CHAPTER III

MATERIALS AND METHODS

MATERIALS

1. Bacterial Strains

1.1 Standard strain of *Mycobacterium avium*, ATCC 25291

The standard strain was obtained from Mr. Somsak Reianthong, TB Division of Ministry of Public Health, Thailand.

1.2 Clinical isolates of *Mycobacterium avium*

Seventy-six isolates *Mycobacterium avium* were obtained from 5 hospitals; 9 isolates from Sanpatong Hospital, 4 isolates from Sansai Hospital, 19 isolates from Nakornping Hospital, 42 isolates from Maharaj Nakorn Chiang Mai Hospital and 2 isolates from Rachavech Hospital. All of these isolates were cultivated at Department of Clinical Microbiology, Faculty of Associated Medical Sciences, Chiangmai University.

2. DNA

2.1 1 kb Plus, Boehringer Mannheim, Germany

2.2 ∅X-174 RF digested by *Hae*III, Boehringer Mannheim, Germany

2.3 Primer 16SC (5'-TCGAAGGTGGGATCGGC-3')

23SG (5'-GCGCCCTTAGACACTTAC-3')

2.4 Primer P1 (5'-GCCGCCGAAACGATCTAC-3')

P2 (5'-AGGTGGCGTCGAGGAAGAC-3')

3. Oligonucleotide

3.1 MV 222 (5'-GGTCTTGGTGGCCGGCGTTCA-3')

3.2 MI 231 (5'-GGCTGATGCGTTCGTCGAAATGT-3')

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4. Enzyme and Restriction Enzymes

The following enzymes were purchased from Roche company

4.1 Lysozyme

4.2 Proteinase K

4.3 *Hae*III

4.4 *Msp*I

4.5 *Bst*XI

4.6 *Pvu*II

5. Instruments

5.1 Automatic pipettes, Gilson, France

5.2 Minicell horizontal gel electrophoresis chamber, Mupid Tokyo Co. Ltd., Japan

5.3 20x24 cm horizontal gel electrophoresis chamber, BRL, USA

5.4 Hybridization oven, SHEL LAB, USA

5.5 Microcentrifuge, Clover Laboratories, USA

5.6 Centrifuge, Kubota, Japan

5.7 Platform shaker, SARSTEDT, Germany

5.8 Gel reader, FOTODYNE, USA

5.9 Transilluminator, VILBER LOURMAT, France

5.10 UV-Visible spectrophotometer, Shimadzu Corp., Japan

5.11 Vortex Genie-2™, Scientific Industries, USA

5.12 Water bath, Memmert, Germany

5.13 Autoclave, Memmert, Germany

5.14 Hot Air Oven, SHEL LAB, USA

5.14 Biohazard Hood, Gelman Sciences, Australia

5.15 GeneAmp PCR System 2400, PERKIN ELMER, USA

5.16 Clean Hood for Master Mix preparation

5.17 CO_2 Incubator, SHEL LAB, USA

5.18 BACTEC 9120 series incubator, Becton Dickinson, USA

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5.19 HYBRI .DOT® MANIFOLD, BRL, USA

6. Media

6.1 Lowenstein-Jensen egg medium (L-J medium)

6.1.1 L-J medium powder, Difco-BBL	9.4	gm.
Deionized water	150	ml.

The solution was autoclaved and allowed it cool at room temperature.

6.1.2 Homogenized whole egg	250	ml.

Mix 6.1.1 and 6.1.2 together, stand for 30 minutes, then dispense 6-8 ml. into steriled McCartney bottle, slant and coagulate by hot-air oven at 80°C for 90 minutes. Store at 2-8°C.

6.2 Drugs containing L-J medium (Ichiyama *et al*, 1988)

Ethambutal (2.5 μg/ml) and ofloxacin (1.0 μg/ml) are added to L-J medium.

6.3 Modified Middlebrook 7H10 agar

6.3.1 Middlebrook 7H10 agar powder, Difco	9.5	gm.
6.3.2 Deionized water	450	ml.

The solution was autoclaved and placed in a water bath at 50 to 56°C.

6.3.3 OADC	50	ml.

Mix them together and add Vancomycin (50 mg/ml), Amphotericin B (50mg/ml), Polymyxin B (500,000 U/ml) and Nalidixic acid (500 mg/ml). Dispense 6-8 ml into steriled McCartney bottle, slant at room temperature. Store at 2-8°C.

