Chapter 8

Pathogenicity study of *Vibrio parahaemolyticus*

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8.1. Introduction

Pathogenic bacteria cause disease when they invade the human body. The pathogenic bacteria possess different virulent genes that cluster together within the genome. The degree of pathogenicity of a bacteria is measured by the ability of bacteria to cause disease in a host organism inspite of its immune response (Peterson, 1996). When bacteria invade and multiply within the animal body cavity, the host immune system becomes activated to eradicate the pathogens from the body. This is achieved through the activation of innate and adaptive immune responses. Bacterial cellular components like polysaccharides, lipopolysaccharide (LPS), peptidoglycans, bacterial DNA were recognized by pattern recognition proteins/receptors of the host that activates host innate immunity by using NF-kB pathway (Magnadóttir et al., 2006). This leads to the production of proinflammatory and anti-inflammatory cytokines (Weighardt et al., 2008; Cua et al., 2010). The immune system of fish is almost similar to that of higher vertebrates. The mouse is widely used as an animal model organsim to study human infectious disease (Zak et al., 1999; Pletzer et al., 2017). Around 3.1 billion base pairs are present in both the mouse and human genomes of which only 5 % of the sequence can encode protein. The protein-coding regions of human and mouse are 85-99 percent identical (Cheng et al., 2014). Homology and degree of genetic similarity are important criteria for the selection of animal models (Swearengen et al., 2018). The whole-genome sequencing of zebrafish has revealed that 70 percent of its protein-coding genes are related to human genes and among that 84 percent of genes are known to be associated with human disease which clearly indicated the importance of zebrafish as

a model (Barbazuk et al., 2000). Therefore, fishes are now widely used as a model to study the immune responses against human pathogens (Rauta et al., 2012), cancer research (Mione and Trede, 2010) and ecotoxicological study (Bambino and Chu, 2017) and evolutionary genomics (Liu, 2003; Gardell et al., 2013).

In the present study, *Labeo rohita* was used as a model organism to understand the pathogenicity mechanism as well as an immune response after infection with *V. parahaemolyticus*.

8.2 Material and Methods

8.2.1 Bacterial culture

The pure colony of bacteria was inoculated in 10 ml of sterile Brain Heart Infusion (BHI) broth and incubation was done for 24 hrs at 37 ℃. After overnight incubation, bacterial cells were collected by centrifugation at 5000 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in normal saline (NS) water and centrifugation again at 5000 rpm for 5 min. The whole process was repeated again. Finally, the pellet was resuspended in 10 ml NS. 1 ml of cell suspension was serially diluted up to 10^{-6} dilution in NS and spread in agar plate to determine the number of colonies per ml of suspension after 24 hrs incubation at 37 ℃.

8.2.2 LD_{50} determination

A total of 200 healthy *Labeo rohita* (mean weight; 15±5 g) were collected from local carp hatchery and were acclimatized under laboratory conditions for 10 days by providing a commercially available fish diet twice daily @ 2 % of their body weight. The fishes were `randomly assigned to five experimental tanks (one tank as control and other

four tanks for the experimental challenge) for challenge study. A total number of 10 fishes were kept in each tank.

The experimental challenge study was conducted to determine the cumulative mortality and the lowest bacterial dose for 50 % mortality (LD_{50}) in *L. rohita*. The fishes were injected with 0.2 ml of bacterial suspension intraperitoneally with a final concentration of 2.0×10^6, 2.0×10^5, 2.0×10^4, and 2.0×10^3 CFU ml^{-1}, respectively. The control fishes were injected with 0.2 ml of saline water. The experiment was carried out in triplicates. Fish mortality was recorded in every 24 hrs interval for 7 days. The injected bacterial isolate as a pathogen was re-isolated from the blood and liver of moribund fish to satisfy Koch's postulates. LD_{50} was calculated following Reed and Muench (1938).

8.2.3 Histopathology

After recording the external clinical signs, the organs like liver, kidney and muscle were collected from the infected fish and fixed in 10 % Neutral Buffered Formalin (NBF) for histopathological studies. The NBF preserved tissues were taken out and wash with water to remove NBF. The tissues were then cut into 1-2 mm size and were dehydrated with a series of different concentrations of alcohol. Finally, the dehydrated tissues were cleared in xylene and embedded into paraffin following the impregnation technique (Leica EG 1140H, Germany). After this, sectioning of the paraffin-embedded tissues at 5-mm thickness using a microtome was done followed by staining with haematoxylin and eosin (Luna, 1968). Histopathological changes manifested in the tissue sections were observed under microphotographs

8.2.4 Experimental challenge

The fish were randomly assigned to three experimental groups for challenge study. A total of 40 fishes were kept in each 200 liter tanks (one tank as control and other two for the experimental challenge) at 30 °C. For the experimental challenge, bacteria were cultured in BHI until the cell density reached an OD 610 of 1.0. The bacterial culture was diluted with PBS to obtain the bacterial concentration of CFU ml^{-1} (LD_{50}: 1.2 X 10^3 CFU ml^{-1}). The fishes were intraperitoneally injected with 200 µl of bacterial suspension and the control group fishes were injected with 200 µl of PBS. Three fishes were sampled after 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs of post challenged. Fishes were euthanized with an overdose of MS22 (> 200 mg/L of water) and liver, kidney and muscle were collected and pooled together, and immediately stored at -80 °C for further analysis. A small part of the liver, kidney and muscle were keeping in RNAlater and stored at -80 °C. Blood was collected and pooled together at a different time interval. One part of the blood was heparinized and store at 4 °C for further study. The serum was collected from the rest of the blood and store at -80 °C for further analysis.

8.2.5 Innate immunity parameters

8.2.5.1 Respiratory burst activity

The respiratory burst activity of serum of *L. rohita* after post-infection was studied using the following method described by Anderson and Siwicki, 1995. 100 µl of heparinized blood was added to 100 µl of 0.2 % nitro-blue tetrazolium (NBT) and mixed well. The mixture was incubated at 25 °C for 30 min in dark condition. After incubation, 50 µl of the reaction mixture was taken out and mixed with 1 ml of dimethylformamide

to solubilize the insoluble granules. Finally, centrifuged at 2000 rpm for 5 min and Optical Density (OD) was taken at 540 nm using a spectrophotometer.

8.2.5.2 Myeloperoxidase activity

Myeloperoxidase activity (MPO) content in serum was analyzed following the protocol described by Quade and Roth, 1997. 10 µl of serum was mixed with 90 µl of Hank's balanced salt solution without Ca^{+2} and Mg^{+2} in a microtitre plate. Now 35 µl of freshly prepared 20 mM 3,3',5,5' tetramethylbenzidine hydrochloride and 5 mM H_2O_2 was added in each reaction. After 2 min of incubation in room temperature, 35 µl of 4 M sulphuric acid was added and the OD was taken at 450 nm.

8.2.5.3 Lysozyme activity

The lysozyme activity was measured by following Shugar (1952). 25 µl of serum sample was added to 800 µl *Micrococcus lysodeikticus* (O.D between 0.6–0.7 at A450, suspended in 66 mM potassium phosphate) and incubated at 25°C for 30 min. Initial OD was taken at 450 nm immediately after incubation. A standard curve was drawn by using lysozyme and serum lysozyme values were expressed as mg ml^{-1} equivalent of lysozyme activity.

8.2.5.4 Total antiproteases activity

Antiproteases activity in fish serum was determined following the protocol as described by Zuo and Woo, 1997. 10 µl of test serum was mixed with 100 µl of trypsin (bovine pancreas type I, Sigma Aldrich). 10 µl PBS was mixed with 100 µl of trypsin as positive control and 110 µl PBS used as a negative control. 10 µl of test serum mixed with 100 µl PBS as serum blank. All the tubes incubated at 25 °C for 30 min. 1 ml of

casein (Sigma Aldrich) dissolved in PBS (2.5 mg/ml) was added in each tube and incubated for 15 min at 25 °C. 500 ml of 10 % trichloroacetic acid was added in each tube to terminate the reaction. All the samples were centrifuged at 10,000 g and the OD of the supernatants was measured at 280 nm. The percentage of trypsin inhibition was calculated by following the equation.

