Materials and Methods

3.1. FISH

3.1.1. Collection of diseased fish

Naturally infected fish Katla (*Catla catla*), Mrigel (*Cirrhinus mrigala*), Shole (*Channa striata*) and Punti (*Puntius* sp.) showing ulcerative lesions (Fig. 2 and 3) were collected during winter months of the year 2005-2006 from different affected ponds in various locations of the Darjeeling and Jalpaiguri districts of West Bengal (Fig. 4 and 5) and were used for the isolation of bacteria. Infected fish were brought alive to the laboratory for experimental work.

Fig. 2 Naturally EUS infected *Cirrhinus mrigala*

Fig. 3 Naturally EUS infected *Catla catla*

3.1.2. Collection and maintenance of healthy fish for experimental works

Healthy air breathing fish (*Channa punctatus*), collected from nearby fish farms of Darjeeling district of West Bengal with no history of EUS infection were used for experimental work. Fish were maintained in the laboratory in glass aquaria measuring 90 × 35 × 35 cm 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.

Fig. 4. An EUS infected pond in Lataguri, Jalpaiguri

Fig. 5. Fishermen netting in the affected pond

3.2. COMPOSITION AND PREPARATION OF 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:

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

All the ingredients were dissolved 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 and mixed to the OF medium to give a final concentration of 1%. It was then distributed ascetically into sterile test tubes.

Tween 80 medium

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 (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

Gelain was socked 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.

Milk Agar

Skimmed milk powder	5 g in 50 mL distilled water
Agar	1 g in 50 mL distilled water

The two ingredients were prepared separately and autoclaved at 115°C for 20 min. They were cooled to 45°C and mixed aseptically.

Lecithovitellin Agar

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

Decarboxylase media (Møller, 1955)

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 (HiMedia Laboratories, Mumbai, India) and distributed into test tubes containing inverted Durham's tubes, and sterilized at 115°C for 20 min.

Nutrient Gelatin

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

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

Peptone Water

Peptone	10 g
NaCl	5 g
Distilled water	1000 mL

The solids 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.

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

3.3. COMPOSITION AND PREPARATION OF BUFFERS AND REAGENTS

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

***TE Buffer*:** 10mM Tris-HCL, 1mM EDTA, Ph 8.0.

50X TAE Buffer (1 lt): Tris base: 242 g, glacial acetic acid: 57.1 mL 100 mL 0.5M EDTA, pH 8.0. The volume is adjusted to 1 Lt with distilled water and autoclaved at115°C for 10 min.

***RBC Lysing Buffer*:** Tris base: 260 mg, NH_4Cl: 770 mg, distilled water: 100 mL, pH 7.2.

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- dimethylaminobenzaldehyde: 5 g, amyl alcohol: 75 mL, cons 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% aqs. Ammonium oxalates soln.: 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.

3.4. BACTERIA

3.4.1. Isolation of bacteria

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 (HiMedia Laboratories, Mumbai, India) 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 (HiMedia Laboratories, Mumbai, India) and mixed thoroughly. The mixture was then poured on sterile petri dish (90 mm diameter) and allowed to solidify for overnight at 30°C. Colonies grown on the agar plates were selected and then streaked on to nutrient agar slants to incubate at 30°C for 24 hrs.

Each isolate was given a particular code name and stored at 4°C. For routine experimental works, the isolates were subcultured by growing in nutrient broth for 24 hrs at 30°C.

3.4.2. 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).

3.4.2.1. 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 turbidly pattern was observed for 5 days.

3.4.2.2. 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 hrs 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 (Barrow and Feltham, 1993).

Production of oxidase

Twenty four hrs grown bacterial culture on nutrient agar (glucose free) were taken and smeared across a filter paper moistened with freshly prepared 1% tetramethy-p-phenylenediaminedihidrochloride with a glass rod. The appearance of a dark purple colour within 30 s indicated a positive reaction (Barrow and Feltham, 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 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 (Barrow and Feltham, 1993).

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. The other tube was left open. Both the tubes were incubated at 30°C for at least 7 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

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 (Barrow and Feltham, 1993).

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).

Resistance to antibiotic cephalothin

The resistance to cephalothin of each isolate was detected by the disc diffusion method. Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India) plates (90 mm diameter) were inoculated with 0.1 mL of 18 hrs old culture of test bacterium in nutrient broth. The antimicrobial discs of cephalothin (HiMedia Laboratories, Mumbai, India) were applied on the bacterial culture plates and incubated at 30°C for 24 hrs. Appearances of clear zones around the discs were noted and the diameters of the clear zones were measured. Zone

diameters were interpreted as sensitive, intermediate or resistant based on the manufacturer's instructions.

