5. MICROBIAL INFECTIVITY AND HISTOPATHOLOGICAL STUDIES IN LABEO ROHITA

5.1. Introduction

Microbial members of the genera belonging to the Enterobacteriaceae family have earned a reputation of placing them among the most pathogenic and most often encountered organisms in clinical microbiology. These large Gram-negative rods are usually associated with intestinal infections, but can be found in almost all natural habitats. They are the causative agents of diseases such as meningitis, bacillary dysentery, typhoid, and food poisoning. Also, they well as being oxidase negative, all members of this family are glucose fermenters and nitrate reducers. In most cases, the pathogenicity of a particular enteric bacterium can be determined by its ability to metabolize lactose. Non-utilizers are usually pathogenic but the lactose utilizers are not. Because many different species in this family can cause similar symptoms, biochemical tests are crucial for the identification, diagnosis, and treatment of infection.

The role of Providencia spp. belonging to the Enterobacteriaceae as a primary pathogen has been questioned in fish pathology since its recovery from diseased animals has been erratic (Austin and Austin, 2007). The Providencia species is currently being reported as one of the main re-emerging pathogens in aquaculture industry in Tamil Nadu, India (Ramkumar et al., 2013; Ramkumar et al., 2014). Since its description in 1976 (Bejerano et al., 1979), infections by this pathogen have reportedly caused considerable mortalities among carp species, and the role of P. stuartii as the causative agent for ulcerative disease has been demonstrated in Labeo rohita fish very recently (Ramkumar et al., 2013). Bacterial diseases are responsible for high mortality in both wild and cultured farm fish (Mastan and Osman Ahmed, 2013). The real role of microorganisms vary from a major pathogen to that of an opportunistic pathogens which makes its host organisms (fish) moribund by commencing infection development. Also the bacterial flora of fish is directly proportional to its environment.
Present experiment pertains to the infection on Labeo rohita fish using the cultured microbe, Providencia vermicola and the description of the resultant pathological changes in the diseased/infected fish.

5.2. Materials and methods

5.2.1. Infectivity of P. vermicola in experimental L. rohita by immersion method, intramuscular method and oral route.

The P. vermicola bacterial suspension was prepared at different concentrations with optical density at 0.1, 0.5 and 1.0 measured using spectrophotometer at 560 nm. The prepared different concentrations of bacterial suspensions were injected intramuscularly to the healthy L. rohita at a concentration of 75 µL per fish (30-35g; 8-10 cm) and observed for 100% mortality and appearance of clinical sign of ulcerative lesions in the experimental fishes. From this experiment, the bacterial isolate responsible for mortality and appearance of clinical signs was selected for further studies (infectivity & histopathological investigation). The bacterial concentration responsible for 100% mortality of fish was diluted further by tenfold serial dilution for 4 times for calculating lethal concentration 50 (LC50) or lethal dose 50 (LD50). The number of bacterial cells in each dilution used in the pathogenicity experiments was calculated (Ducklow et al., 1980).

5.2.2. Experimental infectivity/pathogenicity

Experimental pathogenicity was conducted based on standard protocols (Ducklow et al., 1980; Egidius, 1987).

5.2.2.1. Infection by intramuscular injection

10 fish per tank was taken in individual 100 L FRP tanks with continuous aeration. The animals were fed with commercial fish pellets. 0.1, 0.5 and 1.0 OD concentrations of P. vermicola suspension was used for this experimental setup. 75 µL of P. vermicola bacterial suspension from each of these three concentrations was injected using 1 mL insulin syringes intramuscularly in separate tanks. Control fish received only sterile phosphate buffer. The experiment was repeated thrice for each bacterial concentrations. Fish were monitored twice daily for mortality and ulcerative lesions. Dead animals were removed from the tanks and recorded.
5.2.2.2. Experimental infection by immersion method

10 fish per tank was taken in individual 100 L FRP tanks with continuous aeration. The animals were fed with commercial fish pellets. 0.1, 0.5 and 1.0 OD concentrations of P. vermicola suspension was used for this experimental setup. 25 mL of each concentration P. vermicola bacterial suspensions were immersed in to the water containing 100 L of water. 5 mL of sterilized phosphate buffer alone was added to the control tank. The experiment was repeated thrice for each bacterial concentrations. Fish were monitored twice daily for mortality and ulcerative lesions. Dead animals were removed from the tanks and recorded.

5.2.2.3. Oral route of infection

10 fish were maintained in the aquarium tanks and starved for 24 h. Each fish was fed with a piece of fish meat which was already injected with 1 mL of 1.0 OD concentration of P. vermicola bacterial suspension. The fish were fed thrice a day with an interval of 8 h, after the last feeding with infected meat; the animals were fed with non-infected meat for a week. In the control group, fish were fed with disease-free, healthy meat alone. The experiment was repeated thrice. Fish were monitored twice daily for mortality and ulcerative lesions. Dead animals were removed from the tanks and recorded.

5.2.3. Validation of P. vermicola pathogenicity

The precise action of the P. vermicola isolated from naturally infected fish as a pathogen was confirmed by re-isolating the bacterium from the moribund fish to fulfil Koch's postulates. The homogenized suspensions were inoculated on trypticase soy agar plates by spread plate technique for isolation of bacterial pathogen. The isolated bacteria were recognized using the procedures as described in chapter 4. The LC50 values of the L. rohita fish were calculated by Spearman Karber method based on the method described by Finney (1952).