Reference value = [absorbance of positive control]-[absorbance of negative control]

Control value = [absorbance of test sample]-[absorbance of respective serum blank]

Percent inhibition = [(reference value-control value)/(reference value)]x100

8.2.5.5 Alpha (α)-2 macroglobulin activity

The activity of α-2 macroglobulin (M) in fish serum was studied using the protocol described by Zuo and Woo, 1997. 10 µl of test serum was mixed with 100 µl of trypsin and 90 µl of 0.1 M Tris HCl, pH 8.2. For positive control, 100 µl of trypsin was mixed with 100 µl of Tris HCl and 200 µl Tris HCl was used as a negative control. Again, 10 µl of test serum was mixed with 190 µl of Tris HCl as serum blank. All the reaction tubes were incubated at 25 °C for 40 min. 2 ml of Nabenzoyl-DL-arginine-p-nitroanilide HCl was dissolved in Tris HCl containing 20 mM $CaCl_2$ which was added in each tube and incubated for 20 min at 25 °C. The reaction was terminated by adding 500 ml of 30 % (v/v) acetic acid. The percentage of trypsin inhibition was calculated from the OD value which was measured at 410 nm.

8.2.6 Protein extraction

Frozen fish liver, kidney and muscle were homogenized in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, pH 7.3). The homogenates were centrifuged at 11500 g for 15 min at 4 °C in a high-speed refrigerated centrifuge. The supernatant is collected and stored at -20 °C. The protein content of each supernatant (soluble protein fraction) was determined by the Bradford method (Bradford et al., 1976), using BSA as the standard.

8.2.7 SDS-PAGE

1D SDS-PAGE was used to separate the soluble proteins extracted from the liver, kidney and muscle. The protein was separated using 12 % polyacrylamide gel (W/V) and 5 % stacking gel (W/V) with a stable potential of 200 V on a mini-Protean 3 electrophoresis cell (Bio-Rad) (Laemmli, 1970). A protein molecular marker (Bio-Rad) was run with the samples. After completion of the run, the gel was taken out and protein bands were visualized using Coomassie Brilliant Blue R 250 (CBB).

8.2.8 RNA isolation and cDNA preparation

Total RNA was extracted from liver, kidney and muscle preserved in RNA later at a different time interval from experimentally challenged fish. Tissues were homogenized with ice-cold tri-reagent (Sigma) in cold condition and RNA was extracted from liver, kidney and muscle following the manufacturer's protocol (Figure 8.1). The RNA quality was checked in agarose gel and quantified by spectrophotometer. RNA samples were treated with DNase-1 (Invitrogen) to remove the genomic DNA following the manufacturer's protocol. Total RNA was reverse transcribed using SuperScript III

(Thermo, Fisher) with random hexamers and Poly T-Tail according to the manufacturer's instructions.

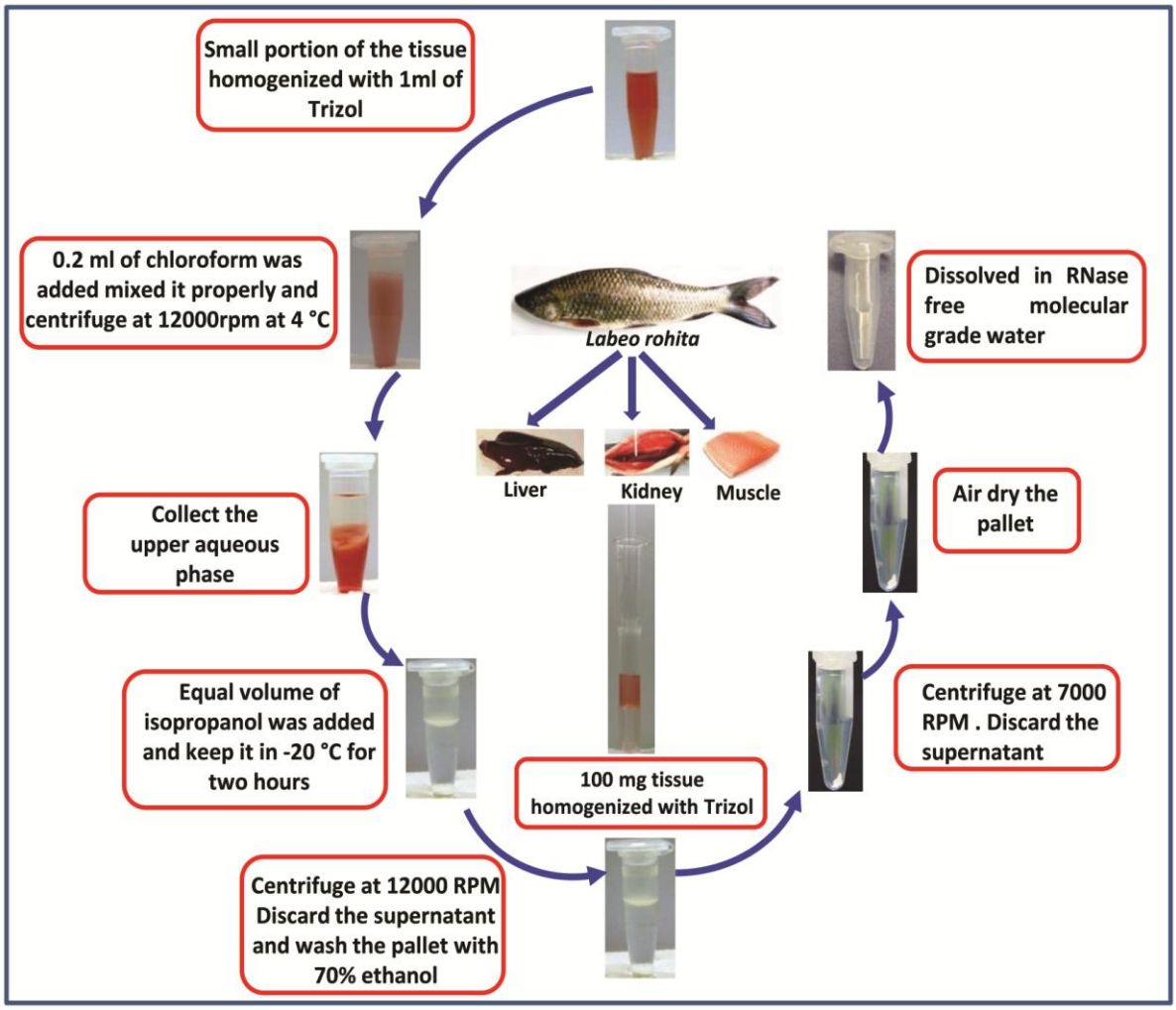

Figure 8.1 Schematic diagram of RNA isolation from different tissue using Trizol

8.2.9 Gene expression analysis

cDNA was prepared from live, kidney and muscle of experimentally challenged fishes used for genes expression analysis. The genes encoding complement factor (C3), cytokines (IL-1β, IL-6 and TNF-α) and pattern recognition receptors [Toll-like receptor (TLR22)] were used for the gene expression analysis. All the primes used in the present study were given in the Table 8.1. The qPCR assays were performed using a Light Cycler 480 (Roach). The cycling conditions were 1 cycle of initial denaturation at 95 °C for 5 min, followed by 40 cycles with denaturation at 95 °C for 10 s and annealing for 15 s and elongation for 10 s. Wells contained 10 µl of 2X Syber green Master mixture, 1 µl forward and reverse primer (5 mM), 2 µl of cDNA and autoclaved MilliQ water to make the total volume 20 µl. In the present study, β-actin was used as the reference gene.

Table 8.1 Primer used in the immune gene expression study

Target Gene	Primer Name	Sequence	Annealing Temp	Amplicon Size	Reference
Beta Actin	β-Actin F	AGACCACCTTCAACTCCATCATG	58	200	Basu et al., 2015
	β-Actin R	TCCGATCCAGACAGAGTATTTACGC			
Interleukin-1β	IL-β F	GTGACACTGACTGGAGGAA	54	164	Dash et al., 2017
	IL-β R	AGTTTGGGCAAGGAAGA			
Interleukin-6	IL-6 F	GGACCGCTTTGAAACTCT	54	212	Robinson et al 2012
	IL-6 R	GCTCCCTGTAACGCTTGT			
Tumor Necrosis Factor α	TNFα F	CTCAACAAGTCTCAGAACAATCAGG	61	181	Giri et al., 2016
	TNFα R	TCCTGGTTCCTTCTCCAATCTAGCT			
Complement factor 3	C3α F	CCCTGGACAGCATTATCACTC	60	155	Ma et al., 2015
	C3α R	GATGGTCGCCTGTGTGGT			
Toll like Receptor 22	TLR F	CAGGTGGCGAGCTTCAGACT	60	134	Kole et al., 2017
	TLR R	CGGAGGTAGGTTCGTTTCTTCA			

8.2.10 Statistical analysis

Statistical analysis was carried out using SPSS (16.0) software. Results were expressed as a mean value ± standard deviation. The differences of expression in immune responsive at different time interval were calculated using one-way analysis of variance and statistically significant differences were reported at $p< 0.05$.