7. Reagents

7.1 Reagents for culture of mycobacteria

- -BACTEC® MYCO/F LYTIC MEDIUM, BDMS
- -4% NaOH
- -NALC
- -NSS
- -Sterile deionized water
- -PBS pH 7.0

7.2 Reagents for isolation of genomic DNA from mycobacteria

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-10 mg/ml lysozyme

-10% SDS/Proteinase K mixture; 5 µl of 10 mg/ml Proteinase K and 70 µl of 10% SDS for each sample

-5M NaCl

-CTAB/NaCl solution

4.1 gm NaCl was dissolved in 80 ml distilled water. While stirring, 10 gm CTAB (N-cetyl-N,N,N,-trimethyl ammonium bromide) were added. If necessary, the solution was heated to 65°C for completely dissolve. The volume was adjusted to 100 ml with distilled water. The solution was stored at room temperature for no longer than 6 months.

-24:1 chloroform/isoamyl alcohol

-Isopropanol

-70% Ethanol

-1xTE Buffer

10 mM Tris/HCl, pH 8.0

1 mM EDTA, pH 8.0

7.3 Reagents for Polymerase Chain Reaction

-*Taq* DNA polymerase

-Reaction buffer: 10 mM Tris-HCl pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% (W/V) gelatin

-Deoxynucleoside triphosphate (dNTP)

7.4 Reagents for agarose gel electrophoresis

-Loading buffer: 40%(w/v) sucrose, 0.25%(w/v) bromphenol blue

-10x TBE buffer per 1000 ml: 108 gm Tris-base, 55 gm boric acid and 9.3 gm Na_2EDTA

7.5 Reagents for DIG DNA labeling

-DIG Labeling Kit (Boehringer Mannhiem, Germany):

10x Hexanucleotide mixture, 10x dNTP labeling mixture, Klewnow enzyme (labeling grade)

-200 mM EDTA, pH8.0

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-4 M LiCl

-Absolute ethanol

-70% ethanol

-TE buffer

7.6 Reagents for DIG Oligonucleotide labeling

-DIG Oligonucleotide 3'-End Labeling Kit: 5x Reaction buffer (1 M Potassium cacodylate, 125 mM Tris-HCl, 1.25 mg/ml bovine albumin; pH 6.6), $CoCl_2$ solution (25mM cobalt chloride), DIG-11-ddUTP, terminal transferase, glycogen solution (20 mg/ml glycogen in re-distilled water)

-200 mM EDTA, pH8.0

-4 M LiCl

-Absolute ethanol

-70% ethanol

-TE buffer

7.7 Reagents for Southern blotting

-Denaturation solution: 1.5 M NaCl, 0.5 M NaOH

-Neutralization solution: 0.5 M Tris-HCl pH 7.5, 3 M NaCl

-20x SSC per 1000 ml: 175.3 gm NaCl, 88.2 gm Na-citrate

7.8 Reagents for hybridization and detection

-Prehybridization solution: 5x SSC (750 mM NaCl, 75 mM Na-citrate), 50%(v/v) Formamide, 0.1%(w/v) N-lauroylsarcosine, 0.02%(w/v) SDS, 2% Blocking reagent.

-Hybridization solution: Prehybridization solution added with 10-100 ng/ml digoxigenin-labelled probe

-2x Wash solution: 2x SSC, 0.1%(w/v) SDS

-0.5x Wash solution: 0.1 xSSC, 0.1%(w/v) SDS

-Buffer 1: 100 mM Maleic acid, 150 mM NaCl; pH 7.5

-Buffer 2: 1%(w/v) Blocking reagent in buffer 1

-Buffer 3: 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$

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-Diluted antidigoxigenin-alkaline phosphatase, Fab fragments (1:5000) in buffer 2

-Coloring solution: 45 μl of 4-Nitroblue Tetrazolium chloride (NBT) and 35 μl of 5-Bromo-4-chloro-3-indolyl-phosphate (X-phosphate) in 10 ml of buffer 3

-Buffer 4: 10 mM Tris-HCl pH 8.0, 1 mM EDTA (1xTE buffer)

