CHAPTER IV

MATERIALS AND METHODS

4. Materials and Methods

4.1. Reagents for water Analysis

Manganous sulphate

48 g of $MnSO_4$ $4H_2O$ was dissolved in 100 mL distilled water. Then the solution was filtered through an ordinary filter paper and kept in a reagent bottle.

Alkaline Iodide-Sodium azide (NaN_3) Solution

500 g of NaOH and 150 g KI (Potasium Iodide) was dissolved in distilled water and after cooling, it was diluted to 1000 mL with distilled water. Then, 10 g of NaN_3 was dissolved in 40 mL distilled water and added to the Alkaline-Iodide Solution.

Sodium thiosulphate ($Na_2S_2O_3$) Solution, 0.025N

6.205 g of sodium thiosulphate was dissolved in 250 mL boiled distilled water and diluted to 1000 mL with distilled water. 5 mL chloroform was added as a preservative and was kept in a brown glass bottle.

Starch Indicator

1 g of starch powder was dissolved in 100 mL of warm distilled water and few drops of toluene were added to the solution as preservative.

Sodium hydroxide, 0.05 N

10 g of NaOH was dissolved in 250 mL of CO_2 free distilled water to prepare 1.0 N NaOH solution. This solution was diluted 20 times to prepare 0.05N solution and standardized with HCl.

Phenolphthalein Indicator (0.5%)

0.5 g of phenolphthalein was dissolved in 50 mL of 95 % ethanol and 50 mL distilled water was added to it. Then 0.05N NaOH solution was added drop wise until the solution turned to pink colour.

Hydrochloric acid (0.1N)

12N concentrated HCl (Specific gravity 1.18) was diluted 12 times with distilled water. Further, it was diluted 10 times. It was standardized against sodium carbonate solution.

Methyl Orange Indicator

0.5 g methyl orange was dissolved in 100 mL distilled water and kept in a reagent bottle.

EDTA solution (0.01M)

3.723 g of disodium salt of EDTA was dissolved in distilled water and volume was made 1000 mL. The solution was stored in polythene bottle.

Ammonia buffer solution

Solution a). 16.9 g of ammonium chloride (NH_4Cl) was dissolved in 143 mL concentrated ammonium hydroxide (NH_4OH).

Solution b). 1.179 g of disodium EDTA and 0.780 g of $MgSO_4.7H_2O$ were dissolved in 50 mL distilled water. Then (a) and (b) solutions were mixed and diluted to 250 mL with distilled water.

Standard ammonia solution

3.819 g of anhydrous NH_4Cl was dissolved in distilled water and diluted to 1000 mL. The solution was diluted 100 times to prepare the solution containing 10 mg/L NH_3-N.

Sulphuric acid (0.04 N)

2 mL of H_2SO_4 was added to 1000 mL distilled water.

Bromocresol green indicator solution, pH 4.5 (0.1%)

100 mg bromocresol green sodium salt was dissolved in 100 mL distilled water.

Methyl red indicator (0.1%)

100 mg of methyl red sodium salt was dissolved in distilled water to prepare 100 mL of solution.

Sodium borate solution

4 g $Na_2B_4O_7.10H_2O$ (Borax decahydrate) was added to 100 mL distilled water and heated to dissolve crystals. Bromocresol green 2 mL, methyl red 0.5 mL and few drops of HCl and NaOH were added to make the solution dark pink.

Eriochrome Black T Indicator

0.40 g Eriochrome Black T was mixed with 100 g NaCl and grinded.

Silver nitrate (0.02N)

3.4 g of $AgNO_3$ was dissolved in distilled water and volume was made 1000 mL. The solution was kept in dark glass bottle.

5 % Potassium chromate (K_2CrO_4) Solution

5 g of K_2CrO_4 was dissolved in 100 mL distilled water.

Sulphuric acid (5N)

70 mL conc. sulphuric acid was added to 500 mL distilled water.

Potassium antimony tartarate solution

1.3715 g of potassium antimony tartarate I [K (SbO) $C_4H_4O_6.H_2O$] was dissolved in 400 mL distilled water.

Ammonium molybdate solution

20 g of ammonium molybdate [$(NH_4)_6MO_7O_{24}.4H_2O$] was dissolved in 500 mL distilled water.

Ascorbic acid solution (0.01 M)

1.76 g of ascorbic acid [$C_6H_8O_6$] was dissolved in 100 mL distilled water.

Salicylic acid-H_2SO_4

5 g of salicylic acid was dissolved in 100 mL of conc. H_2SO_4. The salicylic acid –H_2SO4 reagent was made fresh every week and stored in a brown bottle.

2N NaOH solution

40 g of NaOH pellets was dissolved in 1000 mL of water, transferred to a 500 mL volumetric flask and made up to 500 mL with distilled water to prepare a 2N NaOH solution.

Nitrate standard solution (1 mg N/L)

0.722 g of KNO_3 was dissolved in distilled water and the volume was adjusted to 1000 mL. This solution contains 100 mg N/L. It was diluted 100 times to prepare a solution having 1 mg N/L. Nitrate standard was stored at 4ºC.

