Effects of immunization with *Aeromonas hydrophila* killed by formalin and sonicated Antigen on the Growth and Blood parameters of *Cyprinus carpio*L.

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Abstract

The aim of the study was conducted to investigate the effects of immunization with *Aeromonas hydrophila* killed by formalin and sonicate antigen on some growth parameters (Average body weight, Average feed consumption, average and total average body weight gain, daily gain, relative gain rate, food conversion, food Conversion efficiency) and Blood parameters (RBC, HP, PCV, WBC and differential proportions of leucocyte) of *Cyprinus carpio*L. by used 100 fish were randomly divided into 5 groups (20 fish each group). T1 and T2 killed antigen were given via formaline (T1 0.2 ml IM injection and T2 immersion 5 Minutes), While T3 and T4 were given killed antigen via sonicasion (T3 0.2 ml IM injection and T4 immersion 5 Minutes). The control group were given 0.2 ml phosphate buffer saline intramuscularly. The results after 42 days observation of showed no significant differences al ($P \geq 0.05$) in Growth and Blood parameters between within all Treated group as compare with control group except WBC and differential proportions of leucocyte.

تاثير التمنيع بمستضد *Aeromonas hydrophila* المقتول بالفورمالين أوالمكسر على معايير النمو والدم في اسماك الكارب

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الخلاصة

تهدف الدراسة الى التحقق من تاثير التمنيع لجرثومة *Aeromonas hydrophila* لنوعين من المستضدات مقتوله بالفورمالين ومكسرة بجهاز التكسير على بعض صفات النمو (متوسط نمو الجسم، متوسط استهلاك العلف، متوسط زيادة الوزن، الزيادة اليومية معدل النمو النسبي، كفاءة التحويل الغذائي النسبي) صفات الدم (عدد خلايا الدم الحمراء، عدد خلايا الدم البيضاء، حجم خلايا الدم المضغوطة، خضاب الدم، النسب التفريقية لخلايا الدم البيضاء) على اسماك الكارب *Cyprinus Carpio*. استخدمت في التجربة 100 سمكة وقسمت بصورة عشوائية الى 5 مجاميع بواقع 20 سمكة لكل معاملة اعطيت المعاملة الاولى والثانية مستضد مقتول بالفورمالين حيث المعاملة الاولى حقنت 0.2 مل بالعضله والمعاملة الثانية غطست لمدة 5 دقائق بينما المعاملة الثالثة والرابعة اعطيت مستضد مكسر حيث المعاملة الثالثة 0.2 مل بالعضل والمعاملة الرابعة غطست لمدة 5 دقائق. مجموعة السيطرة 0.2 مل من محلول المتعادل. قيست نتائج النمو خلال مدة التجربة (42 يوم) في حين تم قياس الفحوصات الدموية بعد 14 يوم من الجرعة الثانية حيث اظهرت النتائج عدم وجود اختلاف معنوي بنسبة $P \geq 0.05$ في فحوصات النمو والدم بين جميع المعاملات مقارنة مع مجموعة السيطرة عدا عدد خلايا الدم البيضاء والنسب التفريقية لخلايا الدم البيض خلال مدة التجربة 42 يوم.

Introduction

Common carp (*Cyprinus carpio*) is one of the most cultured fish in the world. In 2008, the world and the European production was 2 987 433 tons and 144 747 tons, respectively (1). This encouraged us to pay attention to this kind of breeding fish because of their economic importance. The common carp is a member of the family *Cyprinidae*. Carp are extensively farmed in Europe, Asia, and the Middle East, and are a very popular angling fish in Europe, but in North America, Canada and Australia they are considered a pest (2). In aquaculture, infectious diseases are the major problems causing heavy loss to fish farmers. Among the different types of disease causing agents, bacterial pathogens are themost important and responsible for severe mortalities in a wide range of fishes at different stages of growth (3). Vaccination is an important prophylactic measure that can be used to prevent diseases. Several studies have shown that different types of vaccines of A. hydrophila stimulate an effective response in fish that protects them against infections (4). Over the last decade, of vaccine has become important for the prevention of infectious diseases in farmed fish (5). Injection or immersion vaccination with heat or formalin-inactivated bacterins provides some protection to a certain extent against A. hydrophila (6 , 7). Our study was conducted on the effects of immunization by formalin and sonicated Antigen of *Aeromonas hydrophila* in some growth and blood parameters in *Cyprinus carpio*L.

