CHAPTER III

MATERIALS AND METHODS

1. Bacterial strains and clinical isolates

M. tuberculosis H37Rv, 6 rifampin-susceptible and 9 rifampin-resistant clinical isolates of *M. tuberculosis* were obtained from Dr. Chiyoji Abe, Research Institute of Tuberculosis, Tokyo Japan. These isolates were known for the sequences of their *rpoB* gene fragment as shown in table 3. One hundred and eight clinical isolates of *M. tuberculosis* were obtained from Tuberculosis Division, Department of Communicable Disease Control, Ministry of Public Health, Thailand and cultured onto Lowenstein-Jensen (L - J) medium.

Table 3. Reference *M. tuberculosis* strains used in this study

Bacterial strain	Phenotype	Amino acid affected	Amino acid change
H37Rv	Susceptible	No	No
Mya-65	Susceptible	No	No
J-5	Susceptible	No	No
H-35	Susceptible	No	No
Kor-1	Susceptible	No	No
O-24	Susceptible	No	No
T-3	Susceptible	No	No
Mya-4	Resistant	526	His→Tyr
Mal-87	Resistant	516	Asp→Val
Mal-90	Resistant	516	Asp→Tyr
Ind-5	Resistant	526	His→Asp
YE-4	Resistant	513	Gln→Pro
Ye-12	Resistant	514-515	Phe insertion
YE-43	Resistant	531	Ser→Trp
YEY-3	Resistant	522	Ser→Leu
can-288	Resistant	526	His→Arg

2. Rifampin susceptibility testing

All *M. tuberculosis* isolates were tested for rifampin susceptibility by the radiometric (BACTEC) method which is based on the modified proportion method (16,55). The critical proportion of resistance was evaluated at the 1% level (16).

2.1 Preparation of inoculum

A suspension of each organism was prepared in a sterile 16X125 mm screw- capped glass tube containing 8-10 glass beads (3-mm diameter) and 3 ml of BACTEC diluting fluid (0.02% polysorbate 80, 0.2% fatty acid free bovine albumin). The tube was capped and vortexed vigorously until large organism clumps were broken and the suspension was allowed to stand until remaining clumps settled at least 30 min. Suspensions were transferred the supernatant into a new tube and adjusted the turbidity with BACTEC diluting fluid to equal the McFarland 0.5 standard (for the nonweekend schedule) or McFarland 1.0 standard (for the daily schedule).

2.2 Preparation of rifampin containing medium

The 5 ml of sterile distilled deionized water was added aseptically into the rifampin bottle (80 μg/ml) and shaken well until the lyophilized drug is completely dissolved. The 0.1 ml of rifampin was added into the 4 ml of BACTEC 12 B medium (7H9 broth base, casein hydrolysis, bovine serum albumin, catase, ^{14}C-fatty acid). The final concentrations of drugs in the medium is 2 μg/ml.

2.3 Drug susceptibility testing

All BACTEC 12 B bottles were then tested on a BACTEC 460 instrument prior to any inoculation to establish the recommended 5% CO_2 atmosphere. Do not use any bottle with a GI of ≥ 20 on initial. A 0.1 ml of inoculum was

inoculated into the BACTEC 12 B rifampin medium (test sample). The inoculum was diluted 1:100 in BACTEC diluting fluid, and then 0.1 ml was delivered to control 12 B medium. The bottles were incubated at 37±1°C, and the growth index (GI) was determined in a BACTEC 460 instrument (Becton Dickinson, U.S.A) until the GI of the controls reached at least 30. All bottles were read at the same time of the day, (± 2 hr) for a minimum of 4 days and a maximum of 12 days, the results were interpreted by comparing the increase in GI (Δ GI) from the previous day in the control with that in the drug bottle. If the difference in the GI values between two consecutive days (Δ GI) in the drug-containing vial is less than the control, the isolated is considered "susceptible" if more, it is "resistant". If the GI in the test sample exceeds 500 and remains >500 on the next day, interpret the result as resistant.

3. DNA preparation

Preparation of DNA by freeze-boiling method (60,61). One or two loopfuls of organisms grown on L-J medium 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. DNA amplification

PCR was done as described by Williams et al. (14), with primers Tb Rif-1 (5'CAG ACG TTG ATC AAC ATC CG) and Tb Rif-2 (5'TAC GGC GTT TCG ATG AAC). Amplification was performed in a 50 µl mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 µM each dATP, dCTP, dGTP, and dTTP, 1.0 µM each primer, and 1.25 U of *Taq* polymerase (Perkin-Elmer Cetus,

Norwalk, Conn.Germany). The reactions were performed in 0.5 ml tubes with 10 µl of target DNA on a Hybaid OmniGene thermal cycler. The PCR cycling parameters were 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min 30 sec, and one cycle of 72°C for 10 min. The amplified product of the *rpoB* gene was 305 bps.

