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Effect of oxytetracycline on immune system of *Cyprinus carpio* during experimental infection with *Areomonas hydrophila*

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Abstract

To investigate the influence of oxytetracycline (OTC) used as feed additives on the immune response to *A. hydrophila* vaccine, the fish inoculation test was applied. Experimental feeds containing OTC, and the basal diet lacking antibiotics were examined. After feeding trial, 40 fish of each treatment were immunized with the vaccine, and 20 fish in each group were then challenge-exposed to a virulent strain of *Areomonas hydrophila* (*A. hydrophila*) 28 days after vaccination. Organs of *Cyprinus carpio* treated with OTC exhibited a varying degree of histopathological changes. There was depletion in hematopiotic and lymphatic tissue besides congestion in the head kidney, trunk kidney, and spleen. Kidney lesions included reducing Bowman's space, atrophy of glomerulus, necrotic renal tubules, and hemorrhage between the tubules. Vaccinated OTC-treated fish showed severe lesions as compared with the vaccinated and untreated fish. *A. hydrophila* challenged OTC-treated groups either vaccinated or non-vaccinated, showed severe lesions compared with the challenged untreated fish. In contrast, the vaccinated and untreated group showed minimal lesions, which revealed that the fish fed antibiotic-free diets developed significant protective responses to live bacterial challenge 28 days post-vaccination. While the fish feed OTC-supplemented diet showed no protection due to the immunosuppression effect of subtherapeutic treatment for 12 weeks.

Keywords: ***oxytetracycline, hematology, histopathology, lymphoid organs, Cyprinus carpio, bacterial infection***

Introduction

Antibiotics are used against a wide range of bacterial species to help prevent many infectious diseases, which improves public health and saves many lives [1]. Antibiotics have many negative impacts on public health, considering; its overuse and misuse [2]. One of its essential hazards is immunomodulatory effects [3]. In animal agriculture, approximately 15.4 million pounds of antibiotics are used each year [4]. There are three categories of use; feed antibiotics, over-the-counter drugs, and veterinary prescriptions. The administration of antibiotics for the treatment of existing disease conditions is termed therapeutic. In contrast, the use of antibiotics when the risk of disease is high is considered prophylactic, and antibiotics administration for enhanced production is termed subtherapeutic [5]. Stokstad and Jukes [6] first reported the benefits of using antibiotics for growth promotion when chickens exposed to small chlortetracycline doses grew more rapidly than non-exposed chickens. At sub-therapeutic levels, antibiotics are helpful in: (I) improving growth, (II) reducing the risk of disease, (III) improving digestion, (IV) fattening domestic animals, and (V) decreasing time and the amount of feed needed to reach slaughter weight [7]. Several antimicrobial classes are approved for use in food animals, including beta-lactams (e.g., penicillin, ampicillin, and cephalosporin), tetracyclines (e.g., oxytetracycline, tetracycline, and chlortetracycline), aminoglycosides (e.g., streptomycin, neomycin, and gentamicin), macrolides (e.g., erythromycin), lincosamides (e.g. lincomycin and pirlimycin), and sulfonamides (e.g., sulfamethazine) [8,9].

Subtherapeutic amounts of tetracyclines are used in certain countries as feed additives for animal husbandry's growth promotion, e.g., in calves, chickens, turkeys, and sheep [10, 11]. Subsequently, they were widely applied in animal husbandry thanks to improving the feed intake ratio [12]. Orally ingested antibiotics promote the growth and efficiency of agricultural animals. The effect can include gain but often is limited to feed efficiency effects only. The mechanism of action must be focused on the gut because some of these antibiotics are not absorbed. Following early demonstrations that oral antibiotics do not have growth-promoting effects in germ-free animals [13], studies of the mechanism for growth promotion have focused on interactions between the antibiotic and the gut microbiota. Thus, the direct effects of antibiotic growth promoters (AGP) on the microflora can be used to explain decreased competition for nutrients and reduction in microbial metabolites that depress growth [14] .

Aeromonas hydrophila is the causative agent of motile aeromonad septicemia, found in a wide variety of freshwater fish species [15, 16]. Motile aeromonad septicemia outbreaks are common all over the world [17]. Outbreaks of motile aeromonad septicemia usually occur only when the fish are immunocompromised by stresses such as overcrowding or concurrent disease [18]. *A. hydrophila* produces several virulence determinants, including cytotoxins and enterotoxins [19] and a repertoire of enzymes that digest cellular components, mostly proteases and hemolysins (Leung and Stevenson, 1988). The usual methods for controlling the fish disease are vaccination for prophylaxis and antimicrobial therapy for the treatment [20]. Like other vertebrates, the long-term use of antimicrobial compounds affects the immune system in fish. The Present study was designed to investigate the effect of long-term exposure of oxytetracycline on health status of *Cyprinus carpio* via hematological and histopathological examination.

Materials and Methods

Experimental animals

Eighty Fingerlings *Cyprinus carpio* of 6 weeks old; the weight of 25±3 g were purchased from Central Laboratory of Aquaculture

Research (CLAR), Suez Canal University, Faculty of Agriculture. The specimens were acclimatized to the laboratory condition in well-aerated dechlorinated water for ten days before beginning the experiment.

Experimental design

As shown in table (1), fishes were divided into 8 groups (n=10). G1: Control group received basal diet, G2: fed pellets medicated with oxytetracycline at a predetermined rate (4% of body weight) every day for 12 weeks, diet, G3: received basal diet and vaccinated with *A. hydrophila* bacterin, G4: fed pellets medicated with oxytetracycline at a predetermined rate (4% of body wieght) every day for 12 weeks and vaccinated with *A. hydrophila* bacterin, G5: received basal diet and experimentally infection with *A. hydrophila*. G6: fed pellets medicated with oxytetracycline at a predetermined rate (4% of body weight) every day for 12 weeks and experimentally infected with *A. hydrophila*. G7: received a basal diet and experimentally infection with *A. hydrophila* after 7 days of vaccination. G8: fed pellets medicated with oxytetracycline at a predetermined rate (4% of body weight) every day for 12 weeks and experimentally infection with *A. hydrophila* after 7 days of vaccination.

