CHAPTER IV

MATERIAL AND METHODS

1. Bacterial strains

Reference mycobacterial strains and other microorganisms used in this study are listed in Table 8.

Fifty-five clinical isolates of *Mycobacterium* sp. (Table 9) were obtained from Department of Medical Microbiology, Faculty of Medicine, Chulalongkorn University, Thailand and National TB Reference Laboratory Center, Tuberculosis Cluster, Bureau of AIDS, TB and STI's, Department of Disease Control, Ministry of Health.

Clinical isolates were identified by biochemical tests and the AccuProbe culture confirmation kit (Gen-Probe, San Diego, California). Clinical isolates from Chulalongkorn University were also identified by DNA sequencing.

2. Clinical specimens

Fifty AFB-positive clinical specimens (including 47 sputum samples, 2 pus samples, and 1 body fluid) and fifty positive-signal hemoculture samples and Kinyoun smear-positive were included in this study.

3. Culture

All mycobacterial strains were cultured on Ogawa medium at 37°C, except *M. marinum* at 30°C. *Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Pseudomonas pseudomalleii, Staphylococcus aureus,* and *Streptococcus pneumoniae* were cultured on blood agar. *Candida albicans, Nocardia asteroids,* and *Nocardia brasiliensis* were cultured on Sabouraud's dextrose agar at 25°C.

For AFB-positive clinical specimens, sputum and pus were decontaminated with N-acetyl-L-cysteine (NALC)-NaOH method. The sample was added to a sterile 50- ml screw-capped plastic centrifuge tube, an equal volume of NALC-NaOH solution (50 ml sterile 4% NaOH , 50 ml sterile 2.6% sodium citrate, and 0.5 g NALC powder) was added. The tube was then vortexed 5-20 s or until liquefied. After vortex, forty milliliters of sterile 0.67M phosphate buffer, pH 6.8 were added. After centrifugation at 6,000 rpm for 15 min, the supernatant was discarded. The sediment was resuspend in sterile water and inoculated on Ogawa medium and incubated at 37°C. Body fluid was concentrated before inoculation by centrifugation at 6,000 rpm 15 min. The sediment was inoculated onto Ogawa medium and incubated at 37°C.

For signal-positive hemoculture samples and Kinyoun smear-positive, a total of 50 µl of broth culture was used to subculture onto 1% Ogawa medium and incubated at 37°C for recovery of mycobacterial colonies. Ogawa slants were visually inspected for growth on the slant.

Table 8. Reference *Mycobacterium* strains and other microorganisms.

Organisms	Number
Mycobacterium	
M. tuberculosis H37Rv KK 11-20	1
M. tuberculosis H37Ra KK 11-05	1
M. bovis ATCC 19210	1
M. bovis BCG KK 12-02	1
M. africanum KK13-02	1
M. asiaticum KK 24-01	1
M. abscessus ATCC 19977	1
M. avium ATCC 25291	1
M. fortuitum ATCC 6841	1
M. gordonae ATCC 14470	1
M. intracellulare ATCC 13950	1
M. kansasii ATCC 12478	1
M. marinum ATCC 927	1
M. nonchromogenicum ATCC 19530	1
M. scrofulaceum ATCC 19981	1
M. terrae ATCC 15755	1
M. xenopi ATCC 19250	1
M. flavescens JATA 67-01	1
M. vaccae KK 66-01	1
M. szulgai KK 32-01	1
Other bacteria and fungus*	
Candida albicans	1
Escherichia coli	1
Haemophilus influenzae	1
Klebsiella pneumoniae	1
Nocardia asteroides	1
Nocardia brasiliensis	1
Pseudomonas aeruginosa	1
Pseudomonas pseudomallei	1
Staphylococcus aureus	1
Streptococcus pneumoniae	1
Total	30

* Clinical isolates from Department of Microbiology, Faculty of Medicine, Chulalongkorn University

