STUDIES ON THE USE OF NEW METHODS IN VIEW OF THE EARLY DIAGNOSIS OF FISH DISEASES

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Abstract**:** *This paper aims to find new methods of laboratory investigations to identify the early stages of parasitic diseases in fish, to reduce mortality and improve disinfecting treatments. Hematology is used in ichthyopathology worldwide, especially in the case of infectious-contagious diseases, but we suggest studying the appropriateness of their widespread use in our country, extending them in the case of parasitic diseases with serious prognosis, to quantify the precise equivalence between the anatomical-pathological changes determined by the degree of fish infestation (resulting from the microscopic examination) and the physio-pathological ones expressed by the biochemical changes (shown by blood tests) that occur in the fish body, in various stages of a parasitic disease. The usefulness of performing a blood analysis, as a precision factor in paraclinical investigations, allows to determine the exact stage of the disease on which the effect of the disinfection treatment shows maximum efficiency and will also reveal the modality in which stocking densities of fish are influencing the immunity mechanism of the fish. From all the parasitic diseases we have chosen a protozoan Ichthyophthirius multifiliis (Fourchet 1876) infestation, which has a mortality of 50-90% on fish, due to a faster propagation rate, an aggressive behavior, it attacks the host tissue, penetrating deep into the subepithelial- skin and gills, thus achieving protection against chemotherapeutic substance used in the treatment, demonstrating the need to treate the disease in the early stages, when the parasite may be destroyed. The experimental results show that the degree of infestation is growing directly proportional to the stocking density, and inversely proportional with the size and immunity of the fish (the immunological parameters are increased by immunoglobulin M), the serological parameters (erythrocytes increase the hematocrit, hemoglobin and the total protein serum decrease), and the biochemical parameters (glucose, ALT and AST) increase due to the stress, disease state and the successive treatments. This study highlights how large losses in the fish farm sector can be effectively decreased, by using new, accurate analysis tools. The losses are caused by parasitic diseases that rapidly evolve and sometimes can be aggravated by the cumulative effect of adaptive stress, as frequent expression of technological errors, that act negatively on the defense mechanisms of fish. By correlating the results of hematologic analysis with those of the clinical and regular microscope examinations, the fish health can be monitored, through the growth period or in the case of biological material transfer from a fish farm to another, aiming to reduce the fish mortality by 89-98%.*

Key words: *fish, ichthyopathology, diagnosis, haematological analyses*

INTRODUCTION

The requirement to provide quality protein for the human consumption to a population that is growing fast worldwide is expressed by the continuous increase of the population ensities used in the current fish farming, which determine the emergence of a diversified and increasingly aggressive pathology, quantified by the increased mortality rate of fish.

Therefore, it is necessary to find new methods for the development of early diagnosis methods of fish diseases, for the urgent use of the treatment, in order to maximize the curative effects and their efficiency [4].

This study aims to develop a range of accurate investigations that can be used to diagnose fish diseases by experimenting with blood, serology and immunology analyses, correlated with the parasitic degree and the size of fish.

The parasitation was induced by the *Ichthyophthyrius multifiliis* parasitic protozoa and was monitored by blood tests as well as by classical microscopy to assess the degree of parasitation throughout the illness period and the three deparazitation treatments performed.

The goal of this study was to determine if the blood analyses, provide accurate data for early diagnosis of some serious parasitic and invasive diseases for preventing major mortalities of fish.

MATERIAL AND METODS

The main concept of this experiment is to demonstrate the evolution of a parasitic disease according to the blood parameters and monitoring them [10], with the final goal to characterize the different stages of the disease, for being able to establish an early diagnosis, for making the treatment more efficient.

The experiment was carried out with one year old carps (*Cyprinus carpio* L.,1758), stocked in four tanks, three experimental tanks BV1, BV2 , BV3 and one control tank BM1 in the Recirculation Aquaculture System of the Institute of Research and Development for Aquatic Ecology, Fishing and Aquaculture-Galați.

Three tanks BM1, BV1, BV2, were equally populated with 32 fish in each one, and the fourth tank with 64 fish.

The health state of the fish stock was evaluated, and their biometrical measurements have been recorded.

The study was conducted over a period of 24 days, initiated by fish stocking on 11/04/2017 and ended on 02/05/2017.

