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Alterations in Some Selected Hematological Parameters and Glucose-6-Phosphate Dehydrogenase (G6PD) Activity of Rainbow Trout (*Onchorhynchus mykiss*), Experimentally Infected with *Aeromonas salmonicida*

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Key words
Aeromonas salmonicida*, G6PDH activity, Experimental infection, Hematology, *Onchorhynchus mykiss

ABSTRACT

An experimental infection with *Aeromonas salmonicida*, in rainbow trout (*Oncorhynchus mykiss*) was performed. In order to compare the daily alterations of the hematological parameters and glucose-6-phosphate dehydrogenase activity, blood samples were taken 1,3,7,14 and 21 days after the infection. Total red blood cell values, were significantly decreased in 7 and 21th day, a significant decrease in hemoglobin concentration noted only in the 21th day, significantly lower values in hematocrit, were observed at 7, 14 and 21th days, total leucocyte and thrombocyte counts, found significantly higher only in the 3th day, comparing with the control group. While, significantly increasing tendency were determined in mean corpuscular volume and mean corpuscular hemoglobin values from 7th day to the end of the experiment, no significant alterations in mean corpuscular hemoglobin concentration values noted. Fish belong to the infected group, revealed significant increase in G6PDH enzyme activity in the first and seventh days of infection.

INTRODUCTION

Disease is one of the major constrains to aquaculture causing economic losses in farms, becase of the mortalities in stocks, cost of treatment and decreased growth. Although, so many bacterial genera present in aquatic environments, only some of them are known to cause systemic infections in fish, due to their pathogenic properties.

A. salmonicida subsp. *salmonicida* is the causative agent of furunculosis, a systemic disease of fish mostly in the salmonid family and characterized by high mortality and morbidity. Since *Aeromonas salmonicida,* was first reported by Emmerich and Weibel (1894) as a fish pathogen from diseased brown trout, more than 100 years have passed and furunculosis has spread worldwide (Munro and Hastings, 1993).

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Information on biochemical, haematological and immunological parameters during challenge with bacterial infections in fish were usually investigated parameters, these are the useful tools for monitoring the physiological and health status of fish (Clauss *et al*., 2008; Mbokane and Moyo, 2018). Hematology tests, combined with other routine diagnostic methods could be used for routine practice for determining the physiologic disturbances in fish and can provide important information for the diagnosis and also prognosis of disease. For *Aeromonas* infections, importance of mentioned parameters in determining fish immune response in case of abacterial disease process was confirmed with the analyses performed by Harikrishnan *et al*. (2003), Lema *et al*. (2021), Korni *et al*. (2017) and Witeska *et al*. (2007).

Deficiency in glucose-6-phosphate dehydrogenase (G6PDH), that catalyzes the first reaction in the pentose phosphate pathway, to produce nicotinamide adenine dinucleotide phosphate (NADPH), results wide range of clinical symptoms like hemolysis. Deficiency of this enzyme is also associated with blood related diseases and disorders, primarily the anemia. NADPH is an important antioxidant that preserve the reduced form of glutathione which removes the oxidative metabolites resulting from oxidative stress (Thachil *et al*., 2014; Gómez *et al*., 2017;

Tiwari, 2017).

This paper reports values for hematological parameters and G6PDH activity of rainbow trout (*Onchorhynchus mykiss*) experimentally infected with *A. salmonicida*. Alterations in some selected hematological parameters such as erythrocyte (RBC), total leukocyte (WBC) and thrombocyte counts, hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) values and also G6PDH activities were analyzed in 1,3,7,14 and 21th days post infection.

MATERIALS AND METHODS

Experimental design

A total of 50 rainbow trout (*Oncorhynchus mykiss*) weighting 50-100 (75±0.255)g, were obtained from a freshwater farm and kept in 300 l circular fiberglass tank with a constant water flow of 1.5 l min^{-1} of aerated, dechlorinated tap water and held 14/10 h light/dark cycle. The observed water quality parameters were: Dissolved oxygen concentration 8-9 mgl^{-1}, pH 7.8±0.2, total hardness 102 mg in $CaCO_3$ and temperature at 11±2 °C during the whole trial. The fish were fed ad libitum once daily with a rate of 1.5% of their body weight. Fish were randomly divided into two groups (25 fish in each tank) one for control group and the other is for experimental infection. Tanks are cleaned daily by siphoning. Fish were acclimated in these conditions for two weeks before the experiment.

