CHAPTER II

MATERIALS AND METHODS

1. Materials

1.1 Organisms

1.1.1 *Staphylococcus aureus* ATCC 1538-P was obtained from the Department of Quality Control, Dumex Ltd., Bangkok, Thailand.

1.1.2 *Pseudomonas aeruginosa* ATCC 27853 was obtained from the Department of Bacteriology and Mycology, Arm Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand.

1.2 Antibiotics

1.2.1 Ampicillin sodium, Lot No. B-953304, was purchased from Dumex Ltd., Bangkok, Thailand.

1.2.2 Gentamicin sulphate, Lot No. 797443, was obtained from Atlantic Trading Co., Ltd., Bangkok, Thailand.

1.3 Media

1.3.1 Tryptic Soy Agar (TSA) (Difco Laboratory, U.S.A.)

1.3.2 Tryptic Soy Broth (TSB) (Difco Laboratory, U.S.A.)

1.4 Chemicals

1.4.1	Alcohol 95%	(The Government Pharmaceutical Organization, Thailand)
1.4.2	Ammonium oxalate GR	(E. Merck Co., Germany)
1.4.3	Collodion 2% in amyl acetate	(Sigma Chemical Company, U.S.A.)
1.4.4	Crystal violet (certified)	(E. Merck Co., Germany)
1.4.5	Dipotassium hydrogen phosphate	(May and Baker Ltd., England)
1.4.6	Immersion oil	(American Optical Company, U.S.A.)
1.4.7	Iodine	(May and Baker Ltd., England)
1.4.8	Phosphoric acid	(May and Baker Ltd., England)
1.4.9	Phosphotungstic acid GR	(E. Merck Co., Germany)
1.4.10	Potassium dihydrogen phosphate	(May and Baker Ltd., England)
1.4.11	Potassium hydroxide	(May and Baker Ltd., England)
1.4.12	Potassium iodide	(May and Baker Ltd., England)
1.4.13	Potassium nitrate	(May and Baker Ltd., England)
1.4.14	Safranin	(Hartmann-Leddon Co., U.S.A.)
1.4.15	Sodium chloride	(May and Baker Ltd., England)
1.4.16	Xylene	(BDH Chemicals Ltd., England)

1.5 Glasswares

1.5.1	Beakers	(Pyrex, U.S.A.)
1.5.2	Erlenmeyer flasks	(Pyrex, U.S.A.)
1.5.3	Glass funnels	(Pyrex, U.S.A.)
1.5.4	Glass slides	(Clay Adams, U.S.A.)
1.5.5	Measuring cylinders	(Pyrex, U.S.A.)
1.5.6	Petri dishes	(Pyrex, U.S.A.)
1.5.7	Pipettes	(Pyrex, U.S.A.)
1.5.8	Test tubes	(Pyrex, U.S.A.)
1.5.9	Volumetric flasks	(Pyrex, U.S.A.)

1.6 Instruments

1.6.1	Analytical balance H6T	(E. Mettler, Switzerland)
1.6.2	Autoclave	(American Sterilizer Company, U.S.A.)
1.6.3	Colony counter	(New Brunswick Scientific, U.S.A.)
1.6.4	Hitachi HU 11 E Electron microscope	(Hitachi, Japan)
1.6.5	Hot plates	(Chromalox, U.S.A.)
1.6.6	Incubator, Precision model 6	(Precision Scientific Co., U.S.A.)
1.6.7	Light microscope	(Hertel & Reuss, West Germany)
1.6.8	Olympus photomicrographic system camera model PM-10-A	(Olympus Optical, Japan)

1.6.9	Oven, Precision model 27	(Precision Scientific Co., U.S.A.)
1.6.10	Tweezers	(Agar Aids, England)
1.6.11	Unicam SP 600 series 2 spectrophotometer	(Pye Unicam Ltd., England)
1.6.12	Vacuum Evaporator, model HUS-4	(Hitachi, Japan)
1.6.13	Vortex cyclomixer	(Clay Adams, U.S.A.)

1.7 Others

1.7.1	Black and White panchromatic film	(Kodak Ltd., England)
1.7.2	Copper grid, square 400 mesh	(Agar Aids, England)
1.7.3	Fuji film for electron microscope	(Fugi Films, Japan)
1.7.4	Kodak photographic paper	(Kodak Ltd., England)

2. Methods

2.1 Determination of minimal inhibitory concentrations (MICs) by broth dilution method[55].

Broth dilution test was used to determine the minimal concentration of an antimicrobial agent required to inhibit or kill a microorganism. Serial dilutions of the antimicrobial agent were inoculated with the organism and incubated. The minimal inhibitory concentration was the lowest concentration without apparent growth

of the organism[56].