Materials and Methods

1. ***Aeromonas hydrophila* isolation:** Healthy carp and carp with haemorrhages and dermal ulcers on their bodies were obtained from different farm around Baghdad. Samples of the, gill, kidney and skin of each fish were collected. Samples were placed on 5% sheep blood agar plates (Oxoid) tryptic soy agar (oxoid) and MacConkey agar (Oxoid) plates and then incubated at 25-30ºC for 1-2 d under aerobic conditions. After incubation, pure hemolytic yellow colonies were isolated from the skin and internal organs from all the carp. The bacteria were identified as *A. hydrophila* on the basis of colony morphology, Gram-staining, and biochemical characteristics. Wet mounts of skin, fin, and gill smears were also examined microscopically Stock cultures Pure cultures were kept in semisolid nutrient medium supplemented with 20% (v/v) glycerol at -20^{o}C. Cultures were routinely grown on TSA or tryptic soya broth (TSB, Oxoid) at 25^{o}C.
2. **Surface viable count by spreading method:** The viable count is calculated from the average colony count per plate(8).
3. **Preparation of Antigen:**

- **Sonicated Antigen:** *Aeromonas Hydrophila* was grown on TSA at 28°C for 24h. Bacterial cells were harvested by Glass boll with phosphate buffered saline and collected in distilled flask. Bacterial cells were collected by centrifugation at 6500×g for 30 min at 4°C and washed three times with sodium phosphate buffered saline and re-suspended in PBS at 10^{10} cells ml^{-1}. The suspension were kept in ice and sonically lysed with 30 min (1 mint power and 1 mint off) bursts using a probe sonicator with power level at 60 W. The sonicated cells were stored at-20°C (9).
- **Formalin killed Antigen:** A formalin-killed vaccine was prepared as previously described (10). *Aeromonas hydrophila* was grown in Tryptic Soy Broth at 28°C for 24h. Bacterial cells were killed by addition of formalin to achieve a final concentration of 0.7% and incubated for 3h at 25°C and then at 4°C overnight. Cells were collected by centrifugation at 6500×g for 30 min at 4°C and washed three times with phosphate buffered saline, and then they were re-suspended in PBS at a final concentration of 1×10^{10} cells/ml. The non-viability of the bacterial cells in formalin killed preparation was checked by inoculating in TSA and TSB.

4. **Experimental Design:** The experimental fish (100 fish) were weighed and randomly divided into 5 treatments, with two replicates (10 fish in each) there groups were immunized with following rations:

- T1 was vaccinated by A.hydrophila killed Antigen by formalin 0.2 ml injection IM 1×10^{10} with posting dose after 14 day.
- T2 was vaccinated by A.hydrophila killed Antigen by formalin by immersion for 5 Minutes 1×10^{10} diluted 1:10 and with posting dose after 14 day.
- T3 was vaccinated by sonecated Antigen for A.hydrophila by 0.2 injection IM 1×10^{10} and with posting dose after 14 day.
- T4 was vaccinated by sonecated Antigen for A.hydrophila immersion for 5 Minutes 1×10^{10} diluted 1:10 and with posting dose after 14 day.
- Control group injection 0.2 ml phosphate buffer saline and immersion 5 Minutes for stress factor.

5. **Growth parameters:**

- **Growth weight:** estimated by weekly throughout the experimental period.
- **Body weight gain:** Final fish weight (g)-Initial fish weight (g) according to (Schmalhusen,1926).
- **Daily gain (D.G):**

$$D.G = \frac{WT-Wt}{T-t} \qquad (11).$$

WF = final weight, Wt= initial weight, (T-t) = time

- **Relative growthratio (RGR)**

$$RGR= \frac{\text{Final fish weight (g)} - \text{Initial fish weight (g)}}{\text{Initial fish weight (g)}} \times 100$$ according to (12)

- **Feed Conversion Ratio (FCR)**

$$FCR = \frac{\text{Total feed consumed by fish (g)}}{\text{Total weight gain by fish (g)}}$$ according to (12)

- **Food conversion efficiency (FCE)**

$$FCE = \frac{\text{Total weight gain by fish (g)}}{\text{Total feed concumed by fish (g)}} \times 100$$ according to (12)

6. **Blood Parameters:** About 2 ml of blood was collected from each fish (five per group) through caudal vein puncture laterally sectioned using a sterile needle and syringe. The blood samples were collected with anticoagulant (EDTA) treated labeled tubes for hematological analysis (Packed Cell Volume, Red Blood Cells Count, Haemoglobin, White Blood Cell Count, and differential proportions of leucocyte (13).
7. **Statistical analysis:** Statistical analysis of means were performed by using statistical package for social science (SPSS, 2008), Version 16, and for determination of a significant differences by using one way analysis ANOVA (14).

