CHAPTER IV

MATERIALS AND METHODS

1. Chemical and reagents

Most of the chemical agents used in this study were molecular biology grade. Name lists of all chemical reagents and instruments were shown in the appendix.

2. Subjects and sample collection

All subjects and samples were obtained at the Siriraj Hospital.

2.1 The subjects of this study were

2.1.1 Swab from patients and environment in Respiratory Care Unit (RCU) and Trauma Intensive Care Unit (TICU).

2.1.2 Water from respirators.

2.1.3 Sputum from patients with respiratory tract infection.

Samples were collected during a 7-month period between July 1997 and February 1997.

a. Throat swabs were collected and cultured on the first day of the patients' admission and subsequently collected and cultured once a week until the *P.aeruginosa* was isolated or patients were moved out of the units. Total of 385 throat swabs samples were included in this study.

b. Swab from surgical wound of tracheostomy were collected on the same day as the throat swab collection and collected subsequently once a week until *P. aeruginosa* was isolated. Totals of 117 wound samples were included in this study.

c. Swabs from 5 sinks in the RCU and 9 sinks in the TICU were collected on the same day as the throat swab collection and collected subsequently once a week. Total of 120 swabs in the RCU and 216 swabs in the TICU were included in this study.

d. Water from respirator. Approximately 10 ml of water was collected on the day of patients' admission in the RCU and the TICU. Total of 20 samples in RCU and 222 samples in the TICU were included in this study.

e. Morning true sputum screened by standard method recommended by John G et al, 1987 (124) from the patient with lower respiratory tract infection was collected. Briefly, sputum sample was smeared on the clean slide and stained with gram staining and was then examined under 100x objective lens of a light microscope over the entire smear. In this study, the criteria were set up that moderate to heavy growth of the organism on the agar plate should indicate *P. aeruginosa* colonization in the patients while the true sputum was identified by the the presence of less than 25 cells of squamous epithelium cell along with equal or less than 25 white blood cells under low power objective lens (X10).

3. Culture and identification

All of the swab samples were plated on tryptose blood agar base medium and incubated at 37°C for 48 hours. Five ml of water from respirator were added to the tube which contained 5 ml of double strength tryptic soy broth and then incubated at 37°C for 48 h. One loop full from broth was streaked on blood agar and incubated at 37°C for 48 h. One loop full of the true sputum were plated on 5% sheep blood agars and incubated at 37°C for 48 hours. Each of the *P. aeruginosa* suspected colonies with different appearance were picked and identified according to Manual of Clinical Microbiology (125) including gram staining, colony morphology, pigment producing test, O-F test, growth at 42 °C, and growth in triple sugar iron (TSI). All test procedures were described as followed;

3.1 Gram staining procedure

The organisms were smeared on a clean slide and allowed to dry. The slide was gentlely heated with a flame to fix the smear. Gram crystal violet was dropped on the smear. After 1 minute, the slide was then washed with water and drained. Next, gram iodine solution was dropped on the smear, and washed with water after 1 minute. The smear was decolorized with ethanol (95%) and then washed with water. Five percent safranin solution was dropped on the smear for 30 second. The smear was washed with water and then allowed to dry.

3.2 Colony morphology

The colonies after 48 hours incubation were examined for their characters on the tryptose blood agar base medium.

3.3 Oxidase test

Each colony of the suspected isolates was streaked on nutrient agar and incubated at 37°C for 24 hr. One percent of tetramethyl-p-phenylenediamine was dropped on the filter paper for 4-5 drops. The colony was picked up with steriled toothpick and lined on this paper. The paper was examined for color, positive test was indicated by a change to purple or blue.

3.4 Pigment producing test

Each colony of the isolates was streaked on Mueller-Hinton agar plate and incubated at 37°C for 24 hr. Pigment was observed by using the standard Woods' lamp at 254 nm and the color was recorded.

