

# Pathogenicity of an Invasive Bacterium, *Aeromonas salmonicida* in Indian Major Carp, *Labeo rohita* – Evaluation of Immune Response Using Effective Molecular Markers

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## Abstract

Effects of *Aeromonas salmonicida* on the non-specific immune response, oxidative stress and subsequent cell death in liver and spleen of *Labeo rohita* exposed to asymptomatic dose ( $2 \times 10^7$  CFU/mL) of pathogen were studied. Evaluation of in vivo non-specific immunity via Nitro blue tetrazolium (NBT) reduction assay, myeloperoxidase (MPO) assay and serum bactericidal activity assay exhibited significant alterations among infected fishes, indicating temporary surge of innate immune responses. Considerable cell death and damage was observed in the histopathological analysis of liver and spleen in the infected fishes. Flow cytometric analysis by FITC-Annexin V/PI conclusively indicated triggering of apoptotic cell death in both organs. Relative expression of pro-inflammatory cytokine (TNF $\alpha$ ) gene, antimicrobial protein producing genes (Lysozyme C and Lysozyme G) and apoptosis effector molecule (Caspase 3) gene were increased significantly both in liver and spleen of *A. salmonicida* treated fishes. In conclusion, infection with *A. salmonicida* at asymptomatic level resulted in severe oxidative stress that, caused apoptotic cell death. Since facultative pathogen eradication is not possible in open water or flow-through aquaculture systems, this remains a crucial area of scientific research and the results obtained in the present study might provide better understanding of prophylactic, diagnostic and therapeutic measures during aquaculture.

## Introduction

Aquaculture industries worldwide frequently suffer heavy financial losses that threaten their growth and sustainability, mainly due to uncontrolled microbial diseases resulting in mass mortalities (Almeida et al., 2009). The most significant factors out of all the microbial diseases are bacterial (Zorilla et al., 2003) and viral (Crane, & Hyatt, 2011). The study of fish diseases requires a wide knowledge, not only of the potential pathogens, but also of the environmental constraints and special adaptations, which govern the inflammatory and immune responses of fishes. Expedient recognition,

treatment and prevention of fish diseases are essential for the improvement of fish culture and subsequent increase in fish resources. The increased frequency of international transfer of exotic fish species in the last few decades have led to introduction of numerous novel and exotic pathogens in many countries (Arthur, 2005). In the Indian perspective, one such key invasive bacterial pathogen is *Aeromonas salmonicida*. According to Evenberg and Lugtenberg (1982) the virulence factor of *A. salmonicida* is a protein layer, composed of 50 kD protein (A-protein) known as A-layer. Although it was previously reported to cause disease exclusively in the salmonids, several cases of *A. salmonicida* infections

have been reported in non-salmonids (Bernoth, Ellis, Midtlyng, Olivier, & Smith, 1997; Arkoosh et al. 2004; Cipriano, & Bullock, 2001; Martinez Murcia et al. 2005). Currently India is the largest producer of the carps, *Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*, which contribute to nearly one third of its total fish production (FAO, 2018). However, during intensification of aquaculture system, outbreaks of disease have become one of the major hurdles in achieving increased production. Although Aeromonads primarily cause gross, irreversible damage, fishes that survive the initial disease outbreaks are recognized as carriers of the disease and effectively continue to infect the remaining population without exhibiting signs of infection. Therefore, it is essential to develop of multiple biomarker approach for early detection at sub-clinical state or at carrier state, in order to adopt suitable preventive, health management measures to reduce the damage caused by *A. salmonicida* is being increasingly felt by Indian hatchery operators. Previous studies have indicated that the disease resistance in fish species is correlated with innate immune parameters, which probably affect the inherent capacity of pathogen resistant fish before the specific immune response act in a multifactorial approach so as to prevent bacterial colonization after invasion within the body (Sahoo et al., 2008; Reyes et al., 2008; Rodríguez, Novoa, & Figueras, 2008). Several authors have documented such changes in innate as well as in adaptive immune parameters in fish exposed to different pathogens (Raida, & Buchmann, 2008; 2009; Mohanty, & Sahoo, 2010).

The objective of this present study was to provide an in depth understanding of the various aspects of the immune system functioning in *L. rohita*, post infection with a pathogenic strain of *A. salmonicida*, particularly at an asymptomatic carrier stage. The analysis of different immune parameters, at molecular and cellular levels, targeted in the current study might also act as important tools for disease prevention, control and management of carp farming.

## Materials and Methods

### Bacterial Culture Collection and Dosage Preparation

The bacterial strain used in this study, *Aeromonas salmonicida* subsp. *salmonicida* (ATCC 33658) was obtained from American Type Culture Collection, USA. After receiving it as a lyophilized culture it was subsequently revived by adding Tryptone soy broth (TSB) and transferring the rehydrated culture to a Trypticase soy agar (TSA) medium and incubated at 22°C for 48 h. Consequently, streak plate method was followed to get isolated bacterial colonies on selective Coomassie Brilliant Blue Agar (CBB Agar) which resulted in bluish colonies suggesting the bacterial strain to be A-layer positive. For dosage preparation, the bacterial strain was cultured in TSB and incubated at 22°C for 48 hours followed by centrifugation at 5000 x g for 5 min

and washing in 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 (Ref:  $OD_{600nm} 1 = 2 \times 10^9$  CFU/mL) following Pal and Pradhan (1990).

### Fish Acclimatization and Maintenance

*Labeo rohita* fingerlings (30.91±2.03 g and 15.26±0.81 cm) were obtained from a local fish farm in Kolkata, West Bengal, India. Ten fishes were held in each glass aquarium (60 cm x 30 cm x 30 cm) in the animal house facility and were acclimatized for seven days. Fish were fed with tubifex and artemia. Dissolved oxygen (DO), ammonia and pH were regularly checked throughout the experiment. Dissolved oxygen and ammonia were monitored every week, ranging from 5.5 to 7.6 mg O<sub>2</sub>/ L and 0.5 to 1 ppm respectively. The pH ranged from 7.5 to 8.5 throughout the experimental period and water temperature was maintained at 25±2°C.

### Asymptomatic Carrier State Dose Determination for *A. salmonicida*

The fishes were injected intra-peritoneally (i.p.) with *A. salmonicida* starting from higher doses (5 x 10<sup>8</sup> CFU/ml) up to subsequent lower doses (2 x 10<sup>6</sup> CFU/ml) until any external symptomatic lesions of the diseases were observed. Bacterial doses for *A. salmonicida*, chosen for experimental challenge studies starting from higher to lower range were: 5 x 10<sup>8</sup> CFU/ml, 3 x 10<sup>8</sup> CFU/ml, 2 x 10<sup>8</sup> CFU/ml, 5 x 10<sup>7</sup> CFU/ml, 3 x 10<sup>7</sup> CFU/ml, 2 x 10<sup>7</sup> CFU/ml, 5 x 10<sup>6</sup> CFU/ml, 3 x 10<sup>6</sup> CFU/ml, 2 x 10<sup>6</sup> CFU/ml. The haematological and serum biochemical parameters were then analyzed in fishes injected with the lowest dose of bacterial pathogen at 7 days post infection (DPI), which resulted in no visible morphological symptoms of the disease but exhibited significant alterations in the haematological and biochemical parameters in comparison to control fishes, which in terms indicated successful establishment of the bacterial infection within the fish body essentially at an asymptomatic level *i.e.* successful production of carrier stages in the *L. rohita* fingerlings.

### Experimental Setup

Fishes were injected intraperitoneally (i.p.) with a carrier state dose (2 x 10<sup>7</sup> CFU/ mL) of *A. salmonicida*, made up in PS (working volume: 0.5 mL 100 gm/body weight of fish). A total number of 180 fishes were used in the course of the overall experiment to study the different parameters. Fishes were divided into three groups as follows: negative control (without any injection), sham operated control (i.p. injection of sterile culture media) and *A. salmonicida* treated. For every group there were 6 replicates, each containing 10 fishes.

The potential health parameters chosen for the study were recorded on the 7<sup>th</sup>, 14<sup>th</sup> and 21<sup>st</sup> day of exposure and compared with corresponding sham-injected control groups. The subsequent studies at cellular and molecular level were performed at 7 DPI depending upon the maximum sensitivity of the health parameters (mentioned afterwards).

