CHAPTER II

MATERIALS AND METHODS

Materials

1. Bacterial Strains

The 90 strains of B. fragilis group were isolated from the clinical specimens which were requested for anaerobic culture, at Microbiology laboratories of Chulalongkorn Hospital, Ramathibodi Hospital, and Siriraj Hospital. These isolates were identified to species by fermentation and biochemical tests according to the method of Virginia Polytechnic Institute Anaerobe Laboratory Manual [96], and were kept for study in 10% skim milk, at -70°C [97].

2. Ingradients and Media

2.1. Vitamin K (Menadione)-hemin solution (VK-H)

Fifty mg. of hemin type III (Sigma) was dissolved in 1 ml. of 1N NaoH and made up to 100 ml. with distilled water. Sterilized at 15 lbs. for 15 minutes then added 1 ampule of sterile vitamin K (10 mg. Konakion, Roche), stored at 4°C.

2.2. Salts solution

$CaCl_2$ (anhydrous) 0.2 gm. and $MgSo_4\ 7H_2O$ 0.2 gm. were dissolved in 800 ml. distilled water and added 500 ml. of

distilled water while swirling slowly then added 1.0 gm. K_2HPO_4, 1.0 gm. KH_2PO_4, 10.0 gm. $NaHCO_3$ and 2.0 gm. NaCl. Continue swirling until all these substances were dissolved. The solution was made up to 1,000 ml. with distilled water, mixed and stored at 4° C.

2.3. Brain heart infusion blood agar (BHIA)

Brain heart infusion blood agar (BBL) was prepared by dissolving 37 gm. brain heart infusion, 5 gm. yeast extract (Difco) and 20 gm. Bacto-agar (Difco) in 1000 ml. distilled water then heated until dissolved completely. Cysteine hydrochloride (Fluka) was added at the concentration of 0.05%, sterilized at 15 lbs. for 15 minutes. When the medium was cooled about 55° C, added 10 ml. of VK-H and 50 ml. of whole expired human blood. The amount of 20-25 ml. of this medium was poured into each sterile petridish.

2.4. Wilkins Chalgren agar [98]

The media was prepared by dissolving 10 gm. trypticase (BBL), 10 gm. gelysate (BBL), 5 gm. yeast extract (Difco), 5 gm. NaCl (BDH), 1 gm. glucose (Difco), 1 gm. L-arginine-free base (Sigma), 1 gm. pyruvic acid sodium salt (Merck), 15 gm. agar in 1000 ml. distilled water, heated until dissolved completely and adjusted pH to 7.0-7.2, then sterilized at 15 lbs. for 15 minutes. When the medium was cooled about 55° C, added 10 ml. of VK-H. Eighteen ml. of this medium was mixed with 2 ml. of antibiotic solution then poured into each sterile petridish.

2.5. Brain heart infusion broth (BHIB)

BHIB was prepared by dissolving 37 gm. brain heart

infusion, 5 gm. yeast extract and 0.5 gm. cysteine hydrochloride in 1000 ml. of distilled water. Swirl until completely dissolved, sterilized at 15 lbs. for 15 minutes. When the medium was cool, added 10 ml. VK-H, and 5 ml. of the medium was dispensed into a 13 x 100 mm. sterile screw-cap tube.

2.6 Chopped meat medium

One tablet of the Oxoid chopped meat was added to 10 ml. of distilled water and soaked for 15 minutes, sterilized by autoclaving for 15 minutes at 15 lbs.

2.7. Biochemical media

2.7.1. Peptone yeast extract broth (PY)

This medium was prepared by dissolving 10 gm. of peptone, 10 gm. yeast extract, and 0.5 gm. cysteine hydrochloride in 1000 ml. of distilled water then added 40 ml. of salt solution. This medium was used for indole test and as the basal medium for other fermentative media.

2.7.2. Fermentative media

Dextrose, lactose, maltose, mannitol, rhamnose, salicin, sucrose, and xylose were added to the PY broth in the final concentration of 1% separately.