Bacteria and bacterial identification

Atypical *A. salmonicida* (A480) strain used in the experiment was obtained from the Central Fisheries Research Institute (Trabzon, Turkey), lyophilized bacteria were grown in tryptic soy broth (TSB) than transferred to tryptic soy agar (TSA) plates supplemented with NaCl to a final concentration of 1% at 25 °C for 24 h (Beaz-Hidalgo *et al*., 2010). After incubation isolates were identified by fatty acid methyl-ester (FAME) gas chromatography analysis using Microbial Identification Systems Software (MIS Delawere, USA).

Experimental infection

After 24-72 h incubation at 25 °C in TSA, *A. salmonicida* colonies were aseptically removed and suspended in phosphate buffered saline (PBS) solution (0.001M PBS, Ph 7.4) in order to obtain approximately 10^6 CFU (colony forming units). About 0.5ml of this solution was injected intramuscularly anterior to the dorsal fin. Fish from the control group was mock injected with the same amount of PBS. At the injection period care also was taken so that the inoculum (bacterial suspension) would not come out after pushing back of the syringe.

Hematological procedures

In order to compare daily alterations of the hematological parameters, blood samples were taken 1,3,7,14 and 21 days after the experimental infection. A total of 10 fish were planned to sampled (5 control and 5 infected) at each day because of two fish from the diseased group died at 21th day, a total of 23 fish from the diseased group could be sampled. To avoid from the sampling stress, fish were anaesthetized with trcaine methane sulfanate (MS222). Blood samples were drown from each fish, by caudal venous puncture with a heparinized disposable sterile syringe. Hematocrit was determined by spinning the blood sample contained in heparinized capillary tubes in a micro hematocrit centrifuge (Carvalho and Femandes, 2006). Hemoglobin concentration was determined spectrophotometrically (at 540nm) using the cianmethemoglobin method (20 µL of blood in 5Ml Drabkin reagent) as described by Smith *et al*. (2007). Standard hematological procedures described by Blaxhall and Daisley (1973) were employed in the assessment of various blood parameters. Erythrocytes, total leucocytes and thrombocytes were counted with a Neubauer hemacytometer with Dacies solution as a diluting fluid. The MCV, MCH and MCHC were also calculated by standard formulae (Kang *et al*., 2005).

Preparation of the haemolysate for enzyme activity determinations

Blood samples were drown from each fish, by caudal venous puncture with a heparinized disposable sterile syringe. Then all samples were separately transferred into heparinized (5 IU/ml) Vacuntainer tubes and centrifuged at 2 500 × g for 15 min. The plasmas were removed by drip. After the packed column of red cells was washed with KCl solution (0.16 M) three times, the samples were centrifuged at 2 500 × g for each time and supernatants were removed. The erythrocytes were haemolysed with 5 vol. of ice-cold water and centrifuged (+4°C, 10 000 × g) for 30 min to remove ghosts and intact cells (Ciftci *et al*., 2004).

G6PDH enzymatic activity

Enzyme activity was performed at 37°C in the spectrophotometer in accordance with the Beutler method This method is based on the fact that NADPH, which is formed as a result of reducing NADP+, yields absorbance at 340 nm. One enzyme unit was described as the enzyme amount reducing 1µmol NADP+ per minute (Beutler, 1971).

Statistical analysis

Results are presented as mean ± Standard Deviations (SD) of means. Results of blood analyses were subjected to

the nonparametric Mann-Whitney U-test and differences were assumed significant at $p<0.05$. All the calculations were carried out using the Statistica (Data Analysis Software System), Version 10.

RESULTS

Hematological alterations

Daily alteration results on hematological parameters obtained from the experimental infection studies are shown in Table I.

The average RBC values were significantly decreased in 7th and 21th days of the experiment, 0.63±0.10 and 0.53±0.02 ($10^6 mm^3$) respectively in the infected group.