Table 9. Clinical isolates of mycobacteria used in this study

Species	Number
Mycobacterium	
*M. tuberculosis**	50
*M. avium**	24
*M. intracellulare**	2
*M. abscessus**	2
*M. chelonae**	2
*M. scrofulaceum**	2
*M. flavescens***	1
*M. kansasii**	1
M. fortuitum **	1
Total	85

* Clinical isolates from Department of Microbiology, Faculty of Medicine, Chulalongkorn University

** Tuberculosis Cluster, Bureau of AIDS, TB and STI's, Department of Disease Control

4. Identification of mycobacteria by conventional method

Routine biochemical methods and the AccuProbe culture confirmation test (Gen-probe Inc., San Diego, Calif) were employed for the conventional identification of isolates.

4.1 Biochemical test

4.1.1 Niacin accumulation test (paper strip method)

4.1.1.1 Inoculum

Culture (> 3 week old) on solid medium showing heavy growth.

4.1.1.2 Reagent

Agent-impregnated paper test strips are commercially available(Niacin Test Strip; Remel, Lenexa, Kans.).

4.1.1.3 Procedure

The direction supplied with the strips should be followed. Sterile distilled water of 1.0 ml was added to the solid medium. The tube was placed horizontally so that the fluid covers the entire surface, and left for al least 15 min for the extraction of niacin. The extract of 0.6 ml was removed to a 12-by 75-mm test tube. The strip was then inserted and the tube was sealed immediately. Then, left the tube for 15 min at room temperature, which occasion agitation. The result was observed the color of the liquid in the tube against a white background, which indicated positive as a yellow color in the liquid (not on the strip) .

4.1.1.4 Control

M. tuberculosis as positive control, and MAC and uninoculated medium as negative control

4.1.2 Nitrate reduction

4.1.2.1 Culture

Culture on solid medium should be 3 to 4 weeks old except for rapid grower, which may to be 2 to 4 weeks

4.1.2.2 Reagent

(i) A 1:2 dilution of concentrated (HCl). (ii) 0.2% aqueous solution of sulfanilamide. (iii) 0.1% aqueous 0.01 M solution of NaNO3 in 0.002 M phosphate buffer pH7 (Nitrate broth, Difco), (v) Powdered zinc.

4.1.2.3 Procedure

Steriled distilled water of a few drop was placed in a screw-capped tube (16 by125 mm). One loopful of mycobacterial growth was inoculated in the tube. $NaNO_3$ solution 2 ml was added and the mixture was shaken and incubated for 2 h in a water bath at 37° C. Reagent ii of 1 drop, regent ii of 2 drops and reagent iii of 2 drops were added into the tube. The test was examined the solution immediately for the development of a pink-to-red color contrasting with the reagent control. The powder zinc was added to all the negative tubes to reduce nitrate to nitrite.

4.1.2.4 Control

M. tuberculosis as positive control, and MAC and uninoculated medium as negative control

4.1.3 Urease (Wayne method)

4.1.3.1 Inoculum

Active growing colonies from solid media

4.1.3.2 Reagent

Mix 1 part of urea agar base concentrate with 9 parts of sterile distilled water. Do not add agar. Dispense 4 ml amount into 16- by 125 mm screw-cap tubes, and store at 4°C.

4.1.3.3 Procedure

A three-mm loopful of growth was emulsified in a tube of substrate and incubated for 3 days at 37o C. The test was observed for a pink or red color as positive result.

4.1.3.4 Control

M. scrofulaceum as positive control and *M. gordonae* as negative control.

4.1.4 Tween hydrolysis

4.1.4.1 Inoculum

Active growing colonies from solid media

4.1.4.2 Reagent

(i) 100 ml of 0.067 M phosphate buffer (pH7), (ii) 0.5 ml of Tween 80, (iii) 0.1% Aqueous neutral red. Mix the three reagents in order. Dispense this substrate in a 4 ml amount in 16-by 125 ml screw-cap tube, and autoclave at 121o C for 10 min. Store in the dark at 4o C for no more than 2 weeks.