Result and Discussion

1. **Growth parameters:**

- The data of average body weight of *Cyprinus carpio* post vaccinated against *Aeromonas hydrophila* during 42 days included initial weight at 0, 14, 28 and 42 days were reported in table (1) at the beginning showed no significant difference at ($P\geq0.05$) were observed in the initial weigh between T1, T2, T3, T4 and control groups that were 124.94, 125.1, 125.28, 124.45 and 125.14 gm respectively. However no significant difference at ($P \geq 0.05$) were observed between all group in day 14, 28, 42 of Experimental period (Table 1).

Table (1) Average body weight of *Cyprinus carpio* post vaccination agonist *aeromonas hydrophila* during 42 days

Weight / Treatment	Zero day	14 day	28 day	42 day
	Weight g	Weight g	Weight g	Weight g
control	125.14 ± 0.25 a	130.93 ± 0.85 a	138.85 ± 1.25 a	146.91 ± 1.43 a
T1	124.94 ± 0.32 a	130.68 ± 0.73 a	138.48 ± 1.32 a	146.45 ± 1.35 a
T2	125.1 ± 0.26 a	130.83 ± 0.75 a	138.74 ± 1.13 a	146.84 ± 1.27 a
T3	125.28 ± 0.18 a	131.03 ± 0.68 a	138.87 ± 0.93 a	146.78 ± 1.74 a
T4	124.45 ± 0.24 a	130.23 ± 0.82 a	138.06 ± 1.78 a	146.08 ± 1.55 a

Values are expressed as mean ± SE means having the different litter in the same column are significantly different at P ≤ 0.05.

- Data on average and total average body weight gain of *Cyprinus carpio* post vaccinated against *Aeromonas hydrophila* during 42 days were reported in table (2) at the beginning no significant difference at (P≥0.05) was observed in body weight gain of experimental groups T1, T2, T3, T4 and control was 21.51, 21.74, 21.74, 21.5 and 21.77 respectively (Table 2).

Table (2) Average and total average body weight gain of *Cyprinus carpio* post vaccinated agonist *aeromonas hydrophila* during 42 days

Weight / Treatment	14 day	28 day	42 day	Total body weight gain
control	5.79 ± 0.32 a	7.92 ± 0.25 a	8.06 ± 0.36 a	21.77 ± 0.24 a
T1	5.74 ± 0.33 a	7.8 ± 0.64 a	7.97 ± 1.40 a	21.51 ± 0.55 a
T2	5.73 ± 0.26 a	7.91 ± 0.71 a	8.1 ± 0.20 a	21.74 ± 0.45 a
T3	5.75 ± 0.28 a	7.84 ± 0.42 a	7.91 ± 0.09 a	21.5 ± 0.50 a
T4	5.78 ± 0.09 a	7.83 ± 0.62 a	8.02 ± 0.32 a	21.63 ± 0.37 a

Values are expressed as mean ± SE means having the different litter in the same column are significantly different at P ≤ 0.05.

- Data on Average and total average feed consumption of *Cyprinus carpio* post vaccination against *Aeromonas hydrophila* during 42 days were reported in table (3) at the beginning with no significant difference at (P≥0.05) was observed in averages feed consumption of experimental groups T1, T2, T3,T4 and control was 153.70, 153.92, 145.12, 153.16 respectively and 154.01 respectively (Table 3).

Table (3) Average and total average feed consumption of *Cyprinus carpio* post vaccination agonist *aeromonas hydrophila* during 42 days

Weight / Treatment	14 day	28 day	42 day	Total feed Consumption
control	48.80 ± 0.65 a	51.06 ± 0.35 a	54.15 ± 0.46 a	154.01 ± 0.94 a
T1	48.72 ± 0.32 a	50.96 ± 0.73 a	54.00 ± 1.80 a	153.70 ± 0.85 a
T2	48.78 ± 0.26 a	51.02 ± 0.75 a	54.10 ± 0.68 a	153.92 ± 0.35 a
T3	48.85 ± 0.18 a	51.10 ± 0.48 a	54.15 ± 0.59 a	154.12 ± 0.90 a
T4	48.53 ± 0.24 a	50.78 ± 0.82 a	53.84 ± 0.78 a	153.16 ± 0.55 a

Values are expressed as mean ± SE means having the different litter in the same column are significantly different at P ≤ 0.05.

