BIOCHEMISTRY

Values obtained from the quantitative estimation of the biochemical composition (haematological parameters such as: haemoglobin, haematocrit, RBCs, WBCs, and in blood serum such as plasma total protein, albumin, globulin, A/G ratio, cholesterol) of the haemoflagellate trypanosome infected fishes are presented in the table-I and II respectively based on the figures I to XIII.

Biochemistry is an important diagnostic tool for different parasites due to the various abiotic environmental differences and biotic interactions such as: changes in water temperature, pH, oxygen concentrations, water pollutants and predator pressure, parasitic invasions respectively (Hansen and Bhelcher, 1970). The gross chemical composition of host differs from place to place and also in different developmental stages. Biochemical parameters can also be used as a tool in disease diagnosis and in guiding the implementation of treatment or preventive measures of haemoflagellates as concluded by Ahmed et al., (2011).

Tandon and Joshi, (1974) studied the effect of trypanosome infection on serum proteins and glucose levels of fishes. Varied observations on serum cholesterol levels of hosts infected with
trypanosomes have been made chiefly on mammals (Launoy and Lagodsky, 1936).

Woo, (1995); Laya, (1994) and Overath et al., (1999) reported that parasites have influence upon host in many ways such as: anemic, dull with slack appearance, physical weakness etc.

It is well known that certain blood parameters serve as reliable indicators of fish health (Bond, 1979) as many parasites can live in a host, sometimes causing damage to it. Therefore, the changes associated with haematological parameters due to various parasites establish a database, which could be used in disease diagnosis and in guiding the implementation of treatment or preventive measures. These measures are essential in fish farming and fish industry (Roberts, 1981).

Various workers like Tandon and Joshi, (1973); Tandon and Chandra, (1977a, 77b); Mukherjee and Haldar, (1982); Woo and Block, (1984); Lom and Dykova, (1992); Stokopf, (1993); Gupta and Gupta, (1994), Gupta et al., (1998); Rauthan et al., (2010) and Ahmed et al., (2012) reported that physiological alterations, host susceptibility, leads to economic losses and mortality. They also reported that interaction and displacement, alter and interfere with the normal physiology of the host. These also bring about a change in biochemical constituents in host body.
It was observed during investigation that parasitized fishes appear pale and lethargic in comparison to healthy one. Some important reported signs of infected fishes were :-

1. In severe infection, trypanosomiasis displayed paleness of the external body surface.

2. Emaciation gasping with dullness.

3. They have slack appearance.

4. They have paleness of gills.

5. Internally, they showed splenomegaly.

6. They have watery blood with paleness of the internal organs.

7. The infected fishes were lethargic.

Thus, it was observed that the presence of haemoflagellates in host body changes the biochemistry of host blood.

**Haemoglobin (Hb)**

The blood parameters have been used as a sensitive indicator of stress in fish exposed to different water pollutants and parasitological infection. The oxygen carrying capacity of blood at 95% saturation, the iron content of the blood and the number of RBCs in fishes often vary
with life history, stage, habits, and environmental conditions. Transport of O$_2$ in the blood depends on the amount of haemoglobin, a respiratory pigment found in the blood of all vertebrates.

Haemoglobin content was found to fell sharply in trypanosomal infected fish as compared to the uninfected host. It was observed 9.85 g/100ml in infected host and for uninfected host was 12.95 g/100ml (Table- I) and (Fig.I).

DISCUSSION

The exposure of *Clarias batrachus* to trypanosome infection lead to anemia, lowered the haematocrit and haemoglobin levels. Anemia is one of the most common feature of infection in fishes along with other clinical signs. This study has confirmed the previous findings that anemia is directly related with the increase in parasitemia during trypanosome infection in fishes (Islam and Woo, 1991; Ahmed and Oilevier, 2001, 2002 and Ahmed et al., 2001).

