CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS

Glass wares

1. Glass rods L-shape
2. Glass-beads
3. Measuring cylinders (Kimax)
4. Petri dishes (Pyrex)
5. Pipettes (Pyrex)
6. Test tubes (Pyrex)

Instruments

1. Autoclave (Fisher & Co.)
2. Colony counter (Arthur H.Thomas)
3. Hot air oven (Thelco)
4. Incubator (Thelco)
5. Refrigerator (General Electric co.,)
6. Test tube shaker (Fisher & Co.)
7. Thermostat water-bath (Thel co.)

Media and Chemicals

1. Baird-Parker-Agar Medium (Difco)
2. Brilliant Green Agar (Difco)
3. Bismuth Sulfite Medium (Difco)
4. Cetrimide Agar (Difco)
5. Disodium Hydrogen Phosphate (Merck)
6. Eosin Methylene-Blue (Difco)
7. Fluid Tetrathionate (Difco)
8. Fluid Lactose Medium (Difco)
9. Mannitol Salt Agar Medium (Difco)
10. Mac-Conkey Agar (Difco)
11. Pseudomonas Agar P (Difco)
12. Pseudomonas Agar F (Difco)
13. Sodium Dihydrogen phosphate (Merck)
14. Selenite Cysteine (Difco)
15. Tryptic Soy Agar (Difco)
16. Tryptic Soy Broth (Difco)
17. Vogel Johnson Agar (Difco)
18. Xylose-Lysine-Desoxycholate Agar Medium (Difco)

2.2 Methods

2.2.1 Preparation of Medium

Soybean Casein Digest Agar Medium

Pancreatic Digest of Casein	15.0 g
Papaic Digest of Soybean Meal	5.0 g
Sodium Chloride	5.0 g
Agar	15.0 g
Water	1,000 ml

Soybean-Casein Digest Medium

Pancreatic Digest of Casein	17.0 g
Papaic Digest of Soybean Meal	3.0 g
Sodium Chloride	5.0 g
Dibasic Potassium Phosphate	2.5 g
Dextrose	2.5 g
Water	1,000 ml

Dissolve the solids in water, warming slightly to effect solution, cool to room temperature, adjust with Sodium Hydroxide T.S. to obtain pH of 7.30 ± 0.2 after sterilization.

pH 7.2 Phosphate Buffer Dissolve 34 g of Monobasic Potassium Phosphate in about 500 ml of water, add Sodium Hydroxide T.S. to adjust pH 7.2 ± 0.1, water add to 1,000 ml mix and sterilize.

Mannitol - Salt Agar medium

Pancreatic Digest of Casein	5.0	g
Peptic Digest of Animal Tissue	5.0	g
Beef Extract	1.0	g
D - Mannitol	10.0	g
Sodium Chloride	75.0	g
Agar	15.0	g
Phenol Red	0.025	g
Water	1000	ml

Mix, then heat with frequent agitation, and boil for 1 minute. Sterilize, pH after sterilization 7.4 ± 0.2

Baird - Parker Agar Medium

Pancreatic Digest of Casein	10.0	g
Beef extract	5.0	g
Yeast extract	1.0	g
Lithium Chloride	5.0	g
Agar	20.0	g
Glycerin	12.0	g
Sodium Pyruvate	10.0	g
Water	950	ml

Heat with frequent agitation, and boil for 1 minute, sterilize, cool to between 45° 50° and add 10 ml of sterile Potassium Tellurite solution (1 in 100) and 50 ml of egg - yolk emulsion, Mix intimately but gently, and pour plates.

Vogel-Johnson Agar Medium

Pancreatic Digest of Casein	10.0	g
Yeast Extract	5.0	g
Mannitol	10.0	g
Dibasic Potassium Phosphate	5.0	g
Lithium Chloride	5.0	g
Glycerin	10.0	g
Agar	16.0	g
Phenol Red	25.0	mg
Water	1000	ml

Boil the solution of solids for 1 minute, Sterilize, cool to between 45° and 50° , add 20 ml of sterile Sodium Tellurite solution (1 in 100)

Cetrimide Agar Medium

Pancreatic Digest of Gelatin	20.0	g
Magnesium Chloride	1.4	g
Potassium Sulfate	10.0	g
Agar	13.6	g
Cetyl Trimethylammonium Bromide (Cetrimide)	0.3	g
Glycerin	10.0	ml
Water	1000	ml

Dissolve solids in water, and add the glycerin heat with agitation boil for 1 minute, sterilize.

