CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Instruments

1.1.1 Analytical balance - H35AR (E. Mettler, Switzerland)

1.1.2 Analytical balance - 1103 (Sartorius, Switzerland)

1.1.3 Bunsen Burner

1.1.4 Gas stove Triplex Universal

1.1.5 Oven - Termaks

1.1.6 Incubator 37°C - 220V 2700W

Memmert TV80b. Western Germany

cool - Termaks

1.1.7 Millipore Filter Holder Millipore Filter Corp. Bedford. Mass. USA.

1.1.8 Refrigerator (Zanussi)

1.1.9 Steam sterilizer (Autoclave) (Omron tateisi electronic Co. Japan).

1.1.10 Steer replicator (Fac of Pharmacy, Chulalongkorn University)

1.1.11 Vortex mixer

1.1.12 Waterbath (Memmert - Rost Frel, Eldelstahl)

1.2 Glasswares

1.2.1	Beakers	(Pyrex, USA)
1.2.2	Burette	(Pyrex, USA)
1.2.3	Bottle	
1.2.4	Erlenmeyer Flasks	(Pyrex, USA)
1.2.5	Funnels	(Pyrex, USA)
1.2.6	Measuring cylinders	(Pyrex, USA)
1.2.7	Pasteur pipetters	(Pyrex, USA)
1.2.8	Petri dishs	(Pyrex, USA)
1.2.9	Screw cap test tube	(Demuth-A, Kimax USA)
1.2.10	Stirring rod	
1.2.11	Syringe	

1.3 Media

1.3.1	Brain Heart Infusion Dehydrate	(Difco Laboratories, Detroit, Mich. USA)
1.3.2	Blood Agar	(Difco Laboratories)
1.3.3	Beef Extract Broth	(Difco Laboratories)
1.3.4	Muller Hinton Agar	(Difco Laboratories)
1.3.5	Muller Hinton Broth	(Difco Laboratories)
1.3.6	Nutrient Agar	(Difco Laboratories)
1.3.7	Trypticase Casein Soy Broth	(Difco Laboratories)
1.3.8	Enrichment Media for Vibrio cholera	(Difco Laboratories)
1.3.9	Enrichment Media for Vibrio para-haemolyicus	(Difco Laboratories)

Media were prepared according to the methods and formulas shown in appendix 1

1.4 Sensitivity disks

1.4.1 Local disk (The Medical Scientific Research Department, Bangkok)

1.4.2 Imported disk (BBL)

Types and potency of disks used in Modified Broth Disk Method and Disk Diffusion Method were shown in Table 5.

1.5 Antibiotics for Minimum Inhibitory Concentration (MIC) Study

Five antibiotic powders and three injectable preparations were shown in Table 6 with the sources and details of individual potency. The stock solution of each antibiotic were prepared in the concentration of 1,000 ug/cc according to the solvent for specific powder (table 7) by the methods in table 8. The stock solutions were filtered through the membrane filter (solutions aseptically prepared from vials of injectable are sterile) and then stored at -10°C in refrigerator.

1.6 Tested Organisms

125 strains of Enteropathogens, employed in this study were obtained from the subculturing of the stock cultures collected at the Microbiology Laboratory, The Medical Scientific Research Department of Thailand.

The tested strains are as follows:

1.6.1 Enteropathogenic *E. coli*		23 strains
	type O-78	13 strains
	O-128	6 "
	O-119	4 "
1.6.2 *Salmonella* spp		49 strains
Salmonella typhi		4 strains
" *paratyphi*		6 "
" *typhimurium*		7 "
" *derby*		6 "
" *krefeld*		21 "
" *weltevreden*		2 "
" *anatum*		3 "
1.6.3 *Shigella* spp.		28 strains
Shigella flexneri 1 b		9 strains
" *sonnei*		19 "
1.6.4 *Vibrio cholerae*		10 strains
Vibrio Cholerae El Tor Inaba		7 strains
" " " Ogawa		3 "

1.6.5 Vibrio parahaemolyticus — 15 strains

01	1 strains
03	4 "
04	5 "
05	4 "
Out	1 "

The stock culture were maintained on nutrient agar within the screw-capped test tube and kept at 4-6°C in refrigerator.

