Chapter II

MATERIALS AND METHODS
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The present investigation dealing with bacterial and parasitic diseases of carps from culture ponds of Kolleru Lake has been undertaken for a period of one year from July 2010 - June 2011. Experimental studies were designed to determine the virulence and pathogenicity of three fish bacterial pathogens i.e. *A. hydrophila*, *P. fluorescense* and *F. branchiophilum*, involved in causing two important bacterial diseases of carps i.e. Haemorrhagic Septicaemia and Bacterial Gill Disease. Observations were made on the dose dependent effect of these pathogens on haemato-biochemical responses of *L. rohita* and *C. catla*.

Study Area: Kolleru Lake (Plates- I&II; Fig.1)

The study area was divided into two regions for convenience to undertake field trips to culture ponds, Krishna Kolleru Lake region (KKLR) falling under Krishna district and Godavari Kolleru Lake region (GKLR) falling under West Godavari district of Andhra Pradesh. The names of the mandalas in both the districts where fish culture is practiced on a large scale and also mandalas from where ponds were selected for collecting fish samples for the present study are given in Table- 3 and marked in Plate- II; Fig.1.

The Kolleru Lake, a wetland eco-system of international repute, is the largest freshwater Lake in India. The Lake, covering an area of 954 sq. kms. is situated between West Godavari and Krishna districts of Andhra Pradesh. The Lake include parts of Eluru, Bhimadolu, Akiveedu, Denduluru, Unguturu, Pedapadu, Nidamarru mandals of West Godavari and parts of Kaikaluru and Mandavalli mandals of Krishna district (Mittal, 1993).
Plate - I

Geographical location and topography of Kolleru Lake in Andhra Pradesh, India

Fig.1: Geographical location and topography of the Kolleru Lake
Fig.1: Culture ponds of Indian major carps located in different Mandals of Kolleru Lake, Andhra Pradesh, India.
Water depth in the Lake varies between 1–1.8 m for most period of the year. However, it reaches 3–4 m during heavy floods. The Lake receives water from the catchment area comprising 3,405 km² upland and 1,360 km² of delta. Freshwater enters the Lake through four rivulets viz. Budameru, Thammileru, Ramuleru and Gunderu, besides this 30 major irrigation canals and agricultural drains flow into the Lake. The Lake empties into the Bay of Bengal through a 63km² long creek, called Upputeru.

Table- 3: Names of the mandala’s in the Kolleru Lake area from where culture ponds were selected for collecting fish samples

<table>
<thead>
<tr>
<th>KRISHNA PART OF KOLLERU, (KKLR)</th>
<th>WEST GODAVARI PART OF KOLLERU, (WGKLR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MANDALS WHERE FISH IS CULTURED (KKLR)</td>
<td>MANDALS WHERE FISH IS CULTURED (WGKLR)</td>
</tr>
<tr>
<td>KAIKALURU MANDAVALLI</td>
<td>KAIKALURU</td>
</tr>
</tbody>
</table>

Traditionally, Kolleru Lake has been a rich source of wild fisheries, showing an average capture fisheries production of about 7000 mts. It was noted that during normal environmental conditions of the year, the production contributed by fish other than carps was about 50%, whereas that by prawns and carps was 30% and 10% respectively (Venkateswara Rao et al., 2003) (Plate- III; Figs.1-4).
Study Area – Kolleru Lake

Figs.1&2: Kolleru Lake

Figs.3&4: Netting of Fish
Species cultured

Initially, fish farmers of Andhra Pradesh adopted the basic composite fish culture techniques by stocking all the six recommended species viz. *L. rohita* (25-30%), *C. catla* (10-15%), *C. mrigala* (15-20%), *H. molitrix* (20-30%), *C. idella* (5-10%) and *C. carpio* (10-20%) (Table- 4). But later, based on the practical experience gained over the years, appropriate changes were made in the fish production practices as per the demand by market preferences, to maximize fish production and profit.

The three Chinese major carps, *C. carpio, C. idella* and *H. molitrix* were almost eliminated from the system because of the problems specific to each of these species. *C. mrigala* has also been gradually excluded due to difficulties in harvesting this bottom dwelling fish. With the total exclusion of the three exotic carps and also *C. mrigala*, by most fish farmers, the culture has now been restricted to only two species, *L. rohita* and *C. catla* (Plate- IV; Figs.1-4).