8.3 Results

8.3.1 Cumulative mortality and determination of LD_{50}

100 % mortality was observed within 48 hrs after the intraperitoneal injection of *V.parahaemolyticus* with $2.0x10^8$ CFU ml^{-1} whereas only 20 % of fishes died in case of intramuscular injection. The intraperitoneally challenged fish had inflammation at the injection site and in the abdomen. Intramuscular injection of *V. parahaemolyticus* with same concentration showed discoloration of skin and loss of scale from the injection site. Mortality was not observed in the control fishes (Figure 8.2).

The cumulative mortality rate of *Labeo rohita* after post-injection with *V. parahaemolyticus* was shown in Figure 8.3. The bacterium was re-isolated from moribund fish and was found to be the same as the injected strain. The LD_{50} value of *V. parahaemolyticus* was estimated to be 1.5×10^5 CFU per fish.

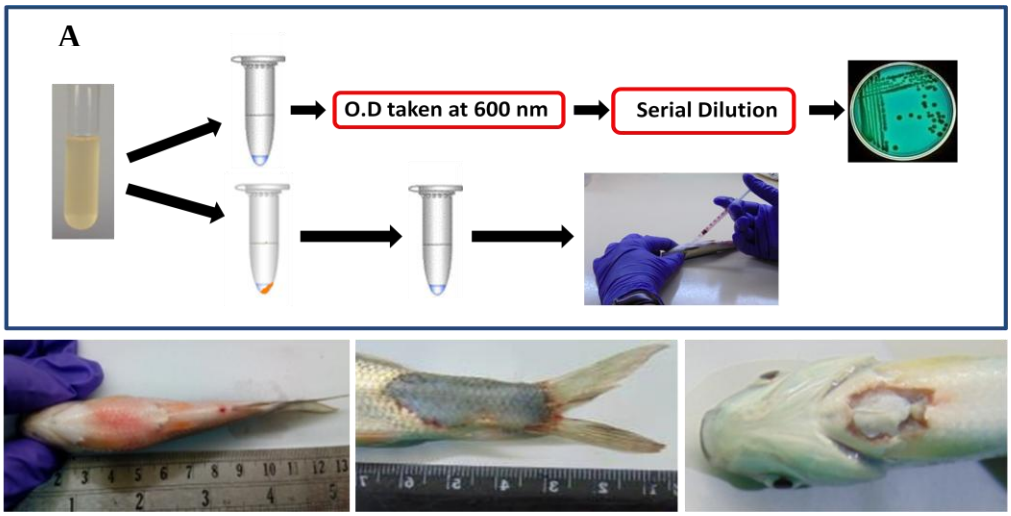

Figure 8.2 Schematic diagram of challenge study (A) and clinical signs that apper in the fish after experimental challenge with *V. parahaemolyticus*

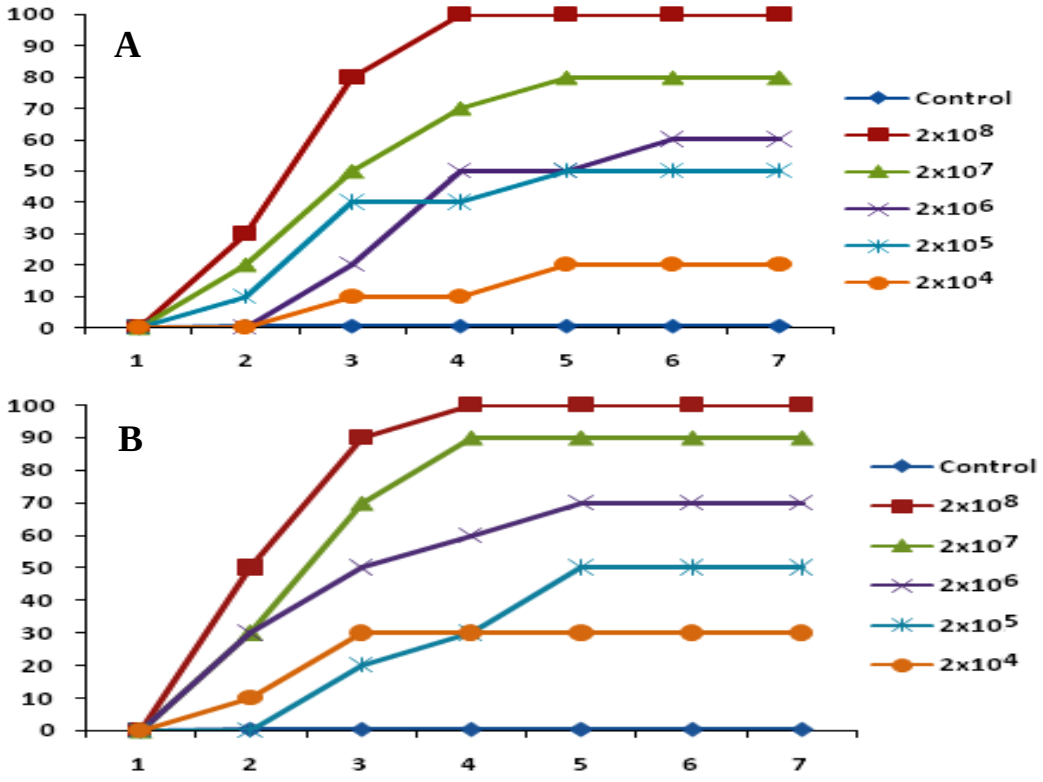

Figure 8.3 Cumulative mortality curves for the determination of LD_{50} values in Indian Major Carps challenged with *V. parahaemolyticus* by intra-peritoneal injection at different concentrations. [A] *tdh* positive stain (S24P132) and [B] *trh* positive stain (AP429)

8.3.2 Histopathology

The clinical symptoms of the infected fishes were observed as a loss of mucus and reddish lesion near the pectoral fin, however, there was no sign in the gill. On post-mortem examination, ascitic fluid was observed in the body cavity, the liver appeared pale and the accumulation of fluid was observed in the intestine. The histopathological examination of liver tissue of the experimentally challenged *Labeo rohita* showed necrosis and degenerated hepatic tissue. Increased hepatocyte vacuolation was also observed in the liver. The kidney section showed changes in the ultrastructural of glomeruli. Vaculation and necrosis of renal tubules were also observed in the kidney section. The intestine section of infected fish showed that the tips of the villi appeared ragged and irregular, necrosis of villus was observed and epithelial cell debris from the disrupted villi was detected in the intestinal lumen (Figure 8.4; Figure 8.5).