#### Haematological and Serum Biochemical Studies

All the haematological parameters were determined following Dacie and Lewis (1984). The corpuscular values *viz.* Mean Corpuscular Volume (MCV) (fl), Mean Cell Haemoglobin (MCH) (pg) and Mean Corpuscular Haemoglobin Concentration (MCHC) (g/dl) were calculated from primary haematological data. Total serum protein (gm%) and serum Glucose (mg/100 ml) was measured by UV-VIS Spectrophotometer (UV 1700 Pharmaspec, Shimadzu) following Biuret's method and O - toluidine method as per the protocols mentioned in the Diagnostic Kits obtained from Nice (Nice Chemicals Pvt. Ltd., Cochin, India).

#### Histopathology of Liver and Spleen:

Sham injected control and *A. salmonicida* treated fishes were dissected aseptically and liver and spleen were carefully fixed in Neutral Formalin buffer for 24 hours. Then, the tissues were 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 5-7  $\mu$ m and stained with Haematoxylin – Eosin following Deivasigamani (2008). Finally, photomicrographs were taken in Olympus BX 51 compound microscope under oil immersion.

#### Nitro Blue Tetrazolium (NBT) Reduction Assay

NBT assay was determined following Gómez-Ochoa, Sabate, Homedes, and Ferrer, (2012). Blood smears were drawn on glass slides and stained with Wright stain for 2 minute and subsequently with Giemsa for 1 minute and eventually rinsed with tap water. Finally, the slides were air dried and mounted with Dibutylphthalate Polystyrene Xylene (DPX) and observed under light microscope (45 X) to measure the percentage of cells containing black formazan deposits.

#### Myeloperoxidase (MPO) Activity in Neutrophil Population

Comparison of MPO activity in neutrophil of control and *A. salmonicida* infected *L. rohita* was analyzed by measuring the MPO activity in the neutrophil population using the Neutrophil Myeloperoxidase activity assay kit (Cayman Chemical) (Prokopowicz et al., 2012). The samples were loaded onto 96 well plate according to the kit and the blue color yielded due to reaction of 3,3',5,5'-tetramethyl

benzidine (TMB) with MPO was observed at an absorbance of 650 nm using ELISA plate reader.

#### Serum Bactericidal Activity Assay

Qualitative and qualitative evaluation of serum bactericidal activity was determined following Pal, Datta Roy and Homechaudhuri, (2016). Serum was collected from both control and *A. salmonicida* treated fish after 7 days of exposure.

#### Flow Cytometric Analysis

Single cell suspensions were prepared from liver and spleen separately, following Datta Ray and Homechaudhuri (2014) with slight modifications and the following assays were performed. For each set, a total of 10,000 even counts were taken. All flow cytometric data were analyzed using BD Accuri C6 Software.

#### Annexin V-FITC / PI Assay

Nature of cell death in liver and spleen was ascertained using FITC conjugated Annexin V and Propidium iodide following Datta Ray and Homechaudhuri (2014) with slight modifications.

#### Gene Expression Analysis of Target Genes

The relative levels of expression of TNF- $\alpha$ , Caspase 3, Lysozyme C and Lysozyme G were analyzed by densitometry using ImageJ software (USNIH, USA). For analysis of gene expression, total RNA was extracted from 50-100 mg tissue samples, taken from sham injected control and *A. salmonicida* treated fishes using Takara RNA – Iso Plus following a standard protocol (Sambrook, Russell, & Russell, 2001). Total RNA concentration and purity was observed by measuring OD at 260 and 280 nm using Microdrop (Multi Skan Go, ThermoScientific, USA). Total RNA (5  $\mu$ g) was used for first strand cDNA synthesis using thermocycler (Applied Biosystems). RNA primer reaction mixture was prepared in a microtube using Random Hexamers (50  $\mu$ M), dNTP mixture (10 mM each), template RNA and RNase free dH<sub>2</sub>O following protocol as provided in the PrimeScript c-DNA Synthesis Kit – 6110A (Takara Bio Inc). The cDNA samples were kept at 0°C for further use. Multiple primers were designed for the genes of interest using Fast PCR software (Informer Technologies, Inc.). Standard desalted primers were custom made from Integrated DNA Technologies (IDT). The sequences of the chosen primers are given below in Table 1. The ratios of target gene/ $\beta$ -Actin product were subsequently calculated after subtraction of the background pixel intensity of each gene of interest and used to assess the differences in expression levels between the control and the infected samples.

**Re-isolation and Detection of *A. salmonicida***

Presumptive identification of bacterial pathogen was evaluated by several biochemical tests. Tissue homolysate prepared from liver of *A. salmonicida* challenged fish was plated onto Coomassie brilliant blue (CBB) agar, MacConkey and Trypticase soy agar (TSA). Genomic DNA was isolated from the bacterial colonies and subsequently PCR was performed for confirmation of the bacterial strain. Four pairs of primers were used viz. AP1/AP2 (Gustafson & Thomas, 1992), PAAS1/PAAS2 (O'Brien et al., 1994), MIY1/MIY2 (Miyata, Inglis, & Aoki, 1996) & 16S rRNA (Dorsch, & Stackebrandt, 1992). 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. Standard desalted primers were custom made from Integrated DNA Technologies (IDT). The sequences of the chosen primers are given in Table 2.

**PCR Reaction Using the Bacterial Genomic DNA**

PCR reaction was carried out after bacterial genomic DNA isolation. 2.5 µl of 10X Taq Buffer (PCR buffer) was mixed with 4 µl of dNTP mixture (dATPs, dTTPs, dGTPs, gCTPs) in PCR tubes. 1 µl of forward primer and 1 µl of reverse primer specific for different genes were then added into the sample followed by addition of 0.5 µl of Taq DNA polymerase. Finally, volume make up was made up by milliQ water to make the final volume of PCR mixture to 25 µl. The initial denaturing temperature, PCR cycle conditions (denaturing temperature, annealing temperature, temperature during extension) and final extension, for screening the different genes, were determined by trial and error methods after multiple trials using a range of temperatures from 90-95°C (for denaturation), 45-60°C (for annealing) and 65°C to 75°C (for extension). The best results were obtained at the specific temperatures and timings mentioned in Table 3.

**Table 1.** Primer sequences for selected genes

Gene	Sequence (5' – 3')	Amplicon size (bp)
β-Actin	F- GACTTCGAGCAGGAGATGG R- CAAGAAGGATGGCTGGAACA	138
TNF-α	F- GGACTACCAGCTGTTGTGCAT R- GCCACCATCGACTGCAAT	377
Caspase 3	F- ATGAACGGAGACTGCGTGGAA R- TGGTGAGCATCGAGACAATGCAGG	285
Lysozyme C	F- CGCTGTGATGTTGTCCGTATCTTC R- GTAACCTCCCGAGGTATCC	244
Lysozyme G	F- CTTATGCAGGTTGACAAACG R- GGCAACAACCATCACTGGAGTAATC	360

**Table 2.** Primer sequences of selected genes for identification of *A. salmonicida*

Gene	Sequence (5' – 3')	Amplicon size (bp)
16S rRNA	F- GAGTTTGATCCTGGCTCAG R- TACGGYTACCTTGTACGACTT	1465
PAAS1/PAAS2	F- CGTTGGATATGGCTCTTCT R- CTCAAACGGCTGCGTACCA	423
AP1/AP2	F- GGCTGATCTCTTCATCCTCACCC R- CAGAGTCAAATCTACCAGCGGGTGC	421
MIY1/MIY2	F- AGCCTCCACGCTCACAGC R- AAGAGGCCCATAGTGTGGG	512

**Table 3.** PCR conditions of selected genes for detection of *A. Salmonicida*

GENE	Initial denaturation	Denaturation	Annealing PCR cycles *	Strand Elongation	Final extension
PAAS1/PAAS2	95°C, 2 minutes	94°C, 2 minutes	57°C, 30 seconds	72°C, 1 min 30 sec	72°C, 3 minutes
AP1/AP2	94°C, 2 minutes	94°C, 15 seconds	57°C, 30 seconds	72°C, 1 min 30 sec	72°C, 3 minutes
MIY1/MIY2	94°C, 2 minutes	94°C, 30 seconds	58°C, 1 min 30 sec	68°C, 1 min 30 sec	68°C, 3 minutes
16S rRNA	94°C, 2 minutes	94°C, 30 seconds	49°C, 30 seconds	72°C, 1 min 15 sec	72°C, 3 minutes