5.2.4. Histopathological studies on experimentally infected animals

5.2.4.1. Tissue preparations and histology

At the end of exposure time after intramuscular injection, the fish samples were sacrificed after 48 h infection by killing. Gill, liver and skin of experimental
and control fish were dissected out and fixed in 10% neutral buffered formalin solution for 24 h and then were processed for paraffin embedding. Paraffin blocks of gill, liver and skin were cut at 6 μm thickness and stretched on decontaminated glass slides. After deparaffinization, sections were stained with Haemotoxylin- Eosine and observed under light microscope. The histopathological changes in the organs were examined in the randomly selected sectors of a fish. Histopathological changes induced by treatments in the tissues were photographed. The histology of these organs in normal fish were also studied.

5.2.5. Biochemical and haematological changes in bacteria injected L. rohita

10 L. rohita fish per tank was taken in an individual 100 L FRP tanks with continuous aeration. The animals were fed with commercial fish pellets. 1.0 OD concentration of P. vermicola suspension was used for this experimental setup. 75 μL of P. vermicola bacterial suspension from each of these three concentrations was injected using 1 mL insulin syringes intramuscularly in separate tanks. Control fish received only sterile phosphate buffer. The experiment was repeated thrice for each bacterial concentrations. Fish were monitored twice daily for mortality and ulcerative lesions. Dead animals were removed from the tanks and recorded.

Collection of blood samples

Each fish was individually caught using a dip net and held the fish by our new device. Then, they were bled from common cardinal vein using 1 mL tuberculin syringe fitted with 24 gauge needle (Michael et al., 1994). In order to sample the blood for serum separation, 200 μL of blood was drawn and the whole bleeding procedure was completed within 2 min. The blood was collected in serological tubes and clot was stored at 20°C overnight. The clot was then spun down at 400 ×g for 10 min. The serum collected by aspiration was stored in sterile Eppendorf tubes at -20°C for further use.

5.2.5.1. Biochemical parameters of L. rohita blood samples

Total protein, albumin, globulin, free amino acids, glucose, total cholesterol, triglycerides, phospholipids, free fatty acids and lysozyme levels were determined in both experimentally intramuscular injected and control L. rohita blood samples.
5.2.5.1.1 Estimation of total protein

Total protein was determined spectrophotometrically at 640 nm based on the method of Lowry et al. (1951).

Reagents used:
- Standard Bovine Serum Albumin (stock solution of 1mg/mL)
- Deproteinizing agent
  - Solution A (1% Copper sulphate)
  - Solution B (2% Sodium potassium tartrate)
  - Solution C (2% Sodium carbonate in 0.1 N NaOH)
- Folin’s reagent.

Procedure

The solutions A, B & C were mixed in the ratio of 1: 1: 98. From this, 2.25 mL was pipetted out into clean, marked glass tubes. The standard BSA was added to the tubes at concentrations of 10, 20, 40, 80 and 100 µL. 10 µL of serum samples from 12, 24, 48, 72 and 96 h infected fish along with control fish samples were added in separate tubes, all the tubes were mixed well and were incubated at room temperature for 10 minutes. Then, 0.1 mL of Folin’s reagent was added to all the tubes and incubated in the dark for 30 minutes and the optical density was read at 640 nm. The readings from protein standards were used to plot a standard graph and to estimate value of the test samples.

5.2.5.1.2. Estimation of albumin

Albumin was estimated by measuring standard and test solutions against blank, spectrophotometrically at 630 nm.

Procedure:

To 100 µL of serum, 1 mL of working reagent was added and incubated for 1 min at 37 °C. After incubation, the absorbance was measured at 600 nm.

Calculation:

Total albumin (g/ dL) = (Absorbance of test/ Absorbance of standard) × Absorbance of std. × 5
5.2.5.1.3. Estimation of Globulin

Serum globulin was calculated by subtracting albumin values from total serum protein

\[
\text{Globulin (g/ dL)} = (\text{Total serum protein} - \text{Total albumin})
\]

5.2.5.1.4. Estimation of Free amino acids

Free amino acids were estimated using ninhydrin according to the method of Moore and Stein (1948).

Reagents used:
- Standard amino acid stock solution (150μg/mL).
- 0.2M Acetate buffer (pH 5.5).
- 8% w/v of Ninhydrin reagent
- 50% v/v ethanol.

Procedure

0.2 – 1.0 mL of standard amino acid solution was added to respective labelled test tubes. 0.5 mL of serum samples from 12, 24, 48, 72 and 96 h infected fishes along with control fish samples were added in separate tubes. The volume of all the test tubes were made up to 4mL using distilled water. To this, 1mL of ninhydrin reagent was added to all the test tubes including the test tubes labelled as blank. The contents of all the tubes were mixed well and boiled in boiling water bath for 15min. The test tubes were cooled in cold water and 1mL of ethanol was added to each test tubes and mixed well. Then, the absorbance at 570 nm of each solution were recorded spectrophotometerically.

Calculation

\[
\text{Free amino acid (mg/ dL)} = (\text{O.D of sample/ O.D of standard}) \times 100
\]

5.2.5.1.5. Estimation of Glucose

Glucose was estimated by the glucose oxidase method followed by Malik and Singh (1980).
Reagents used:

- Gulucostat: A coupled glucose oxidase- peroxidase enzyme preparation
- Standard Glucose (1mg/ mL)
- Deproteinizing agents: as mentioned earlier.

Procedure:

0.2 mL of serum samples from 12, 24, 48, 72 and 96 h infected fish along with control fish samples were added in separate tubes. To these tubes, 1.8 mL of deproteinizing mix was added and mixed well. The tubes were centrifuged at 2500 xg for 5 minutes and the supernatant was collected. 2 mL of the glucostat reagent was pipetted into clean, marked glass tubes. The standard was added to the tubes at concentrations of 10, 20, 40, 80 and 100 µL. 100 µL of test samples from supernatant samples were taken individually. 2 drops of 4N HCl were added to all the tube and the OD was read at 450 nm. The readings from glucose standards were used to plot a standard graph and to estimate values of the test samples.

