Study of some hematological and biochemical parameters of Rainbow trout (*Oncorhynchus mykiss*) fry in western part of Mazandaran province, Iran

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Abstract

This study was done to investigate of some hematological and biochemical factors of rainbow trout *(Oncorhynchus mykiss*) fry in west of Mazandaran in Iran. About 50 pools of blood samples from diseased fry were collected within 30 months from November 2002 till March 2005 from three hatchery farms in western part of Mazanderan province. In addition 30 pools of blood samples as control group were collected randomly from mentioned farms. Each blood samples were examined for whole blood examination and blood enzymes measurement. It consist of total leukocytes (WBC) and erythrocytes counts (RBC), hemoglobin (Hb) content, hematocrits (PCV), leukocytes differential count and blood indices such as MCV, MCH and MCHC. Also blood serums were analysed for total protein (TP) and blood enzymes. All the calculations were made using the SPSS© and t-test statistical method. In hematological findings nine parameters were revealed significant differences ($P<0.05$) with control group in t-test. It consisted of total WBC, Lymph, Neut, Hb and HCT, MCHC, AST, DL and total protein plasma. Also in total white blood cell count, Lymph and Neut had significant differences as compared with the control fish ($P<0.05$). Also blood serum components analysis revealed that only LDH and AST amount showed obvious significant differences ($P<0.05$). Regarding to results it could be concluded that hematological and biochemical studies could be a valuable tool for prognosis and primary diagnosis in some infectious diseases. So it could be recommended for monitoring and surveillance programs in coldwater hatchery health status in Iran.

Keywords: Rainbow trout, Fry, Hematology, Mazandaran province, Iran

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Introduction

Hematological parameters changes would be sign of fish physiological responses against environmental stresses e.g. such as heavy metals in water pollution (Vosyliene, 1996) or bacterial infections (Austin, 1987). Often fish bacterial infectious diseases such as *Aeromonas, Flavobacterium, Vibrio, Edwardsiella,* and some fish viral diseases like IHN, IPN and VHS showed septicemia and hemorrhagic lesions. Thus important internal organs such as kidney, spleen, liver and pancreas that have important duty in fish physiology must be affected acutely by infectious pathogens (Austin & Austin, 1987). Therefore hematological changes would occur subsequently in response to the invading pathogens.

In many cases of fish infectious diseases diagnosis could be assisted by hematological study. Holway *et al*. (1975) reported that hematological examination of Infectious Hematopoietic Necrosis (IHN) disease in rainbow trout revealed important findings. The severe anemia observed in moribund fish was undoubtedly the result of the destruction of hematopoietic tissue. In addition, stained smears of peripheral blood from diseased and sampled fish revealed many abnormalities during the outbreak. Circulating erythrocytes were immature, varied in size and shape and several cells showed cytoplasmic vacuolation. Their nuclei also were varied in size and shape i.e. roundish, swollen, irregular and pyknotic. The researchers also reported that the most distinct and consistent changes in blood cell morphology were bibbed erythrocytes, large numbers of circulating macrophage which contained ingested. Also, degenerating cells and debris were seen freely circulating within the blood.

Clinical pathology of *Aeromonas* infection was investigated by (Rehulka, 2002) which caused mass mortality of rainbow trout, *O. mykiss* at a water temperature of 4°C. Severe anemia was seen and characterized by a reduced erythrocyte count and lower haematocrit and haemoglobin levels. Clinical chemistry analyses in the diseased fish indicated reduced levels of total protein, cholesterol, triacylglycerol and total calcium and an increase in the urea level. Among the five enzymes and isoenzymes analyzed, catalytic concentration reaching multiples of the normal level was found in alanine aminotransferase, lactate dehydrogenase, α-hydroxybutyryl dehydrogenase and γ-glutamyl transferase. Electrophoretic analysis indicated a reduced level of albumin in the diseased fish.

The aim of this study was investigation on some hematological and biochemical aspects of rainbow trout fry in rearing and hatchery centers of cold water fish farm in western part of Mazandaran province in Iran.

