Materials and Methods

3.1. Fishes

3.1.1. Collection of diseased fishes

In 1988, during the first outbreak of the ulcerative disease, Midnapur district was one of the severely affected districts of West Bengal. Since then, the disease has recurred almost every year after the monsoons in various areas of the district. During November 1993, there was a report of the outbreak of this disease from some areas of this district and *Channa punctatus, Puntius* sp. and *Mystus* sp. were among the worst affected. Infected fishes, *Channa punctatus*, *Puntius* sp. and *Mystus* sp. with severe ulcerative lesion were collected from the affected ponds of different areas of this district and used for isolation of the pathogen. Naturally infected fishes, *Channa punctatus*, *Anabas testudineus*, *Clarias batrachus, Heteropneustes fossilis* and *Cirrhinus mrigala* showing ulcerative lesions were obtained from various infected ponds of the Darjeeling, Jalpaiguri and Cooch Behar districts (Figs. 3-6). Infected fishes were also collected from the local markets and brought to the laboratory for a comparative study with experimentally infected fishes.

3.1.2. Collection of healthy fishes for experimental works

Only air breathing fishes were selected for all experimental works because of their easy maintenance and easier availability in comparison to other fishes. Four species of fishes, e.g., *Channa punctatus, Heteropneustes fossilis, Anabas testudineus* and *Clarias batrachus* were brought to the laboratory in various quantities from time to time according to the experimental requirements. The fishes were usually collected from nearby fish farms of Sonapur and Gangarampur of the Darjeeling district of West Bengal that did not have any previous report of ulcerative disease outbreak. Some fishes were also purchased from the local markets after ascertaining their source. The health condition of the fishes was given utmost priority while selecting the fishes,

Plate I

Fig. 3 a, b, c. Netting in an affected pond near North Bengal University campus

Fig. 4. Epizootic ulcerative syndrome affected *Cirrhinus mrigala* showing ulcers on their bodies.

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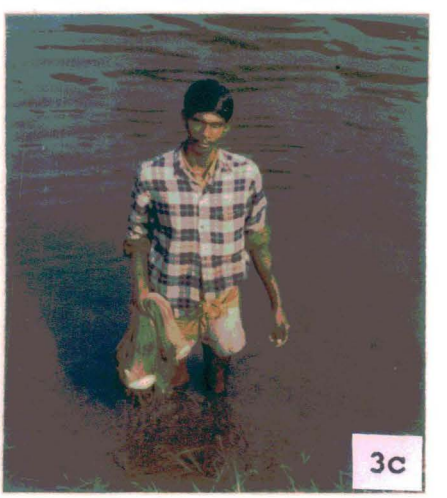

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Plate II

Fig. 5. Naturally infected fishes *Cirrhinus mrigala* showing haemorrhagic ulcers on their body

Fig. 6. Naturally infected *Mystus* sp. showing ulcer


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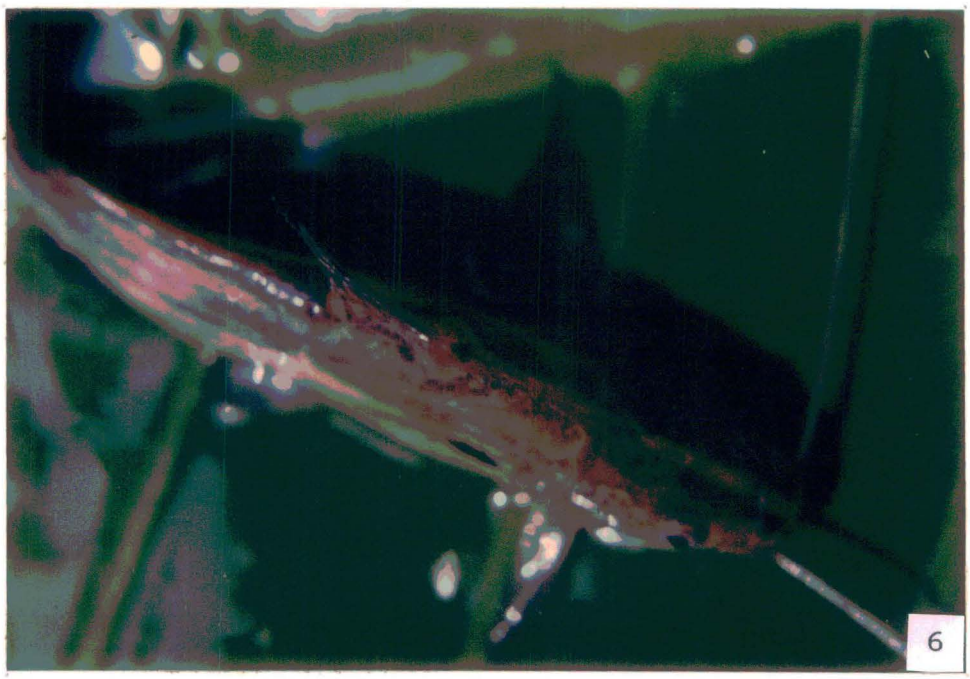
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and inspite of their easier availability, we sometimes had to wait for several weeks and even months to get the right fish in right health.