Regarding the hemoglobin concentration, although fluctuating values from the first day to the end of the experiment, only in the 21th day, a significant decrease noted comparing with the control group. Hemoglobin concentrations in 21th day were observed as 8.46±1.00 in control and 4.80±0.88 (gdL) in *A. salmonicida* infected fish.

Significantly lower values in hematocrit, when compared with the control group, were observed in 7, 14 and 21th. days, during the experimental infection. The values in the mentioned days, were ranged as 36.00±1.87%, 40.00±1.58% and 37.33±2.08%, respectively.

Table I. Mean hematological indices and G6PDH enzyme activity of Control and Infected fish at 1, 3, 7, 14 and 21th days post infection.

Parameters	Days				
	1	3	7	14	21
RBC ($10^6 mm^3$)					
Control	1.23±0.06	1.29±0.15	1.13±0.20	1.21±0.07	1.40±0.13
İnfected	1.14±0.17	1.10±0.21	0.63±0.10*	0.71±0.10	0.53±0.02*
Leukocyte ($10^3 mm^3$)					
Control	57.20±17.12	41.60±9.154	41.40±6.18	62.20±19.89	60.80±7.496
İnfected	56.00±14.14	57.00±7.64*	40.20±9.62	64.00±16.85	54.00±1.00
Thrombocyte($10^3 mm^3$)					
Control	26.00±8.185	19.80±2.683	18.40±3.781	20.80±4.207	29.20±7.563
İnfected	28.60±4.82	25.80±2.86*	22.60±2.19	41.60±12.59	25.66±3.78
Hemoglobin (gdL)					
Control	8.66±0.99	7.52±1.09	7.12±1.22	6.80±0.94	8.46±1.00
İnfected	10.74±3.79	6.96±1.82	7.64±1.44	6.18±1.10	4.80±0.88*
Hematocrit (%)					
Control	39.80±4.38	44.20±2.86	44.00±2.54	48.80±1.30	49.40±4.03
İnfected	38.20±1.92	41.40±4.66	36.00±1.87*	40.00±1.58*	37.33±2.08*
MCV (μ^3)					
Control	321.40±29.8	343.80±33.9	394.60±60.6	402.40±24.3	354.00±42.7
İnfected	338.40±40.9	384.20±79.6	583.4±115.2*	567.2±79.36*	703.66±27.2*
MCH (µg)					
Control	69.80±9.95	58.00±8.33	65.00±23.39	56.00±8.74	60.00±6.55
İnfected	97.00±41.73	63.00±16.26	122.60±25.56*	87.60±21.24*	90.33±20.25*
MCHC (%)					
Control	22.00±2.34	16.80±2.16	15.60±3.71	13.60±1.67	17.20±1.64
İnfected	27.80±10.52	17.00±5.74	20.80±3.96	15.00±2.34	13.33±3.21
G6PDH (EU/mgHg)					
Control	2.48±1.05	6.84±1.51	5.66±1.34	4.14±1.77	4.31±1.51
İnfected	12.66±3.67*	8.20±1.33	9±2.20*	7.53±4.35	3.54±1.06

Values are mean ± standard deviation; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration. *: $p<0.05$

From red blood cell indices (MCV, MCH, MCHC), significantly increasing tendency were determined in MCV and MCH values with the post infection from 7th day to the end of the experiment. No Significant alterations in MCHC values noted, during the experimental infection.

Fluctuating total leukocyte and thrombocyte counts observed in this study. While the total leucocyte (57.00±7.64) and thrombocyte counts (25.80±2.86), in the third day of the infection, were found significantly higher than the control, no significant changes could be observed in the other days.

G6PDH activity

G6PDH enzyme activities of control and *A. salmonicida* infected fishes in different days post infection, were presented in (Table I). Fish belong to the infected group, revealed significant increase in enzyme activity in first and seventh days of experimental infection compared to the control with a value of 12.66±3.67 and 9±2.20, respectively.

DISCUSSION

While, mortalities did not occur any of the fishes in control group, there was a 8 % (2/25) mortality rate in infected group. Two fish from aeromonas infected group, died with the effect of the infection on 21th day.