In the present study it was observed that the decrease in haemoglobin concentration was accompanied with decrease in the number of erythrocytes in the blood of infected fish which was confirmed through the findings of various scientists that significant destruction of erythrocytes has occurred during trypanosome, trypanoplasm or cryptobia
infections in fishes (Khan, 1985; Woo, 1994; Ahmed and Oilevier, 2001, 2002 and Ahmed et al., 2001). This destruction of erythrocytes was positively correlated with the severity of parasitemia during the infection of *T. danilewskyi* in gold fish (Islam and Woo, 1991) because of this, the number of mature erythrocytes in the blood decreased, which ultimately resulted in less haemoglobin per ml of blood. Another factor that is, an increase in immature erythrocytes (small in size, rounded in shape, grayish-blue in colour and contained less haemoglobin) was observed in the infected blood during the infection of *T. danilewskyi* strain FCc 1 in juvenile common carp, that was because of rapid proliferation of erythrocytes from erythrocyte stem cells. The significant decrease in haemoglobin concentration in the blood of infected fish was not only due to an increase in immature erythrocytes but also relative decrease in the number of mature erythrocytes in relation to the number of leukocytes, which eventually lowered the overall relative percentage of mature erythrocytes in the infected blood. Thus, the changes in the final composition of infected blood resulted in lowered level of haemoglobin.

Thus, the average concentration of haemoglobin was decreased in infected fish than the uninfected host. It was reported that parasitization by blood pathogen like trypanosoma metabolically depended on the blood
of their host and altered the host physiology, so decrease in haemoglobin content in fish was due to trypanosome infection.

**Haematocrit (Hct) or Packed Cell Volume**

The micro-haematocrit represents the parameters most often studied perhaps because it is easily undertaken and interpreted. The haematocrit value is not easily altered as other parameters and is used in conjunction with erythrocyte count, haemoglobin content, osmotic fragility and differential leukocyte count (*Wedemeyer et al., 1983*).

Haematocrit (Hct) or packed cell volume was decreased significantly in the infected fish due to parasitization of trypanosoma. It was observed 27.27 % in infected fish and for uninfected fish it was 32.42% (Table-I) and (Fig-II).

**DISCUSSION**

The reduction in packed cell volume in the infested catfish occurred as a result of the parasitic infestation that often leads to anemia. Furthermore, the parasites simply act as a stressor, and during primary stages of stress, the packed cell volume changes due to the released catecholamine, which can mobilize red blood cells from spleen (*Wells and Weber, 1990*) or induced red blood cells swelling as a result of fluid shift into the intracellular compartments (*Chiocchia and Motais,*
1989). Similar results were recorded by Ismail, (2003) in Clarias garipienus naturally infested with Trypanosoma mukasia.

**Red blood cells (RBCs)**

In normal fish, the shape of the RBC was oval in shape but a few irregular circular cells with rounded nucleus were seen at the centre of the cytoplasm. The size was also same. The volume of the cytoplasm was more or less four times higher than the nucleus. The colour of the cytoplasm was reddish and purple respectively.

But in infected fishes, the number of RBCs was decreased gradually and the volume of cytoplasm of these RBCs was also reduced gradually. Most of the nuclei were elongated and some of them were fragmented into two or more equal or unequal parts. In some cases, the cytoplasm was vacuolated, tear drop like or completely lost leaving the nucleus either intact or swelled up (Fig-3a).

The red blood cells or erythrocyte is by far the most prevalent of the formed elements. The number of red blood corpuscles in fish fluctuates greatly during the seasons of the year and also during the life of fish.
Changes in the number of red corpuscles are used for the diagnosis of pathological conditions of diseased fish, when the numbers of red blood cells are reduced significantly, lowered as compared to healthy one. The total count of RBCs in *Clarias batrachus* infected with trypanosomes were 2.19 M/mm³ and for uninfected one it was 2.55 M/mm³ (Table-I) and (Fig.III).

**DISCUSSION**

All the morphological abnormalities of erythrocytes found in trypanosome infected host, might be due to abnormal erythropoesis. Reduction in the volume, vacuolation of cytoplasm and fragmentation of RBCs are the indication of anemia.

