ISSN: 2347-5129
(ICV-Poland) Impact Value: 5.62
(GIF) Impact Factor: 0.352
IJFAS 2015; 3(1): 116-124

www.fisheriesjournal.com
Received: 30-06-2015
Accepted: 02-08-2015

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Evaluation of in vivo non-specific immunity and oxidative stress in *Labeo rohita* (Hamilton, 1822) infected with *Aeromonas hydrophila* as biomarker for early diagnosis

Subharthi Pal, Sriparna Datta Ray, Sumit Homechaudhuri

Abstract
Labeo rohita Hamilton, 1822, was challenged with asymptomatic dose of *Aeromonas hydrophila* to study innate immune responses. Significant ($p > 0.01$) alterations were observed in haematological, biochemical parameters viz. white and red blood cell counts, haemoglobin content, haematocrit, leucocrit, MCV, MCH, MCHC, dWBC % (neutrophil, eosinophil, lymphocytes, monocytes), total protein, albumin, globulin, albumin : globulin ratio, glucose, calcium, bilirubin, alkaline and acid phosphatase activity in serum in treated and control fishes after 7 days exposure. ROS production increased significantly in exposed fishes along with increased SOD and Catalase activities. Aggregation of melanomacrophage centers and modifications were observed in the histopathology of liver. Annexin V/ PI study indicated higher percentage of apoptotic cells in liver of infected fishes. Re-isolation and detection of *A. hydrophila* was done by Duplex PCR. Potent exposure biomarkers have been successfully screened and can be used for the development of rapid diagnostic technique of *A. hydrophila* infection.

Keywords: *Labeo rohita; Aeromonas hydrophila; oxidative stress; flow cytometry; biomarker; melanomacrophage centers.*

1. Introduction
Mass mortalities due to infectious diseases causing heavy loss to the fish farmers are major bottlenecks in the development and sustainability of aquaculture practices. The occurrence of diseases in fish farm is due to several factors concerned with the rearing methods, environmental conditions and variations [1]. But bacterial diseases remain the major problem in the aquaculture industry [2]. *Aeromonas hydrophila* is a gram negative, motile, rod shaped, facultative anaerobic, nonspore forming ubiquitous bacteria present in the aquatic environment causing diseases in fish under stress [3]. These bacteria cause haemorrhagic septicemia also known as Motile Aeromonad Septicemia (MAS). *Aeromonas* species are known as causative agents of a wide spectrum of diseases in man and other animals [4]. *A. hydrophila* is listed in the Contaminant Candidate List and Environmental Protection Agency has validated its detection and enumeration in drinking water system [5].
Sustainable aquaculture depends on perfect balance between growth and health condition of fish. At present extensive use of traditional procedures like antibiotics and chemotherapy increases the selective pressure exerted on the microbial world playing an important role in the natural emergence and amplification of resistant pathogens ultimately leading to residual effect, bioaccumulation and environmental degradation/pollution. Generation of multiple antibiotic resistances (MARs) between *Aeromonas* species, due to excessive use of antibiotics, has been reported globally by different authors [6, 7, 8]. Due to these issues much attention has been paid to immunize the fish with vaccine(s) for better protection. However, till date only a few vaccines are available. Commercial vaccines are also expensive for fish farming practices and are specific against particular pathogens [9]. Nevertheless, it is not possible to immunize the fish with all pathogens due to practical difficulties, stress factors and adverse reaction at injected area [10].
Aeromonads primarily cause gross, irreversible damage in fish. Fishes that survive disease outbreaks are recognized as carriers of the disease and may continue to infect the remaining population without exhibiting signs of infection. So early detection at sub-clinical state or at

carrier state i.e. where, there is successful establishment of bacterial infection, without any external morphological lesion is very much important. A multiple biomarker approach can be highly helpful in this regard as diagnosis based on clinical or external signs of disease is difficult because clinical signs often vary and skin ulcers are often cross-infected with other opportunistic bacteria and fungi.

The main objectives of this study was to evaluate haemato-biochemical parameters to assess non-specific immune response and characterize oxidative stress response in *Labeo rohita* Hamilton, 1822 challenged with *A. hydrophila* at sublethal carrier state and finally to identify the possible mechanism involved in cell death in liver indicated by histopathological study by using flow cytometric method. Cumulatively development of sensitive and rapid diagnostic technique in the form of bio-markers with regard to diagnosis of *Aeromonas* infection at a sub clinical stage was targeted after analyzing several parameters which could be assessed as 'multiple potential exposure biomarkers'.

