MATERIALS AND METHODS

PART 1

III.1 Biochemical Characters

III.1.1 Collection of Wellwater samples

Materials

Sterile Mac Cartney bottles

Methods

Fresh wellwater samples were collected in sterile Mac Cartney bottles and screwcapped. In one hour time the samples were brought to the laboratory.

III.1.2 Isolation of Escherichia coli strains from Wellwater

Materials

- Sterile pipettes
- Mac Conkey broth (Single strength)
- Mac Conkey agar
- Nutrient agar

Methods

From each bottle 4 ml of water sample was pipetted out into 20 ml of sterile Mac Conkey broth in Mac Cartney bottle and were incubated overnight at 37⁰C. Loopful of Mac Conkey broth containing acid and gas production was then
inoculated by streaking on Mac Conkey agar plate and incubated at 37°C overnight. Pure colonies were identified and inoculated on sterile Mac Conkey agar plates by streaking and incubated at 37°C overnight.

Mac Conkey broth was used to find acid and gas production if *E. coli* strains were present in wellwater samples. Mac Conkey agar medium was used to make plates for the cultivation and isolation of pure colonies of *E. coli* strains. Nutrient agar slants were used for stocking *E. coli* isolates.

### III.1.3 Subculturing

From Mac Conkey agar plates pure single colonies of *E. coli* strains were subcultured on nutrient agar slants and stocked for further investigations.

### III.1.4 Identification of *E. coli* isolates

*E. coli* stocked cultures were inoculated on Mac Conkey agar plates to obtain pure single colonies and were then subjected to the following identification procedures:

- Motility test
- Biochemical tests

**Motility test**

A suspension of *E. coli* strains in a suitable medium (Peptone broth) which was maintained alive was made into a thin film for microscopic examination. The culture was placed on the slide and covered and then viewed under low power and then high power of a microscope.
**Materials and Methods**

**Hanging drop method**

The drop to be examined was suspended on the undersurface of the coverslip. The bacterial suspension was taken and placed over the centre of the coverslip. The coverslip was then inverted and placed over the depression slide so that the drop of suspension would be hanging over the cavity of the slide. This was mounted on low and high power with coverslip facing objective.

**Biochemical tests**

(a) **Carbohydrate fermentation tests**

Peptone broth with bromothymol blue as indicator was the medium in which different sugars were added like Glucose, Lactose, Sucrose and Mannitol (GLSM).

The test tubes with sugar medium were inoculated with *E. coli* isolates and incubated overnight at 37°C. Acid formation was shown by change in colour of the indicator. Gas production was noticed by the accumulation of gas collected in the Durham's tubes.

(b) **Indole test**

The medium, peptone water was inoculated and incubated overnight at 37°C. 0.05 ml of Kovac's reagent was added and shaken gently. A red coloured ring indicated a positive reaction.

Xylool was used to extract indole if Ehrlich's reagent was added instead of Kovac's reagent.

(c) **Citrate utilization test**

The test bacteria were cultured on the medium and incubated at 37°C for 24 hours in slants.

Positive test was blue colour with streak of growth. Negative test was original green colour with no growth.
(d) **Urease test**

The test bacteria from a solid medium culture were streaked on the surface of the agar slants of the urease medium, incubated overnight at 37°C. Colour change of the medium to pink was taken as indicative of positive test.

(e) **Methyl red test**

*E. coli* strains were inoculated in 5 ml of sterile glucose phosphate broth. After overnight incubation at 37°C, a few drops of methyl red solution were added. A positive methyl red test was shown by the appearance of a bright red colour indicating acidity.

(f) **Voges Proskauer test**

*E. coli* isolates were cultured in glucose phosphate peptone broth for 24 hours. To the culture 1 ml of 40% KOH was added and then 3 ml of 5% solution of α-naphthol in absolute alcohol. The mixture was aerated by vigorous shaking. A positive reaction was indicated by the appearance of pink colour in 3 minutes and later to crimson red in 30 minutes.