\* Number of PCR cycles for each reaction: 30 cycles

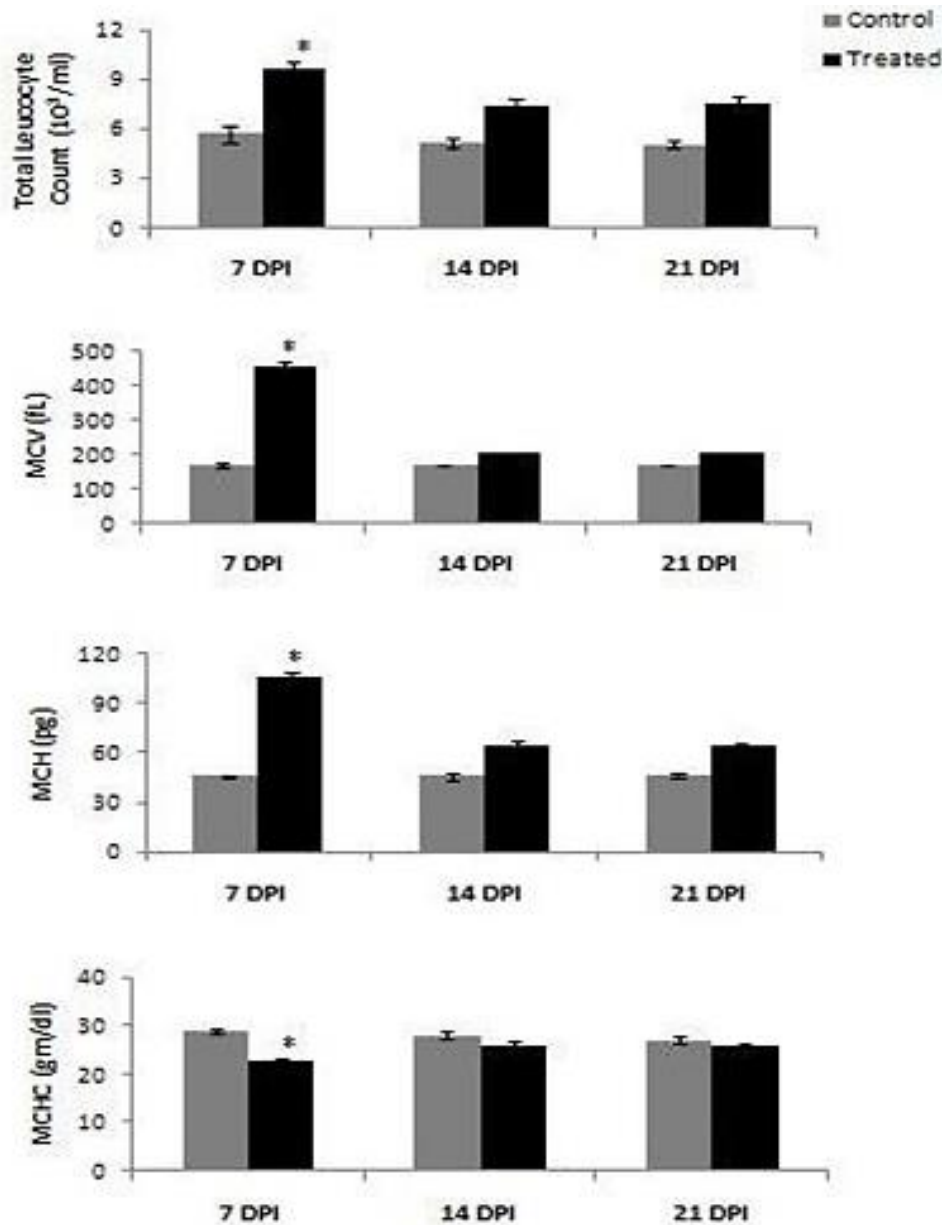
### Statistical Analysis

Means and Standard Error (S.E.) of the means were calculated from the whole range of data following Zar (1999). All data obtained were analysed using Student's t-test ( $P < 0.05$  as significant level). Test of homogeneity of variances by Levene statistics was performed for all the data with respect to different parameters observed ( $P < 0.05$  as significant level). Statistical calculations were performed using SPSS Statistics 17.0.

### Results

Haematological parameters *viz.* total leucocyte count, MCV and MCH (Figure 1) and serum biochemical parameters *viz.* total serum protein and serum glucose level (Figure 2) increased significantly ( $P < 0.05$ ) after *A.*

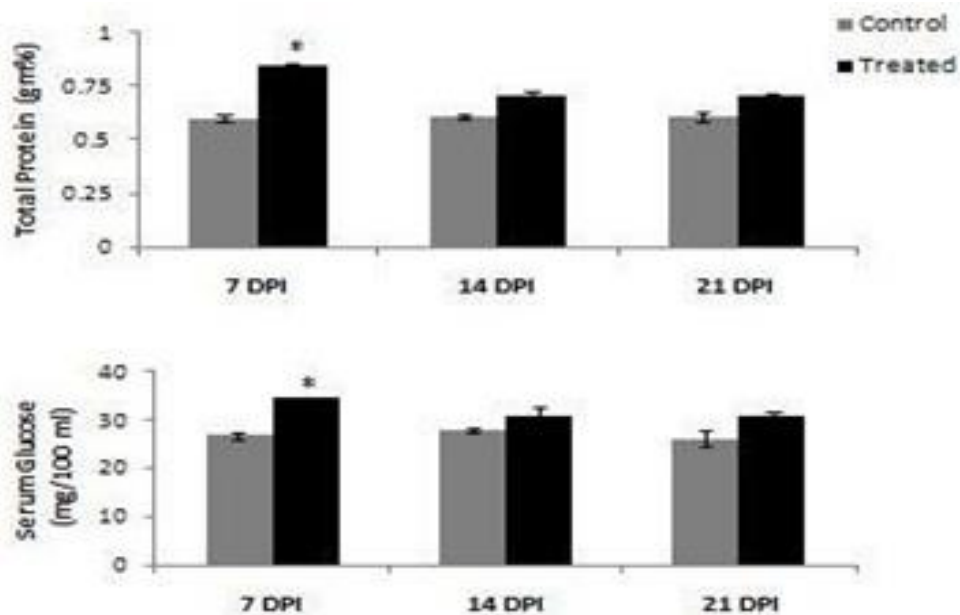
*salmonicida* infection at 7 DPI, 14 DPI and 21 DPI respectively while MCHC value was observed to be decreased significantly ( $P < 0.05$ ) in comparison to control fishes (Figure 1). The changes in all these values were most prominent at 7 DPI compared to 14 DPI and 21 DPI. Percentage of phagocytic cells with formazan deposition in the blood of fishes infected with *A. salmonicida* ( $10.45 \pm 0.48$ ) increased significantly ( $P < 0.05$ ) compared to SHAM operated control fishes ( $4.89 \pm 0.29$ ) after 7 days of exposure period (Figure 3) along with significant ( $P < 0.05$ ) increase in Myeloperoxidase (MPO) activity in the neutrophil population of blood isolated from *A. salmonicida* infected fishes ( $7.54E-06 \pm 9.6E-07$ ) in comparison to SHAM operated control fishes ( $3.08E-06 \pm 9.6E-07$ ) (Figure 4).



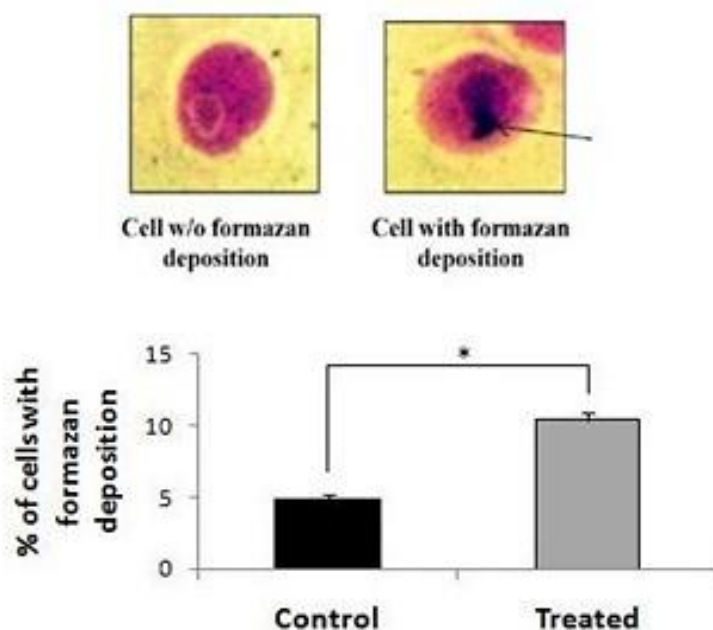
**Figure 1.** Total leucocyte count, MCV, MCH and MCHC of control and infected *L. rohita* after 7, 14, 21 days post infection with *A. salmonicida*. \* Significant ( $P < 0.05$ ).

