)

Preparation of 'H' and 'O' antigens of PF was made following the standard protocols of Edward and Ewing (1996) as follows:

(a) 'H' Antigen

Over night (18 - 24h) BHI broth grown PF was inoculated in freshly prepared BHI broth in the ratio of 1:100 and incubated at 30 ± 5^{0}C for about 18h in a shaker (100 rpm) water bath. The broth culture was diluted with equal amount of 1% formal saline solution and kept at room temperature for about 1 – 2 h. Thereafter, culture was centrifuged at 8000 rpm (Remi CRP-30) for 15 min to harvest the pellets of antigens (bacteria). The harvested cells were resuspended in sterile NSS (0.3%) followed by centrifugation and resuspension in 0.5% formal saline. Finally pelleted (working) antigens were prepared by adjusting the absorbance value 0.6 OD at 540 λ in Spectrophotometer (Systronics UV-VIS 108) and stored at 4^{0}C.

(b) 'O' Antigen

Antigen 'O' prepared in the same way to that of 'H' antigen with slight modifications. In this case harvested pellets were boiled for 2h to destroy H antigen and centrifuged at 8000 rpm (Remi CRP - 30) for 15 min without adding the 1% formal saline. Thereafter, antigens were resuspended in 0.25% formal saline and OD was adjusted to 0.6 at 540 λ (Spectrophotometer, Systronics UV-VIS 108) and stored at 4^{0}C.

Development of sera (Polyclonal Antisera)

Blood samples were collected from *Clarias batrachus* and rabbits through caudal vein at lateral line and heart, respectively by using (1 ml) syringe washed with NSS and kept in 2 ml centrifuge tubes. The collection tubes were shaken gently to prevent clotting of blood during the collection time.

Seperation of sear

Blood samples were allowed to coagulate at room temperature and after coagulation clots were taken off with the help of loop and kept at 40C for overnight. Sera were separated with the help of pipette and put into another 2 ml centrifuge tubes for centrifugation at 3000 rpm for 15 - 30 min few drops of merthulate (1:5000) solution were added in sera as preservative and kept at - 80^{0}C in deep freezer (Remi Ultra Low Freezer) for further applications.

Calculation of lysozyme concentration

The concentration of known standards was regressed on diameter of lysed zones around these standards. The slope of the curve and intercept were determined. The lysozyme concentrations in unknown samples were determined by following regression equation.

$$Y = mx + c$$

Where,

Y = concentration of unknown samples
m = slope of regression equation
c = intercept of regression equation
x = diameter of the lysed zone around unknown samples

Result

Serum lysozyme level of Clarias batrachus

Lysozyme is secreted by leucocytes that inhibit the growth of bacteria hence considered as strong antimicrobial in action. Its activity was judged on the formation of inhibitory zones. Influence of levamisole (injection and immersion), vitamin - E and selenium individually or blend with H / O antigens of PF on the lysozyme activity of challenged CB

were quantified through lysoplate assay method. Results of present investigation revealed the significant ($P<0.01$) variations in lysozyme concentrations. In levamisole injected CB immunized with H / O antigens, the lysozyme mean value elicited in comparison to controlled one in which it was accounted to 21.75 ± 3.18 µg ml-1. However, injection of levamisole showed negative impact on lysozyme activity on challenged CB but it highest value of 34.98 ± 3.25 (60.80%) was accounted in H antigen immunized fish and decreased values were noted in LD1, LD2, HLD2, OLD1 and OLD2 which declined by 19.77, 22.00, 26.18, 24.96 and 20.63%, respectively **(Fig. –1**).

In levamisole immersion experiment, declination in lysozyme level was noted in LD1, LD2, 'O' antigen immunized and OLD1, OLD2 where as elevation was recorded in 'H' antigen immunized, HLD1 and HLD2 treated groups. In negative control the mean value of lysozyme level was 25.36 ± 3.17 which decreased by 4.48% (24.23 ± 1.39) and 7.98% (23.34 ± 3.63) in LD1 and LD2 treated groups, respectively. In 'H' antigen immunized group this value enhanced by 16.39% (29.52 ± 2.63) but in 'O' antigen immunized group this value decreased by 11.89% (22.35 ± 1.07) in comparison to the negative control mean value. OLD1 and OLD2 treated groups showed decreased lysozyme level on 0 - 21d and accounted to 38.76% (15.53 ± 1.29) and 14.67% (21.64 ± 2.99), respectively **(Fig. – 2)**.

