CHAPTER III

MATERIALS AND EXPERIMENTAL
PART I

Section 1

III.1 Avian Fauna Subjected to Investigation

The following common bird species of Kerala were subjected to investigation.

III.1.1 Corvus splendens protegatus (Madarasz), (The Common House Crow)

Class: Aves
Order: Passeriformes
Family: Corvidae

Uniformly glossy, jet-black crow with dusky grey neck, sexes alike. Resident, are very common and abundant, low country, especially in the vicinity of the homesteads and copra drying yards along backwaters. Decidedly urban and gregarious. They are locally known by the name "Kakka." 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) (Plates 1 & 2).
III.1.2 *Phalacrocorax niger* (Vieillot), (Little Cormorant)

Class: Aves  
Order: Pelecaniformes  
Family: Phalacrocoracidae

Locally known as "Neerkakka," this is a glistening black, duck like water bird, larger than crow, with a largish stiff tail and slender compressed bill sharply hooked at the tip. It has a small white patch on throat, sexes are alike, found singly or in flocks, on or near water. They are local migrants. They migrate every year from Ambalavayal to Kumarakom for breeding during June-July and migrate back to Ambalavayal during November-December. Cormorants are almost exclusively fish eaters. They chase and capture their quarry under water, being expert divers and submarine swimmers. The birds nest during the rains on trees often standing in or near water. This is the most abundant bird species present in Kumarakom Tourist Complex near Kottayam (Salim Ali, 1984) (Plates 3 & 4).

III.1.3 *Bubulcus ibis coromandus* (Boddart), (Cattle Egret)

Class: Aves  
Order: Ciconiiformes  
Family: Ardeidae

Local names are 'Kalimunti,' 'Veliru.' Snow-white overall, the colour of the bill is yellow, sexes are alike. It is a resident bird which frequents paddy fields along the backwaters, cultivated and fallow land, chiefly in attendance on grazing cattle. Gregarious birds and food consists of grasshoppers, other insects, frogs, lizards and fishes. Large numbers of cattle egrets collect at night to roost in favourite trees (Salim Ali, 1984) (Plate 5).
III.1.4 *Anas crecca crecca* (Linnaeus), (Common Teal)

Class: Aves  
Order: Anseriformes  
Family: Anatidae

Local name is ‘Yeranda.’ It’s size is that of a half-grown domestic duck. The male is pencil grey, with chest nut head and a broad metallic green band running backward from the front of the eye to the nape, bordered above and below with whitesh. 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, specifically peninsular migrant and winter visitor. It is found during day time on the open Vembanad Lake (Kottayam backwaters), and flies inland at night to 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) (Plates 6 & 7).

III.1.5 *Anas domesticus*, (Domestic Duck)

Class: Aves  
Order: Anseriformes  
Family: Anatidae

Local name is ‘Tharavu.’ These are aquatic birds with broad and depressed beak adapted for feeding on various diet and covered with a soft sensitive membrane. It’s foot is transformed into a swimming organ, with webbed toes. They are good swimmers, omnivorous, and feed on fishes, snails, grains, bugs, etc. Ducks are reared in large numbers in farms for egg and meat (Plate 8).
III.1.6 *Gallus domesticus*, (Domestic Fowl)

Class: Aves  
Order: Galliformes  
Family: Phasianidae

Local name is ‘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 egg and meat. Broilers chickens are reared in large numbers in poultry farms for meat (Plate 9).

III.1.7 *Columba livia intermedia* (Strickland), (Indian Blue Rock Pigeon)

Class: Aves  
Order: Columbiformes  
Family: Columbidae

Locally called ‘Ambalapravu,’ ‘Kuttapravu,’ this is a familiar slaty grey bird with glistening metallic green, purple and magenta sheen on upper breast and round the neck. There are two dark bars on the wings. Sexes are alike. Colonies of these birds can be found on cliffs, on roof of houses, churches warehouses, etc. They are often seen gleaning in cut paddy fields and in the vicinity of towns and villages wherein they live. These are granivorous birds with short, conical beak (Salim Ali, 1984) (Plate 10).
Plate 1. *Corvus splendens protegatus* (Madarasz).
The Common House Crow (Locality: Kottayam)

Plate 2. *Corvus splendens protegatus* (Madarasz).
The Common House Crow (Locality: Ettumanoor)
Plate 3. *Phalacrocorax niger* (Vieillot) Little Cormorant. (Locality: Kumarakom Tourist Complex)

Plate 5. *Bubulcus ibis coromandus* (Boddart)
Cattle Egret (Locality: Paddy fields, Athirampuzha)

Plate 6. *Anas crecca crecca* (Linnaeus)
Common Teal (Locality: Kumarakom - Vembanad Lake)
Plate 7. *Anas querquedula* (Linnaeus)  
Garganey Teal (Locality: Kumarakom - Vembanad Lake)

Plate 8. *Anas domesticus* (Domestic Duck)  
(Locality: Athirampuzha)
Plate 9. *Gallus domesticus* (Domestic Fowl)  
(Locality: Poultry Farm, Kottayam)

Plate 10. *Columba livia intermedia* (Strickland)  
Indian Blue Rock Pigeon (Locality: Kottayam)
III.1.8 Collection of Droppings of Birds

A. Materials

1. Sterile cotton swabs
2. Sterile test tubes with cotton plug

Cotton swabs were prepared using absorbent cotton, covered with brown paper, autoclaved and dried in the oven.