4.1 Detection of amplification products

Five 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 (pH 8.0). The electrophoresis was carried out at 80 volts for 20 min. Gel was photographed with UV transillumination, and band sizes were determined by comparison to ϕX174.

5. Direct PCR sequencing

Sequencing of the 305-bp PCR product was performed with a sequenase PCR Product Sequencing Kit (U.S Biochemical, Cleveland, OH, USA), and sequences were determined using dideoxy chain termination method of Sanger et al. (62). Both strands were sequenced. The regions of compression in sequencing gels were resolved by replacing dGTP with 7-deaza-dGTP (63). The protocols for sequencing, using [α-^{35}S] dATP as a label, were those suggested by the manufacture as follows:

5.1 Preparation of sequencing reaction

5.1.1 Enzymatic pre-treatment of PCR product

Five microliters of PCR product were mixed with 1 µl of Exonuclease I (10.0 units/µl), Shrimp Alkaline Phosphatase (2.0 units/µl) 1 µl and

incubated at 37°C for 15 min. Exonuclease I and Shrimp Alkaline Phosphatase were inactivated by heating to 80°C for 15 min.

5.1.2 Annealing reaction

The 3.5 µl of treated PCR product were mixed with primer Tb Rif-1 or Tb Rif-2 (5-10 pmol/µl) 1 µl, deionized water 5.5 µl. The mixture was heated at 100°C for 3 min and placed on ice for 15 min. The mixture was centrifuged and chilled on ice.

5.1.3 Labeling reaction

The ice-cold annealed DNA mixture (10 µl) was labeled by adding 2 µl of reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1 µl of 0.1 M dithiothreitol (DTT), 2 µl of 1:5 diluted labeling mix (7.5 µM dGTP, 7.5 µM dCTP, 7.5 µM dTTP), 0.5 µl of [α-^{35}S] dATP (1000 Ci/mmole; Amersham Life. Science, Inc. U.S.A), 2 µl of Sequenase Polymerase (1.6 units/µl) and incubated 2 -5 min at room temperature.

5.1.4 Termination reaction

The 3.5 µl of labeling reaction was transferred to 2.5 µl of each termination mixture: ddGTP, ddATP, ddTTP, ddCTP, and incubated at 37°C for 5 min. The reaction was terminated by adding 4 µl of stop solution (95% Formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% xylene Cyanol FF) and heating the sample to 75°C for 2 min immediately before loading onto sequencing gel.

5.2 Preparation of denaturing polyacrylamide gel

The 6% polyacrylamide sequencing gel (see Appendix III) was prepared 2-20 hr prior to perform using the 20X60 cm gel of Model SA Sequencing Gel Electrophoresis System (BRL, Life Technologies, Inc. U.S.A) with sharktooth comb (0.4 mm in thickness) for DNA separation.

5.3 Electrophoresis

Pre-electrophoresis was performed for 15 to 45 min at 2,000 volts using 1XTris-borate buffer (1XTBE), pH 8.3. Then electrophoresis started after loading 3.5 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 (MODEL 583, Biolad, U.S.A) and the DNA bands were visualized by autoradiography using an exposure to X-ray film.

5.4 Analysis of DNA sequence

The DNA sequence was compared to published sequences of 305-bp region of *rpoB* gene in *M. tuberculosis* in the GenBank acession number L27989 (11).

6. Heteroduplex formation analysis (HDF)

Heteroduplex analysis was performed by the procedure described by Williams et al. (14) with some modification as follows: nine microliters of the 305-bp PCR product of each test isolate was mixed with 9 µl of the 305-bp PCR product from rifampin-susceptible *M. tuberculosis* H37Rv and 2 µl of 10 X annealing buffer (1M Nacl, 10 mM tris HCl pH 7.8, 20 mM EDTA) (64). Samples were heated at 95°C for 5 min in a thermal cycle and cooled slowly to room temperature with 2-min ramp time for each 5°C decrease in temperature. The resulting DNA duplexes were analyzed

with the use of 7.5% nondenaturing polyacrylamide gel on a 60-cm gel (BRL, Life Technologies, Inc. U.S.A) and the gel was run at 700 V for 19 hr. Gels were stained with ethidium bromide and observed under ultraviolet illumination. The presence of more than one band was considered to indicate the detection of a mutation in the unknown isolate.