The presence of trypanosome affects the total count of RBCs in host body, it usually decreases as the infection of trypanosomes and the volume of cytoplasm of these RBC was also reduced gradually and most of the nuclei were elongated. Trypanosomes secrete some haemolysins capable of lysing red blood cells in infected host.

According to *Tandon and Joshi, (1973)* and *Gupta and Gupta, (1985)* the parasites which causes anemia in different species of Indian fresh water fishes. Parasitic infection showed conspicuous distortion in the composition of cellular components of blood.
White Blood Cells (WBCs)

White blood cells play a major role in the defence mechanism of the fish and consist of agranulocytes (lymphocytes and monocytes) and granulocytes (neutrophils, eosinophils and basophils). Granulocytes and monocytes function as phagocytes to salvage debris from injured tissue and lymphocytes produces antibodies (Ellis and Roberts, 1978; Wedemeyer and Mcleay, 1981).

Morphologically, in normal fish lymphocytes were rounded in shape and nucleus surrounded by a rim of large amount of cytoplasm. But in infected blood, the amount of cytoplasm was reduced and lymphocyte migrated to periphery, nucleus and cytoplasm were vacuolated occasionally (Fig.3b), but in uninfected host, monocytes were large, spherical in shape and nucleus was indented and surrounded by large amount of cytoplasm, where as in trypanosome infected fish blood, monocytes were reduced in shape and both cytoplasm and indented nucleus was vacuolated (Fig.3c). The normal neutrophils were circular with nucleus. But in infected blood, neutrophils were irregular in shape, both cytoplasm and nucleus were vacuolated and sluggish (Fig.3c). The normal eosinophil was rounded and regular in cell outline, nuclei were bilobed, dumbbell shaped and stained purple colour. But in infected blood, the cytoplasm of eosinophil was vacuolated (Fig.3d) and vacuoles
were enlarged gradually and nucleus was pushed to its periphery. The normal basophil was roughly oval in shape. In infected blood, morphological deformities were observed in basophil (Fig. 3d).

During the present investigation, it was found that differential counts of the white blood cells revealed that the percentage of lymphocytes and monocytes was quite high in infected host as compared to healthy one (Table-I) and (Fig.IV and V respectively). The mean value of lymphocytes and monocytes in uninfected host was 71.99 % and 5.47 % respectively and from infected one it was estimated to be 77.13 % and 8.56 % respectively (Table-I and Fig. IV & V respectively), while the percentage of neutrophils was found to be lower in infected host as compared to healthy one, it was observed 10.79 % in infected host and for uninfected host was 19.27% (Table-I) and (Fig.VI). There were no significant changes in eosinophil and basophil percentages in infected host as compared to uninfected host (Table-I and Fig.VII & VIII respectively).

**DISCUSSION**

Gradual increase in number of WBC was the peculiar effect of anemic condition and depended on trypanosome infection. Increase in WBC count also occurred as a pathological response since these WBCs play a great role during infestation by stimulating the haemopoetic tissues and the immune system by producing antibodies and chemical substances
working as defense against infection (Wedmeyer and Wood, 1974; Lebelo et al., 2001). The neutrophil percentage also fell conspicuously due to trypanosome infection. In fishes, the haemoflagellates are known to cause changes in the various haematological parameters and are also relatively spasmodic (Tandon and Joshi, 1973; Sharma and Joshi, 1991; Gupta and Gupta, 1985 and Akmirza and Tepecik, 2006).

According to Weir, (1983) and Roitt et al., (1996) parasites affects the differential count of white blood cells in host body. Increased lymphocyte count might be associated with production of antibodies by the host against the trypanosome parasites and the monocytes might have increased in number to participate in opsonin mediated phagocytosis of the trypanosomes.

Thus, as a defence mechanism against the parasitic infestation, WBCs count was elevated in the infected fish.

**Protein**

Protein plays a vital role in physiology of living organisms. All biological activities are regulated by enzymes and hormones, which are also proteins. In fishes, proteins are highly digestible. They are the most important nutrient for growth, constitute the bulk of the diet and are usually the most expensive component in artificial fish feeds.
The plasma total protein content was found to be decreased in infected host as compared to healthy one. It was observed 2.73 g/dl in infected host and for uninfected host it was 4.74 g/dl (Table-II) and (Fig.IX).