Test tubes were plugged with non-absorbent cotton and sterilized by keeping in the hot air oven for 1 h at 160°C.

B. Methods

The night resting places of birds under investigation were located and the samples collected early in the morning between 5.30 a.m. and 6.30 a.m. before the birds leave the roosting places. In the case of community roosters like egrets (Bubulcus ibis) and cormorants (Phalacrocorax niger), the freshly voided faecal samples were obtained from the bottom of the trees in which they rest in large numbers, during the night time. Using the sterile cotton swabs, the droppings were collected and kept in labelled sterile test tubes plugged with cotton, and transported to the laboratory.

Pigeons being roof dwellers, samples of droppings were collected from their dwelling places. The faecal samples of domesticated birds like ducks and fowls were collected from the farm. The droppings of crow were easily obtained from the surface of plantain leaves in the premises of the houses, where they used to come and sit in large numbers, during the morning hours in search of food. The faecal
matter of the migratory bird 'teal' was collected by keeping those birds under captivity for a day, during the migratory season, when they come in large numbers.

A minimum of 50 faecal samples were collected, of each species of bird under investigation, on different days, from different localities and transported to the laboratory at the earliest, for further investigation.

III.1.9 Isolation of Bacteria from Faecal Samples of Avian Fauna

A. Materials

Culture media

The following media were employed.

(i) Nutrient agar

Nutrient agar was used as the general purpose medium in routine cultures and basal medium in blood agar preparation.

Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Agar</td>
<td>2 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

All the ingredients except agar were mixed and dissolved by heating to boiling and filtered if necessary. The pH was adjusted to 7.5 and the agar was added, mixed, heated to dissolve, filtered through cotton gauze and autoclaved at 121°C for 15 minutes.
(ii) Blood agar

Blood agar was used as the enriched medium in routine cultures. The medium was prepared by adding sterile blood to sterile nutrient agar that has been melted and cooled to 50°C. The bulk medium was then dispensed in petriplates.

(iii) Mac Conkey agar

Mac Conkey agar medium was used for the cultivation of enteric bacteria. NaCl was excluded from the ingredients to limit spreading of *Proteus* species.

**Composition**

- Peptone: 2 g
- Lactose: 1 g
- Sodium taurocholate: 0.5 g
- Agar: 2 g
- Neutral red solution 2% in 50% ethanol: 0.35 ml

Peptone and sodium taurocholate were dissolved in water by heating, followed by agar, dissolved by steaming, cleared by filtration whenever found necessary. pH was adjusted to 7.5. The medium was sterilized by autoclaving after adding lactose and neutral red solution. The bulk medium was then dispensed into petriplates.

(iv) Peptone broth

- Peptone: 1 g
- Sodium chloride: 0.5 g
- Distilled water: 100 ml
The ingredients were dissolved by heating. The pH was adjusted to 7.4-7.5. The broth was then dispensed into cotton plugged 5 ml tubes, and autoclaved at 121°C for 15 minutes.

B. Culture Methods for Isolation of Bacteria

A small portion of the surface of the well-dried nutrient agar plate, blood agar plate and Mac Conkey's agar plate were inoculated with the specimen. The inoculum was distributed thinly over the plate, by streaking it with the sterile platinum wire loop, in a series of parallel lines in different segments. The inoculum was incubated overnight at 37°C. Colony characters were observed and recorded. Sweep smear was prepared from blood agar plates and nutrient agar plates. Smear was Gram stained and observed under microscope in oil immersion.

C. Subculturing

From the Mac Conkey's agar plate the lactose fermenting (LF) and non lactose fermenting (NLF) colonies and from the nutrient agar plates, the morphologically distinct colonies, were subcultured in respective media till single colonies were obtained.

III. 1. 10 Identification of Bacterial Isolates

The culture and the single colony was subjected to all these identification procedures:

(i) By preparation of direct smear from droppings and sweep smear from culture.
(ii) By Gram staining and microscopy of the smear preparations.
(iii) By motility

(iv) By different biochemical tests

(v) By observing the morphology of the colonies.

(i) Preparation of direct smear from droppings and sweep smear from culture

A. Materials

Normal saline

Physiological saline (0.9%) was made to prepare saline smears of the bacterial strains.

- Sodium chloride 0.9 g
- Distilled water 100 ml

Sterilized by autoclaving at 121°C for 15 minutes.