Table- 4: Species of carps cultured commonly in the Kolleru lake region

<table>
<thead>
<tr>
<th>Name of the Host</th>
<th>Author</th>
<th>Common Name</th>
<th>Taxonomic Status</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Labeo rohita</em></td>
<td>Hamilton, 1822</td>
<td>Rohu</td>
<td>Fam: Cyprinidae Sub-fam: Cyprininae</td>
</tr>
<tr>
<td><em>Catla catla</em></td>
<td>Hamilton, 1822</td>
<td>Catla</td>
<td>Fam: Cyprinidae Sub-fam: Barbinae</td>
</tr>
<tr>
<td><em>Cirrhinus mrigala</em></td>
<td>Hamilton, 1822</td>
<td>Mrigal</td>
<td>Fam: Cyprinidae Sub-fam: Cyprininae</td>
</tr>
<tr>
<td><em>Cyprinus carpio</em></td>
<td>Linnaeus, 1758</td>
<td>Common carp</td>
<td>Fam: Cyprinidae Sub-fam: Cyprininae</td>
</tr>
<tr>
<td><em>Ctenopharyngodon idella</em></td>
<td>Valenciennes, 1844</td>
<td>Grass carp</td>
<td>Fam: Cyprinidae Sub-fam: Leuciscinae</td>
</tr>
<tr>
<td><em>Hypophthalmichthys molitrix</em></td>
<td>Valenciennes, 1844</td>
<td>Silver carp</td>
<td>Fam: Cyprinidae Sub-fam: Leuciscinae</td>
</tr>
</tbody>
</table>
Fig. 1: Fish hauling

Fig. 2: Fish harvesting

Fig. 3: Fish sorting

Fig. 4: Fish before packing
**Biology of two species of Indian major carps**

The Indian major carps *L. rohita* and *C. catla* are two most important species of fish cultured widely and also in culture ponds of Kolleru Lake region situated in Krishna and Godavari districts of Andhra Pradesh.

*Labeo rohita*: (Hamilton, 1822) (Plate- V, Figs.1&2)

It is popularly known as Rohu. It is a natural inhabitant of the riverine system of Northern and Central India, and the rivers of Pakistan, Bangladesh and Myanmar. In India, it has been transplanted into almost all riverine systems including freshwaters of Andaman, where its population has successfully been established. It has also been introduced into many other countries like Sri Lanka, the former USSR, Japan, China, Philippines, Malaysia, Nepal and some parts of Africa (FAO sheets, 2006).

In its early life stages, rohu prefers zooplankton, mainly composed of rotifers and cladocerans, with phytoplankton forming the emergency food. In the fingerling stage there is a strong positive selection for all zooplanktonic organisms and for some smaller phytoplanktons, whereas as adults, they mostly prefer phytoplankton. In the juvenile and adult stages, rohu is essentially herbivorous column feeder, preferring algae and submerged vegetation. It is a fast growing species and attains about 35–45 cm in total length and 700 – 800 gms. in one year under normal culture conditions.
Fig.1: *Labeo rohita* (rohu)

Fig.2: Main producer countries of *Labeo rohita* (FAO Fishery Statistics, 2006)
The minimum age at first maturity for both sexes is two years, while complete maturity is reached after four years in males and five years in females. In nature, spawning occurs in the shallow and marginal areas of flooded rivers. The spawning season of rohu generally coincides with the South-West monsoon, extending from April to September. The fecundity varies from 2,26,000 – 27,94,000 eggs, depending upon fish size and ovary weight. It is a polygamous fish and also seems to be promiscuous. The optimum temperature requirement for spawning is 22–31°C. *L. rohita* is a eurythermal species and does not thrive at temperatures below 14°C.

India is by far the largest producer of rohu, however, Bangladesh and to a lesser extent Myanmar are also major producers.

**Catla catla:** (Hamilton, 1822) (Plate VI, Figs.1&2)

It is commonly known as catla. It is endemic to the riverine system in Northern India, Indus plain and adjoining hills of Pakistan, Bangladesh, Nepal and Myanmar and has been introduced later into almost riverine systems, reservoirs and tanks all over India. The species has also been introduced into other countries like Srilanka, Israel, and Japan.