Pseudomonas Agar Medium For Detection of Fluorescein (F)

Pancreatic Digest of Casein	10.0 g
Peptic Digest of Animal Tissue	10.0 g
Anhydrous Dibasic Potassium Phosphate	1.5 g
Magnesium Sulfate ($MgSO_4$, 7 H_2O)	1.5 g
Glycerin	10.0 ml
Agar	15.0 g
Water	1000 ml

Pseudomonas Agar Medium For Detection of Pyocyanin (P)

Pancreatic Digest of Gelatin	20.0 g
Anhydrous Magnesium Chloride	1.4 g
Anhydrous Potassium Sulfate	10.0 g
Agar	15.0 g
Glycerin	10.0 ml
Water	1000 ml

Dissolve the solid components in water before adding the glycerin, heat with frequent agitation, and boil for 1 minute, sterilize.

Fluid Lactose Medium

Beef Extract	3.0 g
Pancreatic Digest of Gelatin	5.0 g
Lactose	5.0 g
Water	1000 ml

Mix, cool as quickly as possible after sterilization.

Fluid Selenite-Cystine Medium

Pancreatic Digest of Casein	5.0	g
Lactose	4.0	g
Sodium Phosphate	10.0	g
Sodium Acid Selenite	4.0	g
L-Cystine	10.0	mg
Water	1000	ml

Mix and heat to effect solution for 15 minutes. Do not sterilize.

Fluid Tetrathionate Medium

Pancreatic Digest of Casein	2.5	g
Peptic Digest of Animal Tissue	2.5	g
Bile Salts	1.0	g
Calcium Carbonate	10.0	g
Sodium Thiosulfate	30.0	g
Water	1000	ml

Heat the solution of solids to boiling, add solution prepared by dissolving 5 g of Potassium Iodide and 6.0 g of Iodine in 20 ml of water, Then add 10 ml of solution of brilliant green (1 in 1000) and mix. Do not heat the medium after adding the brilliant green solution.

Brilliant Green Agar Medium

Yeast Extract	3.0	g
Peptic Digest of Animal Tissue	5.0	g
Pancreatic Digest of Casein	5.0	g
Lactose	10.0	g
Sodium Chloride	5.0	g
Sucrose	10.0	g
Phenol Red	80	mg
Ager	20.0	g
Brilliant Green	12.5	mg
Water	1000	ml

Boil the solution of solids for 1 minute. Sterilize before use

Xylose-Lysine-Desoxycholate Agar Medium

Xylose	3.5	g
L-Lysine	5.0	g
Lactose	7.5	g
Sucrose	7.5	g
Sodium Chloride	5.0	g
Yeast Extract	3.0	g
Phenol Red	80	mg
Agar	13.5	g
Sodium Desoxycholate	2.5	g
Sodium Thiosulfate	6.8	g
Ferric Ammonium Citrate	800	mg
Water	1000	ml

Heat the mixture of solids and water, just to the boiling point Do not overheat or sterilize.

Bismuth Sulfite Ager Medium

Beef Extract	5.0	g
Pancreatic Digest of Casein	5.0	g
Peptic Digest of Animal Tissue	5.0	g
Dextrose	5.0	g
Sodium Phosphate	4.0	g
Ferrous Sulfate	300	mg
Bismuth Sulfite Indicator	8.0	g
Agar	20.0	g
Brilliant Green	25	mg
Water	1000	ml

Heat the mixture of solids and water, Do not sterilize.

Mac Conkey Agar Medium

Pancreatic Digest of Gelatin	17.0	g
Pancreatic Digest of Casein	1.5	g
Peptic Digest of Animal Tissue	1.5	g
Lactose	10.0	g
Bile Salts Mixture	1.5	g
Sodium Chloride	5.0	g
Agar	13.5	g
Neutral Red	30	mg
Crystal Violet	1.0	mg
Water	1000	ml

Boil the mixture of solids and water for 1 minute, Sterilize

Levine Eosin-Methylene Blue Agar Medium

Pancreatic Digest of Gelatin	10.0	g
Dibasic Potassium Phosphate	2.0	g
Agar	15.0	g
Lactose	10.0	g
Eosin Y	400	mg
Methylene Blue	65	mg
Water	1000	ml

Dissolve the pancreatic digest of gelatin, the dibasic potassium phosphate and the agar in water, allow to cool, just prior to use, liquefy the jelled agar solution, add the remaining ingredients as solutions, and mix, for each 100 ml of the liquefied agar solution 5 ml of lactose solution (1 in 5), 2 ml of eosin Y solution (1 in 50) and 2 ml of methylene blue solution (1 in 300).