4.1.4.3 Procedure

A three-mm loopful of growth was emulsified in a tube of substrate and incubated for 3 days at 37o C without shaken the tube. The tube was observed for a pink or red color after 1, 5, and 10 day. The result was recorded the number of days required for the first appearance of pink or red color. A negative result was indicated by the substrate remaining amber-colored after 10 days.

4.1.4.4 Control

M. kansasii as positive control and MAC as negative control.

4.1.5 Heat-stable (68°C) catalase test

4.1.5.1 Inoculum

Well-developed, isolated colonies from solid media

4.1.5.2 Reagents

Freshly prepared mixture of 10% Tween 80 and 30% H2O2. Phosphate buffer (0.067 M, pH6)

4.1.5.3 Procedure

Several colonies was suspened in a 0.5 ml of phosphate buffer in a screw-cap tube. The tube was placed for 20 min in a 68o C water bath and left at room temperature. Tween-H2O2 mixture was added of 0.5 ml in the tube and observed bubbles (positive). The tube was hold for 20 min before discarding as negative.

4.1.5.4 Control

M. kansasii as positive control and *M. tuberculosis* as negative control.

4.1.6 Sodium chloride tolerance

4.1.6.1 Inoculum

Barely turbid suspension

4.1.6.2 Substrate

Ogawa medium contain 5% NaCl. Ogawa without salt should be used for a control)

4.1.6.3 Procedure

Bacterial suspension of 1 ml was inoculated in the media, and incubated at 37°C. The culture was read the mycobacterial growth or no growth at 4 weeks.

4.1.6.4 Control

M. fortuitum as positive control (growth) *and M. tuberculosis* as negative control (no growth).

4.1.7 Tellurite reduction

4.1.7.1 Inoculum

Several colonies were suspended in 5 ml of 7H9 broth. The tube was incubated for 7 days before test procedure.

4.1.7.2 Reagent

A 0.2% aqueous solution (0.1 g in 50 ml distilled water) of potassium tellutite. Dispense in 2-to5 ml amounts, and autoclave.

4.1.7.3 Procedure

The tellurite solution of 2 drops was added to each culture, and incubated at 37°C. The cultures was examined daily for 4 days or more. A jet black precipitate was shown as positive result .

4.1.7.4 Control

MAC as positive control and *M. kansasii* as negative control.

4.2 AccuProbe (Gen-Probe, Inc., San Diego, Calif.)

The AccuProbe was performed according to the instructions supplied by the manufacturer. The protocol consisted 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. Next step, the tube was incubated at 95° C for 15 min. Chemiluminescent acridinium ester-labeled DNA probes were used to specifically detect the mycobacterial DNA. One hundred microliters from the lysing tube were add 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 (300 µl), the tube was vortex and incubate 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 were considered positive and signal less than cutoff value were considered negative.

5. DNA extraction

5.1 DNA extraction from reference *Mycobacterium* strains and the other microorganisms in Table 8 [105]

DNA extracted by this method was used to determine the sensitivity and specificity of multiplex PCR. Briefly, A loop of mycobacteria grown on Ogawa medium was suspended in 441 µl of Tris-EDTA buffer, pH 8.0 (10 mM Tris-HCL, 1 mM EDTA) and subsequently heated at 100 °C for 10 min in a heating box to inactivate and lyse cells. Forty nine microliteres of lysozyme (1 mg lysozyme/ml) was added to the sample, incubated at 37°C for 90 min. After incubation, the sample was added proteinase K solution (100 µg proteinase K/ml and 1% SDS) , shaked and incubated overnight at 55°C. DNA was extracted three times with an equal volume of Tris-saturated phenol, once with phenol:chloroform (25:24; vol/vol) and once with chloroform. The aqueous phase was made to 1/25 volume of 5 M NaCl (0.2 M NaCl) and 2.5 volume of cold absolute ethanol (70% ethanol). The solution was mixed and DNA was precipitated at -20°C for 1 h. DNA was pelletted by centrifugation at 12,000 rpm for 20 min at 4°C and the supernatant was discarded. The final DNA was dried in a heating box at 37°C for 1 h, and was dissolved in TE buffer and stored at -20°C until needed. The sample was checked for purity and determined for the amount of DNA by measuring the optical density at wavelengths of 260 nm and 280 nm

5.2 DNA extraction from colonies of clinical isolates

In pilot study, ten samples were processed with two DNA extraction methods to compare these methods for preparation of mycobacterial DNA from colonies.