Isolation of bacteria

Shotts and Rimler [21] designed a differential medium for selective isolation of motile aeromonads to facilitate the recovery of motile aeromonads upon primary isolation. The isolated colonies were picked up and streaked onto the surface of tryptic soy agar (TSA). The isolated bacterial was identified by culture morphology, Gram-stain, and biochemically [22, 23]. The colonies that showed a typical TSI reaction (Trible Sugar Iron) and positive for cytochrome oxidase test, oxidation and fermentation reaction of glucose and catalase test were confirmed as *A.hydrophila*.

TABLE 1. **Experimental design**

Group	Type of treatment	Treatment code	Tested conc. of antibiotic	Conc. of challenge species and/or vaccine
1.	Control	Control	---	---
2.	Treated with OTC	OTC	100 mg/ kg diet	---
3.	Vaccination with *A. hydrophila* bacterin	VAC	---	0.1 ml saline/fish I/P 0.1 ml bacterin /fish I/P
4.	Treatment with OTC and Vaccination with *A. hydrophila* bacterin	OTC+VAC	100 mg/ kg diet	0.1 ml bacterin /fish I/P
5.	Experimental infection with *A. hydrophila*	IFN	---	0.1 ml of 16 hours tryptic soy broth (10^8 CFU/ml *A. hydrophila*.)
6.	Treatment with OTC and Experimental infection with *A. hydrophila*	OTC+INF	100 g/ kg diet	0.1 ml of 16 hours tryptic soy broth (10^8 CFU/ml *A. hydrophila*.)
7.	Experimental infection with *A. hydrophila* after vaccination of non-treated group	INF+VAC	---	0.1 ml of 16 hours tryptic soy broth (10^8 CFU/ml *A. hydrophila.)*
8.	Experimental infection with *A. hydrophila* after vaccination of OTC treated group	OTC+VAC+INF	100 / kg diet	0.1 ml of 16 hours tryptic soy broth (10^8 CFU/ml *A. hydrophila.)*

Vaccine preparation

A. hydrophila strain was cultivated in one liter of Trypticase soy broth (TSB) and incubated at 35ºC for 48 hours. The bacterial

cultures were inactivated by formalin's addition to give a final concentration of 0.3% and were held at room temperature overnight. The broth culture was harvested by centrifugation at 4000 r.p.m. for 15 min. and washed three times with sterile saline solution. The preparation was held at 4 ºC until used [24].

The sterility test

It was done by cultivation of the prepared vaccine on Rimler-Shotts agar and Trypticase soya agar and then incubated at 28 ºC for 24 hours. The cultures were examined for bacterial growth [25].

Hematological studies:

Blood was collected at the end of the trial (week 12) from the caudal peduncle of fishes as described by Stoskopf [26] and Joshi et al. (2000). The blood samples were dispensed into tubes containing sodium heparin anticoagulant. Hemoglobin was estimated by cyanomethemglobin method described by Drabkin [27]. Red blood cells (RBC) were counted by Neubauer's improved hemocytometer using Hyem's solution as a diluting fluid [28]. For packed cell volume (PCV) micro-hematocrit centrifuge was employed, the heparinized capillary tubes were centrifuged at 12.00 r.p.m. and PCV was estimated by particular scale [29], mean corpuscular hemoglobin concentration (MCHC); mean corpuscular hemoglobin (MCH) and mean cell volume (MCV) were calculated respectively using a standard formula described by Dacie and Lewis (1991)**.**

White blood cells (WBC) were counted by Neubauer's improved hemocytometer using Dacies solution as a diluting fluid. 4 large (1sq mm) corner squares of the hemocytometer were counted under the microscope (Olympus) at 1000 X. the total number of WBC was calculated in µL x 103 [29]. For the differential count, a dry fixed blood film by methyl alcohol was stained by Giemsa's stain. WBC was counted until 200 WBC on blood smears. The percentage of each WBC type was multiplied by the total WBC count to obtain absolute differential cell counts. This method of manually determining total WBC and the differential count has been recommended for fish blood [26] because nucleated RBC prevents accurate enumeration using automated analysis [30].

Determination of serum IgM (mg/ml):

The IgM level was determined by using Turbox immunoglobulin M assay obtained from Orion Corporation Orion Diagnostica, Finland (Catalog no. 67567).

Histopathological examination

At the end of the experimental period, 5 fish from each group were sacrificed. Pieces of the head kidney, kidney, and spleen were carefully excised, rinsed in physiological saline solution, and fixed in aqueous Bouin´s solution for 24-30 h. Tissues were dehydrated through a graded series of ethanol, cleared in terpineol , and mounted in paraffin wax. Sections of 4-5 µm were prepared from paraffin blocks by using a rotary microtome. The tissue sections were stained with hematoxylin and eosin (H&E) [31], and 3 sections of each tissue from each fish were examined by light microscopy**.**

Data analysis

Statistics were calculated with SPSS for windows version 20.0; the means value obtained in the different groups were compared by one-way ANOVA followed by Duncan's multiple range test. All results were expressed as mean values ± SE, and the significance

level was $p< 0.05$ [32].

Results and Discussion

The performance benefits of oxytetracycline have been established for the major livestock species (bovines, swine, chicken improving body weight gain and feed efficiency [33]. It is presumed that its effects lie in reducing the gastrointestinal tract bacteria [34, 35]. The interpretation of improvement of growth and feed efficiency due to dietary antibiotics supplementation has been subjected to wide speculation by many investigators [36]. Saleh *et al*. [37] reported that antibiotics limit microbial population numbers and their production of toxins and by-products (primarily from Gram-positive bacterial species) in the lumen of birds; they reduce the competition with the host for vital nutrients. They enhance the absorption and utilization of nutrients due to a thinning of the intestinal wall.