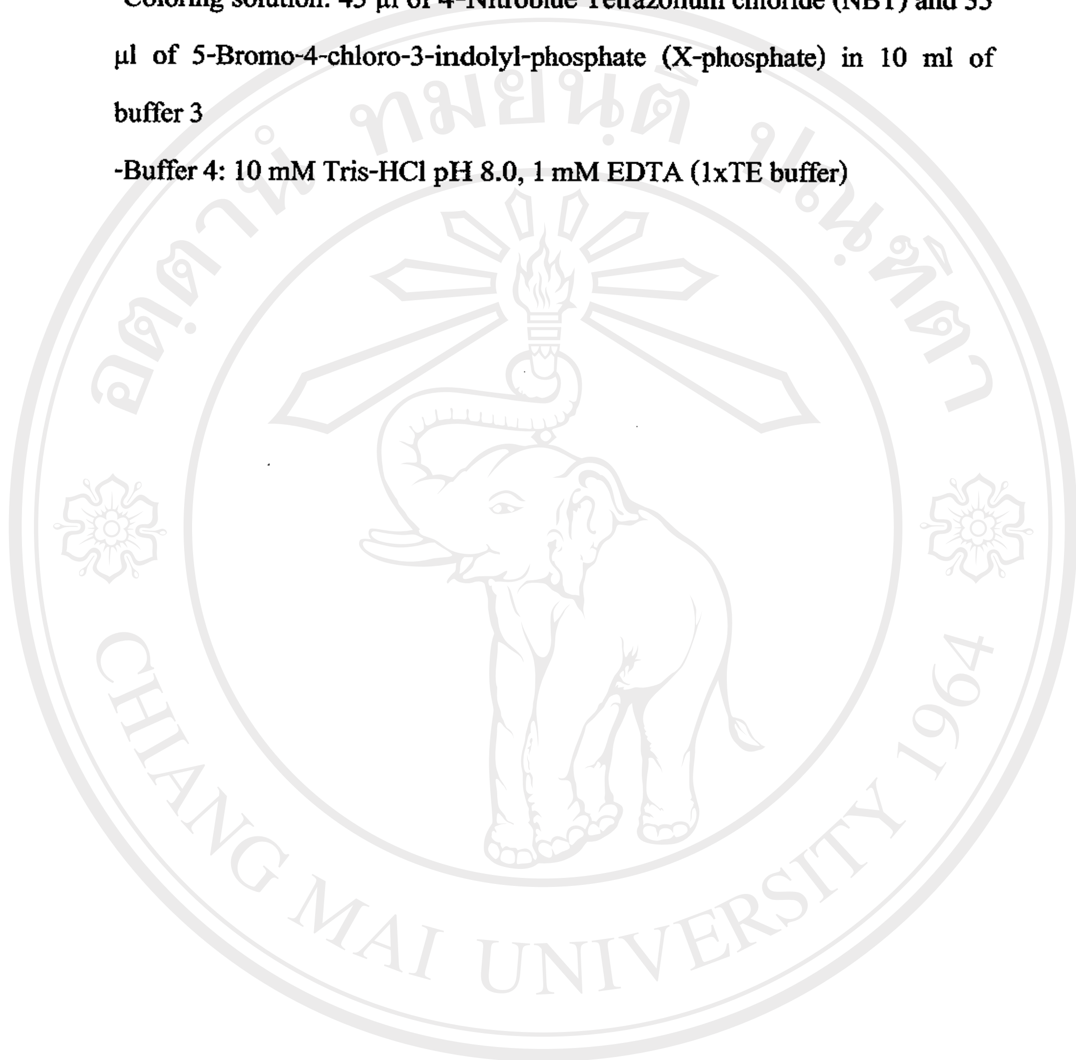
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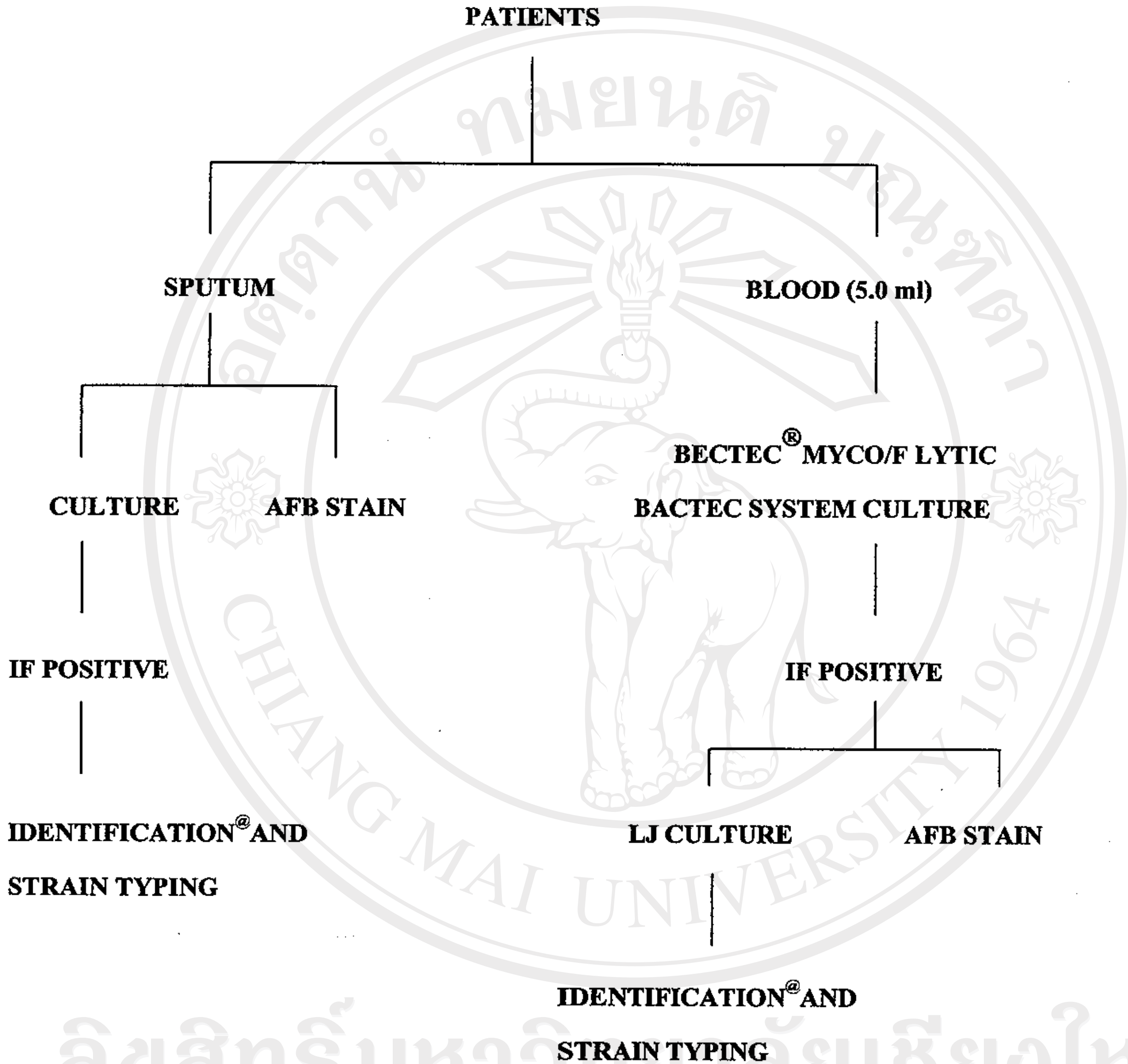
Figure 4 The flowchart of the research