A. Heat lysis method

One large colony or several small colonies of organism was suspended in 0.5 ml of sterile TE buffer (10 mM Tris-HCl and 1 mM EDTA) pH 8.0 containing 4-6 glass beads (3 mm diameter), mixed by vortex and boiled at 100 °C for 10 min for mycobacterial inactivation. The lysate was then centrifuged at 12,000 rpm for 5 min to

precipitate cellular debris. The supernatant was transferred to a sterile eppendorf tube. The lysates were used in amplification reaction or stored at -20 °C until needed.

B. Alkaline wash and heat lysis method [106]

The DNA was extracted from mycobacterial cells by placing one large colony or several small colonies into a 1.5 ml eppendorf tube containing 0.5 ml of alkaline wash solution composed of 0.05 M sodium citrate and 0.5 M NaOH. The eppendorf tubes were vortexed allowed to stand for 5 min, and centrifuged at 12,000 rpm for 5 min; and the cell pellet was washed in 0.5 ml of 0.5 M Tris-HCl (pH 8.0) and resuspened in 100 µl of TE buffer. The suspension was then boiled at 100°C for 10 min, centrifuged at 12,000 rpm for 5 min, and stored at -20 °C until needed.

5.3 DNA extraction from hemocultures [107]

A volume of 0.1 ml of broth culture was added to 1.4 ml of alkali wash solution (0.5 M NaOH and 0.05 M sodium citrate) in a 1.5 ml eppendorf tube, mixed by vortex and centrifuged at 12,000 rpm for 5 min to deposit the bacterial cells. The cell pellet was washed twice with 0.5 ml of 0.5 M Tris-HCl (pH 8.0) and resuspended in 0.1 ml sterile DDW. The suspension was heated at 95°C for 25 min in a heating block and stored at -20 °C prior to PCR.

5.4 DNA extraction from clinical specimens

5.4.1 Decontamination of sputum

Sputum samples were treated with NaOH-citrate *N*-acetyl-L-cysteine (0.25 M NaOH, 25 mM Na-citrate, 15 mM N-acetyl-cysteine), shaked and centrifuged at 12,000 rpm for 10 min and the supernatant was discarded. The cell pellet was resuspended in 200 µl 1XTE buffer and stored at -20°C prior to DNA extraction using QIAamp®DNA Mini kit (Qiagen Corporation, Germany).

5.4.2 DNA extraction by QIAamp®DNA Mini kit

The DNA was purified according to the manufacturer's directions by using the QIAamp®DNA Mini kit (Qiagen Corporation, Germany). In brief, 200 µl of sample was added with 20 µl of proteinase K, 200 µl buffer AL and mixed by vortex for 15 s. After lysis for 10 min at 56°C to reach DNA yield, 200 µl absolute ethanol was added and mixed by vortex for 15 s. To remove drop from the inside of the lid, the tubes was spinned at 8,000 rpm for 1 min . The mixture was transferred to QIAamp spin column and centrifuged at 8,000 rpm for 1 min. QIAamp spin column was then washed with 500 µl buffer AW1 and 500 µl buffer AW2 followed by the spin at 8,000 rpm for 1 min and at 14,000 rpm for 3 min, respectively. After centrifugation the QIAamp spin column was placed in a clean 1.5 ml microcentrifuge tube and 200 µl buffer AE were added and incubated at room temperature for 1 min. After incubation, the suspension was centrifuged at 8,000 rpm for 1 min and the DNA containing eluted buffer was stored at -20°C, ready for the PCR analysis.