Considerable hematological changes, following experimental infection with *A.salmonicida* were observed in the present study. Fish in infected group showed significantly lower RBC values in 7th and 21th days. Significantly lower hematocrit values noted from the 7th day to the end of the experimental infection, a significant decrease in hemoglobin concentrations in infected fish were reported only in 21th day. The results indicated that the total numbers of RBC, Hgb and hematocrit percentages were affected by the experimental infection. Similarly decreased RBC, hemoglobin and hematocrit values in cultured Nile tilapia, infected with *Aeromonas* bacteria (Lema *et al*., 2021), in rainbow trout infected with Aeromonas species (Řehulka, 2002) and in common Carp, infected with *A. hydrophila* (Harikrishnan *et al*., 2003) were previously reported. Significant decrease, observed in red blood parameters, such as erythrocyte count, hemoglobin concentration and hematocrit values are considered as the signs of anemia (Witeska, 2015). In the present study, although a decreasing tendency reported for RBC values, and hematocrit percentages in all days due to the influence of infection, significantly lower values of all these three parameters, RBC, hematocrit and hemoglobin together, that indicates an anemia in infected fishes only occurred 21 days post infection.

In most studies, fluctuating hemoglobin concentrations in infected fish during a bacterial disease were previously reported. Witeska *et al*. (2007) reported a gradually decrease of hemoglobin content on day 1 in juvenile carp after infection with *A. veronii bt. Sobria* in two different diets tested, they also determined extremely low values for hemoglobin in both groups on day 8 which the symptoms were fully developed and finally slightly increasing values on day 15.

Following an artificial infection with *E. tarda* in Koi carp, a significant decrease in the total hemoglobin content in the infected group were detected on 3, 6, 9 and 12 day post infection. On the 15th day of infection, an increasing trend in hemoglobin values observed and values returned to the pre-injection level (Rajapakshe *et al*., 2012).

The fact that the hemoglobin value increased only on the 21st day of the infection and no change was observed on the other days in the present study, could be considered as an indication that the severity of the infection in fish increased on the 21th day. Mortality, occurred on 21th day, supports this situation.

Regarding the erythrocyte indices, it was seen that there were significant increases in MCV and MCH values in infected fish from the seventh day to the end of the experimental infection. No significant changes in MCHC values, during the experimental period were noted. Observed hematological indices in this study indicates a macrocytic normochromic anemia in infected fish from the seventh day to the end of the disease. These findings were in correspondence with those by Altun and Diler (1999) and Aly *et al*. (2021) they reported a macrocytic normochromic anemia after an experimental infection with *Yersinia ruckeri* in rainbow trout (*Onchorhynchus mykiss*) and Nile tilapia (*Oreochromis niloticus*). Macrocytic hypochromic anemia was reported in Nile tilapia, *Oreochromis niloticus* in an outbreak of motile aeromonas septicemia and in rainbow trout (*Oncorhynchus mykiss*), experimentally infected with *Pseudomonas putida* (Korni *et al*., 2017; Bektas and Ayik, 2009). Macrocytosis and hypochromaemia also reported in brook trout *Salvelinus fontinalis* affected by columnaris disease (Řehulka and Minařik, 2007).

Anemic situation in fish resulting from various factors like as toxic agents, viral and bacterial infections and inadequate nutrition cause changes in erythrocyte indices and this indicates alterations in erythrocyte morphology as in the other vertebrates (Witeska, 2015).

Increases in MCV and MCH values together, in infected fish during a bacterial infection in our study, could be sign of a compensatory response of the organism against anemic conditions According to Witeska (2015), a compensatory reaction which increasing oxygen carrying

capacity of fish blood is usually occurs following an anemia. Because of the relatively stable structure of red blood parameters and having an effective compensatory response, fish can improve oxygen transport which allowing the fish to survive during anemia (Vosyliene, 1999).

A. salmonicida produce extracellular exotoxins which have hemolytic, necrotic or lethal action for infected fish. Macrocytic normochromic anemia developed in our study could be associated with the lytic activities of hemolysin caused by the bacteria (Lee and Ellis, 1990; Nomura and Saıto, 1982; Korni *et al*., 2017).