Combined reagent

50 mL of 5N H_2SO_4, 5 mL of potassium tartarate, 15 mL of ammonium molybdate and 30 mL of ascorbic acid solution were mixed and was allowed to stand for few minutes (should be prepared at the time of use).

Stock phosphate standard (50 ppm, PO_4 –P)

0.2195 g of anhydrous potassium dihydrogen orthophosphate [KH_2PO_4] was dissolved in distilled water and the volume was adjusted to 1000 mL in a volumetric flask. This solution contained 50 µg PO_4-P /mL. From this solution, 10 ppm PO_4-P standard solution was prepared by diluting 20 mL of it.

4.2. Temperature Measurment

The air and water temperature were measured by using a standard mercury thermometer graduated up to 50°C with a precision of 0.1°C. The water temperature was recorded by dipping the bulb in water. In each case the thermometer was shaded to prevent direct sunlight on the thermometer and the reading were recorded only after getting constant mercury in thermometer column.

4.3. Methods for Water Analysis

4.3.1. Turbidity

Turbidity was measured using Hanna’s turbidity meter, Model 402000.

4.3.2. Hydrogen ion concentration (pH)

pH was measured by a portable Hanna’s pH meter, Model HI8314.

4.3.3. Dissolved oxygen (DO)

The dissolved oxygen of water samples were estimated by azide modification method. For this 300 mL water sample was taken in a glass stoppered BOD bottle avoiding agitation. 2 mL of manganous sulphate was added to the sample water and after few seconds 2 mL of alkaline iodide azide solution was added. The precipitation was allowed to settle down. After

that 2 mL of conc. H_2SO_4 was added and shaken thoroughly to dissolve the precipitate. Then, 100 mL of sample was titrated against 0.025N sodium thiosulphate solution using starch as an indicator. The calculation was made by following formula:

$$\text{Dissolved oxygen in mg/L} = \frac{(\text{mL} \times \text{N}) \text{ of titrant} \times 8 \times 1000}{V_2 (V_1 - v)/V_1}$$

Where, V_1 = volume of sample bottle after placing the stopper.

V_2 = volume of the part of the contents titrated

v = volume of $MnSO_4$ and alkaline iodide azide solution.

4.3.4. Free Carbon dioxide (FCO_2)

Free carbon dioxide was determined by titrimetric method using phenolphthalein indicator. 100 mL sample water was taken in a conical flask and a few drops of phenolphthalein indicator were added. The sample water did not become pink and remained clear it indicated the presence of free carbon dioxide. Then the titration of sample water was done with 0.05N NaOH until appearance of pink colour. The used volume of NaOH was noted and the final calculation was made as follows:

$$\text{Free } CO_2 \text{ mg/L} = \frac{(\text{mL} \times \text{N}) \text{ of NaOH} \times 1000 \times 44}{\text{Volume of sample water (mL)}}$$

Where, N= Normality of NaOH.

4.3.5. Total alkalinity

The total alkalinity was measured by titration method. 100 mL of sample water was taken in a conical flask and a few drops of phenolphthalein indicator were added to it. The sample did not turn pink. Then, 2 to 3 drops of methyl orange indicator was added to the same sample and the titration was done with 0.1 N HCl until the yellow colour changed to pink. The volume used was recorded and the total alkalinity was calculated as follows:

$$\text{Total alkalinity, mg/L} = \frac{(\text{B} \times \text{N}) \text{ HCl} \times 1000 \times 50}{\text{Volume of sample (mL)}}$$

Where, B = volume of acid used for titration.

N= Normality of HCl.

4.3.6. Total hardness

Total hardness of sample water was determined by EDTA titrimetric method. 50 mL of sample water was taken in a conical flask and 1 mL of buffer solution was added. Then, about 100 mg of Erichrome Black T indicator was added and the solution turned to wine red colour. The solution was titrated with 0.01 M EDTA solution until the colour changed to blue.

$$\text{Total hardness, mg/L as } (CaCO_3) = \frac{\text{Used volume of EDTA} \times 1000}{\text{Volume of water sample (mL)}}$$

4.3.7. Chloride

The chloride of water sample was determined by Argentometric method. 50 mL of sample water was taken in a conical flask and 2 mL of K_2CrO_4 solution was added. That solution was titrated against 0.02N $AgNO_3$ until a persistent reddish brown colour appeared. The volume of titrant was noted and the final calculation was done as follows:

$$\text{Chloride, mg/L} = \frac{(V \times N) \text{ of } AgNO_3 \times 1000 \times 35.5}{\text{Volume of sample}}$$

Where, V= mL of $AgNO_3$ used for titration.

N= normality of $AgNO_3$.

4.3.8. Biological oxygen demand (BOD)

Biological oxygen demand (BOD) was estimated by azide modification method (APHA, 2005), measuring the difference of oxygen concentration between the initial and incubated sample for five days at 20°C in a BOD incubator. The calculation of BOD was done as follows:

$$BOD \text{ mg/L} = D_1 - D_2.$$

Where, D_1 = Initial dissolved oxygen (mg/L).