Relative gene expression analysis of TNF $\alpha$  gene was estimated using semi quantitative reverse transcriptase PCR after isolation of total RNA from liver and spleen of control and *A. salmonicida* infected *L. rohita* fishes. The ratios of TNF $\alpha$  /  $\beta$ -Actin product were subsequently calculated by densitometry using ImageJ software. A significant increase ( $P < 0.05$ ) in the expression of TNF $\alpha$  was observed in *A. salmonicida* infected fish liver ( $1.45 \pm 0.11$ ) and spleen ( $1.18 \pm 0.17$ ) compared to liver ( $0.49 \pm 0.04$ ) and spleen ( $0.25 \pm 0.07$ ) of SHAM operated control fishes (Figure 5).

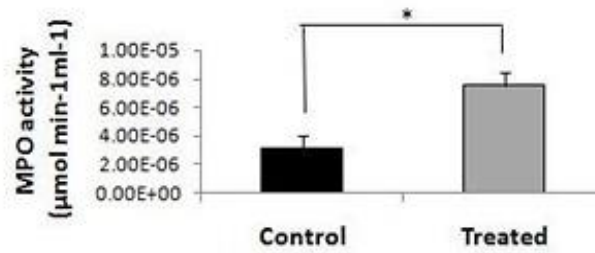
At 7 DPI fish serum was analyzed for bactericidal activity by counting CFU survivors. The results were expressed in two ways: the first one shown in Figure 6, expressed in CFU values, and the second one in Figure 7 expressed in serum bactericidal activity [1- CFU/positive (%)]. The positive group (serum free) displayed the highest values, indicated by the largest bacterial growth, this result was expected once there was no serum to destroy bacteria, however in control and treated groups, the bacterial growth was reduced because of bactericidal ability in serum (Figure 6). Figure 7



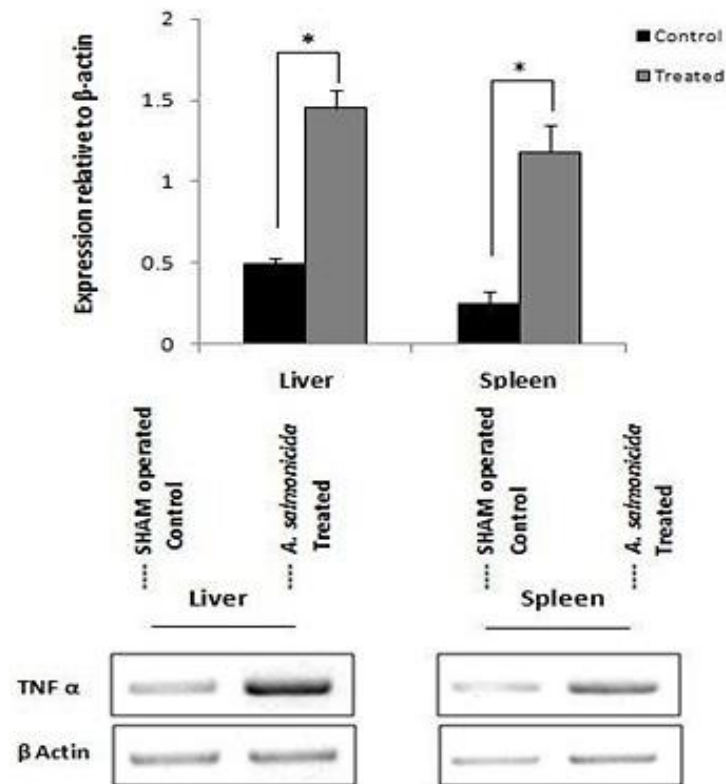
**Figure 2.** Total serum protein and serum glucose level of control and infected *L. rohita* after 7, 14, 21 days post infection with *A. salmonicida*. \* Significant ( $P < 0.05$ ).



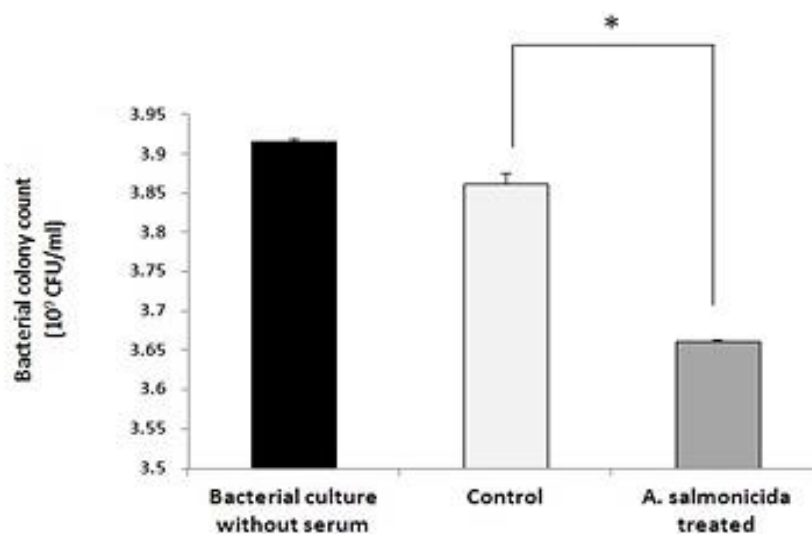
**Figure 3.** Percentage of phagocytic cells with formazan deposition in control and *A. salmonicida* infected *L. rohita*. \* Significant ( $P < 0.05$ ).



**Figure 4.** Determination of MPO activity in neutrophil of control and *A. salmonicida* infected *L. rohita*. \*Significant ( $P<0.05$ ).



**Figure 5.** Differential Expression of  $\text{TNF}\alpha$  in liver and spleen of SHAM operated control and *A. salmonicida* infected *L. rohita*. \*Significant ( $P<0.05$ ).

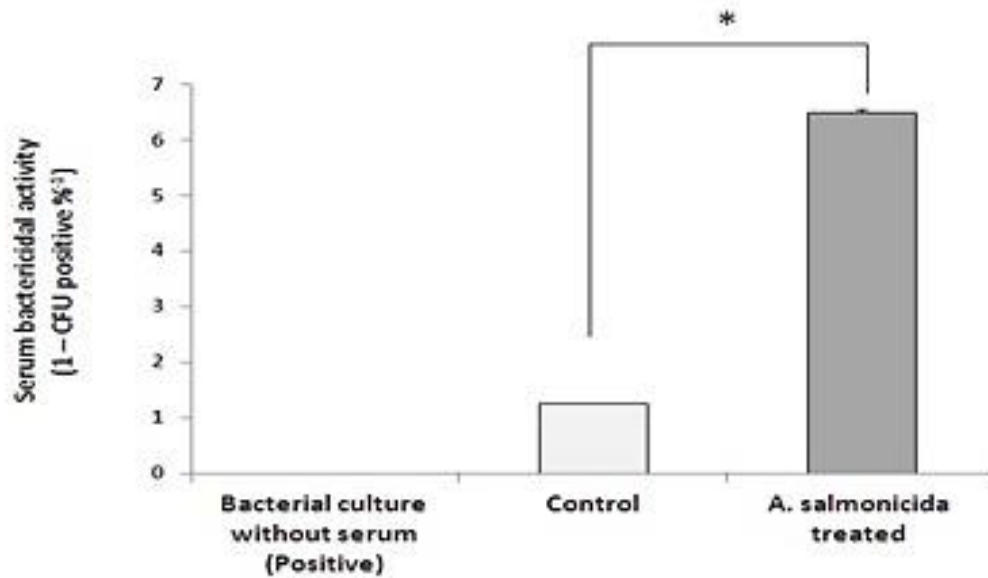


**Figure 6.** Bacterial colony count (CFU/ml) (Mean  $\pm$  SE) after adding serum from SHAM operated control and *A. salmonicida* challenged *L. rohita* by spectrophotometric analysis. (Ref:  $\text{OD } 600_{\text{nm}} 1 = 2 \times 10^9$  CFU/ml).

