CHAPTER 3

MATERIALS AND METHODS

A. In Vitro Study

The organisms used were 351 strains of pathogenic gram negative bacteria. Among these, 66 isolates were obtained from the Department of Medical Sciences, Ministry of Public Health, 215 isolates from Ramathibodi Hospital, 29 isolates from Chulalongkorn Hospital and 41 isolates from Siriraj Hospital.

Determination of Minimal Inhibitory Concentration (MIC)

The agar dilution method was used (7,8). Briefly, the antimicrobial agent was added into agar medium just before it is poured onto a petridish. Strains of pathogenic gram negative bacteria were spot-inoculated simultaneously on to series of petri dishes containing various concentrations of aztreonam. The materials and methods were described as follows.

1. Test medium

Mueller Hinton Agar (Difco, control no. 738974) was used. The ingredients per litre were as follows :

Beef, Infusion form	300	gm
Casamino Acids, Technical	17.5	gm
Starch	1.5	gm
Bacto-Agar	17	gm

To rehydrate the medium, suspended 38 grams in 1000 ml of cold purified water, USP, distilled or deionized water, and heat to boiling to dissolve the medium completely. Dispensed into flasks and sterilized in the autoclave for

15 minutes at 15 pound pressure (121° C). Avoid excessive heat during rehydration or sterilization. Final pH is 7.3 ± 0.1 .

2. The antibiotic diluent

The sterile phosphate buffer pH 6.0 was used. This was prepared by using the following formula :-

1.0 % Potassium phosphate buffer, pH 6.0

Dibasic potassium phosphate	2.0	gm
Monobasic potassium phosphate	8.0	gm
Distilled water to make	1000	ml

Adjust with 18 N phosphoric acid or 10 N potassium hydroxide to yield a pH 5.95 to 6.05 after sterilization.

3. Preparation of the antimicrobial dilutions

The antimicrobial dilutions were prepared as follows :

3.1 Weight aztreonam working standard (potency = 964 μg/mg) 0.2075 gm and dissolved in sterile 1.0% potassium phosphate buffer pH 6.0 to make 100.0 ml solution in a volumetric flask, to obtain a 2000 μg/ml solution.

3.2 Diluted 12.8 ml of the above solution with 7.2 ml of the same sterile buffer to make 1,280 μg/ml solution. It was further diluted to make series of two fold dilutions of aztreonam containing 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40, 80, 160, 320, 640 and 1,280 μg/ml.

4. Preparation of test plates

The agar medium was melted and allowed to cool to 45°C to 50°C in a water bath. Then 18 ml. of it was transferred to each sterile test tube which contained 2 ml of each aztreonam dilution. [The 10 ml. Cornwall continuous pipette (reorder no. 3056, Division of Becton-Dickinson and

company, Rutherford, New Jersey, 07070, USA) was used.]

The tubes were mixed thoroughly but gently and the agar was poured into the sterile 90 mm petri dishes and allowed to harden on a flat level surface.

The agar plates would give the final aztreonam concentrations of 0.0313, 0.0625, 0.125, 0.250, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 and 128.0 μg/ml.

At least one control plate, containing Mueller- Hinton agar without antimicrobial agent, was prepared for every series of dilutions and in every different group of bacteria tested.

The plates were freshly prepared in the day the experiment was performed.

5. Preparation of the inoculum

Pathogenic gram negative bacteria, 351 isolates, obtained from Department of Medical Sciences, Ministry of Public Health and various hospitals, were separately inoculated into 1 ml of sterile nutrient broth in tubes and incubated for 4-6 hours at 37°C. Then the inoculum was standardized to match a 0.5 turbidity standard of Mac Farland when comparing the tubes against a white background with a contrasting black line.

The control strain of E. coli ATCC 25922 was prepared in the same way.

6. Inoculations of agar plates

The inoculum-replicating apparatus (8) was used. About 30 standardized gram negative bacterial suspensions were transferred to the appropriate wells in each seed plate containing 32 reservoirs, the control strain of Escherichia coli ATCC 25922 and sterile nutrient broth were

placed to the other 2 wells in every plate.