The experiment was conducted according to the following protocol: Tp = stocking date (day 1 of the experiment); Ti=parasites inoculation (day 4 of the experiment); Ts = the treatment with natrium chloride (7days after inoculation– day 11of the experiment); TNH_3 = treatment with NH_3 (day 10 after inoculation – day14 of the experiment), T1 is treated with malachite green in BM1 and BV3 (day 17day of the experiment) T2 = treatment with malachite green in BV1 (day 20 of the experiment) T3 = BV2 malachite green treatment (day 24 of the experiment).

The fish were stocked under constant climatological conditions and water quality, and fed in uniform conditions throughout all the experimental period.

After for days from the start of the experiment, all the tanks were inoculated, in equal amounts,with parasites from mud and aquarium sediments (the mud was collected from the ponds where fish presented ectoparasites, from Experimental Base Brateș- Galați, and the sediment was collected from the bottom of 2 aquariums containing fish infested with *Ichthyriophtirius multifiliis* (FOURCHET 1876).

In the 11th day, the ichtyopathologic evaluation, with the Oxion type microscope and the Olympus T2SN 4L00707 type dissecting microscope, identified an important parasitic infestation [7].

Four fish from each tank were sacrificed; after blood samples collection, ectoparasites were identified (11), as shown in Table 1.

Due to a great variety of parasites at this time of the experiment, it was decided to keep a single parasite by making some successive and selective treatments for obtaining a monoparasitosis.

It was chosen to keep the parasite, *Ichthyriophtirius multifiliis*, because it produces a high rate of mortality to the juvenile fish and great losses in fish farming [2].

Treatment recommendations are aimed at establishing two successive treatments every three days, with specific selectivity for different types of parasites [9].

The first treatment with sodium chloride - 5% solution administered by dips for 5 minutes, for trichodiniosis, gyrodactylosis, and the second with ammonia, to combat dactylogyrosis-solution 30% in the dose of 1 ml / 1 liter of water for 30 seconds (Tratat de Ihtiopatologie– Gabriela Munteanu și Dumitru Bogatu, Editura Excelsor Art, 2003), they were administered at an interval of three days, in all tanks, and, as a final result, an Ich.monoparasitosis was obtained.

The parasites found in the skin and gills wet mount, were examined between slide and coverslip, by microscopy method [7], and the results were presented in table no.2

For treating ichthyophthirosis we used malachite green (for scientific purpose, even its use is forbidden in fish farming, due to high toxicity), dosed in an ammount of 3 g / 10 m3 of water, which has been dispersed in the tanks, with an exposure time of 7 hours, by switching off the water supply and with supplementary aeration, to eliminate ciliated protozoa.

The cure bath was repeated three times every 24 hours. (Tratat de Ihtiopatologie– GABRIELA MUNTEANU ȘI DUMITRU BOGATU, EdituraExcelsor Art, 2003).

At the T1 time, (the 17th day of the experiment) the fish were treated with malachite green in BV3 -with fish at double density compared to the control tank and in the BM -control tank (BM versus BV3), the treatment ended on 28.04. 2017 when blood samples were taken and the ihtiopatologic examination was carried out.

Fish from BV1 were treated the same way (on day 20 of the experiment) at T2 time and was finished on 01.05.2017 (after three days since the treatment was carried out in the BM and BV3).

The treatment of BV2 fish was carried out (on the 24 day of experiment) three days after BV1 and six days after the treatment in the BM and BV3 and it was completed on 05/04/2017.

The results of the ihtiopathologic examination performed after treatment are shown in Table 3, was reported also, the mortalities registered during the treatment period.

To demonstrate the relationship between the degree of parasitation of fish and changes in blood, haematological analyses were performed [3] through the collection of biological samples from the blood of 4 fish per tank, randomly chosen, representing 12.5% of each tank's population, at the time of the illness and at the end of the experimental period.

The values of hematocit, hemoglobin, erytrocytes, [1] total proteins, protein fractions of serum, glycaemia, ALT, AST and immunoglobulins were analysed in the present experiment.