2.1.1 Preparation of solvent and diluent.

2.1.1.1 Phosphate buffer, pH 6, 1%[57]

Dibasic potassium phosphate (K_2HPO_4)	2 g
Monobasic potassium phosphate (KH_2PO_4)	8 g
Distilled water q.s.	1000 ml

Dissolved dibasic potassium phosphate and monobasic potassium phosphate in about 750 milliliters (ml) of distilled water, and diluted to 1000 ml. Adjusted the pH with 18 N phosphoric acid or 10 N pocassium hydroxide to 6.0 ± 0.05. Distributed the solution into Erlenmeyer flasks and sterilized at 15 lb/in^2, 121^o for 15 min.

2.1.1.2 Phosphate buffer, pH 8.0, 0.1 M[57]

Dibasic potassium phosphate	16.73 g
Monobasic potassium phosphate	0.523 g
Distilled water q.s.	1000 ml

Dissolved dibasic potassium phosphate and monobasic potassium phosphate in about 750 ml of distilled water, and diluted to 1000 ml. Adjusted the pH with 18 N phosphoric acid or 10 N potassium hydroxide to 8.0 ± 0.1. Distributed the solution into Erlenmeyer flasks and sterilized at 15 lb/in^2, 121^o for 15 min.

2.1.2 Preparation of stock solution of antibiotics.

Ampicillin sodium powder and gentamicin sulphate powder were stored in the desiccator at 4° before using. They were accurately weighed out and dissolved in buffer solutions to give the stock solutions as shown in Table 2 and then were kept frozen at -20°. They were used within 7 days and one month respectively[57].

Table 2 Solvents and diluents for stock solution of antibiotics[56,57].

Antibiotics	Solvent	Diluent
Ampicillin	Phosphate buffer, pH 8.0, 0.1 M	Phosphate buffer, pH 6.0, 1%
Gentamicin	Phosphate buffer, pH 8.0, 0.1 M	Distilled water

2.1.3 Preparation of serial dilution of antibiotics.

2.1.3.1 Preparation of serial two-fold dilutions of antibiotics for preliminary test[55,56].

Each of the stock solution was diluted to twice the highest final concentration desired. Sterile 13 by 100 mm cotton pluged test tubes were used in the preparation of serial dilution of each antibiotic. To the first tube, 2.0 ml of the working solution of antibiotic was added. One ml of TSB was added to each of the

remaining tube. Using the sterile pipette, 1.0 ml was transferred from the first tube to the second tube. After thorough mixing the contents of the second tube, 1.0 ml was transferred to the third tube. This process was continued through the next-to last tube, from which 1.0 ml was removed and discarded. The last tube received no antibiotic and served as a growth control. Each series of the 10 dilutions of antibiotic was prepared duplicately.

2.1.3.2 Preparation of arithmetic serial dilutions of antibiotics[58].

After knowing the limited ranges of minimal concentration of antibiotics that inhibited the bacterial growth from the serial two-fold dilution procedure, the stock solution of each antibiotic was diluted in arithmetic serial concentrations, of 0.2 milligram (mg) interval for ampicillin on *P. aeruginosa*, and 0.2 microgram (μg) interval for ampicillin on *S. aureus* and gentamicin on *S. aureus* and *P. aeruginosa*, to determine the precise minimal inhibitory concentration. This was prepared by pipetting volumes of diluent diminishing by a constant amount. Each was then made up to a standard volume by the addition of increasing volume of antibiotic solution. After thorough mixing the contents of each tube, 1.0 ml of each dilution of the antibiotic in these arithmetic series was added to each

tube that contained 1.0 ml of TSB excepted the growth control tube. After thorough mixing the contents of each tube, 1.0 ml was remove, and discard. Finally, each tube contained 1.0 ml of antibiotic solution in TSB. Each series of the antibiotic dilution was also prepared duplicately.

2.1.4 Inoculum

S. aureus and *P. aeruginosa* were inoculated in TSB and incubated at 37^{o} for 18 hours (hr). They were measured the extinction in a spectrophotometer at 580 nm and adjusted with TSB in order to yield 10^{7}-10^{8} colony forming units (cfu) per ml by comparison with the preliminary data. At last, they were then diluted with TSB to give 10^{5}-10^{6} cfu per ml and 1.0 ml of such preparation was used as standard inoculum for each tube in the serial dilutions of antibiotic understudied.

2.1.5 Incubation

The tubes were incubated at 37^{o} for 16 to 20 hr.

2.1.6 Reading results

The least amount of antibiotic which caused complete inhibition of growth was observed by naked eye and was recorded as the end point.