In vitamin – E supplemented diet experiment, alleviation in the mean values of lysozyme was noted in ED1, ED2 OED1 and OED2 treated groups. The mean lysozyme value of negative control was 17.29 ± 2.55 which decreased by 34.11% (11.39 ± 1.80) and 29.96% (12.11 ± 1.62) in ED1 and ED2 treated groups, respectively. However, immunized groups showed elevation in its value by 94.28% (33.59 ± 1.24) and 110% (36.46 ± 2.80) in O & H antigens inoculated CB. The HED1 and HED2 treated groups showed 25.45% (21.69 ± 5.34) and 11.69% (19.31 ± 4.21) enhancement in the mean value of lysozyme level in comparison to the controlled one. On the contrary, OED1 and OED2 treated groups exhibited decreased mean values and accounted to 11.15% and 6.35% less in OED1 and OED2 treated group, respectively **(Fig. – 3).**

Fig 1: Variation in lysozyme level of CB immunized with plain bacterin (H / O antigen of PF) and challenged with levamisole injection (100µg and 500µg $ml^{-1}fish^{-1}$) individually and its concoction with bacterin. A. -ve control, LD1 and LD2, B. -ve control, H+ and O+, C. -ve control, HLD1 and HLD2, D. -ve control, O LD1 and OLD2.

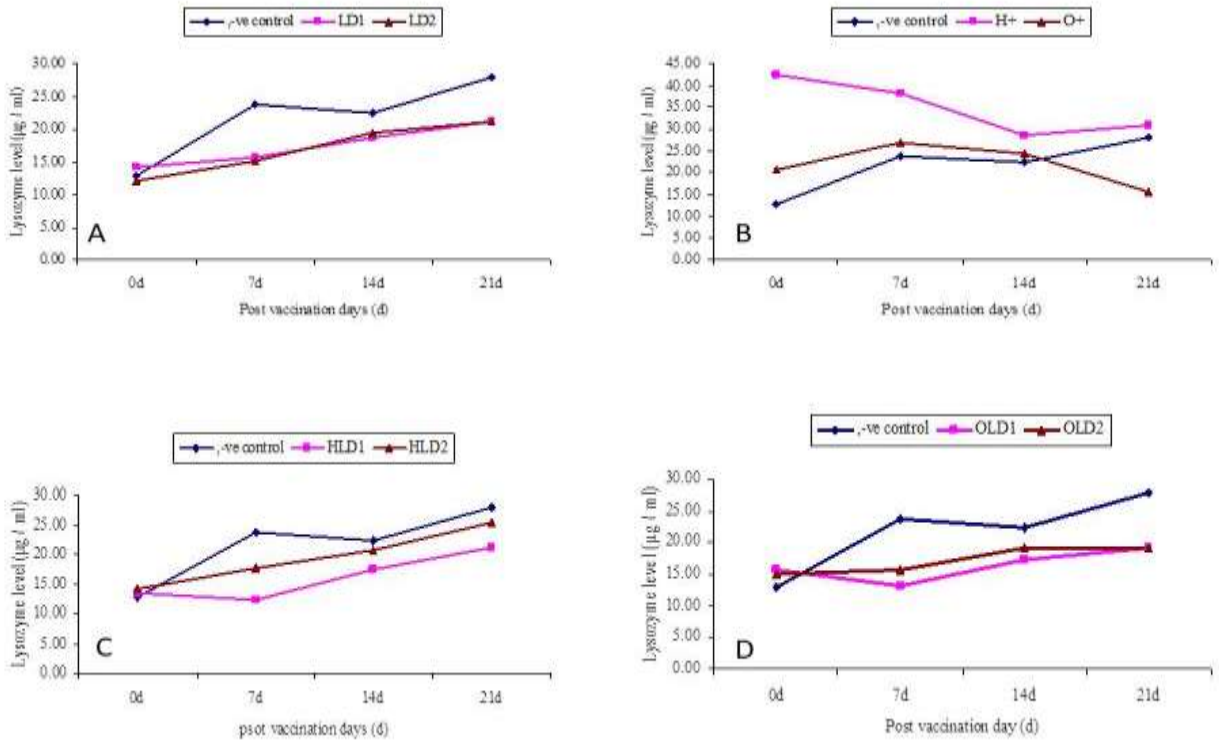


Fig2: Variation in lysozyme level of CB immunized (im) with bacterin (H & O antigens of PF) and subjected tolevamisole hydrogen chloride solutions (1.25 ppm and 2.50 ppm). A. -ve control, LD1 and LD2 B. -ve control, H+ and O+ C. -ve control, HLD1 and HLD2 D. -ve control, OLD1andOLD2