(g) **Differential coliform test**

The Eijkman test is usually employed to find out if coliform bacilli detected in the presumptive test are *E. coli*. After the usual presumptive test, subcultures were made from all tubes showing acid and gas (loopful of culture from broth) to fresh tubes of single strength Mac Conkey medium already warmed to 37°C. They were incubated at 44°C and examined after 24 hours. Incubation at 44°C should be carried out in thermostatically controlled water bath that do not deviate more than 0.5°C from 44°C. Those showing gas in Durham’s tubes contained *E. coli*. Further confirmation of the presence of *E. coli* can be obtained by testing for indole production and citrate utilization.
III.1.5 Collection of clinical *Escherichia coli* isolates

Human (clinical) *E. coli* isolates were procured from Modern Diagnostic Centre, Kottayam and Doctor's Diagnostic Centre, Arpoorkara. The clinical isolates of *E. coli* were inoculated on Mac Conkey agar plates and incubated overnight at 37°C to obtain pure single colonies and were stocked on nutrient agar slants for further investigations.

III.1.6 Isolation of human clinical *Escherichia coli* strains

**Materials**

1. Culture media

The following media were used:

(a) Mac Conkey agar: Mac Conkey agar medium was used to make plates for the cultivation and isolation of pure colonies of *E. coli* strains.

(b) Nutrient agar: Nutrient agar slants were used for stocking *E. coli* isolates.

**Methods**

Fresh fecal samples of human beings were collected using sterile cotton swabs and inoculated on Mac Conkey agar plates and incubated overnight at 37°C. Single colonies of *E. coli* strains were again inoculated on Mac Conkey agar plates by streaking and incubated to purify the isolates.

III.1.7 Subculturing

From Mac Conkey agar plates pure single colonies of *E. coli* strains were subcultured on nutrient agar slants and stocked in refrigerator at 4°C for further investigations.
III.1.8 Identification of *E. coli* isolates

*E. coli* stocked cultures were inoculated on Mac Conkey agar plates to obtain pure single colonies and were then subjected to the following identification procedures:

- Motility test
- Biochemical tests
- Differential coliform test

The above three tests such as motility test, biochemical tests and differential coliform test were carried out as in the identification of *E. coli* isolates from wellwater samples.

III.1.9 *Escherichia coli* isolates from Avian fecal matter

Four Bird species were subjected to investigation for the isolation of *E. coli* strains from the Bird droppings. Two species were terrestrial, i.e., Crows and Fowls while the other two species were aquatic, i.e., Ducks and Teals.

1. **Corvus splendens protegatus** (Madarasz or Common House Crow)

   - Class : Aves
   - Order : Passeriformes
   - Family : Corvidae

   Uniformly glossy, jet-black crow with dusty grey neck, sexes alike. Resident, very common and abundant, especially in the vicinity of the homesteads and copra drying yards along backwaters. Decidedly urban and gregarious. They are locally known as "Kaikka". They are commensal of man, omnivorous and are useful scavenger in towns. An inveterate pilferer of anything that can be eaten and audacious in its methods of acquiring it. It is a ruthless
persecutor of defenceless birds. It has community roosts where large numbers gather from considerable distances each night. Breeding activities are between February and April (Salim Ali, 1984).

2. **Gallus domesticus** (Domestic Fowl)
   
   Class : Aves  
   Order : Galliformes  
   Family : Phasianidae  

   Locally known as “Kozhi”. These are terrestrial birds with short beak. Characteristically omnivorous. They have the peculiar habit of scratching the ground for grain, insects, worms etc. Fowls are reared in houses for eggs and meat. Broiler chickens are reared in large numbers in poultry farms for meat.

3. **Anas domesticus** (Domestic Duck)
   
   Class : Aves  
   Order : Anseriformes  
   Family : Anatidae  

   These are aquatic birds with broad and depressed beak adapted for feeding on various diet and covered with a soft sensitive membrane. Its feet are transformed into a swimming organ, with webbed toes. They are good swimmers, omnivorous and feed on fishes, snails, grains, bugs etc. It’s local name is “Tharavu”. Ducks are reared in large numbers in farms for eggs and meat.