3. Materials & Methods

3.1 Bacterial culture

The bacterial strain used in this study, *A. hydrophila* subsp. *hydrophila*, MTCC 646, was collected from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. This strain was received as lyophilized culture and subsequently revived by adding Nutrient Broth and transferring the rehydrated culture to a Nutrient Agar medium. Consequently, streak plate method was followed to get isolated bacterial colonies.

3.2 Fish and Experimental Condition:

L. rohita an Indian major carp having average body weight ± 30 gm and body length ± 15 cm were collected from a local fish farm, Matsyajibi Samabyay Samity, Anandapur, Ruby, Kolkata. In the laboratory, fishes were kept in glass aquarium (2 ft X 1ft X 1ft) in the Animal House and acclimatized for 7 days. The fishes were given food in the form of live Tubifex. The water quality was regularly monitored. Water temperature was maintained at 26 ± 2°C. Two-third of the water was renewed every day to avoid accumulation of unutilized food or metabolic waste products.

3.3 Artificial inoculation of fishes with *A. hydrophila*:

The bacterial strain MTCC 646 was cultured in nutrient broth (NB) and incubated at 37°C for 24 h prior to artificial inoculation of fishes. Bacterial cells were harvested by centrifugation at 5000 x g for 5 min and washed in physiological saline, PS (0.85% NaCl). The strain was enumerated by correlating the OD value taken at 600 nm of the growing culture with the corresponding colony forming units (cfu) obtained by spread plate dilution method [11] (Ref: OD 600_{nm} 1 = 2 X 10^9 cfu ml^{-1}). For this experiment, fishes were injected intraperitoneally (i.p.) with a sublethal dose, $3x10^7$cfu ml^{-1} of *A. hydrophila*, made up in PS (working volume: 0.5 ml 100 gm^{-1} body weight of fish). The aim was to produce asymptomatic carrier state. Fishes were divided in 2 sets i.e SHAM operated control set and *A. hydrophila* treated set. The potential fish biomarkers chosen for the study were recorded on the 7th day of exposure and compared with corresponding sham-injected control groups (Six replicates, each containing 10 fish injected with sterile PS only).

3.4 Blood and serum collection

On the sampling day, fishes were anaesthetized by dipping for 30 sec in 0.1 ppm of MS 222 suspension in water. After anaesthetizing fish, the blood samples were collected from the caudal vein into plastic Eppendorf tubes with a 2 ml pre-heparinised syringe and 24 gauge needle from six fish of each sample group. For serum, blood samples were withdrawn from caudal veins in the remaining anaesthetized fish into blood collecting tubes or Eppendorf tubes without anticoagulant in the syringe. Blood samples in Eppendorf tubes were allowed to clot for 2 h at room temperature in a slanting position. The clot was then cut with a glass rod and care was taken not to haemolyse the clot. The tubes were kept at 4 °C for an hour and were then centrifuged at 3000 × g for 10 min and the supernatant serum was collected. The serum was stored at -20 °C in Eppendorf tubes till use within two days. During sampling the fish were handled very carefully and aseptically to avoid mortality of fish during handling.

3.5 Haematological studies

Whole blood examination includes total leukocyte count (WBC) and total erythrocytes counts (RBC), hemoglobin (Hb) content, hematocrit value (PCV), leucocrit value (Lct), leukocytes differential count (dWBC%) and blood indices such as mean corpuscular or cell volume (MCV), mean cell hemoglobin content (MCH), and the mean cellular hemoglobin concentration (MCHC). Biochemical studies (total serum protein, albumin, globulin, albumin: globulin ratio, serum glucose, serum cholesterol, serum bilirubin, serum calcium) and plasma enzyme activity of alkaline phosphatase and acid phosphatase were carried out with the serum isolated form fish blood.

Total erythrocyte count (TEC) and Total Leucocyte count (TLC) was done by Neubauer's improved double haemocytometer using Hayem solution as diluting fluid. The haematocrit and leucocrit values were estimated by microhaematocrit method [12]. The amount of hemoglobin in the blood was estimated by Sahli's Haemometer. The MCV (fl), MCH (pg) and MCHC (g dl^{-1}) were calculated from hematological data. The relative abundance of different leucocytes was determined by counting a total of 200 blood cells after staining the blood smear with Leishman's stain. All the haematological parameters were determined following techniques with slight modifications [13].

Alkaline Phosphatase activity (µM mg $protein^{-1}$ min^{-1}) and Acid Phosphatase activity (µM mg $protein^{-1}$ min^{-1}) were determined [14] using p-nitro phenyl phosphate (PNPP) as substrate. Both alkaline and acid phosphatase activities were expressed as µM of p-nitro phenyl produced per mg of water soluble protein in the blood sample.