In the present study, the efficacy of vaccination against *A. hydrophila* was studied in control and OTC- treated *Cyprinus carpio* using challenge tests and monitoring antibody titer, specific antibody production (IgM) and the blood leucocyte pattern. The fish were vaccinated, and the immunity developed during 4 weeks before the challenge with *A. hydrophila* (0.1×10^8 cfu). The challenge was performed by i.p. injection of a virulent strain of *A. hydrophila*. The immune system of *Cyprinus carpio* responded well, and vaccination gave adequate protection against *A. hydrophila* in the control group rather than OTC- treated group. The RLP was 71.4% and 0% following the *A.hydrophila* challenge in control and OTC-treated fish, respectively (Table 2). Figure (1) represents the serum IgM levels. Comparing the ranges of IgM levels among the vaccinated and non-vaccinated fish indicates no statistically significant differences in serum IgM level in the vaccinated OTC-treated fish. Simultaneously, there was a significant decrease in the IgM levels in the non-vaccinated OTC-treated fish. Also, there was a significant increase in the non-treated vaccinated fish. In this experiment, we studied circulatory leucocytes to determine *A. hydrophila* infection's effect on OTC-treated common carp's defense reactions. Circulatory leucocytes were selected to represent defense reactions since it is known that they reflect both tissue damage [38] and antibody synthesis in fish [39]. The results of leucocytic parameters after bacterial challenge are given in Table (3). Before administration of the *A. hydrophila* challenge, control fish had a total leukocyte count (mean±SD) of (24.52±0.59) 10^3/ µL($P>0.05$). After the challenge, there was a significant increase in total leukocyte count in the non-vaccinated (51.67±1.45) and vaccinated (57.50±0.76) control group ($p<0.05$). In the case of vaccinated OTC-treated *Cyprinus carpio*, a significant increase in total leukocyte count was observed after t h e challenge reached (30.00±1.32) compared with the –ve control.

While non-vaccinated OTC-treated fish showed no difference as compared with –ve control. Differential leucocyte counts were characterized by the predominance of lymphocytes in control fish (Table 3). Three types of leucocytes, namely lymphocytes, granulocytes and monocytes were identified in the circulating blood of *Cyprinus carpio*. The number of lymphocytes in un-treated vaccinated and OTC-treated vaccinated fish injected with 0.1×10^8 CFU/mL of *A. hydrophila* was significantly higher than that of the control group ($p<0.05$). There was a significant increase in the number of granulocytes in unvaccinated and vaccinated untreated fish. On the other hand, a significantly increased number of monocytes was found after injection with 1 x 108 CFU/mL of *A. hydrophila* in all treatments ($p< 0.05$). The present study revealed that challenged vaccinated non-treated *Cyprinus carpio* showed a significantly higher number of circulatory leucocytes, mainly lymphocytes, as compared to the control. However, challenged non-vaccinated fish displayed a higher number of Granulocytes. These results agree with those of Harikrishnan et al. [40] where they demonstrated that *Cyprinus carpio* injected with 10^8 cfu/ml of *A. hydrophila* showed a significant increase in white blood

cells. In respect to the OTC-treated group, the vaccinated fish showed a slight increase in lymphocytes, decreased the number of granulocytes and IgM level (p<0.005), however, in the non-vaccinated fish there was a significant decrease in lymphocytes and monocytes.

Table 2: Mortalities and relative level of protection after challenge in *Cyprinus carpio* treated with OTC and vaccinated against *Aeromonas hydrophila* as compared to non-immunized control

Treatments	Type of Inoculate	Challenge level (cfu/fish)	Died fish during 7 days after injection.								Mortality (%)	RLP (%)*
			0	1	2	3	4	5	6	7		
-ve control	Saline	0.1 ml	-	-	-	-	-	-	-	-	0	_
INF	*A. hydrophila*	0.1X 10^8 *A. hydrophil*	-	1	2	_	2	1	-	-	70	_
VAC	*A. hydrophila*	0.1 X 10^8	-	1	1	-	-	-	-	-	20	71.43
OTC+INF	*A. hydrophila*	0.1 X 10^8	-	2	3	1	1	1	1	-	90	-28.57
OTC+VAC+INF	*A. hydrophila*	0.1 X 10^8	-	1	2	-	2	2	-	-	70	0.0

***RLP (Relative level of protection) =**				
	percent of immunized mortality			
100 -	percent of control mortality		X	100

These results confirm those reported by Kuzin et al. [41], who evaluated the effect of antibiotics on suppressing lymphocyte function *in vitro*. They concluded that doxycycline caused a significant depression of the mitogenic response of both B and T lymphocytes. Antibody production by lymphocytes incubated with doxycycline was utterly depressed. Lymphocyte and granulocyte counts were elevated in vaccinated non-treated compared to non-vaccinated fish at 4 weeks post-vaccination, but not after OTC-treated group. This result agrees with Lönnström et al. [42], who investigate RLP of vaccinated European whitefish (*Coregonus lavaretus* L.) against *Aeromonas* challenge. The authors found that RLP of the vaccinated group was 99% following the *Aeromonas* challenge. The antibody levels were significantly increased after the vaccination. These results parallel those reported by many authors on different fishes and different bacterial organisms [43-45]. Moreover, Woo et al. [46] expounded that tetracyclines are known to have immunomodulatory activities. For the mice receiving the pneumococcal polysaccharide vaccine, the total antibody and IgM levels of the doxycycline group at day 7 were significantly lower than the control group.