3.5. PATHOGENICITY TEST OF THE ISOLATED BACTERIA

All the isolates were tested for their ability to induce ulcers in healthy *C. punctatus* fish weighing of 40-50 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 side of the fish with a

Fig. 6. Intramuscular administration of bacterial suspension to fish, *Channa punctatus*

28 gauge needle attached with a 1.0 mL insulin syringe (Fig. 6). The needle was inserted from behind to the front at an angle of 20° to the body axis.

3.6. CHARACTERIZATION OF ISOLATED BACTERIA BASED ON SOME VIRULENCE FACTORS

The presence of extracellular virulence factors such as haemolysin, lipase, amylase, caseinase, lecithinase and gelatinase in the isolated bacteria were determined by the following tests

Lipid hydrolysis

Tween 80 hydrolysis was conducted for determining the presence of lipase in the bacteria. Tween 80 medium plate was streaked and incubated at 30°C. The plate was examined each day for an opaque halo of precipitation around the growth that indicated hydrolysis of Tween 80 (Barrow and Feltham, 1993).

Haemolysin production

Tryptone soya agar (HiMedia Laboratories, Mumbai India) plate containing 5% defibrinated sheep erythrocytes was spot inoculated and incubated for 24-48 hrs at 30°C. The presence of haemolysin was indicated by a clear zone around the bacterial growth (Santos *et al.*, 1988).

Starch hydrolysis

Starch hydrolysis was conducted to determine the presence of extracellular enzyme amylase in the test bacterium. Plates of nutrient agar containing 0.2% soluble starch were streaked and incubated at 30°C for 5 days. The plates were then flooded with Burke's iodine solution. A positive result was indicated by the clear colourless zone around the growth (Barrow and Feltham, 1993).

Casein hydrolysis

Casein hydrolysis was carried out to determine the presence of caseinase in the bacteria. Plates of milk agar were streaked with the bacteria and incubated at 30°C and examined daily upto 14 days. A clear zone around the bacterial growth indicated the positive result (Barrow and Feltham, 1993).

Lecithinase production

The lecithovitellin agar plate was streaked and incubated for 5 days. The plate was observed daily and the presence of lecithinase in the test bacterium was indicated by a clear zone of opalescence under and around the growth (Barrow and Feltham, 1993).

Gelatin hydrolysis

Gelatin hydrolysis was conducted to determine the presence extracellular enzyme gelatinase in the test bacteria. Nutrient gelatin was inoculated with the sample bacteria and incubated at 30°C for 24- 48 hrs. The medium was chilled in a refrigerator for about 2 hours before examination. A positive result was indicated by the liquefaction of gelatin medium whereas a gelled medium after chilling represented a negative result (Barrow and Feltham, 1993).

3.7. CYTOTOXIC EFFECT OF BACTERIAL ISOLATES ON HEAD KIDNEY CELLS OF HEALTHY FISH

For analysis of the cytotoxic effect, bacterial isolates were grown in nutrient broth at 30°C for 24 hrs in an orbital shaking incubator. The bacterial culture was then centrifuged at 10.000 rpm for 10 min at 4°C and the supernatant was collected carefully. The cell free culture was obtained by passing the supernatant through a 0.45 μm cellulose acetate membrane filter (Sartorius). The total protein content of the filtrates was estimated by Lowry's method (Lowry *et al.*, 1951).

Serial double dilutions of filtrates (1; 1/2; 1/4) were prepared using sterile nutrient broth to judge the cytotoxic affect on head kidney cells separated from a healthy *Channa punctatus* fish. Head kidney was collected aseptically from healthy *Channa* fish and dissociated in phosphate buffered saline (PBS, pH 7.2) (HiMedia Laboratories, Mumbai, India) with the help of stainless steel wire mesh. The cell suspension was then washed twice with PBS by centrifugation at 1500 rpm for 5 min and further kept at 4°C with RBC lysis buffer for 15

min. After lysis of the RBC, cell suspension was further centrifuged and suspended in L-15 medium Leibovitz (Sigma, USA). Final volume of cells was adjusted to 10^5 in 2 mL culture medium.

Bacterial cultural filtrates (100 µL) was added to10^5 head kidney cells, on a sterile culture plastic petri plate supplemented with 10% heat inactivated sterile fetal calf serum (Sigma, USA). Control set was prepared using 100 µL of sterile nutrient broth instead of culture filtrates. Culture plates were then incubated at 25°C for 6 hrs in humidified atmosphere with 5% CO_2 in air. Cell survival after 6 hrs of culture was judged by trypan blue dye exclusion test which indicated uptake of blue dye by death and dying cells. Counting of the cells was made with haemocytometer.