6. Determination of sensitivity of multiplex PCR

In order to determine the multiplex PCR sensitivity, purified DNAs of all mycobacteria listed in Table 8 were diluted to a concentration of 2 ng/µl and then further diluted in TE buffer to provide samples with concentration ranging from 200 pg to 200 fg of DNA per µl. Five microliters of these samples were amplified by multiplex PCR. The amplified products (5 µl) were electrophoresed in 2% agarose gel containing ethidium bromide and visualized by UV fluorescence. For negative control, DDW was used as the template for multiplex PCR

7. Determination of specificity of multiplex PCR

In order to determine the multiplex PCR specificity, 10 ng of DNA from microorganisms in Table 8 were tested in multiplex PCR amplification and DDW was used as a negative control.

8. DNA amplification by multiplex polymerase chain reaction (multiplex PCR)

A multiplex polymerase chain reaction (multiplex PCR) with primers which detect the 16S rRNA gene of members of the genus *Mycobacterium* and which distinguish between *M. avium* and *M. intracellulare* and the MPB 70 gene of *M. tuberculosis* as described by Wilton and Cousins [37] and Kulski [38] was performed with modification. Primers MYCGEN-F (positions 8 to 28) and MYCGEN-R (positions 1047 to 1027) [37, 38] used for amplification of genus *Mycobacterium* were replaced by primers AFB-F (positions 29 to 50) and AFB-R (positions 694 to 673) described by Han et al. [108]. Primers AFB-F and AFB-R permitted efficient amplification of 640 to 665 bp PCR products from almost all AFB species (95 of 98), thereby enabling universal amplification of AFB by PCR. The multiplex PCR primers were 5'-biotinylated primer AFB-F (5' GCGTGCTTAACACATGCAAGTC 3') (29-50, forward), AFB-R (5'TCCTCCTGA TATTGCGCATTC3') (694-673, reverse), MYCINT-F (5' CCTTTAGGCGCA TGTCTTTA 3'), MYCAV-R (5' ACCAGAAGACATGCGTCTTG 3'), TB1-F (5' GAACAATCCGGAGTTGACAA 3'), and TB1-R (5' AGCACGCTGTCAATCATGTA 3'). An amplification product of 640-665 bp (AFB-F and AFB-R) depending on species, was indicative of the genus *Mycobacterium*, and smaller fragments of 490 bp (MYCINT-F and AFB-R), 183 bp (MYCAV-R and AFB-F), and 372 bp (TB1-F and TB1-R) were positive signals for *M. intracellulare, M. avium*, and *M. tuberculosis*, respectively (Figure 6).

Amplification was performed in 50 µl mixture containing 1x buffer, 2 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), 1.25 U of *Taq* polymerase, primers (25 pmole each of AFB-F, AFB-R and MYCINT-F, and 12.5 pmole each of MYCAV-R, TB1-F and TB1-R), 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 cyclers. The PCR cycling parameters were 94 °C for 10 min, followed by 35 cycles of denaturation at 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 [38].

Sample of 5 µl were used for PCR in a total volume of 50 µl. Each clinical specimens was tested in duplicate. One part was spiked with 1 ng of *M. tuberculosis* DNA, and one part was not spiked. If all two sample were positive, the sample was considered positive. The sample was scored as negative if the nonspiked reaction was negative and the spiked reaction was positive. If the results for the nonspiked reaction were ambiguous, the PCR was repeated. If all two reactions were negative, then there were inhibitors in the samples.