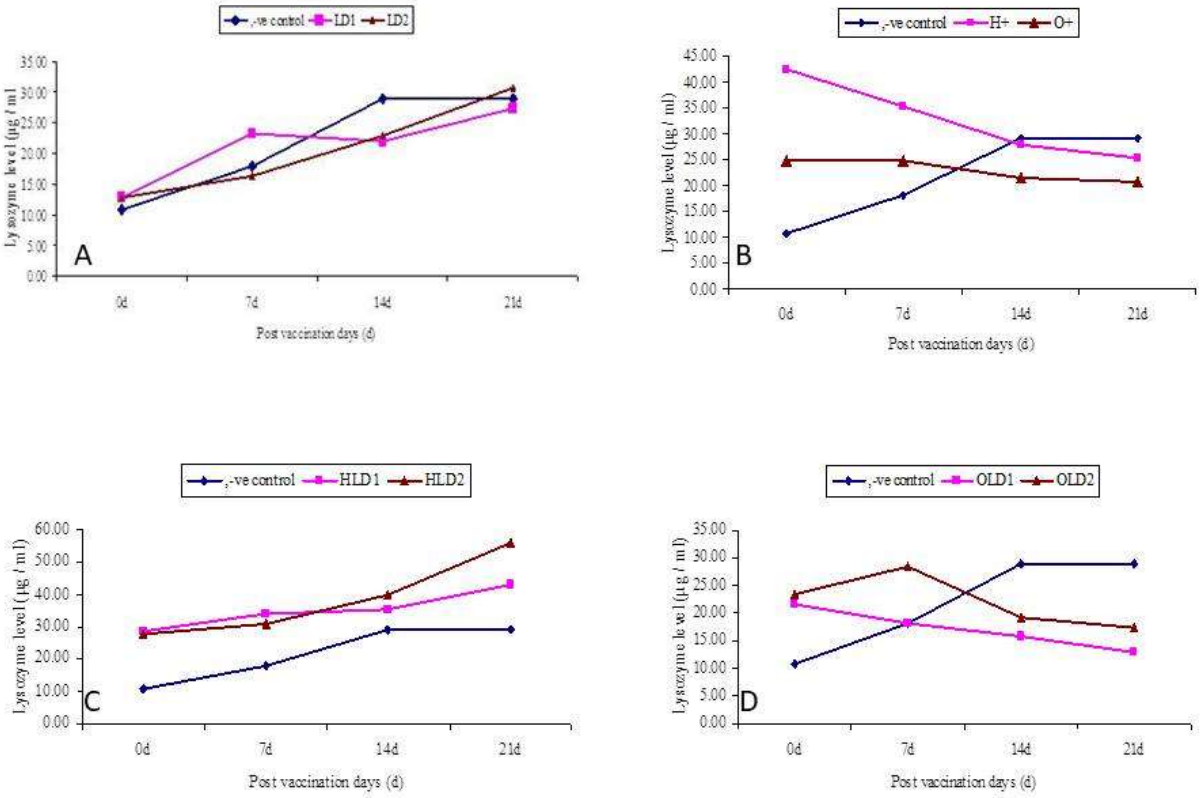
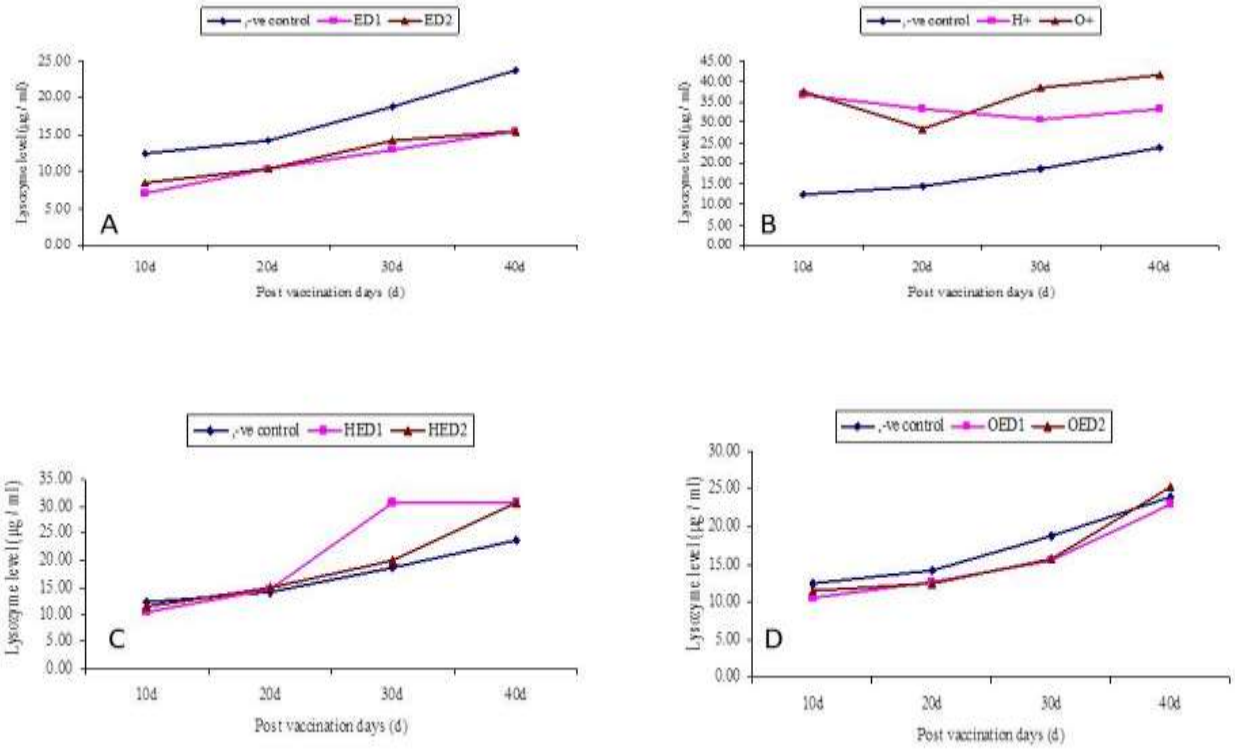


