CHAPTER IV

MATERIALS AND METHODS

1. Bacterial strain

1.1 *Mycobacterium avium* complex (MAC) clinical isolates

One hundred MAC clinical isolates obtained from Chulalongkorn hospital, during June 2002 to September 2004. The isolates were from hemoculture.

1.2 Control strain

Mycobacterium avium ATCC 25291 was used as quality control strain.

2. Culture and identification

All clinical isolates were cultured on Ogawa medium and identified at the Mycobacteriology laboratory, Department of Microbiology, Faculty of Medicine, Chulalongkorn University by using the AccuProbe culture confirmation kits (Gen Probe, San Diego, California) and biochemical tests. The criteria for MAC identification are positive of tellurite reduction and negative for niacin test, arysulfatase, 10 days Tween-80 hydrolysis, urease and nitrate reduction (38).

2.1 AccuProbe (Genbrobe, San Diego, California)

The AccuProbe was performed according to the instructions supplied by the manufacturer. The protocol consisted of the following steps. For lysis, one loopful of mycobacterial colonies was added to 200 µl of specimen dilution buffer in a lysing tube, and the mixture was sonicated for 15 min in a model 1200 water bath sonicator (Branson Ultrasonics Corporation, Danbury, Conn.) at room temperature. Tube was then incubated at 95°C for 15 min. Chemiluminescent acridinium ester-labeled DNA probes were used to specifically detect the mycobacterial rRNA. One hundred microliter from the lysing tube was

added to the reaction tube of specific probe and the tube was incubated at 60°C for 15 min in a water bath to allow hybridization. After addition of selection reagent (300µl), the tube was vortexed and incubated for an additional 10 min. After the tube was cooled at room temperature for at least 5 min, the result was read in a luminometer. Sample producing signals greater than or equal to the cutoff value 30,000 relative light units was considered positive and signal less than cutoff value was considered negative.

3. Clarithromycin susceptibility testing

All MAC isolates were tested for clarithromycin susceptibility by the broth microdilution, BACTEC MGIT 960 and Epsilometer (E) test method.

3.1 Preparation of clarithromycin

Clarithromycin powder (Abbott Laboratories, Cham, Switzerland) was stored at room temperature prior to testing. The drug was dissolved in methanol and prepared according to the manufacturer's instructions as follow: A stock solution of clarithromycin was prepared in methanol, and sonicated for up to 30 minutes in a water bath sonicator to bring into solution. After the powder was in solution, the solution was diluted with 0.1 M phosphate buffer pH 6.8.

For the broth microdilution, serial two-fold dilution of the stock solution was prepared with 7H9 broth supplemented with 5% oleic acid–albumin-dextrose-catalase (OADC) enrichment to the concentration range 512 to 0.5 µg/ml. For the BACTEC MGIT 960, working solution, whose concentration was 77-fold greater than the required concentration was made from stock solution in sterile distilled water, i.e. the working solutions were 4928 and 1232 µg/ml for the final concentration of 64 and 16 µg/ml, respectively.

3.2 Broth microdilution method

This was performed according to the NCCLS guidelines (26) as follow.

A. Preparation of inoculum

Each isolate was inoculated into 4 ml of 7H9 broth supplemented with 5% OADC enrichment (pH6.8) and incubated at 37°C for 7 days. The isolates were then adjusted to equal the turbidity of a 0.5 McFarland standard (1.5×10^8 CFU/ml). The final inocula ($\sim5\times10^5$ CFU/ml) were prepared by transferring 14 µl of the suspension to tubes containing 4 ml of 7H9 broth supplemented with 5% OADC enrichment (pH6.8). Each of the tubes was then inverted 8 to 10 times prior to use.

