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Haematobiochemical and Histopathological Changes in *Labeo rohita* Infected with *Aeromonas hydrophila* by Immersion Challenge

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Haematobiochemical and histopathological variations in *Labeo rohita* subjected to immersion challenge with *Aeromonas hydrophila* by smearing a virulent bacterial culture (10^8 cfu/ml) over descaled portions (approximately $1cm^2$) near operculum and caudal peduncle of the fish were studied for a period of 15 days, with a view to assess the pathogenesis of the bacteria. The changes were recorded on 3rd, 5th, 7th, 10th and 15th days post infection (pi) and compared with a control and the results were compared statistically using Student's *t*-test. The symptoms of the infected fish include sluggishness with pale red gills, liquefaction of the internal organs and marked blackening at the descaled areas. Tissue sections revealed dermal oedema and muscular degeneration focal hemorrhage and necrosis of liver necrotic changes of haematopoietic tissue, glomerulii and renal tubules in kidney hyperplasia of gill lamellae sloughing of intestinal mucosal epithelium and mild degenerative changes of myocardium. The total erythrocyte count (TEC) increased on 5th and 10th day post infection (pi) while haemoglobin (Hb) values decreased and total leucocyte count (TLC) increased during the period of study. Alkaline phosphatase (ALP), aspartate amino transferase (AST) and alanine amino transferase (ALT) activity of liver tissue were highest on 3rd day pi. There was no set pattern in the differential leucocyte count (DLC) between the infected and uninfected fish. Histological studies confirmed that the fishes exposed were infected with *A. hydrophila* and the variations recorded in the enzyme activities and cell counts of blood in the infected fish revealed their impaired health status, massive systemic damage and anaemia due to invasion of the bacteria.

Keywords: **Haematobiochemical, histopathology, *Labeo rohita*, *Aeromonas hydrophila*, immersion challenge**

Aeromonas hydrophila has been identified as one of the most significant microbial pathogen affecting fish culture globally. It is a ubiquitous, free-living, gram-negative bacterium, prevalent in freshwater, marine and brackish water systems. Although it is a component of the superficial and intestinal flora of fish, the bacterium very often behaves as an outrageous secondary invader (Tonguthai, 1985). This organism has been reported to cause mass mortality in several groups of fish including carps, gouramies, murrels and catfishes. It is also the etiological agent of several fish diseases including dropsy, hemorrhagic septicemia, asymptomatic septicemia, ulcerative infections, tail rot and fin rot (Rahman *et al.*, 2001). The epidemics triggered by Aeromonas have been reported to take a heavy toll of Indian major carps and produce ulcerative forms of infection in fishes (Karunasagar *et al.*, 1986; Kumar, 1989; Mukherjee, 1991). The consistent association of this pathogen with fish affected by epizootic ulcerative syndrome (EUS), the rearing waters as well as with the laboratory inducement of dermo-muscular lesions in snakehead and catfish has been demonstrated (Lio-Po *et al.*, 1992).

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Once the infection is established, rapid growth of the bacterium and elaboration of its toxic products cause irreparable systemic damage, which ultimately leads to death (Brenden & Huzinaga, 1986). The variation in the degree of haematological responses that may vary according to stress or stimulus, treatment, parasitic or infectious disease etc. is an important tool in fish health diagnosis (Rehulka, 2002; Chen *et al*., 2004; Silveira-Coffigny *et al*., 2004; Martins *et al*., 2004) Changes in haematological indices also depend on the fish species, age, cycle of sexual maturity and health status (Hrubec *et al*., 2000). Moreover, haematological tests and analysis of serum constituents provide useful information in detection and diagnosis of metabolic disturbances and diseases in fishes (Aldrin *et al*., 1982).

The present study was aimed to evaluate the pathogenesis of *A. hydrophila* and the haematobiochemical and histopathological changes associated with infection by immersion challenge of *A. hydrophila* in rohu, *Labeo rohita*, a species which affected by EUS outbreak suffered widespread mortality in India.