D_2 = Dissolved oxygen after five days (mg/L).

4.4. Physico-chemical parameters of water samples

Temperature was estimated on the site by glass thermometer. pH of water was estimated on the site by Hanna's pocket pH meter. Turbidity of the water was measured by Hanna's turbidity meter. All other physico-chemical analysis of water like dissolved oxygen (DO),

biological oxygen demand (BOD), free carbon dioxide (free CO_2), chloride ions (Cl^-), total alkalinity and total hardness was determined following standard methods (Trivedy and Goel, 1986; APHA, 2005).

4.4.1. Statistical Analysis

Standard deviation, correlation coefficient were calculated by using Microsoft excel statistical function of computer software. The significance of correlation coefficient was tested by applying t-test by using SPSS-V20 computer software. Two way ANOVA was used to test the significant and insignificant difference among sites and seasons and it was done by using Microsoft excel statistical function of Computer software.

4.5. Collection of diseased fish

Naturally infected fish *Catla catla*, *Cirrhinus mrigala*, *Channa striata* and *Puntius* sp., *Labeo rohita*, *Labeo bata*, *Clarias batrachus*, *Heteropneustes fossilis*, *Mystus tengara* and *Lepidocephalichthys guntea* showing ulcerative lesions were collected during winter months of the year 2008-2015 from different affected ponds in various locations of the Sunsari and Morang districts of eastern Nepal and were used for the isolation of bacteria and fungi. Infected fish were brought alive to the laboratory for further studies.

4.6. Collection and maintenance of healthy fish for experimental works

Healthy air breathing fish (*Heteropneustes fossilis*) collected from nearby fish farm with no history of EUS infections were used for experimental work. Fish were maintained in the laboratory in glass aquaria measuring 90 × 35 × 35 cm^3 in which the depth of the static water was 20 cm. Water temperature was maintained at 28 - 30°C. The fish were fed with chopped earthworms and acclimatized under laboratory conditions for at least 15 days before using them for experimental work. The diseased fish were diagnosed and the type of disease was determined using the key for the diagnosis of fish diseases laid down by Lucky, 1977.

4.7. Isolation of microorganisms from the ulcers of diseased fishes

4.7.1. Isolation of bacteria and culture

The ulcerated area of the diseased fish was dissected aseptically following Pal and Pradhan (1990) and placed in a conical flask containing 15 mL of nutrient broth (Hi Media Lab., Mumbai) supplemented with glucose. The flask was incubated at 30°C for 72 hrs. Then 1 mL

of each bacterial culture grown on nutrient broth was inoculated in a conical flask containing 20 mL of molten *Aeromonas* isolation medium supplemented with *Aeromonas* selective supplement (Hi Media Lab., Mumbai) and mixed thoroughly. The mixture was then poured on sterile petridish (90 mm diameter) and allowed to solidify for overnight at 30°C. Some colonies grown on the agar plates were selected and then streaked on to nutrient agar slants to incubate at 30°C for 24 hrs. Bacterial cultures grown on nutrient broth were also streaked on nutrient agar slants to incubate at 30°C for 24 hours. Each isolate was given a particular code name (Cm_1, Cm_2, Cm_3,C m_4 ,Cc_1, Cc_2, Cc_3, Cc_4, Cs_1, Cs_2, Cs_3, Mt_1,Mt_2,Mt_3 Mt_4, P_1, P_2 P_3, P_4, Lb_1, Lb_2,Lb_3 and Lb_4) and stored at 4°C. For routine experimental works, the isolates were sub cultured by growing in nutrient broth for 24 hrs at 30°C.

4.7.2. Isolation of fungus and culture

Fungi were isolated from the infected fish following the method of Willoughby and Roberts, 1994 and Lilley *et al.,* 1998 and cultures of the same were maintained by aseptically transferring the fungus to freshly prepared fungal media. Similarly, sporulation of fungus was also done by growing the mycelium in GPY broth. Code names (A_1, A_2, A_3 and A_4) have been assigned to each of the isolate until identification.

4.8. Composition of bacterial media

Several media were used during the present study for isolation and maintenance of isolates and for biochemical tests. The compositions of all media used in the study are given below:

Nutrient Broth

Peptone	10 g
Beef extract	10 g
NaCl	5 g
Water	1000 mL

The ingredients were dissolved by heating and pH was adjusted to 8.0-8.4 with 10 (N)/ NaOH. Then it was filtered to remove the precipitated phosphates. The pH was again adjusted to 7.2-7.4 and sterilized at 115 ºC for 20 minutes.

Nutrient Agar

Nutrient Agar was prepared by adding 2% agar powder to the final nutrient broth. The agar was melted with media and sterilized.

OF medium (Hugh and Leifson, 1953)

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	3 g
Distilled water	1000 mL
Bromothymol blue,	15 mL
0.2 % aqueous solution.	