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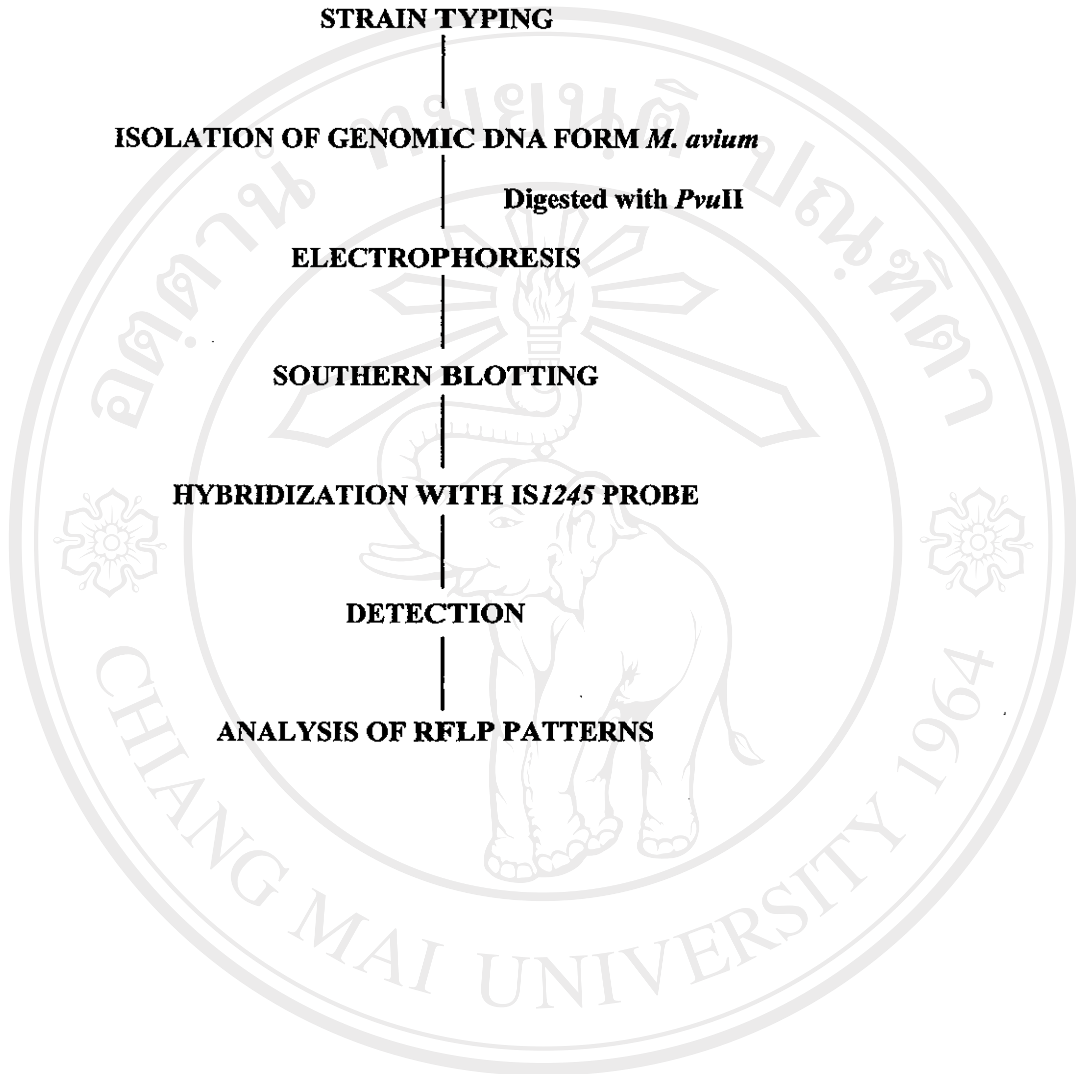
@ Identification with PCR-Restriction Enzyme Analysis and Biochemical test