3.1.3. Maintenance of fishes

The healthy fishes were maintained in the laboratory in glass aquarium measuring 90 x 35 x 35 cms in which the depth of static water was 20 to 25 cm. Water temperature was maintained at 28-30^{0}C. The fishes were fed regularly with chopped earthworms. Fifteen fishes in the weight range of 10-25 gms were kept in one aquarium while 10 fishes in the weight range of 25 to 35 grams were kept in an aquarium. Fishes of higher weights were not considered for carrying out experiments due to difficulties in handling. Mortalities if any were recorded daily. All fishes were acclimatized under laboratory conditions for at least 15 days before using them for experimental works. If significant amount of fish died within 10 days of acclimatization, the set of fishes was discarded.

3.2. Bacterial cultures

3.2.1. Source of culture

In our laboratory, three types of fish pathogenic bacteria, R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*) were isolated from naturally infected air breathing fishes of North Bengal region (Pal and Pradhan, 1990; Pradhan, 1992). These bacterial isolates were used for different experimental works along with other bacteria isolated during the course of this study.

3.2.2. Maintenance of cultures

Cultures were maintained by asceptically transferring the bacteria to freshly prepared sterile nutrient agar slants after every three week. The stock cultures were stored at 4^{0}C. For experimental works, subcultures were

made from the stock cultures in suitable media before use. The cultures were also examined at regular intervals to test their pathogenicity.

3.3. Composition of Media

A number of culture media was used during this study. The names and composition of these media are given below:

Nutrient Broth

Peptone	10 g
Beef Extract	10 g
NaCl	5 g
Water	1000 ml

The ingredients were dissolved by heating. The pH was adjusted to 8.0-8.4 with 10 (N) NaOH and steamed for 10 min. It was then filtered to remove the phosphates precipitated at the alkaline stage. The pH was again adjusted to 7.2-7.4 and sterilized at 115^{0}C for 20 min.

Nutrient Agar

Nutrient agar was prepared by adding 2% agar powder to the final nutrient broth solution. The agar was melted by heating the media before sterilization.

Glucose Peptone Broth (GPB)

Glucose	3 g
Peptone	1 g
$MgSO_4 .7H_2O$	0.128 g
KH_2PO_4	0.014 g
$CaCl_2$	8 mg

$FeSO_4$	0.5 mg
$MnSO_4$	0.5 mg
$CuSO_4.5H_2O$	0.1 mg
$ZnSO_4$	0.1 mg
Distilled water	1000 ml

All the ingredients were dissolved and after dispensing into tubes and 100 ml conical flasks (containing 30 ml of broth), the media was sterilized at 115°C for 20 min.

Glucose Peptone Agar (GPA) (for growth of fungus only)

This medium was prepared by adding 1.5% w/v agar to the GP broth. 100 mg /lit of streptomycin and nalidixic acid was added to the medium after sterilization to obtain bacteria free cultures.

Glucose Peptone Yeast Extract Broth (GPYB) / Glucose Peptone Yeast Extract Agar (GPYA)

This medium was prepared by adding 1.0 g per litre yeast extract to the GPB medium. 1.5% agar was added to GPYB to obtain GPYA medium. 100 mg / litre of streptomycin and nalidixic acid were added to check the growth of bacteria if required.

Nitrate Broth

KNO_3	1 g
Nutrient Broth	1000 ml

Potassium Nitrate was dissolved in the broth and distributed into test tubes containing inverted Durham's tubes, and sterilized at 115°C for 20 min.

Gelatin agar

Gelatin	4 g
Distilled water	50 ml
Nutrient agar	1000 ml

The gelatin was soaked in water for 30 min and added to the molten nutrient agar. It was then mixed and sterilized at 115°C for 20 min and distributed into sterile petridishes.

Milk Agar

Skimmed Milk Powder	5 g in 50 ml distilled water
Agar	1 g in 50 ml distilled water

The two ingredients were separately autoclaved at 115°C for 20 min, cooled to 45°C and mixed together.

Glucose-Phosphate medium

Peptone	5 g
K_2HPO_4	5 g
Distilled Water	1000 ml

The medium was steamed until the solids were dissolved, filtered and the pH was adjusted to 7.4.

Glucose	5 g

The glucose was then added and 5 ml volumes of media were distributed into tubes. It was then sterilized at 115°C for 10 min.

Hugh and Leifson's 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, 0.2% aqueous soln.	15 ml

The solids were dissolved by steaming. The pH was adjusted to 7.1, filtered, and the indicator was added. Then it was sterilized at 115^{0}C for 20 min.

The carbohydrate solution was sterilized separately, cooled to 45^{0}C and mixed to the OF media to give a final concentration of 1%. It was then distributed asceptically into sterile test tubes.