Materials and Methods

Ten advanced fingerlings each of *Labeo rohita* (20 cm and 30 g) stocked in 60 l of good quality freshwater in rectangular fibreglass tanks and fed with a prepared feed at the laboratory of Central Institute of Fisheries Education (CIFE), Mumbai, India were used for the experiment. Water quality parameters and health condition of the experimental fishes were regularly monitored throughout the study.

The microbial culture obtained from Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India which was cultured in Tryptose soy agar (TSA) and Aeromonas isolation medium(AIM) and confirmed as *Aeromonas hydrophila* by means of biochemical tests (Olivier *et al*., 1981) was used for the challenge. The isolate was exhaulted serially four times in rohu and reisolated for immersion challenge studies. The clinical signs recorded during exhaultation were similar to Aeromonad infection. The fishes in the experimental tanks were descaled on the right side behind the operculum and also near the caudal peduncle, one day prior to the commencement of the study. The virulent bacterial culture was quantified to a final concentration of 10^8 cfu/ml by measuring absorbance, and using sterile swabs the bacterial suspension was smeared over the descaled area of the fish which were later released back into the tanks. Another group of ten fishes descaled and kept in identical conditions, but were not smeared with the bacterial suspension formed the control.

The infected fish were selected randomly from both control and treatment tanks for analysis on 3rd, 5th, 7th, 10th and 15th day post infection (pi). Individual fish was anaesthetized using clove oil and blood was drawn from the caudal vein and collected in EDTA (10 %) coated glass vials. To analyse haemoglobin (Hb) content using cyanmethemoglobin method (Blaxhall & Daisley, 1973), 20µl of blood was used. Another 20µl was used to determine total erythrocyte count (TEC) and total leucocyte count (TLC). Blood smear was prepared for determining differential leucocyte count (DLC) after staining the smear in Field stain A & B (Houston 1990). During necropsy 0.2 to 0.3 g of liver tissue was collected and stored at –4^{0}C for enzymatic studies, which was later analysed for alkaline phosphatase (ALP), aspartate amino transferase (AST) and alanine amino transferase (ALT) activities (Anderson & Siwicki, 1994; Reitman & Frankel, 1957). A portion of the descaled area was dissected and fixed in Carnoy's fluid. Tissues of liver, heart, spleen, gills, kidney and intestine were collected and fixed in 10% neutral buffered formalin. The tissues were later dehydrated in alcohol gradations, wax embedded, sectioned using microtome and stained with hematoxylin and eosin (H&E) for preparing the slides. Duplicate sections were stained by

Brown Hopps modification of Grams stain (Bancroft & Stevens, 1996) for demonstration of microorganisms. Photographs of these sections were taken using Olympus CX-31 camera to record the pathological changes. Similar observations were also made in the control group for statistical comparison of the data. The data of various parameters studied were subjected to statistical analysis using Student's *t*-test assuming equal variances ($p<0.05$).

Results and Discussion

The results of the present study show marked variation in blood cell counts, haemoglobin content and also enzymatic activity of alkaline phosphatase (ALP), aspartate amino transferase (AST) and alanine amino transferase (ALT) of liver tissue during the period of study when compared to the control revealing systemic damage and anaemic condition of the infected fish.

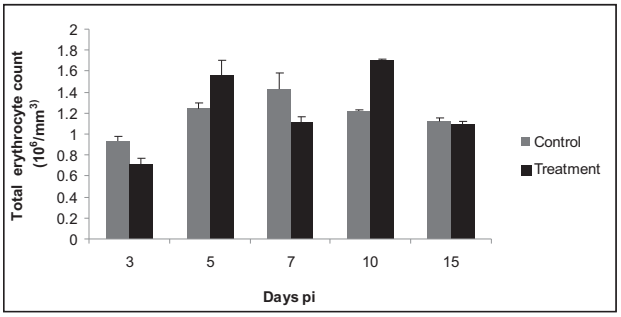

Fig. 1. Total erythrocyte counts (10^6/mm^3) of *Labeo rohita* challenged with *Aeromonas hydrophila* compared to control