B. Procedure

A drop of normal saline was placed on a clean slide. The swab with faecal samples was applied on the drop of saline and a thin smear was prepared. This was allowed to dry.

(ii) Staining solutions and reagents

Gram staining

(a) Methyl violet stain

- Methyl violet 6B 10 g
- Distilled water 1000 ml

The dye was dissolved in distilled water and filtered for use.
(b) Gram's iodine

Iodine crystals 10 g
Potassium iodide 20 g
Distilled water 1000 ml

Potassium iodide was dissolved in 250 ml water and then iodine was added, when dissolved, made up to 1 litre.

(c) Acetone 100%

(d) Safranine, Counter stain

Safranine O 20 g
Distilled water 1000 ml

Safranine was dissolved in distilled water and filtered.

Procedure

The smear was covered with a few drops of crystal violet and allowed to stain for 2 minutes. The dye was drained off and the smear rinsed in tap water. The smear was then covered with a few drops of Gram's iodine solution (mordant) for 1 minute. The iodine solution was drained off, and the smear was washed in a slow stream of water.

The smear was then exposed to a few drops of acetone (or absolute alcohol) for about 15-20 sec., ensuring effective contact of the solvent and the smear. The smear was rinsed in tap water until the decolourising solvent was completely removed. The smear was counterstained with safranine (1 min.), dried and examined under the microscope in oil using 100x objective.

Gram positive organisms appeared purple and Gram negative organisms red in colour. The morphology of the bacterial cells were observed and recorded.
(iii) Motility

A suspension of the microorganisms in a suitable medium which is maintained alive was made into a thin film for microscopic examination. The culture was placed on the slide and coverslip was placed over it. The slides were covered with petroleum jelly and viewed under low power and high power of phase contrast microscope.

Hanging drop method

The drop to be examined was suspended on the undersurface of the coverslip.

Preparation

A clean glass slide was taken and a small bead of plasticene was rolled into a ring of 1 cm diameter. The ring was placed over the surface of the slide. A clean coverslip was taken using a sterile wire loop. The bacterial suspension was taken and placed over the centre of coverslip. The slide was inverted with plasticene ring over the coverslip so that the drop remained in the centre of the ring. Slight pressure was applied so that the ring made contact with the coverslip. The slide and the coverslip was reverted by rapid movement. This was mounted on low and high power with coverslip facing objective.

(iv) Biochemical Tests for Identification of Bacteria

A. Preparation of indicators

(1) Bromothymol blue - 0.2% aqueous

Bromothymol blue 1 g
0.1 N NaOH 25 ml
Made upto 500 ml with distilled water.
(2) Methyl red - 0.05% aqueous
   Methyl red                   0.1 g
   Ethanol                     300 ml
   Distilled water             200 ml

(3) Neutral red
   2% in 50% ethanol

(4) Phenol red
   1 in 500 aqueous solution

B. Media and Reagents for Biochemical Tests

Commercially prepared reagents purchased from Hi Media Laboratories Pvt. Ltd., Bombay, were used for the study. The reagents were prepared according to the standard procedures (Cruickshank et al., 1975).

1. Carbohydrate Fermentation Tests

(i) Medium

Composition

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Bromothymol blue (0.2%)</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>Sugar</td>
<td>1 g</td>
</tr>
</tbody>
</table>

Sugars used were Glucose, Lactose, Sucrose and Mannitol (G, L, S, M).
Peptone and sodium chloride were dissolved in distilled water. The pH was adjusted to 7.5. The test carbohydrate and bromothymol blue was then added. Durham's tubes, one in each, were placed in test tubes with sugar medium and autoclaved at 10 lbs for 25 minutes.

**Test procedure and inference**

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

(ii) **Indole Test**

**Medium**

(a) Peptone water  
(b) Kovac's reagent

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amyl or Isoamyl alcohol</td>
<td>150 ml</td>
</tr>
<tr>
<td>p-dimethyl aminobenzaldehyde</td>
<td>10 g</td>
</tr>
<tr>
<td>Conc. HCl</td>
<td>50 ml</td>
</tr>
</tbody>
</table>

Aldehyde was dissolved in alcohol and acid was added slowly. The reagent was then stored in amber coloured bottles.

**Test procedure and inference**

This was tested on a 48 h culture of the organism in peptone water. Indole production was detected by Kovac's or Ehrlich's reagent which contains
4-p-dimethyl aminobenzaldehyde. This reacts with indole to produce a red coloured compound.

The medium peptone water was inoculated and incubated for 48 h at 37°C. 0.5 ml of Kovac’s reagent was added, and shaken gently. A red coloured ring indicated a positive reaction. Xylol was used to extract indole if Ehrlich’s reagent was used instead of Kovac’s reagent.