M. avium species are confirmed by Dot blot hybridization assay using MV222 probe. The strain typing is identified by RFLP using IS*1245* probe. The water supply, stool of their livestock, and soil around accommodation of *M. avium* patients will be analyzed as well.

Figure 5 Flowchart of strain typing



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METHODS

1. Samples

1.1 Blood

After the patient's skin has been cleaned first with tincture of iodine then 70% ethanol, venous blood was drawn about 5.0 ml. The venepuncture must be done twice at an hour interval. The blood was injected into MYCO/F lytic bottle. All blood culture bottles were incubated in BACTEC 9120. The culture was going on up to six weeks. The positive and negative cultures were examined with AFB and Gram's stain, and planted on Chocolate agar, Sabouraud dextrose agar, LJ medium and Middlebrook 7H10 medium.

1.2 Sputum

Three cups of sputum were collected in the early morning lasting for 3 days. The sputa of each patient were digested with NALC-NaOH in the same amount and mixed them until homogeneous. After 15 minutes, PBS was added until the total volume reach 50 ml. The mixture was spun at 3,000xg for 15 minutes. The supernatant was discarded then left for about 0.5 ml above the pellet. The pellet suspension was examined with AFB stain and planted on LJ medium and Middlebrook 7H10 medium.

1.3 Water

One litre of the water was collected from varieties of water supply. The water was filtered by 0.45 μm Millipore filter membrane. The membrane was soaked in 10 ml of 0.85% NaCl. The solution was treated with 10 ml of 4% NaOH for 10 minutes then PBS was added until the total volume 50 ml. The mixture was spun at 3,000xg for 15 minutes. The supernatant was discarded then left for about 0.5 ml above the pellet. The pellet suspension was examined with AFB stain and planted on drugs containing LJ medium and Middlebrook 7H10 medium to inhibit contaminant.

1.4 Soil and Stool

Ten to twenty grams of livestock's stool and soil around each patient's accommodation were collected. Only two to five grams of livestock's stool and soil around each patient's

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accommodation were soaked in 10 ml of 0.85% NaCl. Left stand for 30 minutes at room temperature for precipitation of large particles. The upper supernatant amount 5.0 ml was transferred to steriled centrifuge tube. The same amount of 4% NaOH was added and the solution was mixed briefly. The solution was left for 10 minutes then PBS was added until the total volume 50 ml. The mixture was spun at 3,000xg for 15 minutes. The supernatant was discarded and left for about 0.5 ml above the pellet. The pellet suspension was examined with AFB stain and planted on drug containing LJ medium and Middlebrook 7H10 medium.

The optimal temperature for incubating all medium inoculated with most specimens is 35 to 37°C for 8 weeks. The cultures were observed at day1, day3, day 5, day7, and every week.

2. Isolation of genomic DNA

2.1 Crude extraction of genomic DNA from mycobacteria

The genomic DNA of mycobacteria was prepared by mechanical lysis method as previously described by Sansila *et al* (1998). Briefly, a loop of mycobacteria grown on LJ metium was added to 1.5 ml microcentrifuge tube containing 0.5 ml of distilled water. After the cell suspension was boiled at 80°C for 20 minutes. About 0.5 ml of siliconized acid-washed glass bead (425-600 µm in diameter) was added. Then, the tube was vortexed vigorously for 1 minute and placed in ice bath for another minute. After repeated 10 times, the suspension was centrifuged at 10,000 rpm for 5 minutes. The aqueous phase was used for PCR reaction or stored at -20°C until used.