Decreased red blood cells (RBC) were found in coho salmon (*Oncorhynchus kisutch*) infected with *V. anguillarum* (Harbell *et al*., 1979); in pearl spot (*Etroplus suratensis*) with epizootic ulcerative syndrome (Pathiratne & Rajapakshe, 1998); in rainbow trout (*Oncorhynchus mykiss*) with ulcerous dermatitis (Rehulka, 1998); in rainbow trout experimentally infected with *Aeromonas sobria* and *A. caviae* (Rehulka, 2002); in carp (*Cyprinus carpio*) experimentally infected with *A. hydrophila* (Harikrishnan *et al*., 2003), and in Nile tilapia experimentally infected with *Streptococcus iniae* (Chen *et al*., 2004). The haemoglobin and RBC count were significantly low in all moribund rainbow trout injected with infectious haematopoietic necrosis virus (IHNV) (Donald & Smith, 1975), in chum salmon infected with *V. anguillarum*, and in rainbow trout infected with Aeromonas/Streptococcus (Harbell *et al*., 1979; Barham *et al*., 1980). Diseased rainbow trout *Oncorhynchus mykiss* infected with viral hemorrhagic septicemia virus (VHSV) compared to healthy fish, had a significantly lower RBC and haemoglobin levels (Rehulka, 2003). Boon *et al*. (1989) and Genc *et al*. (2005) found that the erythrocyte count of fish infected with parasite was significantly lower in comparison to those in non-infected fish. The lower value of RBC in fungal infected Caspian salmon was reported by Jamalzadeh *et al*. (2009).

The results obtained in the present study also concur with the available reports in respect of the changes in the Hb and RBC levels in different species of fishes. The total erythrocyte counts (TEC) of fishes in the treatment tanks were significantly low when compared to the control group on 3rd and 7th day post infection (pi). There was significant difference between TEC of the treatment and control fish on all days of sampling except on the 15th day (Fig. 1). The haemoglobin content (Hb) in the treatment group was significantly lower than the control group throughout the period of study, the lowest Hb values being on the third day (Fig. 2).

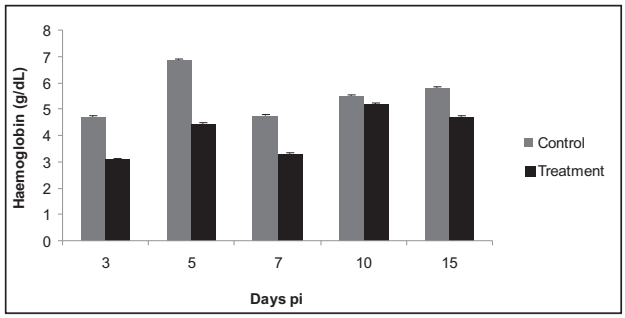

Fig. 2. Hemoglobin content (g/dL) of *Labeo rohita* challenged with *Aeromonas hydrophila* compared to control

Anaemia associated with *A. hydrophila* is the result of the ability of the bacteria to haemolyze erythrocytes (Olivier *et al.,* 1981). Since hemorrhage is a conspicuous feature of Aeromonas infection and because these haemorrhagic effects are evident *in vitro* and *in vivo*, haemolysis definitely contributes to the pathogenecity of this bacterium (Aruna *et al.,* 2001).