In studying of the susceptibility of microbial species, pure culture are essentially obtained by the streak plate method (6) as in Fig. 1.

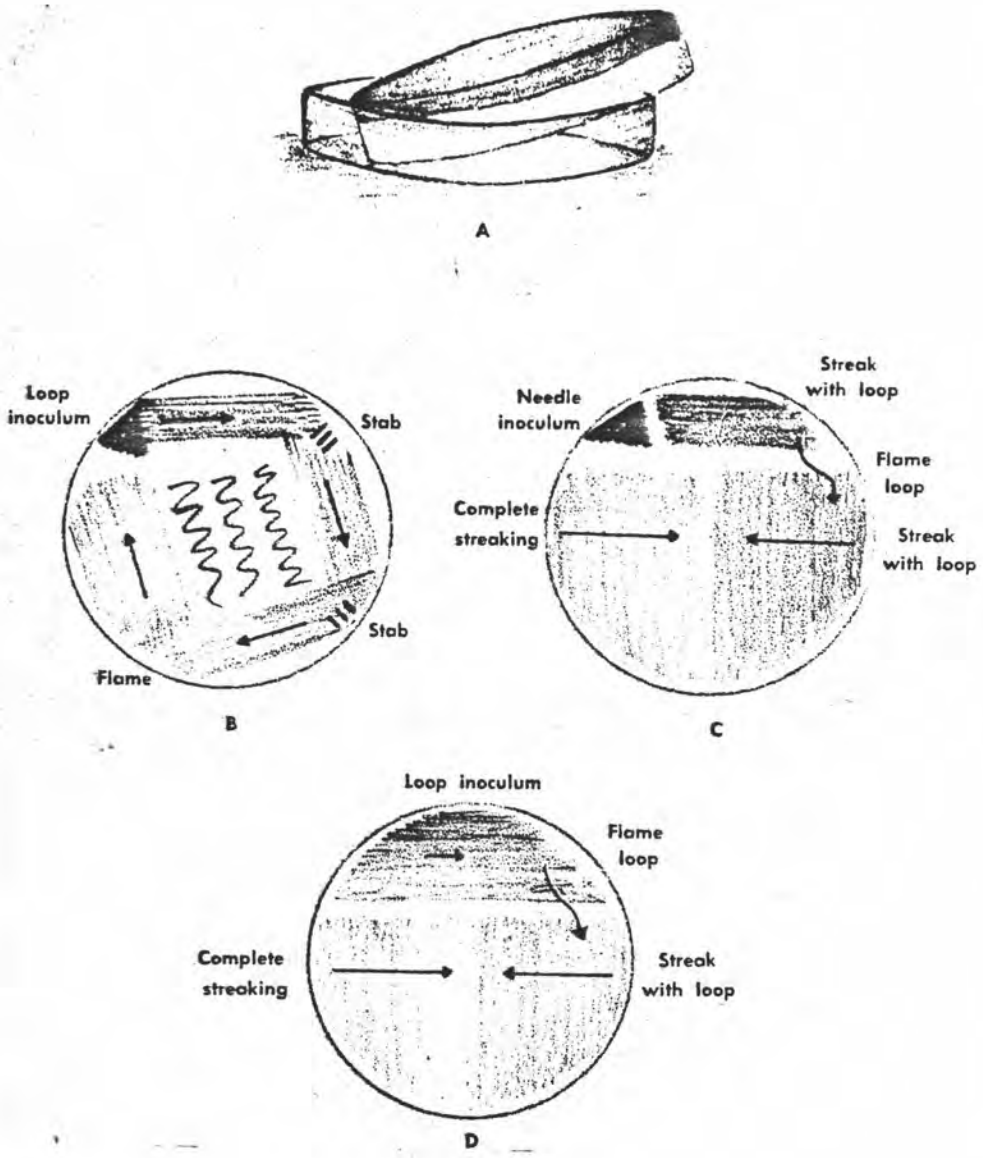

Figure 1 Streak plate technique

A. Drying a plate of agar in the laboratory

B. Method 1

C. Method 2

D. Method 3

1.7 Chemicals

1.7.1 Ethyl Alcohol

1.7.2 Phosphate buffer pH 6.0

1.7.3 Phosphate buffer pH 8.0

1.8 Others

1.8.1 Dropper

1.8.2 Forcep

1.8.3 Inoculating loop

1.8.4 Millipore membrane

1.8.5 Test tube rack

Table 5 **Antibiotic disks in Modified Broth Disk and Disk Diffusion** methods

Antibiotic disk	LABELLED DISK CONTENT (µg)	
	IMPORTED DISK[@]	LOCAL DISK[@@]
Streptomycin	10	10
Tetracycline	30	30
Chloramphenicol	30	30
Colistin	10	10
Neomycin	30	30
TMP/SMX	1.25/23.75	1.25/23.75
Kanamycin	30	30
Penicillin	10[@@@]	10[@@@]
Polymyxin-B	300[@@@]	-
Gentamicin	10	-
Ampicillin	10	-
Sulphadiazine	250	-

@ BBL DISKS

@@ Manufactured by the Medical Scientific Research Department of Thailand.

@@@ Units

Table 6 Antibiotic powder and injections used in Dilution Methods

Antibiotics	Potency	Source
Antibiotic powders		
Streptomycin Sulphate $6H_2O$	MW 1457.4	Med. Sc Research Dept.
Polymyxin B Sulphate	7580 IU/mg	" " "
Chloramphenicol	980 µg/mg	" " "
Neomycin Sulphate	660 µg/mg	" " "
Ampicillin	85.7 %	Saha Prat Bhaesaj
Injectable antibiotic		
Penicillin-G	1 million units	Commercially available