Total serum protein (gm %) and Serum Albumin (gm %) was measured from serum colorimetrically following Biuret method and Bromocresol green method respectively. Serum Globulin (gm %) was calculated by simply subtracting Serum albumin from Total protein. Albumin: Globumin ratio (A: G ratio) was also calculated later. Serum Glucose (mg 100 ml^{-1}), serum cholesterol (mg 100 ml^{-1}), serum bilirubin (mg 100 ml^{-1}) and serum calcium (mg 100 ml^{-1}) were measured colorimetrically following O - toludine method, Ferric chloride method, Malloy and Evelyn method and O- cresolphthalein method respectively. Diagnostic Kits obtained from Nice (Nice Chemicals Pvt. Ltd., Cochin) were used for the determination of all indices. Also for controls, same kits were used. Colorimetric measurement was done in UV-VIS Spectrophotometer (UV 1700 Pharmaspec, Shimadzu).

3.6 Histopathology
Specimens were dissected out aseptically and liver was carefully fixed in Neutral Formalin buffer for 24 h. Then, the tissue was dehydrated in upgraded alcoholic series, immersed in cedar wood oil followed by embedding in molten paraffin (melting point: 56° - 58° C). Serial sections were cut at 5-7µm. Sections were stained in Haematoxylin – Eosin. Finally photomicrographs were taken in Olympus BX 51 compound microscope under oil immersion.

3.7 Estimation of Intracellular Reactive Oxygen Species (ROS)
Single cell suspensions were prepared from liver by treatment with Collagenase I (2mg ml^{-1}) at 37ºC with constant shaking. The cells were then stained for 30 min at room temperature in the dark with cell permeable fluorescent and chemiluminescent probes, 2′-7′- Dichlorodihydrofluorescein diacetate (DCFDA) in a Ca^{2+} enriched binding buffer and analyzed by a BD Accuri C6 Flow cytometer (DCFDA) in a Ca^{2+} enriched binding buffer and analyzed by a FACS Calibur flow cytometer [15]. For each set a total of 10,000 even counts were taken. ROS was detected in the FL1 channel using emission filter at 489 nm. The mean values were considered. Data were analysed using BD Accuri C6 Software.

3.8 Antioxidant enzymes activity in Liver
Superoxide dismutase (SOD) and Catalase (CAT) in liver cytosol were estimated [16, 17]. The protein concentrations of samples were determined [18]. The enzyme activity was measured spectrophotometrically in UV-VIS Spectrophotometer (UV 1700 Pharmaspec, Shimadzu).

3.9 Annexin V/PI study
To understand the probable mechanism of cell death Annexin V/PI study was done. Single cell suspensions were prepared from liver and the cells were then stained for 30 min at room temperature in the dark with (FITC) - conjugated with Annexin V and PI in a Ca^{2+} enriched binding buffer and analyzed by a BD Accuri C6 Flow cytometer [15]. For each set a total of 10,000 even counts were taken. Annexin V and PI emissions were detected in the FL1 and FL 2 channels using emission filters at 508 and 643 nm, respectively. The Annexin V – negative / PI negative population was regarded as normal and healthy, while Annexin V – positive / PI negative and Annexin V – positive / PI positive cells were taken as a measure of early and late apoptosis respectively. The data were analysed using BD Accuri C6 Software.

3.10 Re-isolation and detection of pathogen
Presumptive diagnosis of *A. hydrophila* was done by selective Rimler Shotts (R-S) media supplemented with Novobiocin and isolation of Genomic DNA from bacteria was done [19]. Two pairs of primers *viz.* primer for conserved regions 16S rRNA [20] and aerolysin gene [21] were used in the duplex PCR. After gel electrophoresis the PCR products were observed under an UV transilluminator and the results obtained were analyzed by Gel Doc, Bio-Rad Quality One Software, Version 4.6.5.

3.11 Statistical analysis
Means and Standard Error (S.E.) of the means were calculated from whole range data [22]. Student's t-test was done to distinguish between significant differences using SPSS Statistics 17.0. Treatments were taken to be differing significantly where ($p < 0.01$).

4. Results and Discussion
The haematological parameters *viz.* TEC, haemoglobin content, haematocrit value and MCHC value decreased significantly ($p < 0.01$), whereas TLC, MCV and MCH tended to increase significantly ($p < 0.01$). The leucocrit value also increased in accordance with increased TLC. Neutrophil, large lymphocyte, eosinophil and monocyte % increased in a significant manner ($p < 0.01$). All these parameters were observed in *A. hydrophila* infected fishes compared with SHAM operated control fishes after 7 days of exposure period (Table 1).

