CHAPTER IV

MATERIALS AND METHODS

PART I : SUSCEPTIBILITY TESTING BY RADIOMETRIC METHOD (BACTEC) (98)

1. Chemical reagents and instruments

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

2. Preparation of organism

2.1 Reference strain : *Mycobacterium tuberculosis* H37Rv KK 11-20 was kindly provided by Dr. Chiyoji Abe, Research Institute of Tuberculosis, Tokyo, Japan.

2.2 Clinical isolates : 20 isolates of ciprofloxacin-susceptible *M. tuberculosis* were from Department of Microbiology, King Chulalongkorn Memorial Hospital and 21 isolates of ofloxacin-resistant *M. tuberculosis* were kindly provided by Dr. Charoen Chuchottaworn, Central Chest Hospital, Department of Communicable Disease Control. All resistant isolates were subcultured on 7H11 supplemented with

OADC containing either ofloxacin (Dichi, Japan) and ciprofloxacin (Bayer, Germany) at concentration of 2 μg/ml.

3. Preparation of inoculum from culture on a solid medium

A small quantity of microorganism was removed from the culture tube or plate by scraping numerous representative colonies with the help of a sterile spatula. The cells were transferred to a sterile 16x125 mm screw cap tube containing 8-10 glass beads(1-2 mm diameter) and 1-2 drops of the special diluting fluid. The culture was homogenized by shaking the sealed tube on a vortex mixer until the suspension is well dispersed with as few clumps as possible. 3 ml of the special diluting fluid were added into the tube. The suspension should be moderately turbid. The suspension was standed for at least 30 minutes to allow the large particles to settle. The homogeneous suspension of supernatant was transfer carefully into a separate sterile test tube and adjust the turbidity to a McFarland No.1 standard by adding more special diluting fluid. This suspension was used to inoculate drug susceptibility tests.

4. Inoculation Procedure for Susceptibility Test

The drug medium was prepared by adding 0.1 ml of ciprofloxacin or ofloxacin into individual 12B medium vials for final concentration 2 μg/ml. The 12B vials were arranged in a rack and label properly (one vial for each drug concentration and one for the control). 0.1 ml of the bacterial suspension was inoculated into each of the BACTEC 12B vials containing a drug. A disposable tuberculin syringe was used with a

permanently attached needle for this inoculation. The top of each inoculated vial was followed by cleaning with 70% alcohol swab.

For the control vial, the suspension was inoculated by diluting 1:100 before transferring 0.1 ml of the suspension into 9.9 ml of special diluting fluid. After mixing thoroughly(inverting at least 10 times), 0.1 ml of this dilution was inoculated into the control 12B vial(without a drug). *M. tuberculosis* H37Rv was tested in parallel with the clinical isolates as a control.

5. Incubation

The vials were incubated at 37°C±1°C. Incubation temperature is very critical. It must be not below 36°C. Incubator temperature was checked daily.

6. Reading Schedule

The vials were tested daily approximately at the same time of the day(± 2 hours) on a BACTEC instrument until a GI of 30 or more is achieved in the control vial. The minimum of testing were 4 days and the maximum of testing were 12 days.

7. Interpretation of Results

The GI readings of a susceptibility test require more interpretation than do the primary isolation vials. For the first two to three days, the GI in the control vial will be low but then it will start increasing by a factor of two or three. Since the vials containing drugs were inoculated with a one-hundred-fold larger inoculum, the GI readings are usually higher than the control for the first day or two. If the strain is susceptible to the test drug, the GI output levels off or decreases on the subsequent days. However, the GI value continues to increase for resistant strains and is much higher than the control. This is the reason that a resistant strain can be detected and reported earlier than a susceptible strain. The difference in the GI values from the previous day is designated as ΔGI. Negative ΔGI values indicate a decrease while positive ΔGI values indicate an increase in growth and GI output. When the control vials reach a GI of 30 or more, the results should be interpreted as follows: if the ΔGI is less in the drug vial than the control, the population is susceptible; if more, it means resistant.

ΔGI (Control) > ΔGI (Drug) --------- "susceptible"

ΔGI (Control) < ΔGI (Drug) --------- "resistant"

ΔGI (Control) = ΔGI (Drug) --------- "borderline"

PART II : POLYMERASE CHAIN REACTION (PCR)

1. Extraction of DNA (72)

All isolates were subcultured on Ogawa slants for 4 weeks. The cells were suspended and adjust the turbidity to a McFarland No.1 in 100 µl of sterile deionized H_2O and added 10 µl Proteinase K solution (10x). The cells were incubated at 60°C overnight. The enzyme was inactivated by boiling for 15 min. The protein was extracted by adding 200 µl of phenol-chloroform-isoamyl alcohol (25:24:1) into the mixture and mixed extensively on the vortex mixer. Then, it was centrifuged at 12,000 rpm for 10 min. The aqueous phase was transfered to a new eppendorf tube, followed by adding 20 µl of 3M sodium acetate and 1 ml of cold absolute ethanol. The solution was mixed and DNA was precipitated at -70°C for 30 min. DNA was pelleted by centrifugation at 12,000 rpm for 10 min. at 4°C and the supernatant was discarded. The pellet was washed once with 1 ml of cold 70% ethanol. After centrifugation and supernatant discarded, the pellet was vacuum dried for 10 min. The final DNA was dissolved in Tris-EDTA buffer (TE buffer) and stored at 4°C.

2. Amplification of *gyr*A gene by PCR (12)

2.1 Oligonucleotide primers

The primers were generated from the 5′ conserved region of the *gyr* A gene as described by Takiff (12). Primer GyrA1 (5′-CAGCTACATCGACTATGCGA) and primer GyrA2 (5′-GGGCTTCGGTGTACCTCAT) were purchased from the National Center for Genetic Engineering and Biotechnology, Bioservice Unit (BSU, Thailand). Nucleotide sequence of the *gyr*A fluoroquinolone resistance region was amplified with primers GyrA1 and GyrA2 corresponding to nucleotides 78 to 397 in *M. tuberculosis gyr*A.

