CHAPTER II

MATERIALS AND METHODS

MATERIALS

1. Microbiological Culture Media
2. Chemical Reagents
3. Glasswares
4. Instruments

Microbiological Culture Media

1. Media for Total Aerobic Microbial Count
   - 1.1 Nutrient Agar (Difco)
   - 1.2 Sabouraud Agar (Difco)
2. Media for identification of the pathogenic microorganisms.
   - 2.1 Selective Media
     - 2.1.1 Brilliant Green Agar (Difco)
     - 2.1.2 Bismuth Sulfite Agar (Difco)
     - 2.1.3 Cetrimide Agar (Difco)
     - 2.1.4 Eosine Methylene Blue Agar (Difco)
     - 2.1.5 MacConkey Agar (Difco)
     - 2.1.6 Mannitol Salt Agar (Difco)
     - 2.1.7 Selenite Broth (Difco)
     - 2.1.8 Xylose-Lysine Desoxycholate (XLD) Agar (Difco)

2.2 Differential Medium

2.2.1 Triple Sugar Iron (TSI) Agar (Difco)

Chemical Reagents

1. Alcohol 95 %
2. Gram crystal violet solution
3. Gram iodine solution
4. Gram safranin solution
5. 1 N HCl
6. 1 N NaOH
7. Lacto-phenol cotton blue solution
8. N, N -dimethyl - p - phenylenediamine dihydrochloride

Glasswares

1. Beaker 250 ml, 500 ml, 1000 ml (Pyrex)
2. Bottle with cover 120 ml
3. Centrifuge tube
4. Cover glasses
5. Measuring cylinder 10 ml, 100 ml (Pyrex)
6. Petri dishes
7. Pipette 1 ml, 10 ml
8. Stirring rod
9. Volumetric flask (Pyrex)
10. Water bath

Instruments

1. Autoclave (Amsco International, S.A.)
2. Bacterial colony counter (New Brunswick Scientific)
3. Bunsen Burner
4. Centrifuge (Clay Adams)
5. Cyclo-Mixer (Clay Adams)
6. Hot air oven (Precision Thelco Model 27)
7. Incubator (Precision Thelco Model 6)
8. Microscope

METHODS

1. Preparation of samples

For a fluid sample that consists of a true solution, or a suspension in water or a hydroalcoholic vehicle containing less than 30 percent of alcohol, and for a sample that dissolves readily and practically completely suspend 10 ml in 90 ml of distilled water, proceed as directed under Total Aerobic Microbial Count and under test for *Staphylococcus, Pseudomonas, Salmonella* species and *Escherichia coli*.

For a solid sample that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend 10g sample in 90 ml distilled water, and proceed as directed under Total Aerobic Microbial Count, and under test for *Staphylococcus, Pseudomonas, Salmonella* species and *Escherichia coli*.

2. Total aerobic microbial count

Plate Method

Dissolve or suspend 10.0 g of sample if it is a solid, or 10 ml, accurately measured, if the sample is a liquid, in 90 ml distilled water to make 100 ml. Dilute further, if necessary, the fluid so that 1 ml will be expected to yield between 30 and 300 colonies. In the experiment the dilutions are 10^{-1}, 10^{-2} and 10^{-3}.

Pipet 1 ml of each dilution onto each of four sterile petri dishes. Promptly add to each two dishes 15 to 20 ml of Nutrient Agar

Medium and another two dishes of Sabouraud Agar Medium, that previously has been melted and cooled to approximately 45 C. Cover the Petri dishes, mix the sample with the agar by tilting or rotating the dishes, and allow the contents to solidify at room temperature. Invert the Petri dishes, and incubate at 37 C for 24 to 48 hours of Nutrient Agar Medium plates and at room temperature for 5 days of the Sabouraud Agar Medium plates.

Following incubation, examine the plates for growth, count the number of colonies, and express the average for the two plates in terms of the number of microorganisms per g or per ml of sample. If no microbial colonies are recovered from the plates representing the initial 1 to 10 dilution of the sample, express the results as "less than 10 microorganisms per g or per ml, of sample".

3. Isolation for pure colony

Pick up one colony from the mixed populations and transfer to the respective media with a streak plate technique for isolation of pure culture. Find the well isolated colony and transferred to agar slants.

4. Gram stain and microscopic observation

Gram - staining procedure

1) Prepare a thin film smear of young culture (12 to 18 hours) and allow to air-dry on a slide. Then passed through the flame

of Bunsen burner 3 times.

2) The slide is flooded with crystal violet solution (a primary stain) allow it to react for 1 min. The excess liquid is then allowed to run off the slide.

3) Any crystal violet remaining on the slide is washed off with tap water and apply Gram's iodine solution as mordant, and allow to react for 1 min, wash off with tap water.

4) The slide, being held almost vertically, is washed with alcohol 95% (a decolourizer) until the latter runs colorless from the slide. Rinse it in tap water to stop the decolorizing action.