Hematological parameters have been considered as an essential indicators of fish health [47, 48]. In the current study, the erythrocytic parameters after bacterial challenge are given in Table (4). Before the challenge with *A. hydrophila*, control fish had red blood cell count (mean±SD) of 2.43±0.1210^6/ µL (p>0.05). After the challenge there was a significant decrease in red blood cell count in the non-vaccinated group while there was no difference between the control and the vaccinated group (p>0.05). After the challenge of non-vaccinated and vaccinated OTC-treated groups, there were significant decreases in red blood cell count as compared with the –ve control. Prior to challenge with the *A. hydrophila* inoculum, control fish had a PCV (mean±SD) of 27.80±0.73. Subsequently after challenge there were a significant decrease in packed cell voulme in the non-vaccinated control group while there

was no difference between the control and the vaccinated and non-treated group (p>0.05). After a challenge, there were a significant decrease in PCV in non-vaccinated and vaccinated OTC-treated groups compared with the –ve control. Before challenge with *A. hydrophila* inoculum, vaccinated and control fish had a mean hemoglobin content of 11.93±0.09. after challenge there were a significant decrease in hemoglobin content in the non-vaccinated control group while there was no difference between the control and the vaccinated group fed on antibiotic free diet (p>0.05).

Table 3: Leucocytic parameters after challenge in *Cyprinus carpio* treated with OTC and vaccinated against *Aeromonas hydrophila*

Groups	Total leucocyte count (10^3/µL)	Lymphocyte (10^3/µL)	Monocytes (10^3/µL)	Granulocytes (10^3/µL)
Control	24.52±0.59 [a]	13.23±1.33 [a]	0.96±0.05 [a]	10.33±0.25 [a]
OTC	16.27±0.10 [b]	8.84±0.81 [b]	0.59±0.01 [b]	6.84±0.60 [b]
VAC	41.21±0.64 [c]	16.65±1.38 [c]	1.29±0.13 [c]	23.28±1.31 [c]
OTC+VAC	20.97±0.31 [a]	10.60±1.17 [a]	0.50±0.04 [a]	9.87±1.28 [a]
INF	51.67±1.45 [d]	10.33±0.29 [a]	2.58±0.07 [d]	38.75±1.09 [d]
OTC+INF	20.17±0.60 [a]	7.21±1.47 [b]	1.14±0.06 [c]	11.83±1.09 [a]
VAC+ INF	57.50±0.76 [d]	37.95±0.50 [d]	2.30±0.03 [a]	17.25±0.23 [c]
OTC+VAC+INF	30.00±1.32 [c]	21.00±0.92 [c]	1.50±0.07[c]	7.50±0.33 [a]

Data represent the mean value ± SD from 10 fish/group. For each column: the same letter displayed, the difference between the means is not statistically significant. a,b,c,d different letters, means statistically significant difference (p<0.05) between different treatments. using One way ANOVA, followed by Duncan's multiple rangetest.

Table 4: Erythroctic parameters after challenge in *Cyprinus carpio* treated with OTC and vaccinated against *Aeromonas hydrophila*

Groups	Red blood cell count (10^6/ µL)	Hb (g/dL)	PCV (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)
Control	2.43±0.12 [a]	11.93±0.09 [a]	27.80±0.73 [a]	114.96±3.31 [a]	49.47±1.82 [a]	43.02±0.86 [a]
OTC	1.82±0.08 [b]	9.13±0.08 [b]	21.17±0.48 [b]	116.63±2.13 [a]	50.48±1.75 [a]	43.21±0.75 [a]
VAC	2.51±0.08 [a]	11.82±0.08 [a]	26.16±0.70 [a]	105.14±5.76 [b]	47.34±1.45 [a]	45.33±1.19 [a]
OTC+VAC	1.73±0.05[b]	9.14±0.07[b]	20.67±0.67 [b]	119.00±4.26 [a]	53.02±1.56	44.46±1.50 [a]
INF	1.42±0.05 [c]	7.65±0.08 [c]	16.67±0.84 [c]	117.54±6.10 [a]	53.98±1.44 [b]	46.41±2.05 [b]
VAC+ INF	2.25±0.2[a]	11.19±0.11 [a]	24.33±0.76[b]	108.27±3.59[b]	49.76±0.65 [a]	46.21±1.65 [b]
OTC+INF	1.16±0.04 [b]	7.42±0.15 [c]	14.50±0.43 [d]	125.58±4.92[c]	64.13±1.23[c]	51.31±1.36 [c]

OTC+VAC+INF	1.74±0.02 [b]	8.98±0.18[c]	17.67±0.67 [c]	101.82±3.55 [b]	51.79±0.96 [b]	51.13±1.77 [c]

Data represent the mean value ± SD from 10 fish/group. For each column: the same letter displayed, the difference between the means is not statistically significant. a,b,c,d different letters, means statistically significant difference ($p<0.05$) between different treatments. Using One way ANOVA, followed by Duncan's multiple range test.