5.2.5.1.6. Estimation of Cholesterol

Cholesterol was estimated by the method of Zlatkes et al. (1953). The lipid extract was treated with ferric chloride-acetic acid reagent to precipitate proteins. The protein free supernatant was treated with concentrated sulphuric acid. A reddish purple colour formed was read at 560 nm.

Reagents used

- 0.05% Ferric chloride-acetic acid reagent
- Concentrated sulphuric acid
- Cholesterol working standard-40 mg/mL in ferric chloride-acetic acid reagents.

Procedure

0.1 mL of serum samples from 12, 24, 48, 72 and 96 h infected fishes along with control fish samples were evaporated to dryness. 5.0 mL of ferric chloride – acetic acid reagent was added to all the tubes. To this, 3.0 mL of concentrated sulphuric acid was added and the absorbance was read after 20 min at 560 nm against blank solution. The serum cholesterol was expressed as mg/dL.
5.2.5.1.7. Estimation of Triglycerides

Tissue triglycerides were estimated by the method of Foster and Dunn (1973). Neutral lipids were extracted from the tissue lipid aliquot with isopropanol in the presence of alumina. Glycerol was released by saponification and oxidized to from formaldehyde. The later was condensed with ammonia and acetylacetone and absorbance of the product formed was measured at 405 nm.

Reagents used
- Isopropanol
- Activated alumina: Activated grade–I alumina for chromatography was washed with distilled water till all the fine particles were removed and then dried in an oven over night.
- Saponifying agent: 1.0 g of potassium hydroxide was dissolved in 12 mL of distilled water and 0.8 mL of isopropanol was added to the solution and mixed thoroughly.
- Sodium meta peroxidate reagent: To 7.7 g of anhydrous ammonium acetate in 70 mL of distilled water, 6 mL of glacial acetic acid was added followed by the addition of 65 mg of sodium meta peroxidate and made up to 100 mL with distilled water.
- Acetyl acetone reagent: 0.75 mL of redistilled acetyl acetone was added to a mixture of 20 mL of isopropanol and 80 mL of distilled water.
- Standard tripalmitin solution: 10 mg of tripalmitin was dissolved in 100 mL of isopropanol.

Procedure

To 0.1 mL of serum samples from the infected and control fish, 3.0 mL of isopropanol was added, mixed well and 400 mg of activated alumina was added to all the tubes. The tubes were mixed well in a vortex mixer and the proteins were centrifuged off. To 2.0 mL of the supernatant, 0.6 mL of saponifying reagent was added and kept in a water bath at 60 - 70°C for 15 min. Standard tripalmitin (20 -100 µg) solution was also saponified in a similar manner. The tubes were cooled, 1.0 mL of sodium meta peroxidate solution and 0.5 mL of acetyl acetone were added and mixed well. The tubes were cooled and the colour developed was read at 405 nm.
against the reagent blank. The amount of tissue triglycerides was expressed as mg/ g tissue. Serum triglycerides were expressed as mg/dL.

5.2.5.1.8. Estimation of Phospholipids

The Phospholipids were estimated by the method described by Zilversmith and Davis (1950).

Reagents used

- Concentrated sulphuric acid (Conc. H₂SO₄)
- Concentrated Nitric acid (Conc. HNO₃)
- Ammonium molybdate in 2.5% Sulphuric acid.
- 1-amino 2-naphthal 4-sulphonilic acid (ANSA): 100 mg ANSA was dissolved in a mixture of 195 mL 15% bisulphate and 5.0 mL 20% sodium sulphite solution.
- Tricholoro acetic acid (TCA).

Procedure

0.1 mL of serum samples from the infected and control fish were mixed with 1.9 mL of distilled water and 1.5 mL of 10% TCA individually. The precipitated proteins were sedimented by centrifugation, the supernatant was discarded. 1.0 mL of each Conc. HNO₃ and Conc. H₂SO₄ were added to the residue and digested on a sand bath till the solution became colourless. After cooling, the volume was made up to 5 mL with water. Then 1mL of ammonium molybdate solution followed by 0.4 mL of ANSA reagent was added. The absorbance was read at 680 nm after 5 min.

5.2.5.1.9. Estimation of free fatty acids

Fatty acids were determined according to the method of Cox Pearson (1962).

Reagents used:

- 1% phenolphthalein: 1 g in 100 mL of distilled water.
- 1N Potassium hydroxide: 400 mg in 100 mL of distilled water.
- Neutral solvent: Mixed 25 mL of ether, 25 mL of 95 % alcohol and 1 mL of 1% phenolphthalein solution.
- NaCl 0.9%: 900 mg in 100 mL distilled water.
Procedure

50 µL of serum samples from infected and control fish were dissolved in 2 mL of 1% alcoholic potassium hydroxide and heated on a steam bath for 15 min in separate tubes. Then 3 mL of distilled water was added to each tubes and the mixture was acidified with 2 to 3 drops of 6N HCl using Congo red. Following this, 15 mL petroleum ether was added to this acidified solution. The acidified petroleum ether was decanted in a small 50 mL separating flask and washed with 5 mL of distilled water. The contents were shaken vigorously and the lower layer was discarded. The washing was repeated twice with 5mL-distilled water and each time the lower layer was discarded. The petroleum ether was evaporated to dryness at 6-65 °C and the residue in each was dissolved in 2 mL of 95% ethanol and titrated against blank (2 mL of 95% ethanol) with N/50 KOH using 0.1% phenolphthalein (in alcohol) as an indicator. 1 mL micro burette was used for the titrations.

5.2.5.1.9. Estimation of serum lysozyme

The turbidimetric assay for lysozyme activity was carried out according to Hutchinson and Manning (1996), in combination with microplate adaptation of Christybapita et al. (2007) methods.