Arabinose, trehalose and esculin were added in the concentration of 0.5% in the same basal madium separately. Five ml. of each medium were dispensed into a 13 x 100 mm. screw-cap tube and sterilized at 10 lbs. for 10 minutes.

2.7.3. Bile medium

One gm. of dextrose and 2 gm. of ox gall were dissolved to 100 ml. PY broth then dispensed 5 ml. of the medium into a 13 x 100 mm. screw-cap tube and sterilized at 15 lbs. for 15 minutes.

2.7.4. Motility medium

The motility medium was prepared by suspending 20 gm. of motility medium (Gibco)in 1000 ml. of distilled water, mixed thoroughly, heated to boiling, to completely dissolved the medium. The medium was dispensed into 13 x 100 mm. screw-cap tube, 2 ml. each and sterilized at 15 lbs. for 15 minutes.

3. Antibiotic Solutions

3.1. Ampicillin (Gist Bocades) Ampicillin 2560 µg./ml. was prepared by dissolving 0.0256 gm. of standard ampicillin powder in 10 ml. of sterile 0.1 M phosphate buffer pH 8.0.

3.2. Benzylpenicillin (Glaxo) Benzylpenicillin 2560 µg./ml. was prepared by dissolving 0.0256 gm. of penicillin powder in 10 ml. of sterile distilled water.

3.3. Cefoxitin (Merck Sharp and Dome) Cefoxitin 2560 µg./ml. was prepared by dissolving 0.0256 gm. of the powder in 10 ml. of sterile distilled water.

3.4. Rifampicin (Atlantic Lab. Co., Ltd.) Rifampicin 5000

μg./ml. was prepared by dissolving 0.05 gm. of rifampicin powder in 1 ml. dimethylsulfoxide and made up to 10 ml. with phosphate buffer pH 7.0.

4. Nitrocefin Solution

Five mg. of nitrocefin powder (Glaxo) was dissolved in 0.5 ml. of dimethylsulfoxide, then added 9.5 ml. of 0.1 M phosphate buffer pH 7.0, stored in a dark bottle at 4°C, for about 2 weeks.

5. Reagents for Plasmid Extraction

5.1. Lysozyme solution

The solution was prepared by dissolving 0.86 gm. of glucose, 0.27 gm. of Tris (hydroxymethyl) aminomethane, 0.37 gm. of EDTA in 100 ml. of distilled water. Then 2 mg./ml. of lysozyme were added to the solution before use.

5.2. NaOH 0.4 N

The solution was prepared by dissolving 1.6 gm. of NaOH in 100 ml. of distilled water.

5.3. Sodium dodecyl sulfate (SDS) 2%

Two gm. of sodium dodecyl sulfate was dissolved in 100 ml. distilled water.

5.4. Sodium acetate 3M pH 4.8

The solution was prepared by dissolving 40.8 gm. of sodium acetate in minimal volume of distilled water then adjusted to

pH 4.8 with glacial acetic acid and the volume was made up to 100 ml. with distilled water.

5.5. Tris-EDTA, pH 7.5 (TE)

The solution was prepared by dissolving 1.21 gm. of Tris (hydroxymethyl) aminomethane and 0.37 gm. of disodium EDTA in 1000 ml. distilled water then adjusted the pH to 7.5.

5.6. Tris-borate, pH 8.0

This buffer was prepared by dissolving 10.7 gm. of Tris (hydroxymethyl) aminomethane, 5.5 gm. of boric acid and 0.74 gm. of EDTA in 700 ml. distilled water, adjusted to pH 8.0, then the volume was made up to 1000 ml. with distilled water.

5.7. Loading dye

The dye solution was prepared by dissolving 60 gm. of sucrose, 0.068 gm. of sodium acetate trihydrate, 0.05 gm. of bromphenol blue and 0.01 gm. of SDS in 100 ml. distilled water.