2.2 Isolation of genomic DNA from *M. avium*

The genomic DNA of *M. avium* was prepared by an enzymatic method, which described previously by van Soolingen *et al.* Briefly, a loopful of cells was transferred to a microcentrifuge tube, which contained 400 µl TE buffer, the cell were killed at 80°C for 20 minutes. Fifty microlitres of 10 mg/ml lysozyme was added. The suspension was vortexed and incubated at 37°C overnight. After adding 75 µl of 10% SDS/proteinase K mixture, the suspension was incubated for 10 minutes at 65°C. One hundred microlitre of 5 M NaCl and 100 µl of CTAB/NaCl solution (which was prewarmed at 65°C) were added. The mixture was vortexed until the liquid became white ("milky") and then incubated for 10 minutes at 65°C. For

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protein separation, 750 µl of chloroform/isoamyl alcohol was added, vortexed for at least 10 second and then centrifuged for 5 minutes at 12,000xg. The aqueous phase was transferred to a new microcentrifuge tube. After that, 0.6 volume of isopropanol was added to precipitate the nucleic acids. The mixture was incubated at -20°C for 30 minutes, and then spun at room temperature for 15 minutes at 12,000xg. The pellet was washed with cold 70% ethanol. The pellet was dried at room temperature and re-dissolved with 50 µl of TE buffer. The concentration of DNA was estimated by measuring the absorbance at the wavelength of 260 nm. DNA solution was stored at -20°C until used.

3. DNA amplification by PCR

3.1 Primers

3.1.1 Primers for 16S-23S rRNA gene spacer

A single pair of primers 16SC and 23SG (synthesized by Gibco,BRL®) was used to amplify spacer sequences of all *Mycobacterium* species. The 16SC (5'-TCGAAGGTGGGATC GGC-3') anneals to a highly conserved region in 16S rRNA gene 63 bp upstream of the spacers of most mycobacteria. The 23SG (5'-GCGCCCTTAG ACACTTAC-3') anneals to a sequence in the 23S rRNA gene 2 bp downstream from the spacers of most mycobacteria (Figure 6).

3.1.2 Primers for IS*1245*

A pair of primers P1 and P2 (synthesized by Gibco,BRL®) was used to amplify IS*1245*. The P1 (5'-GCCGCCGAAACGATCTAC-3') and P2 (5'-AGGTGGCGTCGAGGAAG AC-3') anneal to the position of 135 to 152 and 543 to 561, respectively (Figure 7).

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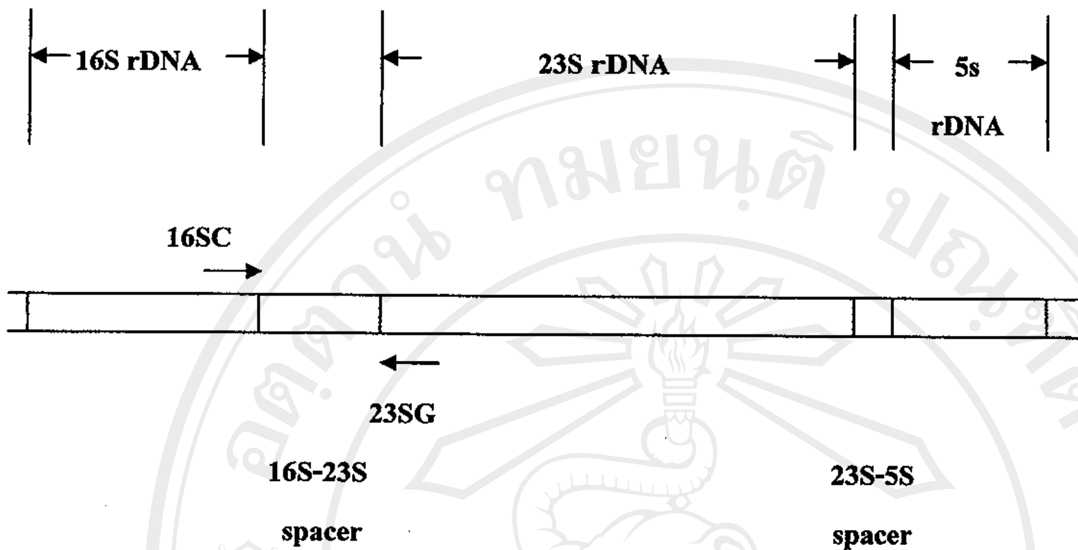

Figure 6 Schematic representation of rDNA operon showing the priming sites (16SC and 23SG) for the PCR amplification of the 16S-23S rDNA spacer region.

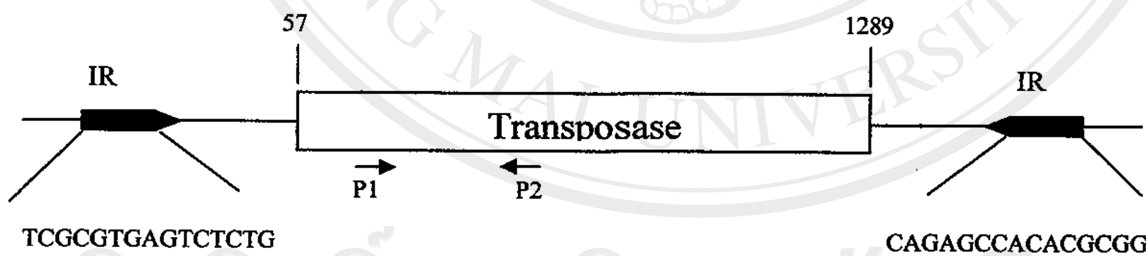

Figure 7 Schematic representation of IS*1245* element showing the inverted repeats (IR), the priming sites of P1 and P2.