Medium A of King (King *et al*, 1954)

Peptone	20 g
Glycerol	10 g
K_2SO_4, anhyd.	10 g
$MgCl_2$, anhyd.	1.4 g
Agar	20 g
Distilled water	1000 ml

pH - 7.2

The constituents except agar were dissolved by steaming and the pH was adjusted. The agar was then added and dissolved. The medium was sterilized by autoclaving at 121^{0}C for 10 min.

Medium B of King - for Fluorescin (King *et al*, 1954)

Proteose peptone	20 g
Glycerol	10 g
K_2HPO_4	1.5 g
$MgSO_4$, $7H_2O$	1.5 g
Agar	20 g
Distilled water	1000 ml

pH - 7.2

Procedure as stated for medium A was followed.

Tween 80 medium (Sierra, 1957)

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

The ingredients were dissolved by steaming, and the pH was adjusted to 7.4. Volumes of 500 ml were sterilized in flasks, which were cooled to 45°C.

Tween 80 was sterilized separately at 121°C and 5 ml added asceptically to each flask to give a final concentration of 1%. The final media was dispensed into sterile petridishes.

Simmon's Citrate media

NaCl	5 g
$MgSO_4,7H_2O$	0.2 g
$NH_4H_2PO_4$	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 salts solution and the pH was adjusted to 6.8 with 1(N) NaOH. Twenty ml of 0.4% aqueous solution of bromothymol blue indicator was prepared separately and added to the media. The light green colored media was heated to dissolve all the ingredients and was dispensed in tubes and sterilized at 115°C for 20 min.

Modified motility media (Hajna, 1950)

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 heated to dissolve and sterilized at 115^{0}C for 20 min.

Arginine media

Peptone	1.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
Phenol red, 1.0 % aq. soln	1.0 ml
L(+)Arginine hydrochloride	10.0 g
Agar	3.0 g
Distilled water	1000 ml

The solids were dissolved in the distilled water and the pH was adjusted to 7.2. The media was distributed into tubes to a depth of about 20 mm and sterilized at 121^{0}C for 15 min.

Lecithovitellin Agar

Egg yolk saline suspension

Hen eggs	4
NaCl, 0.85% soln	1000 ml

The yolks were separated from the whites and beaten in sterile saline to form a homogeneous mixture.

Lecithovitellin agar

Egg yolk saline suspension	100 ml
Nutrient Agar	900 ml

The sterile nutrient agar was melted and cooled to 50°C. The egg yolk-saline suspension was added asceptically, mixed and poured into plates.

3.4. Inoculation Techniques

The fishes were inoculated by either intramuscularlly or intraperitoneally injecting 0.5 ml/100 gm body weight of the bacterial suspensions in desired doses with a 24 gauge needle attached to a graduated glass syringe. Before applying injection to the fishes, all injection appliances were thoroughly cleaned, disinfected with formalin and then repeatedly rinsed with sterile distilled water. If necessary, the fishes to be injected were anaesthetized by keeping in benzocaine solution (25 mg/litre) for 1 to 2 min for easier handling.

Intramuscular injection was given at the trunk region on the right side of the fish (Fig.7). The needle was inserted from behind to the front at an angle of 20° to the body axis. Intraperitoneal injection was also given similarly over the centre of the left pelvic fin at the upper level of the pectoral fin attachment.

3.5. Histopathological techniques

3.5.1. Sampling and fixation

Live fishes were removed from the water with fine nets and quickly transferred to a container of benzocaine solution (25 mg / lit). After two min, the fish was removed from the solution and placed in a dissection tray. The external lesions were first excised from the fish body and immediately placed in Bouin's fixative (75 ml saturated aqueous picric acid solution, 25 ml formalin, and 5 ml glacial acetic acid). The internal organs were carefully dissected out from the body, cut into small pieces of 3 to 5 mm and immediately placed in the fixative. All tissues were kept in a volume of fixative at least 20 times the volume of the tissue and kept overnight.

3.5.2. Processing

The fixative was washed out of the sample by 70% alcohol. Next the tissues were subjected to routine processing (Schäperclaus, 1986). Dehydration of the tissues was done by passing through a graded series of alcohol solutions (70%, 90% and 100%). The tissues were then placed in xylene and infiltrated with molten paraffin (melting point 58-60^{0}C) and allowed to cool and harden.

3.5.3. Sectioning

The hardened paraffin containing the tissues were trimmed into rectangular blocks, mounted on the 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 stretched on warm distilled water and the slides were dried by keeping them overnight at 40^{0}C.

3.5.4. Staining

Before staining, the sections were completely dewaxed by placing in xylene for 5 to 10 min. For Haematoxylene-Eosin staining, the sections were then transferred to water by first removing the xylene in absolute alcohol for 5 min and passing the sections through descending grades of alcohol, e.g. 90%, 70%, 50% and 30%. The sections were then stained with hematoxylene and washed thoroughly with water to remove the excess stain and observed under the microscope to check the differentiation level. The nuclei were stained blue. The sections were then passed through ascending grades of alcohol and stained with alcoholic eosin. The excess eosin was washed in 90% alcohol and again observed under the microscope to check the differentiation level. If the counterstaining was satisfactory, the tissues were further dehydrated in absolute alcohol, cleared in xylene and mounted in DPX mountant.