B. Procedure

Testing was performed within 30 minutes following final inoculum preparation as described by Brown et al. (106). One hundred microliters of clarithromycin working solution was dispensed into each well of microtiter plate and 100 µl of final inoculum was inoculated into each well. The final concentration of the drug was therefore ranged from 0.25 to 256 µg/ml. Each microtiter plate also contained a positive-growth control well. The microtiter plate was sealed in plastic bag and incubated aerobically at 35-37 °C. A Middlebrook 7H11 agar plate was also inoculated with a loopful of the final inoculum to check for purity. Readings were made after 8 days of incubation. If growth (appearing as turbidity or a deposit of cells at the bottom of the well) in the growth control well was sufficient, the MICs was recorded. If growth in the control well was insufficient, the microtiter plate was reincubated to achieve better growth and read again after the additional 7 days of incubation. The MIC was recorded as the lowest concentration of clarithromycin that inhibited visible growth. If no growth was detected in control wells following reincubation of microtiter plate, result was invalid.

Interpretative criteria of broth microdilution suggested by NCCLS (pH6.8):

Susceptible : MIC ≤16 µg/ml

Intermediate : MIC 32 µg/ml

Resistance : MIC ≥64 µg/ml

3.3 BACTEC MGIT 960 method

A. Preparation of inoculum

Each isolate was inoculated into 4 ml of 7H9 broth supplemented with 5% OADC enrichment (pH6.8) and incubated at 37°C for 7 days. The isolates were then adjusted to equal the turbidity of a 0.5 McFarland standard. Then 0.5 McFarland suspension was homogenized thoroughly and diluted 1:50 by adding 0.1 ml to 4.9 ml of normal saline solution (NSS); this dilution was the working suspension.

B. Procedure

MGIT OADC enrichment of 0.5 ml and 0.1 ml of clarithromycin working solution were added into the MGIT tubes which contain 7.0 ml of Middlebrook 7H9 broth. Then 0.1 ml of the working suspension was inoculated to each of the test tube. Such an inoculum provides an initial bacterial concentration to ~10^4 CFU/ml. The final concentration of clarithromycin in MGIT tubes were 16 and 64 μg/ml. The working suspension was then diluted 1:100, and 0.1 ml was used to inoculate MGIT drug-free tube (containing a 1:100-diluted control), which contained 1% of the bacterial population. All tubes were incubated in BACTEC MGIT 960 automated system. The MIC was considered interpretable when the 1:100 diluted control became positive. This requirement was usually fulfilled between days 5 and 7 of incubation. The MIC defined as the lowest drug concentration in the presence of which the tube remain negative after the incubation period (28).

3.4 E test

A. Preparation of inoculum

Each isolate was inoculated into 4 ml of Middlebrook 7H9 broth supplemented with 5% OADC enrichment (pH6.8) and incubated at 37°C for 7 days. On the day before the MIC test was performed, this culture was adjusted to equal the turbidity of a 0.5 McFarland standard in fresh 7H9 broth supplemented with 5% OADC, and incubated overnight at 35-37°C. This diluted culture was then mixed by tilting 10 times, and diluted 1:100 in Middlebrook 7H9 broth supplemented with 5% OADC. This produced a concentration of ~10^7 CFU/ml.

B. Procedure

Muller-Hintor agar plates enriched with 10% OADC were used for the E test. Each of the plates were flooded with the 1:50 diluted bacterial suspension and pre-incubation for 18 hr at 35-37°C in a 5% CO_2 containing atmosphere before putting the E test strips which contained a gradient of clarithromycin ranging from 0.016 to 256 μg/ml on the plate. Plate were then incubated in plastic bags at 35-37°C in a 5% CO_2 containing atmosphere until growth was visible and the MIC was deduced after six days of incubation at the point of intersection between the zone edge and the E-test strip (27).

C. Analysis criteria

Because the E-test strips contain a continuous gradient of each drug tested instead of the $\log_2$ drug dilution scheme of the broth microdilution, the E-test MIC were elevated to the next drug concentration which matched the broth microdilution scheme to facilitate comparison of the results (e.g. 0.75 μg/ml was rounded up to 1 μg/ml). The percentage of agreement between the E test and the reference broth microdilution method was defined as the proportion of E test results which fell within ± 1 or 2 $\log_2$ dilution of the standard MIC results.