5.8. Ethidium bromide for staining gel

One gm. of ethidium bromide was added to 100 ml. of distilled water, then stirred by a magnetic stirrer for several hours to ensure that the dye had dissolved, stored in a dark bottle at 4 °C.

6. Reagents for Isoelectric Focusing Study

6.1. Stock acrylamide

The stock acrylamide was prepared by dissolving

17.5 gm. of acrylamide (Sigma) and 0.5 gm. of N, N'-methylene bisacrylamide (Sigma) in 250 ml. of distilled water then filtered through Whatman filter paper No. 1 and stored the filtrate in a dark bottle at 4°C, not more than one week.

6.2. Riboflavine solution (20 µg./ml.)

The solution was prepared by dissolving 2 mg. of riboflavine in 100 ml. distilled water, stored in a dark bottle at 4°C.

6.3. Ammonium persulfate (50 mg./ml.)

The 0.5 gm. of ammonium peroxydisulfate was dissolved in 10 ml. distilled water. This reagent was prepared just before use.

6.4. Hemoglobin solution

The 5 ml. of anticoagulant blood were centrifuged to get the pack red cell. The cells were washed three times with normal saline solution, and lysed with equal volume of distilled water. The cells debris was extracted with 0.5 volume of toluene, then the tube was vigorously shaken for 5 minutes and centrifuged at 3000 rpm. for 15 minutes. The toluene layer was removed.

7. Equipments

7.1. Steers' inoculum replicating apparatus

7.2. Microcentrifuge (Sigma 101 M)

7.3. Horizontal slab electrophoresis unit

7.4. Transilluminator (Ultra-Violet Products, Ltd.)

7.5. Refrigerated superspeed centrifuge (Sorvall RC-5B, Du Pont Instruments)

7.6. Ultrasonic disintegrator (MSE Soniprep 150, MSE Scientific Instruments)

7.7. LKB 2117 Multiphor analytical electrofocusing

7.8. Flat bottom electrode

Methods

1. Detection of β-lactamase

The method used for detection of β-lactamase activity was the cell suspension method with nitrocefin, a chromogenic cephalosporin as the substrate [99].

The cell suspension was prepared from cells grown on BHIA plates, emulsified in normal saline solution. An aliquot (50 μl.) of cell suspension was placed in a well of a microtiter plate containing 50 μl. of nitrocefin solution. The plate was incubated at 37°C. Readings for color change were performed at immediately, 10 minutes and 30 minutes (Fig. 3, 4).

Staphylococci aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 27853 were used as negative and positive control respectively.

Interpretation

The rate of β-lactamase activity was expressed semiquantitatively as followings [100] :

-, no color change at 30 minutes,

+, orange at 30 minutes,

++, red at 30 minutes,

+++, red at 10 minutes.

2. Determination of Antibiotic Susceptibility

The broth-disc method of Wilkins and Thiel was used for antibiotic susceptibility test [101].

The medium used for testing was BHIB (5 ml.). The appropriate number of standard high concentration discs was added to each tube by the used of flamed forceps (Table 4). One drop of culture in chopped meat broth was inoculated in each tube. The control tube consisted of the media and the culture without antibiotics. All tubes were incubated in anaerobic chamber at 37°C overnight.

Interpretation was based on a comparison of the turbidity of each tube to the turbidity of the control tube. The tested strain was considered to be susceptible when the turbidity was not greater than 50% of the control culture.

3. Determination of Minimum Inhibitory Concentration (MIC)

The MICs of ampicillin, penicillin G, and cefoxitin of each isolate were determined by the agar dilution technique [102].

Preparation of antimicrobial plates

The antimicrobial agents were prepared in a concentration ten times the highest working concentration. The highest concentration for ampicillin, penicillin G and cefoxitin were 256

µg./ml. Two fold dilution of the antimicrobial agent was made with sterile distilled water. Two ml. of the prepared antimicrobial solution were added to 18 ml. sterile Wilkins Chalgren agar which was cooled to about 55°C, mixed together then poured in each sterile petridish. Four control plates containing 20 ml. of Wilkins Chalgren agar without antimicrobial agent were prepared.