The TLC and dWBC % (*viz.* large lymphocytes, neutrophils, monocytes and eosinophils) in the *A. hydrophila* treated fishes were found to be significantly higher as compared with the control fishes ($p < 0.01$) (Table 1). When infectious disease agents such as bacteria enter the fish body the non-specific (cellular) defense system gets stimulated during the first stage of disease manifestation. In this situation, the leucocytes get increased (leucocytosis) initially in order to protect the fish body by phagocytosis and produce antibacterial chemicals to stop the agent from spreading. The significant increase in the total WBC count and the number of different leucocytes observed in this study signifies the fact that the innate immunity of the fish was stimulated to fight against the bacterial pathogen as the primary line of defense.

Aerolysin, produced by some strains of *A. hydrophila*, is an extracellular, soluble, hydrophilic protein exhibiting both hemolytic and cytolytic properties. It binds to specific glycoprotein receptors on the surface of eukaryotic cells before inserting into the lipid bilayer and forms pores [23]. Hemolysins are exotoxic proteins produced by bacteria and their lytic activities on red blood cells are reported to be important for nutrient acquisition or for causing certain conditions such as anemia [24]. Aerolysin and hemolysin genes are reported to be the putative virulence genes of *A. hydrophila.* The significant decrease ($p < 0.01$) in total erythrocyte count in the A. hydrophila infected fish compared to the control fishes, in this study (Table 1), could be attributed to the severe hemolytic activity of aerolysin protein. Similarly, infection of *A. hydrophila* producing strong β-hemolysin was reported among common carps [25].

Table 1: Haematological and serum biochemical parameters in *A. hydrophila* treated *L. rohita* and SHAM operated control samples after 7 days exposure.

Parameters	SHAM operated Control (Mean ± SE)	*A. hydrophila* Treated (Mean ± SE)	*t* - values
(A) Haematological			
Total erythrocyte count (10^6 ml^{-1})	1.263 ± 0.108	0.411 ± 0.036	6.211*
Total leucocyte count (10^3 ml^{-1})	5.717 ± 0.513	16.033 ± 1.612	5.951*
Hemoglobin (%)	9.95 ± 0.159	6.825 ± 0.264	10.372*
Haematocrit (%)	34.728 ± 0.455	27.972 ± 0.96	6.661*
Leucocrit (%)	1.23 ± 0.066	1.757 ± 0.044	5.540*
MCV (fL)	242.831 ±12.311	585.809 ± 24.545	16.107*
MCH (pg $cell^{-1}$)	70.195 ± 3.213	151.795 ± 6.601	14.794*
MCHC (g dL^{-1}))	28.96 ± 0.224	25.981 ± 0.32	7.332*
Differential WBC count (%)			
Neutrophil (%)	26.76 ± 1.4	34.97 ± 1.09	14.63*
Eosinophil (%)	0.44 ± 0.1	4.56 ± 0.7	18.42*
Basophil (%)	1.19 ± 0.3	3.26 ± 0.7	8.59*
Large lymphocyte (%)	14.84 ± 3.2	25.15 ± 1.08	9.65*
Small lymphocyte (%)	56.52 ± 4.2	26.31 ± 1.9	20.72*
Monocyte (%)	0.25 ± 0.17	5.75 ± 1.8	9.62*
(B) Serum Biochemical			
Alkaline phosphatase (µM mg protein $^{-1}$min $^{-1}$)	34.575 ± 4.15	107.639 ± 4.15	39.36*
Acid phosphatase (k mg protein $^{-1}$ min $^{-1}$)	5.402 ± 0.69	9.204 ± 0.69	12.32*
Total protein (gm %)	0.663 ± 0.01	0.849 ± 0.01	307.626*
Serum Albumin (gm %)	0.387 ± 0.02	0.692 ± 0.04	41.962*
Serum Globulin (gm %)	0.216 ± 0.01	0.598 ± 0.02	36.668*
Albumin : Globulin ratio	1.788 ± 0.07	1.161 ± 0.1	53.673*
Serum Glucose (mg 100 ml $^{-1}$)	36.276 ± 1.14	59.021 ± 0.91	223.067*
Serum cholesterol (mg 100 ml $^{-1}$)	195.57 ± 7.2	246.75 ± 9.2	3.271
Serum calcium (mg 100 ml $^{-1}$)	3.485 ± 0.03	8.049 ± 0.06	79.013*
Serum bilirubin (mg 100 ml $^{-1}$)	6.908 ± 0.03	5.713 ± 0.045	63.351*

***** *Significant (p < 0.01)*

4.1. MCV, Mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cellular hemoglobin concentration; WBC, white blood cell; SE, standard error.