Materials and methods

About 50 pools of blood samples from 750-1000 diseased fry were collected within 30 months from November 2002 till March 2005 from western part of Mazanderan province for hematological and biochemical studies.

In recent years, unknown mortalities were observed in many coldwater hatchery farms in several regions in Iran (Zorriehzahra *et al.,* 2005). To study the disease outbreaks as been

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reported in rainbow trout farms in Mazandaran province with darkening of the body, exophthalmia, ascites, erratic swimming and whirling symptoms, three important hatchery farms were selected for fry blood sampling:

1. Shahid Bahonar Hatchery Centre in Kelardasht region (20 pools of blood samples)*
2. Sarshar Hatchery Center in Tonekabon region (10 pools of blood samples)
3. Kousar Hatchery Centre in Tonekabon region (20 pools of blood samples)

*15-20 pieces of rainbow trout fry were considered as one pool.

About 450-600 normal fry (as 30 pools of blood samples) like control group were collected from mentioned farms. These samples were selected randomly from mentioned three hatchery farms in western part of Mazandaran province. They revealed no pathognomonic clinical signs of any infectious disease problem such as abdominal distention, exophthalmia, erratic pattern of swimming and white fecal casts. Both samples groups (control and diseased fry) were collected based on similar criteria such as age, size, pond, and were gathered at the same time.

150 environmental samples were collected for examination of some important environmental factors that gathered from water supply river of mentioned farms. These samples were examined according to routine and standard methods.

The blood collection was followed as indicated by OIE Diagnostic Manual for Aquatic Animal Disease (2003).The blood samples were collected by severing the caudal peduncle.

Bloods was allowed to flow freely from the vein of caudal peduncle to capillary tube and by allow the tube to fill by means of capillary action. In some cases surface of the tubes are covering by heparin solution as anticoagulant to prevent the clotting of the blood sample. In other side blood was allowed to clot for 10 min and then it was centrifuged at 4100 ×g for 10 min at 4°C. Serum was collected, visually examined to exclude haemolysis, then immediately analysed in hematology laboratory of Caspian Sea Ecology Research Center (Leloup *et al*.,1998). Clotted blood was used for blood enzymes measurement, while whole blood was collected for hematology study using sodium heparin (0.2ml heparin, equals to 5000I.U, was used for 1ml blood sample) as anticoagulants (Svobodova *et al.*, 2008).

For follow up mentioned purpose, each blood sample was divided two parts as follows:

a) Whole blood examination:

Whole blood examination includes total leukocytes (WBC) and erythrocytes counts (RBC), hemoglobin (Hb) content, hematocrits (PCV), leukocytes differential count and blood indices such as mean corpuscular or cell volume (MCV), mean cell hemoglobin content (MCH), and the mean cellular hemoglobin concentration (MCHC). Haematocrits (PCV) were determined immediately after sampling, using a microhaematocrit centrifugation (10, 500 ×g for 5 min) in mentioned hematology laboratory.

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Capillary tube was centrifuged for 5 minutes at 10, 500rpm in a micro-haematocrit centrifuge (SESAN MEDICAL EQUIPMENTS IND®) and volume PCV was measured using microhaetocrit reader. The blood plasma was obtained by centrifuging the heparinized blood at 4100 ×g for 10 min at 4°C and the blood cells (erythrocytes and leucocytes) were separated into eppendorf tubes. All blood parameters were performed within 12h. Blood smears were air-dried and stained by the Giemsa Romanowski method. Erythrocyte counts were determined using a Bürker counting chamber and Hayem solution. The counts were made in 2 × 20 rectangles per sample. Hemoglobin concentrations (gl^{-1}) were determined by the cyanhaemoglobin method using a wavelength of 540 nm. The mean corpuscular volume (MCV, fl), mean corpuscular hemoglobin (MCH, pg) and mean corpuscular hemoglobin concentration (MCHC,g/dl) were calculated from hematological data. The leucocytes were differentiated according to Ivanova (1983) and the relative abundance of all cell types was determined by counting a total of 200 blood cells.