2.2 The PCR mixture

DNA lysate preparation (10 µl) were used for amplification of the *gyr*A region . The PCR mixture(50 µl) contained 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$; 10% glycerol; 200 µM(each) dATP, dTTP, dCTP, and dGTP; 0.5 µM(each) primer; and 1.25U of *Taq* DNA polymerase (Pharmacia). Mineral oil was added on surface and amplification was performed for 40 cycles (1 min. at 94°C, 1 min. at 55°C, 1 min. at 72°C); this was followed by a 10 min. extension at 72°C to generate a 320-bp PCR product that was analyzed by agarose gel electrophoresis. The gels were stained with 0.1 µg of ethidium bromide per mL for 15 min, and bands were visualized by UV transillumination.

PART III : DNA SEQUENCING (99)

DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. using doubled-stranded DNA. Both strands were sequenced by synthesizing oligonucleotide primers for the remaining region of the fragments. This study used Sequenase™ version 2.0 DNA sequencing kit, Cleaveland, Ohio, USA. The protocols for sequencing, using [α-^{35}S] dATP as a label, were those suggested by the manufacturer as follows.

3.1 Preparation of sequencing reaction

3.1.1 Treated PCR product :

The PCR product (5 µl) was treated by 1 µl of exonuclease and 1 µl of alkaline phosphatase. After mixing, the product was incubated at 37°C for 15 min and then incubate at 80°C for 15 min.

3.1.2 Annealing mixture :

2 µl of H_2O and 1 µl of primer (5-10 pmole) were added into treated PCR product. Annealing step was started by heating at 100 °C for 2-3 min and then cool rapidly in wet ice. The annealing mixture was centrifuged for use in step 3.1.6.

3.1.3 The cap tubes were labeled and filled with 2.5 µl of each termination mixture (G,A,T and C). The termination mixture were kept at room temperature for steps 3.1.5 and 3.1.7

3.1.4 Labeling mix was diluted 5-fold to working concentration of needed, dGTP. Retain for use in step 3.1.7

Labeling mix	2 µl
H_2O	8 µl

Labeling mix (7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP, 7.5µM [α-^{35}S] dATP (1,000 Ci/mmol at 10µCi/µl, Amersham Corp.,UK)

3.1.5 4 termination tubes were pre-warmed from step 3 (G,A,T and C) in 37°C bath.

3.1.6 LABELING REACTION

The ice-cold annealed DNA mixture (10 µl) was added

DTT, 0.1 M	1 µl
reaction buffer	2 µl
Diluted labeling mix	2 µl
[^{35}S] dATP	0.5 µl
Diluted sequenase polymerase	2 µl
TOTAL	17.5 µl

The labeling reaction was mixed and incubated at room temperature for 2-5 min.

3.1.7 TERMINATION REACTIONS

3.5 µl of labeling reaction was transferred to each termination tube (G,A,T and C), mixed and continue incubation of the termination reactions at 37°C for 5 min.

3.1.8 The reactions were stopped by adding 4 µl of stop solution.(95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% Xylene cyanol FF)

3.1.9 The samples were heated to 80°C for 2 min immediately before loading onto sequencing gel.

3.2 Preparation of denaturing polyacrylamide gel

The 6% polyacrylamide sequencing gel (see Appendix II) was prepared 2-20 h prior to perform using the 20x60 cm gel of Model SA Sequencing Gel Electrophoresis System (BRL, Life Technologies, Inc.) with sharktooth comb (0.4 mm in thickness) for DNA separation.

3.3 Electrophoresis

Pre-electrophoresis was performed for 15 to 45 min at 2,000 volts using 1x Tris-borate buffer (1xTBE), pH 8.3. The well was rinsed with a syringe with needle, or disposable plastic transfer pipette (to remove urea) before applying the samples to the gel. Then electrophoresis started after loading 2-3 µl of sample per well in the same voltage. The progress of electrophoresis was determined by migration of

a marker dye front. After running, the gel was soaked in 5% acetic acid and 15% methanol for 15 min to remove the urea, subsequently dried at 80°C for 90 min using the gel dyer and the DNA bands were visualized by autoradiography using an exposure to X-ray film. Clinical isolates with doubtful results obtained from our manual sequencing protocol were analyzed by automate sequencing service of the National Center for Genetic Engineering and Biotechnology, Bioservice Unit (BSU, Thailand).

PART IV : HETERODUPLEX MOBILITY ASSAY IN POLYACRYLAMIDE GEL (84)

1. The heteroduplexes were performed by using 5 µl of PCR product from the test strain and 5 µl of PCR product from the analogous fragment from a reference strain and 1.1µl of heteroduplex annealing buffer (10x).

2. The mixtures were heated at 94°C for 2 min in ThermoCycler (or in boiling water bath).

3. Then the tubes were cooled rapidly by transferring to wet ice. The heteroduplexes can be kept at room temperature before loading or stored at -20°C.

4. Before loading, the heteroduplex reaction was mixed with 3 µl of Ficoll/loading dye (5x) and was loaded onto a 5% non-denaturing polyacrylamide gel for 6 hours at 200V in 0.6x Tris-borate-EDTA buffer at room temperature. The gels were stained with 0.1 µg of ethidium

bromide per ml for 15 min, and bands were visualized by UV transillumination.

5. For interpretation of results : If the test strain shows pattern of band the same as the reference strain (ciprofloxacin-susceptible *Mycobacterium tuberculosis* H37Rv), the population is susceptible; if different, it is resistant.