*C. catla* is a eurythermal species that grows best at water temperatures ranging from 25–32°C. The minimum temperature tolerance limit is approximately 14°C. At larval stage, these are planktophagic in nature feeding mainly on zooplanktons like rotifers and cladocerans. Adults feed only on surface and mid-waters and are planktophagous in nature, with a preference towards zooplankton, including crustaceans, rotifers, insects and protozoa, as well as a considerable share of algal and plant material.
Fig.1: *Catla catla* (catla)

Fig.2: Main producer countries of *Catla catla* (FAO Fishery Statistics, 2006)
It attains maturity in its second year, performing a spawning migration during the spawning season towards the upper stretches of rivers, where males and females congregate and breed in shallow marginal areas. The spawning season coincides with the South-West monsoon in northern India and Bangladesh, which lasts between May and August and in north India and Pakistan from June to September. Its fecundity generally varies from 10,000–2,00,000 eggs/kg body weight depending on fish length and weight. Since a riverine environment is required, natural breeding does not occur in ponds, even though the species attains maturity. Under normal conditions it grows to 1–1.2 kgs. in the first year. India is by far the largest producer of *C. catla*, followed by Bangladesh.

**Diseases encountered in *L. rohita* and *C. catla***

During the present investigation data has been collected on bacterial and parasitic diseases of carps from culture ponds of Kolleru Lake. The four bacterial diseases, commonly encountered in these fishes are identified as Haemorrhagic Septicaemia (HS), Bacterial Gill Disease (BGD), Abdominal Dropsy (AD) and Columnaris Disease (CD). Whereas the parasitic diseases responsible for causing major damage to carp species in the study area were identified as Dactylogyrosis (monogenoideans), Argulosis (copepods) and Myxosporidiosis (protozoans). These diseases were found to influence the survival and productivity of both *L. rohita* and *C. catla* cultured in the Kolleru Lake.

**Survey on fish diseases**

A survey on various parasitic and bacterial diseases of *L. rohita* and *C. catla* has been conducted by random sampling method for a period of 12 months from July 2010 to June 2011. 100 ponds covering an area of 1345 ha.
in the Krishna Kolleru basin and 135 ponds covering 1404 ha. in Godavari Kolleru basin were selected for collecting fish samples. Priority was given to ponds where mortality or any other health problem was reported in fishes. Altogether, the total number of ponds covered in the entire study area was 235, covering an area of 2749 ha. The area covered in the survey formed only 3.44% of the estimated 80,000 ha. of the land under fish culture in the Kolleru Lake.

Based on the information collected from local fish farmers, and other sources, culture ponds located at Kaikaluru (Krishna – Kolleru Zone) and Bhimadolu (Godavari – Kolleru Zone) were selected to collect monthly fish samples (Table-3). All the ponds were selected on the basis of their accessibility and feasibility to collect samples. Fish sampling was done by cast netting; usually in the morning hours between 6 am to 9 am and a minimum of 200 fish were netted each time for examination and data collection. Healthy fish without any symptoms were again dropped back into the ponds, whereas fish showing symptoms of any disease were collected and subjected to a detailed investigation. For this purpose fish samples collected from culture ponds were transported to a nearby fisheries laboratory in live or moribund condition to conduct autopsy and to take samples for microbiological studies. Data on monthly prevalence of infection, seasonal distribution and severity and type of infection were recorded. A standard data sheet was prepared to take details like total number of fish collected, number of infected and uninfected fish, type of infection, clinical signs and symptoms and data on single infections, double infections, and multiple infections. Besides, information on stocking density, pond details etc. were obtained from fish farmers.
Myxosporean cysts, monogenoidean parasites i.e. (Dactylogyrus sps.) and copepod parasites (Argulus sps. and Lernaea sps.) were examined with naked eye. Myxosporean cysts and spores, ciliates and monogenoidean gill flukes were first observed live under the microscope by preparing wet mounts of gills immediately at the pond site itself. Gills and other external surfaces including buccal cavity were thoroughly examined to the relevant data was recorded.

Identification of individual parasite species belonging to different groups was not done due to shortage of time.