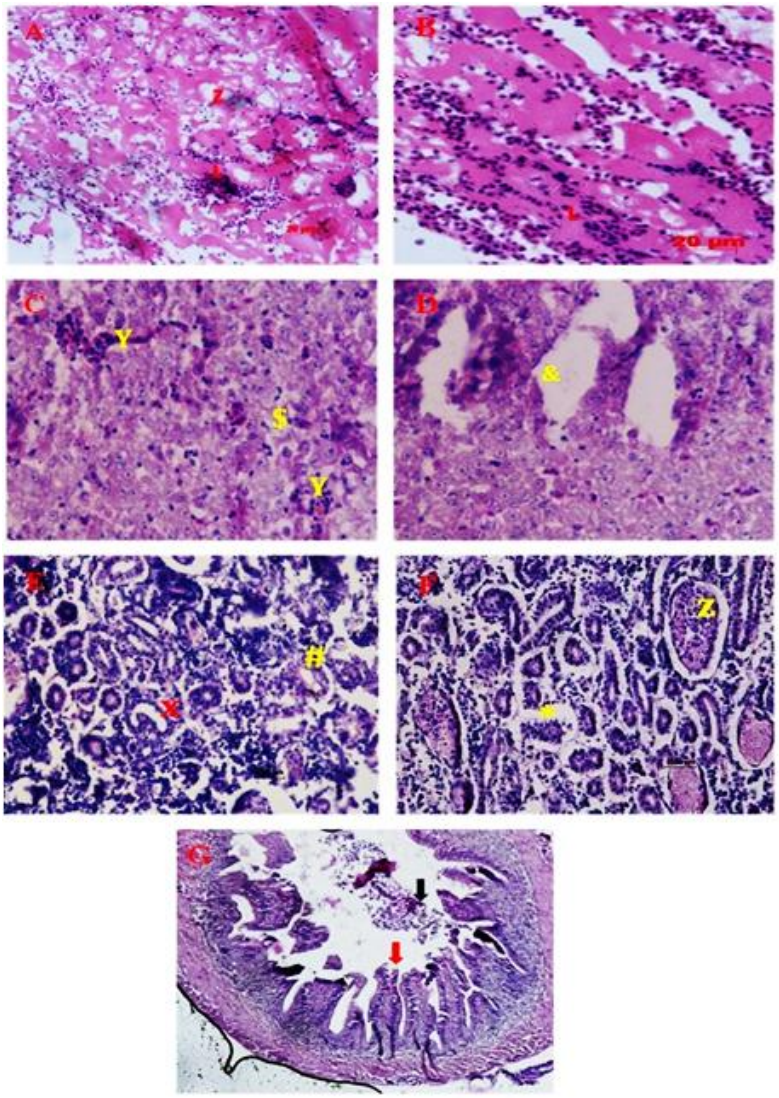

Figure 8.4 Histopathological examination of tissues of *Labeo rohita* infected with *tdh* positive *V. parahaemolyticus* (S24P132) through intraperitoneal injection.

[A-B] Photomicrograph of muscle of *Labeo rohita* [A]: Infiltration of the lymphocyte (L), Myonecrosis (Z). [B]: Infiltration of the lymphocyte (L).

[C-D] Photomicrograph of liver of *Labeo rohita* [C]: Infiltration of the blood cells (Y), Degeneration of the hepatocytes ($). [D]: Vacuole formation in the liver (&).

[E-F] Photomicrograph of kidney of *Labeo rohita*.[E]: Glomerular degeneration (X), Necrosis of Bowman's capsule (#). [F]: Necrosis of the renal tubules (*), Sclerosis of glomerulus with atrophic tubules (z).

[G] Photomicrograph of intestine of *Labeo rohita*. [G]: Intestinal mucosal epithelial cell falling off (⬇) and intestinal bleeding (⬇).

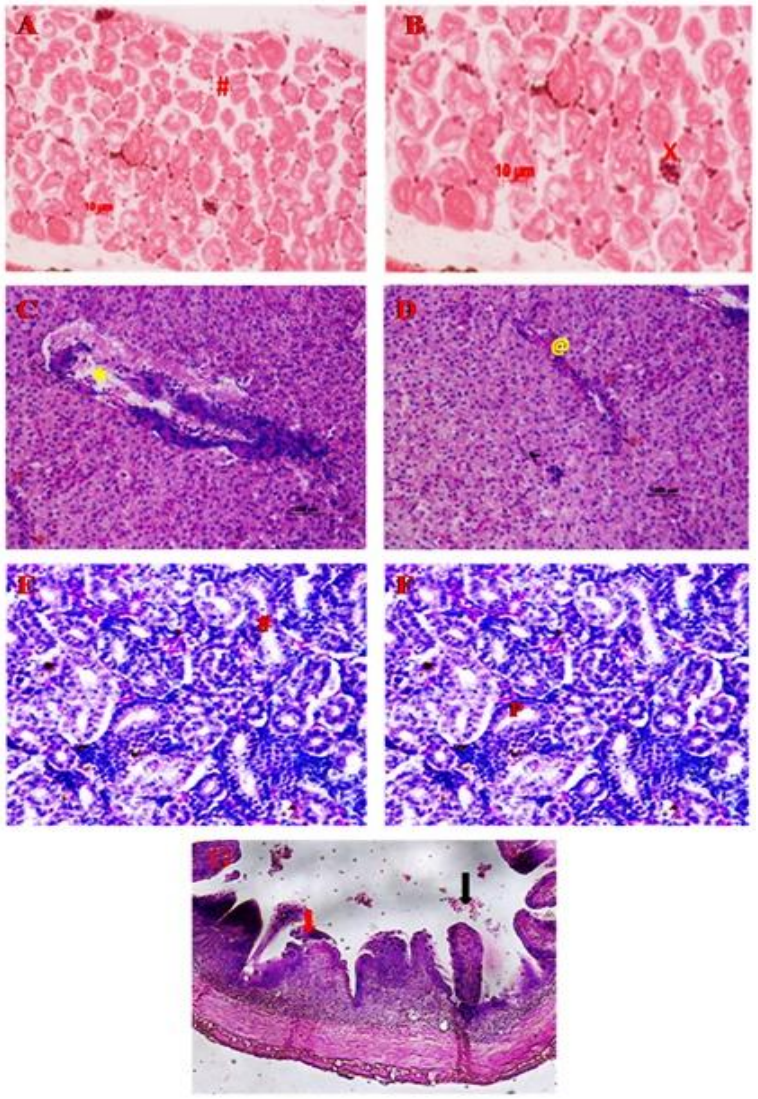

Figure 8.5 Histopathological examination of tissues of *Labeo rohita* infected with *trh*-positive *V. parahaemolyticus* (AP429) through intraperitoneal injection.

[A-B] Photomicrograph of muscle of *Labeo rohita* challenged with *trh*-positive *V. parahaemolyticus*[A]: Myonecrosis (#). [B]: Infiltration of the lymphocyte (X).

[C-D] Photomicrograph of liver of *Labeo rohita* challenged with *trh*-positive *V. parahaemolyticus* [C]: Necrosis of liver tissue and degeneration of hepatocytes (*). [D]: Blood cell infiltration (@).

[E-F] Photomicrograph of Kidney of *Labeo rohita* challenged with *trh*-positive *V. parahaemolyticus* [E]: Ultrastructural changes in the glomeruli. [F]: Vacuolation and necrosis of tubule (P).

[G] Photomicrograph of Intestine of *Labeo rohita* challenged with *trh*-positive *V. parahaemolyticus*. [G]: Intestinal mucosal epithelial cell falling off (⬇) and intestinal bleeding (⬇).

8.3.3 1D profile of different tissue

8.3.3.1 1D profile of liver

1-D gel profile of the protein prepared from liver tissue of control and experimentally challenged fishes is showed in Figure 8.6. CCB-stained 12 % SDS-polyacrylamide gels separated the liver protein in approx 27 bands in the molecular weight range of 10 to >250 kDa. The electrophoretic profiles of liver protein of control and 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs 72 hrs of post challenged fishes showed differential express protein bands. The band with a molecular weight of 34 kDa and 29 kDa was present in control and in 12 hrs of post-challenge fishes. Similarly, a band with molecular weight 32 kDa was absent in control but present in the entire infected group. A faint band with molecular weight 17 kDa was present in control but absent in entire post-challenge groups.

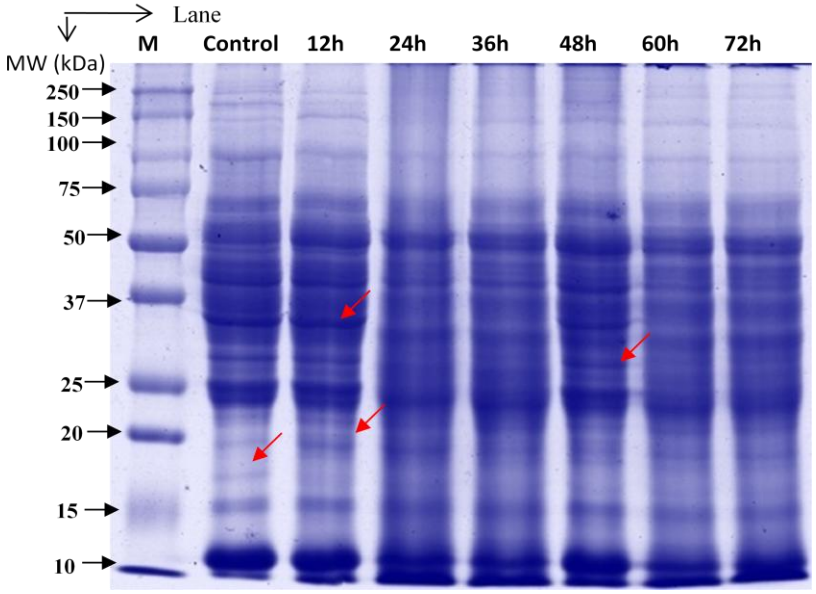

Figure 8.6 SDS-PAGE profile of proteins prepared from liver tissue of control and experimentally challenge *Labeo rohita*. Red arrows indicate the differentially express protein bands.