Fig3: Variation in lysozyme level of CB immunized (im) with bacterin (H / O antigens of PF) and challenged with vitamin-E (800 mg & 1600 mg Kg^{-1} feed) individually and with antigens. a. -ve control, ED1 and ED2, b. -ve control, H+ and O+, c. -ve control, HED1 and HED2, d. -ve control, OED1 and OED2



In selenium supplemented diet experiment, except OSeD2 treated and 'H' and 'O' antigens immunized groups other treated ones exhibited declination in lysozyme level. The mean value of lysozyme level was 20.31 ± 2.87 in negative control. In SeD1 treated group, lysozyme level decreased by 5.83% (19.12 ± 2.36) in comparison to the negative control. SeD2 treated group also showed declination in lysozyme level. The HSeD1 & HSeD2 treated groups also showed decreased mean values of lysozyme level which was 24.30% (15.37 ± 1.81) and 15.81% (17.09 ± 2.41) less in comparison to the controlled one. In OSeD1 treated group there was declination in mean value of lysozyme by 23.64% (15.51 ± 1.14). H & O antigens immunized groups showed enhancement in the mean value of lysozyme by 40.71% (28.57 ± 4.13) and 17.53% (23.86 ± 1.68), respectively and in OSeD2 treated group this value enhanced by 8.91%, 22.12 ± 1.36 **(Fig. – 4).**

In mixed diet experiment, all the treated and immunized groups showed enhancement in lysozyme level. In negative control the mean value of lysozyme level was 21.63 ± 2.85 which increased by 10.90% and 41.76% in MD1 and MD2 treated CB, respectively. In 'H' antigen immunized group there was 61.85% enhancement in the mean value of levamisole level from the controlled one. HMD1 & HMD2 treated groups showed 61.55% (34.93 ± 1.20) and 110.76% (45.58 ± 5.02) elicitation in lysozyme mean value. Highest mean value of 50.16 ± 5.61 (131.94%) of lysozyme was observed in 'O' antigen immunized group. The OMD1 and OMD2 treated groups also exhibited enhancement in mean values by 60.38% (34.68 ± 1.91) and 91.47% (3.80 ± 1.90), respectively **(Fig. – 5)**.