4. **Anas crecca crecca** (Linnaeus)
   
   Class : Aves  
   Order : Anseriformes  
   Family : Anatidae
It is locally named as "Eranda". Its size is that of a half grown domestic duck. The male is pencil Grey with chestnut head and a broad metallic green band running backward from the front of the eye to the nape, bordered above and below with whitish. A tricoloured wing-bar, black, green and buff is particularly conspicuous in flight. The female is mottled dark and light brown, with pale underparts and black and distinctive green wing speculum. The bird is migratory, especially peninsular migrant and winter visitor. It is seen during daytime on the open Vembanad lake (Backwaters of Kerala) and feed on paddy fields. Its food consists largely of vegetable matter. Garganey or blue winged teal, Anas querquedula (Linnaeus) is an international migrant visiting India during winter from Siberia and is the commonest and most abundant of the migratory ducks in Kerala (Salim Ali, 1984).

III.1.10 Collection of samples of droppings of Birds

Materials

- Sterile cotton swabs
- Sterile test tubes, cotton plugged

Cotton swabs were prepared using absorbent cotton, covered with brown paper and autoclaved.

Test tubes were plugged with nonabsorbent cotton and sterilized by keeping in the hot air oven at 160°C for one hour.

Methods:

Using the sterile cotton swabs, samples of fresh droppings were collected and kept in labelled sterile test tubes plugged with cotton and brought to the laboratory. Sufficient number of avian fecal samples were collected in order to get a minimum of 30 E. coli isolated of each bird species under investigation.
### III.1.11 Isolation of *Escherichia coli* from fecal samples of Avian fauna

**Materials**

- Culture media

The following media were used.

**Mac Conkey agar**: Mac Conkey agar medium was used to make plates for the cultivation and isolation of pure colonies of *E. coli* strains.

**Nutrient agar**: Nutrient agar slants were used for stocking *E. coli* isolates. Culture methods for isolation of *E. coli* strains

**Methods**

Fecal samples were inoculated on Mac Conkey agar plates by swabbing and kept in incubator at 37°C overnight. Single colonies of *E. coli* strains were again inoculated on Mac Conkey agar plates by streaking and incubated overnight.

### III.1.12 Subculturing

From Mac Conkey agar plates pure single colonies of *E. coli* strains were subcultured on nutrient agar slants and stocked for further investigations.

### III.1.13 Identification of *Escherichia coli* isolates

*E. coli* stocked cultures were inoculated on Mac Conkey agar plates to obtain pure single colonies and were then subjected to the following identification procedures:

- Motility test
- Biochemical tests
- Differential coliform test
The above three tests such as Motility test, Biochemical tests and differential coliform test were employed as in the identification of *E. coli* isolates from wellwater and human fecal samples.

### III.1.14 Identification of biochemically Atypical *E. coli* strains from different sources

**Materials**

Same as for carbohydrate fermentation tests.

**Methods**

Peptone broth with bromothymol blue as indicator was the medium in which different sugars were added like glucose, lactose, sucrose and mannitol (GLSM).

The test tubes with sugar medium were inoculated with *E. coli* isolates from wellwater, human and avian sources and incubated overnight at 37°C. Acid formation was shown by change in colour of the indicator. Gas production was noticed by the accumulation of gas collected in the Durham's tubes. All sucrose non-fermenting *E. coli* isolates were identified as Classical *E. coli* strains and all sucrose fermenting *E. coli* isolates were identified as Atypical *E. coli* strains.
PART 2

III.2 Haemagglutination Property

Escherichia coli isolates from three different sources, namely, Wellwater, Clinical and Avian sources were examined for their Haemagglutination property.