An aluminium replicating device (8)(the multipoint inoculator apparatus) was dipped into the wells of inoculum in the seed plate and then the inoculum suspensions were spotted onto the previously dried surface of each antimicrobic-containing plate, by touching the ends of the inoculators on the agar surface. The plates were incubated at 37°C for 16 to 20 hours.

There were 1 or 2 control plates without aztreonam which were inoculated as a control in every set of tests.

7. Reading of test results

The agar dilution plates were examined for growth, after incubation. First, the control plates without aztreonam were checked to be sure that each test strain was capable for providing adequate growth. Then the remaining plates were examined to determine the minimal concentration of drug required for inhibition of growth. The end points were judged when there was a definite dense film of growth in the next plate of lesser concentration. The control strains end points were checked in each set of tests to confirm the reproducibility of the test.

B. Clinical Study

1. Drug used

Aztreonam for injection (Azactam$^{(R)}$) was obtained from E.R. Squibb & Sons, Inc. Each vial contains 1 gm of aztreonam with approximately 780 mg of L-arginine (Control No. 7D01928, Expiry date : April 1, 1990).

1.1 Method of reconstitution and route of administration

1.1.1 For intravenous injection

Add 3 ml of sterile water for injection, shake immediately and vigorously to make a clear solution and inject direct intravenously or by intravenous infusion.

1.1.2 For intramuscular injection

Add 3 ml of sterile water for injection, shake immediately and vigorously to make a clear solution.

1.2 Dose of aztreonam

multiple doses of 30 mg. per kilogram body weight of aztreonam were given intravenously or intramusculary every 6-8 hours.

2. Criteria for selecting the patients

2.1 The patients were children less than 15 years of age from both sexes with a history of gram negative bacterial infections.

2.2 The patients were allowed to recieve other antibiotics with activity against aerobic gram positive or anaerobic bacteria, but not against the infecting aerobic gram negative organisms.

2.3 Number of patients to be studied was not less than 10 evaluable cases.

2.4 Most of the patients were the in-patients who were admitted in Ramathibodi Hospital for at least 5 days or until the symptoms were improved.

3. Antimicrobial Susceptibility Test

Using the disc agar diffusion method (7,8) with the following details.

3.1 Medium and preparation of plates

Medium : Mueller Hinton Agar (see title no.1 p.33) was prepared in 500 ml flasks and sterilized at 121°C under 15 pounds per square inches pressure for 20 minutes.

In the preparation of plates, 20 ml of sterile Mueller Hinton Agar pH 7.2-7.4 was dispensed into sterile glass petri dishes of 90 mm. diameter with the same brand and lot to produce the uniform thickness of the agar. The agar was allowed to harden on a flat level surface. The plates were dried for 1 hour at 37°C.

3.2 Discs

The antibiotic discs tested were aztreonam (Azactam$^{(R)}$) 30 μg/disc obtained from The E.R. Squibb & Son, Inc.

3.3 Preparation of the Inoculum

Samples (Urine or pus) taken from the patients were cultured appropriately before initiation of therapy with aztreonam. The isolates were prepared and standardized as described in title no. 5 (page 35).

3.4 Preparation of test plates

Spread 0.1 ml. of standardized suspension of the causative gram negative bacteria (described in 3.3)over the agar surface (from 3.1) in several directions. After that the discs of antibiotics were placed. The plates were left in room temperature for 30 minutes, then incubated at 37°C for 16 - 18 hours.

3.5 Interpretation of the test results

The diameters of the inhibition zones of the antibiotic were measured with a sliding calipers with an accuracy of nearest 0.1 mm. Faint growth or tiny colonies near the edge of the inhibition zones were ignored if they were presented. Organisms were considered susceptible if the zone of inhibition was $\geq$22 mm , intermediate if the zone of inhition was between 16-21 mm and resistant if the zone of inhibition was $\leq$ 15 mm (12).

4. Determination of patients' serum levels of aztreonam after injection

The method used was the microbiological assay of antibiotics which was adapted from the USP method (10). The materials and methods were set out as follows:

4.1 Assay medium : Muller Hinton Agar (see title no.1 page.33) was used. The medium was dispensed in 500 ml conical flask with cotton wool plugs and sterilized at 15 pounds pressure (121°C) for 15 minutes.