2.2.2 Procedure

Prepare the sample to be tested, by treatment that is appropriate to its physical characteristics and that does not alter the number and kind of microorganisms originally present, in order to obtain a solution or suspension of all or partof it in a form suitable for the test procedure(s) to be carried out.

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 solid that dissolves readily and practically completely in 90 ml of pH 7.2 Phosphate Buffer or the media specified, proceed as directed under Total Aerobic Microbial Count, and under Test for Staphylococcus and Pseudomonas and Test for Salmonella Species and Escherichia coli.

For a solid that dissolves to an appreciable extent but not completely, reduce the substance to a moderately fine powder, suspend it in the vehicle specified, and proceed as directed under Total Aerobic Microbial Count, and under Test for Staphylococcus and Pseudomonas and Test for Salmonella Species and Escherichia coli.

For water-immiscible fluids, ointments, creams, and waxes, prepare a suspension with the aid of a minimal quantity of a suitable, sterile emulsifying agent (such as one of the polysorbates), using a mechanical blender and warming to a temperature not exceeding 45°, if necessary, and proceed with the suspension as directed under Total Aerobic Microbial Count, and under Test for Staphylococcus and Pseudomonas and Test for Salmonella Species and Escherichia coli.

2.2.3 Total Aerobic Microbial Count-For samples that are sufficiently soluble or translucent to permit use of the Plate Method, use that method; otherwise, use the Multiple-tube Method. With either method, first dissolve or suspend 10.0 g of the sample if it is a solid, or 10 ml, accurately measured, if the sample is a liquid, in pH 7.2 Phosphate Buffer, Fluid Soybean-Casein Digest Medium, or Fluid Casein Digest Soy Lecithin-Polysorbate 20 Medium to make 100 ml. Perform the test for absence of inhibitory (antimicrobial) properties as described under Preparatory Testing before the determination of Total Aerobic Microbial Count. Add the sample to the medium not more than 1 hour after preparing the appropriate dilutions for inoculation.

Plate method - Dilute further, if necessary, the fluid so that 1 ml will be expected to yield between 30 and 300 colonies. Pipet 1 ml of the final dilution onto each of two sterile Petri dishes. Promptly add to each dish 15 to 20 ml of Soybean Casein Digest Agar Medium that previously has been melted and cooled to approximately 45°. 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 for 48 to 72 hours. 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"

Multiple-Tube Method

Into each of fourteen test tubes, place 9.0 ml of sterile Fluid Soybean-Casein Digest Medium. Arrange twelve of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as the controls. Into each of three tubes of one set ("100") and into a fourth tube (A) pipet 1 ml of the solution or suspension of the sample, and mix, From tube A, pipet 1 ml of its contents into the one remaining tube (B) not included in a set, and mix, these two tubes contain 100 mg and 10 mg of the sample, respectively. Into each of the second set ("10") of three tubes pipet 1 ml from tube A and into each tube of the third set, ("1") pipet 1 ml from tube B incubate all of the tubes. Following the incubation period, examine the tubes for growth, the three control tubes remain clear, and the observation in the tubes containing the sample. When interpreted by reference to Table 1, indicate the most probable number of microorganism per g or per ml of sample.

Table 1.* Most Probable Total Count by Multiple-Tube Method.

Observed Combinations of Numbers of Tubes Showing Growth in Each Set			Most Probable Number of Micro Organisms per g or per ml.
No. of mg (or ml) of sample per Tube			
(A) 100 (100 µL)	(B) 10 (10 µL)	1 (1 µL)	
3	3	3	1100
3	3	2	1100
3	3	1	460
3	3	0	240
3	2	3	290
3	2	2	210
3	2	1	150
3	2	0	93
3	1	3	160
3	1	2	120
3	1	1	75
3	1	0	43
3	0	3	95
3	0	2	64
3	0	1	39
3	0	0	23

*Extracted from U.S.P. XIX p 591

Test for Staphylococcus aureus and Pseudomonas aeruginosa To the sample add Fluid Soybean-Casein Digest Medium to make 100 ml, mix, and incubate. Examine the medium for growth, and if growth is present, use an inoculating loop to streak a portion of the medium on the surface of Vogel-Johnson Agar Medium (or Baird-Parker Agar Medium, or Mannitol-Salt Agar Medium) and of Cetrimide Agar Medium, each plated on Petri dishes. Cover and invert the dishes, and incubate. If, upon examination, none of the plates contains colonies having the characteristics listed in Tables 2 and 3 for the media used, the sample meets the requirements for freedom from Staphylococcus aureus and Pseudomonas aeruginosa.