Preparation of inoculum

An overnight culture in BHIB was diluted with the same broth to match the turbidity of a 0.5 Mac Farland standard.

Inoculation of antimicrobial plates

A portion of diluted culture was transferred to the appropriate well in the seeded plate and then the inocula were picked up and gently transferred to the agar surface with Steers' inoculum replicating apparatus.

Control plates without antimicrobial agent should be inoculated before and after the inoculation of antimicrobial plates.

The plates were allowed to dry without spreading and incubated 48 hours at 37°C in an anaerobic jar. A couple of control plates (before and after the inoculation of the antimicrobial plates) were incubated anaerobically to serve as growth control and the others were incubated aerobically to detect aerobic contamination.

Escherichia coli ATCC 25922 and Staphylococci aureus ATCC 25923 were included as reference controls.

Interpretation

The MIC of each strain was determined as the lowest concentration of antibiotic yielding no growth.

4. Bacterial Conjugation

Bacterial conjugation was done by the filter mating technique [71]. Twenty-four isolates of Bacteroides strains which had MIC of ampicillin equal or more than 64 µg./ml. served as genetic donors and an isolate of β-lactamase non-producing Bacteroides vulgatus which resisted to rifampicin (50 µg./ml.) served as recipient. The donor and recipient strains were grown separately in BHIB overnight in anaerobic chamber at 37°C. The culture was diluted 10 fold and allowed to grow to mid exponential phase (ca. 10^8 cells/ml.). One ml. of recipient and 0.5 ml. of donor were then mixed and collected by suction on to a sterile membrane filter (25 mm. diameter, 0.45 µm. pore size; Millipore Corp.). The filters were placed cell-side up onto BHIA (containing no antibiotics) and incubated anaerobically for 24 hours. Following the incubation, the filters were transferred to a sterile screw cap tube containing 1 ml. of phosphate buffer saline, then the tube was agitated vigorously. The amount of 0.1 ml. of the cell suspension was cultured on BHIA containing 32 µg./ml. and 50 µg./ml. of ampicillin and rifampicin respectively. The plates were incubated 48 hours in anaerobic jar at

37°C. After incubation, many colonies were transferred to the second plate of the same selective BHIA and reincubated in the same condition. About ten colonies of transconjugant from second plate were confirmed for its species by biochemical tests and tested for antibiotic susceptibility and β-lactamase activity (Fig. 5).

In a control experiment for checking the reliability of the filter mating technique used, the transfer of ampicillin resistance from E. coli A170 to E. coli C600 rifampicin resistance was successfully repeated.

Control experiments in which either donor or recipient strains alone was plated on selective media showed no growth, except for the infrequent occurrence of spontaneous rifampicin resistant mutants of a few strains of B. fragilis.

5. Preparation of Partially Purified Plasmid DNA

The plasmid DNA of transconjugants was prepared by the method described by Birnbaum and Doly [93].

One ml. of overnight culture in BHIB was transferred to a 1.5 ml. microcentrifuge tube and centrifuged for 5 minutes at the speed of 10,000 rpm. The supernatant was removed then suspended the cell pellet with 100 µl. of lysozyme solution incubated at 37°C for 10 minutes. Added 200 µl. of lysis solution (containing equal volumes of 2% SDS solution and 0.4N NaOH) mixed gently and leaved at room temperature for 5 minutes then added 150 µl. of 3M sodium acetate pH

4.8, mixed gently and leaved on ice for 60 minutes to allow the most of proteins, high molecular weight RNA and chromosomal DNA to precipitate. The tube was then centrifuged at 10,000 rpm. for 10 minutes and 400 µl. of supernatant was transferred to a new microcentrifuge tube. Added 1 ml. of cold absolute ethanol, mixed well and leaved at -20°C overnight. The tube was centrifuged at 10,000 rpm. for 5 minutes then the supernatant was removed by aspiration and the pellet of plasmid DNA was washed with 70% ethanol. Then the pellet was dried in air for about 2 hours and dissolved in 20 µl. of Tris EDTA, pH 7.5.