5) Finally, it is flooded with Gram's safranin solution (a contrasting counterstain) and rocked gently for 10 - 20 sec.

6) The slide is rinsed with tap water and gently blotted dry.

And by the use of the microscope with the magnification of about 1000 - fold we can identify that microorganism is a gram positive or gram negative, morphology of the cell and arrangment.

From microscopic observation three groups of bacteria were found:

1. gram positive and negative cocci
2. gram positive bacilli with or without spore.
3. gram negative rods

5. Identification for pathogenic bacteria by biochemical test

5.1 Test for *Staphylococcus aureus*

Transfer the unknown pure culture to Mannitol-Salt Agar medium and incubate at 37 C for 24 hours. If, upon examination, the plate does not contains colony having the characteristics listed in TABLE 1 the sample meets the requirement for freedom from *Staphylococcus aureus*.

TABLE 1

Characteristics of *Staphylococcus aureus* on selective agar medium

Selective Medium	Mannitol-Salt Agar Medium
Characteristic colonial morphology	Yellow colonies with yellow zones
Gram stain	Positive cocci (in clusters)

Coagulase test

With the aid of an inoculating loop, transfer representative suspected colonies from agar surfaces of Mannitol-Salt Agar Medium to tubes containing 0.5 ml of mammalian plasma, with or without additives.

Incubate in water bath at 37 C, examine the tubes at 3 hours subsequently at suitable intervals up to 24 hours. If no coagulation in any degree is observed, the sample meets the requirements of the test for absence of *Staphylococcus aureus*.

Other confirmatory tests

Zones of hemolysis are found on streaked blood agar plates. Microorganisms can oxidize mannitol, decolorize litmus milk, reduce nitrate to nitrite and indole is not formed in peptone water.

5.2 Test for *Pseudomonas aeruginosa*

Transfer the unknown pure culture to Cetrimide Agar Medium and incubate at 37 C for 24 hours. If, upon examination, the plate does not contain colonies having the characteristic listed in the TABLE 2, the sample meets the requirements for freedom from *Pseudomonas aeruginosa*.

TABLE 2

Characteristics of *Pseudomonas aeruginosa*

on selective agar medium

Selective Medium	Cetrimide Agar Medium
Characteristic colonial morphology	Generally greenish
Fluorescence in ultraviolet light	Greenish
Oxidase test	Positive
Gram stain	Negative rods

Oxidase test for *Pseudomonas aeruginosa*

Transfer colonies from the agar surface of Cetrimide Agar to strip or disk of filter paper that previously has been impregnated with N, N - dimethyl - p - phenylenediamine dihydrochloride, if there is no development of a pink color, changing to purple, the sample meets the requirements of the test for the absence of *Pseudomonas aeruginosa*.

The presence of *Pseudomonas aeruginosa* may be confirmed by inoculating in TSI agar slant. If after 24 hours, both slant and butt show an alkaline reaction the presence of *Pseudomonas aeruginosa* is indicated.

Other confirmatory tests

Motility test is positive. Fermentation test on different sugars; glucose, lactose, sucrose, mannitol is negative. Hydrogen sulfide is produced. Indole test is negative.

5.3 Test for *Salmonella* species

Transfer colonies from the pure culture of unknown to a tube with Selenite Broth, mix, incubate at 37 C for 24 hours. By means of an inoculating loop, streak portions from the Selenite Broth on the surface of Brilliant Green Agar Medium, Xylose-Lysine-Desoxycholate Agar Medium, and Bismuth Sulfite Agar Medium contained in Petri dishes. Cover and invert the dishes, and incubate. Upon examination, if none of the colonies conformed to the description given in TABLE 3, the sample meets the requirements of the test for absence of the genus *Salmonella*.

TABLE 3

Characteristics of *Salmonella* species on selective agar media

Selective Medium	Description of Colony
Brilliant Green, Agar Medium	Small, transparent, colorless or pink to white opaque (frequently surrounded by pink to red zone)
Xylose - Lysine Desoxycholate Agar Medium	Red, with or without black centers
Bismuth Sulfite Agar Medium	Black or green

If colonies of gram - negative rods matching the description in TABLE 3 are found, proceed with further identification by transferring representative suspected colonies colonies individually, by means of an inoculating needle to a butt-slant tube of Triple-Sugar-Iron-Agar Medium by first streaking the surface of the slant and then stabbing the wire well beneath the surface. Incubate at 37 C for 24 hours. If examination discloses no evidence of the formation of acid (color change) and/or gas bubbles (with or without concomitant blackening) beneath the surface, without a change of color (from red to yellow) on the slant, the sample meets the requirements of the test for absence of the genus *Salmonella*.

Other confirmatory tests

Motility test is positive. Fermentation test on different

sugars-acid and gas is produced from glucose, mannitol is positive; lactose, sucrose and salicin are negative. Hydrogen sulfide is produced, while methyl red and citrate test are positive. Indole and Voges-Proskauer test are negative.