Gentamicin Sulphate	40 mg/ml	" "
Kanamycin Sulphate	250 mg/ml	" "

(Penicillin-G 1 unit = 0.6 µg
Polymyxin 1 unit = 0.127 µg) (50)

Stock solutions of antibiotic are prepared from concentrated, dehydrated sterile material of known potency that obtained from the Pharmaceutical manufacturers.

Table 7 Solvents and Diluents for Stock Solutions of Antimicrobials (2)

Antimicrobial	Solvent	Diluent
Ampicillin	Phosphate buffer, pH 8.0, 0.1 M	Phosphate buffer, pH 6.0
Carbenicillin	Water	Water
Cephalothin	Phosphate buffer, pH 6.0, 0.1 M	Water
Chloramphenicol	Ethanol	Water
Clindamycin	Water	Water
Cycloserine	Water	Water
Erythromycin	Ethanol	Water
Ethambutol	Water	Water
Flucytosine	Saline, 0.85%	Saline, 0.85%
Gentamicin	Phosphate buffer, pH 8.0, 0.1 M	Water
Isoniazid	Water	Water
Kanamycin	Phosphate buffer pH 8.0, 0.1 M	Water
Nalidixic acid	NaOH, 1 N	Water
Nitrofurantoin	Dimethylformamide	Water
Oxacillin	Water	Water
p-Aminosalicylic acid	Water	Water

Table 7 Continue

Antimicrobial	Solvent	Diluent
Penicillin	Water	Water
Polymyxin B	Water	Water
Rifampin	Dimethylsulfoxide	Phosphate buffer, pH 7.0
Streptomycin	Water	Water
Sulfonamides	Hot water + minimal amount of 10% NaOH to dissolve	Water
Tetracycline	Water	Water
Vancomycin	Water	Water

Table 8 Precedure for preparing stock solutions (5,50)

The stock solution of antibiotics employed in Dilution Mrthods were prepared in the concentration of 1,000 µg/ml.