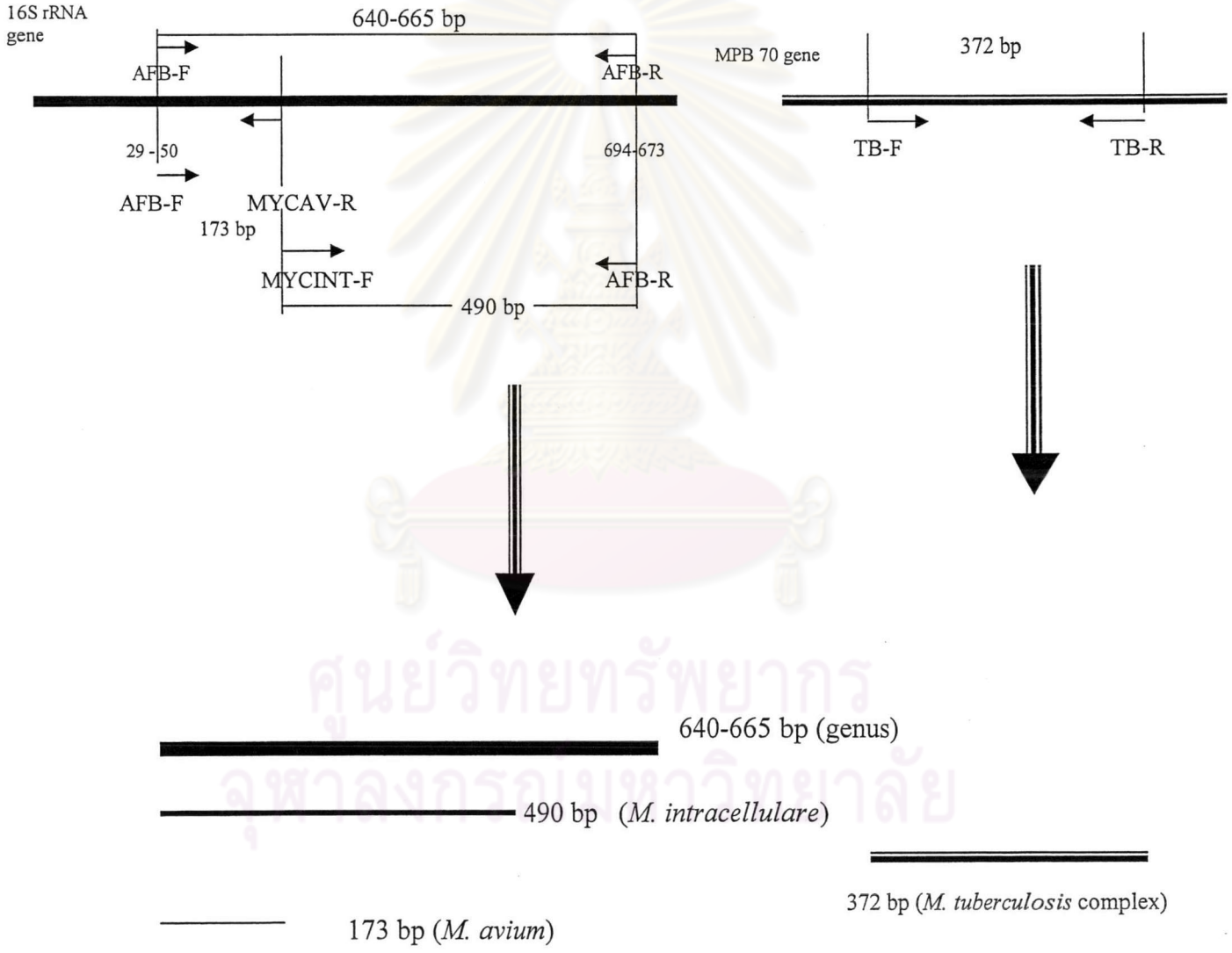

Figure 6. Schematic overview of 16S rRNA gene and MPB 70 gene to amplify PCR product by multiplex PCR