Some of the sections were also stained with Giemsa in order to observe the presence of bacteria in the tissues.

3.6. Haematological techniques

For microscopic examination of the blood and to determine the total erythrocyte count and haemoglobin content of blood of normal and infected fishes, the following methodologies were followed.

3.6.1. Blood sampling

Fishes were netted out from their aquariums and held in benzocaine solution at a dilution of 25 mg/litre in water for 1 to 2 min for anesthetization. The caudal region of the fish was cut (Fig. 8a) and the blood was collected in a tube rinsed with EDTA solution (1mg/ml). Both RBC counting and haemoglobin content measurement was done immediately after collection of the blood.

3.6.2. Smear preparation and staining of blood

For preparation of blood smears, a fresh drop of blood obtained by cutting the caudal region of the fish (Fig. 8a) was placed on a grease free slide (Fig. 8b). The grease free slide was prepared by keeping the slide in chromic acid for 48 hours after which it was thoroughly rinsed with tap water and dried and kept in alcohol and benzene (1:1). Before using, the slide was wiped with a clean cloth and drawn through flame (Schäperclaus, 1986). A thin film of the blood was drawn with the help of another slide held at 45^0 (Fig. 8c). The smears were stained by Leishman's stain and Wright's stain.

3.6.3. Blood studies

For RBC counting, the clean and dry erythrocyte pipette was first rinsed with 1% Sodium Citrate to prevent clotting of the blood in the tube. Blood was then sucked to the 0.5 mark. The blood sticking to the tip of the pipette was wiped off. Hendrick's fluid (sodium sulfate 10g, sodium chloride 2.5g, sodium citrate 1.5g, glacial acetic acid 50ml, distilled water to make upto 500ml) was then sucked into the pipette upto the 101 mark. The ends were closed with the thumb and the contents of the tube were mixed thoroughly. The dilution of blood : fluid was 1: 200. The fluid was then blown out from the lower stem upto 1.0

Plate III

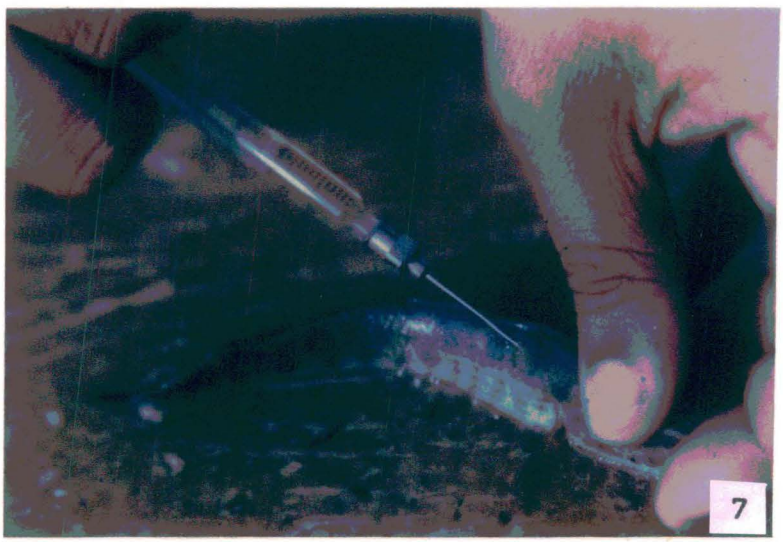
Fig. 7. Administration of intramuscular injection in fish, *Channa punctatus*.

Fig. 8. Blood smear preparation :

a. Cutting the caudal region of the fish

b. Placing a drop of blood on a slide

c. Smearing technique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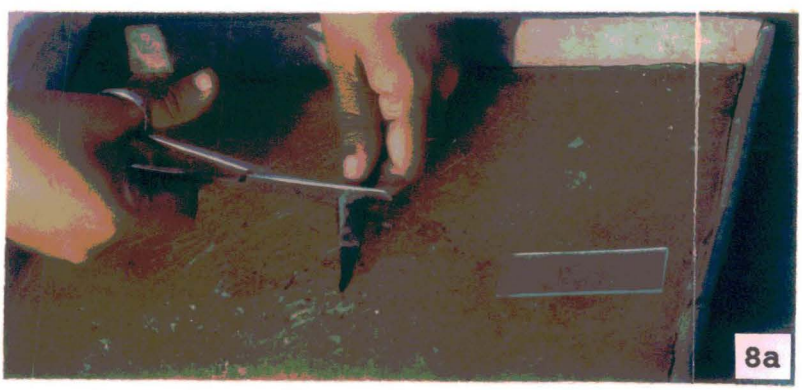
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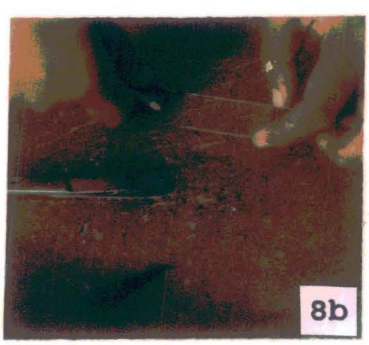
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8c

mark. The pipette was then held on the platform of the haemocytometer (Fein-Optik, Blankenburg, Germany) so that it makes 45^0 angle with it. A drop of the diluted blood from the tube was placed very carefully. Blood overflowed into the grooves of H. The coverslip was placed over the counting chambers and after 5 min counting was done (Swarup *et al*, 1981).