Materials

1. Screw-capped bottle
2. Anticoagulant
3. Blood cell suspension of
   (a) Human blood (Venous blood)
   (b) Fowl blood (Collected from wing vein)
4. Glass slides
5. White glazed tiles
6. Normal saline

Cell suspension: 25% suspension of washed RBC in saline was used for slide agglutination test.

Methods

Slide agglutination test: Three drops of saline were taken separately on a large slide (3"x 2") or on a white glazed tile. Bacterial growth from a pure culture was emulsified in the saline drops to make homogeneous suspensions. One drop of human and fowl cell suspensions were mixed with 1st and 2nd drop respectively. The mixture was examined for aggregation of cells within 1 minute. Development of cell aggregates within one minute was taken as positive. The intensity of agglutination was graded as +, ++, or ++++. Autoagglutination of RBC suspensions was periodically checked with saline.
PART 3

III.3 Serotyping

Materials

- Nutrient Broth
- Formalin
- Antisera
- Buffered Formal Saline (BSF)
- Stock BSF 2.5%
- Working BSF 0.25%
- Mercuric iodide Stock Solution (SMIS)
- Bridge’s solution
- Acriflavine solution
- Merthiolate solution
- Thioglycollate media

Methods

Serotyping of *Escherichia coli* is achieved when all the three antigens viz., O, H and K are identified. If only O antigen has been identified and if it is O1 then it should be stated that the given strain belongs to serogroup O1. It is incorrect to say that the strain belongs to serotype O1. Similarly even when both O&H antigens have been identified but not K antigen, the word serogroup should be used. When all the three antigens have been identified then it is correct to refer to as a serotype. O1:K51:H7 is a serotype.

III.3.1 Determination of O Antigen

3.1a Inoculate the test strain into 5 ml of nutrient broth. Incubate overnight at 37°C (with shaking). Boil the broth with growth for 1 hour at 100°C in a
water bath. Add formalin to obtain a final concentration of 0.3%. Now it acts as antigen.

3.1b Take a microtitre plate and distribute the 16 pools (Table III.1) of O antisera in 25 μl quantities. Add 75 μl/ml of antigen prepared in 3.1a to each of the wells. Set up a control well containing 25 μl antigen and 75 μl saline. Incubate at 37°C overnight after packing the plate in tin foil.

Table III.1. *E. coli* 'O' Sera Pools

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Prepare the pools such a way that individual constituent sera of each pool will have final titre of 1:16 to 1:32 in the pool.

3.1c Observe for agglutination. Control well should not show any agglutination. If agglutination is seen in any one of the pools and no agglutination or only minor reaction is seen in other wells, assume that the test strain belongs to any of the serotypes included in the pool that has given agglutination. If agglutination is seen in more than one well, refer to 3.1e. (If agglutination is seen in none of the wells, refer to 3.1f. If agglutination is seen in all the wells or in the control well then the strain is rough).

3.1d When the agglutination is seen in only one well and no or minor reaction is seen in other wells put up the test as given in 3.1b and use factor sera constituting the agglutination pool instead of different pools and determine the O antigen of test strain.

3.1e If agglutination is seen in more than one well, then the antigen has to be tried against the factor sera constituting the different pools. If more than one factor sera shows agglutination with the test antigen, all these sera are to be titrated against the test antigen. The serum in which the test antigen gives agglutination matching the homologous titre determines the O antigen of the test strain.

3.1f When the test antigen shows no agglutination with any of the pools, it means that the strain in question is a) untypable b) possesses K antigen which has not been inactivated by heating at 100°C for 1 hour. Hence, reprocess the strain again at 121°C for 2.5 hours instead of at 100°C for 1 hour to determine the O antigen. If even then it does not show agglutination it is truly untypable.
III.3.2 Determination of H Antigen

To identify H antigen it is necessary to use cultures in which majority of cells are motile. Such cultures can be obtained by passaging strains in Cragie's tube containing semisolid agar few times. If it is difficult to obtain motile cultures at 37°C, improved motility can be obtained at 30°C.

3.2a Grow the test strain in nutrient broth for 4.5 hours in a shake culture. Then add formalin for a final concentration of 0.3%.