8.3.3.2 1D profile of kidney

CCB-stained 12 % SDS-polyacrylamide gels separated the kidney protein in approx 27 bands in the molecular weight range of 10 to >250 kDa. The differential express protein bands were observed in the control and challenge groups (Figure 8.7). The protein band with a molecular weight of 150 kDa was present in control and in 12 hrs of the post-challenge group but absent in other groups. A new band with molecular weight 75 kDa appears in 36 hrs of the post-challenge group. Another protein band with molecular weight 50 kDa absent in control but present in entire post-challenge groups. A protein band with molecular weight 17 kDa present in control but absent in entire post-challenge groups.

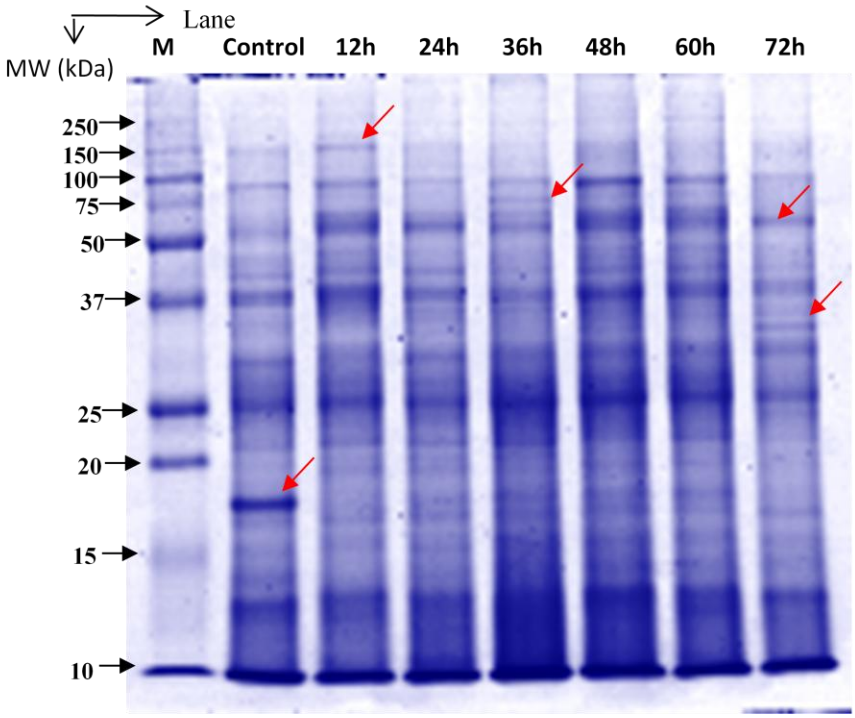

Figure 8.7 SDS-PAGE profile of proteins prepared from kidney tissue of control and experimentally challenge *Labeo rohita*. Red arrows indicate the differentially express protein bands.

8.3.3.3 1D profile of muscle

1D SDS-PAGE revealed a large dynamic range of protein expression 10 to >250 kDa. The protein patterns of control and other challenge groups are shown in Figure 8.8. 1D image analysis revealed that the 12 % SDS-polyacrylamide gels separated the muscle protein extracts into 20 different bands with a molecular weight range from 10 to > 205 kDa. The protein bands with a molecular weight of 100 kDa were overexpressed in the muscle of 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs of the post-challenge group. Similarly, protein bands with molecular weight 75 kDa, 25 kDa and 23 kDa were expressed in all the treated groups though it is absent in control. A faint band with molecular weight 20 kDa and 16 kDa was present in control but absent in muscle tissue of all the post-challenge fish collected at a different time interval.

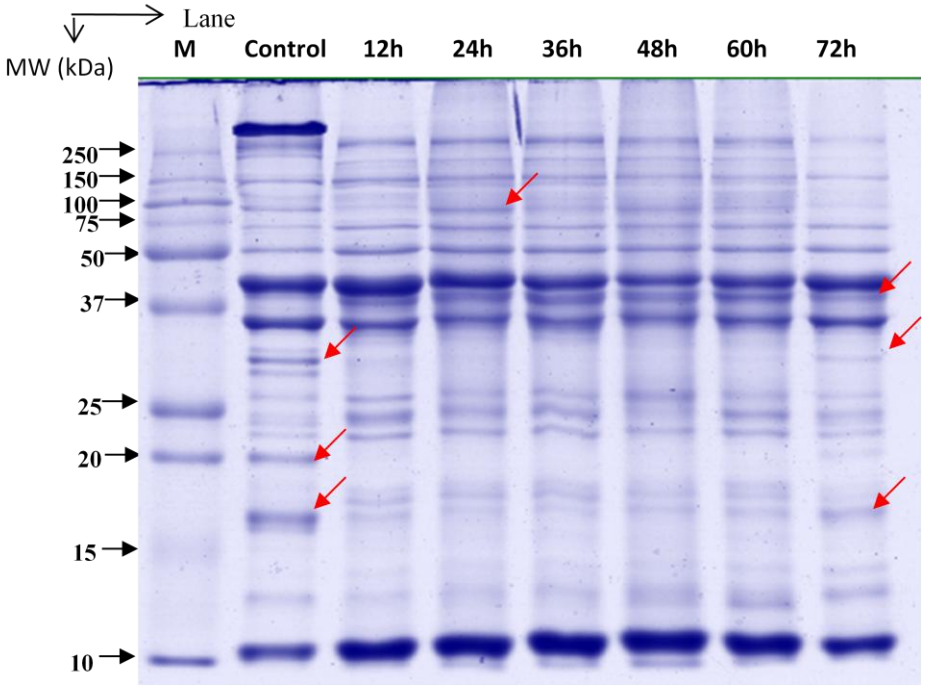

Figure 8.8 SDS-PAGE profile of proteins prepared from muscle tissue of control and experimentally challenge *Labeo rohita*. Red arrows indicate the differentially express protein bands.

8.3.3.4 1D profile of serum

The Figure 8.9 depicts a representative of 1D gel profile of serum protein collected from control and 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs and 72 hrs of post challenged fishes. CCB-stained 12 % SDS-polyacrylamide gels separated the serum protein in 14 different bands in the molecular weight range of 10 to >250 kDa (Figure 8.10). The electrophoretic profiles of serum proteins of control and 12 hrs, 24 hrs, 36 hrs, 48 hrs, 60 hrs, 72 hrs of post challenged fishes showed major changes. The protein band with a molecular weight of 150 kDa was absent in serum of 24 hrs of post-challenge fishes. A protein band with molecular weight 37 kDa was absent in control and 12 hrs of the post-challenge group whereas it was present in the serum of all the other challenge groups. A minor band with molecular weight 32 kDa was present only in 24 hrs and 36 hrs of post infected group. A major band with molecular weight 25 kDa was disappeared from 24 hrs post-challenge serum group.

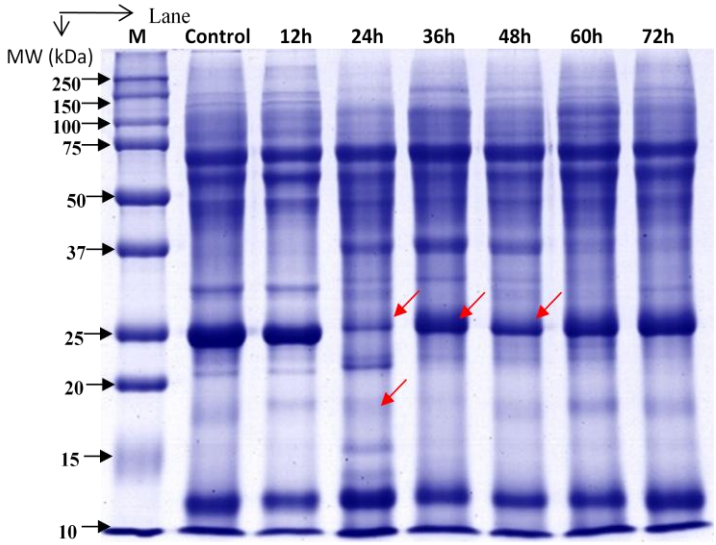

Figure 8.9 SDS-PAGE profile of proteins prepared from serum of control and experimentally challenge *Labeo rohita*. Red arrows indicate the differentially express protein bands.