3.5 O-F test

The 24 hrs old colony on the tryptose blood agar base plate were picked up with straight wire and stabbed at least four times into O-F base medium. Stabs should extend approximately 5 mm deep into the test medium. One of each of the two identical tubes was overlayed with sterile melted paraffin combined with approximately 1 cm. The tubes were then incubated at 37°C for as long as 4 days and examined daily for production of acid, as indicated by a change in the bromthymol blue indicator from green to yellow.

3.6 Growth at 42°C

Each colony was streaked on nutrient agar and incubated at 42°C for 24-48 hr. The plates were examined for ability to growth after incubation.

3.7 Growth in triple sugar iron (TSI)

The 24 hrs old colonies of the isolates on tryptose blood agar base medium plate was streaked on the surface of medium and then also stabbed into the test medium. The tube was incubated at 37°C for 24-48 hr and examined for the production of base from butt and slant, as indicated by a change in the indicator from red to yellow.

4. Antimicrobial Susceptibility Test

All the *P. aeruginosa* isolates were tested for antimicrobial susceptibility pattern against 15 commonly used antimicrobial agents in the treatment of respiratory

tract infections. The antimicrobial discs were ampicillin 10 μg, gentamicin 10 μg, amikacin 30 μg, cefazolin 30 μg, co-trimoxazole 25 μg, ceftazidime 30 μg, tetracyclin 30 μg, piperacillin 100 μg, imipenem 10 μg, ciprofloxacin 5 μg, cefotaxime 30 μg, ceftriazone 30 μg, netilmicin 30 μg, sulbactam/cefoperazone 30 μg, and ampicillin/sulbactam 20 μg. The test procedure was performed according to Kirby and Bauer Disk Diffusion method (126). Briefly described as followed:

4.1 Inoculum preparation

At least 3 to 5 well-isolated colonies of all *P. aeruginosa* including control strain, *P. aeruginosa* ATCC 27853 were inoculated into a tube containing 5 ml of tryptic soy broth. The broth culture was incubated at 37°C until it achieved or exceeded the turbidity of the 0.5 McFarland standard (usually 2 to 6 hours). The turbidity of the actively growing broth culture was adjusted with sterile saline to obtain the 0.5 McFarland standard which should contain the bacteria of approximately 1-$2x10^8$ cfu/ml.

4.2 Inoculation of test plates

Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculum from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the swab over the entire sterile agar surface and repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed.

4.3 Application of disks to inoculated agar plates

The antimicrobial disks were dispensed onto the surface of the inoculated agar plate. Each disk was pressed down to ensure complete contact with the agar surface. No more than 5 antimicrobial disks was placed on each plate. The plates were inverted and placed in an incubator set to 37°C within 15 minutes after the disks were applied.

4.4 Plate reading and result interpreting

After 16 to 18 hours of incubation, each plate was examined. The diameters of the zones of complete inhibition were measured. Zones were measured to the nearest whole millimeter, using a sliding caliper, which was held on the back of the inverted petri plate. The sizes of the zones of inhibition were interpreted by referring to table of zone diameter standard of National Committee of Clinical Laboratory Standard (NCCLS) (127). The organisms were reported as either susceptible, intermediate susceptible, or resistant to the agents tested.

5. Preparation and separation of restriction fragments length polymorphism of chromosomal DNA by pulsed-field gel electrophoresis (PFGE)

Restriction fragments of chromosomal DNA of all *P. aeruginosa* isolates were prepared and separated by PFGE according to the method recommended by Maslow et al. (128) The method was as followed :