4.2 Assay microorganism : Escherichia coli ATCC 25922

The microorganism was maintained and grown on the nutrient agar slants and had been subcultured every 2 weeks and kept at 4-10°C

The culture which was freshly inoculated on nutrient agar slants and incubated at 37°C for 16-18 hours before each experiment, was washed out with sterile NSS and was adjusted to give a turbidity that would permit 25% light transmission.

4.3 Preparation of plates

Sterile glass petri dishes of 90 mm diameter were used and were of uniform size. They were chosen from the same lot of the same brand (Pyrex).

One-tenth milliliter of the standardized inoculum was added to each 100 ml of the assay medium which had been melted and cooled at 45-50°C. The seeded medium was then swirled to obtain a homogenous suspension. Twenty milliliter of the seeded medium were added to each sterile petri dish, and allowed to harden on a flat level surface. Six isolated holes were made in the hardened agar using a sterile cork borer (4 mm diameter) and a sterile needle.

4.4 Working standard

The working standard was obtained from the E.R. Squibb & Sons Inc. as sodium-free, crystalline powder of aztreonam with the potency 964 μg/mg. It was kept in tightly closed vial which was placed in the desiccator over silica gel and stored in the refrigerator.

The standard solution was freshly prepared on the day of the experiment by the following method :

4.4.1 Preparation of the diluting solution

Sterile phosphate buffer pH 6.0 was used for standard and sample dilutions. It was prepared as described in the title no.2 page 34

4.4.2 Dilution of the standard

1. Aztreonam working standard 0.1037 gm, was dissolved in sterile 1.0 % phosphate buffer pH 6.0 to make 100.0 ml solution in a volumetric flask. This gave a 1,000 μg/ml solution, solution 1.

2. One milliliter of solution 1 was further diluted with 1.0 % phosphate buffer pH 6.0 to make 100.0 ml solution in a volumetric flask. This gave a 10 μg/ml. solution, solution 2.

3. Further diluted solution 2 with 1.0 % phosphate buffer pH 6.0 to make series dilution as follows, using aseptic technique.

No. of solution	proportion of standard solution to buffer solution	Final concentration, μg/ml.
3	0.64 ml of solution 2 + 9.36 ml of buffer pH 6.0	0.64
4	0.8 ml of solution 2 + 9.20 ml of buffer pH 6.0	0.80
5	1.0 ml of solution 2 + 9.0 ml of buffer pH 6.0	1.0
6	1.25 ml of solution 2 + 8.75 ml of buffer pH 6.0	1.25
7	1.56 ml of solution 2 + 8.44 ml of buffer pH 6.0	1.56

4.5 Preparation of the samples

The sera were obtained from the patients at $\frac{1}{2}$-1 hour after injection of the drug and immediately before the next dose. They were kept at -20°C until each assay within one week. The serum had to be diluted to give the appropriate size of zone diameter, but their final concentrations had to be within the standard curve concentrations (0.64-1.56 μg/ml). This was done by making various experimental dilutions of the sera each time blood was drawn after injection.

The results obtained were the followings :-

Sera of the $\frac{1}{2}$-1 hour after injection were diluted to make 1 : 70 dilution. Sera obtained immediately before the next dose were used in the undiluted form or were diluted 1 : 2, 1 : 10 or 1 : 20 as necessaries.

4.6 Assay procedures :-

4.6.1 Preparation of the standard plates

In each experiment, one set of standard curve determination plates was included. It contains 4 set of standard dilutions with the triplicate plates. Solution no. 5 was used as the reference

solution in every plate of assay to check the variations of the zone diameters.

Every inoculated agar plates from 4.3 contained 6 holes,3 of them were filled with 25 μl of one standard solution and 3 holes with the reference dilution using alternate holes.

The volume of the solution added to each hole was accurately measured and transferred with micro-pipette (Eppendorf Digital Pipettes, capacity 10-100 μl, Catalog no. 21-728-43B, Fisher Scientific, Pittsburgh, USA).