DISCUSSION

These pathogenic parasites affect the productivity of the catfish through mortalities, by decreasing growth rate, reducing the quality of the meat and utilize proteins to a different degree for energy production and amount of total proteins vary with species to species.

The decrease in proteins level may be a result of consumption of nutrient by the parasites.

Proteins make up about 7-8% plasma and are classified into general subdivisions serum albumins (4.2%), serum globulins (2.6%), fibrinogen (0.3%) and the remainder constituting 1% or less of the plasma.

These compounds endow blood with some of its most important regulating functions, including the function of chemical body defence.

These maintain the osmotic pressure of the blood, and since most of them are ionized, they also play a role in maintaining the pH of the blood, like mineral ions.
Serum albumin

Serum albumin not only maintains osmotic pressure needed for proper distribution of body fluids between intravascular compartments and tissues but also acts as a plasma carrier protein to transport steroid hormones, fatty acids and also compounds like drugs.

Serum albumin content was found to be decreased in infected host as compared to healthy one. It was observed 0.62 g/dl in infected fish and from uninfected fish it was 1.33 g/dl (Table- II) and (Fig.X).

DISCUSSION

In the blood serum of trypanosoma infected *Clarias batrachus*, we found the serum albumin was diminished as compared to uninfected fish. Decrease in albumin indicates liver insufficiency and malnutrition.

According to Gupta, (2006) trypanosomiasis in fish caused generalized odema and haemodilution. So the decrease in serum albumin is may be due to haemodilution.

Serum globulin

These serum proteins or globulins are very complex and are in immense range of physiological importance.
Serum globulin was found to be decreased in infected host as compared to healthy one. It was observed 2.11 g/dl in infected fish and from uninfected fish it was 3.41 g/dl (Table-II) and (Fig.XI).

In the blood serum of trypanosoma infected *Clarias batrachus*, we found the serum globulin was also diminished as compared to uninfected fish.

**Albumin-globulin ratio (A/G ratio)**

A/G ratio is a measurable humoral component at the non-specific defences. Shell, (1961), who surveyed the nutritive, osmotic and other functions of blood proteins in fish found a cyclic reversal of the albumin–globulin ratio in the fish.

A/G ratio was found to be decreased in infected fish as compared to healthy one. It was observed 0.30 g/dl in infected fish and from uninfected fish it was 0.41 g/dl (Table-II) and (Fig.XII).

The reduction of A/G ratio, might be due to the increase value of total serum globulin level as compared to A/G ratio with significance protective mechanisms for fish.
Cholesterol

Cholesterol content in the blood is linked to lipid metabolism and depends on the calorific value of the feed of fishes. Cholesterol is a major lipid found in the blood and has significant function on general physiology of cell membrane integrity and metabolic functions as formation of bile. Cholesterol holds a key and central position in body metabolism of the organism (Shell, 1961).

Brand, (1966, 1973) observed occurrence of disturbances in the carbohydrate metabolism of hosts during protozoan infections. He also suggested that pathogenic trypanosomes consume so much sugar in the blood stream that carbohydrate reserves of the host become exhausted, causing heavy strain on liver. Hypocholesterolemia was caused in Clarias batrachus infected with trypanosomes.

Serum cholesterol content was found to be decreased in infected host as compared to healthy one. It was observed 115.52 g/dl in infected fish and from uninfected fish it was 191.51 g/dl (Table-II) and (Fig.XIII).

DISCUSSION

Brand, (1966, 1973) also suggested that due to involvement of liver in such a condition its function is greatly disturbed, since liver is the
chief centre of cholesterol metabolism and its origin, the trypanosome infection disturbed the metabolism of cholesterol also and resulted in hypocholesterolemia in the infected fishes.