Clinical Examination of Fish

Fishes were examined thoroughly for any external disease symptoms like haemorrhages on the body, loss of scales, excessive mucus secretion, and clubbing of gills and the details were recorded. For internal examination, fish were autopsied and details on the nature and condition of the internal organs were noted. Autopsy of the diseased fish was conducted according to the techniques mentioned in the Asia Diagnostic Guide to Aquatic Animal Diseases (Bondad - Reantaso et al., 2001; Hoole et al., 2001). For recording data on clinical signs and detection of parasites, thorough external and internal examination was conducted. External parts like mouth, lips, oral cavity, eye, gills, skin and scales and internal organs including body cavity, intestine, liver, spleen, gall bladder and kidneys were examined thoroughly and the data was recorded. The clinical signs of each of the diseases was photographed and presented whenever required.

Sample collection for isolation and identification of Bacteria

For isolation of bacteria from diseased fish, the infected portion of the fish body was first cleaned with a cotton swab dipped in 70% ethyl alcohol or spirit and cut with a sterile blade. The cut pieces were then immediately
placed in 2ml of nutrient broth, stored in an ice box and transported to lab. Wet mount preparations of the smears from the external lesions and internal organs i.e. kidney, liver, spleen and gill of each species were examined under the microscope for the presence of pathogens. Blood and abdominal fluid samples were also collected from diseased fishes and inoculated in nutrient broth.

**Isolation of Bacteria**

To isolate bacterial pathogens, ten fold serial dilutions were made with 24 hrs. old broth culture to avoid over growth of bacteria as suggested by Bullock (1971). The diluted samples were inoculated on nutrient agar and trypton soya agar (TSA) by spread plate or pour plate technique and incubated at 28°C for 24–48 hrs. Morphologically similar and dominant bacterial colonies were selected and streaked on nutrient agar plates for further isolation and purification. Besides, specific media like Rimler-shotts medium (RS medium) for the rapid identification of *A. hydrophila* (Shotts and Rimler, 1973), Pseudomonas agar for isolation of *P. fluorescence* and FLPA medium for selective isolation of *F. branchiophilum* (Daskalov *et al.*, 1999, Madsen *et al.*, 2005) were used to produce pure cultures. Bacterial isolates were identified according to the taxonomic keys given in Bergey's Manual of Systematic Bacteriology (Baumann and Schubert, 1984) and Cowan and Steels Manual of Medical bacteria (Barrow and Feltham, 1993). After obtaining pure cultures by repeated streaking of colonies, they were stored at room temperature (20°C– 25°C) by preparing slants of cultures and by covering them with wax to prevent moisture loss. These pure cultures were maintained in the lab to conduct a number of biochemical tests for identifying and characterizing the bacteria upto species level.
Procedures for Phenotypic Characterization of Bacteria

A number of tests were conducted to identify the bacteria. Tests such as Gram staining, motility, oxidase, catalase, acid production from glucose and OF tests were employed to identify up to generic level. A series of tests including acid production from carbohydrates like glucose, adonitol arabinose, cellobiose, fructose, inositol, lactose, maltose, mannitol, sorbitol, sucrose, and nitrate reduction, citrate utilization, urease production, starch hydrolysis, β-galactosidase, H₂S production, growth on KCN medium, decarboxylase (Arginine, Lysine and Ornithine), sensitivity to O/129, MRVP reaction, indole production and NaCl tolerance were used for the identification up to species level.

Presumptive tests

Gram staining:

Gram stain is a very useful stain for classifying bacteria in to two major groups, the gram positive and the gram negative which was developed by Dr. Hans Christian Gram, a Danish Physician in the year 1884. In this staining procedure, bacterial smear is subjected to four different reagents i.e. crystal violet (primary stain), iodine solution (mordant), alcohol (decolorizing agent) and safranin (counter stain). The bacteria which retain the primary stain (appear dark blue or violet) are called gram positive and those that are stained by safranin are referred to as gram negative.

Procedure:

1. Thin smears of bacterial isolates were prepared, air dried and fixed by gently warming the slide.

2. The bacterial smear was flooded with crystal violet solution and left for one minute.
3. The slides were washed for a few seconds and covered with gram iodine solution for 30 seconds.

4. The slides were washed for 15 seconds, decolorized with 95% ethyl alcohol incorporating 5% acetone.

5. The slides were washed again and counter stained with safranin for ten seconds.

6. The stained and dried slides were observed under oil immersion microscope.

**Motility test:**

Hanging drop preparation is useful to observe the motility of the bacteria. Young broth culture of the organism, incubated at or below the optimum growth temperature was examined in hanging drop preparation, using a high power dry objective and reduced illumination.