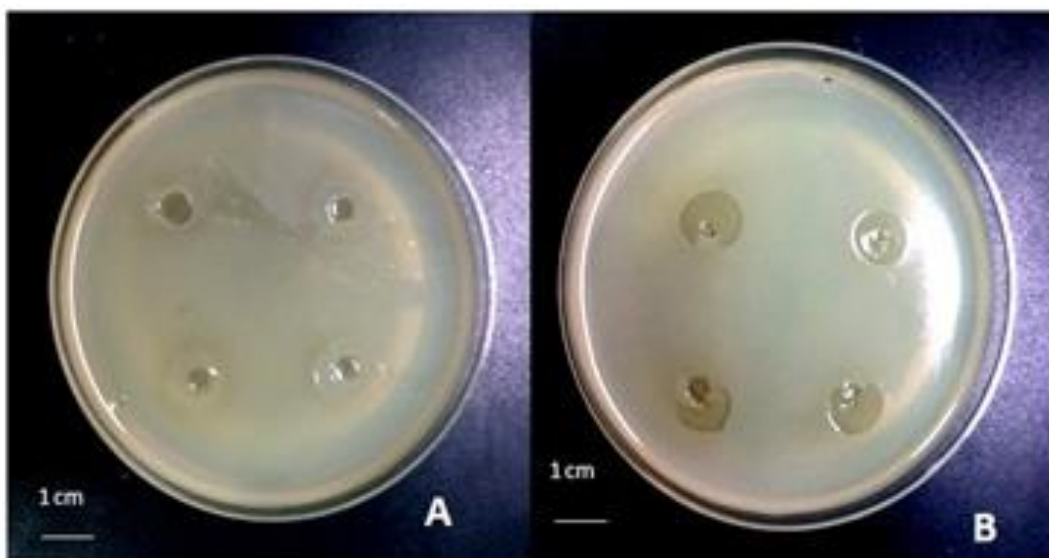
represents the serum bactericidal activity of *L. rohita* after *A. salmonicida* challenge. In that, the positive group (serum free) displayed zero activity once there was no serum, but in the control and treated groups, the bacterial activity increased significantly ( $P < 0.05$ ), indicated by the higher serum bactericidal activity profile (Figure 7). Serum bactericidal activity was also studied qualitatively, by adding serum drops from control and *A. salmonicida* challenged fish onto soft agar overlay mixed with *A. salmonicida* ( $3.92 \times 10^9$  CFU/ ml) after incubating at  $25^\circ\text{C}$  overnight and subsequently

observing the zones of inhibition (Figure 8) around each drop (one drop per quadrant). Zones created by serum from treated fish (Figure 8B) appear significantly larger than the zones created by serum from control fish (Figure 8A).

A significant ( $P < 0.05$ ) increase was observed in the expression of Lysozyme C (liver  $2.08 \pm 0.24$ ; spleen  $3.05 \pm 0.11$ ) and Lysozyme G (liver  $1.27 \pm 0.31$ ; spleen  $1.04 \pm 0.26$ ) among *A. salmonicida* infected fishes compared to SHAM operated control fishes in liver and spleen (Figure 9 and Figure 10) for Lysozyme C (liver

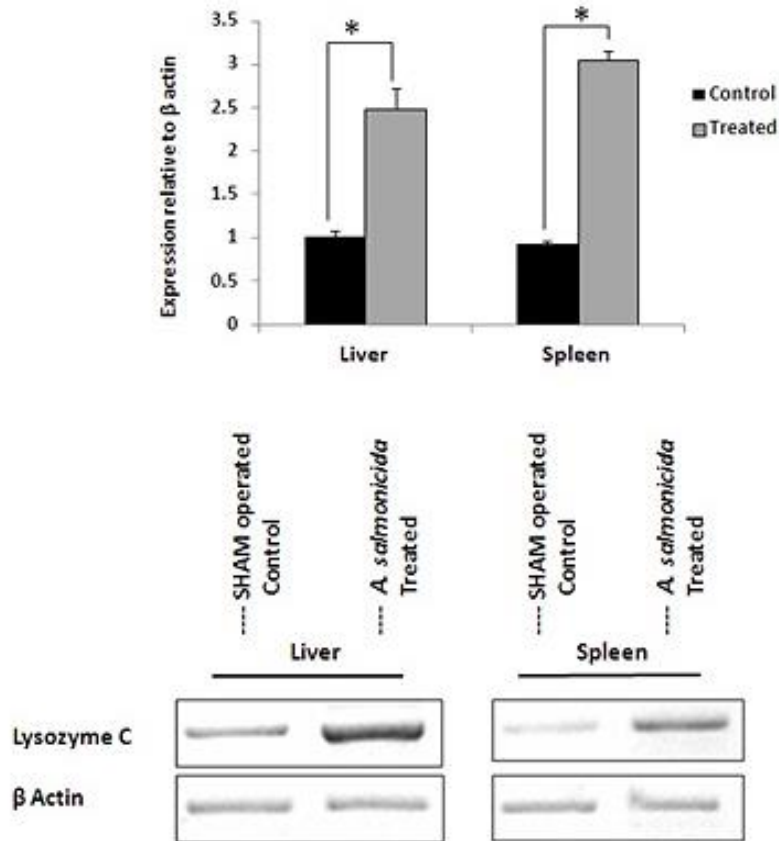


**Figure 7.** Serum bactericidal activity in *L. rohita* (1-CFU/positive %) after challenge with *A. salmonicida* and in SHAM operated control samples after 7 days of exposure.

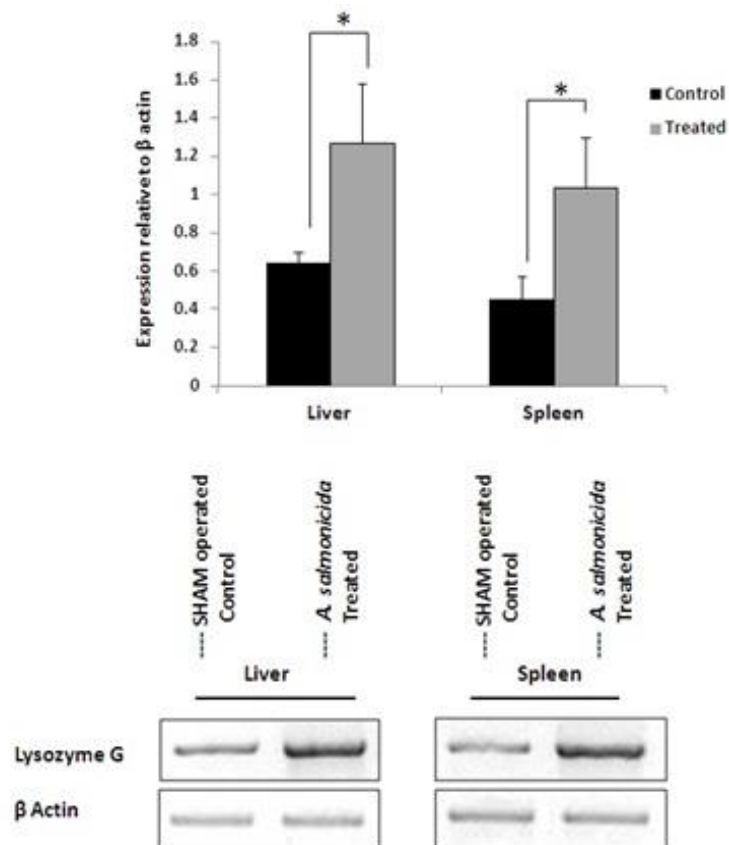


**Figure 8.** Observation of zones of bacterial inhibition after adding serum drops in soft agar overlay containing bacterial culture ( $3.92 \times 10^9$  CFU/ ml) after 24 hours incubation at  $25^\circ\text{C}$ . Fig 8A showing no inhibition of bacterial growth around serum drops from SHAM operated control samples and Fig 8B showing zone of bacterial inhibition around serum drops from *A. salmonicida* challenged *L. rohita* at 7 days post infection.





**Figure 9.** Differential Expression of Lysozyme C in liver and spleen of SHAM operated control and *A. salmonicida* infected *L. rohita*. \*Significant ( $P < 0.05$ ).



**Figure 10.** Differential Expression of Lysozyme G in liver and spleen of SHAM operated control and *A. salmonicida* infected *L. rohita*. \*Significant ( $P < 0.05$ ).