8.3.4 Innate Immunity Parameters

8.3.4.1 Respiratory burst activity

The respiratory burst activity of the infected group at different hours of post-injection (hpi) was significantly higher ($p < 0.05$) with respect to their control group. The highest respiratory burst activity was observed after 12 hrs of post-injection. The respiratory burst activity was decreased in 24 hrs of post-injection but again it was gradually increasing and reach a maximum in 48 hrs of post-injection (Figure 8.10A).

8.3.4.2 Myeloperoxidase activity

The myeloperoxidase activity of the infected group was high in compared to their respective control group and was statistically significant ($p > 0.05$) except 72 hrs. The myeloperoxidase activity was gradually increased and the highest activity was observed at 48 hrs of post-infection (Figure 8.10B).

8.3.4.3 Lysozyme activity

The lysozyme activity of the serum of the infected group and the control group showed a significant difference. Lysozyme activity gradually increases up to 36 hrs of post infected group and again decreased. The lowest lysozyme activity was observed at 60 hrs of hpi group (Figure 8.10C).

8.3.4.4 Total antiproteases activity

The antiproteases activity of the infected group was high compared to their respective control group and was not statistically significant ($p > 0.05$). The highest antiproteases activity was observed at 36 hrs of post-infection (Figure 8.10D).

8.3.4.5 Alpha(α)-2 macroglobulin activity

The activity of α-2 macroglobulin of the infected group and the control group did not show any statistically significant ($p > 0.05$) change. No significant differences were observed in α-2 macroglobulin activity in control group and infected group at different hpi (Figure 8.10E).

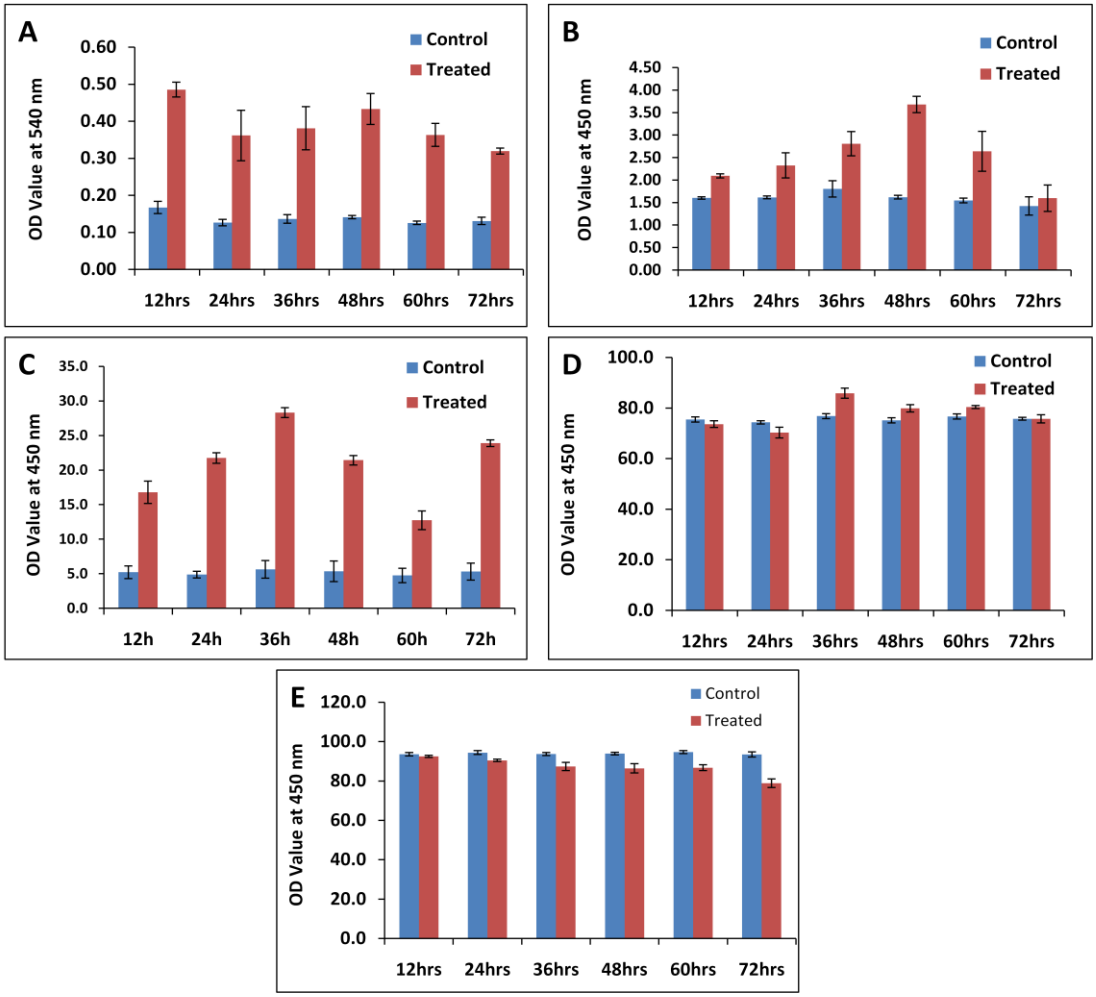

Figure 8.10 Inateimmune parameters in *Labeo rohita* at different time interval after experimentally challenge with *V. parahaemolyticus*. Respiratory burst activity (A); Myeloperoxidase activity (B); Lysozyme activity (C); Antiproteases activity (D); 2-Macro globulin activity (E). Bars represent mean ± SD (n =6). Except the values of 2-Macro globulin activity and Antiproteases activity all the values are significantly different in respect to control ($P < 0.05$)

8.3.5 Gene expression profile

Differential expression in immune responsive genes was observed after experimental challenge with *V. parahaemolyticus* (Figure 8.11). The expression of IL-β was up-regulated 6 fold in liver tissue after 12 hrs of post-infection where as in kidney it was 5 fold and in muscle it was 2 fold upregulated. The expression of IL-β was increased in kidney and muscle and highest level of expression was observed after 24 hrs and 48 hrs of post-infection in kidney and muscle, respectively. IL-6 and TNF-α gene were gradually upregulated in liver and highest level of expression was observed after 72 hrs and 48 hrs of post-infection, respectively. In kidney, highest level of expression of IL-6 and TNF-α was observed after 24 hrs of post-infection. The highest level of expression of C3a in the liver was observed after 48 hrs of post-infection. However, the expression of C3a in kidney and muscle was observed after 24 hrs and 36 hrs of post challaenge. The highest expression of all the immunoresposive genes was observed in kidney after 24 hrs post-infection. High expression of all immunoresposive genes was observed after 24 hrs of post-infection in kidney. However in case of muscle the high expression of all immunoresposive genes was observed after 36 hrs of post-infection