5.4 Test for *Escherichia coli*

By means of an inoculating loop, streak a portion from the pure culture of unknown on the surface of MacConkey Agar Medium. Cover and invert the dishes and incubate at 37 C for 24 hours. Upon examination, if none of the colonies conforms to the description given in TABLE 4 for this medium, the sample meets the requirements of the test for the absence of *Escherichia coli*.

TABLE 4

Characteristics of *Eschrichia coli* on MacConkey Agar Medium

Characteristic colonial morphology	Brick-red : may have surrounding zone of precipitated bile.
Gram stain	Negative rods (cocco-bacilli)

If colonies matching the description in TABLE 4 are found, proceed with further identification by transferring the suspected colonies individually, by means of an inoculating loop, to the surface of Eosin-Methylene Blue Agar Medium, plated on petri dishes. Cover and invert the plates, and incubate. Upon examination, if none of the colonies exhibits both characteristic metallic sheen under reflected light

and a blue-black appearance under transmitted light, the sample meets the requirements of the test for the absence of *Escherichia coli*.

Other biochemical tests

Test for indole, motility and methyl red test are positive. Voges-Proskuer and citrate test are negative.

5.5 Test for Bacillus species

Cells rod-shaped, straight or nearly so, 0.3-2.2 by 1.2-7.0 μ. Majority motile; flagella typically lateral. Heat resistant endospores formed, not more than one in a sporangial cell. Spore formation has proved to be a generally reliable diagnostic character of the genus *Bacillus*. Majority gram-positive. Strict aerobes or facultative anaerobes, and catalase usually produced.

6. Examination of fungus cultures

6.1 Gross or colonial morphology

Observe the gross or colonial morphology over a period of 5 days, for the following characteristics.

1. Rate of growth (rapid or slow)
2. General topography (flat, heaped, regularly or irregularly folded)
3. Texture (yeast-like, glabrous, powderly, granular, velvety or cottony)
4. Surface pigmentation
5. Pigmentation on the reverse side

6.2 Microscopic morphology

Using sterile technique, remove a small portion of the colony with a stiff nichrome wire needle (22 guage). Place the material in a drop of lacto-phenol cotton blue mounting fluid and teasing apart with 2 sterile inoculating needles, place the coverslip onto the slide. Examine the slide under the low power microscope and then with the high power for the diagnostic characters of the fungus such as :

1. Mycelium : septate or non-septate (coenocytic); pigmented or non-pigmented (hyaline); if pigmented mycelium are observed, record the color.
2. Conidiophores : shape, color and arrangement of conidiophores on the hyphae.
3. Conidia (spores) : shape, color and size of conidia to the mycelium (sessile along the hyphae or terminal at the hyphal tips or on the conidiophore); arrangement of conidia (single, clusters or both).

Aspergillus

Macroscopic features:

Growth of this fungus varies considerable from rapid to slow. Also, color ranges from, at first, white to shades of blue-green, yellow-green, black or tan. The surface may be velvety or cottony.

Microscopic features:

The mycelium is septate and hyaline with unbranched conidiophores arising from the hyphae. The tips of the conidiophores are

enlarged forming vesicles of varied shapes and sizes. On the surface of the vesicles are produced many flask-shaped sterigmata, either primary or both primary and secondary. The sterigmata may cover the vesicles partly or completely and produce chains of conidia, which may be spherical to elliptical smooth or rough-walled.

Curvularia

Macroscopic features :

Rapidly growing colony, grayish-brown, becoming darker or black in center, velvety to wooly surface. Black reverse.

Microscopic features :

Mycelium branched, septate, brown or dematecacious. Conidiophores are brown, septate, unbranched with spirally arranged conidia borne at the tips. Conidia are brown, ellipsoid or cylindrical, curved or bent (rarely straight), containing three or four cells-one of the central cells being distinctly larger and darker than the terminal cells.

Paecilomyces

Macroscopic features :

This is a rather rapidly growing fungus, which is flat and powdery to velvety. It may vary in color from yellowish-brown to grayish-green, violet or white.

Microscopic features :

The mycelium is hyaline and septate with both single and "penicillus" or brush-like conidiophores arising from the hyphae.

Both types produce characteristic long tapered sterigmata with elliptical conidia in chains.

Penicillium

Macroscopic features :

The colonies produced by different species of *Penicillium* differ in color, texture, rate of growth, etc. They are commonly rapid-growing, at first white then later becoming shades of bluish-green and other colors. They usually show a very powdery surface texture due to the abundant spores produced on the aerial mycelium.

Microscopic features :

Spore bearing hyphae characteristically form a "penicillus" or brushshaped structure. The conidia occur in unbranched chains cut off from the tip of flask-shaped sterigmata which arise from branches of the conidiophore.

The various species are identified on the basis of the manner of branching of the conidiophore, shape and color of the conidia and the characteristics of the perithecium and asci, if present.