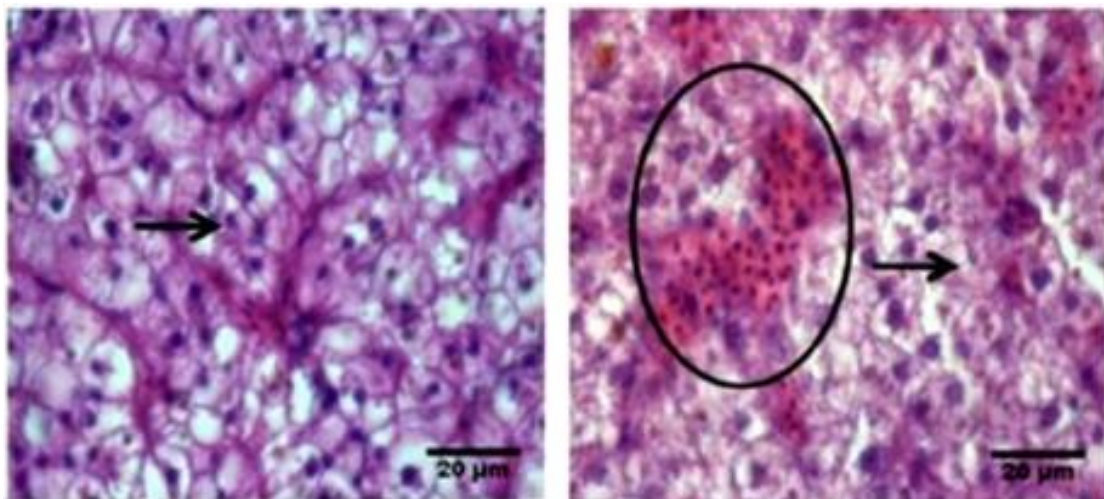
1.01±0.07; spleen 0.93±0.04) and Lysozyme G (liver 0.64±0.06; spleen 0.45±0.12) respectively.

Histological examination in *L. rohita* liver and spleen, after *A. salmonicida* infection exhibited hepatic and splenic damage characterized by development of granulomatous inflammation, aggregation of melanomacrophage centres (MMC) and other considerable modifications *i.e.* ruptured and irregular cells, granulation in cytoplasm, necrosis, rupturing and cytolysis of hepatocytes (Figure 11) structural damage of connective tissue and necrosis of splenic pulp (Figure 12).

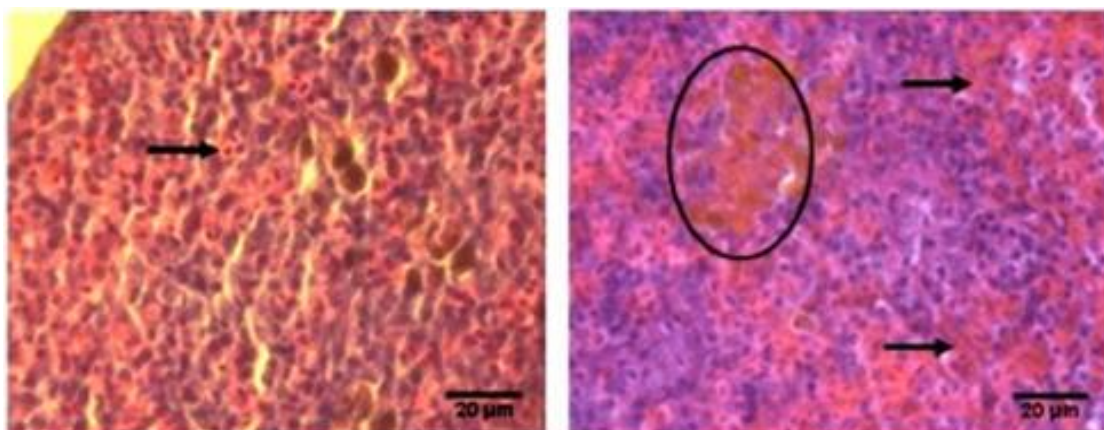
Figure 13 depicted the induction of apoptosis by Annexin V- FITC / PI staining in liver and spleen of control and *A. salmonicida* infected *L. rohita*. Significant ( $P<0.05$ ) increase in the percentage of early apoptotic cells (18.2±0.12 %) and late apoptotic cells (0.5±0.08 %) were observed in liver of infected fish in comparison to

control samples (early apoptotic: 5.0±0.21 %; late apoptotic: 0.205±0.05 %). The normal cells (81.3±0.56 %) decreased significantly in the infected fishes compared to control (87.2±0.25 %). A similar trend was also observed in the spleen of infected fishes where early apoptotic cells (17.11±0.16 %) and late apoptotic cells (0.51±0.08 %) increased significantly ( $P<0.05$ ) when compared to the control (early apoptotic: 11.0±0.11 %; late apoptotic: 0.23±0.02 %). The normal cells (82.18±0.72 %) in spleen also decreased in the treated fishes in comparison to the control (83.0±0.21 %), in a pattern similar to that of fish liver.

Relative gene expression analysis of Caspase 3 gene was studied by semi quantitative reverse transcriptase PCR after isolation of total RNA from liver and spleen, which exhibited a significant increase in the expression of Caspase 3 in the *A. salmonicida* infected fishes compared to SHAM operated control fishes both



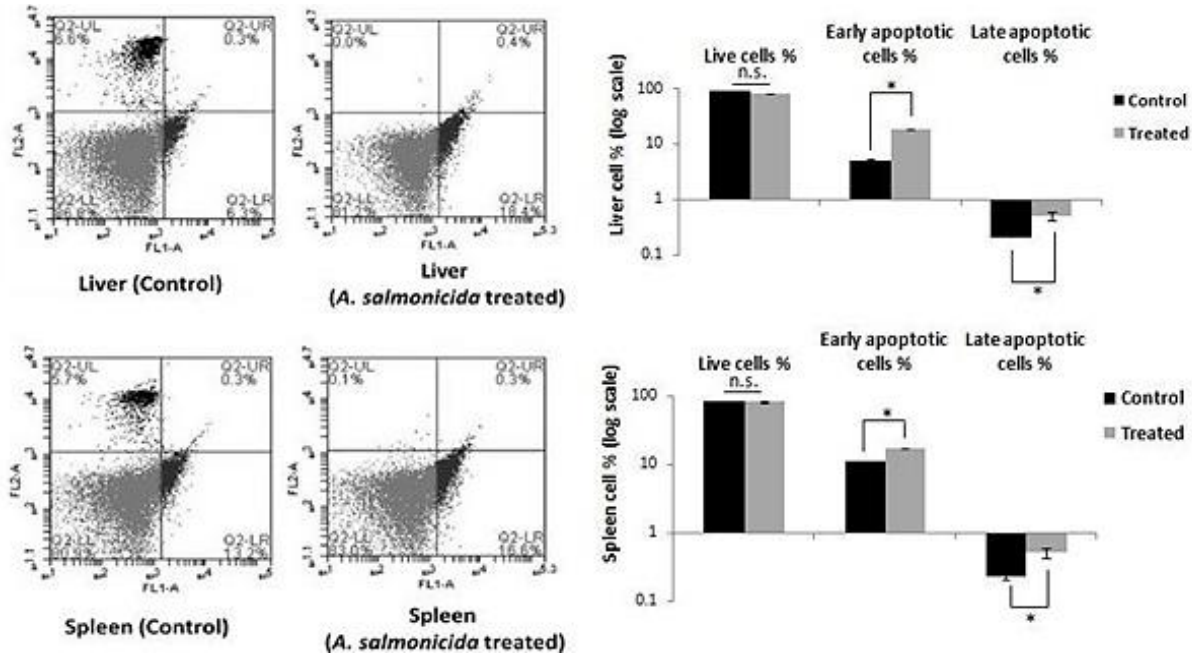
**Figure 11.** Histopathology of control liver (Figure A) and liver in the *A. salmonicida* (Figure B) infected *L. rohita* after 7 days of exposure. [x 400]. Figure A: depicting normal tissue architecture with compact hepatocytes (marked by arrow). Figure B: clearly showing bacterial infestation (marked by circle) and tissue damage (marked by arrow).