0.03% lyophilized, Micrococcus luteus in 0.05 mM sodium phosphate buffer (pH 6.2) was used as substrate.

Procedure

10 µL of different hours infected and controlled fish serum were added to 250 µL of bacteria l suspension in a ‘‘U’’ bottom microtitre plate and the reduction in absorbance at 490 nm was determined after 0.5 and 4.5 min incubation at 22 °C using a microplate reader (Microplate H4 synergy). One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 per min. Lyophilized hen egg white lysozyme, HEWL (Sigma), was used to develop a standard curve.

5.2.6. Estimation of Hematological parameters

5.2.6.1. Hemoglobin level

The hemoglobin content of the blood was analysed by cyanmethaemoglobin method using Drabkins fluid (Azizoglu and Cengizler, 1996).
Procedure:

20 µL of blood was transferred with the help of hemoglobin pipette into a test tube containing 5 mL of Drabkin’s solution. Drabkin’s diluent was used as a blank. The absorbance was measured by using a spectrophotometer (UV-vis Schimadzu 1600 Spectrophotometer) at 540 nm and the final concentration was calculated by comparing with the standard cyanmethaemoglobin (Qualigens Diagnostics). The hemoglobin concentration was calculated by using the formula:

\[ \text{Hb (g/dL) = 60 [OD (T) / OD (S)]} \times 251/1000 \]

Where, OD (T) = absorbance of test; OD (S) = absorbance of standard

5.2.6.2. Total RBC and WBC count

RBC and WBC diluting fluids were used for total erythrocytes count and total leukocyte counts. 20 µL of blood with 3.98 mL of appropriate diluting fluid in a clean test tubes were shaken well to suspend the cells uniformly in the solution. A small drop of this mixture were transferred to Neubauer’s counting chambers of haemocytometer and the cells were counted (Larsen and Snieszko, 1961).

No. of cells (cu. mm⁻¹) = (No. of cells counted × dilution) / (Area counted × depth of fluid)

5.2.6.3. Packed Cell Volume (PCV)

The packed cell volume (PCV) is the proportion of blood volume that is occupied by red blood cells. It is considered to be an integral part of a person's complete blood count results. The procedure was performed by following the methods of Snieszko (1960) and Sarder et al. (2001).

Procedure

Heparinized blood in a capillary tube was centrifuged at 10,000 xg for 5 min to separate blood as layers. The volume of packed red blood cells, divided by the total volume of the blood sample gave the PCV.

Formula

\[ \text{PCV = Packed red blood cells / Total volume of the blood} \]
5.2.6.4. Mean Corpuscular Volume (MCV)

The hematological MCV was calculated according to Seiverd (1964).

Calculation:

It was calculated by dividing the PCV by the red blood cell count. The result is being typically reported in femtolitres/ cell.

Formula:

\[ \text{MCV} = \frac{\text{PCV}}{\text{RBC}} \]

5.2.6.5. Mean Corpuscular Hemoglobin (MCH)

The mean corpuscular hemoglobin (MCH) is the average mass of hemoglobin per red blood cell in a sample of blood. It is reported as a part of standard complete blood count.

Calculation

It was calculated by dividing the total mass of hemoglobin by the number of red blood cells in a volume of blood. The result is being typically reported in picograms/cell

Formula:

\[ \text{MCH} = \frac{\text{Hb}}{\text{RBC}} \]

5.2.6.6. Mean Corpuscular Hemoglobin Concentration (MCHC)

The mean corpuscular hemoglobin concentration (MCHC), is a measure of concentration of hemoglobin in a given volume of packed red blood cells. It is being reported as a part of standard complete blood count.

Calculation:

It was calculated by dividing the hemoglobin by the hematocrit.

Formula:

\[ \text{MCHC} = \frac{\text{Hb}}{\text{PCV}} \]
5.3. Results

Experimental pathogenicity of P. vermicola by immersion, intramuscular and oral methods in healthy L. rohita

The immersion method administration of P. vermicola (30 x 10^6 CFU mL\(^{-1}\)) caused 30, 50, 60 and 70% of mortalities at 24, 48, 72 and 96 h of exposure, respectively, and the LC\(_{50}\) value of Providencia vermicola was found to be 1.687 x 10^6 CFU mL\(^{-1}\), at 96 h of post infection (p.i.) (Table-5).

Table 5. Cumulative percentage mortality of L. rohita exposed to different concentrations of P. vermicola by immersion challenge at different time intervals.

<table>
<thead>
<tr>
<th>No. of bacterial cells / ml of rearing medium</th>
<th>Accumulated % mortality at different time intervals (h p.i.)</th>
<th>LC(_{50}) value for 96 h (LCL-UCL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24    48    72    96</td>
<td>0.0±0.00  0.0±0.00  0.0±0.00  0.0±0.00</td>
</tr>
<tr>
<td>30 x 10^3</td>
<td>0.0±0.00  10±0.57  30±0.57  30±0.57</td>
<td>1.687×10^6</td>
</tr>
<tr>
<td>30 x 10^4</td>
<td>10±0.57  20±0.57  30±0.57  40±0.57</td>
<td>(1.017×10^5 - 2.797×10^7)</td>
</tr>
<tr>
<td>30 x 10^5</td>
<td>20±0.57  30±0.57  30±0.57  50±0.57</td>
<td></td>
</tr>
<tr>
<td>30 x 10^6</td>
<td>30±0.57  50±0.57  60±0.57  70±0.57</td>
<td></td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SD.