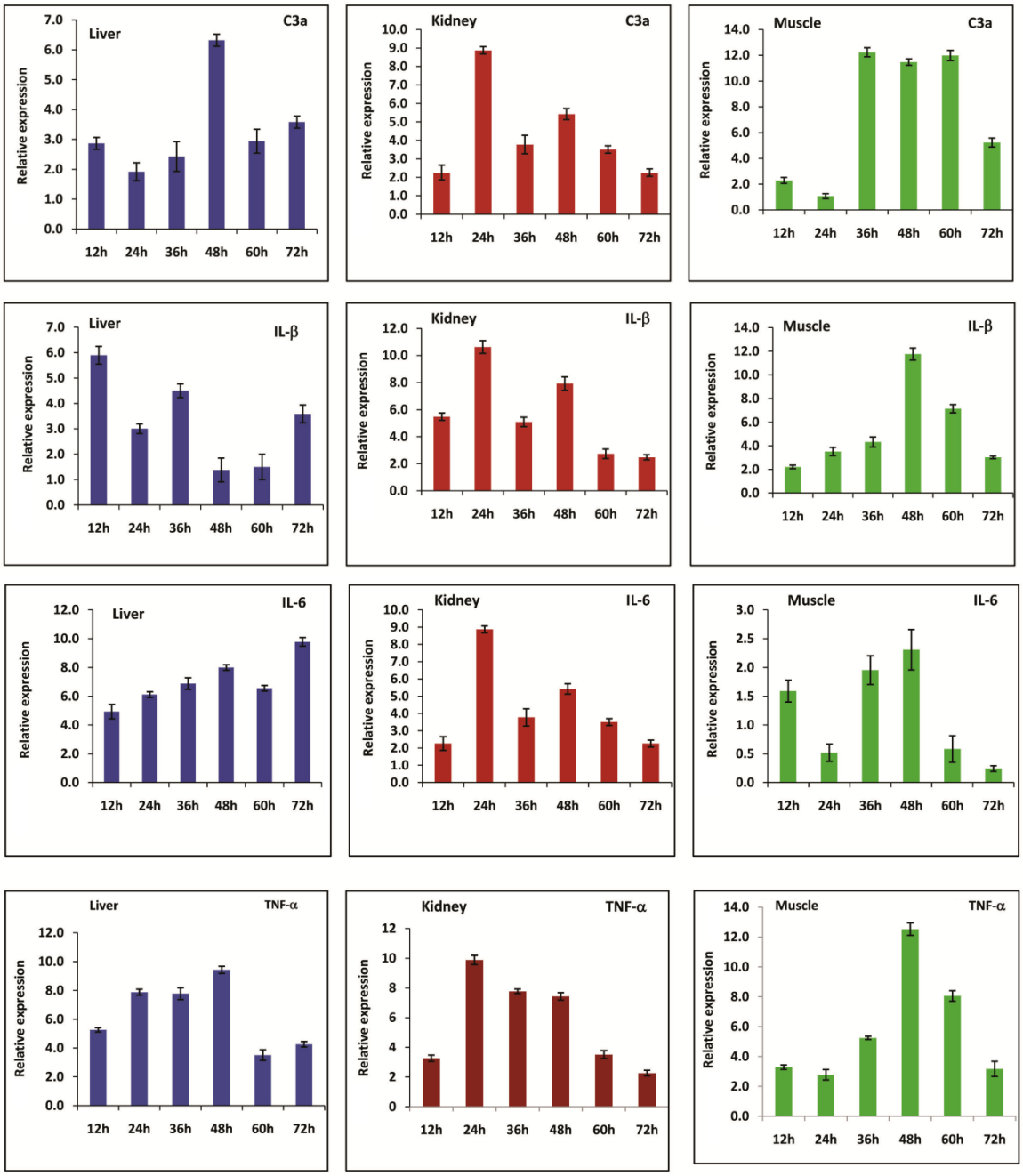

Figure 8.11 Expression profiles of four different immuno genes (C3a, IL β, IL 6 and TNF α) in three different tissue (liver, kidney and muscle) during experimental challenge with *V. parahaemolyticus*. Bars represent mean ± SD (n =6)

8.4 Discussion

V. parahaemolyticus is a zoonotic pathogen, widely distributed in marine and estuarine environments throughout the world (Daniels, 2000; Denner et al., 2002 and Heidelberg et al., 2002). The most of the clinical strain of *V. parahaemolyticus* carries either Tdh or Trh or both protein along with other virulence factors that modulates the host immune system to create an own ecological niche within the host cell. The present study was conducted to understand the pathogenic mechanism of this bacteria using *L. rohita* as a model organism. Several workers (Paranjpye, et al., 2013; Dong et al., 2015) used to fish as a model to compare the virulence of *V. parahaemolyticus* isolated from the environment and clinical isolates. In the present study, *V. parahaemolyticus* (AP429) was isolated from shrimp farm of West Bengal and the cytotoxicity and enterotoxicity were studied in *Labeo rohita*. 30 % motility in fish was recorded when *V. parahaemolyticus* was injected intramuscularly. Similarly, less mortality was observed by Dong et al., 2015 when they challenged zebrafish with *V. parahaemolyticus*. 100 % mortality was observed when *V. parahaemolyticus* was administrated intraperitoneally. Intraperitoneal had more significant effects than the other infection route (Paranjpye, et al., 2013 and Dong et al., 2015). Intraperitoneal injection with *V. parahaemolyticus* in *Labeo rohita* showed hemorrhages in the abdomen. Similar type of clinical signs was observed when fishes were challenged with *V. parahaemolyticus* (Paranjpye, et al., 2013; Peng et al., 2016) or other pathogenic bacteria (Behera et al., 2017; Das et al., 2017) The histopathological examination of liver tissue of the experimentally challenged *Labeo rohita* showed necrosis and degenerated hepatic tissue. Increased hepatocyte vacuolation was also observed in the liver. The kidney section showed changes in the ultrastructural of

glomeruli. Vaculation and necrosis of renal tubules were also observed in the kidney section. The accumulation of intestinal fluid was observed in challenged fish. A similar type of clinical symptoms was observed in the intestine of rabbit, mouse and zebrafish when they were challenged with *V. parahaemolyticus* (Daniels et al., 2000; Park et al., 2004; Dong et al., 2015).

When fishes are challenged with bacteria, the immunosystem of fishes become activated and released immunoglobulins, cytokine and interferon etc. in the blood, therefore, serum is an important sample for the immunological study. The study carried out by other workers revealed that, in teleost IgM was comprised of a 70-81 kDa heavy chain and 22-32 kDa light chain consistent with these findings the SDS-PAGE profile of serum in the present study consisted two major bands 72 kDa and 25 kDa (Shamsudddin et al., 2011). In the teleost, the immunoglobulins molecule appears primarily as a tetramer composed of four monomeric subunits. The serum protein bands in the present study, *viz*., 85, 72 and 70 kDa are regarded as $\alpha 1$, α 2 and γ protein fractions of the H chain and 25 kDa are their corresponding L chains. The albumin proteins significantly form a thick band with the molecular weight 64 kDa (Shamsudddin et al., 2011). In an earlier study, the molecular weight of heavy chains and light chains was estimated at about 67 and 29 kDa, respectively (Chanphong and Adams, 1994). Zhong et al. (1999) observed H and L chain of 71 and 24- 26 kDa for common carp Ig. However, Sood et al. (2007) reported MW of H and L chain of *C. carpio* Ig as 73.7 and 25.3 kDa, respectively. Rathore et al. (2006) reported that MW of H chain in *Clarias gariepinus* was 74.8 kDa while that of L chain was 27.2 kDa.

Liver proteome composed of proteins with a wide range of molecular weight, relative abundance, different hydrophobicity, acidity and basicity. It would be ideal to analyze the entire liver proteome (Wang et al. 2007). The liver plays a dual role by helping the host defense response during infection and also plays an important role in the metabolic process (Causey et al., 2018). It is well known that liver has role in regulation of physiologic response to inflammatory stimuli by synthesizing acute phase proteins upon activation by cytokines (Castell et al. 1989). Comparative analysis with SDS-PAGE profile of liver tissue carried out by Heinemann and Ozols.(1998) and with the SDS-PAGE profile of liver tissue of *Labeo rohita* showed similar kind of band pattern. The protein band with molecular weight 64 kDa may be serum albumin. The protein band with molecular weight 45 kDa may be a argininosuccinate synthases. The protein band with molecular weight 40 kDa may be a fructose-bisphosphate aldolase B. The protein bands with 130.5 kDa, 110 kDa, 98 kDa and 17.2 kDa were differentially expressed in liver tissue of challenged fish. Further study with two-dimensional gel electrophoresis and mass spectrometry (MS) will be providing better information about the protein.

During the challenge study, hemorrhage spots were observed in the muscle tissue. Bacterial infection in tissue increases local blood flow and release histamine from mast cell that increases the vascular permeability and recruit lymphocyte in the infection site to destroy the bacterial infection. Recruitment of lymphocyte is critical to initiate inflammatory responses in macrophages, dendritic cells, and mast cells to release the initial components of cellular innate immune responses, including the proinflammatory cytokines TNF-α, IL-1, and IL-6; chemokines; prostaglandins and histamine. Therefore, any kind of changes in the muscle could be identifying in the muscle proteome.