6. Agarose Gel Electrophoresis for Plasmid DNA [95]

The mixture of 10 µl. of the DNA solution and 10 µl. of loading dye was subjected to electrophoresis in 0.7% agarose dissolved in Tris borate buffer. Electrophoresis was carried out at 100 volts for 3-4 hours. The gel was then placed in a solution of ethidium bromide for an hour and destained in distilled water for 15 minutes. The red fluorescence was visualized in the dark with a long wave ultraviolet light source.

Lambda phage DNA treated with restriction endonuclease Hind III was used as reference DNA fragments.

7. Determination of the Isoelectric Point of β-lactamases

The isoelectric point of β-lactamases produced by Bacteroides were determined by the method described by Matthew [27].

Preparation of crude β-lactamase

Organisms were grown overnight in 15 ml. of BHIB then transferred to 300 ml. broth incubated overnight in an anaerobic chamber at 37°C. The bacterial cells were harvested by centrifugation at 4,000 g for 20 minutes at 4°C. The pellets were washed once in 0.05 M sodium phosphate buffer pH 7.0 then centrifuged and finally resuspended in 0.5 ml. of the same buffer. The suspension was then disrupted with an ultrasonic disintegrator for 3 minutes while keeping in an ice bath. After centrifugation at 20,000 g for 20 minutes at 4°C, the supernatant constituted the crude β-lactamase preparation.

Preparation of polyacrylamide gel

The gel mixture was composed of 7.2 gm. of saccharose, 57 ml. of stock acrylamide, 2 ml. of riboflavine solution, 2.4 ml. of ampholine (LKB Bromma, pH 3.5-10 or pH 4-6.5), 60 ul. of N,N,N',N'--tetramethylethylenediamine (TEMED) and after degassed for 10 minutes, 150 µl. of ammonium persulfate solution was added. The gel was then poured in the cassette as shown in the Fig. 6. Ensure that no air bubbles were trapped in the mould. The gel was kept at room temperature for about an hour to allow complete polymerization. This could be noticed by looking for a change in refractive index which became visible when a line could be seen all around the gel about 2 mm. in from the gasket. After polymerization was complete, removed the clamps and kept the sets at 4°C for an hour then carefully pulled out the gasket. Introduced a spatula between the top thin glass plate

and the thicker plate and gently raised of the top plate, by carefully allowing air to enter the gap. Removed the top glass plate and leaved the gel on the other plate.

Isoelectric focusing technique

Pieces of 0.5 x 1 cm. of the Whatman filter paper No. 1 immerged in the crude β-lactamases were placed on the surface of the gel near the cathode. Hemoglobin solution which was used as indicator of focusing was placed on the gel near both the anode and the cathode. Strips of 30 x 0.5 cm. filter paper were immerged in 1M H_3PO_4 and 1N NaOH placed on the surface of the gel near the anode and the cathode respectively. Crude β-lactamases type TEM-1, TEM-2, OXA-1, OXA-2, OXA-3, PSE-1, PSE-2, PSE-3, PSE-4, SHV-1, HMS-1 were used as reference controls. Isoelectric focusing was carried out at about 25 °C, 200 volts, 20 watts, 48 milliampares for an hour then the pieces of filter paper were discarded and further isoelectric focusing was continued until hemoglobin was seen as sharp band. As soon as the current had been switched off, the pH gradient of the gel was read at 1 cm. intervals from the cathode to the anode using a miniature flat ended combined glass electrode. After the pH measurements had been recorded, the gel was overlayed with nitrocefin solution and observed the development of red bands.