3.2b Distribute 6 H antisera pools (Table III.2) in 50μl quantities in a microtitre plate and to each add 50 μl of antigen prepared as per 3.2. Set up a control well containing 50μl of antigen and 50μl of saline. Cover the plate with tin foil and incubate at 52°C for 2 hours. Note the wells which show agglutination. Control well should not show any agglutination. Test carried out in Drayer's tube using 0.2 ml amounts of reagents gives better results than microtitre plate test.

3.2c Test the antigen against the individual factor sera that go to make the pool showing agglutination to arrive at the H antigen identity.

3.2d Titration usually is not required in determination of H antigen.

Table III.2. E. coli H serum pools

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Prepare the pool such a way that individual constituent sera of each pool will have a final titre of 1:16 to 1:32 in the pool.

**III.3.3 Determination of K Antigen**

Slide agglutination method has been used all along for the detection of *E. coli* K antigens, wherein, the live culture picked up from a nutrient agar plate is tried against the OK serum pools and individual OK sera by slide agglutination method to detect the K antigen. The same culture after being boiled at 100°C for one hour is again tried by slide agglutination against the OK sera to determine the O antigen. Because of some inherent drawbacks of the slide agglutination method, the WHO Collaborating Centre for Research & Reference on *E. coli* recommends counter current immunoelectrophoresis technique (CIEP) for the detection of K antigen.

3.3a Inoculate the test strain on two 14 cm nutrient agar plates by lawn method and incubate overnight at 37°C. Scrape the growth and suspend in 5 ml of normal saline. Heat suspension at 100°C for 1 hour in water bath. Centrifuge the heated suspension at 5000g for 30 minutes and take the supernate to conduct CIEP.

3.3b Prepare a 2"x3" slide for CIEP by overlying it with 1% agarose in veronal buffer, pH 8.6, punch holes as per the diagram. Charge the wells on the cathodic side using 20 µl of antigen diluted 1 in 100. Charge the wells on the anodic side using 20 µl quantities of different K sera pools.* Run the electrophoresis using 2.5 volts per cm for 1 hour at room temperature using Whatman filterpaper No. 7 as wicks and veronal buffer pH 8.6 in the trough.

* No standard pattern of pooling has emerged till now. 18 pools of 4 sera each according to their serial numbers have been used in WHO collaborating Centre for Research & Reference on *E. coli*. K88 and K99 have been omitted from pools.
3.3c Look for the precipitation which appears as a neat line between the antigen and any of the pools. Check the antigen against the constituent of the pools to arrive at K antigen identification.

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3.3d K88 and K99 being proteins and heat labile cannot be determined by CIEP. Slide agglutination against relevant K sera will easily detect these antigens.

III.3.4 Preparation of *Escherichia coli* diagnostic sera

Three types of antigens i.e. Somatic (O antigens), Capsular (K antigens) and Flagellar (H antigens) are known to be present in *Escherichia coli*. So, for complete serotyping, identification of all the antigenic components is necessary. About 170 different types of O antigens, 103 K antigens and 56 H antigens are known in *E. coli*. As such 170 O sera, 103 K sera and 56 H sera are required to be raised, absorbed and finally tested for any cross reactions, before attempting to serotype *E. coli* strains. To avoid confusion as a result of cross reactions it is appropriate to pay close attention to the minute details at all stages in the process.
The sera must be produced **ONLY IN RABBITS** and in no other animal species.

The production of *E. coli* diagnostic factor sera were considered under the following heads:

- Selection of immunising strains
- Preparation of immunising suspension
- Immunizing and bleeding schedules
- Preliminary testing of sera
- Absorption
- Final testing
- Preservation and storage
- Labelling
- Maintenance of records

**Publications consulted**


PART 4

III.4 Antibiotic Resistance Studies

Isolates of *Escherichia coli* from different sources, namely, Wellwater, Clinical and Avian sources were screened for resistance to Amikacin, Amoxycillin, Ceftazidime, Cefuroxime, Cephotaxime, Cloxacilline, Cephaloridine, Gentamicin, Kanamycin, Nalidixic Acid, Penicillin, Chloramphenicol, Nitrofurantoain, Norfloxacin, Ofloxaclin, Pefloxaclin, Streptomycin, and Tetracycline using commercial discs (HI MEDIA, Mumbai, BEACON, Navsari, VIBLES BIOTECH, Faridabad).