For haematological analysis methods, current methods applied in veterinary haematology have been used. (BLAXHAL 1973 GHERGARIU ET AL. 1985 SVOBODOVÁ, Z., 1991)

The dosage of total serum protein (proteinemia) was made by the colorimetric method which is based on the biuret reaction used for the determination of total protein in the fish serum. (Method AKSiwieki and DPAnderson 1993).

The dosage of protein fractions was performed by the standard electrophoresis method and the glucose analysis of the collected fish blood samples, based on the enzymatic method with oxidase-peroxidase, used in human medical analysis laboratories.

Collection of blood samples for glucose analysis was performed in three important stages: during stocking phase of the experiment, in the beginning, the first day of the

experiment, 7 days after inoculation and after drug treatment carried out in three stages: T1, T2, T3.

The last blood parameters studied were immunoglobulins analyzed by two methods:first,by the CIC precipitation with polyethylene glycol (PEG) and the determination of Immunoglobulin M(IgM) in the serum according to the Haskova method, modified [10] by PATRICHE T. (2007)

RESULT & DISCUSSION

Table 1.

Parasites identified after 7 days from inoculation

No.	*Parasite species identified*	*ControlTank Treatament T1*		*Tank V1 Treatment la T2*		*Tank V2 Treatment la T3*		*Tank V3 Treatment la T1*	
		affected organ	*infestation degree*	*affected organ*	*Degree of infestation*	*affected organ*	*Degree of infestation*	*affected organ*	*Degree of infestation*
1.	*Apiostoma piscicola*	O	-	-	-	-	-	*B*	isolated
2.	*Argulus foliaceus*	T	-	-	-	-	-	*T*	isolated
3.	*Cryptobia branchialis*	B	-	-	-	-	-	*B*	isolated
4.	*Dactylogyrus sp.*	B	isolated	B	isolated	B	isolated	B	isolated
5.	*Gyrodactylus sp.*	T	medium	T	medium	T	medium	T	isolated
6.	*Ichthyophthirius multifiliis (foront)*	T,B	isolated	T,B	isolated	T,B	isolated	T,B	isolated
7.	*Trichodina sp.*	T,B	isolated	B	isolated-low	B	isolated-low	B	isolated-low

O=eyes, T=tegument, B=gills

Table 2

Recorded of identified parasites after the treatment with the salt and ammonia

No.	*Parasite species identified*	*Control Tank Treatment T1*		*Tank V1 Treatment T2*		*Tank V2 Treatment la T3*		*Tank V3 Treatment la T1*	
		affected organ	*degree of infestation*	*affected organ*	*degree of infestation*	*affected organ*	*degree of infestation*	*affected organ*	*degree of infestation*
1.	*Dactylogyrus sp sp.*	B	-	B	isolated	B	isolated	***B***	isolated
2.	*Ichthyophthirius multifiliis (foront)*	T,B	low-medium	T,B	medium	T,B	medium	***T, B***	massive
3.	*Trichodina sp.*	B	isolated	B	-	B	-	B	isolated

For ichthyophthirosis, prevalence (expressed in %) and parasitic intensity (low <5 parasites per microscopic field, medium 5-10 parasites per microscopic field, high >10 parasites per microscopic field) were assessed [6].

Table. 3

Parasites identified after the treatment with malachite green

No.	*Parasite species identified*	*Control Tank Traatment T1*		*Tank V1 Teratment T2*		*Tank V2 Teratment la T3*		*Tank V3 Treatment la T1*	
		affected organ	*degreeof infestation*	*affected organ*	*degree of infestation*	*affected organ*	*degree of infestation*	*affected organ*	*degree of infestation*
1	*Ichthyophthirius multifiliis multifilis (foront)*	-	-	B	medium-massive	T,B	massive	B	isolated-low

The final results of fish losses through the experimental period was in BM- 6 dead fish with a survival rate of 70%, in BV3- 34 dead fish with 34,6% survival, 11 dead fish in BV1 with 45% survival percentage and 18 dead fish in BV2 with an achieved percent of 10% survival (excepting the sacrified fish for analyses).

The variation of the haematological values, at Tb, (7 days after the inoculation, when easily perceptible changes have been occurred in the behavior of the biological material) from the time of stocking (Tp) and after treatment with malachite green applied at different times (T1, T2, T3) for the BM and the three experimental variants (BV1, BV2, BV3), was analysed.