b) Blood enzymes measurement:

Clotted blood in capillary tubes was centrifuged for 10 minutes using a high-speed centrifuge (Xiangyi® Centrifuge Instrument Co., Ltd, Model: TG16-W) 4100 ×g for 10 min at 4°C. The separated serum was then analysed for total protein (TP) and blood enzymes such as Lactate dehydrogenase (LD), Alkaline phosphatase (ALP), Alkaline transeaminase (ALT) and Aspartate transeaminase (AST). An automatic blood enzyme analyzer (Hitachi 704) was used for the following determinations: Total protein (TP,g l^{-1}), Lactate dehydrogenase (LD,μkat l^{-1}), Alkaline phosphatase (ALP, μkat l^{-1}), Alkaline transeaminase (ALT, μkat l^{-1}) and Aspartate transeaminase (AST, μkat l^{-1}). The apparatus is based upon dry chemical technology and colorimetric reaction. Kits obtained PLIVA-Lachema and DIALAB ®, were used for the determination of all indices. Also for controls, same kits were used. The hematological and biochemical parameters are expressed in international units (SI).

All the calculations were made using the SPSS© program version 13.0.1 (SPSS Inc., Chicago, IL, U.S.A.) and t-test statistical method (Mela *et al.,* 2007).

Results

Regarding to fry samples collection schedule, two models were selected. First of all, 50 pools of infected fry were selected as affected samples and 30 pools were collected from normal fry as control group. They revealed no pathognomonic clinical signs of any infectious disease problem but infected fry showed signs of lethargy, stayed at the periphery of the pond or gathering near the outlet of the raceway and showed pale appearance in gills. Some had a dark body color and a unilateral or bilateral exophthalmus (Fig. 1), and erratic swimming. The gut was swollen at the end, and the anus was reddened and slightly prolapsed. Also ascites and presence of the faecal casts were observed.

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Average of some morphology aspects of obtained fry samples were revealed in table 1.

Hematological findings from 80 pooled fry blood samples (30 normal fry, 50 infected fry) were analyzed and presented in tables 3, 4 & 5.

Components of water supply of three hatchery farms were summarized in table 2.



Figure 1: Clinical signs of affected fry

Table 1: Some morphology features of collected *(Oncorhynchus mykiss)* fry samples in Mazandaran province

Morphology Features / Hatchery Farm	Age (days) (± SD)	Weight (g) (± SD)	Length (cm) (± SD)
Control group *(Oncorhynchus mykiss)* fry	36±4	2.14±0.08	5.73±0.251
Shahid Bahonar Hatchery Center (Kelardasht)	35±4	2.15±0.14	5.62±0.363
Sarshar Hatchery Center in Tonekabon	37±3	2.16±0.04	5.83±0.152
Kousar Hatchery Center in Tonekabon	34±4	2.08±0.12	5.73±0.057

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Table 3: Average hematology parameters from rainbow trout *(Oncorhynchus mykiss*) fry obtained from hatchery centers in Mazandaran province

Blood index / Hatchery name	Total Leucocytes ×10^4 No / mm^3 (±SD)	R.B.C× 10^4 No/mm^3 (±SD)	Hct (%) (±SD)	Hb (gl^{-1}) (±SD)	M.C.V (fl) (±SD)	M.C.H (pg) (±SD)	M.C.H.C (g/dl) (±SD)
Control group *(Oncorhynchus mykiss)* fry	2.0±0.2	1.33±0.13	47±1	9.4±0.2	353±11	71±2	20±0.4
Shahid Bahonar Hatchery Center (Kelardasht)	2.8±0.27	1.26±0.12	35.2±1	9.1±0.2	279±7	72.2±2	25.8±0.6
Sarshar Hatchery Center in Tonekabon	4.8±0.4	1.18±0.11	42.2±1	8.2±0.17	357±11	69.5±1.8	19.4±0.4
Kousar Hatchery Center in Tonekabon	3.86±0.38	1.30±0.11	41.6±1	9.4±0.2	319±9	72.3±2	22.6±0.5