(iii) **Citrate utilization test**

Simmon’s Citrate Medium

**Composition**

- Sodium chloride \((\text{NaCl})\) 0.5 g
- Magnesium sulphate \((\text{MgSO}_4)\) 20 mg
- Ammonium dihydrogen phosphate \((\text{NH}_4\text{H}_2\text{PO}_4)\) 100 mg
- Potassium dihydrogen phosphate \((\text{KH}_2\text{PO}_4)\) 100 mg
- Sodium citrate \(2\text{H}_2\text{O}\) 500 mg
- Agar 2 g
- Bromothymol blue 0.2% 4 ml
- Distilled water 100 ml

The ingredients were dissolved in warm water. The pH was adjusted to 6.8 before adding the indicator bromothymol blue and filtered. This was then distributed in test tubes and autoclaved at 121°C for 15 minutes. The tubes were kept in inclined position to form slants.
Test procedure and inference

The test organism was cultured on the medium. Positive test was blue colour with streak of growth. Negative test was original green colour with no growth.

(iv) Urease Test

Medium

Christensen's urease agar

Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Sodium chloride (NaCl)</td>
<td>5 g</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate (K₂HPO₄)</td>
<td>2 g</td>
</tr>
<tr>
<td>Agar</td>
<td>20 g</td>
</tr>
<tr>
<td>Phenol red (1 in 500 aqueous)</td>
<td>6 ml</td>
</tr>
<tr>
<td>Glucose</td>
<td>10 g</td>
</tr>
<tr>
<td>Sterile urea solution (20%)</td>
<td>100 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1 litre</td>
</tr>
</tbody>
</table>

The ingredients other than urea were dissolved in warm water. The pH was adjusted between 6.8 and 6.9 and filtered. The medium was autoclaved at 121°C for 15 minutes after adding the indicator. To the sterilized molten (56°C), medium urea solution was added and dispensed into slants.
Test procedure and inference

The organism from a solid medium culture was streaked on the surface of the agar slant of the urease medium, incubated at 37°C for 24 h. Colour change of the medium to pink was taken as indicative of positive test.

(v) Methyl Red Test

Medium

Glucose phosphate peptone water

Composition

Peptone 5 g
Dipotassium hydrogen phosphate (K$_2$HPO$_4$) 5 g
Distilled water 1 litre

The ingredients were dissolved in water. The pH was adjusted at 7.6 and filtered. This was sterilized by autoclaving and 50 ml of sterile 10% solution of glucose was added.

Indicator Solution

Methyl red 0.1 g
Ethanol 300 ml
Distilled water 200 ml

Test procedure and inference

The test organism was inoculated in 5 ml of sterile glucose phosphate broth. After overnight incubation at 35-37°C, few drops of methyl red solution was added.
A positive methyl red test was shown by the appearance of a bright red colour, indicating acidity.

(vi) Voges Proskauer Test (VP)

Medium
Glucose phosphate peptone water

Reagents
Solution A Sodium hydroxide 400 g/l (40% w/v)
Solution B 5% solution of α-naphthol in absolute ethanol

Test procedure and inference

The test organism was cultured in a glucose phosphate peptone water for 48 h. To the culture 1 ml of 40% KOH was added followed by 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 development of pink colour in 2.5 minutes, becoming crimson red in 30 minutes.

(vii) Catalase Test

Hydrogen peroxide 3% H₂O₂ (10 volume solution)
Glass slide
Sterile wooden stick
Test procedure and inference

Hydrogen peroxide solution was taken on a clean slide and a small portion of bacterial growth from non-inhibitory solid medium was mixed to it with a sterile wooden stick, instead of platinum wire. Presence of effervescence indicated positive test.

(viii) Oxidase Reaction Test

Oxidase reagent

\[
\begin{align*}
\text{Tetramethyl-p-phenylene diamine dihydrochloride} & \quad 0.1 \text{ g} \\
\text{Distilled water} & \quad 10 \text{ ml}
\end{align*}
\]

Prepared fresh before use.

Test procedure and inference

A piece of filter paper was soaked with a few drops of oxidase reagent, a colony of the test organism from a non-inhibitory medium was then smeared on the filter paper. Development of purple colouration at the site of the smear within 30 seconds indicated positive test.

(ix) Hydrogen Sulphide Production Test

(a) Lead acetate paper strips

The strips of filter papers were soaked in a 50 g/l (5% w/v) solution of lead acetate (trihydrate) and allowed to dry. The dried strips were stored in an air tight container.
(b) Medium

Nutrient broth

Composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

All the ingredients were mixed and dissolved by heating. The pH was adjusted to 7.5 and the mixture was autoclaved at 121°C for 15 minutes.

Test procedure and inference

A filter paper soaked in 10% lead acetate solution was inserted into a tube containing the culture medium with inoculum. After overnight incubation the paper was examined. Blackening of paper indicated H₂S production.