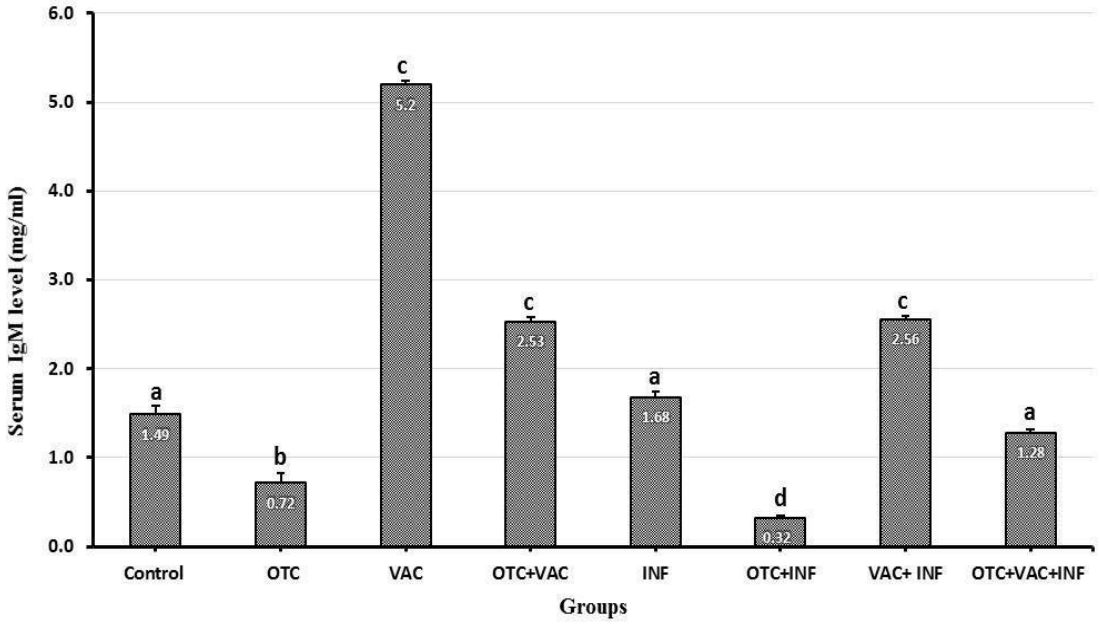

Figure (1): Serum IgM level after challenge in *Cyprinus carpio* treated with OTC and vaccinated against *Aeromonas hydrophila* as compared to non-immunized control. a,b,c,d different letters, means statistically significant difference ($p<0.05$) between different treatments. using One way ANOVA, followed by Duncan's multiple range test.

After challenge there were a significant decrease in Hb content in non-vaccinated and vaccinated OTC-treated groups as compared with the –ve control. Within the red blood cell indices after bacterial challenge, significantly lower MCV and higher MCH and MCHC values were reported in vaccinated and OTC-treated fish, however no significant changes in MCV and MCHC values between vaccinated untreated and control groups and higher MCH were reported. Previous studies demonstrated that the reduction in the number of erythrocytes in the blood and in the hematocrit percentage may be signs of bacterial infection [49-52]. In the present study a significantly lower number of erythrocytes, hemoglobin level and hematocrit value ($p<0.05$) were observed in infected non-vaccinated treated with OTC either vaccinated or non-vaccinated fish as compared to the control group. The decreased hemoglobin content may be brought about due to the swelling of RBC and poor mobilization of hemoglobin from the spleen and other hemopoeitic organs in *Ictalurus punctatus* [53]. These facts supported by the present finding that the significant decrease in erythrocyte and hemoglobin content is possibly due to hypochromic microcytic anemia caused by the bacteria. Decreased RBC counts, hematocrit and hemoglobin concentration indicate that RBCs are being destroyed by the leucocytosis activity in an erythrocytic

anemia with subsequent erythroblastosis [40].

In our experiments, the hematocrit level significantly decreased (p<0.05) in infected fish. In addition, other studies have reported that there is a significant reduction in many different parameters. For instance, the pearl spot fish *Etroplus suratensis*, when infected with (epizootic ulcerative syndrome) EUS becomes anemic, followed by a significant reduction in RBC, Hb and PCV [54]. Since fish have no lymph nodes and their bones usually have no medullary cavity, hematopoietic tissue is located in the spleen's stroma, head kidney (pronephros), and interstitium the kidney. The histopathological alterations in the hematopoietic organs of fish in response to the vaccination were observed mainly in the head kidney, kidney, and spleen. The present results revealed that the control group's head kidney consists primarily of hemopoietic and lymphoid tissue. Besides, there are interrenal and chromaffin cells (Fig. 2A) closely associated with the posterior cardinal veins. The lymphoid cells are spherical in shape; their nuclei are large round, deeply stained with hematoxylin, and central. Their cytoplasm is delicate and stains faintly with eosin. There is also a moderate number of erythrocytes and erythroblasts. These are scattered amongst the lymphoid cells, being oval in shape and possess centrally located nuclei.

Histopathologic evaluation of the primary hematopoietic compartment of *Cyprinus carpio* fed pellets medicated with a sub-therapeutic dose of oxytetracycline, the pronephros, demonstrated increased focal cell death of both stromal and parenchymal cell, reductio'n of hematopiotic elements and necrosis in lymphoid tissue. Also, chromaffin and interrenal cells showed hydropic degeneration (Fig. 2E). The pronephros of *Cyprinus carpio* experimentally infected with bacteria (Fig. 2C), revealed hyperplasia in lymphoid tissue and congestion. Pronephrous *Cyprinus carpio* treated with OTC and experimentally infected with *Aeromonas hydrophilla* showed sever degeneration in interrenal and hematopoietic tissue (Fig. 2G). Besides, fibrotic bands are associated with inflammatory cell infiltration. The vaccinated non-treated fish revealed hyperplasia of lymphoid tissue and activation of melanomarophages (Fig. 2B). While vaccinated and OTC- treated fish showed sever degenerative changes (the loss of cellular architecture). Besides, depletion of lymphatic and hematopoietic tissue (Fig. 2F). Such findings were met with those reported by Kumar and Day [55]; Soliman and Hasseib [56], Abdel-Fadeel [57].

In contrast, vaccinated and experimentally infected fish (Fig. 2D) showed mild lesions represented by hyperplasia of lymphatic and hematopiotic tissues in pronephrous. This result confirme those reported by Miyazaki [58] who reported that the histological responses of *Plecoglossus altivelis*, given an intramuscular injection of a formalin-killed bacterin *of Vibrio anguillarum* showed bacterial phagocytosis by infiltrated neutrophils and slight tissue necrosis. The histological examination of the trunk kidney of *Cyprinus carpio* treated with oxytetracycline revealed mild renal tubule damage leading to the gradual disappearance and occasional replacement of the tubule with interstitial mononuclear cells (Fig. 3E). In addition, some renal corpuscles degenerated displayed atrophy or dilatation in glomerular capillary, and areas of hemorrhage were detected between renal tubules.