ANTIBIOTIC	METHOD OF PREPARATION
Streptomycin	1. Weigh out 22.9969 mg. of powder. 2. Add 20 ml. of sterile distilled water to make a stock solution of 1,000 µg/ml.
Chloramphenicol	1. Stated activity equals 980 µg/mg. 2. Weigh out 50 mg. (total activity equals 49,000 µg). 3. Add 1 ml. of ethyl alchohol to dissolve drug and then sufficient water qs to 49 ml.
Neomycin	1. Stated activity equals 660 µg/mg. 2. Weigh out 50 mg. (total activity equals 33,000 µg). 3. Add 33 ml. of steriled distilled water to give 1,000 µg/ml.
Polymyxin-B	1. Stated activity equals 7580 IU/mg. (=7580 x 0.127 = 962.66 µg/mg). 2. Weigh out 50 mg. (total activity equals 48,133 µg). 3. Add 48.1 ml. of sterile distilled water to give 1,000 µg/ml.

Table 8 Continue

ANTIBIOTIC	METHOD OF PREPARATION
Penicillin-G	1 Injection contains 1 million units or 600,000 µg. 2 Add 60 ml of sterile distilled water, this makes a stock solution of 10,000 µg/ml. 3 Dilute 1:10 to give the stock solution of 1,000 µg/ml.
Gentamicin	1 Injection 1 ml contains 40,000 µg. 2 Add 39 ml of water to make 1,000 µg/ml.
Kanamycin	1 Injection 1 ml contains 250,000 µg. 2 Add 24 ml of sterile distilled water to make a stock of 10,000 µg/ml. 3 Dilute 1:10 to give a stock solution 1,000 µg/ml.
Ampicillin	1 Stated activity equals 85.7%. 2 Weigh out 1 mg powder (contains 85,700 µg activity). 3 Add 0.1 ml of pH 8.0 phosphate buffer to dissolve. 4 Dilute with 85.6 ml of pH 6.0 phosphate buffer to make the final stock solution of 1,000 µg/ml.

2. Methods

2.1 Modifieds Broth Disk Method (36)

A sery of 125 rows of, 13 screw capped test tubes each, was arranged. Twelve tubes for individual antibiotic disks-SM, TC, CM, CL, NM, CO, KM, PN, PM, GM, AM and SDZ - and the thirteenth as control tube.

Label tubes 1-12 with specific antibiotic disks, in each row with the tested strains.

Fill in each tube, 5 ml of Brain Heart Infusion Broth, then sterile in autoclave 121°C 15 lbs for 15 mins and keep cool.

The appropriate BBL disks were added aseptically in each row, one disk into one tube according to the labelled antibiotic. In the same manner, complete the 125 rows. The tubes were then simultaneously inoculated from a Plasteur pipette with one drop of an overnight (18-24 hrs.) culture of the tested organism in Trypticase Caesein Soy Broth, one strain for each row of 13 tubes. (One drop of culture per 5 ml will give approximately a 1:100 dilution of culture. This was an inoculum of $10^6 - 10^7$ bacteria per ml). The final concentrations of individual antibiotic in each tube were as in Table 9 of which will above or approximately equal the normal attainable blood levels (Table 4). The inoculated tubes were then incubated at 37°C. The result was detected and read at the period of 3, 6, 8, 18 hrs. after incubation. Susceptibility to the test antibiotic was defined as either abscence of turbidity or less than 50% of the turbidity of the control culture.

Another sery of 125 rows of 13 tubes were proceeded in the same manner as the already described procedure but using the local disks instead of the BBL disks (Table 9). The results from BBL and local disk were then compared.

Table 9 **Final antibiotic concentration in Modified Broth Disk Method**

Antibiotic disk	Labeled Disk Content (µg)		Final Concentration per ml broth (µg).
	Local disk[@]	Imported disk[@@]	
Streptomycin	10	10	2
Tetracycline	30	30	6
Chloramphenicol	30	30	6
Colistin	10	10	2
Neomycin	30	30	6
Cotrimoxazole(TMP/SMX)	1.25/23.75	1.25/23.75	0.25/4.75
Kanamycin	30	30	6
Penicillin	10[@@@]	10[@@@]	2[@@@]
Polymyxin-B	300[@@@]	-	60[@@@]
Gentamicin	10	-	2
Ampicillin	10	-	2
Sulphadiazine	250	-	50

@ - Medical Scientific Research Department

@@ - BBL disk

@@@ - Units

2.2 Disk Diffusion Method (49, 50)

The principle of diffusion method is dependent upon the inhibition of the growth of a microorganism on the surface of an inoculated agar plate, by the antimicrobial agent that diffuses into the surrounding medium from a impregnated disk on the surface of the medium.