III.1.11 Stock Medium Preparation and Stocking of the Identified Bacterial Isolates for Further Investigation

A. Materials

Semisolid nutrient agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone</td>
<td>1 g</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1 g</td>
</tr>
<tr>
<td>Agar</td>
<td>0.8 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
</tbody>
</table>
All the ingredients were mixed and dissolved by heating. The pH was adjusted to 7.5. The medium was poured into Bijou bottles, about one third, screw capped and autoclaved at 121°C for 15 minutes. After autoclaving the agar slants were prepared by keeping the bottle with the medium in a slanting position and allowing to solidify.

B. Stock Preparation

Stab Culture

The single colony was inoculated into the labelled Bijou bottle containing the stock medium, by stabbing, using a sterile straight wire loop. Incubated overnight for growth, the stock culture could be kept in the refrigerator for three months. Subcultured at intervals of three months for maintenance of the stock.

III.1.12 Human Bacterial Isolates

(a) Collection of human clinical bacterial isolates from hospitals

Human clinical bacterial isolates were procured from the Medical Centre, Kottayam, and stock was kept in refrigeration below 4°C.

(b) Collection and identification of faecal coliforms from human faecal samples

Fresh faecal samples of human beings were collected using cotton swabs, cultured, identified and made into stock for further investigation. The materials and methods are the same as for the bacterial isolation from avian faecal samples.
The following were the specimens obtained and isolated from human sources.

<table>
<thead>
<tr>
<th>Name of organism</th>
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<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Clinical</td>
</tr>
<tr>
<td>Urine</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>E. coli</td>
</tr>
<tr>
<td>Klebsiella sp.</td>
</tr>
<tr>
<td>Citrobacter sp.</td>
</tr>
<tr>
<td>Enterobacter sp.</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
</tr>
<tr>
<td>Alcaligenes faecalis</td>
</tr>
<tr>
<td>Providencia sp.</td>
</tr>
<tr>
<td>Aeromonas sp.</td>
</tr>
<tr>
<td>Proteus sp.</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>
PART II

Section 2

III.2  Antimicrobial Sensitivity Tests

A.  Materials

III.2.1  Antimicrobial Drugs and Disc Potency Used for the Present Study

Penicillin G (P)  10 U
Ampicillin (A)  10 µg
Tetracycline (T)  30 µg
Chloramphenicol (C)  30 µg
Streptomycin (S)  10 µg
Kanamycin (K)  30 µg
Gentamycin (G)  10 µg
Amikacin (Ak)  10 µg
Cephalordine (Cr)  30 µg
Cephalexine (Cp)  30 µg
Cotrimoxazole (Co)  10 µg
Trimethoprim (Tr)  10 µg
Nalidixic acid (Na)  30 µg
Norfloxacin (Nx)  30 µg
Nitrofurantoin (Nf)  50 µg
Furazolidone (Fr)  30 µg
Cephotaxime (Ce)  10 µg
Sterile discs

Antibiotic incorporated sterile discs, commercially available from Hi Media were used.

**Preparation of discs when standard discs are not available**

Penicillin, Ampicillin discs 10 U/disc or 10 mg/d
Benzyl Penicillin 1 mg = 1000 Units

**Penicillin**

Dissolved 10 mg of Benzyl Penicillin in 12 ml of sterile distilled water. Dipped sterile discs so as to soak in the solution and applied on the lawn.

**Ampicillin**

Dissolved 10 mg of Ampicillin in 20 ml of sterile distilled water. Dipped the sterile discs so as to soak in the solution and applied on the lawn.

**Growth medium**

Simple non-inhibitory media supporting the growth of test organisms were used. The medium suitable for antibiotic sensitivity test is Mueller Hinton Agar Medium of Hi Media.
B. Methods

III.2.2 Stokes Method

Emulsified the colonies of the test organism in a small volume of sterile peptone water or nutrient broth. Incubated at 37°C for 2-4 h (or until faint turbidity). This was the inoculum noted.

Lawned the inoculum over the surface of the petridish 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. Commercially available discs are provided with labelling, for example, Penicillin discs are with label (P). Ensured proper contact of disc to the surface by applying a gentle pressure. Incubated the plate 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).

Section 3A

III.3A Haemolysis

III.3A.1 Materials

(i) Anticoagulants

(a) Sodium citrate - 3.8%

(b) Alsever's Solution
<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
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</tr>
<tr>
<td>Sodium citrate</td>
<td>8.0 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.55 g</td>
</tr>
<tr>
<td>Glucose</td>
<td>20.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Dissolved the above chemicals in distilled water and sterilized by Arnold steam sterilizer for 20 minutes on three successive days. The solution was stored in tightly stoppered bottle for 2 to 3 weeks.

(ii) Normal saline

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.5 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

(iii) Sterilized syringe, needle, spirit, cotton

(iv) Screw capped sterile bottles

(v) Peptone water

(vi) Human blood

Venous blood, O, A and B groups

(vii) Rabbit blood - Collected by heart puncturing

(a) Preparation of blood cell suspension

Sterilized the screw capped bottles with a few drops of normal saline. Removed the cap and poured out the saline. Pipetted out 0.5 ml of 3.8% sterilized, filtered sodium citrate into the sterilized bottle.
The rabbit and human blood were collected into the bottles with anticoagulants. The citrate, blood ratio was 0.5:5 ml.