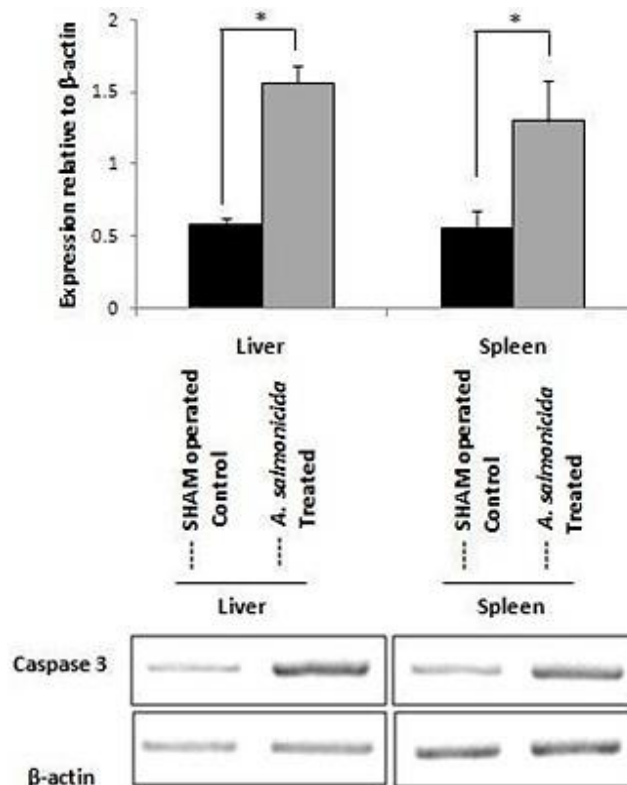
**Figure 12.** Histopathology of control spleen (Figure A) and *A. salmonicida* (Figure B) infected *L. rohita* after 7 days of exposure. [x 400]. Figure A: depicting normal tissue architecture with compact splenocytes (marked by arrow). Figure B: clearly showing bacterial infestation (marked by circle) and tissue damage (marked by arrow).

in liver and spleen (Figure 14). In case of liver a significantly ( $P < 0.05$ ) higher value of Caspase 3 expression was observed in *A. salmonicida* infected fishes ( $1.57 \pm 0.11$ ) compared to control fishes ( $0.58 \pm 0.05$ ). Similar trend was also observed in case of spleen, where significantly ( $P < 0.05$ ) higher value was found in *A. salmonicida* infected fishes ( $1.30 \pm 0.28$ ) in comparison to control fishes ( $0.56 \pm 0.12$ ) (Figure 14).

Finally, to confirm the fact that successful establishment of the infection was caused by the bacterial pathogen *A. salmonicida* and also to conclusively state that all the changes evident in the present study have occurred due to the pathogen, re-isolation of the bacterial colonies from the fish tissue was targeted. Presumptive identification of *A. salmonicida* was carried out by several biochemical



**Figure 13.** Cell death analysis by FITC - Annexin V /PI study in liver and spleen of control and *A. salmonicida* infected *L. rohita*. \*Significant ( $P < 0.05$ ).



**Figure 14.** Differential Expression of Caspase 3 in liver and spleen of SHAM operated control and *A. salmonicida* infected *L. rohita*. \*Significant ( $P < 0.05$ ).

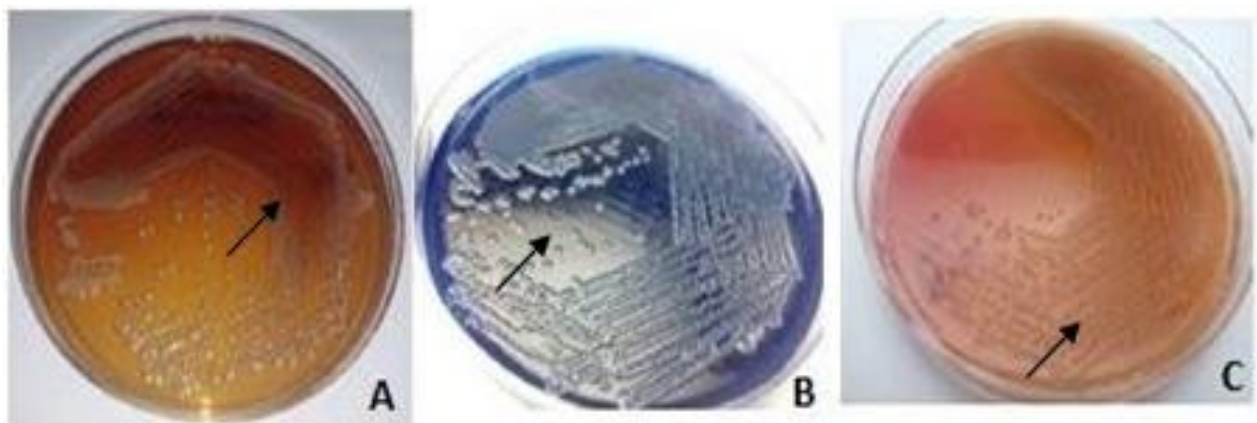
tests. At 7 DPI tissue homogenates were prepared from liver and spleen of the clinical isolates and inoculated and plated onto different selective, biochemical growth media *viz.* MacConkey agar, CBB agar and TSA for confirmation (Figure 15) following Teska, & Cipriano (1993), Popoff (1984), Wiklund, & Bylund (1993). Production of brownish diffusible pigment (Figure 15A), bluish colonies (Figure 15B) and yellowish colonies (Figure 15C) respectively in TSA, CBB and MacConkey agar indicated and confirmed *A. salmonicida* to be present in the treated fish after 7 days of artificial challenge study and successful re-isolation of the bacterial pathogen.

Further confirmation, at molecular level was performed by PCR amplification with DNA isolated from presumptive *A. salmonicida*, using 4 pairs of primers *viz.* AP1/AP2, PAAS1/PAAS2, MIY1/MIY2 & 16S rRNA. After gel electrophoresis the PCR products were observed under an UV transilluminator (Figure 16) and identification of *A. salmonicida* was confirmed with

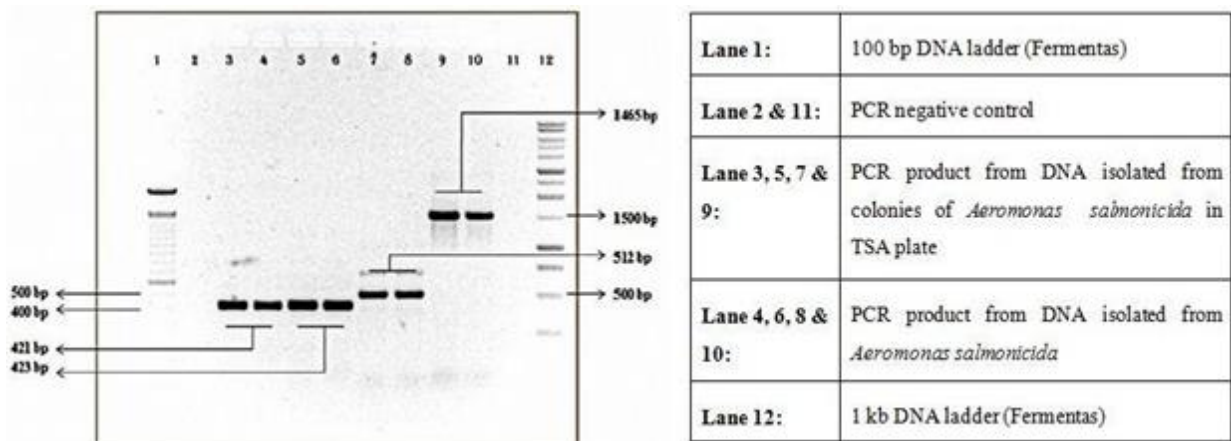
observation of specific bands at length 421 bp, 423 bp, 512 bp and 1465 bp respectively as per Mooney, Powell, Clabby, & Powell (1995).

## Discussion

The trend of haematological and serum biochemical parameters observed in our earlier studies (Pal, Datta Roy & Homechaudhuri, 2015) suggested that alterations in these parameters could be used as potential exposure biomarkers for Aeromonad infection at asymptomatic level. As per our earlier reports *L. rohita* injected with asymptomatic dose of *A. salmonicida* showed no external and internal lesion of the diseases even after 7 days of exposure. But significant alterations in fish health indicators *viz.* haematological and serum biochemical parameters in comparison to control fishes indicated successful establishment of the bacterial infection within the fish body (Pal, & Homechaudhuri, 2016). In the present



**Figure 15.** Re-isolation and confirmation of *A. salmonicida* from clinical isolates by biochemical tests. Fig 16A. Production of brown soluble pigment in TSA; Fig 16B. Bluish colonies in CBB agar; Fig 16C. Yellowish colonies in MacConkey agar all indicating *A. salmonicida* culture isolated from infected fish tissue after 48 hours incubation at 22°C.