Quality and quantity of leukocyte cells are generally used in the determination of immune reactions and diseases (Cagirgan, 1990). It is known that leukocyte cells which are normally lower in healthy fishes could be used as a significant indicator for infectious diseases. In the present study, the total leucocyte counts (TLC) in the treatment group were higher than those in the control group on all the days of observation with the highest TLC on 5th day (Fig. 3). Increase in TLC is mainly due to response of fish immune system against the bacterial invasion (Ikeda *et al.,* 1976). Martins *et al*., 2008 reported that white blood cells (WBC) and lymphocyte number increased significantly in Nile Tilapia injected with *Enterococcus* spp. at a concentration of 10^6 cfu/ml. An increase in the WBC and glucose was observed by Haney *et al*. (1992) in chum salmon (*Oncorhynchus keta*) infected with erythrocytic necrosis virus. An increase in the number of WBC with induction of inflammatory processes during acute intestinal coccidiosis of carp was reported from differential WBC counts of parasitized carp (Steinhagen, 1997). Sahan *et al*. (2007) reported an increase in leukocyte cells of fish infected with the parasite in European eel, *Anguilla anguilla.* In the present study the differential leucocyte counts (DLC) show almost a constant percentage of neutrophils (Fig. 4). Although the number of neutrophils in the infected fish was higher than those in the control groups during 5th and 7th day pi, these values were not significantly different. The lymphocyte count, which was higher than control group on 3rd and 15th day pi, showed a decreasing trend during 5th, 7th and 10th day pi. In the present study though the percentage of total leucocytes in the blood smear remained unchanged, the percentage of lymphocytes in the differential leucocyte count significantly increased and the percentage of neutrophils decreased. The percentage of monocytes was significantly higher in the treatment group on 7th and 10th days pi, and was higher than control group on all days except on 3rd and 15th days pi. However, Donald & Smith (1975) found no difference in the percentage of monocytes during IHNV infection of rainbow trout. In another study, neutrophil and monocyte counts increased in Yellow tail infected with bacteria (Ikeda *et al*., 1976) while a decrease in lymphocyte count and an increase in neutrophil count were seen in *C. auratus* infected with *A. hydrophila* by intramuscular injection for 36 h pi (Brenden & Huzinaga, 1986). The counts of neutrophils, WBC, and eosinophils have been higher in fungal infected fishes than in healthy Caspian

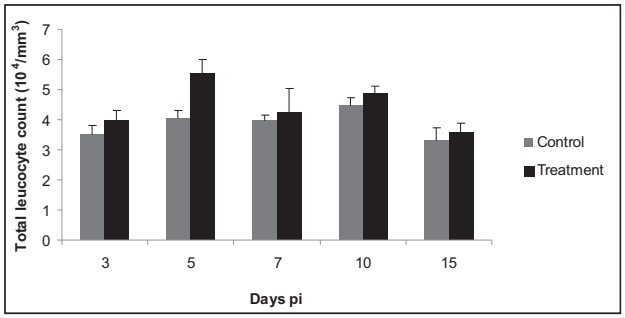

Fig. 3. Total leucocyte counts (10^4/mm^3) of *Labeo rohita* challenged with *Aeromonas hydrophila* compared to control

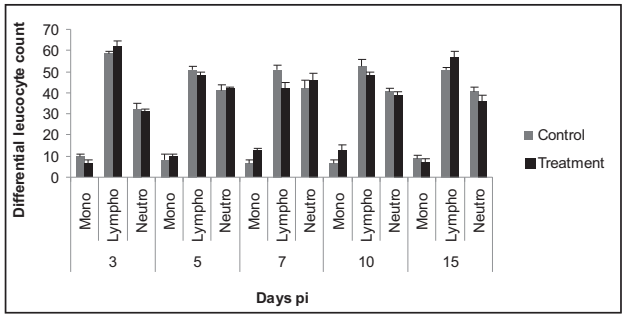

Fig. 4. Differential leucocyte counts of *Labeo rohita* challenged with *Aeromonas hydrophila* compared to control

salmon (Jamalzadeh *et al*., 2009). Changes in WBC and differential counts play important roles in the assessment of the state of health of *Clarias gariepinus* (Gabriel *et al*., 2004).

In the present study ALP activity was higher in the treatment group except on 10th day pi. The highest ALP activity was observed on 3rd and 15th day pi (Fig. 5). The ALP activity might increase even upto 40 times during disease conditions such as infective hepatitis, cholestasis etc. (Jiro, 1989; Donald, 1992; Luxton, 1999). Hepatocyte necrosis increases ALP activity (Noonan & Meyer, 1979). The higher ALP activity in the present experiment was the result of a systemic damage of the liver.