All the ingredients were dissolved by steaming and the pH was adjusted to 7.1. The solution was filtered and the indicator was added to it. It was sterilized at 115°C for 20 min.

The carbohydrates solution was sterilized separately, cooled to 45ºC and mixed to the OF media to give a final concentration of 1%. It was then distributed aseptically into sterile test tubes.

Simmon's Citrate media

NaCl	5 g
$MgSO_4 .7H_{2O}$	0.2 g
$NH_4 H_2 PO_4$	1 g
K_2HPO_4	1 g
Citric acid	2 g
Distilled water	1000 mL

The salts were dissolved in water, citric acid was added to the salt solution and the pH was adjusted to 6.8 with 1(N) NaOH.20 mL of 0.4% aqueous solution of bromopthymol blue indicator prepared separately was added to the media and was dispensed in tubes then sterilized at 115 ºC for 20 minutes.

Tween 80 medium (Barrow and Feltham, 1993**)**

Peptone	10 g
NaCl	5 g
$CaCl_2.2H_2O$	0.1 g
Agar	20 g
Distilled water	1000 mL

The ingredients were steamed until the solids were dissolved. The pH was adjusted to 7.4 and sterilized at 115°C for 20 min. Tween 80 was sterilized separately and added aseptically to

the solution to give a final concentration of 1%. The final medium was distributed into sterile Petri plates.

Modified motility media (Barrow and Feltham, 1993**)**

Peptone	10 g
Beef extract	3 g
NaCl	5 g
Agar	4 g
Gelatin	80 g
Cystein	0.2 g
Ferrous ammonium sulphate	0.2 g
Sodium Citrate	2 g
Distilled water	1000 mL

Gelatin was soaked in water for 30 min. The other ingredients were added and dissolved to sterilize at 115°C for 20 min.

Glucose-Phosphate medium

Peptone	5 g
K_2HPO_4	5 g
Distilled water	1000 mL
Glucose	5 g

The first two ingredients were steamed to dissolve, filtered and the pH was adjusted to 7.4. The glucose was then added to it and distributed into tubes containing 5 mL of solution. It was then sterilized at 115 ºC for 10 minutes.

VP test medium

Glucose-peptone medium (Barrow and Feltham, 1993)

Peptone	10 g
Glucose	5 g
Distilled water	1000 mL

By gentle heating the solution was mixed and dissolved. Then filtered it and pH was adjusted 7.6 and distributed into tubes and sterilized at 115ºC for 10 minutes in a solid bottomed container.

Egg yolk saline suspension

Hens egg	4 pc
NaCl, 0.85 % soln.	1000 mL

The yolks were separated aseptically from the whites and thoroughly mixed with sterile saline to form a homogeneous mixture.

Lecithovitelline Agar

Egg yolk saline suspension	100 mL
Nutrient Agar	900 mL

The sterile molten nutrient agar (Hi Media Laboratories, Mumbai, India) was added aseptically to the mixture at a temperature of 50°C and mixed thoroughly to pour into plates.

Decarboxylase media (Barrow and Feltham, 1993)

Peptone	5 g
Beef extract	5 g
Pyridoxal	5 mg
Glucose	0.5 g
Bromothymol purple 0.2 % aq. Soln.	5 mL
Cresol red, 0.2 % aq. soln	2.5 mL
Distilled water	1000 mL

The ingredients were dissolved by heating and the pH was adjusted to 6.0. The indicators were added and the mixture was distributed into four equal volumes to sterilize at 115°C for 20 min.

L-arginine hydrochloride	1%
L-lysine hydrochloride	1%
L-ornithine hydrochloride	1%
No addition	

The above ingredients were added separately and the four media were distributed into small rimless tubes with a volume of 1-1.5 mL containing sterile medicinal grade liquid paraffin to height of about 5 mm above the medium. Then, the tubes were autoclaved at 115°C for 10 min

Nitrate Broth

KNO_3	1 g
Nutrient Broth	1000 mL

Potassium nitrate was dissolved in the Nutrient Broth (Hi Media Laboratories, Mumbai, India) and distributed into test tubes containing inverted Durham's tubes, and sterilized at 115°C for 20 minutes.

Nutrient Gelatin

Beef extract	3 g
Peptone	5 g
Gelatin	120 g
Distilled water	1000 mL

Gelatin was soaked in water for 30 min. The other ingredients were added and dissolved. Then it was sterilized at 115°C for 20 min.

Peptone Water

Peptone	10 g
NaCl	5 g
Distilled water	1000 mL

The solid mixture of peptone and NaCl were dissolved and the pH was adjusted to 8.0-8.4. It was boiled for 10 min., filtered and again the pH was adjusted to 7.2-7.4 and autoclaved at 115°C for 10 min.

Triple Sugar Iron Agar (TSI) (Barrow and Feltham, 1993)

Peptone	20 g
Meat extracts	3 g
Yeast extracts	3 g
NaCl	5 g
Glucose	1 g
Lactose	10 g
Sucrose	10 g
$FeSO_4.7H_2O$	0.2 g

$Na_2S_2O_3.5H_2O$	0.3 g
Agar	20 g
Distilled water	1000 mL
Phenol red, 0.2% aq. Solution	12 mL

The ingredients were dissolved by steaming; indictor was added and then dispensed into tubes. The solution was sterilized at 115ºC for 20 minutes and cooled to form deep butts about 3 cm long.