Haemoglobin content of the blood samples was determined by the acid haematin method (Swarup *et al*, 1981).

3.7. Characterization of the pathogenic bacteria based on some virulent factors

The virulence of a bacteria is often found to be related to certain characteristics like ability to hydrolyze gelatin, protein, lipid etc. The presence of such virulent factors in the pathogenic strains was therefore tested according to the following methodology.

Lipid hydrolysis

Tween 80 hydrolysis test was done for determining lipolytic activity of the bacteria. Tween 80 is the oleic acid ester of a polyoxyalkylene derivative of sorbitan. Tween 80 nutrient agar was streak inoculated with the test culture and incubated at 30^0C . The plate was observed each day for an opaque halo of precipitation around the growth that indicates hydrolysis of the Tween.

Haemolysin production

Tryptone Soya agar plates containing 2% fish blood was spot inoculated with the pathogenic bacteria and incubated for 24 hours at 30^0C. A clear zone around the colonies indicated the presence of haemolysin in the bacteria.

Lecithinase production

The lecithovitellin agar plates were streaked with the pathogenic bacteria and incubated for 5 days. The plates were observed daily for growth and opalescence under and around the growth.

Casein hydrolysis

Plates of milk agar were inoculated with the pathogenic bacteria and incubated at 30°C and examined daily for upto 14 days for clearing of the medium around the bacterial growth.

3.8. Determination of LD_{50} values of the pathogenic bacteria R1, R2 and R3

The three pathogenic bacteria, R1, R2 and R3 were grown in Brain Heart Infusion Broth at 30°C for 48 hours. These cultures were used to make five 10 fold dilutions of bacterial cell suspension in sterile 0.85% NaCl (w/v). The number of cells was calculated by the spread plate method. 0.05 ml of each of these diluted cell suspensions were injected intraperitoneally to 10 healthy fishes (*Heteropneustes fossilis*). The doses received by the fishes ranged from $5x10^7$ to $5x10^3$ cfu. Each of the 10 control fishes received 0.05 ml of sterile 0.85% NaCl solution. Following injection, the fishes were observed for a 15 day period to record the appearance of disease symptoms and mortality. The dead fishes were immediately sacrificed and parts of liver and kidney were incubated in nutrient broth for re-isolation of the bacteria. The lethal dose-50% end point (LD_{50}) was calculated from the relationship between the probits of percentage mortalities and the logs of the dilution series of bacterial suspension.

3.9. Preparation of cell free culture filtrates from bacteria

Brain heart infusion broth was prepared, taking 1000 ml for each bacteria by dissolving 37 grams of broth powder in 1000 ml distilled water. The media were distributed into ten 250 ml Erlenmeyer's flasks each containing 100 ml of the media and sterilized in an autoclave at 115°C for 20 min. The three virulent bacteria were first inoculated into 10 ml of brain heart infusion broth. After 24 hours incubation at 30°C, 0.5 ml of this culture was used to inoculate each Erlenmeyer's flask containing 100 ml of fresh BHIB and incubated in a rotary shaker at 30°C at a speed of 120 revolutions per min. After 48 hours, the broth cultures were centrifuged at 10,000 g for 30 min at 4°C. The supernatant

solution was filter sterilized by passing through a G5 sintered filter. The filtrate was tested for presence of bacteria by streaking in nutrient agar slants. Absence of any growth in the slants after 96 hours confirmed that the filtrate was free from bacteria.

3.10. Method for protein estimation

The soluble proteins were estimated following the method of Lowry *et al* (1951). Initially an alkaline mixture was prepared by mixing of 0.5 ml of 1% $CuSO_4$, 0.5 ml of 2% sodium potassium tartarate, 50 ml of 2% Na_2CO_3 dissolved in 0.1(N) NaOH. Finally, reaction mixture was prepared by mixing 0.1 ml of the protein sample, 0.9 ml of water and 5 ml of the alkaline mixture and incubated for 10 min. Subsequently, 0.5 ml of folin-phenol reagent (Folin-phenol : water :: 1:1) was added and again incubated for 15 min. In case of blank, distilled water was used instead of protein sample. At the end of the incubation period, O.D. values of each sample were determined by Systronics spectrophotometer at 710 nm. Quantity of the protein was estimated following the standard curve made with bovine serum albumin (BSA).