Coagulase test (for Staphylococcus aureus)- With the aid of an inoculating loop, transfer representative suspect colonies from the agar surfaces of the Vogel-Johnson Agar Medium (or Baird-Parker Agar Medium, or Mannitol-Salt Agar Medium) to individual tubes, each containing 0.5 ml of mammalian, preferably rabbit or horse, plasma with or without suitable additives. Incubate in a water bath at 37° examining the tubes at 3 hours and subsequently at suitable intervals up to 24 hours. Test positive and negative controls simultaneously with the unknown samples. If no coagulation in any degree is observed, the sample meets the requirements of the test for absence of Staphylococcus aureus.

Oxidase and pigment test (for Pseudomonas aeruginosa) With the aid of an inoculating loop, streak representative suspect colonies from the agar surface of Cetrimide Agar Medium on the agar surfaces of Pseudomonas Agar Medium for Detection of Fluorescin and of Pseudomonas

Agar Medium for Detection of Pyocyanin contained in Petri dishes. If numerous colonies are to be transferred, divide the surface of each plate into quadrants, each of which may be inoculated from a separate colony. Cover and invert the inoculated media, and incubate at 35 ± 2° for not less than three days. Examine the streaked surfaces under ultraviolet light. Examine the plates to determine whether colonies having the characteristics listed in Table 3 are present.

Confirm any suspect colonial growth on one or more of the media as P. aeruginosa by means of the oxidase test. Upon the colonial growth place or transfer colonies to strips or disks 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 other suitable cultural and biochemical tests, if necessary.

Test for Salmonella Species and Escherichia coli To the sample, contained in a suitable vessel. add a volume of Fluid Lactose Medium to make 100 ml, and incubate. Examine the medium for growth, and if growth is present, mix by gently shaking. Pipet 1-ml portions into vessels containing, respectively, 10ml of Fluid Selenite-Cystine Medium and Fluid Tetrathionate Medium, mix, and incubate for 12 to 24 hours. (Retain the remainder of Fluid Lactose Medium.)

Test for Salmonella species By means of an inoculating loop, streak portions from both the selenite-cystine and tetrathionate media 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 conforms to the description given in Table 4, the sample meets the requirements of the test for absence of the genus Salmonella.

If colonies of Gram-negative rods matching the description in Table 4 are found, proceed with further identification by transferring representative suspect colonies individually, by means of an inoculating wire, 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. 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 surface, the sample meets the requirements of the test for the absence of the genus Salmonella.

Test for Escherichia coli By means of an inoculating loop, streak a portion from the remaining Fluid Lactose Medium on the surface of Mac Conkey Agar Medium. Cover and invert the dishes, and incubate. Upon examination, if none of the colonies

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conforms to the description given in Table 5 for this medium, the sample meets the requirements of the test for absence of Escherichia coli.

If colonies matching the description in Table 5 are found, proceed with further identification by transferring the suspect colonies individually, by means of an inoculating loop, to the surface of Levine Eosin-Methylene blue Agar Medium, plated on Petri dishes. If numerous colonies are to be transferred, divide the surface of each plate into quadrants, each of which may be seeded from a separate colony. Cover and invert the plates, and incubate. Upon examination, if none of the colonies exhibits both a 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. The presence of Escherichia coli may be confirmed by further suitable cultural and biochemical tests.

Table 2. Morphologic Characteristics of Staphylococcus aureus on Selective Agar Media.

Selective Medium	Vogel-Johnson Agar Medium	Mannitol-Salt Agar Medium	Baird-Parker Agar Medium
Characteristic Colonial Morphology	Black surrounded by yellow zone	Yellow colonies with yellow zone	Black, shiny, surrounded by clear zones 2 to 5 mm
Gram Stain	Positive cocci (in clusters)	Positive cocci (in clusters)	Positive cocci (in clusters)

Table 3. Morphologic Characteristics of Pseudomonas aeruginosa on Selective and Diagnostic Agar Media.

Medium	Cetrimide Agar Medium	Pseudononas Agar Medium for Detection of Fluorescin	Pseudomonas Agar Medium for Detection of Pyocyanin
Characteristic Colonial Morphology	Generally greenish	Generally colorless to yellowish	Generally greenish
Fluorescence in Ultraviolet Light	Greenish	Yellowish	Blue
Oxidase Test	Positive	Positive	Positive
Gram Stain	Negative rods	Negative rods	Negative rods

Table 4. Morphologic Characteristics of Salmonella Species on Selective Agar Media.

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

Table 5. Morphologic Characteristics of Escherichia coli on MacConkey Agar Medium.

Characteristic Colonial Morphology	Brick-red; may have surrounding zone of precipitated bile
Gram Stain	Negative rods (cocco-bacilli)