Fig4: Variations in lysozyme level of CB immunized with bacterin (H and O antigens of PF) and fed with selenium (@ 1.8 mg and 3.6 mg / Kg) supplemented feed individually and with antigens. A. -ve control, SeD1 and SeD2 B. -ve control, H+ and O+ C. -ve control, HSeD1 and HSeD2 D. -ve control, OSeD1 and OSeD2

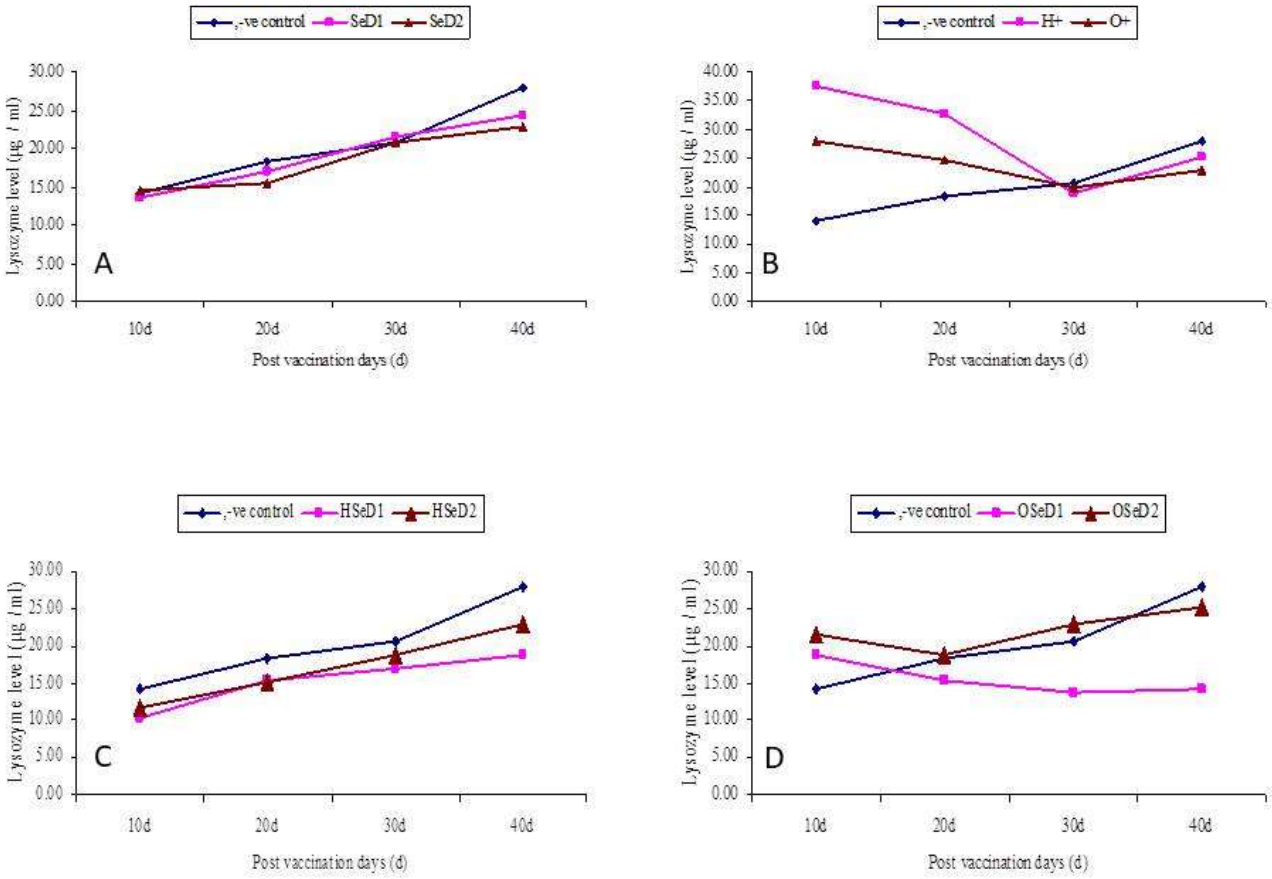
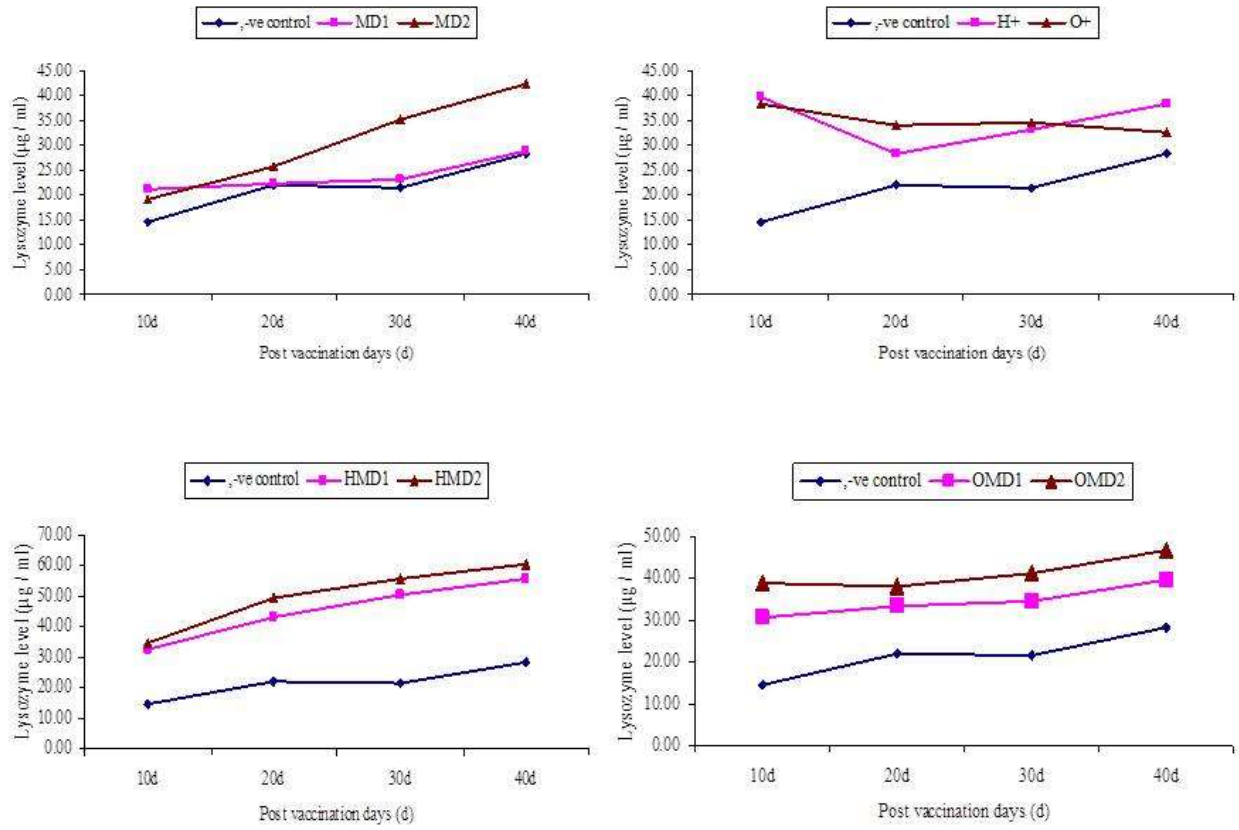