3.11. Gel filtration

Eight grams of Sephadex G-200 (Pharmacia) was soaked in 300 ml Tris-HCl buffer (pH- 7.4) for 72 hours. The swelled beads were then loaded in a chromatography column fitted with a G2 filter at the base. Buffer was allowed to run through the column for 12 hours. When the column was not in use, 0.02% Sodium azide was added to the buffer to prevent growth of any bacteria. The size of the column was 20 cm x 2 cm. Two ml of the sample was loaded at the top of the column. The sample was eluted with 0.5 M Tris-HCl buffer, pH 7.4, and fractions, 3 ml each were collected at a flow rate of 0.6 ml per min. The fractions were assayed immediately or stored at $4^{o}C$ for not more than 24 hours.

3.12. Assay of extracellular products

3.12.1. Protease assay

Protease assay was quantified as described by Thune *et al* (1982). A sample of 0.1 ml was added to 0.9 ml of 0.1 M tris-HCl buffer pH 7.2 followed by 0.5 ml of 0.5% azocasein substrate (Sigma). After 30 min incubation at 37^{0}C, the undigested azocasein was precipitated at 4^{0}C with 3.5 ml of 5% trichloroacetic acid. The precipitate was pelleted by centrifugation at 10,000 g for 10 min at 4^{0}C. Equal volumes (1 ml) of the supernatant and 1(M) NaOH were mixed and the released azo dye was measured by reading the absorbance at 440 nm in a Spectrophotometer (Systronics). A blank was prepared by using sterile BHIB media as the sample. Protease activity was expressed as direct absorbance at 440 nm.

3.12.2. Haemolysin assay

Fish blood was collected from a 20 cm fish (*Clarias batrachus*), weighing 35 to 40 gms, by cutting the tail region. The blood was taken in a tube containing equal volume of Alsever's solution (20.5 g glucose, 8 g sodium citrate. $2H_2O$, 0.55 g citric acid. H_2O, and 4.2 g NaCl in 1 L distilled water). The contents of the tube were mixed and used immediately or stored at 4^{0}C for no longer than 24 hour before use. Before use the red cells were pelleted by centrifugation, then washed three times in 0.01M Sodium phosphate buffered saline (PBS), pH 7.2. The assay mixture was 3.7 ml PBS, 0.8 ml of sample, and 0.5 ml of red blood cell suspension. The assays were incubated in round bottomed polypropylene centrifuge tubes at 25^{0}C for 1 hour. The unlysed cells and debris were removed by centrifugation at 12000 g for 10 min at 4^{0}C, and the absorbance of the supernatant was read at 541 nm. A unit of haemolytic activity was defined as the activity required in a sample volume of 0.8 ml to produce a supernatant with an absorbance of 1.0 at 541 nm under the assay conditions described (Allan and Stevenson, 1981).

3.13. Isolation of Microorganisms from the ulcers of diseased fishes.

3.13.1. Isolation of Bacteria

The ulcerated area was dissected out asceptically from the fishes and then surface sterilization was done with 0.1% mercuric chloride. The dissected tissues were placed in 100 ml conical flasks containing 15 ml nutrient broth medium supplemented with glucose. The fishes were then dissected asceptically and parts of liver and kidney were similarly placed separately in nutrient broth media. The flasks were incubated at 37^{0}C for 72 hours. The tissues were then removed and the cultures were observed under microscope. Only bacteria were found to be present in the cultures. Then the cultures were diluted by serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}) and 1 ml of each of these dilutions were added to 20 ml of nutrient agar media in 90 mm diameter sterile petridishes. Single separated colonies on agar plates were selected and streaked in nutrient agar slants and incubated for 24 hours at 30^{0}C. Thus isolated pure cultures were obtained. Code names were given to each of the isolates and stored at 4^{0}C for characterization and identification.

3.13.2. Isolation of fungus

The ulcer portion of the skin was dissected out asceptically and placed in sterile Glucose-Peptone broth without antibiotics and incubated for 24 hours. Next the growing fungal hyphae were transferred to GPB containing antibiotics. After 72 hours, the fungal culture was transferred to GPYA medium and after incubation for 72 hours it was maintained at 4^{0}C in GPYA.

3.14. Morphological and biochemical tests for characterization of the isolated bacteria

For characterization of the bacteria that were isolated from the ulcers of epizootic ulcerative syndrome positive fishes, a number of specific physiological and biochemical examinations were performed. The methodologies followed during these tests are described below. The three pathogenic bacterial isolates R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*) which were already identified (Pradhan, 1992) were used as type strains for comparing the pathogenic *Pseudomonas* and *Aeromonas* isolates.

3.14.1. Morphology of cells

Shape of cells

To examine the shape and size of cells , a drop of cell suspension of the test organism was placed on a clean grease-free slide, air dried and stained with carbol fuchsin (phenol 85g, basic fuchsin 15g, ethanol 250 ml, distilled water 1250 ml : 1 volume diluted with 10 volumes of distilled water) and observed under microscope. Diameter was measured with a standard occular micrometer.

Motility

To detect the motility, tubes of motility medium were stab inoculated to a depth of about 5 mm. The tubes were incubated at 28^{0}C and the turbidity pattern of the medium was observed for 5 days. The bacterial cultures in nutrient broth were also observed under a phase contrast microscope to determine their motility.