**Hanging drop preparation:**

1. Hanging drop slide (cavity slide) was cleaned and a little vaseline or petroleum jelly was spread around the cavity of the slide.

2. A clean cover slip was taken and vaseline was applied on each of the corners of the cover slip.

3. A drop of the culture was placed at the center of the cover slip and cavity was placed on the cover slip, with the cavity facing down so that the depression covers the suspension.

4. The slide was pressed gently so as to form a seal between the coverslip and cavity. The hang drop preparation was quickly turned right side up so that the drop is suspended. The depression slide is inverted over the cover slip in such a way that the suspension does not touch the surface of the concavity at any point.
5. The prepared slide was examined under high power microscope with reduced light.

**Catalase test:**

Many microorganisms produce catalase in order to counteract the effect of toxic hydrogen peroxide that is produced in large quantities under aerobic conditions. Catalase is an enzyme that converts hydrogen peroxide into water and oxygen. Therefore, presence or absence of catalase is used to differentiate aerobic and anaerobic forms. The bacteria that contain this enzyme are usually aerobic (need oxygen) or facultative anaerobes (can live with or without oxygen). A positive reaction is indicated by a continuous bubble formation when the catalase is introduced into bacterial colonies.

\[
\text{Catalase} \\
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

**Procedure:** A loopful of 24–48 hrs. culture grown on nutrient agar plate was taken and placed in 1% H₂O₂ on a glass slide. The slide was observed for the production of bubbles which indicates the presence of catalase.

**Oxidase activity:**

The oxidase test identifies organisms that produce the enzyme cytochrome oxidase. The oxidase reagent contains a chromogenic reducing agent that changes colour when it becomes oxidized. If the test organism produces cytochrome oxidase, the oxidase reagent will turn blue or purple within 15 seconds.

**Procedure:** A loopful of 18–24 hrs. culture grown on nutrient agar plate was taken and placed on wet oxidase disc (commercially available: Himedia) and the appearance of a dark purple colour on the disc within 30 seconds indicates a positive reaction.
OF test (Oxidation and Fermentation of Glucose):

Organisms use carbohydrate differently depending upon their enzyme complement. The pattern of fermentation is characteristic of certain species, genera or groups of organisms and for this reason this property has been extensively used as a method for biochemical differentiation of microbes. Glucose after entering a cell can be catabolized either aerobically (oxidative metabolism) or anaerobically (fermentative metabolism) or both. The metabolic products of carbohydrate fermentation can be either organic acids (eg. Lactic or formic or acetic acid) or inorganic acid and gas (eg. H₂ or CO₂). Whether an organism is oxidative or fermentative can be differentiated using Hugh and Leifson’s medium (OF medium), which contains desired carbohydrate (1% glucose), tryptone and bromothymol blue (an pH indicator).

Procedure

Preparation of OF glucose agar medium:

Peptone–2g., Sodium chloride–5g., Di-potassium hydrogen phosphate–0.4 g., Bromothymol blue solution–15 ml. Agar–3 g. and Distilled water–1000 ml.

The weighed constituents were dissolved in distilled water and bromothymol blue solution was added. The basal medium was poured into tubes and sterilized at 121°C for 15 minutes. 1 ml of sterile glucose solution (10%) was added to the molten base to produce a final concentration of 1% and the tubes were allowed to cool. Two tubes containing OF-glucose medium were incubated with the culture and in one of them, paraffin was poured over the medium to keep the air out. Two un-inoculated tubes were used as controls. All the tubes were incubated at 35°C for 24–48 hrs. and observed for the presence of bacterial growth and colour development.
Growth of the bacteria in this medium is either by utilizing the tryptone which results in an alkaline reaction (dark blue color) or by utilizing the glucose, which results in the production of acid (turning green to yellow). Those bacteria which produce acid in both closed and open tubes are described as fermentative, while those which produce acid only in the open tubes are called oxidative.