The hemoglobin (Hb) and hematocrit (Hct) values manifested significant decrease ($p < 0.01$) in *A. hydrophila* treated fishes in comparison with control group (Table 1) along with obvious decrease in erythrocyte counts. The significant decrease in the Hct observed in *Aeromonas hydrohila* treated samples, in this study, could be attributed to multiple factors viz. the destruction of the erythroblast, thereby, limiting their synthesis; gill damage or impaired osmoregulation. Moreover the extreme reduction in total erythrocyte count alone might have also caused for the observed low haematocrit value. The decreased hemoglobin content could be a result of the swelling of RBCs as well as poor mobilization of hemoglobin from the spleen and other hematopoietic organs [26]. Decreased RBC counts, Hct and Hb concentration indicate that RBCs are being destroyed by the leucocytosis activity during erythrocytic anemia with subsequent erythroblastosis [27]. Several other studies have reported similarly. For instance, the pearl spot fish *Etroplus suratensis* when infected with EUS became anemic followed by a significant reduction in RBC, Hb and PCV [28], Viral Erythrocytic Necrosis (VEN) disease in Chum salmon (*Oncorhynchus keta*) resulted in decreased TEC, Hct and Hb but increased TLC [27], in many bacterial and viral diseases such as IHN and VEN diseases, septicemia was observed and anterior portion of kidney which is responsible for hematopoiesis function was damaged resulting in severe decrease in TEC, Hct and Hb level [29, 30]. Similar trends in erythrocytes in fishes exposed to various toxicants and pathogens have been observed by other researchers [31. 32, 33, 34, 35].

Mean corpuscular volume (MCV) gives an indication of the status or size of the erythrocytes and reflects an abnormal or normal cell division during erythropoiesis. MCV is elevated or decreased in accordance with average RBC size; i.e, low MCV indicates microcytic, normal MCV indicates normocytic and high MCV indicates macrocytic. On the other hand, the increase in MCV ($p < 0.01$) observed in this study after *A. hydrophila* infection, in comparison with control fishes (Table 1), could be further attributed to intense damage or cirrhosis to the liver, the largest organ in the body, responsible for filtering (removing) harmful chemical substances, producing important chemicals for the body, and other important functions.

Significant increase ($p < 0.01$) which was observed in Mean corpuscular hemoglobin (MCH) value in *A. hydrophila* treated fishes compared to control ones (Table 1), could be attributed to macrocytic anemia, a blood disorder in which not enough red blood cells are produced, but the ones that are present are large thus fitting more hemoglobin. The macrocytosis is probably an adaptive response through the influx of immature erythrocytes from the hematopoietic tissues to the peripheral blood to make up for the reduced number of erythrocytes and decreased hemoglobin concentration [36].

Mean corpuscular hemoglobin concentration (MCHC) is a superior indicator of erythrocytes swelling [37]. 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 [38]. The significant decrease in the MCHC ($p < 0.01$) of *A.*

hydrophila infected fishes compared to the control fishes in this study (Table 1) was an indication of erythrocytes swelling and/or due to a decrease in hemoglobin synthesis leading to hypochromic anemia.

Changes in plasma enzyme activity are used as indicators of tissue injury, environmental stress, or a diseased condition. The rate of increase of plasma enzyme activity depends on the concentration of an enzyme in cells, the rate of leakage of it caused by injury and the rate of its clearance from plasma [39]. Alkaline phosphatase is a membrane bound enzyme found at bile pole of hepatoctyes and also found in pinocytic vesicle and Golgi complex. It is present on all cell membranes where active transport occurs and hydrolase and transphosphorylase are in function. It is often employed to access the integrity of plasma membrane since it is localized predominantly in the plasma membrane of microvilli at the bile canaliculi [40]. Acid phosphatase is known to be associated with lyososomal activity and thus it is speculated that acid phosphatase elevation reflects proliferation of lysosomes in attempt to sequester the toxic pathogens. In the present study, activities of both serum alkaline phosphatase and acid phosphatase increased significantly ($p < 0.01$) in *A. hydrophila* treated fishes in comparison to SHAM operated control samples (Table 1). The increase in the plasma alkaline phosphatase might be a consequence of increased intra and extra hepatic obstructions of biliary passage [41]. On the other hand, the increase in acid phosphatase activity might be associated either with the decrease in stability of liver lysosomal membrane or with liver damage [42].