Fig5: Variation in lysozyme level of CB immunized with bacterin (H / O antigens) and fed with mixed diet (levamisole, vitamin-E & selenium, impregnated with H and O antigens of PF). A. -ve control, MD1 and MD2 , B. -ve control, H+ and O+, C. -ve control, HMD1 and HMD2, D. -ve control, OMD1 and OMD2



Discussion

Lysozyme is antibacterial in action and abundantly found in mucus, skin, gills, serum and intestine of fish and prevents adherence and colonization of microorganisms (Alexander and Ingram, 1992). Lysozyme (N-acetylmuramide glycanhydrolase) is mainly secreted by leucocytes distributed in various tissues and blood serum (Fletcher and White, 1973; Fange *et al.*, 1976 and Lindsay, 1986) and cleaves the glycosidic bonds in the peptidoglycan layer of bacterial cell wall and hence acts as a strong antibacterial agent against several Gram-negative bacteria (Grinde, 1989 and Lie *et al.*, 1989). Robertson *et al.*, (1994) noted an increased protection against bacterial fish pathogens, increment in serum lysozyme levels, phagocytic activity and bactericidal activity of head kidney leucocytes. In present study, prevalence of the elicited values of lysozyme activity in H antigen immunized and levamisole (injection / bath), vitamin – E and mixed diet subjected fishes in comparison to the controlled one. Enhancement in lysozyme activity by 60.80% in H antigen (injection), 16.39% in H antigen (bath) 94.28% in vitamin – E & O antigen, 110% in vitamin – E & H antigen, 25.45% in HED1, 11.69% in HED2, 8.91% in OSeD2, 10.90% in MD1, 41.76% in MD2, 61.55% in HMD1, 110.76% in HMD2, 60.38 in OMD1 & 91.47 in OMD2 revealed that vaccination of fish by attenuated antigen influences the activation of leucocytes which ensue enhancement in lysozyme concentrations. It is also vivid that when vitamin - E was supplemented along with H antigen it caused maximum elicitation as it has been account to 110% enhancement in ED1. Interestingly mixed diet proved to be highly significant because augmentation in lysozyme has been noted in all the groups. Conversely, selenium has not been given any good impact on lysozyme activity. In most of the selenium treated CB, declination in this value was noted. Foregoing results concord to the progression made in Atlantic salmon, *Salmo salar* (Findlay

and Munday, 2000) subjected to levamisole bath treatment (@ 2.5 mg / l for 40 d) in which it was reported that levamisole pretenses stress on fish in response to that lysozyme activity of levamisole immersed fishes gets elevated. It might be possible that transfer from normal water to a solution of levamisole causes stress which ensue enhancement in lysozyme concentration. Kubilay and Ulukoy (2002) also suggested that in stress conditions generally elicit in serum cortisol, serum glucose and lysozyme in rainbow trout (*O. mykiss*). Mock and Peters (1990) also reported that lysozymic activity in fish is affected by stress such as handling, transportation etc.

Results of levamisole injection individually or with 'H' and 'O' antigens (PF) explicated that there was enhancement in lysozyme activity for a period of 21d and which is in accordance with the progression of Ispir and Dorucu (2004) who suggested that levamisole injection increases the lysozyme activity and acts as an immunomodulator. Sakai *et al.* (1992) and Verlhac *et al.* (1996) also reported immunostimulatory effects of immunostimulents on the lysozyme activity of fish. Although, injection is the most rapid and effective way to administering immunostimulent (Esteban *et al.* 2001 and Cuesta *et al.* 2002) but their supplementation in diet also enhance lysozyme activity in present case. In one of the experiment significant enhancement in lysozyme activity in *Labeo rohita* fed with levamisole @ 5 mg / Kg body weight has been recorded (Sahoo and Mukherjee, 2001). Gopalakannan and Arul (2006) has also explicated that levamisole supplemented feed enhances the lysozyme activity in *Cyprinus carpio*. Moreover, Leano *et al.* (2003) suggested that supplementation of levamisole does not change lysozyme activity in blood plasma of cobia (*Rachycentran canadum*) fingerlings which may be true in his case.

Minimum elicitation in lysozyme activity of HED1 and HED2 treated CB divulges that vitamin E individually and with H or O antigen (PF) suppress the lysozyme formation and concur to the report of Thompson *et al.* (1995) who suggested that vitamin A supplemented diet did not affect the lysozyme activity in Atlantic salmon (*Salmo salar*). Verlhac *et al.* (1996) also did not record any change in lysozyme activity of *O. mykiss* fed with Yeast glucan along with vitamin C. Due to scarcity of literatures depicting the lysozymic activity on vitamin – E supplemented feed fed fishes, the present observation and its significant impact could not be compared adequately.

Declination in lysozyme activity of SeD1, SeD2, HSeD1and HSeD2 treated groups advocate that supplementation of selenium in feed does augment mean values of lysozyme. More or less similar views have been put forward in salmon, *Salmo salar* (Engstad *et al.*, 1992); trout, *O. mykiss* (Jorgensen *et al.,* 1993 and Thompson *et al.*, 1995); carp, *Cyprinus carpio* (Matsuyama *et al.*, 1992). Moreover, enhancement in lysozyme activity of *Cyprinus carpio* subjected to chitosan, selenium and levamisole supplemented feed in which significantly higher value was noted in chitosan than the control (Gopalakannan and Arul, 2006) and has not conformity with present findings.

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