Nitrate reduction:

The bacterial culture was inoculated lightly on nitrate broth and incubated for up to 5 days. Then 1 ml of nitrite reagent-A and 12 ml of nitrite reagent-B were added to 5 ml of the medium. Development of deep red colour shows the presence of nitrate and thus reduction of nitrate, indicating a positive reaction.

Preparation of nitrate broth:

1 gm of KNO₃ dissolved in 1000 ml nutrient broth, distributed into tubes containing inverted Duhram's tubes and sterilized at 115°C for 20 minutes.

Nitrite test reagents:
Solution A: 0.8 % sulphanilic acid dissolved in 5N acetic acid by gentle heating.
Solution B: 0.5% of naphthylamine dissolved in 5N acetic acid by gentle heating.

Decarboxylase reaction or Decarboxylation of aminoacids:

Moller's decarboxylase broth base (commercially available) was prepared and divided into four aliquots. To three of the aliquots, 10 ml of 10% solution of an appropriate amino acid (L-arginine, L-lysine or
L-ornithine) was added per 100 ml of the medium. The fourth aliquot is used as a control with no amino acid being added. All the aliquots were dispensed into tubes and autoclaved at 15 lbs for 15 min. After cooling they were inoculated heavily into TSA with 2.5% NaCl and 2-3 mm thickness paraffin oil was poured on the inoculated aliquots. All the tubes were incubated for 14 days, checking daily for the reaction. Tubes of organisms producing carboxylate become purple or they first turn yellow and then will revert to purple in 48 hrs, indicating a positive reaction. Whereas tubes of organisms not producing decarboxylase turn yellow and remain so without any change, indicating a negative reaction. The controls should remain yellow.

**NaCl tolerance:** 24 hrs. culture inoculated into tryptone broth containing varying concentrations of NaCl (0% – 8%) was observed for growth within 48 hrs.

**Indole production:**

This test demonstrates the ability of certain bacteria to decompose the amino acid tryptophane to indole which accumulates in the medium. The test organism was inoculated into tryptone broth and Kovac's reagent strip was hung on the edge of the test tube containing inoculated tryptone broth. Appearance of purple colour on the Kovac's reagent strip indicates the production of indole.

**B - galactosidase:**

TSA was prepared and the test organism was streaked on to the agar. After 24 hrs. growth, ONPG differentiation disc was placed on the culture and observed for change in colour. A yellow colour change of the disc indicates the reaction or 0.2– 0.5 ml of 2.5% saline was dispensed in a small test tube and a small amount of culture material was suspended aseptically in
the saline. ONPG differentiation disc was placed in the tube and incubated for 20–30 minutes at 25–30°C and observed for colour change for 4 hrs. Appearance of yellow colour indicated positive reaction.

**Methyl Red and Voges - Proskaur test:**

The MRVP test was used to distinguish between bacteria that produce large amounts of acid and those that produce the neutral product acetone as an end product.

5 ml of MRVP broth (commercially available) poured into test tubes was sterilized by autoclaving at 15 lbs pressure for 15 minutes. The tubes were inoculated with the test organisms at 35°C for 48 hrs. along with suitable controls. Then 5 drops of methyl red indicator was added and observed for colour change. The development of yellow colour indicates negative reaction, while no change in colour indicates a positive reaction.

**VP test:**

The MRVP broth was inoculated with the bacterial isolates and 12 drops of VP reagent-I and 2–3 drops of VP reagent-II were added to the broth. The tubes were shaken gently and the reaction was allowed to complete for 15–30 minutes. The development of crimson to ruby pink (red) colour is indicative of positive reaction and no change in colouration indicates a negative test.

**Starch hydrolysis:**

The ability to degrade starch is used as the criterion for determination of amylase production by a microbe. In the laboratory it is tested by performing the starch test in which the absence or presence of starch in the medium was tested by using iodine solution as an indicator. If starch in the
presence of iodine produces a dark blue colony or blue medium it is indicative of amylolytic activity.

Starch agar medium was prepared, cooled to 45°C and poured into sterile petridishes. A single streak inoculation was made at the center of the plate and incubated for 48 hrs. at 37°C. The surface of the plate was flooded with iodine solution with a dropper for 30 seconds. A typical positive starch hydrolysis reaction is indicated by the formation of a clear zone surrounding the microbial colonies. A negative reaction is indicated by dark blue coloration of the medium.