Table 5: Comparison of hematological and biochemical indices between infected fry and uninfected fry as control group

No.	Hematological test and unit of measure	Control (Mean ±SD)	Infected fry (Mean ±SD)
	Whole blood:		
1	Hemoglobin (g/100 ml)	9.40±0.2	(8.90±0.2)[a]
2	Erythrocytes (RBC) (x10^6/ml)	1.33±0.13	(1.25±0.11)[b]
3	Hematocrits (Packed cell volume)(%)	47±1	(39.67±6.87)[a]
4	Mean corpuscular volume (fl)	353±11	(318.33±89.73)[b]
5	Mean corpuscular hemoglobin (pg)	71±2	(71.33±17.01)[b]
6	Mean corpuscular hemoglobin concentration (g/dl)	20±0.4	(22.60±8.07)[a]
7	Total leukocytes (%)	2.0±0.2	(3.59±1.45)[a]
	Differential Leukocyte count:		
8	Lymphocytes (%)	90.7±1.7	(88.5±1.45)[a]
9	Neutrophils (%)	4.0±1.0	(1.18±0.55)[a]
10	Total Plasma Protein (g/dl)	4.45±1.0	(5.2±1.48)[a]
	Blood enzymes:		
11	Aspartate transeaminase (AST, µkat l^{-1})	112.27±65.8	(79.10±44.04)[a]
12	Alkaline transeaminase (ALT, µkat l^{-1})	7.27±2.05	(7.7±2.44)[b]
13	Alkaline phosphatase (ALP, µkat l^{-1})	194.55±91.48	(213.5±99.6)[b]
14	Lactate dehydrogenase (LD, µkat l^{-1})	869.64±372.65	(997.7±495)[a]

a = Significant differences (P<0.05) with control group b = Non- significant differences (P>0.05) with control group

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Discussion

In this study, fourteen hematological and biochemical indices were measured. Consequently nine parameters were revealed significant differences (P<0.05) with control group in t-test. It consisted of total WBC, Lymph, Neut, Hb and HCT, MCHC, AST, DL and total protein plasma.

For the total white blood cell count, Lymph and Neut in the three investigated farms significant differences were observed as compared with the control fish (P< 0.05).

Fry mortalities were observed in mentioned farms several times in recent years. Both samples groups (control and diseased fry) were collected based on similar criteria such as age, size and pond, and at the same time.

Statistical analysis of hematological findings revealed significant differences (P<0.05) using t-test. In the determination of total leucocytes count of the three investigated farms, some significant differences (P<0.05) were observed when compared with the control group. In the fish, infectious diseases agents such as bacteria or virus, in the first stage of disease the non-specific defense system (cellular) was first stimulated. In these situation, first of all the leucocytes will be initially increased (leucocytosis) in order to protect the fish body with phagocytosis mechanism and produce antibacterial or antiviral chemicals to stop the agent from spreading.

Haney & Winton (1992) observed that in Viral Erythrocytic Necrosis (VEN) disease in Chum salmon (*Oncorhynchus keta*), the total erythrocyte blood count, hematocrit and hemoglobin were decreased but total white blood cell count was increased.

In the current study, hemoglobin and hematocrits manifested significant differences (P<0.05) in examined infected fry samples in comparison with control group. Also all samples revealed obvious decreased in erythrocytes counts.

Wedemeyer and Nelson (1978) previously reported that in IHN disease, the mean values for hemoglobin, hematocrits, and erythrocyte could be reduced significantly lower than control values.

In many bacterial and viral diseases such as IHN and VEN diseases, septicemia was observed and anterior portion of kidney that responsible for hematopoiesis function could be damaged. Therefore total erythrocyte blood count, hematocrit and hemoglobin were showed severe decreased (Haley & Weiser, 1985).

In fish obtained from three mentioned farm the (Mean ±SD) of MCV in infected fry (318.33±89.73) was less than control group (353±11). Although no significant differences were observed in result but it could be concluded that infected fry suffered from hypochromic microcytic anaemia.