King's media- for Pseudomonas (Barrow and Feltham, 1993)

Medium A of King- for pyocyanin

Peptone	20 g
Glycerol	10 g
K_2SO_4, anhydrous	10 g
$MgCl_2$, anhydrous	1.4 g
Agar	20 g
Distilled water	1000 mL

The constituents except agar were dissolved by steaming and pH was adjusted to 7.2. Agar was added and dissolved then it was sterilized 121 ºC for 10 minutes.

Medium B of King- for fluorescin

Proteose Peptone	20 g
Glycerol	10 g
K_2HPO_4	1.5 g
$MgSO_4$.7H2O	1.5 g
Agar	20 g
Distilled water	1000 mL

The constituents except agar were dissolved by steaming and pH was adjusted to 7.2 Agar was added and dissolved then it was sterilized 121 ºC for 10 minutes.

Rapid Diagnostic procedure

L-lysine hydrochloride	5.0 g
L-ornithine hydrochloride	6.5 g
Maltose	3.5 g
Sodium thiosulfate	0.3 g
Bromthymol blue	0.03 g
Iron ammonium sulfate	0.8 g
Sodium deoxycholate	1.0 g
Novobiocine	0.05 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Agar	13.5 g
Distilled water	1000 mL
pH	7.0

All ingredients were kept in a 500 ml Erlenmeyer flask then dissolved thoroughly and sterilized at 115°C for 20 min. in autoclave.

After incubation at 26ºC for 3 days, production of yellow coloured colony confirms *Aeromonas punctata* (94 %).

Esculin Broth

Esculin	1 g
Ferric citrate	0.5 g
Peptone Water	1000 mL

The ingredients were dissolved in the peptone water and sterilized at 115°C for 10 min.

4.9. Composition and preparation of buffers and reagents

The compositions of buffers and reagents used in the present investigation are enlisted below:

TE Buffer

10 mM Tris-HCL, 1 mM EDTA, pH 8.0.

50X TAE Buffer (1 L) Tris base

242 g, glacial acetic acid: 57.1 mL, 100 mL 0.5M EDTA, pH 8.0. The volume was adjusted to 1 L with distilled water and autoclaved at115°C for 10 min.

Oxidase reagent

Freshly prepared 1% tetramethy-p-phenylenediamine dihydrochloride.

Nitrite reagent A

0.33% sulphanalic acid in 5N acetic acid.

Nitrite reagent B

0.5% dimethyl -α- naphthylamine in 5N acetic acid.

Kovács' reagent

p- dimethylamino benzaldehyde: 5 g, amyl alcohol: 75 mL, conc. HCl: 25 mL.

Burke's iodine

Iodine: 1 g, potassium iodide: 2 g, distilled water: 100 mL.

Carbol fuchsin

Phenol: 85 g, basic fuchsin: 15 g, ethanol: 250 mL, distilled water 1250 mL. One volume is diluted with 10-20 volumes of distilled water.

Crystal violet

Crystal violet: 2 g, 95% ethyl alcohol: 20 mL, 1% aqueous ammonium oxalates solution: 80 mL.

Methyl red

Methyl red: 0.04 g, ethanol: 40 mL. Methyl red was dissolved in ethanol and diluted with distilled water to 100 mL.

Safranin reagent

Safranin (2.5% solution in 95% ethyl alcohol) 10 mL and distilled water 100 mL.

4.10. Characterization of the isolated bacteria

To identify the bacteria, a number of physiological and biochemical tests (Barrow and Feltham, 1993) were conducted following the identification scheme described by Popoff (1984), Carnanhan *et al*. (1991) and Abbott *et al.* (1992).

Morphological characterization

Shape and size of the bacteria

To examine the shape and size of the cells, a drop of cell suspension of the test bacterium was placed on a clean grease-free slide, air dried and stained with carbol fuchsin and observed under microscope. Diameter was measured with standard ocular micrometer.

Morphology of the colony

Morphology of the bacterial colony on nutrient agar plates like texture (smooth or rough), appearance (glistening or dull), optical property (opaque, translucent, transparent) etc. were examined.

Motility

To detect the motility of sample bacteria, tubes of modified motility medium were stab inoculated to a depth of about 5 mm. The tubes were incubated at 28°C and the turbidity pattern was observed for 5 days.

Physiological and Biochemical tests

Gram reaction

Smear prepared on slide from 24 hrs old culture of sample bacterium in nutrient broth was heat fixed, air dried and flooded with crystal violet stain for 1 min and then rinsed off with water. Burke's iodine was then added to the smear and allowed to stand for 1 min and rinsed

off with water. The smear was then decolorized with 95% ethanol, which was poured drop by drop holding the slide in a slanting position against white background till no colour came out from the lower edge of the slide. The smear was then counterstained with safranin for 1 min and rinsed off with water. The slide was air dried and observed under microscope (Bartholomew, 1962).