3.14.2. Physiological and biochemical tests

Gram Staining

Smears of test organisms made from 24 hour old cultures on nutrient agar slant with sterile distilled water were made on the centre of clean grease-free slides. The smears were air dried, heat-fixed and flooded with crystal violet (crystal violet 2g, 95% alcohol 20 ml, 1% aqs. ammonium oxalate soln. 80 ml) stain for 1 minute, washed for 3 seconds with tap water, flooded with Burke's iodine (Iodine 1 g, potassium iodide 2 g, distilled water 1000 ml) and allowed to react for 1 minute. Slides were washed in 95% ethanol, which was poured drop by drop holding the slides in slanting position against white background till no color came out from the lower edge of the slides. After washing with tap water, the smears were stained with saffranin for 1 minute and rinsed with water. The slides were air dried and observed under microscope (Bartholomew, 1962).

Production of Catalase

The organisms were incubated for 24 hours on a slope of nutrient agar and 1 ml of 3% Hydrogen Peroxide solution was poured down the slope. Immediate evolution of gas indicated the presence of catalase activity. (Cowan and Steel, 1993).

Production of Oxidase

A 24 hour old culture of the bacterial isolates grown on Nutrient Agar (glucose free) were taken and smeared across a filter paper moistened with freshly prepared 1% tetramethyl-p-phenylenediamine dihydrochloride with a glass rod. The appearance of a dark purple colour within 30 seconds indicated a positive reaction (Cowan and Steel, 1993).

Oxidation or Fermentation of glucose

Oxidation or fermentation of glucose was done by inoculating duplicate tubes of Hugh and Leifson's medium containing glucose as the carbohydrate, 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. The tubes were incubated at 30°C and observed for 14 days. If the blue color of the medium changes to yellow in the open tube only, it indicated acid production from glucose by oxidation. If both tubes change to yellow it indicated that glucose can also be broken down fermentatively (Hugh and Leifson, 1953).

Acid and gas production in media containing different carbohydrates

The medium used to detect acid production from carbohydrates was a modified Hugh and Leifson's medium in which agar was not added. The following carbohydrates were tested : L-arabinose, D-glucose, D-fructose, sucrose, D-lactose, adonitol, D-sorbitol, mannitol and meso-inositol. The liquid medium was inoculated and incubated at 30°C for 14 days. If the color of the medium changed from blue to yellow, it indicated acid production. To test for gas production, inverted Durham's tubes filled with the medium was inserted into the broth. The gas, when produced, was accumulated at the top of the inverted Durham's tube (Cowan and Steel, 1993).

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 (0.8% Sulphanalic acid in 5N Acetic acid) followed by 1 ml of reagent B (0.5% α-napthylamine in 5N Acetic acid). Appearance of red color indicated presence of nitrite. Zinc dust was added to the culture tubes showing negative test for nitrite; appearance of red color indicated presence of nitrate in culture tubes that was not reduced to nitrite by the test organisms. Any gas production in the Durham's tubes was noted (Cowan and Steel, 1993).

Indole production

Tryptone broth was inoculated and incubated at 30°C for 48 hours. Indole production was tested by adding 0.5 ml of Kovac's reagent (p-dimethylaminobenzaldehyde 5g, Amyl alcohol 75 ml, Conc. HCl 25 ml) and examined for 1 min. Appearance of a red color in the reagent layer indicated indole production (Cowan and Steel, 1993).

Levan formation

Nutrient agar plates and slants containing 4% sucrose were inoculated and observed for production of levan after incubating the plates and slants at 30°C for 72 hours (Cowan and Steel, 1993).

Methyl red reaction

Glucose Phosphate medium was inoculated and incubated at 30°C for 5 days. Two drops of Methyl red solution (Methyl red 0.04g, Ethanol 40 ml : methyl red was dissolved in ethanol and diluted with distilled water to 100 ml volume) was then added, shaken and examined. A positive MR reaction was shown by the appearance of a red color at the surface.

Acetylmethylcarbinol production : the Voges-Proskauer test

After completion of the methyl red test, 0.6 ml of 5% α-naphthol solution in ethanol and 0.2 ml of 40% Potassium hydroxide aqueous solution was added and well shaken. The tubes were then held in a sloping position to increase the area of the air-liquid interface and examined after 15 min and 1 hour. A positive reaction was indicated by a strong red colour (Cowan and Steel, 1993).

Pigment production

To test for pigment production by Pseudomonads, King, Ward and Raney's media (A and B) were used. After inoculating, medium A was incubated at 30°C for 24 to 96 hours and examined daily for pigment production. Medium B was incubated at 30°C for 24 hours followed by room temperature (22-25°C) 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.

Hydrolysis of gelatin

Gelatin agar plates were inoculated and incubated for 3 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 stab inoculated with a straight wire, incubated at 28^{0}C and observed daily for 30 days for presence of liquefaction.