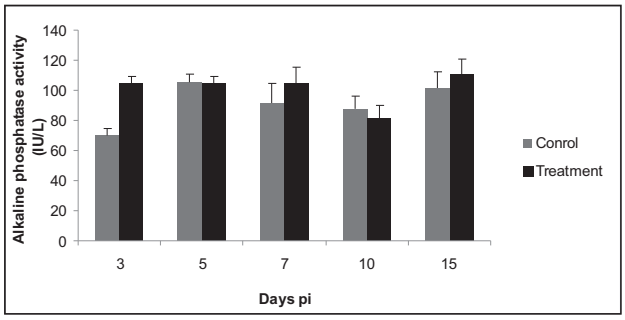

Fig. 5. Alkaline phosphatase activity (IU/L) of *Labeo rohita* challenged with *Aeromonas hydrophila* compared to control

In rainbow trout *Oncorhynchus mykiss* infected with viral hemorrhagic septicemia virus (VHSV) compared to healthy fish, increased levels were recorded in the catalytic concentration of ALT and AST while the catalytic concentration of ALP decreased (Rehulka, 2003).

On the 3rd, 5th 7th and 15th day pi in the present experiment, AST activity of the treatment group was significantly higher compared to the control. The ALT activity was significantly high only on 3rd day pi (Fig. 6, 7). It is well known that the amino transferases carry out the basic energy maintaining mechanisms during different physiological conditions like stress, starvation, liver dysfunction etc. and their degrees of elevation mark the extent of hepatocellular necrosis (Vasudevan & Sreekumari, 1998). During *A. hydrophila* infection of *C. auratus* AST activity increased while ALT activity decreased when compared to control during the first 36 h pi (Brenden & Huzinaga, 1986). The AST and ALT levels were highest during toxic or viral hepatitis and ischemia (Jiro, 1989; Donald, 1992; Luxton, 1999). Necrosis of skeletal muscles combined with focal necrosis of liver parenchymal cells may account for the elevated AST activity (Brenden & Huzinaga, 1986).

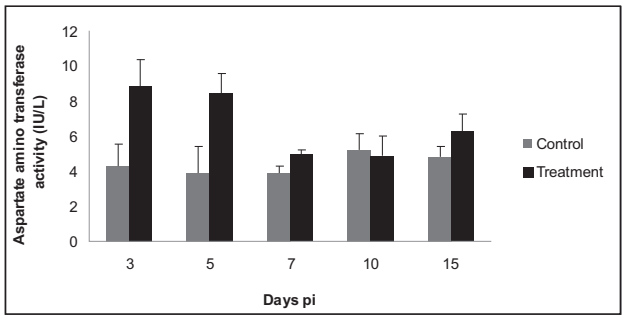

Fig. 6. Aspartate amino transferase activity (IU/L) of *Labeo rohita* challenged with *Aeromonas hydrophila* compared to control

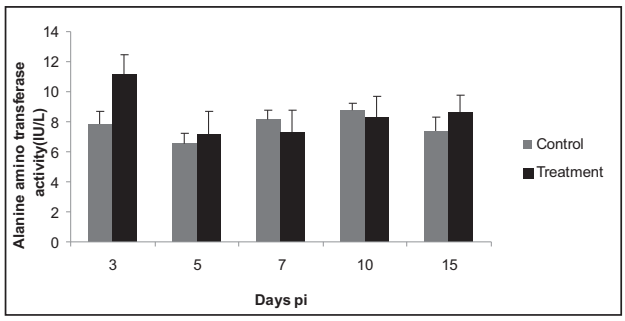

Fig. 7. Alanine amino transferase activity (IU/L) of *Labeo rohita* challenged with *Aeromonas hydrophila* compared to control

Although noticeable external lesions were not present on fish upon infection in the present study, a dark pigmentation was observed over the area of descaling. Epidermis was found markedly sloughed off in tissue sections (Fig. 8). Stratum spongiosum and stratum compactum exhibited derangement of muscle layers. Separation of dermal