The blood was poured into the centrifuge tubes.

1. Centrifuged at 3000 rpm for 10 minutes—discarded the supernatent.
2. Resuspended, deposited in saline (excess)
3. Centrifuged again - discarded the supernatent-Resuspended, deposited in saline
4. Centrifuged again - discarded supernatent
5. Prepared 20-25% suspension i.e., 5 times dilution in normal saline.

III.3A.2 Methods

Test for Soluble Haemolysin

The pure culture of the test strain was inoculated in about 5 ml of peptone water or nutrient broth. It was incubated overnight and centrifuged at 3000 rpm for 30 minutes. To about 1 ml of the supernatent transferred into a separate tube, 0.1 ml of 25% RBC suspension was added and incubated at 37°C for half an hour. The tubes were then centrifuged and the supernatent examined for haemolysin. Majanta colouration of the supernatent was taken as positive test for haemolysin production. Observation was recorded. Cell control of the test contained peptone water (1 ml) + 0.1 ml of 25% RBC suspension (Cruickshank et al., 1975).
Section 3B

III.3B Haemagglutination Reaction

III.3B.1 Materials

1. Screw capped bottle
2. Anticoagulant
3. Blood cell suspension of
   (a) Human blood (Venous blood)
   (b) Rabbit blood (Collected from heart)
   (c) Fowl blood (Collected from the 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 tests.

III.3B.2 Methods

Slide Agglutination Tests

Three drops of saline were taken separately on a large glass 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, rabbit and fowl cell suspensions were mixed with 1st, 2nd and 3rd drop respectively. The mixture was examined for aggregation of cells for 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.

Section 4

III.4 Test for Bacteriocin Production

III.4.1 Materials

1. Mac Conkey agar
2. Peptone broth
3. Sterile cotton swab
4. Indicator strains of bacteria (Selected from the local isolates)
   14B *E. coli* atypical
   59B *E. coli* atypical
   22A *E. coli* classical
   26B *E. coli* classical
   51C *Enterobacter* sp.
   4B *Enterobacter* sp.
   6A *Klebsiella* sp.
   13A *Klebsiella* sp.
   15A *Citrobacter* sp.
   17B *Citrobacter* sp.
III.4.2 Methods

Procedure

Bacteriocin producing ability of the strains were tested using 10 susceptible strains belonging to coliform group, as indicator strains. The method employed was the plate diffusion technique.

The test bacterium was inoculated into peptone water and incubated for 3-4 h. From this the agar plate was inoculated as a broad streak on the centre of the culture medium with the help of the cotton swab. The plates were then incubated overnight at 37°C. The bacterial growth was scraped off with the edge of a clean glass slide, and the remaining residual growth were killed by exposure to chloroform vapour. Indicator strains of bacteria from broth culture were then streaked at right angles to the original inoculum with the wire loop. This was incubated overnight and the spectrum of inhibition of the indicator strains by the test strain was recorded (Ananthanarayanan and Paniker, 1990).

PART III

Section 5

III.5 Effect of Pesticides on Soil Microorganisms

III.5.1 Materials

Pesticides subjected to the present study

1. Nuvacron (Monocrotophos 36%)
2. Foratox (Phorate 10%)
3. Cuman (Ziram zinc dimethyl dithio carbamate 27%)
4. Carbofuran (Furadan)
5. BHC 50 WP

Working concentrations (in use concentrations) of the pesticides in agricultural practice.

1. Nuvacron 0.2 ml/100 ml
2. Foratox 1 g/100 ml
3. Cuman 0.3 ml/100 ml
4. Carbofuran 1 g/litre
5. BHC 5 g/litre

Medium

Soil extract stock

1000 g of sewed garden soil was mixed with 1000 ml of tap water and steamed in the autoclave for 30 minutes. A small amount of CaCO₃ was added and the whole was filtered through a double filter paper.

Soil extract agar

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1.0 g</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Soil extract</td>
<td>100 ml</td>
</tr>
<tr>
<td>Tap water</td>
<td>900 ml</td>
</tr>
</tbody>
</table>
The agar was dissolved in 900 ml of water by steaming it for an hour or more. 100 ml of the stock soil extract solution was added. Glucose was added first, prior to tubing. The pH was adjusted at 6.8 (Subba Rao, 1977a).

The medium was sterilized by autoclaving at 15 lbs pressure for 15 minutes.

Soil infusion

The fertile soil samples--50 different samples--from different localities were collected during a period of three months. The infusion of soil extracts were made using sterile normal saline. The infusion of soil extracts were filtered to get free from other particulate contamination.

Control Medium

Soil extract agar without pesticide was incorporated.