2.2 Determination of the effects of subinhibitory antibiotic concentrations on bacteria.

2.2.1 Inoculum

Prepared the inoculums according to the method described in 2.1.4

2.2.2 Concentrations of antibiotics

Ampicillin sodium and gentamicin sulfate, each was diluted to give the effects of final antibiotic concentrations equivalent to one-fourth, one-half and one time the MIC on the test organisms.

2.2.3 Duration of exposure to the antibiotics.

Cultures of *S. aureus* and *P. aeruginosa* were exposed to ampicillin and gentamicin for 4, 16 and 24 hr. These were studied comparing to the controls which contained no antibiotics.

2.2.4 Effects on growth of bacteria.

All antibiotic-treated bacterial cultures were quantitated by serial 10-fold dilution in normal saline and 0.1 ml aliquot portion of each dilution was incorporated into TSA pour plates for enumeration of cfu/ml after 18 hr incubation at 37°.

2.2.5 Effects on microscopic morphology.

2.2.5.1 Gram staining for light microscopy[56].

2.2.5.1.1 Preparation of solutions for staining.

Modified Hucker's crystal violet:

Solution A

Crystal violet (certified)	2	g
Ethyl alcohol, 95%	20	ml

Solution B

Ammonium oxalate	0.8	g
Distilled water	80.0	ml

Mixed solution A and B. Stored the solution for 24 hr before using and filtered through filter paper into staining bottle.

Gram's iodine:

Iodine	1	g
Potassium iodide	2	g
Distilled water	300	ml

Grinded the dry iodine and potassium iodide in a mortar. Added water, a few milliliters at a time, and grinded thoroughly after each addition until

solution was achieved. Rinsed the solution into an amber glass bottle with the remainder of the distilled water.

Counterstain:

Stock Solution

Safranin O (certified)	2.5	g
Ethyl alcohol, 95%	100	ml

Dissolved the safranin in the ethyl alcohol and mixed thoroughly.

Working solution

Stock solution	10	ml
Distilled water	90	ml

Mixed the solution and water thoroughly.

2.2.5.1.2 Staining procedure

- Smeared the bacterial culture on cleaned glass slide, allowed to dry in the air and fixed the slides with gentle heat.

- Flooded the smears with crystal violet solution and let standing for 1 min. Washed the smear briefly with tap water and drained off excess water.

- Flooded the smears with iodine solution and let standing for 1 min. Washed the smears with tap water.

- Decolorized the smears with 95% ethyl alcohol until the solvent appeared colourlessly from the slide. Washed briefly with tap water.

- Counterstain with safranin for 10 sec. Washed briefly with tap water and then blot dry.

2.2.5.1.3 Examined by the light microscope and photographed.

2.2.5.2 Negative staining for electron microscopy[54].

2.2.5.2.1 Preparation of collodion grids.

- A dish about 20 centimeters (cm) in diameter was filled with distilled water.

- A large circle of fine wire gauze (about 100 meshes/in^2) was placed on the bottom of the dish.

- A number of 400 meshes grids were placed on the wire guaze.

- Two drops of a 2% solution of collodion in amyl acetate were allowed to fall on the surface at the center of the dish from a dropping pipette.

- When the solvent had evaporated to leave a solid film which was then removed from the surface with a forcep. The purpose was to clean the water surface.

- A second film was now formed in the same way and it could be mounted on the grids.

- The wire gauze, which carried the grids and film with it, was lifted from the dish and allowed to dry.

- Put the collodion grids in the vacuum evaporator to make carbon replica.

2.2.5.2.2 Electron Stain

One drop of bacterial suspension was mixed with one drop of 2% phosphotungstic acid at pH 6.5. The prepared 400 meshes carbon-collodion coated grids were touched to the mixture of bacterial suspensions, and the excess fluid was removed by filter paper. After drying, the grids were examined in a Hitachi HU 11 E electron microscope at 75 KV and photographed.

2.3 Determination of MICs and morphology of microorganisms subsequently grown from the antibiotic-treated cells.

2.3.1 Subcultured the antibiotic-treated bacteria in drug-free TSB, incubated at 37° for 18 hr.

2.3.2 Gram stained the growth culture according to 2.2.5.1 and examined with light microscope.

2.3.3 Prepared arithmetic serial dilutions of antibiotics according to 2.1.3.2. Finally, each tube contained 1.0 ml of diluting antibiotic solution in TSB excepted growth control tube. Each series of the antibiotic dilutions was prepared duplicately.

2.3.4 To each tubes of a series, 1.0 ml of the standardized inoculum from 2.3.1, as described in 2.1.4, was added and mixed. The tubes were then incubated at 37° for 16 to 20 hr.

2.3.5. The least amount of antibiotic which caused complete inhibition of growth was observed by naked eye and was recorded as the end point.