Production of catalase

The bacterial culture was incubated for 24 hours on nutrient agar slant and 1 mL of 3% Hydrogen peroxide solution was poured down over the slant. Immediate evolution of gas indicated the presence of catalase activity.

Production of oxidase

Twenty four hours grown bacterial culture on nutrient agar (glucose free) were taken and smeared across a filter paper moistened with freshly prepared 1% tetramethy-p-phenylenediamine dihydrochloride with a glass rod. The appearance of a dark purple colour within 30 s indicated a positive reaction.

Nitrate reduction

Nitrate broth was inoculated and incubated for 5 days. Inverted Durham's tube was inserted into the medium. The presence of nitrite (after reduction of nitrate to nitrite) was tested by addition of 1 mL of nitrite reagent A followed by 1 mL of reagent B. Appearance of red colour indicated a positive reaction. Zinc dust was added to the tubes which were not showing positive reaction and allowed to stand for 5 min. Appearance of red colour reported the presence of nitrate in the culture medium which indicated a negative reaction. Any gas production in the Durham's tube was noted.

Oxidation or Fermentation of glucose

Oxidation or fermentation of glucose was carried out by inoculating duplicate tubes of Hugh and Leifson's medium containing 1% glucose by stabbing. After inoculation, sterile molten paraffin was poured on to the top of one of the tubes to a depth of 10 cm to seal it from air. The other tube was left open. Both the tubes were incubated at 30°C for at least 7-14 days. The appearance of yellow colour in the open tubes only indicated the acid production from glucose by oxidation. Yellow colour in both the tubes indicated oxidation and fermentation of glucose (Hugh and Leifson, 1953).

Indole production

Peptone water / nutrient broth was inoculated and incubated at 30°C for 48 hrs. Indole production was judged by adding 0.5 mL of Kovac's reagent for 1 min. Appearance of red colour in the reagent layer indicated indole production (Barrow and Feltham, 1993).

Acid and gas production in media containing different carbohydrates

The acid production of glucose was tested by the Hugh and Leifson's medium without agar. The following carbohydrates were tested: L-arabinose, sucrose, mannitol and salicin. The medium was incubated at 30°C for 14 days. If the colour of the medium turned into yellow, it indicated acid production. To determine the gas production, inverted Durham's tubes filled with medium was inserted into the tube. Generation of gas at the top of the Durham's tube indicated positive result.

Methyl red reaction

Glucose phosphate medium was inoculated and incubated at 30°C for 5 days. Two drops of Methyl red (MR) solution was then added, shaken and examined. A positive MR reaction is shown by the appearance of a red colour at the surface (Barrow and Feltham, 1993).

Voges- Proskauer (VP) test

After completion of the methyl red test, 0.6 mL of 5% α-napthol solution in ethanol and 0.2 mL of 40% potassium hydroxide aqueous solution was added and mixed thoroughly. The tube was then placed in a slanting position to increase the air-liquid interface and examined after 15-60 min. The appearance of strong red colour indicated positive result (Barrow and Feltham, 1993).

Esculin hydrolysis

Esculin broth was inoculated with sample bacteria and examined for 5 days. A positive result was indicated by the blackening of the medium (Barrow and Feltham, 1993).

Decarboxylase tests

The three Decarboxylase media (argine, lysine and ornithine) and control were heavily inoculated with bacteria through the paraffin layer and incubated at 30°C for 24-48 hrs. The media first turned into yellow due to acid production from the glucose. The acidic environment in the media caused decarboxylation that raised the pH of the media and turned

bromocresol purple from yellow to purple. A purple colour represented the positive test (Barrow and Feltham, 1993).

Pigment production

To test pigment production by Pseudomonads, King, Ward and Raney's media (A and B) were used. After inoculating, medium A was incubated at 30ºC for 24 to 96 hours and examined daily for pigment production. Medium B was incubated at 30ºC for 24 hours followed by room temperature (22-25ºC) for 72 hours. To test for pigment production by Coccus, organisms were grown on nutrient agar plates at room temperature and were kept under diffused day light.

Gelatin Hyrolysis

Gelatin agar plates were inoculated and incubated for three days. The plates were then flooded with 30% trichloroacetic acid, clear zones indicated areas of gelatin hydrolysis.Gelatin hydrolysis was also tested in the modified motility medium along with testing of motility and H_2S production. The medium was stabbed inoculated with a straight wire, inoculated at 28ºC and observed daily for 30 days for presence of liquefaction.

4.11. Pathogenicity test of the isolated bacteria

All the isolates were tested for their ability to induce ulcers in healthy *Heteropneustes fossilis* fish weighing of 50-60 g by intramuscular application of 0.5 mL of bacterial cell suspension (1×10^7 c.f.u /mL) per 100 g of body weight in 0.85% NaCl. Each isolate was injected into a set of five fish. The control set of fish received 0.05 mL sterile saline. Fish were observed for changes in their behavioral patterns as well as development of hemorrhagic ulcers and tissue necrosis (Pradhan and Pal, 1990). Intramuscular injection was given at the trunk region on the right/left side of the fish with a 28 gauge needle attached with a 1.0 mL insulin syringe. The needle was inserted from behind to the front at an angle of 20° to the body axis.