- Body weight gain, Daily gain (DG), relative growth ratio (RGR). Food conversion rate (FCR) and food conversion efficiency (FCE) were reported in table (4). Feed Conversion Ratio showed significant difference at (P.≥0.05) between T1, T2, T3, T4 and control group was (7.14, 7.08, 7.16, 7.08, 7.07) and (17.39) respectively. So that food conversion efficiency there were no statistically significant difference at (P≥0.05) between T1, T2, T3, T4 and control group was (13.99, 14.12, 13.95, 14.12) and (14.13) respectively (Table 4). While the Relative gain rate% there were no statistically significant difference at (P≥0.05) between T1, T2, T3, T4 and control group was (17.21, 17.37, 17.16, 17.38) and (17.39) respectively. While daily weight showed no significant difference at (P≥0.05) between T1, T2, T3, T4 and control group was (0.512, 0.517, 0.511, 0.515) and (0.518) respectively (Table 4).

Table (4) Growth parameters of *Cyprinus carpio* post vaccination aganist *aeromonas hydrophila* during 42 days

Parameters / Treatment	Body Weight gain g	Daily gain g/d/fish	Relative gain rate %	Food Conversion	Food Conversion efficiency
Control	21.77± 0.01 a	0.518± 0.002 a	17.39± 0.23 a	7.07± 0.09 a	14.13± 0.26 a
T1	21.51± 0.32 a	0.512± 0.003 a	17.21± 0.41 a	7.14± 0.11 a	13.99± 0.34 a
T2	21.74± 0.19 a	0.517± 0.002 a	17.37± 0.26 a	7.08± 0.06 a	14.12± 0.67 a
T3	21.5± 0.21 a	0.511± 0.011 a	17.16± 0.30 a	7.16± 0.06 a	13.95± 0.28 a
T4	21.63± 0.08 a	0.515± 0.013 a	17.38± 0.19 a	7.08± 0.08 a	14.12± 0.24 a

Values are expressed as mean ± SE means having the different litter in the same column are significantly different at P ≤ 0.05.

Vaccination is an important prophylactic measure that can be used to determine the effect of A.hydrophla vaccine upon fish production. No significant differences were discerned between treatment groups for FCR, or weight growth indicating that treatment had no impact upon fish performance. This finding contrasts to the observation of others who had employed oil-based vaccine preparation. In general, the use of oil aluminum, and other types of adjuvant has been reported to impact negatively fish growth and appetite. Nevertheless, studies with other species also indicate that vaccination has varying negative impacts upon farmed fish but do not affecting growth, vaccination via injection produce local lesion in cold water marine species (15, 16) an apparent reduction in appetite was also noted although this feature was no explicity monitored. Negative observed growth responses immediately observed by (17) following period of our vaccination but over the entire study no negative growth impact was recorded. It has been hypothesized that the adjuvant component is not responsible for observed growth reductions in vaccinated fish. Rather, it is the antgen or antigen vis adjuvant interaction that is liable (18) a suggestion that appears to be supported by the findings of (19) Others have speculated that growth reduction and loss of appetite following vaccination results due to irritation of the gut or intrusion upon normal swim bladder function (20). Our results agreed with (21) who observed that there was no difference in final weight, CF, FCR. And also agreed with studies with rainbow trout *Oncorhynchus mykiss* (22), common whitefish *Coregonus lavaretus* (23) and Atlantic Salmon who observed that there were no effects of vaccination upon growth.

2. Hematological Parameters:

- **Red blood cells count (RBC):** Red blood cell count of *Carpiol* L post vaccinated against *Aeromonas hydrophila* during 42 days was revealed no significant differences at (P≥0.05) for the treatment T1, T2, T3, T4 and control group (1.82, 1.83,1.82,1.81 and 1.82 $\times 10^6$ cells/ mm^3 respectively) (Table 5). This result within normal range of RBCs count according to (24) who regestered that number of erythrocytes in 1 L circulating blood in fish is most often within 0.5-3.0 T/L. under normal condition the values of red blood cells count are stable. External conditions have a considerable effect on fish blood composition. The RBC count may change significantly between season (25). The increase in number of RBC per unit blood volume may decrease oxygen deficient during transport or acculimation (26).
- **Haemoglobin and packed cell volume (Hb, PCV):** For Hb and PCV% there was no significant difference at (P≥0.05) in all treated (10.82, 10.76, 10.73, 10.65 and 10.62 g/100 ml respectively and for PCV 30.4, 29.85, 30.20, 29.56 and 29.6% respectively (Table 5). This data indicated that vaccine didn't effect the Hb and PCV defect is attributed to the fact that *A.hydrophila* causes hypochromic microcytic anemia and decreased hematocrit, hemoglobin concentration which indicates that RBCs are being destroyed by the leuococytosis activity in an erythrocytic anemia with subsequent erythroblastosis (27).
- **White blood cells count (WBC):** For WBC count there were significant increase observed in treatment T1, T2, T3 and T4 (32.89, 29.24, 34,26 and 29.81×10^3) respectively as compared to control group (27.12×10^3) and also there was no significant difference between T2 and T3 which recorded the highest group as followed by treatment T2 and T4 respectively (Table 5). The result indicated that the stimulation increase in the total leukocyte count after vaccination of *C.Carpio* by IM injection and immersion which also revealed by (28) who recorded a significant increase at (p≥0.05) in WBC count of the immunized group compared with control group. Therefor this result is in line with (29) who observed the significant increase in WBC count of the vaccinated groups by immersion, orally and interaperetoneal vaccine as compared with control group after vaccination by 7 and 21 days. In crease in WBC count in vaccinated *C.carpio* found in this study was observed in sturgeon and rainbow trout which was vaccinated intraperitoneally against *A.hydrophila* and three pathogenic species for the trout reactively (30, 31) in the study by (32) in carp (*C.carpio* L.) immunized with Lps of *A.hydrophila* and the study by (33) carp immunized with a ylucano plus Lps of *A.hydrophila* presented higher total leukocytes counts in neutrophil and monocyte numbers but the number of lymphocytes remained constant.