Table 4

Changes in haematological indicators at the stocking time (Tp) and at the moment of illness (Tb)

Exp. Mom.	*Tank*	*Weight (g)*	*Hematocrit %*	*Hemoglobin (g/dl)*	*Erythrocyte (mil/µl)*	*MCV (μm^3)*	*MCH (pg)*	*MCHC (g/dl)*
Tp		105,65±76.3	21.21±1.5	6.81±0.9	1.41±0.1	150.43±10.4	48.30±13.2	32.11±5.4
Tb	BM	87.3±57.1	20.9±1.3	6.6±0.7	1.6±0.2	130.6±14.1	41.3±3.2	31.6±1.7
	BV1	107.1±60.0	20.6±2.4	6.6±0.3	1.7±0.2	121.0±15.4	38.8±3.6	32.1±2.3
	BV2	108.9±56.0	19.9±1.4	6.4±0.7	1.9 ±20.1	104.7±10.1	33.6±3.3	32,13±1.9
	BV3	115.5±64.3	23.6±3.1	9,8±0.5	1.6±0.4	147.3±16.2	61.5±17.1	41,7±8.4

The hematocrit (Ht) under the stressing effect of stocking density and the identified parasites recorded at time Tb, increased in the BV3 group (where the density is doubled) compared to the initial moment Tp and compared to the other experimental groups.

The concentrations of hemoglobin at stocking time and 7 days after the parasitation of the biological material can be found within normal limits (7.8 to 9.5 g / dl) (GHITTINO, 1983; POP et al, 1991; MUNTEANU, 1970; Pieter, 1981; Molnar 1983), both for the control group and the experimental groups BV1 and BV2 (table no. 7), but smaller than the stocking ones. The exception is BV3's biological material whose values are greater than the upper range of normal limits (9.8 ± 0.5).

Significant reductions in the amount of hemoglobin and hematocrit occurs at the onset of the disease resulting in a decreased food apetite and severe anemia states [5].

Table 5

Changes in haematological indicators after treatment with malachite green at the time T1, T2 and T3

Exp. mom.	*Tank*	*Weight (g)*	*Hematocrit %*	*Hemoglobin (g/dl)*	*Erythrocyte (mil/µl)*	*MCV (μm^3)*	*MCH (pg)*	*MCHC (g/dl)*
T1	BM	78.87±59.1	21.1±2.3	7.6±0.7	1.6±0.2	183.9±24.1	49.0±13.2	26.7±11.7
T2	BV1	120.87±50.0	20.5±2.4	6.5±1.3	1.3 ±0.2	157.3±15.4	49.6±13.6	31.5±2.3
T3	BV2	159.37±36.0	19.9±1.4	5.7±0.7	0.7 ±20.1	284.3±20.1	81.4±13.3	28.6±11.9
T1	BV3	92.37±24.3	19.5±3.1	6.5±1.5	0.8±0.4	243.8±16.2	81.3±17.1	33.3±8.4

Increase in erythrocyte numbers and decreased values of hemoglobin and hematocrit values at the time of the acute disease, is the natural response to the parasitic disease of fish [13].

After the usage of the experimental treatments, that occurred at different times, haematological indices have been modified differently, depending on the density and the moment of chemotherapeutic intervention.

Hematocrit value suffered a slight increase in BM and BV2 after treatment, while in the other two tanks the values decreased.

At the Tb time, the amount of hemoglobin decreases, with the exception of the double-density tank, BV2, where there is a slight value increase. After treatment, hemoglobin continued to decline except the BM group, where treatment occurred at time T1 and the amount of hemoglobin slightly increased.

The amount of erythrocytes increased at Tb time and decreased significantly after the treatment.

In the parasited fish, the disturbance of haematological parameters can be interpreted as a defensive reaction to the action of parasites, either by stimulation of erythropoiesis and increase of hemoglobin or decrease in hematocrit due to some disturbances that occur in the metabolic and hence in the hematopoietic activity.

Analysing the statistical values obtained in the case of the erythrocyte amounts [11] it is observed that the stocking density has determined a significant erythrocytar hemoglobin increase - MCH ($p < 0.05$) and the hemoglobin concentration per erythrocyte - MCHC ($p = 0.001$ and highly significant). So, with increasing stocking density it is observed an enrichment of erythrocyte in hemoglobin characterizing the effort of the fish to adapt to low oxygen conditions [1].