The results obtained from this study showed an interesting pattern of response in the hematological parameters. Studies have shown that when the water quality was contaminated by toxicants, any physiological changes will be reflected in the values of one or more of the hematological parameters (Nussey *et al.,* 1995). In the light of the

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present study, the significant decrease in the HCT observed in collected fry specimen could be attributed to the destruction of the erythroblast, thereby, limiting their synthesis. Similar trends in erythrocytes in fishes exposed to various toxicants and pathogens have been observed by other researcher (Mc Leay, 1975; Smit *et al.* 1979; Koyama & Ozaki, 1984; Srivastava & Narain, 1985; Van der Merwe, 1992). At present, the distinct decreased in the level of hemoglobin and MCV observed could induce anaemia condition in fry mortality syndrome in Iran. Its clearly suggests that a hemodilution mechanism has occurred. The MCV gives an indication of the status or size of the erythrocytes and reflects an abnormal or normal cell division during erythropoiesis.

On the other hand, the decrease in MCV that observed in this study after infection-coupled with low hemoglobin content indicated that the erythrocytes have shrunk, either due to hypoxia or a microcytic anaemia. Thus, microcytosis might be due to the decrease in the hematocrit during exposure. Similar pattern has been detected in Moggel fish *(Labeo umbratus)* after exposure to various pollutants (Nussey *et al.,* 2000).

In this study the fluctuation in the MCH was observed but MCHC had increment (22.60±8.07). It was indicated that the concentration of hemoglobin in the red blood cells were much lower in the infected fry than in the control group, thereby, depicting an anaemic condition. Anaemia can be caused by a number of pathological conditions. The macrocytosis is probably an adaptive response through the influx of immature erythrocytes from the hemato-poietic tissues to the peripheral blood to make up the reduced erythrocytes number and decreased hemoglobin concentration (Rehulka *et al*., 2005). The condition was observed to occur in infected fry from examined hatcheries centers.

These findings further support the hypothesis that hemodilution is a probable cause for decrease in hemoglobin content in infected fry in mentioned farms. The MCHC is a superior indicator of erythrocytes swelling (Wepener *et al.*, 1992). The MCHC, which is the ratio of blood hemoglobin concentration as opposed to the hematocrit, was not influenced by the blood volume nor by the number of cells in the blood but could be interpreted incorrectly only when new cells, with a different hemoglobin concentration, were released into blood circulation (Sovlo *et al.*, 1981). The significant decrease in the MCHC in this study was an indication of erythrocytes swelling and/or due to a decrease in hemoglobin synthesis. Buckley *et al.* (1976) reported that prolonged reduction in hemoglobin content was deleterious to oxygen transport and any blood dyscrasia and degeneration of the erythrocytes could be ascribed as pathological conditions in fishes exposed to toxicants or infectious diseases.

In this study blood serum components analysis revealed that in comparison of serum enzymes between LDH, ALP, ALT and AST, only LDH and AST amount showed obvious significant differences ($P<0.05$).

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Turnbull (1999) previously reported that Aspartate transeaminase (AST) was often raised in other viral salmonid viral diseases that revealed anemia such as Infectious Salmon Anemia (ISA).

In addition, in the current study, marked increment were observed in mean ±SDs of blood serum total protein (TP) in three examined farms (4.8 ±1.6gL^{-1}), (4.9 ±1.3gL^{-1}) & (6.5±1.4gL-^{1}) in comparison with control group in raceway culture (4.45 ±1.0g L^{-1}). This could be an indicator sign of antibody production activity in moribund fish in infectious diseases (Rehulka *et al*., 2005).

In opposite diminished levels of serum proteins have been reported for several diseases of fish (Hunn, 1964; Hodgins *et al.,* 1965). A reduction of total protein in salmonids with infectious disease is described by many other researchers. Mulcahy (1969) studied such a decrease in the serum of the brown trout and Atlantic salmon with ulcerative dermal necrosis (UDN) and single fungal infection *Saprolegnia ferax*, and in salmon fingerlings with fin rot and furunculosis.