It was indicated that the trypanosomes lowered the serum cholesterol levels of fish *Clarias batrachus*. In some other organs like pancreas, supra renals, and thyroid was observed to be involved during trypanosome infection and lipid depletion of adrenals was seen in mice infected with trypanosomes (*Brand, 1973*). The protozoan parasites are said to contain endotoxins which cause pronounced metabolic disorders in animals in which liver is mainly involved (*Brand, 1966, 1973*). The cholesterol levels of blood of *C.batrachus* may have been reduced due to the involvement of either liver or adrenals or both in the metabolism of cholesterol under pathological conditions produced by trypanosome infection.

Antigen- antibody reactions are techniques using which antigen and antibodies are measured. Mixing of antibodies with their matching antigens on a surface such as animal cell, erythrocytes, or bacteria results in antibodies cross lining the particles forming visible clamps. The reaction is termed as agglutination. The specificities of the agglutinins elicited by the variants are studied by adsorption and agglutination method.
Fish respond to parasitic infections by the production of antigen specific IgM-like antibodies as well as by the elaboration of non-specific soluble factors and phagocytic cells. Fish infected with the haemoflagellates Trypanosoma generally elicit antibody and complement dependent responses. The levels of these responses vary depending on ambient temperature fluctuations. Below 10-15 °C there is an almost complete depression of immune responsiveness. Both primary and secondary antibody responses are produced in fish to this parasites. These cells (non-specific cytotoxic cells) may provide an important component of anti-parasitic resistance.

The agglutination test which uses formaldehyde fixed freeze dried trypanosomes stained with coomassie blue showed that agglutination test showed higher sensitivity (83%) but low specificity (96%) (Bajyana and Hamers, 1988; Reid and Copeman, 2003). When cattle serum was diluted 1/4 compared to a lower sensitivity (72%) but higher specificity (98%) when serum was diluted 1/8. Both IgG and IgM antibodies are produced in response to trypanosome infection. IgM is produced early in infection followed by more specific IgG, which plays a key role in the control of parasitaemia (Buza and Nassens, 1999; Magez et al., 2006).

Autoantibodies produced in response to repeated contact with trypanosome antigens may have contributed to the pathogenecity of the disease (Landsteiner and Vander Scheer, 1927).
Trypanosome-specific antibodies are predominantly of the IgG subclass, where as autoantibodies usually belong to the IgM subclass (Vincendeau and Bouteille, 2006). These so-called “natural” IgM antibodies produced by CD5+B cells provide innate protection against pathogens and toxins (Buza et al., 1997; Ochsenbein and Zinkernagel, 2000). The large increase in polyclonal antibodies observed during trypanosome infection could therefore be an expansion of non-specific antibodies normally associated with the immune system rather than direct stimulation of B cells by the trypanosomes (Buza and Nassens, 1999).

Serum levels of IgM remained throughout infection, and are not related to parasite load (Baltz et al., 1981). There may be a possible link between high IgM levels and immunodepression when suboptimal anti-parasite responses allow the parasite to evade host immunity (Hudson et al., 1976). It has been suggested that trypanosome induced polyclonal B cell activation in the presence of a continuous trypanosome infection could result in depletion of antigen-reactive B lymphocytes, which possibly become secretory cells without proliferating (Hudson et al., 1976). Immunodeficiency appears to be influenced by the level of parasitaemia (and living parasites) rather than B cell clonal exhaustion (Baltz et al., 1981).
**TABLE-I SOME HAEMATOLOGICAL PARAMETERS IN INFECTED HOST AND UNINFECTED HOST. (THE VALUES REPRESENT MEANS ± S.D.)**