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3.2 Amplification

The PCR method was performed with an automated thermocycler. Amplification was done in a total volume of 50 µl with 2 ng of template DNA, 1 µM of each primer, and 1µl of *Taq* DNA polymerase in a reaction buffer containing 10 mM Tris-HCl pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% (w/v) gelatin, 200 µM of each dNTP and 1 µM of each primer (16SC and 23SG). The reaction mixture was placed into the thermal cycle and the condition of the reaction was incubated for 3 minutes at 94°C, and running for 30 cycles of denaturation of DNA for 1 minutes at 94°C, 3 minutes of primers annealing at 62°C and 1 minutes of extension reaction at 72°C. The final cycle was followed by 7 minutes of completely extension at 72°C.

3.3 Agarose gel electrophoresis

Ten microlitre of amplified products of the primers, 16SC and 23SG, were mixed with 2 µl of loading buffer, loaded onto a 1.5% agarose gel in TAE buffer, and electrophoresed in MUPID At 100V for 35 to 40 minutes. The amplified products were visualized by ethidium bromide staining and photographed. The sizes of amplified products were estimated by comparison with the molecular weight marker (1 kb plus marker). For probe preparation, the amplified products of IS*1245* were loaded on to 0.8% agarose gel.

4. Restriction analysis of the amplified products

4.1 Restriction enzyme digestion

For identification of mycobacteria, the PCR product was digested with *Hae*III restriction enzyme. The reaction mixture composed of PCR product 10 µl, *Hae*III (10U/µl) 1 µl, 10xBuffer2 1.5 µl, and distilled water 2.5 µl. The mixture was incubated at 37°C for at least 1 hour.

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4.2 Evaluation of restriction patterns

After digestion, 3 µl of 6xloading buffer was added, and the mixture was loaded on a 3.5% Nusieve 3:1 agarose gel in TAE buffer. Electrophoresis was run at 100 Volts for 30 to 45 minutes, Visualization and interpretation of restriction patterns were described by Sansila *et al* (1998).

4.3 Digestion of Chromosomal DNA by Restriction enzyme

All chromosomal DNA samples of *M. avium* including ATCC 25291 strain were digested with a restriction enzyme, *Pvu*II. The reaction was as following 3 μg of DNA, 3 μl of 10x buffer2, 10 units of *Pvu*II and a volume of sterile distilled water to adjust the volume to 30 μl. The mixture was incubated overnight at 37°C. The electrophoresed DNA fragments were used for Southern blotting hybridization.

4.4 Separation of DNA fragments by electrophoresis for Southern blotting

Twenty-five microlitre of digested chromosomal DNA sample was electrophoresed in 0.8% agarose gel in the 24-cm long electrophoresis set hybridization with IS*1245* probe. Electrophoresis was done for 10 minutes of 100 Volts and thereafter at low voltage (0.5 volts/cm) for 20 hours (overnight) until the 872-bp fragment of *Hae*III-digested ϕX174 DNA has reached a distance of 19 cm from the slot of the gel. Finally, the gel was stained with ethidium bromide and photographed on an UV transilluminator.

5. Extraction and purification of PCR products of *M. avium* ATCC 25291 from agarose gel

The 427-bp PCR products of P1 and P2 primers were extracted from the 0.8% agarose gel by using Agarose Gel DNA Extraction kit (Boehringer Mannheim). The procedure was done as followed: an agarose block containing DNA band was excised from ethidium bromide-stained agarose gel with a surgical blade and 3 volume of solubilisation buffer containing sodium perchlorate was added. Ten microlitre of silica suspension was added and incubated at 56-60°C for 10 minutes. Then, the mixture was spun at 12,000 g for 30 second and removed the supernatant. Five hundred microlitre of nucleic acid binding buffer was added, vortexed, centrifuged and discarded supernatant as before. Washed the pellet twice with 500 μl of washing buffer and removed all liquid. Let it dry at room temperature. The DNA was eluted from the pellet by TE buffer or distilled water.

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6. Hybridization assays

6.1 Probe Labeling

6.1.1 Oligonucleotide probes labeling

Each oligonucleotide probe (Table 2) was 3'-end labeled with digoxigenin-11-ddUTP. The probes were labeled by incubating in the reaction mixture containing 5x reaction buffer, $CoCl_2$ solution, oligonucleotide probes, DIG-ddUTP, and terminal transferase at 37°C for at least 15 minutes. 0.2 M EDTA was added to terminate the reaction. The labeled oligonucleotide probes were stored at –20°C until used without any precipitation.