The intramuscular injection of the maximum concentrations of 52 x 10^6 feasible cells of Providencia vermicola caused 100% death at 96 h. But the application of lowest concentrations of 52 x 10^3 and 52 x 10^4 feasible study-bacterial cells per animal caused 40 and 60% caused death at 96 h of booster. The LC\(_{50}\) value of Providencia sp. like bacterium for intramuscular course was defined at 96 h of booster and was found to be 9.48 x 10^4. There was no mortality in the oral route administered L. rohita fish. The clinical signs of ulcerative lesions were observed in experimentally infected rohu. The signs observed in the experimentally infected rohu were lethargy and rude smell at the time of mortality (Table-6).
Table 6. Cumulative percentage mortality of L. rohita exposed to different concentrations of P. vermicola by intramuscular challenge at different time intervals.

<table>
<thead>
<tr>
<th>No. of bacterial cells /animal</th>
<th>Accumulated % mortality at different time intervals (h p.i.)</th>
<th>LC50 value for 96 h (LCL-UCL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>Control</td>
<td>0.0±0.00</td>
<td>0.0±0.00</td>
</tr>
<tr>
<td>52 x 10³</td>
<td>0.0±0.00</td>
<td>10±0.57</td>
</tr>
<tr>
<td>52 x 10⁴</td>
<td>10±0.57</td>
<td>30±0.57</td>
</tr>
<tr>
<td>52 x 10⁵</td>
<td>20±0.57</td>
<td>40±0.57</td>
</tr>
<tr>
<td>52 x 10⁶</td>
<td>20±0.57</td>
<td>40±0.57</td>
</tr>
</tbody>
</table>

The values are expressed as mean ± SD

Koch’s postulates

The infectivity of P. vermicola was confirmed by satisfying Koch’s postulates. P. vermicola was isolated from moribund and dead animals subjected to experiments. The samples were isolated from the liver, gill and ulcerative skin of experimentally infected rohu fish. The characters of these reisolates resembled the original isolates isolated from naturally infected rohu fish, which were confirmed by procedures described earlier.

Histopathological studies on experimentally infected fishes

Histopathological investigation was carried out on experimentally infected rohu fish to study the structural changes in different organs such as gill, liver and skin tissues. The histological results of 96 h infected fish samples at concentrations of 30 x 10⁶ and 52 x 10⁶ of intramuscular and immersions methods respectively were presented (Plates 6 & 7).
Plate 6. Gill, liver and skin histology of L. rohita in control (A, C, E) and intramuscular exposed organs after 48 h (B, D, F). A: Micrograph of gill tissue from control showing the thickness of primary lamellar epithelium, appearance of secondary lamellar epithelium, normal presence of central vein. B: Gill of exposed fish showing epithelial lifting, necrosis and desquamation. C: Control group showing hepatocytes with central nucleus, converging sinusoids RBC and vacuolation. D: Liver of exposed fish showing hepatic necrosis, irregular vacuolation and jamming of central vein. E: Control group of skin showing mucus gland with architectural structures. F: Skin of exposed fish showing the disrupted mucus gland and architecture.
Plate 7. Gill, liver and skin histology of L. rohita in control (A, C, E) and immersion exposed organs after 48 h (B, D, F). A: Micrograph of gill tissue from control showing the thickness of primary lamellar epithelium, thin and squamous appearance of secondary lamellar epithelium, normal appearance of central vein. B: Gill of exposed fish showing epithelial lifting, necrosis, cartilaginous disruption and desquamation. C: Control group showing central vein in hepatocytes with central nucleus. D: Liver of exposed fish showing hepatic necrosis, irregular vacuolation and converging blood sinusoids. E: Control group of skin showing mucus gland with architectural structures. F: Skin of exposed fish showing the disrupted mucus gland and shrinked architecture.
Gill

The gill of L. rohita is normally composed of primary lamellae arranged in rows, bulged on the lateral sides of which there are alternately arranged secondary lamellae with periodic distributions as pillars. Mainly there was cartilaginous cores with traces of sinusoidal blood spaces and these structures are evenly distributed. The gill epithelium was thin with a large surface area to facilitate a high level of exposure of gill capillaries to water (Plates 6A & 7A). The experimentally infected fish showed epithelial hyperplasia, lamellar fusion (shrink), epithelial lifting, epithelial necrosis and desquamation. And the cartilaginous rod at the core of primary lamella was found to be disrupted. Over all, in experimentally infected rohu, severe gill damage and destruction of secondary lamellae were observed (Plates 6B & 7B).

Liver

Liver is a relatively large organ. It is composed of branching and anastomosing, two cell thick laminae or cords of hepatocytes. The hepatic parenchymatous appearance was observed with homogenous cytoplasm, increased cytoplasmatic vacuolation, blood sinuoids, central nucleus, converging sinuoids and RBC (Plates 6C & 7C). In experimental fish, hepatic necrosis, irregular cytoplasmic vacuolation and jamming of central vein were observed with converging sinuoids (Plates 6D & 7D).

Skin

The skin is composed of two layers, the epidermis and the dermis. A filament containing malpighian cell is the primary parenchymal cell of fish skin. Mucus-secreting cells are found in the epidermis of fish. L. rohita showed the presence of mucus gland with architectural structures in both experimental rooted animals (Plates 6E & 7E). In the experimental fish skin, the mucus gland and epidermis were disrupted and ruptured very much (Plates 6F & 7F).

Biochemical and hematological changes in P.vermicola injected L. rohita

Biochemical changes:

The biochemical components such as total protein, albumin, globulin, free amino acids, glucose, cholesterol, triglycerides, phospholipids, free fatty acids and lysozyme levels were determined in both experimentally intramuscular injected and control L. rohita blood samples. There was a significant increase in total protein, albumin, free amino acids, glucose, total cholesterol, triglycerides, phospholipids,
free fatty acids and lysozyme levels in experimentally P. vermicola injected L. rohita when compared to normal fish (Table-7 & Fig. 2).