Hemoglobin values at the time Tb, highlights the differences between the two versions of both BV and BV3 in comparison with the density at the initial moment- Tp time. Hemoglobin values recorded in fish, for both experimental variations, are in concordance with the literature data for this species (7 g / dl, Ghittino, P., 1983, Munteanu G., Cristea V., 1995).

From the statistical point of view, the amount of hemoglobin was significantly increased ($p < 0.05$) in BV3, and it can be explained by the fact, that in the case of higher stocking densities [12] there was an increased oxygen requirement.

Therefore, the decrease in the amount of dissolved oxygen and the onset of the disease, the fish show an adaptive response, thus hematopoetic organs are stimulated to produce more respiratory pigment – hemoglobin [8]. In fish lots of similar density hemoglobin has decreased according to the time in which the treatment was applied.

Increased stocking density influences haematological constants (MCV, MCH, MCHC). As in the experimental variant the stocking density was double (BV3), the hemoglobin of erythrocytes (MCHC) increased compared to baseline and compared to the experimental variants BM, BV1, BV2.

On the other hand, the blood parameters change and overall plasma protein levels rations betwen protein fractions such as the albumin / globulin, or one of various protein fractions that have important implications, especially as on proper functioning of the immune mechanisms of fish.

The causes leading to decreased albumin in serum levels are essentially the same as those produced and the decrease of total serum proteins. While lowering total serum proteins, indicates inadequate protein intake food, lowering the albumin / globulin (A / G) ratio, indicates a possible disease state.

As a result of the experiment, the following medium values were determined for the average total serum proteins and serum protein fractions, as determined by electrophoretic method (table 6).

Table.6

The values of serum proteins (g%) and the serum protein fractions in different stages of disease

Tank	*Exp.moment*	*Total serum protein (g%)*	*Albumin (g)*	*Albumin (%)*	*α (g)*	*α (%)*	*β (g)*	*β (%)*	*ϒ (g)*	*ϒ (%)*
	Tp	2.95	1.21	41	0.65	22	0.74	25	0.35	12
BM	Tb	1.8	0.5	28	0.54	30	0.49	27	0.27	15
	T1	2.95	1.21	41	0.65	22	0.74	25	0.35	12
BV1	Tb	1.55	1.74	49	1.03	29	0.64	18	0.14	4
	T2	2.75	1.38	50	0.74	27	0.52	19	0.11	4
BV2	Tb	1.75	0.25	14	0.38	22	0.35	20	0.37	21
	T3	1.2	0.23	19	0.6	25	0.3	25	0.07	16
BV3	Tb	1.55	0.75	49	1.93	29	0.64	18	0.14	4
	T1	2.75	0.9	50	1.74	27	0.52	19	0.11	4

The experiment registers a significant decrease of total serum proteins for BV2 group where the treatment was applied late, at T3 moment.

High values, but much lower than the BM group, were recorded in BV3, where although the fish density was double, the treatment was done at T1 time, when the disease was in the early stages.

The decrease in total serum protein is also reflected by the low level of albumin and globulins in the serum, in the ill carp compared to the healthy carp visibly varying, depending on the stage of the disease.

The value of the albumin / globulin ratio fell below 0.3 in the BV2 group where the treatment was applied at T3 time, and in BV3 where the density was double and stress had a destructive impact on the immunity of the biological material. The decrease below the normal value of albumin indicates a disturbance of the protein synthesis function, in the liver due to the lack of appetite for food, when the disease installs.

Glucose levels in fish blood, constitutes an indicator marker of the stress conditions (KEBUS ET AL., 1992; DE DOMINIS ET AL., 1993; BAU ET AL., 1994; REHULKA, 1996).

Dosage of blood glucose is an indicator for the prognosis and diagnosis of disease states. The results regarding the dosage of glycaemia in blood by the enzymatic method have shown that the blood glucose declined in all four versions at the time Tb (acute stress disease) in comparison with the stocking value (table no. 7).