9. Detection of amplification product

Five microliters of PCR product were mixed with 2 µl of gel loading buffer (20% ficoll, 0.05% bromophenol blue), analyzed by electrophoresis on 2.0% agarose gel, consisted of 0.5 µg/ml ethidiun bromide, in 1XTris-borate-EDTA (1XTBE) buffer (pH8.0). The electrophoresis was carried out at 80 volts for 40 min. Gel was visualized with UV fluorescense. The results were recorded by Camera Gel Doc™ MZL (Bio-RAD, USA)

10. Reverse dot blot hybridization [109]

In reverse dot blot hybridization, the amplified product of 16S rRNA gene was hybridized to a set of oligonucleotide probes which were covalently bound to Biodyne C membrane by dot blotting. Since one primer was biotinylated, the PCR product was biotin-labeled as well. Positive hybridization was detected on film after streptavidin-peroxidase incubation and enhanced chemiluminescence.

Oligonucleotide probes were designed based on alignment of the sequences of 16S rRNA gene region A of mycobacterial species. By comparing the aligned sequence obtained, species-specific sequence for mycobacteria can be defined. The complementary strands of these sequences were used as species specific probes listed in Table 10.

10.1 Preparation of the reverse dot blot

The Biodyne C membranes were prehybridized in 10 ml freshly prepare 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in demineralized water at room temperature for 15 min, and the membranes were placed in plastic container and shaked with demineralized water for 2 min. After the membranes were activated, they were placed on support cushion in a clean dot blotter apparatus (BBL, USA). Dilutes of the oligonucleotides solution in 100 µl 0.5 M $NaHCO_3$, pH 8.4 (Table 10) were added to the dot of dot blotter and incubated in 200 ml freshly prepared 0.1 M NaOH for 10 min (maximum) at room temperature in a plastic container and rinsed with demineralized water. The membranes were washed in 50 ml 2xSSPE/0.1% SDS for 10

min at 50°C and finally washed in 50 ml 20 mM EDTA, pH 8.0 for 15 min at room temperature

Table 10 Sequences and concentration of the 16S rDNA amino-link oligonucleotides

Specific for	Oligo name	Sequence (5'→ 3')**	Position*	Tm (°C)	Concen-tration (pmole/ 100µl)	Reference
M. tuberculosis complex	MTb	ACC ACA AGA CAT GCA TCC CG	168-148	67.9	6.25	[31]
M. avium	MAv	ATG CGT CTT GAG GTC CTA TC	156-137	60.7	25	This study
M. intracellulare	MInt 1	ACC TAA AGA CAT GCG CCT AA	168-148	62.1	12.5	This study
M. intracellulare	MInt 2	ACC AAA AGA CAT GCG TCT A	168-147	61.1	12.5	This study
M. chelonae and *M. abscessus*	MChe-Ab 1	ATG AAG TGT GTG GTC CTA TCC	156-138	60.3	12.5	This study
M. chelonae and *M. abscessus*	MChe-Ab 2	GGA AGT GCG CGG TCA TAT T	156-137	65.1	12.5	This study
M. flavescens	MF1a 1	AGG CCA TGC GAC CAA TAG	204-186	63.2	25	This study
M. flavescens	MFla 2	CCA GGC CAT GCG ACC A	202-186	66.3	25	This study
M. fortuitum	MFor 1	ACC ACA CAC CAT GAA GCG C	168-149	66.4	25	This study
M. fortuitum	MFor 2	ACC ACA CCC CAT GAA GAG C	168-149	64.3	25	This study
M. fortuitum	MFor 3	ACC ACG CAC CAT GAA GC	168-151	61.6	25	This study
M. gordonae	MGor	CAT GTG TCC TGT GGT CCT ATT	158-137	61.3	100	This study
M. kansasii, M. gastri, M. scrofulaceum, and M. simiae	MKGSS	CCA AGT GGT CCT ATC CGG T	152-133	63.6	12.5	This study
M. xenopi	MXe 1	ACC ACC CCA CAT GCG C	168-152	65.0	3.125	This study
M. xenopi	MXe 2	ACC ACC ACC ACA TGC GC	168-152	65.1	3.125	This study
Genus *Mycobacterium*	Myc (AFB-R)	TCC TCC TGA TAT CTG CGC ATT	694-673	66.4	3.125	[108]
	Control 5' aminolink and 3' biotinylated	GGT TGC ACG CAT TTTCCC TTAA		70.5	0.1	This study