The total protein in control fish was found to be 3.27±0.02 g/dL whereas in P.vermicola injected rohu it was found to be 4.96±0.02 g/dL at 24 h and 3.63±0.03 g/dL at 96 h p.i.

The albumin level in control fish was found to be 2.13±0.05 g/dL whereas in P.vermicola injected rohu it was found to be 3.29±0.01 g/dL at 24 h and 2.93±0.01 g/dL at 96 h p.i.

The globulin level in control fish was found to be 1.08±0.01 g/dL whereas in P.vermicola injected rohu it was found to be 1.78±0.01 g/dL at 24 h and 0.73±0.01 g/dL at 96 h p.i.

The free amino acids in control fish were found to be 3.06±0.0 mg/dL whereas in P.vermicola injected rohu it was found to be 5.2±0.15 mg/dL at 24 h and 4.54±0.01 mg/dL at 96 h p.i.

The glucose level in control fish was found to be 59.37±0.01 mg/dL whereas in P.vermicola injected rohu it was found to be 96.8±0.05 mg/dL at 24 h and 97.7±0.01 mg/dL at 96 h p.i.

The cholesterol level in control fish was found to be 3.26±0.01 mg/dL whereas in P.vermicola injected rohu it was found to be 5.61±0.01 mg/dL at 24 h and 5.37±0.01 mg/dL at 96 h p.i.

The triglycerides level in control fish was found to be 3.11±0.01 mg/dL whereas in P.vermicola injected rohu it was found to be 4.46±0.01 mg/dL at 24 h and 4.11±0.01 mg/dL at 96 h p.i.

The phospholipid level in control fish was found to be 0.59±0.01 mg/dL whereas in P.vermicola injected rohu it was found to be 0.91±0.01 mg/dL at 24 h and 0.77±0.01 mg/dL at 96 h p.i.

The free fatty acids in control fish was found to be 0.40±0.01 mg/dL whereas in P.vermicola injected rohu it was found to be 2.89±0.01 mg/dL at 24 h and 3.13±0.01 mg/dL at 96 h p.i.

The lysozyme level in control fish was found to be 2.29±0.01 units/mL whereas in P.vermicola injected rohu it was found to be 5.10±0.01 units/mL at 24 h and 4.0±0.01 units/mL at 96 h p.i.
Table 7. Biochemical changes in experimentally (P. vermicola) infected (intramuscular) L. rohita at different time intervals.

<table>
<thead>
<tr>
<th>Biochemical Parameter</th>
<th>Control</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (g/ dL)</td>
<td>3.27±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.96±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.31±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.69±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.63±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Albumin (g/ dL)</td>
<td>2.13±0.05&lt;sup&gt;e&lt;/sup&gt;</td>
<td>3.29±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.16±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.00±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.93±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Globulin (g/ dL)</td>
<td>1.08±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.78±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.32±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.55±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.73±0.01&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amino acids (mg/ dL)</td>
<td>3.06±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>5.23±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.94±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.54±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>59.37±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>96.8±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>98±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.99±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.7±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cholesterol (mg/ dL)</td>
<td>3.26±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.61±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.94±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.83±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.37±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triglycerides (mg/ dL)</td>
<td>3.11±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.46±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.93±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.13±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.11±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phospholipids (mg/ dL)</td>
<td>0.59±0.01&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.91±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.01±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.83±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.77±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Free fatty acids (mg/ dL)</td>
<td>0.40±0.01&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.89±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.57±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.21±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.13±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lysozyme (Units/ mL)</td>
<td>2.99±0.01&lt;sup&gt;e&lt;/sup&gt;</td>
<td>5.10±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.63±0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.89±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.0±0.01&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values represented are the mean±SD of 10 fish/group (assayed in triplicate). Mean values bearing same superscript among the groups are not statistically significant, (P < 0.05)
Fig. 2. Biochemical changes in experimentally (P. vermicola) infected (intramuscular) L. rohita at different time intervals. The values represented are the mean ±SD of 10 fish/group (assayed in triplicate). Mean values bearing same superscript among the groups are not statistically significant, (P < 0.05).
Hematological changes:

Hematological parameters such as hemoglobin, RBC, WBC, PCV, MCV, MCH and MCHC were determined in both experimentally intramuscular injected and control L. rohita blood samples. There was a significant increase in WBC, MCH and MCHC levels (Table-8 & Fig. 3).

The hemoglobin level in control fish was found to be 61±1.0 g/dL whereas in P.vermicola injected rohu it was found to be 61.21±0.26 g/dL at 24 h and 57±0.17 g/dL at 96 h p.i.

The RBC count in control fish was found to be 1.26±0.03 (x 10^6 cells mm^-3) whereas in P.vermicola injected rohu it was found to be 1.39±0.01 (x 10^6 cells mm^-3) at 24 h and 1.01±0.03 (x 10^6 cells mm^-3) at 96 h p.i.

The WBC count in control fish was found to be 586±5.56 (x 10^4 cells mm^-3) whereas in P.vermicola injected rohu it was found to be 598±1.15 (x 10^4 cells mm^-3) at 24 h and 609±2.0 (x 10^4 cells mm^-3) at 96 h p.i.

The PCV level in control fish was found to be 0.21±0.01% whereas in P.vermicola injected rohu it was found to be 0.24±0.01% at 24 h and 0.16±0.02% at 96 h p.i.

The MCV level in control fish was found to be 160±1.73 (fL cell^-1) whereas in P.vermicola injected rohu it was found to be 151.33±2.30 (fL cell^-1) at 24 h and 151.33±0.57 (fL cell^-1) at 96 h p.i.