III.4.1 Antibiotic resistance studies

Materials

Antimicrobial Susceptibility Test Discs and Disc Petency used for the present study are:

- Amikacin (Ak) : 30 mcg
- Amoxycillin (Am) : 10 mcg
- Ceftazidime (Ca) : 30 mcg
- Cefuroxime (C8) : 30 mcg
- Cephotaxime (Ce) : 30 mcg
- Cloxacilline (Cx) : 5 mcg
- Cephaloridine (Cr) : 30 mcg
- Gentamicin (G) : 10 mcg
- Kanamycin (K) : 30 mcg
- Nalidixic Acid (Na) : 30 mcg
- Penicillin (P) : 10 mcg
- Chloramphenicol (C11) : 30 mcg
Nitrofurantoin (Nf) : 30 mcg
Norfloxacin (NF) : 10 mcg
Ofloxacin (OF) : 5 mcg
Pefloxacin (Pfs) : 5 mcg
Streptomycin (S) : 10 mcg
Tetracycline (T) : 30 mcg

Growth Medium: Simple non-inhibitory media supporting the growth of E. coli strains were used.

The medium suitable for antibiotic susceptibility test is Mueller Hinton agar medium of HI MEDIA or Nutrient agar medium.

Methods

Stokes method: Emulsified the colonies of E. coli isolates in a small volume of sterile peptone water or nutrient broth. Incubated at 37°C for 4 hours (or until faint turbidity obtained). This was the inoculum noted.

Lawned the inoculum over the surface of the plates of medium, using a sterile cotton swab. Dried the surface by allowing to remain at room temperature for 2-3 minutes. Aseptically, with the help of forceps, placed different antibiotic discs over the surface of the lawn. Care was taken to keep a distance of 1 cm in between the discs. Ensured proper contact of disc to the surface by applying a gentle pressure. Incubated the plates at 37°C overnight. Examined for zone of inhibition around each disc. Measured the diameter of zone of inhibition around each disc. A zone of inhibition of diameter 13 mm or more was taken as sensitive and below it as resistant (Monica Cheesbrough, 1989).
PART 5

III.5 Plasmid Profiles

Among 320 *Escherichia coli* isolates from three different sources like Wellwater, Clinical and Avian sources, 45 isolates (15 each from every source) were selected for Plasmid profile study.

III.5.1 Isolation of Plasmid DNA

Materials

- 25% Sucrose
- Lysozyme
- 10% SDS
- 10 N NaOH
- Ethidium bromide (10 mg/ml)
- 7.5 M Ammonium acetate
- TE pH 7.5
- RNase A (10 mg/ml)
- 100% Ethanol

Methods

The selected *E. coli* cultures from different sources like Human, Wellwater and Avian sources were streaked on LB agar plate to get single colonies. For the extraction of plasmids, single colony from the plate was inoculated in 1 ml LB medium containing 50 μg/ml ampicillin and grown by keeping overnight in 37°C shaker. 100 μl of overnight culture was inoculated in 5 ml LB medium containing 50 μg/ml of ampicillin and grown for five hours by keeping in 37°C shaker. The plasmid was extracted from the culture as per the method of Daniel *et al.* which is indicated in the flow chart.
Overnight culture (5 – 10 ml)

Resuspend pellet in 25% sucrose containing 30 mg/ml lysozyme, to a final volume of 200 μl

↓ Incubate at 37°C for 15 minutes

Add 400 μl of alkaline SDS solution

↓ 3% SDS
↓ 0.2 N NaOH
↓ Mix immediately, incubate 7 min at room temperature

Add 300 μl ice cold 3 M sodium acetate (pH 4.8)