Experimental Medium

Soil extract agar with pesticide was incorporated.

Soil extract agar was prepared in six different conical flasks. The first one was taken for control and to the remaining flasks 2-6, added the working concentrations of the pesticides namely Nuvacron (Flask 2), Foratox (Flask 3), Cuman (Flask 4), Carbofuran (Flask 5) and BHC (Flask 6). All the six flasks were autoclaved.
III.5.2 Methods

Procedure

Pour plate method

0.1 ml each of the soil infusion of each sample was poured into the sterile petridishes numbered 1 to 6. The molten autoclaved medium (1-6) were kept in the waterbath adjusted to 40°C and poured into the petridishes with soil infusion. The first one was taken as control and numbers 2-6 experimental. These were incubated for 24 h. The colony count of the Gram positive as well as Gram negative organism of the control as well as experimental were taken, and from the data, percentage difference between the control and the experimental was calculated in the case of each pesticide. The experiment was repeated using 50 different soil sample infusions, using all the five insecticides.

Appropriate dilution of the infusion was decided by trials so as to get countable numbers of colonies (200-400) in the control plates.

Section 6

III.6 Bacteriological Studies on Water Samples

III.6.1 Materials

Collection localities of water samples

1. Well water (drinking water) from 10 different wells around Athirampuzha.
2. Pond water: 10 perennial ponds around Athirampuzha and Kottayam.
3. Paddy fields: Agricultural (10) around Kottayam and Athirampuzha.
4. Rivers: Meenachil (Pala) and its major tributaries: Kavanar (Kumarakom), Kodoor river (Kodimatha), Pennar (Athirampuzha), Mundar (Kottayam): 2 samples each (Total 10).

5. Minor tributaries of Meenachil river

(a) Streams of Kavanar (Kumarakom) 4 Nos.
(b) Streams of Kodoor river (Kodimatha) 2 Nos.
(c) Streams of Pennar (Athirampuzha) 4 Nos.

A total of 50 samples during monthly intervals in each season.

III.6.2 Methods

(i) Collection, preservation, transport and storage

Water samples for bacteriological analysis were aseptically collected into sterile Mac Cortney bottles, about 50 cms below the water surface. Bottles were pre-wrapped with filter paper to minimise contamination through handling. Samples after collection were immediately taken to laboratory for examination. The samples were preserved at 4°C upto 6 h, if immediate analysis was not possible.

(ii) Standard Plate Count (SPC)

1. Appropriate dilutions were selected according to the expected SPC. Generally undiluted (1:0) and diluted 100 times (1:100) are prepared. 1 ml and 0.1 ml, if plated from each of them will give a dilution of 1:0, 1:10, 1:100 and 1:1000 respectively. For this, the sample was shaken vigorously for 10 seconds and prepared an initial dilution 1:100 by pipetting 1 ml of the original sample into 99 ml dilution water (sterile distilled water) or 0.1 ml +
9.9 ml of distilled water. Additional dilutions were prepared in sterile dilution bottles using a separate pipette every time.

2. The nutrient agar medium and Mac Conkey agar medium were prepared. The medium after melting was placed in a waterbath maintained at 44-46°C for 1 h and the temperature was monitored.

3. 1 ml and 0.1 ml from the diluted samples were transferred in aseptic conditions to already marked plates (2 sets each) (sterilized). After delivering the sample, the tip of the pipette was touched to a dry spot in the plates.

4. About 20 ml of liquified media (44°C) were poured to these petriplates by gently opening the plates. The medium was mixed thoroughly with the sample in petriplates. When the media was solidified, the plates were inverted and kept for incubation at 37°C ± 0.5°C for 48 ± 3 h.

5. The colony counts were taken after incubation. The results were recorded and SPC/ml of water or CFU/ml of water was calculated.

Total bacterial count was obtained from nutrient agar plates and the total coliform count and the count of the non-lactose fermenting colonies were obtained from Mac Conkey agar plates.

IIII.6.3 Confirmatory Test for Faecal Coliforms

Eijkman Test

Eijkman elevated temperature test separates the organisms of the coliform group into those of faecal origin and non-faecal origin. A loopful of culture from
broth were transferred to Mac Conkey broth with durham’s tube, prewarmed to 37°C in a waterbath. The inoculated tubes were incubated at 44.5°C in a thermostatically adjusted waterbath for 24 h. Those showing gas in Durham’s tube contain *E. coli* of faecal origin.

**III.6.4 Physico-chemical Parameters**

(a) **pH**: Using pH meter. The pH of the water samples were determined by using syntronics digital pH meter 335.

(b) **Determination of salinity percentage of water samples.**

**Materials**

(i) **0.1 N AgNO₃**

16.96 g AgNO₃ in 1 litre water.