The levels of total protein and cholesterol are considered to be major indices of the health status of teleosts. Total serum protein, albumin and globulin values were significantly ($p < 0.01$) higher in the *A. hydrophila* infected fishes compared to control fishes (Table 1). Increased concentrations of total protein can be caused by structural liver alterations reducing aminotransferase activity, with concurrent reduction of deamination capacity [43]; impaired control of fluid balance [44]; due to the destruction of RBCs and the resultant release of cell contents into the blood stream [27]. Increased level of serum total protein could also be an indication of antibody production in moribund fish with infectious diseases [36]. As the serum proteins include various humoral elements of the non-specific immune system, high concentrations of total serum protein, albumin and globulin might be due to the functioning of non-specific immune response of fishes against the bacterial infection. Although not significant but an increase ($p > 0.01$) in serum cholesterol level in *A. hydrophila* infected fishes compared to control fishes (Table 1) was observed in this study. Elevated levels of cholesterol indicate disorders of lipid and lipoprotein metabolism, especially liver dysfunction [45]. *Aeromonas* induced ulcerous dermatitis in rainbow trout (*Oncorhynchus mykiss*) [46] and *Aeromonas* infected red tilapia [47] resulted in an increase in total protein and cholesterol levels in the plasma which were in accordance with the present study.

Under the condition of stress, immediate responses of the fish body are recognized as primary and secondary responses. The primary response is the perception of an altered state by the central nervous system (CNS) and the release of stress hormones like cortisol and catecholamines (adrenalin and noradrenalin) into the blood stream [48]. Secondary responses occur as a consequence of the released stress hormone [49] causing changes in the blood and tissue chemistry e.g. an increase in plasma glucose [50]. Cortisol facilitates glycogenolysis and gluconeogenesis processes in fish and chromaffin cells that release catecholamines, further increase glycogenolysis and modulate cardiovascular and respiratory functions [51, 52]. This entire metabolic pathway increases the substrate level (glucose) to produce enough energy according to the demand of the fish for an emergency situation [53]. It is apparent that, a significant increase ($p < 0.01$) in serum glucose level in *A. hydrophila* infected fishes compared to control fishes is primarily due to acute stress caused by bacterial infection (Table 1). Similar observations have been made in several other fishes [54]. It has also been demonstrated that, elevation of plasma glucose is a part of generalized stress response in fish [55], mobilization of readily available energy in the form of glucose enhances the survival of fish [49, 56], and glucose level increases in the infected or stressed fishes to ward off the infection or stress [57]. Significantly higher serum bilirubin value ($p < 0.01$) in *A. hydrophila* infected fishes compared to control fishes (Table 1) also indicated liver dysfunction as it is the organ responsible for the elimination, via the bile, of the products of heme breakdown, primarily bilirubin. Impairment of this process causes a condition of jaundice and is reflected in a rise in bilirubin in the plasma [58].

The histopathological changes in several tissues of fish naturally and experimentally infected with different strains of bacteria have been described [59]. Histopathological changes in *A. hydrophila* infected catfish, *Clarius batracus* [60], *Salmo gairdneri* [61], *Cirrhinus mrigala* [15], *Catla catla* [62] have also been reported earlier. In this study, *L. rohita* after *A. hydrophila* infection exhibited hepatic damage characterized by developed granulomatous inflammation, aggregation of melanomacrophage centres (MMC) and considerable modifications i.e. rupturing and cytolysis of hepatocytes leading to distortion of hepatic cords (Fig. 1b and 1c).

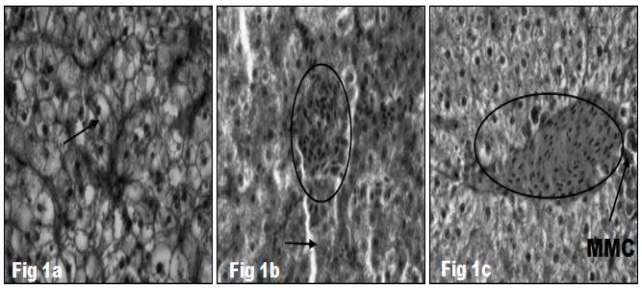

Fig 1: Histopathology of Liver in the *A. hydrophila* infected *L. rohita* after 7 days of exposure. [X 600]. Fig 1a depicting normal tissue architecture with compact hepatocytes (marked by arrow). Fig 1b clearly showing bacterial infestation (marked by circle) and tissue damage (marked by arrow). Fig 1c depicting bacterial infestation (marked by circle) and formation of Melano Macrophage centers (marked by arrow).

The possible explanation for this cytolysis might be due to leakage of hepatocytes's biomembrane causing efflux of K^+ and influx of Na^+ along with water into the cell leading to cytotoxic edema. No prominent change in the normal architecture was observed in the liver of control fishes (Fig. 1a). A similar type of tissue destruction and the affinity of these bacteria to the liver were also reported in case of *I. punctatus* [59]. The histopathological changes observed were similar to those in channel catfish and rainbow trout infected with *A. hydrophila*. Internally, the liver is the primary target organ of an acute septicemia and is apparently attacked by bacterial endotoxins and loses its structural integrity. The severity of lesion in the experimental fishes attributed to toxic substances produced by *A. hydrophila* evoked hemorrhage and

necrosis [63]. Those *in vivo* and *in vitro* studies confirmed that the bacterial toxin contains enzymes such as protease and elastase.