The petri dishs of which poured by Muller-Hinton Agar to a depth of approximately 4 mm (24)were allowed to dry for, at least 30 minutes before being inoculated and were used within 7 days.

The inoculum was then prepared by diluting an overnight broth culture, of which was prepared with an noculum from at least 3 colonies of the organism to be tested in Trypticase Soy Broth, with sterile water to the density equivalent to Mac Farland NO.5 standard (equivalant to 10^5 CFU/ml) or diluted with sterile water to the density of opacity standard (about 100 fold (49)

Dip a sterile cotton swab into the inoculum. Express excess broth by pressing swab against the inside of the tube. Streak the entire surface of the plate evenly with the swab in three directions. Allow the plate to dry at room temperature for 3-5 minutes.

Sensitivity disks were then applied with sterile flamed forceps onto the surface of the medium. Disks should not be less 3 centrimetres from each other and not less than 2 centrimetres from the edge of the plates. Plates were then incubated invertly at 37°C aerobically overnight

(18-24 hrs.).

The plates were read by measuring the size of inhibition zone from the underside of the plate with a ruler. The entire diameter of the zone including the disk (approximately 6.0 mm in diameter) was measured. The end point of the reading was taken as complete inhibition of the growth to the naked eye. The zone diameters for individual antibiotics were translated in term of susceptibility intermediate and resistant by referring to an interpretative chart (Table 10).

Table 10 Zone Sizes and Their Interpretation for Frequently Used Chemotherapeutics (49)

Antibiotic or Chemotherapeutic Agent	Disk Potency	Inhibition Zone Diameter to Nearest Millimeter		
		Resistant	Intermediate	Sensitive
Ampicillin				
S. aureus	10 µg.	20 or less	21–28	29 or more
All other organisms	10 µg.	11 or less	12–13	14 or more
Bacitracin	10 units	8 or less	9–12	13 or more
Cephalothin	30 µg.	14 or less	15–17	18 or more
Chloramphenicol	30 µg.	12 or less	13–17	18 or more
Colistin	10 µg.	8 or less	9–10	11 or more
Erythromycin	15 µg.	13 or less	14–17	18 or more
Kanamycin	30 µg.	13 or less	14–17	18 or more
Lincomycin	2 µg.			17 or more
Methicillin	5 µg.	9 or less	10–13	14 or more
Nalidixie acid	30 µg.	13 or less	14–18	19 or more
Neomycin	30 µg.	12 or less	13–16	17 or more
Nitrofurantoin	300 µg.	14 or less	15–16	17 or more
Novebiocin	30 µg.	17 or less	18–21	22 or more
Oleandomycin	15 µg.	11 or less	12–16	17 or more

Table 10 Continued

Antibiotic or Chemotherapeutic Agent	Disk Potency	Inhibition Zone Diameter to Nearest Millimeter		
		Resistant	Intermediate	Sensitive
Novobiocin	30 µg.	17 or less	18-21	22 or more
Oleandomycin	15 µg.	11 or less	12-16	17 or more
Penicillin-G	10 units	20 or less	21-28	29 or more
Polymyxin-B	300 units	8 or less	9-11	12 or more
Streptomycin	10 µg.	11 or less	12-14	15 or more
Sulfonamides	300 µg.	12 or less	13-16	17 or more
Tetracycline	30 µg.	14 or less	15-18	19 or more
Vancomycin	30 µg.	9 or less	10-11	12 or more