(ii) **8% solution of potassium chromate**

<table>
<thead>
<tr>
<th>Potassium chromate</th>
<th>80 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

**Procedure**

The salinity was determined by titration using standard silver nitrate. Potassium chromate was used as the indicator. The end point was determined by the appearance of slight red colour. Chlorosity and chlorinity and hence salinity could be calculated by the following equations (Trivedy and Goel, 1984).
Chlorosity \( = \) \( N_1 \times 3.5 \) W

Chlorinity \( = \) \( \frac{\text{Chlorosity}}{\text{Density}} \)

Salinity \( = \) \( (1.805 \times \text{Chlorinity}) + 0.03 \)

(c) Determination of organic content of water samples

**Materials**

(i) Glucose standard solution

- Glucose: 100 g
- Distilled water: 1000 litre

Glucose solution was prepared by dissolving 100 g of glucose in 1000 litre. It was freshly prepared every time when it was needed. Further solutions of various dilutions 10, 25, 50, 100 mg/ml glucose were prepared.

(ii) 0.1% anthrone solution

0.1 g of anthrone was dissolved in 500 ml of conc. analar sulphuric acid.

This solution was very carefully added to 200 ml of distilled water. 1 g thiourea was also added as preservative. This solution could be stored only for a maximum period of 2 weeks in refrigerator.

**Procedure**

The estimation of dissolved organic matter was done by colorimetric estimation. The method was based on the principle that on hydration with conc.
sulphuric acid at 100°C, sugars form coloured furan derivatives with anthrone reagent in strong sulphuric acid. Different carbohydrates reacted at different rates with the reagent.

25 ml anthrone solution was taken in a long test tube into which 15 ml of the water sample was added. The test tube was stoppered loosely. The tubes were well shaken and placed in boiling water bath for exactly 15 minutes. The blanks of standard glucose solutions were also treated in the same way. After cooling, the optical density was measured at wavelengths between 620 and 630 nm.

A standard graph was prepared. This graph was used to read off glucose concentrations of unknown samples.

Section 7

III.7 Survival of Coliforms in Water Samples

III.7.1 Materials

(i) Sterile water samples

10 ml quantities of water samples were tubed and autoclaved at 121°C for 15 minutes.

(ii) Broth culture of bacteria

Peptone broth was inoculated with the following bacteria and was incubated for two hours.

1. *E. coli*

2. *E. coli* atypical
3. *Klebsiella* sp.
4. *Citrobacter* sp.
5. *Enterobacter* sp.
6. *Salmonella typhi*
7. *Salmonella paratyphi*
8. *Vibrio cholerae*
9. *Proteus* sp.
10. *Pseudomonas* sp.

### III.7.2 Methods

The water from different sources were collected in sterile screw capped bottles. Water from wells, ponds, rivers streams, paddy fields, backwaters and sea were collected.

Loopful of two hours incubated broth culture was inoculated into sterile water samples. The inoculated water samples were incubated at room temperature.

Survival of bacteria in water samples was detected by subculturing from water samples on Mac Conkey agar plates. Absence of growth in the plate indicated the death of the organism in the tube.

### III.7.3 Environmental parameters

(a) **pH**: Using pH meter. The pH of the water samples was determined by using syntronics digital pH meter 335.

(b) Determination of salinity percentage of water samples.
Materials

(i) 0.1 M AgNO₃
16.96 g AgNO₃ in 1 litre water.

(ii) 8% solution of potassium chromate
Potassium chromate 80 g
Distilled water 1000 ml

Procedure

The salinity was determined by titration using standard silver nitrate. Potassium chromate was used as the indicator. The end point was determined by the appearance of slight red colour. Chlorosity and chlorinity and hence salinity could be calculated by the following equations (Trivedy and Goel, 1984).

\[
\begin{align*}
\text{Chlorosity} &= N_1 \times 3.5 W \\
\text{Chlorinity} &= \frac{\text{Chlorosity}}{\text{Density}} \\
\text{Salinity} &= (1.805 \times \text{Chlorinity}) + 0.03
\end{align*}
\]

(c) Determination of organic content of water samples

Materials

(i) Glucose standard solution
Glucose 100 g
Distilled water 1000 litre
Glucose solution was prepared by dissolving 100 g of glucose in 1000 litre. It was freshly prepared every time when it was needed. Further solutions of various dilutions 10, 25, 50, 100 mg/ml glucose were prepared.

(ii) 0.1% anthrone solution

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25 ml anthrone solution was taken in a long test tube into which 15 ml of the water sample was added. The test tube was stoppered loosely. The tubes were well shaken and placed in boiling waterbath for exactly 15 minutes. The blanks of standard glucose solutions were also treated in the same way. After cooling the optical density was measured at wavelengths between 620 and 630 nm.

A standard graph was prepared. This graph was used to read off glucose concentrations of unknown samples.
III.7.4 Statistical analysis of the data

The data obtained from the various experiments were statistically analysed for significance. The 'Z' test, 'Chi-square' test and Analysis of Variance were applied for the analysis and interpretation of the results (Gomez and Gomez, 1984).