Table (5) Haematological parameters of common carp *Cyprinus carpio* L. post vaccination

Parameters / Treatment	RBC $\times10^6/mm^3$	PCV %	Hb g/100ml	WBC $\times10^3/mm^3$
Control	1.82 ± 0.02 a	29.60 ± 0.44 a	10.62 ± 0.31 a	27.12± 0.61 c
T1	1.81 ± 0.05 a	30.40 ± 0.74 a	10.82 ± 0.23 a	32,89± 1.43 a
T2	1.83 ± 0.02 a	29.85 ± 0.54 a	10.76 ± 0.51 a	29.24 ± 0.90 b
T3	1.82 ± 0.07 a	30.20 ± 0.66 a	10.73 ± 0.30 a	34.26 ± 0.81 a
T4	1.81 ± 0.03 a	29.56 ± 0.30 a	10.56 ± 0.27 a	29.81 ± 0.81 b

Values are expressed as mean ± SE means having the different litter in the same column are significantly different at (P ≤ 0.05)

- **Differential white blood cell (WBC):** This result of differential leukocyte counts of Cyprinus Carpio L. of the experimental treatments post vaccinated by Aeromonas hydrophila Antigen during 42 days was shown in table (6) The number of lymphocyte decreased significantly at level P≤0.05 in treatments T1, T2, T3 and T4 (51.9%, 56.15%, 51.02% and 57.01%) respectively as compared with control treatment (63.5%). The number of monocyte and neutrophil was significantly increased at level of (P≤0.05) in the treated groups T1, T2, T3 and T4 (13.67%, 12.22%, 14.20% and 11.6% respectively and 34.21%. 41.83%, 34.58 and 31.18 respectively as compared with control treatment was 8.95% and 27.31%. No significant difference was observed in esinophile and basophile among all treated groups as compared with control treatment.

Table (6) Differential proportions of leucocyte in *Cypriuns carpio* post vaccination

Parameters / Treatment	Lymphocyte %	Monocyte %	Neutrophil %	Eosinophil %	Basophil %
Control	63.50±1.10 a	8.95±0.92 c	27.31±0.95 c	0.22±0.02 a	0±0 a
T1	51.90±1.29 c	13.67±0.50 a	34.21 ±1.17 a	0.20±0.01 a	0±0 a
T2	56.15±0.77 b	12.22±0.43 b	31.38±0.66 b	0.23±0.06 a	0±0 a
T3	51.02±0.72 c	14.20±0.64 a	34.58±1.01 a	0.18±03 a	0±0 a
T4	57.01±0.48 b	11.60±0.48 b	31.18±0.61 b	0.20±0.01 a	0±0 a

Values are expressed as mean ± SE means having the different litter in the same column are significantly different at (P ≤ 0.05)

This result is supported by (34) who assessed increased in number of monocytes and neutrophile, decreased in number of esinophile. The study agreed with the findings in Pacu (piaractus mesopotamicus) following infection with A. hydrophila (35) and common carp injected with A.hydrophila (36). The obtained result could be attributed I.M injection and immersion vaccine to cyprinus carpio L suggesting stimulate C.M.I and humoral immunity such as increase in the proportion of monocytes and enhanced phagocytic activity. Our study agree (37) who showed significant incease in lymphocyte count 74% as comparated to non vaccinated fish 64%.

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