The plates were left at room temperature for 1 hour to allow the antibiotic to diffuse into the medium. They were then incubated at 37^{o}C for 16-18 hours. The diameters of the inhibition-zones (produced by the varied concentrations of the standard dilutions) were measured by zone reader (Fisher Scientific company, model 290, USA).

4.6.2 Standard curve determination

To prepare the standard response line, the diameters of the standard reference concentration and the diameters of the standard response line concentration test for each set of three plates were averaged. All 36 diameters of the reference concentration for all four sets of plates were also averaged. The averaged value of the 36 diameters of the reference concentration was used as the correction of the response line. Correct the average diameter obtained for each concentration with the corrected value. Thus, if in correcting the highest concentration of the response line, the average of the 36 diameters of the reference concentration is 16.5 millimeters and the average of the reference concentration of the set of three plates (the set containing the highest

concentration of the response line) is 16.3 millimeters, the correction is + 0.2 millimeter. If the average reading of the highest concentration of the response line of these same three plates is 16.9 millimeters, the corrected diameter is then 17.1 millimeters. Plot these corrected diameters, including the average of the 36 diameters of the reference concentration on 2-cycle semilog paper using the concentration of aztreonam in micrograms per milliliter as the ordinate (the logarithmic scale), and the diameter of the zone of inhibition as the abscissa. The response line is drawn through points plotted for highest and lowest zone diameters obtained by means of the following equation.

$$L = \frac{3a + 2b + c - e}{5}$$

$$H = \frac{3e + 2d + c - a}{5}$$

where :

L = Calculated zone diameter for the lowest concentration of the standard response line ;

H = Calculated zone diameter for the highest concentration of the standard response line ;

C = Average zone diameter of 36 readings of the reference point standard solution ;

a, b, d, e = Corrected average values for the other standard solution, lowest to highest concentration, respectively.

To estimate the potency of the sample, average the zone diameters of the sample and the zone diameters of the standard on the three plates used. If the average zone diameter of the sample is lower than that of the standard, subtract the difference between them from the reference

concentration diameter of the standard response line. From the response line, read the concentrations corresponding to these corrected values of zone diameters. Then the exact serum concentrations were obtained by multiplying the above values with their dilution factors.

5. Method of clinical evaluation

The clinical evaluation (after multiple doses injection of aztreonam) was based on the following criteria :

5.1 The reduction of body temperature (pyrexia) after recieving aztreonam along with paracetamol.

The criterion was set that the patient had a fever if the body temperature was over 37.5°C. The body temperature was recorded every four hours during hospitalization by nurses.

5.2 Other symptoms such as frequency, urgency and/or dysurea in urinary tract infections and improvement of the swelling at the infected sites in skin infection .

The criteria for determining the clinical responses were as follows :

Cure : signs and symptoms of the infection were subsided completely.

Improve : signs and symptoms were substantially improved without complete clinical resolution.

Failure : Substantial improvement in signs and symptoms were absent.

During drug administration, recorded adverse reactions and side effects such as pain at the injection site, skin rash, diarrhea etc. were recorded.

6. Study for bacteriological efficacy of aztreonam

The sample taken from the patients once a day in the morning were inoculated by direct plating on Mc Conkey agar and/or blood agar and were incubated at 37°C for 18 - 24 hours. The colonies were picked up and were inoculated by streaking and stabbing into the Triple Sugar Iron Agar (TSI-agar) slants. The colonies were also transfered to different kinds of media, to determine patterns of biochemical activities, as follows

1. Motility test Semisolid motility agar was inoculated by stabbing with a 24 hours Triple Sugar Iron agar culture. Incubated the culture tubes at 37°C, 24 hours and growth from the stab line (motile) was observed.

2. Urease test Culture was heavily inoculated over the entire surface of the Christensen urease test medium and was incubated at 37°C. Urease-positive culture was determined at various time intervals of incubation. Negative tubes were observed daily for 4 days in order to detect delayed reactions given by members of certain groups other than Proteus, Urease-positive cultures produce an alkaline reaction evidenced by a pink-red color.