* Numbering is according to the alignment of Rogall et al.[110]

** Complementary sequences of the non-coding strand

10.2 Reverse dot blot hybridization

All buffers were prewarmed before use. Twenty microliters of the PCR product were added to 1,500 µl 2xSSPE/0.1% SDS, and heated for 10 min at 100°C and cool on ice immediately. The membrane was washed for 5 min at 50°C in 50 ml of 2xSSPE/0.1% SDS. The membrane was then placed into hybridization bag and the bag filled with 1,500 µl of diluted PCR product (avoid air bubbles). Hybridization was performed with shaking for 45 min at 50°C. the membrane was then removed from hybridization bag using forcep and washed twice in 50 ml 2XSSPE /0.5% SDS for 10 min at 59°C.

10.3 Detection of hybridization signals

The membrane with the hybridized PCR product was placed in a plastic container and allowed to cool down to prevent inactivation of the peroxidase in the next step. Solution of 2.5 µl streptavidine-peroxidase conjugate (500U/ml) was added to 10 ml of 2XSSPE /0.5% SDS, and the membrane was incubated in this solution for 30 min at 42°C in the plastic container. The membrane was washed twice in 50 ml of 2XSSPE /0.5% SDS for 10 min at 42°C to remove excess streptavidine-peroxidase, and rinsed twice in 50 ml of 2XSSPE for 5 min at room temperature to remove SDS. For chemiluminescent detection of hybridized DNA, the membrane was incubated for 1 min in 20 ml ECL detection liquid (10 ml detection reagent 1and 10 ml detection reagent 2). The membrane was placed on a carrier by using forcep and covered with a transparent plastic sheet and a light sensitive film was exposed to the membrane for 15 min. If the signal was too weak or too strong, the membrane can be used again to expose another film for a shorter or longer period.

11. Determination of sensitivity of reverse dot blot hybridization

In order to determine the sensitivity of reverse dot blot hybridization, DNA of *Mycobacterium* species to be tested by reverse dot blot hybridization were prepared as described in 6. These samples were amplified by multiplex PCR. The amplified products were detected by gel electrophoresis and reverse dot blot hybridization.

12. Determination of specificity of reverse dot blot hybridization

In order to determine the specificity of reverse dot blot hybridization, 10 ng of purified DNA of the microorganisms in Table 8 were amplified by multiplex PCR and detected by agarose gel electrophoresis. The biotinylated amplified products were hybridized with oligonucleotide probes by reverse dot blot hybridization.

13. Sequencing of the 16S rDNA region A

An ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystem) 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 DNA concentration was determined by measuring the A_{260} of 1 corresponds to a DNA concentration of 50 ug/ml). The sequencing reaction required 4 ml of Premix, 3.2 pmol of sequencing primer; primer pB: 5' TAA CAC ATG CAA GTC GAA CG 3' corresponding to *Mycobacterium* 16S rDNA position 50-70 (26), and 150 ng of PCR product template in a total volume of 10 ul. The sequencing reaction was performed using Perkin Elmer GeneAmp PCR system 9600 with cycling parameters were 25 cycles of 96° C for 30 sec, 55° C for 10 sec, 60° C for 4 min and hold 4° C until ready to purify. The products were purified with ethanol/sodium acetate precipitation before capillary electrophoresis was run for sequence analysis.

The amount 300 bp 16S rDNA fragment corresponding to hypervariable region A of *Mycobacterium* was analyzed in an ABI Prism™ 310 Automate Sequencer (PE Applied Biosystem) and the DNA sequences were then compared to database in GenBank (http://www.ncbi.nlm.nih.gov/) by the Basis Local Alignment Search Tool (BLAST).