2.3 Agar Plate Dilution Method (50)

Working antibiotic solutions 10 times the final concentration desired in the plates were prepared from the 1,000 µg/ml stock solution by setting up a sery of 11 sterile plugged test tubes in rack. Label 1-11. Aseptically add 4 ml of sterile distilled water to tube 1 and 2.5 ml to each remaining tube. Thaw the stock solution and aseptically add 1.0 ml to tube 1. The concentration will be 200 µg/ml. With a fresh sterile pipette, mix contents of tube 1 and transfer 2.5 ml to tube 2; discard the pipette complete the two-fold serial dilution by repeating the preceeding step out to and including tube 11. Discard the used pipette after each transfer. Leave the 2.5 ml added from tube 10 in tube 11. A sery of working solutions ranging from 200 ug/ml down to 0.195 µg/ml thus has been prepared.

In preparation the plates, each plate used 18 ml of Muller Hinton Agar. Melt agar and cool to 45°- 50°C and hold in a water bath. Add 2 ml of working solution per plate to a cooled 18 ml agar. The same pipette may be used if transfer are made from the most dilute to the most concentrated working solution. Mix and pour plate. Allow plate to solidify and dry before inoculation. The concentration of tested antibiotic was as in Table 11. Prepared plates may be stored up to 7 days without detectable loss in activity. A control plate containing no antibiotic must be included in each sery. The final plate concentrations were as in table 11.

The inoculum was prepared by inoculate about five colonies of each organism to be tested to a 5.0 ml tube of Muller Hinton Broth. Incubate for 18–24 hrs. at 37°C, then dilute the broth culture in Muller Hinton Broth to contain 10^5 - 10^6 organisms per ml. The inoculum was applied on to the agar surface of the prepared plate by the Steer replicator according to the instruction described in appendix 2. Incubate plates for 18–24 hrs. at 37°C, aerobically. The plate was read as the least concentration of antibiotic that completely inhibit growth of which called MIC and reported in µg/ml. The individual MIC value was then interpreted as resistant or susceptible according to the Table 12.

Table 11 Final antibiotic concentration in plate

Plate Number	Final concentration (µg/ml)
1	20
2	10
3	5
4	2.5
5	1.25
6	0.625
7	0.313
8	0.156
9	0.078
10	0.039

Table 12 Zone-Diameter Interpretive Standards and Approximate MIC Correlates (2)

(National Committee on Clinical Laboratory Standards - NCCLS, 1974)

Antimicrobial Agent	Disk Content	Zone-Diameter (Nearest Whole mm)			Approximate MIC Correlates	
		Resistant	Intermediate	Susceptible	Resistant	Susceptible
	(ug)				(ug/ml)	(ug/ml)
Amikacin	10	≤11	12-14	≥15	32	8
Ampicillin -when testing gram-negative enteric organisms and enterococci	10	≤11	12-13	≥14	32	8
Ampicillin -when testing Staphylococci and penicillin G-susceptible microorganisms	10	≤20	21-28	≥29	32 penicillinase	02
Ampicillin -when testing Haemophilus species	10	≤19	-	≥20	-	2.0
Carbenicillin-when testing Proteus species and Escherichia coli	100	≤17	18-22	≥23	32	16

Table 12 Continue

Antimicrobial Agent	Disk Content	Zone-Diameter (Nearest Whole mm)			Approximate Resistant	MIC Correlates Susceptible
		Resistant	Intermediate	Susceptible		
	(µg)				(µg/ml)	(µg/ml)
Carbenicillin- when testing Pseudomonas aeruginosa	100	≤13	14-16	≥17	250	125
Cefamandole	30	≤16	15-17	≥18	32	10
Cefoxitin	30	≤16	15-17	≥18	32	10
Cephalothin	30	≤14	15-17	≥18	32	10
Chloramphenicol	30	≤12	13-17	≥18	25	12.5
Clindamycin- when reporting susceptibility to clindamycin only	2	≤14	15-16	≥17	2	1
Colistin	10	≤ 8	9-10	≥11	-	-
Erythromycin	15	≤13	14-17	≥18	8	2
Gentamicin	10	≤12	13-16	≥17	16	4
Kanamycin	30	≤13	14-17	≥18	25	6
Methicillin - when testing Staphylococci	5	≤ 9	10-13	≥14	-	3