Dorson (1972) reported previously that in IHN disease no apparent overall reduction in total plasma protein occurred, but specific alterations in some of the serum proteins was found. Klontz *et al*. (1965) showed an increase in the beta-2 serum fraction of Chinook salmon surviving an IHN infection and suggested it might be of immunological significance. The antibody production activity of fish serum resides in the slowest migrating macroglobulin.

Rehulka *et al.* (2005) showed that proteinaemia could be explained as a metabolic response to feed content, infections of bacterial or viral aetiology or to intoxicaions.

Regarding to clinical signs and hematological findings (blood and bio-chemical parameters) and observation of leucocytosis in first stages and neutrophylia as two important pathognomonic indicators in fish viral diseases in duration of diseased fry, it could be concluded that there was an acute infectious disease occurring in farm investigated, a kind of viral septicemia.

It is clear that comparison of hematology findings with other confirmatory diagnostic methods such as virology, serology and histopathology examinations could be used to identify and confirmed the presence of infectious causative agents of fry mortality syndrome in the country. Also regarding to obtained evidences of hematological and biochemical findings in this study it could be concluded that hematological and biochemical studies could be a valuable and available tool for prognosis and primary diagnosis in some infectious diseases in first stages of new probably outbreaks. So it could be recommended for using in monitoring and surveillance programs in coldwater hatchery health status in Iran.

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بررسی خونشناسی و بیوشیمیایی بچه ماهیان قزل آلای رنگین کمان(*Oncorhynchus mykiss*) در ناحیه غرب استان مازندران ایران

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چکیده

این مطالعه به منظور بررسی برخی عوامل بیوشیمیایی و خون شناسی بچه ماهیان قزل‌آلای رنگین کمان در ناحیه غرب مازندران صورت گرفت. در حدود ۵۰ نمونه خون بچه ماهیان بی‌مار طی ۳۰ ماه از آبان ۱۳۸۱ تا اسفند ۱۳۸۴ از مزارع این ناحیه جمع‌آوری گردید. همچنین ۳۰ نمونه خون نیز از مزارع مشابه بعنوان گروه کنترل تهیه شد. هر نمونه برای آزمایشات کامل و اندازه‌گیری آنزیمهای سرمی خون مورد آزمایش قرار گرفت که شامل سنجش کل گلبولهای سفید، قرمز، هموگلوبین، هماتوکریت، شمارش تفریقی و شاخصهایی مانند MCV، MCH و MCHC بود. همچنین سرم خون آنان برای سنجش پروتئین کل و آنزیمهای سرمی مورد آزمایش قرار گرفت. نتایج توسط نرم‌افزار SPSS و آزمون t-test مورد تجزیه و تحلیل آماری قرار گرفت. نتایج مویید آن بود که در آزمون t-test ، ۹ پارامتر خونی نمونه‌های بی‌مار در مقایسه با گروه شاهد اختلاف معنی‌دار نشان می‌داد ($P<0.05$). همچنین گلبولهای سفید، لمفوسیت‌ها و نوتروفیل‌ها اختلاف معنی‌داری نشان می‌دادند ($P<0.05$). تجزیه و تحلیل اجزای سرمی خون نشان داد که لاکتات دهیدروژناز و آسپارات ترانس آمیناز دارای اختلاف معنی‌دار می‌باشند($P<0.05$). براساس نتایج این بررسی می‌توان نتیجه‌گیری کرد که مطالعات خونشناسی و بیوشیمیایی برای پیش‌بینی و تشخیص اولیه در برخی بیماریهای عفونی ماهیان یک ابزار با ارزش می‌باشد. همچنین این مطالعات می‌تواند برای برنامه‌های پایش و مراقبت وضعیت بهداشتی مراکز تکثیر ماهیان ایران نیز توصیه شود.

کلمات کلیدی: قزل‌آلای رنگین کمان، بچه ماهی، خونشناسی، استان مازندران، ایران

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