4. Detection of resistance mutation within the 23S rRNA gene

4.1 DNA preparation

Preparation of DNA by freeze-boiling method (120). One or two loopful of organisms grown on ogawa slant for 4 weeks were transferred to a sterile 16x125 mm screw-capped tube containing 6-8 glass beads (3 mm. diameter) and 50 µl of 1% tween-80. The tube was capped and vortexed vigorously until large organism clumps were broken. The suspension was approximated to McFarland standard No.4 in 100 µl of sterile deionize water and subjected to three cycles of snap freeze-boiling (-70°C for 30 min and then 100 °C for 15 min for one cycle). The suspension was used in amplification reaction or stored at -20 °C until needed.

4.2 DNA amplification by polymerase chain reaction (PCR)

Polymerase chain reaction was performed by the method of Jamal, et al. (22) with primers designed according to the sequence of the 23S rRNA gene of *M. avium* (GenBank accession number:X74494). The primers 23SFII (5'-CCGTAACTTCGGGAGAAGGG-3', position 1682 to 1701, *E. coli* numbering) and 23SRI (5'-CCAAACCATCCCGTCGATAT-3', position 2494 to 2475, *E. coli* numbering) was used for amplification to span domain V region of the 23S rRNA gene. The amplified product in the domain V of 23S rRNA gene was 813 bp. Amplification was performed in 50 µl mixture containing 10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 200 µM deoxynucleoside triphosphate (dNTPs; dATP, dCTP, dGTP, dTTP) and 25 pmol of each primer, and 50 µL of mineral oil (Sigma St. Louis, Mo.) to prevent evaporation. The reaction was performed in 0.5 ml eppendorf tube with target DNA on a Hybaid OmniGene Thermal cycles. The PCR cycling parameters were 94°C for 10 min, followed by 35 cycles of denaturation 94°C for 1 min, primer annealing at 55°C for 1 min, extension at 72°C for 1 min, and one cycle of 72°C for 10 min.

4.3 Detection of amplification product

Ten microliters of PCR product were mixed with 3 μl of gel loading buffer (20% ficoll, 0.05% bromophenol blue), analyzed by electrophoresis on 1.5% agarose gel, consisted of 50 μg/ml ethidium bromide, in 1xTris-acetate-EDTA (1xTAE) buffer (pH8.0). The electrophoresis was carried out at 80 volts for 40 min. Gel was visualized with UV transillumination and the positive result of PCR showed a single band of 813 bp fragment for domain V of 23S rRNA gene compared with the 3 Kb molecular size marker.

5. Sequencing of the 23S rRNA gene

Sequencing in the domain V of 23S rRNA gene by the method of Jamal, *et al.* (22) with internal primers 23SFI (5'-TTTAAGCCCCAGTAAACGGC -3', position 1886 to 1905, *E. coli* numbering) and 23SRIII (5'-GTCCAGGTTGAGGGAACCTT-3', position 2305 to 2286, *E. coli* numbering). An ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystem, Foster City, Ca.) was used for the sequencing of the PCR product. Sequencing was performed on 30-90 ng of purified PCR product using QIAquick PCR Purification Kit (Qiagen Corporation, Germany). The sequencing reaction required 4 ml of Premix, 3.2 pmol of primer , and 150 ng of PCR product template in a total volume of 10 μl. The sequencing reaction was performed using Perkin Elmer GeneAmp PCR system 96000 with cycling parameters of 25 cycles of 96°C for 30 sec, 50°C for 10 sec, 60°C for 4 min and hold at 4°C until ready to purify. The product was purified with ethanol/sodium acetate precipitation before capillary electrophoresis was run for sequencing analysis.

The DNA sequence was compared to published sequences of 420 bp region in domainV of 23S rRNA gene in *M. avium* GenBank accession number X74494.