The MCH level in control fish was found to be 56.46±0.14 (pg cell^-1) whereas in P.vermicola injected rohu it was found to be 44±0.09 (pg cell^-1) at 24 h and 56.74±0.23 (pg cell^-1) at 96 h p.i.

The MCHC level in control fish was found to be 290±12.28 g/dL whereas in P.vermicola injected rohu it was found to be 255.33±4.04 g/dL at 24 h and 382.33±6.65 g/dL at 96 h p.i.
Table 8. Hematological changes in experimentally (P. vermicola) infected (intramuscular) L. rohita at different time intervals.

<table>
<thead>
<tr>
<th>Biochemical Parameters</th>
<th>Control</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/ dL)</td>
<td>61±1.0 a</td>
<td>61.21±0.26 a</td>
<td>60.13±0.09 b</td>
<td>58.13±0.08 c</td>
<td>57.31±0.17 c</td>
</tr>
<tr>
<td>RBC (x 10^6 cells mm^3)</td>
<td>1.26±0.03 b</td>
<td>1.39±0.01 a</td>
<td>1.21±0.02 b</td>
<td>1.13±0.02 c</td>
<td>1.01±0.03 d</td>
</tr>
<tr>
<td>WBC (x 10^4 cells mm^3)</td>
<td>586±5.56 c</td>
<td>599±1.14 d</td>
<td>608±2.08 b</td>
<td>614±1.0 a</td>
<td>609±2 ab</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>0.21±0.01 b</td>
<td>0.24±0.01 a</td>
<td>0.20±0.01 b</td>
<td>0.18±0.01b c</td>
<td>0.16±0.02 c</td>
</tr>
<tr>
<td>MCV (fLcell-1 )</td>
<td>160±1.73 b</td>
<td>151.33±2.30 c</td>
<td>165±3.60 a</td>
<td>150.33±13.33 c</td>
<td>151.33±13.33 c</td>
</tr>
<tr>
<td>MCH (pg cell^-1)</td>
<td>56.46±0.14 a</td>
<td>44.03±0.09 d</td>
<td>49.66±0.37 c</td>
<td>51.44±10 b</td>
<td>56.74±0.23 a</td>
</tr>
<tr>
<td>MCHC (g/ dL)</td>
<td>290±12.28 c</td>
<td>255.33±4.04 d</td>
<td>364±5.56 b</td>
<td>387±6.24 a</td>
<td>382.33±6.65 a</td>
</tr>
</tbody>
</table>

The values represented are the mean±SD of 10 fish/group (assayed in triplicate). Mean values bearing same superscript among the groups are not statistically significant, (P < 0.05)
Fig. 3. Hematological changes in experimentally (P. vermicola) infected (intramuscular) L.rohita at different time intervals. The values represented are the mean ± SD of 10 fish/group (assayed in triplicate). Mean values bearing same superscript among the groups are not statistically significant, (P < 0.05).
5.4. Discussion

Intramuscular, immersion and oral administration were the three methods followed in the infectivity experiments to evaluate the pathogenic nature of the inoculum prepared from infected rohu in the present study. Mortality was noted in rohu injected intramuscularly and by immersion challenge. No mortality was observed through oral route. A similar method was followed by Angka (1990) who reported that Aeromonas hydrophila injected intraperitonially into Clarius batrachus fingerlings became pathogenic and caused 93% mortality in fish infected with bacteria containing 10 CFU/ mL with peak mortalities occurred on days 14 and 15.

From the results of our pathogenicity experiments, it may be concluded that the main portal of entry of the pathogen is by means of penetration of tissue at the site of lesions or wounds. The mortality data in the pathogenicity experiments showed that the pathogenicity of the present isolate depends on the dosage and period of exposure.

A wide range of putative virulence factors have been detected and studied in several Aeromonas spp. (Kingombe et al., 1999; Albert et al., 2000; Gonzalez-Serrano et al., 2002; Sechi et al., 2003) where they play a pivotal role in the establishment of infection. Indeed, several studies have reported the detection and characterization of virulence factors in Aeromonas spp. isolated from tilapia fish, humans, meat-producing animals and potable water (Granum et al., 1998; Gonzalez-Serrano et al., 2002; Escarpulli et al., 2003).

The gill of experimentally infected fish showed epithelial hyperplasia, lamellar fusion (shrinking), epithelial lifting, epithelial necrosis and desquamation. And the cartilaginous rod at the core of primary lamella was found to be disrupted as reported earlier by various workers (Karlsson-Norrgren et al., 1985&1986; Hemalatha and Banerjee, 1997a & b; Devi and Banerjee 2003; Banerjee and Chandra 2005). Deformity of the lamellar elements, haemorrhage due to necrosis, and sloughing of the respiratory epithelia followed by hyperplasia and fusion of the neighbouring secondary lamellae with consequent decreased xenobiotic diffusion distance have also been reported. Ramaswamy et al. (2009) reported that in gold fish (Carassius auratus) intramuscularly infected with Aeromonas hydrophila showed by
day 36 after injection, the disease resulted in degeneration of lamellar epithelial cells and formation of hemorrhagic necrosis.

In our experimental fish, hepatic necrosis and irregular cytoplasmic vacuolation were seen with converging sinusoids. The organ most associated with the detoxification and biotransformation process is the liver and due to its function, position and blood supply it is also one of the organs most affected by contaminants in water (Camargo and Martinez, 2007). The liver also exhibited considerable damages like enlargement of sinusoids, vacuolation and necrosis of hepatic cells. These damages probably diminish the detoxification function of the liver (Solem et al., 2003). Marina et al. (2007) have reported focal necrosis and cellular rupture in Prochilodus lineatus exposed to various pollutants in a disturbed urban stream. Olurin et al. (2006) have also reported focal necrosis and the presence of large vacuoles within the cytoplasm in Clarius gariepinus fingerlings exposed to the herbicide glyphosate. Angka (1990) reported that Clarias batrachus infected intraperitoneally with Aeromonas hydrophila showed focal necrosis in liver.