Table 7

Changes in blood glucose (mg / dl) during the experiment

Tank	*Time Exp.*	*Glycaemia (mg/dl)*		
		minimum	maximum	average± Sd
	Tp	25,8	68	34,84±4,83
BM	Tb	23,70	37,50	33,56±3,57
	T1	36,00	90,00	54,75±13,12
BV1	Tb	23,70	31,50	27,56±3,57
	T2	27,90	41,50	34,20±2,57
BV2	Tb	22,5	24,8	23,65±0,70
	T3	36,04	90,00	63,15±14,72
BV3	Tb	20,5	21,8	21,15±0,80
	T1	28,00	43,00	34,17±4,09

After treatment, the fish organism, in order to face the aggression of pathogen agents, reconsiders their oxygen reserves leading to increased glycaemia values, as an adequate response to the installation of the stress state, as follows: BM- 63%; BV1-increase by 26%; BV2 - increase by 74%; BV3 - increase by 62%.

The effect of overcrowding BV3 tank is expressed by glycaemia levels increase in direct proportion to the density of the fish by 62% after treatment.

In disease states the tests carried out on blood collected from the ill carp (especially the acute phase of stress disease) or fish with a high degree of parasitary infestation, showed a decrease in blood glucose up to 38% compared to the average stocking value.

Normally, there are small amounts of blood transaminases. When these values rise, it means that the aggressive factor increased the liver transaminases, causing hepatocyte lysis and discharges their contents into the bloodstream.

The effort of the body to overcome the illness leads to increases in ALT and AST.

In all groups ALT values have increased due to the treatment that attacks the liver cells, determining an increase of the ALT and AST values in comparison with the moment of stocking.

Tests on immunochemical diagnosis of parasitic disease on imunocirculante complexes and determination of serum immunoglobulin M were made.

The test results made on biological material showed normal levels of circulating immune complexes (ICC) (reve no.8)

Table 8

Evolution of values of immune circulating complexes

Tank	*The experiment moment*	*ICC(UF)*
	Tp	84,84±14,83
BM	Tb	73,56±13,57
	T1	64,75±13,12
BV1	Tb	97,56±10,57
	T2	74,20±20,57
BV2	Tb	163,65±21,70
	T3	63,15±14,72
BV3	Tb	381,15±10,80
	T1	94,17±14,09

Seven days after the inoculation (time Tb), the total number of specimens analyzed, 25% had recorded values within the range of normality, 25% had elevated and 25% had very high values, in particular specimens of carp presenting, at the macrocopic and microscopic clinical examination, a high degree of infestation with parasites.

After treatment with malachite green, 56% of all analyzed samples showed values within the range of normal values, 44% had elevations in values and 0% had very high values.

The tests conducted on the biological material have shown that fish reacted positively to encounter antigenic stimulus at the time of harvesting samples of blood, serum immunoglobulin levels being directly reflected by the massive attack, and corelated with the antigen in each specimen which has undergone inpart.

The test results of biological material showed normal levels of immunoglobulin IgM.

After obtaining monoparazitosis, the analysed specimens that showed, during microscopical clinical examinations, a maximum degree of parasitic for ichtyophthiriosis in all the 4 tanks, registered high values, and very high values of immunoglobulins (IgM).

It is obvious the interrelation between the degree of fish infestation and the adaptive stress (BV3) which is closely linked to the evolution of blood parameters and the immune mechanisms correlated with the size of the fish.

CONCLUSIONS

A 3-day or 72-hour delay in diagnosing and applying the treatment can lead to loss of up to 50% of the biological material (table no. 19).

Delay of fish treatment within 6 days (144 hours) after the diagnosis, cancels the curative effect of the treatment, in the case of massive infestation with Ich., because the parasites are penetrating deep under the epitelium of the skin and gills, obtaining protection against the chemotherapeutic substances.

Size variations correlated with the immune response of the fish indicate a tendency to illness depending on the fish weight.

Blood analyses deemed appropriate for early diagnosis of serious parasitic disease in fish, giving maximum treatment efficacy.

We finally conclude that it is appropriate to use new methods to quantify the pathophysiological changes expressed by haematological analyses, as a new paraclinical investigation method correlated with the classic clinical methods, which are microscopic examinations, of the anatomo-pathological changes recorded in various fish diseases.

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