**Figure 16.** *Aeromonas salmonicida* (ATCC 33658) showing products obtained from primer for AP1/AP2 (421 bp), PAAS1/PAAS2 (423 bp), MIY1/MIY2 (512 bp) & 16S rRNA (1465 bp).

study, haematological parameters (TLC, MCV, MCH, MCHC) and serum biochemical parameters (total serum protein and serum glucose level) were studied over three different exposure durations and it was found that these parameters varied most significantly in the treatment groups at 7 DPI. The treatment groups also exhibited an overall decrease in all these values when compared to the control groups over the period of 21 days. The significant increase in the total WBC count after bacterial infection observed in this study signified the fact that the innate immunity of the fish was stimulated to fight against the injected bacterial pathogen as the primary line of defense. The increased level of serum total protein observed in this current study could be an indication of elevated level of functioning of nonspecific immune response of fishes against the bacterial infection, as the serum proteins include various humoral elements of the non-specific immune system. Similar observations were also reported by several authors (Rehulka, & Minarik, 2005; Zhou, Li, Abbas, & Wang, 2009; Singh, Singh, Bhartiya, & Yadav, 2010). Significant increase in serum glucose level in *A. salmonicida* infected fishes compared to control fishes was primarily due to acute stress caused by bacterial infection where mobilization of readily available energy in the form of glucose enhanced the survival of fish and helped to ward off the infection induced stress. After the initial screening of the above-mentioned parameters in *L. rohita* against *A. salmonicida* infection at a carrier state it was found that the immune reaction and resultant stress was most prominently exhibited at 7 DPI in comparison to 14 and 21 DPI. As the objective of the current study was to provide an insight on the immune system functioning of *L. rohita* in response to *A. salmonicida* infection and also to provide an overview on the disease mechanisms, thus further detailed studies on the immune response at cellular and molecular level have been targeted at 7 DPI only.

In fish, phagocytosis has been recognized as one of the important elements in the host defense against invading micro-organisms (MacArthur, & Fletcher, 1985) and constitutes an important characteristic of the innate immune system (Seeley, Gillespie, & Weeks, 1990). The NBT assay is a very good indicator of the health status of fish and further indicative of oxidative radical production from neutrophils and monocytes for use in defense against pathogens (Anderson, Moritomo, & de Grooth, 1992; Anderson, & Siwicki, 1995). The significantly higher number of NBT-positive cells *i.e.* cells with dark bluish formazan deposit within the cytoplasm observed in the current study indicated that upon pathogenic bacterial infection, the phagocytic activity and respiratory burst activities of neutrophils were significantly induced due to the innate immune system functioning.

Myeloperoxidase (MPO) is an important enzyme which utilizes hydrogen peroxide during respiratory burst to produce hypochlorous acid acting as

antimicrobial agent (Dalmo, Ingebrigsten, Sveinbjornsson, & Seljelid, 1996). An increased MPO activity was recorded in the neutrophil population of *L. rohita* infected with *A. salmonicida* at asymptomatic levels in comparison to sham operated control fishes in the present study. Several authors have documented similar changes in fish exposed to different pathogens (Sahoo et al., 2004; Rodríguez, Novoa, & Figueras, 2008; Raida, & Buchmann, 2008; 2009; Mohanty, & Sahoo, 2010).

TNF $\alpha$  is mainly involved in the recruitment of leukocytes to the inflammatory foci rather than in their activation (Roca et al., 2008). The results indicated that *A. salmonicida* infection, even at asymptomatic carrier stage, might modulate the host immunity as relative expression of TNF $\alpha$  gene was found to be significantly up-regulated both in liver and spleen tissue of *L. rohita* post infection in comparison to sham operated control fishes. The expression levels were normalized with  $\beta$ -actin as internal control to avoid any errors in the initial RNA concentration. The cytokine TNF- $\alpha$  is one of the crucial mediators of pro inflammatory responses (Hanington, Hitchen, Beamish, & Belosevic, 2009). Similarly, up-regulation of TNF $\alpha$  differential expression have been reported in rainbow trout leucocytes in response to different pro-inflammatory stimuli (Hardie, Chappell, & Secombes, 1994; Iliiev, Liarte, MacKenzie, & Goetz, 2005). These findings pointed out that at 7 DPI with *A. salmonicida*, the innate immune system of *L. rohita* was significantly stimulated in terms of respiratory burst activity and a pro-inflammatory response was also initiated at major immune competent organs like liver and spleen.

The increased non-specific immune response in terms of serum bactericidal activity in the present study was a result of pathogen detection and activation of innate system. These results w.r.t. serum bactericidal activities were in accordance with various studies on different fish species (Hernandez, Teshima, & Koshio, 2007; Aly, Ahmed, Ghareeb, & Mohamed, 2008; Rao, Das, Jyotirmayee, & Chakrabarti, 2006; Misra, Das, & Mukherjee, 2009; Das, Pradhan, & Sahu, 2009).

Lysozyme, an important defence molecule, is one of the several antimicrobial enzymes, associated with the first line immune defense of both invertebrates and vertebrates playing a role in mediating protection against microbial invasion. It is bactericidal in nature that catalyzes the hydrolysis of  $\beta$ -1, 4 linked glycoside bonds of peptidoglycan layer resulting in lysis of bacterial cell wall (Brown et. al., 1997). In the present study, relative expression of lysozyme C was found to be significantly increased in liver and spleen tissue of *L. rohita*. Similar pattern was also observed in case of lysozyme G where significant increase was observed both in liver and spleen of infected fishes when compared to control ones. Also, liver exhibited the higher degree of up-regulation in the expression of these genes compared to spleen.

Histological sections of liver and spleen in *A. salmonicida* infected *L. rohita* when examined under microscopy at 7 DPI indicated significant alteration and modification of normal tissue architecture, induced by oxidative stress were observed. Similar type of tissue destructions in the liver and spleen were also reported in several instances viz. in *I. punctatus* (Ventura & Grizzle, 1988), *Clarius batracus* (Angka, 1990), *Salmo gairdneri* (Candan, 1990), estuarine Cat fish (Alagappan, Deivasigamani, Kumaran, & Shaktivel, 2009), *Cirrhinus mrigala* (Datta Ray & Homechaudhuri, 2014) and *Catla catla* (Datta Ray & Homechaudhuri, 2013) post infection with *A. hydrophila*. However, the spleen was found to be less susceptible than liver in terms of histological alterations in the present study.

Since significant cellular damage and death in liver and spleen were observed at the histopathology level, further study to ascertain the nature of such reported cell death by the Annexin V – FITC/ PI study was employed. In this study, *A. salmonicida* infection resulted in significantly higher values of early and late apoptotic cell percentage both in liver and spleen compared to control fishes. The finding was in accordance with results obtained in histopathological observations earlier. Similar studies in *Cirrhinus mrigala* challenged with *A. hydrophila* showed results in support of the present findings (Datta Ray & Homechaudhuri, 2014), thus indicating that production of intracellular ROS (liver > spleen) takes place in an organ specific pattern and that excess ROS is a potent inducer of apoptotic cell death.

Consequently, it was aimed to determine the fate of caspase-3 gene expression in *L. rohita* upon infection with *A. salmonicida*. PCR analysis of the cDNA prepared from liver and spleen using primers designed for Caspase-3 resulted in a 285 bp product. From the results obtained it was found that in the liver and spleen tissue the expression levels showed a significant increase after infection with *A. salmonicida* in comparison to the control fishes. Activated caspase-3 is a central effector of apoptosis that cleaves and inactivates a number of molecules contributing to the typical morphology of apoptosis (Wolf & Green, 1999; Fischer, Jänicke & Schulze-Osthoff, 2003). Increased gene expression of caspase-3 has been described earlier in cells under apoptosis (Miller et al. 1997, Chiang et al. 2001). In this case, it was obvious that upon bacterial infection and subsequent ROS production the caspase-3 mRNA levels shot up significantly in order to meet the increasing demand for the execution of apoptosis.

## Conclusion

The overall results obtained from the present study indicated that exotic, invasive pathogen *A. salmonicida* infection at an asymptomatic, carrier state acted as strong physiological stressors for *L. rohita* fingerlings resulting in the activation of alarm–stress response leading to deterioration of health status. It induced

oxidative stress related inflammation and ultimately resulted in apoptotic cell death both in liver and spleen (Liver > Spleen). Expression of antimicrobial protein producing genes (Lysozyme C and Lysozyme G), pro-inflammatory cytokine (TNF $\alpha$ ) producing gene and expression of apoptosis effector molecule (Caspase 3) were also found to be significantly higher both in liver and spleen of *A. salmonicida* treated fishes compared to control ones. From the overall findings, it could be stated that a multi parametric bio-marker approach for evaluating the pathogenic and physiological impact of *Aeromonas* infection can be highly convenient in *L. rohita*, especially at a carrier state dose i.e. without obvious symptomatic manifestation of the disease.

Since facultative pathogen eradication is not possible in open water or flow-through aquaculture systems, this remains a crucial area of scientific research and the results obtained in the present study might provide a better understanding of prophylactic, diagnostic and therapeutic measures during aquaculture.

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