Fig.4.1. Intramuscular injection of bacterial suspension to healthy fish, *Heteropneustes fossilis.*

4.12. Composition of fungal media

The names and composition of fungal media are mentioned below which were used during this study.

Glucose- peptone broth (GPB) (Lilley *et al*., 1998)

Glucose	3 g
Peptone	1 g
Mg $SO_4.7H_2O$	0.128 g
KH_2PO_4	0.014 g
$CaCl_2.2H_2O$	0.029 g
$FeCl_3.6H_2O$	2.4 mg
$MnCl_2.4H_2O$	1.8 mg
$CuSO_4.5H_2O$	0.4 mg
Distilled water	1000 mL

All the above ingredients were dissolved in distilled water and then autoclaved at 121ºC for 15 minutes.

Glucose-Peptone Agar (GPA)

12 mg/L agar powder was added to GPB to prepare GPA.

Glucose- Peptone-Yeast extracts Broth (GPY)

0.5 g/L of yeast extract was added to GPB to prepare GPY medium.

Glucose- Peptone-Yeast extracts Agar (GPYA)

0.5 g yeast extract and 12 g/L of agar powder were added to GPB to obtain GPYA medium. Penicillin-K and Oxonilic acid were added at a concentration of 100 mg/L to the media after autoclaving and cooling to 50ºC (Roberts *et al.,* 1993). Streptomycin was used at a concentration of 100 mg/L in this experiment.

Autoclaved Pond water

The pond water was first filtered through Whatman filter paper. Filtered pond water and distilled water was mixed in the ratio of 1:2 and then autoclaved. The pond water is known to promote the growth of fungi.

Material for Fungal stain

1% Lactophenol cotton blue

Cotton blue powder	1 g
Lactophenol Solution	100mL

Composition of Lactophenol

Phenol	20 mg
Glycerol	33.3 mL
Lactic acid	1.68 mL
Distilled water	20 mL

4.13. Methods for fungal isolation (Willoughby and Roberts, 1994; Lilley *et al.*, 1998)

Slightly ulcerated tissues of fish lesions are most suitable for the isolation of fungi and following techniques were adopted.

In scaly fishes, the scales around the periphery of lesion of infected fishes were removed and underlying skin was seared with red hot spatula to sterilize the surface. The anesthetized fish (scaly or without scales) was pinned on a dissecting tray in a laminar flow cabinet. The underlying muscles of the lesion were then exposed by cutting the superficial tissues. The affected muscles were then excised into 4 mm pieces and placed on a petridish containing the isolation media. Inoculated media were incubated at 25ºC after examination under phase contrast inverted microscope and first transfer was made after six hours. The emerging hyphal tips were repeatedly transferred to fresh plates of GP medium containing antibiotic until the cultures were free of bacterial contamination. Isolates were then sub-cultured on GP agar and transfer was given at an interval of five days.

4.13.1. Sporulation methods of isolated fungus

An agar plug containing actively growing mycelium was placed in a petridish containing GPY broth and was incubated for 4 days at about 20 ºC for sporulation of fungus. The resulting mat was washed by sequential transfer through 5 petri dishes containing autoclaved pond water finally washed and left overnight at 20 ºC in autoclaved pond water. After about 12 hours motile secondary zoospores were observed under microscope.

4.13.2. Microscopical examination of isolated fungus

A portion of ulcerated tissue was taken and smeared on a clean glass slide. The smear was stained with Lactophenol cotton blue and was observed under the microscope. The fungal hyphae and sporangium from the pure culture maintained in GPYA were observed under microscope after staining with cotton blue.

4.14. Histopathology

4.14.1. Materials for histopathological techniques

Bouin's Fixative

Saturated aqueous picric acid solution	75 mL
Formalin	25mL
Glacial acetic acid	5 mL

Materials for Grocott hexamine (methenamine) Silver staining for fungi (Grocott, 1955)

i. 5 % Borax solution: Aqueous Sodium Tetraborate.

ii. 5 % Silver nitrate solution with 5mL distilled water.

3 % methenamine / hexamethelene tetramine in distilled water 100 mL.

Silver nitrate was added to the methenamine solution and shaked until the precipitation and this mixture was kept for 1-2 months at 4 ºC.

iii. Incubating solution.