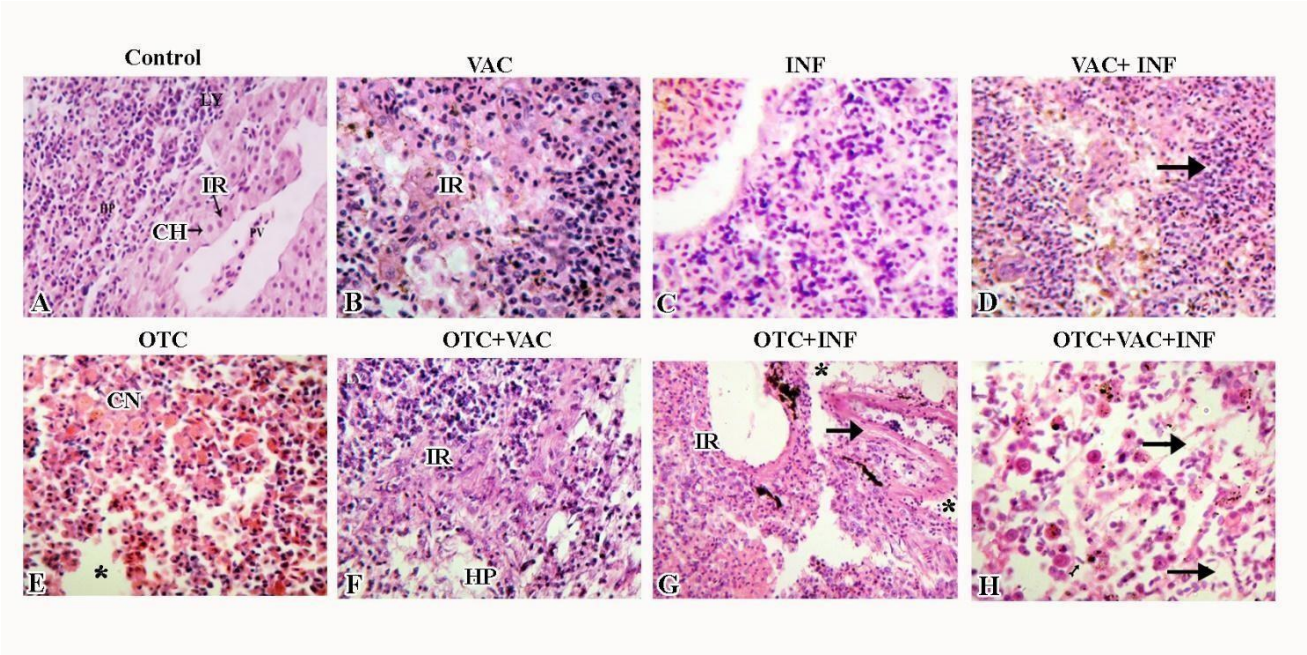

Figure (2): Section of head kidney of *Cyprinus carpio* (A) control group, showing lymphoid (LY), hemopoietic (HP) tissue, and interrenal tissue (IR). (B) *Cyprinus carpio* immunized with *Aeromonas hydrophilla* vaccine displays proliferation lymphocytes and normal interrenal tissue (IR). (C) *Cyprinus carpio* experimentally infected with Aeromonas hydrophilla, revealed hyperplasia in lymphoid tissue (D) *Cyprinus carpio* during challenge test after vaccination revealed proliferation of lymphocytes (arrow) (E)Section of head kidney of *Cyprinus carpio* fed pellets medicated with sub-therapeutic dose of oxytetracycline, showing reduction of lymphoid elements and focal cell death (*). Besides, coagulative necrosis (CN) of hematopoietic tissue. (F) Section of the head kidney of OTC-treated *Cyprinus carpio* immunized with *Aeromonas hydrophilla* vaccine. The revealed proliferation of lymphoid tissue and normal appearance of renal tubules besides mild depletion in hematopoietic tissue (HP). (G) Section of a head kidney of *Cyprinus carpio* treated with OTC and experimentally infected with *Aeromonas hydrophilla*, revealed fibrotic bands associated with inflammatory cell infiltration (arrow). Besides, sever atrophy in interrenal tissue (IR) and depletion of hematopoietic and lymphoid tissue (*).(H)OTC-treated *Cyprinus carpio* during the challenge test after vaccination. focal depletion of the hematopoietic tissue and hypocellularity of lymphoid tissue beside mutiblication of bacteria (arrow). (Magnification:400X)

The kidney of *Cyprinus carpio* experimentally infected with bacteria (Fig. 3C) revealed necrotic renal tubules hemorrhagic areas between degenerated renal tubules. Besides, marked mononuclear cell infiltration and Hyaline material (amyloidosis): orange-red deposits of amyloid, an abnormal accumulation of breakdown products of proteinaceous material that can collect within cells tissues. The most marked lesions detected in kidney tissue of *Cyprinus carpio* treated with OTC and experimentally infected with

Aeromonas hydrophilla were focal depletion of hematopoietic tissue and atrophy in renal corpuscle (Fig. 3G). The vaccinated non-treated fish showed that some renal tubules disappeared and were replaced with interstitial and lymphoid tissue (Fig. 3B).