Table 12 Continue

Antimicrobial Agent	Disk Content	Zone-Diameter (Nearest Whole mm)			Approximate Resistant	MIC Correlates Susceptible
		Resistant	Intermediate	Susceptible		
Neomycin	30	≤ 12	13–16	≥ 17	-	10
Oxacillin	1	≤ 10	11–12	≥ 13	-	
Penicillin G – when testing staphylococci	10 (U)	≤ 20	21–28	≥ 29	penicillinase	0.1(g/ml)
Penicillin G – when testing other microorganisms	10 (U)	≤ 11	12–21	≥ 22	32(g/ml)	1.5(g/ml)
Polymyxin B	300 (U)	≤ 8	9–11	≥ 12	50 (U)	-
Streptomycin	10	≤ 11	12–14	≥ 15	15	6
Tetracycline	30	≤ 14	15–18	≥ 19	12	4
Tobramycin	10	≤ 12	13–16	≥ 17	16	4
Vancomycin	30	≤ 9	10–11	≥ 12	-	5
Sulfonamides	250 or 300	≤ 12	13–16	≥ 17	350	100
Trimethoprim-Sulfamethoxazole	1.25 / 23.75	≤ 10	11–15	≥ 16	200	35
Nitrofurantoin	300	≤ 14	15–16	≥ 17	100	25
Nalidixic acid	30	≤ 13	14–18	≥ 19	32	12

2.4 Broth dilution test tube method (50)

The principle of the tube or broth dilution method is the inhibition of the growth of the test organism by an antimicrobial incorporated in a broth medium.

The inoculum was prepared by inoculating about five colonies of each organism to be tested to 5.0 ml of Trypticase Casein Soy Broth, incubate 37°C overnight and then dilute 1:1000 in the same broth.

Arrange a series of 12 sterile test tubes in a test tube rack to prepare a serial dilution of the working stock. To all but the first tube in the series, aseptically add 0.5 ml of the broth to be used in the test. To the first and second tubes of the series, add 0.5 ml of the working stock previously removed from the deep freeze and thawed and prepared in concentration of 100 µg/ml.

Using a sterile 1.0 ml pipette, mix and transfer 0.5 ml from tube 2 to tube 3. Discard the pipette after each transfer to the next tube. Repeat the procedure to tube 11. Leave in tube 11 the 0.5 ml transferred from tube 10. (This is a control on the titration).

With a sterile 5.0 ml pipette, add 1.5 ml of the 1:1,000 dilution of the overnight broth culture to each of tube 1 to 10. Skip tube 11, and add 1.5 ml to tube 12 of which is the culture control as shown in Fig. 2. The final concentration of antibiotic in tube 1, having started with 0.5 ml

of a solution containing 100 µg/ml; will be 25 µg/ml; tube 2, 12.5 µg/ml; tube 3, 6.25, ... etc. as in Table 13. The inoculated tubes were incubated at 37°C overnight.

The end point of the inhibition, the MIC, is the greatest dilution of the antibiotic which exhibits no visible growth. (Tube 11 should be clear and tube 12, the culture control, should be macroscopically cloudy). The MIC was reported as µg/ml.

The obtained MIC values were then interpreted as resistant or susceptible according to the Table 12.

Figure 2 Line drawing of the broth dilution susceptibility test. The minimal inhibitory concentration for the test illustrated here is 1.56 µg/ml.

Tube No	1	2	3	4	5	6	7	8	9	10	11	12
Conc. of antibiotic (µg/ml)	25	12.5	6.25	3.12	1.56	0.78	0.39	0.2	0.1	0.05	0.03	0

Table 13 The final antibiotic concentrations in tube

Tube No.	Final concentration (µg/ml)	
1	25	
2	12.50	
3	6.25	
4	3.12	
5	1.56	
6	0.78	
7	0.39	
8	0.20	
9	0.10	
10	0.05	
11	0.03	(antibiotic control)
12	0	(culture control)