When examined, ın terms of total leukocyte counts, only the third day results of infected fish showed a significant increase, compared to control fish in this study. In other words, leukocytosis developed in infected fish on the third day of the infection. Similarly, Afiyanti *et al*. (2018) reported an increase in total leukocytes counts, in *A. salmonicida* infected carp (*Cyprinus carpio* Linn) and also Sebastião *et al*. (2011) reported significantly greater mean total leukocyte counts in the Tilapia (*Oreochromis niloticus*) with columnaris disease.

Leukocytes are one of the important components of the immune system and they are involved in defending the body against invasion by foreign substances (Adeyemi *et al*., 2013). İncreasing total leukocyte count results, in the present study, confirmed the fact that immune system of infected fish is activated by pathogen bacteria so as to fight against the bacterial invasion. This is in accordance with the opinion of Lema *et al*. (2021) which states that the increase in total leukocytes in *Aeromonas* infected Nile tilapia, caused by migration of white blood cells from the spleen to the blood circulation and cause leukocytosis, and also they claimed that the defense mechanism of the infected fish is enhanced with the more production of leucocytes.

Thrombocytes in fish, are analogues of platelets in mammals and they have an active role in antimicrobial host defense, tissue repair and mediating the clotting response. They can also bind and internalize pathogens and with releasing the microbicidal proteins can deactivate the certain bacteria and fungi (Klinger and Jelkmann, 2002; Stosik *et al*., 2019).

Regarding the thrombocytes counts, a significant increase in infected fish reported only on day 3 after the infection in the present study. Similar trends in thrombocytes counts in Nile tilapia experimentally infected with *Enterococcus* sp. reported by Martins *et al*. (2008).

Platelets in mammals and thrombocytes in lower vertebrates like as fish, known as mediators in hemostatic functions and also these cells have a role in inflammatory responses upon stimulation with various microbial stimulants (Ferdous and Scott, 2014). Increase in total thrombocyte count of infected fish, in the present study might be due to fish cell response against *A. salmonicida* infection.

Experimental infection with *A. salmonicida*, was resulted in a significant increase in G6PDH enzyme activities on the first and seventh days of the present study, comparing to the control fishes.

Deane and Woo (2005) reported an increased G6PDH expression in both liver and kidney at early- and mid-stage during vibriosis in sea bream and they argued that this increase may be related to improved protection against oxidative stress via production of more rising power in the form of NADPH in fish as a result of vibriosis.

Many researchers emphasized a positive relationship between bacterial infection and stimulation of oxidative stress in fish (El-Sayed *et al*., 2019; Chen *et al*., 2020). Basically oxidative stress can be explained as an imbalance between the production of oxidant and antioxidants components, in other words if synthesis of free radicals is faster than their removal by antioxidant mechanisms, oxidative stress occurs. If free radicals, mainly reactive oxygen species (ROS) increases and reaches toxic levels for the host in many ways, such as phagocytosis most organisms activate the antioxidant defense system to limit the pro-oxidant activity of ROS (Biiler and Takahashi, 2018; Rodríguez-Quiroga *et al*., 2017).

The possible explanation of increase in G6PDH enzyme activities on the first and seventh days, following an experimental infection with *A. salmanicida* in the present study, might be due to activated antioxidant defense system of fishes, against free radicals resulted from oxidative stress. Being an important antioxidant, NADPH preserve the reduced form of glutathione which removes the oxidative metabolites and levels of NADPH mostly determined by the enzyme glucose-6-phosphate dehydrogenase (Nóbrega-Pereira *et al*., 2016).

CONCLUSIONS

Analysis of the blood parameters, were indicated blood disorders occured, following *A. salmonicida* infection in Rainbow trout (*Oncorhynchus mykiss*). As in a number of other bacterial fish disease, infection in our study, resulted with macrocytic normochromic anemia (increases in MCV and MCH values). Fluctuating hematological values, during the course of the infection were observed in the present study. While, the decreasing blood parameters on some days of infection were, RBC, hemoglobin and hematocrit, In the values of leukocyte,

thrombocyte, MCV and MCH, an increasing tendency was observed. Increases, observed in G6PDH enzyme activities of infected fishes, on the first and seventh days, of infection might be due to activated antioxidant defense system of fishes against free radicals resulted from oxidative stress. Our overall results, indicated that blood disorders resulted from a bacterial infection, are important tools for monitoring the disease and also provides useful information in early diagnosis and treatment.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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