The role of *A. hydrophila* as a potent inducer of apoptosis in *L. rohita* was analyzed by flow cytometry with Annexin V/ PI study. Results clearly indicate higher percentage of cell death in the liver of infected fishes in comparison to SHAM operated control samples (Fig. 2).

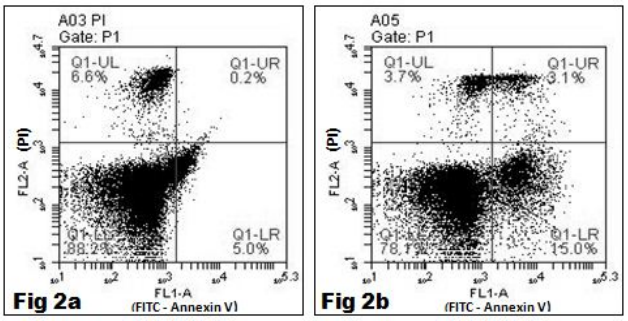

Fig 2: Annexin V/PI study in liver of *A. hydrophila* infected *L. rohita* after 7 days of exposure. Fig 2a showing 88.2% live, 5% early and 0.2% late apoptotic cells in lower left, lower right and upper right quadrants respectively in control fishes. Fig 2b showing 78.1% live, 15% early and 3.1% late apoptotic cells in the lower left, lower right and upper right quadrants respectively in infected fishes.

A. hydrophila deploys an array of virulence determinants, which interact with the key components of the cell death pathway of the host or interferes with the regulation of transcription factors monitoring cell survival, to avoid, negate or suppress host defenses. Cell death due to pathogenic infection was identified as apoptosis and thus chosen serotype of *A. hydrophila* (MTCC 646) can be inferred as a potent inducer of apoptosis in fish liver.

Flowcytometric results in this present study, showed a 4.76 fold increase of the oxidized DCF mean peak in case of fish injected with *A. hydrophila* (Fig. 3b) compared to the SHAM operated control ones (Fig. 3a). Thus, there was a clear indication of higher H_2O_2 production in the treated fish in comparison to control. *A. hydrophila* virulence is frequently associated with production of several extracellular toxins and enzymes [64, 65]. In this study, bacterial infection was able to induce the release of ROS in liver.

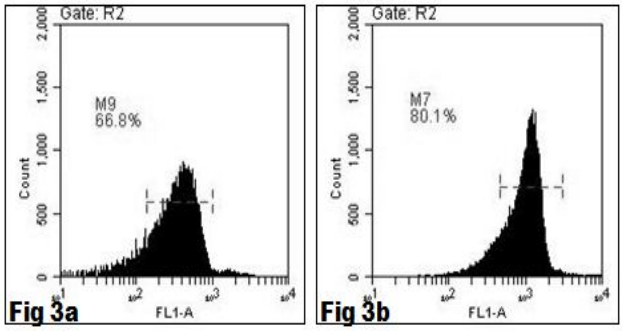

Fig 3: Estimation of Intracellular ROS, where oxidized mean peak has been plotted along the FL1 channel. Fig 3a depicting the mean FL1-A value to be 342.42 in the control sample. Fig 3b showing a higher mean FL1-A value of 1631.12 i.e. a 4.76 fold increase of the oxidized DCF mean peak in the liver of infected fishes.

These results are in conformation with the detection of respiratory burst related genes on an SSH library conducted on *A. salmonicida* and *Staphylococcus aureus* infected zebrafish [66]. Furthermore, cytotoxic enterotoxin (Act) isolated from *A. hydrophila* increased ROS production in murine macrophages [67]. A dose dependant increase in percentage of cell death in liver along with concomitant increase in ROS production in hepatocytes was recorded in *Aeromonas* infected *Cirrhinus mrigala* [15] and *Catla catla* [62] and such results are in conformity with our current findings.

Since many environmental contaminants exert toxic effects related to oxidative stress, this phenomenon may be an important feature for biomarker development. Their function in detoxification processes motivates continuous research on the potential use of superoxide dismutase (SOD), catalase (CAT), in monitoring programs. Species differences in the efficacy of antioxidant defenses may partly explain the prevalence of pathological lesions observed in certain species of fish [68].