**Urease test:**

It is performed by growing the test organisms on urea broth containing the pH indicator phenol red (pH 6–8). During incubation, microorganisms possessing urease will produce ammonia that raises the pH of the medium (to pH 8.4). The colour of phenol (indicator) changes from brownish red to deep pink. Failure of the development of a deep pink colour due to lack of ammonia production is an indication of the absence of urease production by the microorganisms.

Maslen's urea broth was prepared and inoculated heavily with bacterial culture of not more than 18 hrs. old and incubated at 37°C for 5–18 hrs. The rapid development of a red colour indicates urease activity.

**Acid production from carbohydrates:**

Phenol red broth base was prepared in test tubes and inoculated with 18–24 hrs. old bacterial culture. Different carbohydrate discs were placed in the test tubes and after incubation for 24–48 hrs. they were observed for the production of acid. A change in colour of broth to yellow is indicative of a positive reaction.
**H₂S Production:**

The test organism inoculated into a tube of TSA by stabbing the butt and streaking the slope and was observed daily for up to 7 days for blackening due to H₂S production.

**Gelatin hydrolysis:**

Plates of gelatin agar were inoculated and incubated for 3 days. The surface was flooded with 30% trichloro acetic acid. Clear zones indicate areas of gelatin hydrolysis.

**Phenylalanine deaminase test:**

This test was performed to test the ability of the organism to deaminate phenylalanine to phenyl pyruvic acid.

Phenylalanine agar was prepared and poured on slants and the bacterial culture was inoculated and incubated for 18-24 hrs. at 37°C. After incubation, 4–5 drops of fresh 10% ferric chloride solution was flooded over the growth of slant. Development of green colour indicates a positive reaction.

**Citrate utilization test:**

A single streak was made over the surface of a slope of Simmon's citrate agar and examined daily upto 7 days for growth and colour change. Blue colour of the medium indicates that citrate is utilized.

**Tween 80 hydrolysis:**

On the surface of Tween 80 nutrient agar, the test culture was inoculated and incubated at optimal temperature. Appearance of an opaque halo of precipitation around the growth indicates hydrolysis of the tween.
**Tween medium:**

Typtone – 10 g, Nacl – 5 g, Cacl$_2$ 2H$_2$O - 0.1 g, Agar – 20 g, distilled water 1000 ml. All the ingredients were dissolved and adjusted to pH 7.4. The solution was taken in 500 ml flasks, sterilized at 121°C and 5 ml of medium was added aseptically to each flask to give a final concentration of 1%. Then the medium was dispensed into petridishes.

**Pigment production:**

Nutrient agar plates were inoculated with a drop of a light suspension of the organism and incubated for 24 hrs. at 37°C and then transferred to room temperature and observed for 5 days. Development of colours such as red, orange, yellow, green, violet, brown indicates positive reaction whereas no colour development indicates negative reaction.

**Experimental Studies**

Experimental studies were carried out with the fingerlings of *L. rohita* and *C. catla* to determine the LD$_{50}$ dose, bacterial loads and pathogenicity of the bacterial isolates identified during the present study. Fish required to conduct the studies were collected from culture ponds at Vizianagaram district. They were held in well aerated tanks of 100 liters and 200 liters capacity and were fed on rice bran and oil cake. Prior to setting up of any experimental study fish were acclimatized to the laboratory conditions for a period of 10 days. Aeration was given throughout the experiment.

**Preparation of bacterial inoculum**

For preparing the inoculum, 200 μl (0.2 ml) of 12–18 hour static TSB broth culture was inoculated on TSA and incubated for 24 hours. Cells were harvested and washed in PBS, centrifuged at 3000 rpm for 15 min. The supernatant was removed and the pellet was again resuspended in PBS and
the process was repeated three or four times until a homogenate bacterial suspension without any clumps was formed. The cell suspension was then adjusted to a turbidity of 1 OD at 540 nm, which was equivalent to a cell suspension of $1 \times 10^9$ cfu/ml. Treating this as a stock solution, 10 fold serial dilutions in PBS were prepared, and viable counts were made by plating the dilution (0.025 ml aliquots) on TSA using the drop inoculation method. Three sets of experiments were carried out with three batches of fish separately to determine the lethal dose ($LD_{50}$).