During bacterial infection, innate and adaptive immunity of the host activates to clear the pathogen from the host body. The innate immunity starts responding first during microbial infection. The bacterial cell wall product like lipopolysaccharide activates macrophage and neutrophils resulting in the production of superoxide anion and hydrogen peroxide that clear the invaded pathogen (Slauch, 2011). PMNs mediated signaling or signaling from G protein-coupled receptors (GPCRs) and cytokine receptors are responsible for more robust activation of NADPH oxidase complex (El-Benna et al., 2016). Deficiency in the production of reactive oxygen species causes severe bacterial infections in human (Nguyen et al., 2017). Myeloperoxidase is an important indicator of the innate immune response and is released mostly upon the activation of neutrophils. Myeloperoxidase used hydrogen peroxide (H_2O_2) to produce hypochlorous acid (HOCl) which kills the pathogen (Nauseef, 2014). The activation and release of myeloperoxidase closely linked with respiratory burst activity (Khan et al., 2018). The generation of reactive oxygen species and release of myeloperoxidase are required to eliminate the invading pathogenic bacteria (Nauseef et al., 2014). MPO deficient mice are highly susceptible to infection caused by *Klebsiella* and *Candida* (Khan et al., 2018). Lysozyme is a conserved antibacterial protein play an important role in host defense. Lysozyme is present in tears, milk, mucosa, saliva and urine which play an important role in innate immunity. It also produced by phagocytic cells like macrophages, neutrophils, and dendritic cells. Elevated expression of lysozyme was reported by several workers during bacterial infection in fish (Saurabh et al., 2008), Mice (Akinbi et al., 2000). Fish antiproteases are also an important component in the nonspecific humoral immune system which includes antiprotease, Alpha (α)-2 macroglobulin and antiplasmin. The

antiproteases inhibit the protease enzyme secreted by the pathogen when pathogens invade into the host body (Chuang et al., 2013). No significant changes were observed in control and experimentally challenge group. Lähteenmäki et al. (2005) identified a protein (*PgtE*) in *Salmonella enterica* that can inactivate the antiprotease α 2-antiplasmin. *V. parahaemolyticus* might be using similar mechanism against antiproteases activity. α-2 macroglobulin is also induced in human when protease secreting pathogen invades into the human body (Chuang et al., 2013). α-2 macroglobulin is a broad-spectrum protease inhibitor. But no statistically significant variation was observed in control and experimentally challenge group though the bacteria produce proteases. Bacteria are producing protein homologous to α-2 macroglobulin that also has the ability to bind with proteases which might reverse the block in host antimicrobial defenses (Budd et al., 2004)

Cytokines act as a chemical messenger for regulating the innate and adaptive immune systems in response to antigen. IL-β is a primary cytokine in the signaling cascade induced during infection. The mammalian IL-β gene shared structural similarities with cyprinids (Secombes et al., 2011). During the pathogen attack, IL-β plays an important role in host immunity. It increased the leukocyte phagocytosis, synthesis of lysozyme, migration of leukocyte and macrophage proliferation (Dash et al., 2015). IL-β also induces the expression of COX2 and MHC II in macrophage. The overexpression of IL-β had already been reported in fish during bacterial infection or in the presence of LPS (Wang et al., 2009). The prominent expression of IL-β was observed in muscle, heart and spleen of *L. rohita* (Dash et al., 2015). In our study, we also found prominent expression in liver and kidney along with other tissues. Increased expression

of IL-6 and IL-1β was reported in head kidney, spleen and peripheral blood of *L. rohita* after challenge with formalin-killed *Aeromonas hydrophilia* (Dash et al., 2015). IL-1β secreted from monocytes and macrophages is a key regulator of inflammation and increase phagocytosis of macrophages.

TNFα is another proinflammatory cytokine and expressed at the initial stage of infection in fish. It also activates the phagocytic activity of leukocytes. TNFα produced from macrophages, lymphocytes, NK cells and adipocytes in response to external stimuli increase the migration of neutrophil and proliferation of lymphocyte in rainbow trout (Zimmerman et al., 2014). The expression of TNFα was reported in the kidney, head kidney, muscle, liver, spleen, gill and heart of *Megalobrama amblycephala*. However, the highest expression of TNFα was detected in immune organs like the spleen and head kidney (Lv et al., 2017). However, Xiao et al. (2007) did not find any significant changes in the expression of TNFα after post-infection in mandarin fish (*Siniperca chuatsi*). Poor upregulation of TNFα by immune challenge in vitro and in vivo was reported by several workers (Zou et al., 2003; Praveen et al., 2006; Sepulcre et al., 2007).

IL-6 produced during inflammatory events promotes the proliferation of macrophage, induce the expression of antimicrobial peptide and at the same time down-regulate the expression of IL-1β and TNFα which reduces the inflammation. Overexpression of IL-6 was observed in the spleen of fugu and flounder, head kidney and leukocytes of seabream after injection with LPS or poly I:C or peptidoglycan (Secombes et al., 2011). Increased expression of IL-6 was observed in muscle, heart and spleen of *L. rohita* (Dash et al., 2015). In our study, we also found prominent expression in liver and

kidney. Dash et al. (2015) reported that expression of IL-6 was also increased in response to different stimuli in immune tissues of different fish species like Japanese pufferfish, flounder, gilthead seabream and rainbow trout. Increased expression of IL-6 and IL-β was reported in head kidney, spleen and peripheral blood of *L. rohita* after challenge with formalin-killed *Aeromonas hydrophilia* (Dash et al., 2015).

The C3 component of the complement system plays a central role in the complement activation pathway. In the complement activation pathway (Classical, Lectin and Alternative), C3 component cleaved into C3a and C3b. C3a involve in the activation of inflammation and cell migration. C3a also has antibacterial activity, efficiently killed the Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, and the Gram-positive *Enterococcus faecalis* (Nordahl et al., 2004). C3a defective mice reduce the expression of chemokines like IFNγ and TNF α in the host response to *Listeria monocytogenes* (Mueller-Ortiz et al., 2014). Up regulation in the expression of C3a was observed in the liver and kidney tissue of *L. rohita* at 6, 12, 24, 48 and 72 hrs of post-infection with *Aeromonas hydrophila* (Pushpa et al., 2014). Overexpression of C3 component of complement pathway was reported in skin of *O. mykiss* (Sigh et al., 2004) and *C. carpio* infected with parasite *Ichthyophthirius multifiliis* (Saeij et al., 2003)

Myeloid differentiation factor 88 (MyD88) and Toll-interacting protein are two important factors in the signaling cascade of the Toll-like receptor (TLR). Knock out of MyD88 gene from zebrafish increased the susceptibility to bacteria and reduced the expression of proinflammatory cytokines interleukin (IL-1β) (Liu et al., 2010; van der Vaart et al., 2013). A separate experiment carried out by Adachi et al., 1998; Takeuchi et al., 2000 showed that MyD88-deficient mice are highly susceptible to *Listeria*

monocytogenes and *Staphylococcus aureus* infection which clearly indicates that MyD88 plays a crucial role in the innate immunity signaling cascade. MyD88 binds to the TIR domain of the receptors, initiates a signaling cascade, ultimately leading to the activation of proinflammatory genes by transcription factor NF-κB (Fitzgerald et al., 2001). Overexpression of MyD88 was reported in Rock bream infected with Rock bream iridovirus (RBIV) (Jung and Jung 2017), *Rachycentron canadum* (Cobia) infected with *Photobacterium damselae* (Tran et al., 2018).

8.5 Conclusion

Pathogenic bacterial isolates of *V. parahaemolyticus* were isolated from environmental samples in India and their pathogenic potential was tested by the experimental challenge in *L. rohita* using as a model organism. The histopathological changes confirmed the tissue-specific infection of *V. parahaemolyticus*. The gene expression study showed that, after experimental challenge the expression of toll-like receptor, proinflammatory cytokines like IL-β, IL-6 and TNFα was upregulated during infection. Overexpression of complement factor 3 and Heat shock proteins 70 was also observed after post-infection. The presence of these virulent strains in the aquaculture farms increases the risk of spread of disease in aquatic animals and in the human.