Arginine hydrolysis

The arginine agar was inoculated by stabbing with a straight wire and a layer of sterile molten paraffin was poured with a pipette to a depth of about 2 cm. The tubes were incubated at 30^{0}C and observed daily for 5 days for a color change of the medium. A positive reaction was indicated by change of the yellow-orange color of the medium to red.

Citrate utilization

The bacteria were inoculated as a single streak over the surface of the slope of Simmon's citrate medium. The tubes were examined daily for 7 days for growth and color change. Utilization of citrate was shown by a streak of growth and change of the color of the medium to blue.

Hydrogen sulphide production

Tubes of the modified motility medium containing cystein and ferrous ammonium sulfate were stab inoculated with the bacteria. A lead acetate paper was inserted between the cotton plug and the tube and incubated at 28^{0}C. The tubes were examined daily for blackening of the media and the paper.

3.15. Microscopical examination of the isolated fungus

A portion of the ulcer tissue was taken and smeared on a clean slide. The smear was stained with cotton blue and was observed under the microscope.

During the isolation of the observed fungus, growing hyphae from the tissue in GPB was separated and placed on a slide and again stained with cotton blue and observed under the microscope.

Similarly, fungal hyphae from the pure culture maintained in PDA and GPYA was placed on a slide and after staining with cotton blue, it was observed under the microscope.

3.16. Antimicrobial sensitivity test by the disc diffusion method

The following commercially available antimicrobial sensitivity discs were used: Penicillin (10 units), Streptomycin (10 μg), Kanamycin (30 μg), Ampicillin (10 μg), Chloramphenicol (30 μg), Gentamycin (10 μg), Norfloxacin (10 μg), Co-trimoxazole (25 μg), Erythromycin (15 μg), Tetracyclin (30 μg), Nalidixic acid (30 μg), and Amoxicillin (30 μg) (Hi-media Laboratories, India). One ml of 18 hour culture of the test bacterium was inoculated in a conical flask containing 20 ml of sterile Mueller Hinton Agar cooled to 45^{0}C and shaken to mix completely. Then, it was poured on sterile petridishes of 9 cm diameter and allowed to solidify. Subsequently, antimicrobial sensitivity discs were placed asceptically on the solidified medium keeping a distance of approximately 4 cm between each disc. The plates were incubated at 30^{0}C for 18 hours. Appearance of clear zones surrounding the discs were noted and the diameters of the clear zones were measured. Zone diameters, less than 12 mm was denoted as resistant, higher than 22 mm as highly sensitive and between 12 to 22 mm as sensitive.

3.17. Determination of Minimum Inhibitory Concentration (MIC) of oxytetracycline

The minimum inhibitory concentration of oxytetracycline against the fish pathogenic bacteria was determined by serial tube dilution method. R1, R2 and R3 were first transferred from their stock cultures to 5 ml Mueller Hinton broth and incubated for 48 hrs at 30^0C giving a final cell density of $1x10^9$ cells/ml. Using inoculation loops, bacteria from the broth cultures were transferred to a series of tubes containing 5ml Mueller-Hinton broth and oxytetracycline. Oxytetracycline were added to the tubes in 2 fold dilution from freshly prepared concentrated stock solution asceptically. The concentration of oxytetracycline ranged from 0.005 μg / ml to 163.84 μg / ml. The tubes inoculated with R1, R2 and R3 were incubated for 24 hours at 30^0C. The minimum concentration showing no visible growth of bacteria was recorded to be the minimum inhibitory concentration.

3.18. Bath treatment of experimentally infected fishes with oxytetracycline

3.18.1. Preparation of bath solution

Bath treatment of infected fishes was carried out in round plastic treatment tanks measuring 35 cms in diameter and 45 cms in height. Each tank was filled with 20 litres of water having the same temperature (28^0 to 30^0C) and other water parameters as that of the maintenance aquariums. The tanks were labeled according to the concentration of oxytetracycline and time of exposure. A concentrated oxytetracycline solution (100 mg/ml) was then prepared in sterile distilled water. Required amount of this solution was added in the buckets in order to get the desired final concentrations of 50 μg/ml, 100 μg/ml, 200 μg/ml and 500 μg/ml of oxytetracycline.

3.18.2. Inoculation of fishes

Bacterial suspensions of R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*) were injected intramuscularlly (0.5 ml / 100 gm body

weight) to healthy *Channa punctatus* in pure and mixed condition. The concentration of bacterial suspension in each case was $1x10^7$ cfu/ml. Following injection, the fishes were kept in experimental aquaria measuring 45 x 30 x 30 cm and routine maintenance was continued. A control set of fishes received only sterile saline suspension.

3.18.3. Treatment

Fishes inoculated with the pathogenic bacteria were netted out of their experimental aquaria after 24 hr and placed in the treatment tanks. Ten fishes were placed in one tank at a time and exposed to fixed concentrations of oxytetracycline for the desired time periods. Separate nets were used for R1, R2 and R3 in order to avoid any contamination. After the treatment period, the fishes were released into clean aquaria without being subjected to any further stress. In each set, a group of 10 infected but untreated fishes were used as control.