Table 2 Species-specific oligonucleotides probes sequence

Probe	Sequence	Position	*Mycobacterium* species.	Tm* (°C)
MV222	GGTCTTGGTGGCCGGCGTTCA	222-242	*M. avium*	70
MI231	GGCTGATGCGTTCGTCGAAATGT	231-253	*M. intracellulare*	70

Tm* (melting temperature) = 2(A+T) + 4(G+C)

6.1.2 Random primed DNA labeling

The 427-bp DNA of IS*1245* was labeled with DIG DNA Labeling and Detection Kit (Roche). Firstly, DNA was denatured by heating in boiling water bath for 10 minutes and then chilled quickly in icebath. The labeling reaction was done by adding the reagents in the following order: 10 ng of denatured DNA, 2 µl of hexanucleotide mixture, 2 µl of dNTP labeling mixture, sterile distilled water to adjust the volume to 19 µl and 1 µl of Klenow enzyme. The mixture was centrifuged briefly and incubated 20 hours at 37°C. The reaction was stopped by adding of 2 µl of 0.2M EDTA solution.

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6.2 Blotting

6.2.1 PCR-amplified DNA dot blotting

The PCR products of all *M. avium* isolates were denatured at 95°C for 10 minutes and chilled directly on ice. One microlitre of each RCR product was dotted onto the nylon membrane. The DNA was fixed to the membrane by UV-crosslink. The blotted membranes were stored in seal bag at room temperature until used.

6.2.2 Southern blotting

The DNA in an agarose gel was transferred to a nylon membrane by capillary method as described by Southern. Firstly the gel was denatured for 1 hour in 300 ml of denaturation solution, and neutralized foe 1 hour in 300 ml of neutralization solution. After blotting, the membrane was baked 80°C for 1 hour in a hot air oven.

7. Hybridization

7.1 Hybridization with IS*1245*-labeling probe

Membrane filters were prehybridized with prehybridization buffer (with formamide) with the ratio of 20 ml per 100 cm^2 filter at 42°C for 30 minutes. The prehybridization buffer was then replaced with 3.5 ml per 100 cm^2 filter of hybridization buffer containing 10 to 100 ng of denatured labeling probe DNA and incubated overnight at 42°C. Then, the filter was washed twice with 2x wash solution at room temperature for 5 minutes and twice with 0.1x wash solution at 68°C for 15 minutes. The filter was used directly for detection of hybridized DNA or stored air-dried for later detection.

7.2 Hybridization with oligonucleotide-labeling probe

The procedures were the same as hybridization with IS1245-labeling probe except for changing the washing temperature to 42°C.

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8. Detection

The detection was done following the manual of Roche, Germany. The filter was washed 2 minutes with washing buffer and then for 30 minutes with 100 ml of blocking solution. The filter was incubated with 20 ml of 1:5,000 antidigoxigenin-alkaline phosphatase conjugated

solution for 30 minutes. The unbound antibody-conjugate was removed by washing twice with 100 ml of washing buffer for 15 minutes. The filter was incubated with 10 ml color-substrate solution tin the dark. The membrane was washed for 5 minutes with 50 ml of TE buffer to stop the reaction.

9. Analysis of RFLP patterns

RFLP analysis was performed at least twice with each isolates. The best images were selected and analyzed with the GelCompar*II* (version 2.0) program (Applied Maths, Kortrijk, Belgium). The whole process was consisted of **1. Strips** (lane finding), **2. Curves** (defining densitometric curves), **3. Normalization** and **4. Bands** (defining bands and quantification). The Dice coefficient of similarity for all pairwise comparison of patterns was calculated. A dendrogram of pattern relatedness among the isolates was constructed by the unweighted pair group method using arithmetic averages clustering method (UPGMA).

10. Multiplex PCR

The PCR method was performed with an automated thermocycler. Amplification was done in a total volume of 50 µl with 2 ng of template DNA, 1 µM of each primer, and 1µl of *Taq* DNA polymerase in a reaction buffer containing 10 mM Tris-HCl pH 8.9, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% (w/v) gelatin, 200 µM of each dNTP and 1 µM of each primer set (16SC, 23SG and P1, P2). The reaction mixture was placed into the thermal cycle and the condition of the reaction was incubated for 3 minutes at 94°C, and running for 30 cycles of denaturation of DNA for 1 minutes at 94°C, 3 minutes of primers annealing at 62°C and 1 minutes of extension reaction at 72°C. The final cycle was followed by 7 minutes of completely extension at 72°C. The amplified products were loaded to 2.5% agarose gel.

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