3. Fermentation test Carbohydrate media were inoculated for fermentation tests (lactose, dextrose, mannitol, maltose, sucrose, dulcite and xylose) with overnight broth culture. The media were incubated for 18 to 24 hours at 37°C and were examined for acid or acid and gas production.

4. Tests for Indole Tryptophan broth were inoculated and incubated for 48 hours at 37°C. Five drops of Kovac's reagent were added to the broth. A deep red color of the broth indicated the presence of indole

5. Methyl red test Five drops of methyl red solution were added to 5 ml of the culture in Methyl Red-Voges-Proskauer broth (MR-VP); A positive reaction is indicated by a distinct red color, showing the

presence of acid. A negative reaction is indicated by a yellow color.

6. Voges-Proskauer test for acetyl-methylcarbinol Three milliliter of 5% alpha-napthol in absolute ethyl alcohol and 1.0 ml of 40% potassium hydroxide solution were added to 5 ml of 48 hours culture in Methyl Red-Voges-Proskauer broth. The broth was shake well and allowed to stand for 10 to 20 minutes. The presence of acetylmethylcarbinol was shown by a bright orange-red color which gradually extend throughout the broth.

7. Citrate test Simmons Citrate Agar was inoculated. A positive test is indicated by the development of a Prussian blue color in the medium, showing that the organism can utilize citrate as a sole source of carbon.

8. Malonate test Malonate broth was inoculated with a 3 mm loopful of a broth culture and was incubated at 37°C. The result was observed at both 24 and 48 hours. Positive results aer indicated by a change in the color of the indicator from green to prussian blue.

9. Phenylalanine deaminase test Phenylalanine broth was inoculated with a 3 mm loopful of a broth culture and incubated at 37°C for 18 to 24 hours. Five drops of a 10% (W/V) ferric chloride solution were added to the broth. If phenylpyruvic acid has been formed, the broth developed a green color.

10. Potassium cyanide medium Potassium cyanide broth was inoculated with one loopful (3-mm loop) of a 24 hours broth culture and incubated at 37°C. The result was observed daily for 2 days. Positive results aer indicated by growth in the presence of potassium cyanide.

11. Decarboxylase tests Inoculate lysine, ornithine and arginine decarboxylase broth (Falkow) and control tubes from an agar slant culture and overlay with 4-5 mm sterile mineral oil. Incubated at 37°C and examined daily for 4 days. Positive reactions is indicated by

alkalinization of the medium with a change in color from yellow (due to initial fermentation of glucose) to violet (due to decarboxylation of the amino acid). Most positive reaction occur in 1 or 2 days.

12. Phenol red tartrate agar The medium was inoculated by stabbing with a straight wire and incubated at 37°C for 24 hours. Positive test is indicated by the development of a yellow color in the medium.

13. Catalase test An 18 to 24 hours pure colony were picked at the center with an inoculating needle and placed on a clear, glass slide. A drop of 3 % H_2O_2 was added over organism on slide by using a dropper or Pasteur pipette. The result was observed for immediate bubbling (gas liberation).

14. Oxidase test Two drops of Kovac's reagent were added directly to a few suspected colonies growing on plate medium. Positive test is indicated by the development of a dark purple-black color within 10 to 15 seconds.

The results of the test were recorded everyday after treatment with aztreonam for at least 5 days or until the patients were discharge. The organisms were identified as described in Appendix 1, II.

Criteria for determining the microbiological response were as follows.

Elimination : The original causative microorganism was eradicated during therapy without any relapse in the follow-up period.

Relapse : The reappearance of the original causative organism during the follow-up period, ie., after completion of treatment was observed.

Reinfection : The recurrence of infection due to a different organism after completion of treatment was observed.

Failure : The causative organism persisted during

treatment was observed.

Superinfection : The development of infection due to a resistant organism, other than the original pathogen was present during treatment with recurrence of signs and symptoms of infection.

Colonization : The new organism, which was not present in the original clinical specimens, was isolated from clinical material during/or after therapy, but was not associated with signs or symptoms of infection.