<table>
<thead>
<tr>
<th>Blood Parameter</th>
<th>Infected host</th>
<th>Uninfected host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin content</td>
<td>9.85±0.26*</td>
<td>12.95±0.23</td>
</tr>
<tr>
<td>(g/100ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haematocrit or PCV %</td>
<td>27.27±0.96*</td>
<td>32.42±1.24</td>
</tr>
<tr>
<td>RBC s (M/mm³)</td>
<td>2.19±0.10*</td>
<td>2.55±0.17</td>
</tr>
<tr>
<td>Lymphocyte percentage</td>
<td>77.13±1.35*</td>
<td>71.99±2.24</td>
</tr>
<tr>
<td>Monocyte Percentage</td>
<td>8.56±0.47*</td>
<td>5.47±0.91</td>
</tr>
<tr>
<td>Neutrophil Percentage</td>
<td>10.79±0.55*</td>
<td>19.27±1.32</td>
</tr>
<tr>
<td>Eosinophil Percentage</td>
<td>2.08±0.10</td>
<td>2.08±0.17</td>
</tr>
<tr>
<td>Basophil percentage</td>
<td>0.75±0.04</td>
<td>0.64±0.06</td>
</tr>
</tbody>
</table>

* Significantly different from the value in uninfected fishes at 1 percent level.
TABLE-II SOME SERUM BIOCHEMICAL PARAMETERS IN INFECTED AND UNINFECTED HOST. (THE VALUES REPRESENT MEANS ± S.D.)

<table>
<thead>
<tr>
<th>Blood Parameter</th>
<th>Infected host</th>
<th>Uninfected host</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma total protein (g/dl)</td>
<td>2.73±0.11*</td>
<td>4.74±0.39</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>0.62±0.14*</td>
<td>1.33±0.31</td>
</tr>
<tr>
<td>Globulin (g/dl)</td>
<td>2.11±0.21*</td>
<td>3.41±0.58</td>
</tr>
<tr>
<td>A/G Ratio</td>
<td>0.30±0.10*</td>
<td>0.41±0.16</td>
</tr>
<tr>
<td>Cholesterol (g/dl)</td>
<td>115.52±3.57*</td>
<td>191.51±4.26</td>
</tr>
</tbody>
</table>

* Each value represents mean ± S.D.; n=10. * Significantly different by student t-test at P<0.01.
Fig.I. Diagram showing haemoglobin content (g/100ml) in infected and uninfected host (Acc. to table-I).

Fig.II. Diagram showing haematocrit percentage (g/100ml blood) in infected and uninfected host (Acc. to table-I).
Fig. III. Diagram showing RBCs (M/mm³) in infected and uninfected host (Acc. to table-I).

Fig. IV. Diagram showing lymphocyte percentage (g/100ml blood) in infected and uninfected host (Acc. to table-I).
Fig. V. Diagram showing monocyte percentage (g/100ml blood) in infected and uninfected host (Acc. to table-I).

Fig. VI. Diagram showing neutrophil percentage (g/100ml blood) in infected and uninfected host (Acc. to table-I).
Fig.VII. Diagram showing eosinophil percentage (g/100ml blood) in infected and uninfected host (Acc. To table-I).

Fig.VIII. Diagram showing basophil percentage (g/100ml blood) in infected and uninfected host (Acc. to table-I).
Fig. IX. Diagram showing plasma total protein content (g/dl) in infected and uninfected host (Acc. to table-II).

Fig. X. Diagram showing albumin content (g/dl) in infected and uninfected host (Acc. to table-II).
Fig. XI. Diagram showing globulin content (g/dl) in infected and uninfected host (Acc. to table-II).

Fig. XII. Diagram showing A/G ratio content in infected and uninfected host (Acc. to table-II).
Fig.XIII. Diagram showing cholesterol content (g/dl) in infected and uninfected host (Acc. to table-II).
Fig. 3a. Abnormal RBC in trypanosoma infected host *Clarias batrachus*. (f-fragmented RBC, vc-vacuolated cytoplasm, en-excruded nucleus, td-tear drop like RBC and nwc- nucleus without cytoplasm).

Fig. 3b. Abnormal lymphocyte (L) in trypanosome infected host *Clarias batrachus* (cr-cytoplasm reduced, vn&c-vacuolated nucleus and cytoplasm).
Fig. 3c. Abnormal monocyte (M) and neutrophil (N) in trypanosome infected host *Clarias batrachus*.

Fig. 3d. Abnormal eosinophilic (E) and basophilic (B) granules in trypanosome infected host *Clarias batrachus*. 