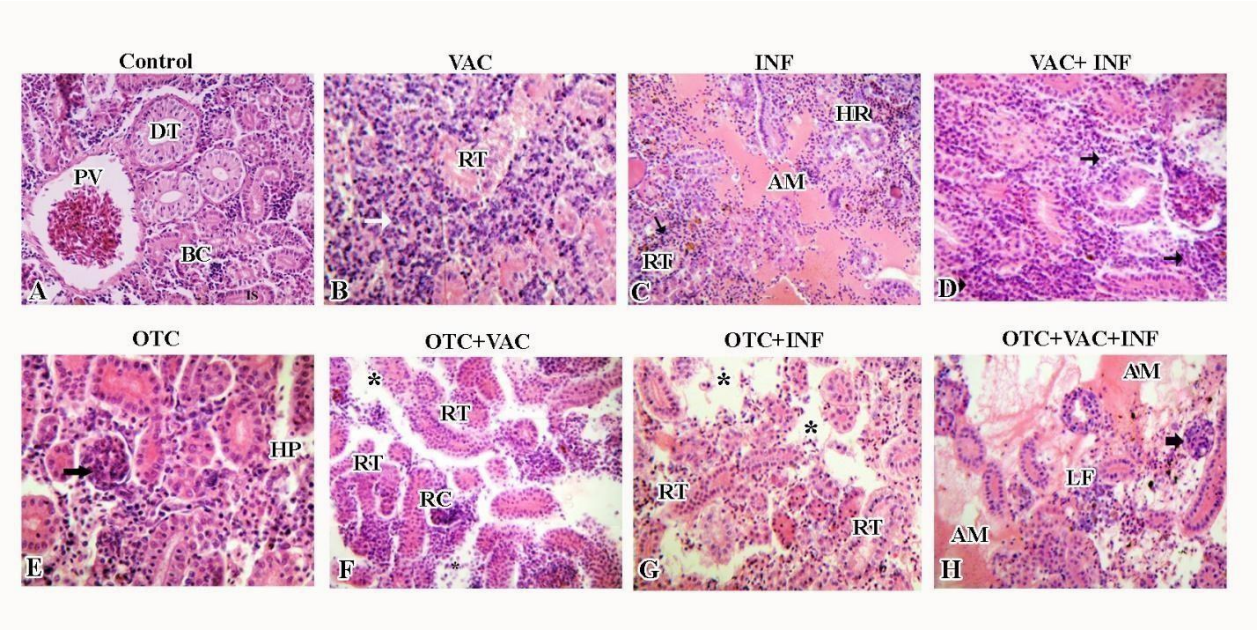

Figure (3): Section of trunk kidney of *Cyprinus carpio* (A) control *Cyprinus carpio* shows Bowman's capsule (BC) enclosing the glomerulus and section in the posterior cardinal vein (PV). Also, a portion of the distal tubule (DT). (B) fish immunized with *Aeromonas hydrophilla* vaccine. Showing Infiltration of mononuclear cells (arrow) between renal tubules (RT). (C) *Cyprinus carpio* experimentally infected with Aeromonas hydrophilla, showing hemorrhagic areas (HR) between degenerated renal tubules (RT) and marked amyloidosis (AM). Besides marked mononuclear cell infiltrations (arrow). (D) *Cyprinus carpio* during the challenge test after vaccination. revealed marked hyperplasia in lymphatic elements (arrow). (E) *Cyprinus carpio* fed pellets medicated with a sub-therapeutic dose of oxytetracycline, showing atrophy glomerulus with renal space reduction (arrow). Besides, depletion of hematopoietic tissue (HP). (F) *Cyprinus carpio* immunized with *A. hydrophilla* vaccine and treated with OTC, showing severe degenerated renal corpuscles (RC) and necrotic renal tubules (RT). Besides depletion of hematopoietic tissue (asterisk) (G) *Cyprinus carpio* treated with OTC and experimentally infected with *A. hydrophilla*, revealed severe degeneration of renal tubule (RT), focal depletion of hematopoietic tissue (*) (H) OTC-treated *Cyprinus carpio* during challenge test after vaccination. Revealed marked amyloidosis (AM), atrophy of renal corpuscle (arrow), and lymphatic infiltration (LF) between the renal tubules. (Magnification:400X)

Besides, focal activation of melanomacrophages (Fig. 3B). While vaccinated and OTC-treated fish revealed marked degeneration in renal tubules and focal depletion of the hematopoietic tissue. Besides, severe amyloidosis (Fig. 3F). The present work's result coincided with those of Ellis [5 9] and Bromage et al. [60], who observed the proliferation of lymphocytes and plasma cells in the kidney after vaccination. Also, Badran et al. [61] investigated A. hydrophila bacterin's effect

on the head kidney and spleen of Nile tilapia. They found activation of Melanomacrophage centers and an increase in melanin density within macrophages with a proliferation of lymphocytes. Moreover, Lin et al. [6 2] reported that a single vaccination of three combined inactivated bacteria into cobia elicited specific antibodies and proliferation of lymphatic tissue, leading to the protection of fish against the antigen. Concerning the OTC-treated group's immunization, the results revealed depletion of hematopoietic tissue, congestion and hemorrhage, and deposition of the hyaline cast in the head kidney and posterior kidney. The histopathological alteration of the spleen revealed congestion and atrophy of melanomacrophage centers. This is in accordance with the results obtained by Costa et al. [63], who evaluated the impact of tetracycline administered orally in daily doses on the immune and hematopoietic systems of rabbits and concluded that this antibiotic causes the depletion of the immune system which was intensified if the drug was used for a prolonged period and in higher doses.

Spleen from control fish revealed no clear distinction between the red pulp and white pulp (Fig. 4A). The parts rich in erythrocytes and those rich in lymphocytes are intermingled. Granules, stained yellowish-brown by HE are frequently present. These granules are produced after the degradation of senescent erythrocytes and are known as hemosiderin. In OTC-treated fish, lymphoid cells were mildly depleted from many white pulp areas. Hemorrhages in the spleen were noted as a dark pigment (hemosiderin pigments) derived from hemoglobin and nucleated erythrocytes (Fig. 4E). The spleen of *Cyprinus carpio* experimentally infected with bacteria revealed degenerative changes associated with marked lymphatic infiltration and depletion in white pulps (Fig. 4 C). The most marked lesions detected in splenic tissue of *Cyprinus carpio* treated with OTC and experimentally infected with *Aeromonas hydrophilla* were mild degeneration in spleen architecture accompanied by inflammatory infiltrate (Fig. 4 G). The vaccinated non-treated fish Revealed mild activation on melanomacrophage centers. Besides, hyperplasia of lymphoid tissue (Fig. 4B). While vaccinated and OTC-treated fish revealed sever degenerative changes in spleen (the loss of cellular architecture). Besides lymphatic infiltration and hemorrhages in spleen where dark pigment derived from hemoglobin and nucleated erythrocytes was detected (Fig. 4F).