To quantify $LD_{50}$ dose, fingerlings of *L. rohita* and *C. catla* measuring 8–10 cm in length and 10–12 gms in weight were selected and maintained in separate tanks as three sets, with 100 fish in each set. Each set was divided into 10 batches with 10 fish in each batch to be used at each dilution. All 10 batches of fish in each set i.e. set-I, set-II, set-III were intraperitoneally inoculated with 0.2 ml of live suspension of *A. hydrophila*, *P. fluorescens*, and *F. branchiophilum* in PBS at serial dilutions of $1 \times 10^1$, $1 \times 10^2$, $1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$, and $1 \times 10^9$ cfu/ml respectively. Three control batches, with 10 fish in each batch, inoculated intraperitoneally with PBS (pH 7.2), were maintained simultaneously for each pathogen set. Both control and experimental fish were kept under observation for a period of 96 hours and mortality was recorded for every 24 hours. The dilution of the pathogen that kills exactly 50% of fish is taken as $LD_{50}$ dose of that pathogen and is calculated by following the method of Reed and Muench (1938).

Moribund fish were sampled and bacterial reisolation was performed on TSA plates. The isolated bacteria were characterized by various morphological, physiological and biochemical tests.
Quantification of Bacterial loads

Experimental infections with bacterial isolates from infected tissues of the hosts were carried out, to determine their pathogenicity and to test whether they fulfill Koch's postulates or not.

Bacterial loads were estimated for tissue samples of visceral organs viz. kidneys, spleen, liver, gills including blood collected from the fingerlings of *L. rohita* and *C. catla*, inoculated with 200 μl of three different doses of bacterial pathogens i.e. *A. hydrophila, P. fluorescence* and *F. branchiophila*. Doses were determined based on the LD$_{50}$ dose as lower dose (less than LD$_{50}$), LD$_{50}$ dose and higher dose (more than LD$_{50}$) for all the three pathogens. A batch of 10 fingerlings was used at each dose for each respective pathogen. Control batch of 10 fish were injected with 200 μl of PBS. Both control and experimental batches were maintained for 96 hours at room temperature and at the end of the experiment, moribund and live fish were autopsied under highly aseptic conditions. Weighed tissue samples (<100 mg) of visceral organs i.e. kidneys, spleen, liver, gills including blood were aseptically taken into a known volume of sterilized and cooled peptone broth. The tissue samples were homogenized and 1ml of the homogenate was transferred into a sterile petridish aseptically and molten nutrient agar was poured onto it. The dish was rotated in both clock directions to allow uniform distribution of the sample and was inverted and incubated for 24 hrs. at 28$^0$C in an incubator. Triplicates were maintained at each dilution. The numbers of colonies were counted with the help of a colony counter and are expressed in colony forming units/ml (cfu/ml).

Haematological and Serum Biochemical Parameters

Blood samples collected from fish exposed to different doses of the three pathogens, viz. *A. hydrophila, P. fluorescence* and *F. branchiophilum*
were used for evaluating haematological and serum biochemical parameters. Part of the collected blood was used for analyzing hematological parameters and the remaining part was centrifuged to separate serum. Serum samples were stored immediately after centrifugation at \(-4^\circ\text{C}\) until further analysis.

Hematological Parameters like RBC, WBC and PCV were estimated following the standard methodology (Blaxhall and Daisley, 1973) whereas Hb\% was estimated by Cyanomethemoglobin method using Drabkin’s reagent (Dacie and Lewis, 1968).

Lowry’s method was used to estimate total serum proteins (Lowry et al., 1951); Glucose (enzymatic GOD-POD method of Trinder, 1969) and Albumins (Bromocresol Green method of Corcoran, 1977) were estimated using kits purchased from Span Diagnostics Ltd., INDIA.

Transaminase enzymes like Serum Glutamate Oxaloacetate Transaminase (SGOT), Serum Glutamate Pyruvate Transaminase (SGPT), and Blood Urea Nitrogen (BUN) were analyzed by spectrophotometric method using commercial kits purchased from Span Diagnostics Ltd., INDIA.

**Statistical Analysis**

The data generated during the present study from various natural and experimental studies was analyzed employing descriptive statistics and one-way and two-way analysis of variance; Computer statistical software programmes like SPSS 16.0 and Statistica 7.0 were used for data analysis. Entire data is presented in the form of tables and graphs.