5.1 Sample Preparation

A single well-isolated colony was inoculated into 0.5 ml tryptic soy broth and grown for 2 h or until turbid, then streaked onto a plate and incubated at 37°C

overnight. Each well-isolated colony of *P. aeruginosa* was then again inoculated into 5 ml of tryptic soy broth and incubated overnight at 37°C in shaking bath. One and a half millilitre of overnight culture was dispensed into each 15 ml snap-top tube which was previously filled with 5 ml cold PIV buffer, and placed on ice. The tubes were then centrifuged at 1,100xg for 15 min at 4°C. PIV buffer were then decanted from the cell pellet resuspended the cells throughly in 1.5 ml of cold PIV. All the tubes were placed on ice. Low melting point agarose (Difco) in PIV buffer was prepared in a 15 ml tube to make the final concentration of 1.3% w/v. This agarose tube was revortexed prior to aliquoting. One ml of agarose was used for each strain by dispensing into 5 ml snap-top tube which was kept in 50°C heat block. One ml of bacterial cell suspension was added into this tube, which was, then lightly vortexed. One hundred and five microlitre of the mixture were dispensed into each well of the cold plug mold, and placed at 4°C for 30 min to solidify. The cold plug molds were prepared prior to the dispension of the bacteria-agarose mixture by placing the mold in metal tray which was filled with ice and placed the tray in the refrigerator for at least 30 minutes.

Fresh lysis solution was made by adding RNase (final concentration 10 mg/ml) and lysozyme (final concentration 50 mg/ml) to stock lysis buffer. Four ml of lysis solution were dispensed into each labeled 15-ml round-bottom tube. For each strain, the plug was pushed out into the tube containing the lysis solution and incubated overnight at 37°C on a roller (Life Science, US). The lysis step was repeated three times. After that, the tubes were chilled on ice for at least 15 min to harden the plugs. The lysis solution was carefully aspirated. Four ml of ESP solution were dispensed into each chilled tube and incubated overnight at 50°C, shaking gently in a shaker water bath. This step was repeated twice. The plugs as ready for the restriction enzyme digestion. ESP solution was removed and each plug was then washed by adding 5 ml of 1xTE into the plug tube. The tube was incubated at 37°C on a roller. The washing step was repeated four times, 1h, 1h, 2h, and overnight, respectively.)

5.2 Restriction enzyme digestion

For each plug, a labelled microcentrifuge tube containing 1x restriction enzyme buffer, bovine serum albumin (final concentration, 100μg/ml.), and water to a final volume of 250μl were prepared. After that restriction enzyme (*Spe*I) was added into the tube, mixed gently, and incubated overnight at 37°C.

5.3 Gel preparation and loading

The running gel was prepared using Ultrapure agarose (Gibco BRL) dissolving in 0.5x TBE to make the final concentration of 1% w/v. It was pre-electrophoresed at 200v (6v/cm), for 1.5 hr, with the temperature of the buffer in the electrophoresis box at 14°C. Initial switch time of 1s, a final switch time of 40s were used. Each prepared plug including lambda ladder in agarose plug which was used as DNA size standard was cut into the size of approximately 1 mm thick off the end of it by using a glass coverslip and loaded into a well of the gel. All the wells were then filled with 1% low-melting-point agarose (Bio-Rad, US), and the gel was placed on a PFGE box with 0.5x TBE. The remainder of the plug was stored at 4°C in microcentrifug tube containing ESP.

5.4 Gel running

The contour-clamped homogeneous electric field (CHEF) system (Bio-Rad, US) was used. The gel running condition were the same as the pre-electrophoresis condition except that the running time was longer, 23 hr.

5.5 Gel visualization

The gel was stained for 30 min with ethidium bromide solution (0.5μg/ml in water). It was destained with the deionized water for at least 2 h, and then photographed under UV illumination by using the Gel Doc™ 1000 Computerized documentation system (Bio-Rad, US).

5.6 Interpretation of results

The electrophoretic restriction patterns were analyzed by Molecular Analysist™/PC Windows Software for Bio-Rad's Image Analysis Systems Version 1.5.(Bio-Rad,US). The program provided the discrimination of each different patterns based on number and sizes of the fragments. According to Tenover et al,(15) the patterns which were different from each other more than 3 bands were classified as different pulsotypes while the patterns which differed from each other less than 3 bands were classified as subtypes.