On the whole, our results show that infection had a severe histopathological alteration on lymphoid organs even in vaccinated. Moreover, non-vaccinated OTC-treated fish revealed extensive bacterial multiplication and tissue necrosis. These results agreed with those reported by Rijkers et al. [64] and Gaikowski et al. [65] , who revealed that Oxytetracycline causes depletion of the immune system, which makes the fish more susceptible to infection.

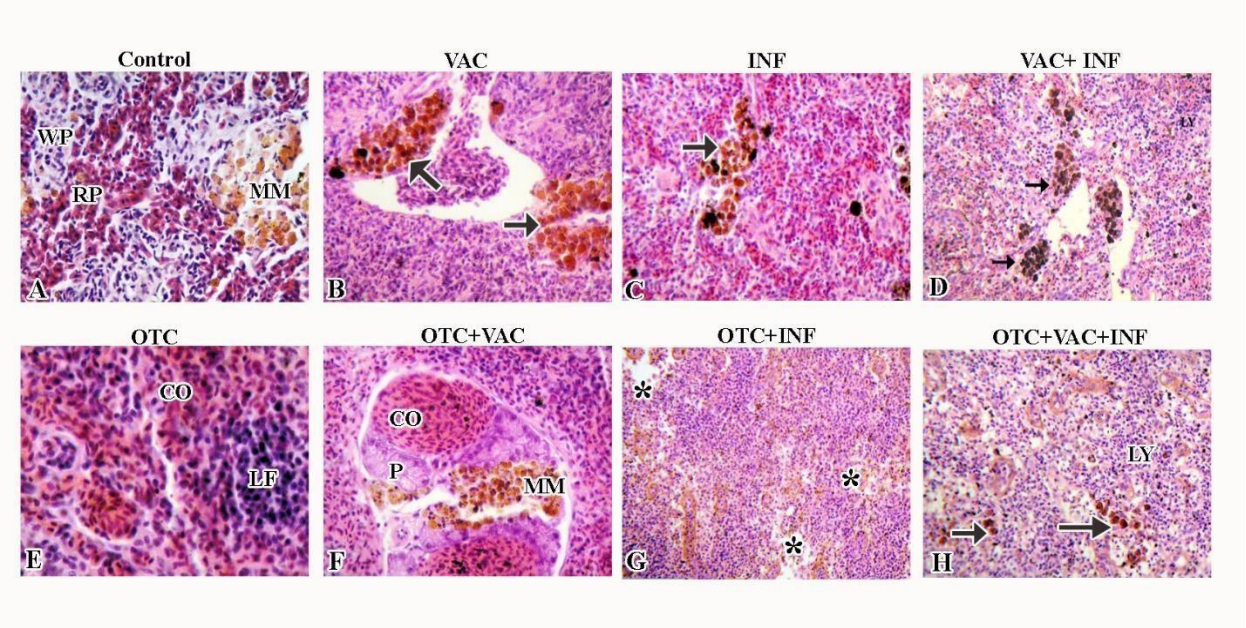

Figure 4: Section of spleen Cyprinus carpio, (A) control group showed white pulp (WP), red pulp (RP), melanomacrophage centers (MM). (B) *Cyprinus carpio* immunized with *Aeromonas hydrophilla* bacterin revealed marked hyperplasia of lymphocytes and mild activation of melanomacrophage centers (arrows). (C) *Cyprinus carpio* experimentally infected with Aeromonas hydrophilla, displayed mild depletion in white pulp and melanomacrophage centers' activation (arrow). (D) *Cyprinus carpio* during the challenge test after vaccination. Revealed marked lymphatic infiltration. (E) *Cyprinus carpio* fed pellets medicated with a sub-therapeutic dose of oxytetracycline, showing hemorrhages in the spleen. Besides, depletion in lymphatic elements. (F) *Cyprinus carpio* immunized with *Aeromonas hydrophilla* vaccine and treated with OTC, displays severe congestion (CO) and mild activation of melanomacrophage centers (MM). (G) *Cyprinus carpio* treated with OTC and experimentally infected with Aeromonas hydrophilla, revealed severe degeneration in spleen architecture. (H) Section of spleen of OTC-treated *Cyprinus carpio* during challenge test after vaccination. Revealed degenerative changes in the spleen (the loss of cellular architecture). Besides hemorrhages in the spleen, where dark pigment derived from hemoglobin and nucleated erythrocytes are visible (arrows). (Magnification: 400X; except G: 200X)

Conclusion

From the present study, it could be concluded that the use of sub-therapeutic levels of oxytetracycline in fish production to enhance growth performance showed many alterations in immunological, hematological, and histological parameters under investigation. It is clear that fish fed on OTC supplemented diet showed a significant decrease in erythrocyte and leukocyte numbers plasma IgM level. Moreover, histopathological changes in most tissues may be due to the enhancement of lipid peroxidation by OTC. Also, the present results suggest that OTC interferes with the vaccine's immune effect if given at the usual concentrations used for feed additives. A deep concern must be taken to reduce the use of antibiotics in the feed of animals. This excessive use of antibiotics is developing an increase in microbial resistance to antibiotics and the presence of antibiotic residues in animal products is a matter of public health importance.

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