Borax solution	5mL
Distilled water	25 mL
Methenamine Silver solution (ii)	25 mL

The above solution was heated to 56ºC and then mixed prior to use as the silver solution to degenerate when the borax was added.

iv. Arzac's counter stain

Orange Green	0.25 g
Light green	1.00 g
Tungstophosphoric acid	0.50 mg
50% alcohol	100 mL
Glacial acetic acid	1.25 mL

Sodium metabisulphate	1%
Gold chloride	0.1%
Sodium thiosulphate	3%
Chromic acid	5%

Materials (Chemicals) for Periodic acid Schiff stain (PAS)

i. Basic fuchsin solution

Basic fuchsin	0.1g
95% alcohol	5.0 mL
Water	95 mL

ii. Zinc / Sodium hydrosulphite solution

Sodium hydrosulphite (Sodium dithionite)	1.0 g
Tartaric acid	0.5 g
Water	100 mL

iii. Light green stain

Light green	1.0 g
Glacial acetic acid	0.25 mL
80% alcohol	1000 mL

Haematoxylin

Haematoxylin	2.0 g
Glacial acetic acid	10.0 g
Absolute alcohol	100 mL
Glycerol	100 mL
Potasium alum	in excess
Distilled water	100 mL

Aqueous Eosin

Eosin powder	1.0 g
Distilled water	100 mL

4.14.2. Histopathological Techniques

4.14.2.1. Sampling and Fixation

Live fishes were quickly put into a container of benzocaine solution (25 mg/L) for two minutes. Then the fish was taken out of the solution and pinned on a dissecting tray. The external lesions were first excised from the fish body with sterilized scissors and forceps and put in Bouin's

fixative. The internal organs were carefully dissected out, cut into small pieces of 3 mm to 5 mm and placed in fixative as early as possible .The volume of fixative was always at least 20 times of the volume of the tissues. The tissues were kept in fixative for overnight.

4.14.2.2. Processing

The fixative was first washed out of the samples by 70% alcohol. The dehydration of tissue was done by passing through a graded series of alcohol solutions (70%, 90% and 100%) (Schäperclaus,1986). After dehydration the tissues were then placed in xylene and infiltrated with molten paraffin (melting point 58-60ºC) and allowed to cool and harden. Some tissues were preserved in cedar wood oil for the future use following fixation and dehydration.

4.14.2.3. Sectioning

The hardened paraffin containing the tissues were trimmed into rectangular blocks, mounted on microtome and sections of 6µ thickness were cut. The sections were placed on grease free slides with albumin serving as an adhesive. The sections were properly stretched on warm distilled water and the slides were dried by keeping them overnight at room temperature/in incubator.

Before staining the stretched and dried sections were completely dewaxed by keeping them in xylene for 5-10 minutes.

4.14.2.4. Procedures for Haematoxylin –Eosin stain

After completely dewaxing the slides with stretched section were kept in absolute alcohol for 5 minutes and passed through descending grades of alcohol eg. 90%,70%, 50% and 30% to water. The sections were then stained with haematoxylin and washed thoroughly with water to remove the excess stain. Next the sections were examined under microscope to check the differentiation level. If the differentiation was up to the mark, the section were then passed through ascending grades of alcohol and stained with alcoholic eosin. The excess eosin was washed off by 90% alcohol and the tissues were further dehydrated in absolute alcohol, cleared in xylene and mounted in DPX mountant.

4.14.2.5. Grocott hexamine (methenamine) silver staining for fungi

The section was hydrated through graded series of alcohols to water after dewaxing then oxidized in 5% aqueous chromium trioxide (Chromic acid) for 1 hour. The section was

washed in tap water and rinsed in 1% sodium metabisulphite solution and rinsed in distilled water then placed in preheated (56ºC) incubating solution in the dark up to 1 hour.
Afterwards it was rinsed well in distilled water and toned in 0.1% gold chloride for 4 minutes following which the section was rinsed in distilled water and fixed in 3% sodium thiosulphate for 5 minutes. It was finally counterstained with Arzac's stain for 15-30 seconds, blotted, dehydrated and mounted in DPX mountant.

4.14.2.6. Periodic Acid-Schiff stain (PAS)

The slides were slowly brought to water following downgrades of alcohol (100%, 90%, 70%, 50% and 30%). Then the slides were kept in 10% periodic acid for 5-8 minutes. The slides were washed with running water for 3 minutes then with distilled water for 1 minute. Afterwards the slides were kept in Schiff's reagent for 15 minutes in dark and washed with tap water for 10 minutes and then dried and mounted with Euparol mountant.

4.15. Pathogenicity test of isolated fungus

The *Aphanomyces* sp. isolated from naturally infected fish, *Cirrhina mrigala* was inoculated into the healthy fish *Heteropneustes fossilis* to test its pathogenicity. Healthy *Heteropneustes fossilis* weighing about 55-95 g were collected from local pond (Itahari sub- metropolitan pond) and acclimatized in the glass aquarium in laboratory at temperature 25±1ºC for 15 days. Then 10 fish were injected zoospore suspension of *Aphanomyces* sp. at the rate of 0.5 mL/100 g of body weight intramuscularly as described by Robert *et al.* (1993). The controlled set was injected with sterile saline solution (0.85% NaCl) @ 0.5 mL/100 g of body weight. Three sets of such experiments were carried out simultaneously.

Fig.4.2. Intramuscular injection of fungal zoospore suspension to healthy fish, *Heteropneustes fossilis.*