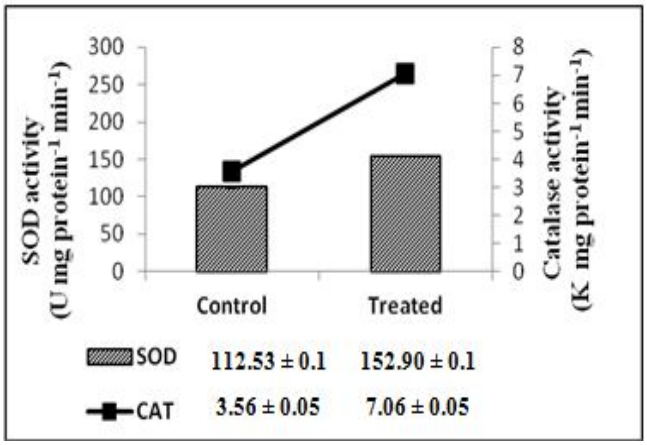

Fig 4: Antioxidant enzymes activity in Liver. Superoxide dismutase (SOD) and Catalase (CAT) activities have been recorded along primary and secondary Y- axis respectively in the liver cytosol of treated fishes in comparison to SHAM operated control samples.

The results obtained in the present study indicate that, there was a significant increase ($p < 0.01$) in enzymatic activity of Catalase and Superoxide dismutase in the liver cytosol of *A. hydrophila* treated fishes in comparison to SHAM operated control samples (Fig. 4). Observation of biochemical changes of enzymatic defense system after phenanthrene exposure to *Paralichthys olivaceus* showed marked elevation in hepatic catalase activity [69]. A dose dependant increase in hepatic catalase and SOD was again observed after *Aeromonas* infection in *Cirrhinus mrigala* [15] and in *Catla catla* [62] and such results are in conformity with our present findings.

Liver of *A. hydrophila* infected fish were dissected out aseptically and liver cytosol was incubated in NA plate. Two bands have been obtained at length 599 bp and 252 bp respectively when Duplex PCR was done with DNA isolated from presumptive *A. hydrophila*, identified and isolated from R-S media.

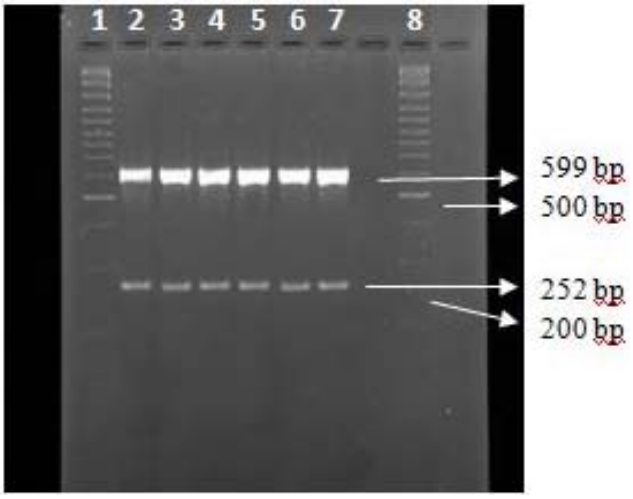

Fig 5: Re-isolation and detection of pathogen by Duplex PCR. *A. hydrophila* (MTCC 646) showing product obtained from primer for 16S rRNA (599 bp) and primer for aerolysin (252 bp) from bacteria isolated from fishes.

Electrophoretic analysis of the PCR product revealed the specific amplifications of 599 bp and 252 bp fragments without any spurious product for both the primers targeted against 16S rRNA and the aerolysin gene (Fig. 5). These findings are in accordance with the previous results obtained [70].

5. Conclusion

Summarizing the present findings, this study establishes the potentiality of *A. hydrophila* in generation of oxidative stress in *L. rohita* and revealed that it induces apoptotic cell death in liver which was indicated by histopathology. Further, it can be said that changes in hemato-biochemical parameters could be used for development of rapid diagnostic technique in the form of fish biomarkers with regard to diagnosis of *Aeromonas* infection at a sub clinical and/or asymptomatic stage. In conclusion it could be assumed that this kind of multi-parametric bio-marker approach can be highly convenient as rapid diagnostic technique with regard to diagnosis of *Aeromonas* infection at a sub clinical stage or even in an immuno-compromised stage, without obvious symptomatic manifestation of the disease. This approach once validated and standardized for a particular aquatic system, could be a useful management tool because it can lead to frequent cost-effective early detection which would have its importance both in aquaculture/economic sector and public health sector eventually leading to economic growth.

6. Acknowledgements

The authors are thankful to the Head of the Department of Zoology, University of Calcutta, India for the facilities provided. Financial support from the University Grant Commission-National Eligibility Test project is gratefully acknowledged.

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