3.8. ANTIBIOTIC SUSCEPTIBILITY TEST BY DISC DIFFUSION METHOD

The antibiotic susceptibility of each isolate was detected by the disc diffusion method. The following antimicrobial sensitivity discs (HiMedia Laboratories, Mumbai, India) were used to determine the antibiotic sensitivity of the bacterial isolates: ampicillin (10µg), erythromycin (15µg), streptomycin (10µg), tetracycline (30µg), nalidixic acid (30µg), gentamycin (10µg), kanamycin (30µg), chloramphenicol (30µg), novobiocin (30µg), sulphadiazine (15µg) and rifampicin (10µg). 0.1 mL of 18 hrs old culture of test bacterium grown on nutrient broth was inoculated in a conical flask containing 20 mL of sterile Muller Hinton Agar cooled to 45°C and mixed thoroughly. The mixture was then poured on sterile Petri dish (90 mm diameter) and allowed to solidify. Antibiotic sensitivity discs were put aseptically on the solid medium maintaining a distance of approximately 4 cm between each disc and incubated at 30°C for 24 hrs. Appearances of clear zones around the discs were noted and the diameters of the clear zones were measured. Zone diameters were interpreted as sensitive, intermediate or resistant based on the manufacturer's instructions.

3.9. ISOLATION OF DNA

3.9.1. Isolation of bacterial genomic DNA

A single c.f.u. of test bacterium was picked up and grown in 20 mL Luria-Bertani (LB) medium (HiMedia Laboratories, Mumbai, India) at 30°C for 18-24 hrs. A volume of 1 mL bacterial cell culture was taken in a centrifuge tube and spun at 10,000 rpm for 10 min. The supernatant was discarded and the pellet was mixed with 20 µL of TE (pH 8.0), 30 µL 10% SDS and 13 µL of proteinase K (10 mg /mL). The mixture was then incubated at 56°C for 16 hrs. The solution was then thoroughly mixed with 0.80 µL of CTAB (1% CTAB, 1M NaCl) and 100 µL of 5M NaCl. It was incubated at 65°C for 10 min and then centrifuged at 12,000 rpm for 10-15 min. The supernatant was gently taken to a fresh vial and 0.6 volume 70% ethanol was added to it. The mixture was spun at 12,000 rpm for 10 min and the supernatant was discarded. The precipitate DNA was dried and finally resuspended in 50 µL of TE, pH 8.0. The DNA was electrophoresed on 1.0 % agarose gel and visualized after staining with 0.2 % ethidium bromide (Ausubel *et al.*, 1992).

3.9.2. Isolation and purification of plasmid

Plasmids were isolated from bacterial cultures with a mini preparation kit (Bangalore Genei, India) according to manufacturer's instructions. A single colony of test bacterium was picked up and grown in 20 mL of LB medium at 30°C for 18-24 hrs. A volume of 1.5 mL bacterial cell culture was taken in a 1.5 mL eppendorf tube and centrifuged at 6,000 rpm for 10 min. The supernatant was discarded and 100 µL of ice cold solution I. was added to the pellet. The pellet was resuspended completely by vortexing and kept on ice for 5 min. To this mixture, 200 µL of solution II was mixed thoroughly at room temperature by inverting the tube several times. Then, 150 µL of chilled solution III was added and mixed thoroughly by inverting the tube. The mixture was kept on ice for 5 min and centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant was removed carefully into a fresh eppendorf tube and 450 µL of solution IV was added to it. It was kept at room temperature for 15 min and thereafter centrifuged at 10,000 rpm for 25 min. The

supernatant was discarded and the precipitate DNA was dried and resuspended in 20 µL TE, pH 8.0. The plasmids were electrophoresed on 0.8% agarose gel along with molecular weight marker (Lambda DNA Hind III digest, Bangalore Genei) and visualized after staining with 0.2 % ethidium bromide. Molecular weight analysis of plasmids was carried out by Image Aid software package (Spectronics, New York)

For purification, sample DNA was mixed with equal volume (1:1) of saturated phenol and centrifuged at 8,000 rpm for 2-5 min. The upper aqueous phase was removed into a fresh eppendoerf tube and an equal amount of 24:1(v/v) chloroform- isoamyl alcohol was added to it. To this mixture, 0.1 volume of 3M sodium acetate (pH 5.5) and 2 volumes of absolute ethanol was added to incubate at -20°C for 12-16 hrs. The mixture was then spun at ≥ 10,000 rpm for 25-30 min and the supernatant was discarded carefully. The precipitate DNA was washed with 70% alcohol at 10,000 rpm for 10 min to remove excess salts. The DNA was dried and resuspended in TE, pH 8.0 (Brown, 2000).