↓ Mix immediately, spin at 15000 rpm for 15 min (4°C)

Transfer supernatant to new microfuge tube and add 650 μl of isopropanol (room temperature)

↓ Mix well, spin max. speed for 15 min (4°C)

Remove all liquid and resuspend pellet in 320 μl sterile distilled water

Add 200 μl of 7.5 M Ammonium acetate containing 0.5 mg/ml Ethidium bromide

↓ Add 350 μl Phenol chloroform (25:24)

↓ Mix well, spin at 15000 rpm for 5 min (room temperature)

Transfer aqueous phase to new microfuge tube and add 1 ml ice cold absolute alcohol

Keep at -70°C for 1 hour or at -20°C overnight

↓ Mix well, spin at 15000 rpm for 15 min (4°C)

Wash pellet in 70% ethanol (room temperature). Remove all liquid and resuspend in 40 μl TEl RNase 0.1 mg/ml
III.5.2 Agarose gel electrophoresis

Materials

- Agarose
- 5× TBE buffer
- Ethidium bromide
- Gel loading dye (0.25% Bromphenol blue in 40% glycerol).

Methods

The isolated plasmid DNA was loaded on 0.7% agarose gel electrophoresis prepared in 0.5xTBE. The gel was run for 4 hours at 100 volts. Then the gel was stained in 0.5 mg/ml of Ethidium bromide in water by keeping for 30 minutes. The gel was washed in sterile distilled water, viewed under UV transilluminator and photographed.

III.5.3 Transformation

The gel containing the plasmids which are common in majority of the strains in different sources were eluted and transformed to E. coli (Mos blue) strains and checked for the antibiogram.

- The band of interest from the agarose gel was cut and made into slices. Then it was kept in a 0.5 ml microfuge tube containing hole at the bottom packed with sterile glasswool. The tube was placed above 1.5 ml microfuge tube and spun for 2 minutes at 12000 rpm. The DNA from the collected solution was extracted once with phenol:chloroform: isooamy alcohol (25:24:1) and once with chloroform: isooamy alcohol (24:1). The DNA was precipitated by the addition of 1/10 volume of 3M sodium acetate pH 4.8 and 2.5 volume of 100% Ethanol and keeping at -70°C for 1 hour. Then the tube was spun at 12000 rpm for 10 minutes and the pellet was washed once with 70% Ethanol. The pellet was dried
and dissolved in 20 µl TE buffer and checked in 0.7% agarose gel electrophoresis and used for transformation.

**Reagents required**

- 100 mM CaCl$_2$ (Filter sterilized)
- LB Agar
- LB broth
- Mos blue cells
- Ampicillin
- SOC medium

Single colony of Mos blue cells was inoculated in 3 ml of LB medium and grown for overnight. 50 µl of overnight culture was inoculated into 3 ml LB broth again and grown till reaching 0.6 OD A600. The tube was kept in ice for 20 minutes and transferred to prechilled 1.5 ml sterile microfuge tube. It was then centrifuged for 5 minutes at 3000 rpm and the supernatant was discarded. The pellet was suspended in 0.5 ml of prechilled 100 mM CaCl$_2$. The tube was kept in ice for 20 minutes. Again it was centrifuged by keeping at 3000 rpm for 5 minutes at 4°C. The pellet was resuspended in 100 µl of ice cold 100 mM CaCl$_2$. The cells were used for transformation.

One microlitre of the eluted plasmid was added to the competent cells and kept in ice for 30 minutes. Heat shock was given by keeping at 41°C for two minutes. Immediately then kept in ice for 5 minutes. Then 450 µl of SOC medium was added and incubated in 37°C shaker with slow shaking (90 rpm). Then cells were screened in LB agar medium containing ampicillin.

The colonies appeared after overnight incubation at 37°C were inoculated and checked for plasmid as well as antibiotic resistance pattern.
Chapter IV

Results