The observed marked degeneration of the gill and other organs in 48 h infected fish would be prone to other complications. As the gill of fish are responsible for respiratory and osmoregulatory processes, oxygen uptake and disruption of osmoregulatory function might have occurred. Further, toxicity induced liver-damage might prevent its detoxification role, as opined earlier by Figueiredo-Fernandes et al. (2007) and Mishra and Mohanty (2008). Importantly, the infection caused skin damages were largely facilitating the microbial invasion and further colonization (Mac Law, 2001).

During the experimental infectivity tests, the fish were seen to exhibit several behavioral responses, such as fast jerking, frequently jumping, erratic swimming, spiraling, convulsions and tendency to escape from the aquaria. Following this state of hyper excitability, the fish became inactive and loss of orientation. There was loss of equilibrium and paralysis which ultimately resulted in death of the fish as reported by previous workers (Chattopahay et al., 2006; Thenmozhi et al., 2011).
Analyses of biochemical and hematological changes were carried out in normal and P.vermicola injected L. rohita. There was significant increase in total protein, albumin, free amino acids, glucose, total cholesterol, triglycerides, phospholipids, free fatty acids and lysozyme levels. The changes in biochemical parameters such as carbohydrates, proteins and lipids are important indication of the susceptibility of organ systems to pollutants by altering their function as indicated by Verma and Tonk (1983).

Our results indicate time and dose dependent decrease in PCV and Hb throughout the duration of exposure. There was also a significant decrease in RBC recorded from 48-96h post infection. The decrease in these haematological variables may be due to hemolysis of red blood cells, haemodilution resulting from impaired osmoregulation across the gill epithelium or due to an appreciable decline in hematopoiesis. A similar such reduction in these haematological parameters was reported in different fish species exposed to different concentrations of diazinon (Banaee et al. 2008 & 2011; Ramesh and Saravanam 2008; Yekeen and Fawole, 2011; Mohammad et al., 2012). Contrary to our results, Ahmad (2011) reported a significant increase in RBC, Hb and PCV in the common carp specimens exposed to the sublethal concentration of diazinon, relating it to hyperactivity in reaction to the pesticide induced physiological stress. Total serum protein also increased due to the destruction of RBCs and the resultant release of cell contents into the blood stream (Haney et al., 1992). Contrary to our results, some earlier researchers (Köprücü et al., 2006; Ahmad 2011 & 2012) have reported a decrease in WBC, MCH and MCHC in different fish species exposed to pesticides of various concentrations (Mohammad et al., 2012). The analysis of haematological profiles of blood is widely used for the assessment of the toxic, functional status of animal health and the internal environment of organisms (Saravanan et al., 2012). In addition, blood is a pathophysiological reflector of the body because it is highly susceptible to internal and external fluctuations (Tilak et al., 2007). Thus physiological changes in blood indicate changes in the quality of the environment. Blood parameters are, therefore, important in diagnosing the functional status of the fish exposed to toxicants.
At 96 h experimental infectivity the intramuscular injected fish samples showed marked reduction in the total protein level which was due to that proteolysis was intended to increase the role of proteins in the energy production during P.vermicola induced stress on the L.rohita. Similar findings were earlier reported by De Smet and Blust (2001).

During stressful situation, higher blood glucose level is being maintained in fish through breakdown glycogen from liver, mainly through glycogenolysis (Vijayan et al., 1997). The glucose level was thus increased in the infected or stressed animals (Yoganandan et al., 2003). It was also reported that the glucose level elevated in stress condition (David et al., 2005). Injection of P. vermicola was a stress indicator, which was indirectly confirmed by the glucose level during that period and the entire metabolic pathway produces a burst of energy to prepare the fish for an emergency situation (Rottmann et al., 1992). Hall and Van Ham (1998) reported a significant elevated level of blood glucose in P. monodon in stress condition.

Enzyme regulates the addition of glucose to proteins by glycosylation, which is often essential for the body function. Globulins like gamma globulin are essential for maintaining a healthy immune system. Increased level of serum protein and albumin levels were thought to be associated with a stronger innate response in fish (Wiegertjes et al., 1993). The fatty acid level increased in hepatopancreas and decreased in hemolymph and muscle (Bowser et al., 1981).

Lysozyme hydrolyzes β-1-4-glucosidic linkages between N-acetyl muramic acid and N-acetyl-D glucosamine residues are present in the mucopolysaccharide cell wall of a variety of bacterial pathogens. It is an important fish defense factor, which prevents biofilm formation by adherence and colonization of microorganisms by stimulating the complement system and phagocytes (Alexander and Ingram, 1992; Magnadottir, 2006).

Similarly, researchers have assessed effects of pesticides on the behavior, biochemical and haematological responses of various fish species (Köprücü et al. 2006; Banaee et al., 2008 & 2011; Ahmad, 2011; Mohammad et al., 2012) and have recorded different responses of the fish exposed to varying concentrations of the pesticide.
Waagbo et al. (1988) analysed the hematological and biochemical parameters in the Atlantic salmon suffering from coldwater vibriosis (Hitra Disease). They reported that the level of protein, albumin, triglycerides and cholesterol were all significantly reduced in infected fish compared to healthy fish which are in agreement with our findings at 96 h p.i. Rehulka (1998) reported that Aeromonas induced ulcerous dermatitis in rainbow trout, O. mykiss resulted in an increase in total protein and cholesterol level in the plasma as observed in the present study.