Spectrophotometric analysis of DNA: Sample DNA was used for the spectral reading at two different wavelenghs, 260 nm and 280 nm. The spectral reading at 260 nm has been calculated for the concentrations of DNA and dilution factor of the sample. An O.D value of 1 corresponds to 50 µg of double stranded DNA. A pure sample of standard DNA has O.D of 1.8 at 260 nm and 2.0 at 280 nm.

3.10. TRANSFORMATION OF PLASMID DNA TO *E. Coli* DH5α STRAIN

To detect the antibiotic resistance determinants in the plasmid, artificial transfer of plasmid content to the *E. coli* DH5alpha strain was done by $CaCl_2$ activation method (Sambrook *et al.*, 1989). Artificial transformation includes the following steps:

3.10.1. Preparation of competent cell

A single colony of *E. coli* DH5α was transferred into 100 mL of LB broth. The same was incubated in an orbital shaking incubator at 200 rpm at 37^0C until the O.D reached 0.3.

Culture was then cooled to 0^0C and centrifuged at 4,100 rpm for 10 min to discard the supernatant. Pellet was then resuspended in 30 mL chilled $MgCl_2$-$CaCl_2$ solution (80mM $MgCl_2$, 20mM $CaCl_2$) by gentle vortexing and centrifuged at 4100rpm for 10 min at 4^0C. Finally the supernatant was removed and the pellet was resuspended in 2mL of ice cold 0.1M $CaCl_2$.

3.10.2. Uptake of DNA by competent cell

DNA to be transformed was then added (≤50ng in 10 µL) to 200 µL of competent cells in chilled microcentrifuge tubes. Tubes were then transferred to circulating water bath preheated at 42°C for 90 s and then to ice for 2 min. After this 800 µL of LB broth was added and incubated at 37°C for 45 min in water bath. Transformed competent cells were then transferred onto LB agar plates with appropriate antibiotics and incubated at 37°C for the growth of the transformed colonies.

3.11. CONJUGATIONAL GENE TRANSFER

Each potential donor was incubated overnight in LB broth at 30°C and *E. coli* DH5α nalidixic acid resistant and plasmid free recipient strains were incubated in the same medium at 37°C to an equal density. Broth conjugation was performed by mixing equal (1:1) volume of donor and recipient strains and incubated at 25°C for 24 hrs without shaking. A 10 fold serial dilution of each mating mixture were spread on LB agar plates supplemented with 10 µg/mL nalidixic acid and an inhibiting concentration of antimicrobial agent to which the potential donors were resistant. Colonies growing on these double selective plates after 24 to 48 hrs of incubation at 37°C were treated as putative transconjugants. Suspected transconjugants were replica plated on antibiotic containing media and screened for plasmids. Transfer frequencies were calculated as the mean number of transconjugants per initial number of recipients.

3.12. PCR DETECTION OF THE CYTOLYTIC ENTEROTOXIN GENE AND EXTRACELLULAR AEROLYSIN GENE

Detection of both cytolytic enterotoxin and aerolysin genes in the various *Aeromonas* isolates were investigated by using the PCR primer combination strategy of Kingombe *et al.* (1999) with the primers AHCF1 (5´-GAG AAG GTG ACC ACC AAG AAC A-3´) and AHCR1 (5´-AAC TGA CAT CGG CCT TGA ACT C-3´). These primers were used to simultaneously detect the presence of cytolytic enterotoxin gene and extracellular aerolysin gene both in bacterial genome and plasmids of *Aeromonas* bacteria isolated from affected fish. The primers were synthesized by Sigma, USA. The PCRs used here were performed in a final volume of 25 µL containing of 5 µL DNA, 0.5 µL of a mixture containing 10 mM deoxyribonucleotide triphosphate, 2.5 µL of 50 mM $MgCl_2$ solution, 2.5 µL of 10X PCR buffer, 1.0 µL of a 20 µM solution of each primer, 1.0 µL of *Taq* DNA polymerase (Bangalore Genei, India) at 3 U/µL, and 11.5 µL of double-distilled sterile water. PCR was performed under the following conditions: Denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 64°C for 30 s, and extension at 72°C for 30 s. After the final cycle, an extension at 72°C was allowed for 7 min. PCR was performed in an automated thermal cycler (Peqlab, South Korea) and the amplicons were visualized after electrophoresis in a 1.5% agarose gel stained with 0.2 % ethidium bromide along with 500 bp DNA marker (Bangalore Genei, India). The specificity of the primer combination was corroborated with negative PCR results obtained by using *E. coli* DH5α strain.