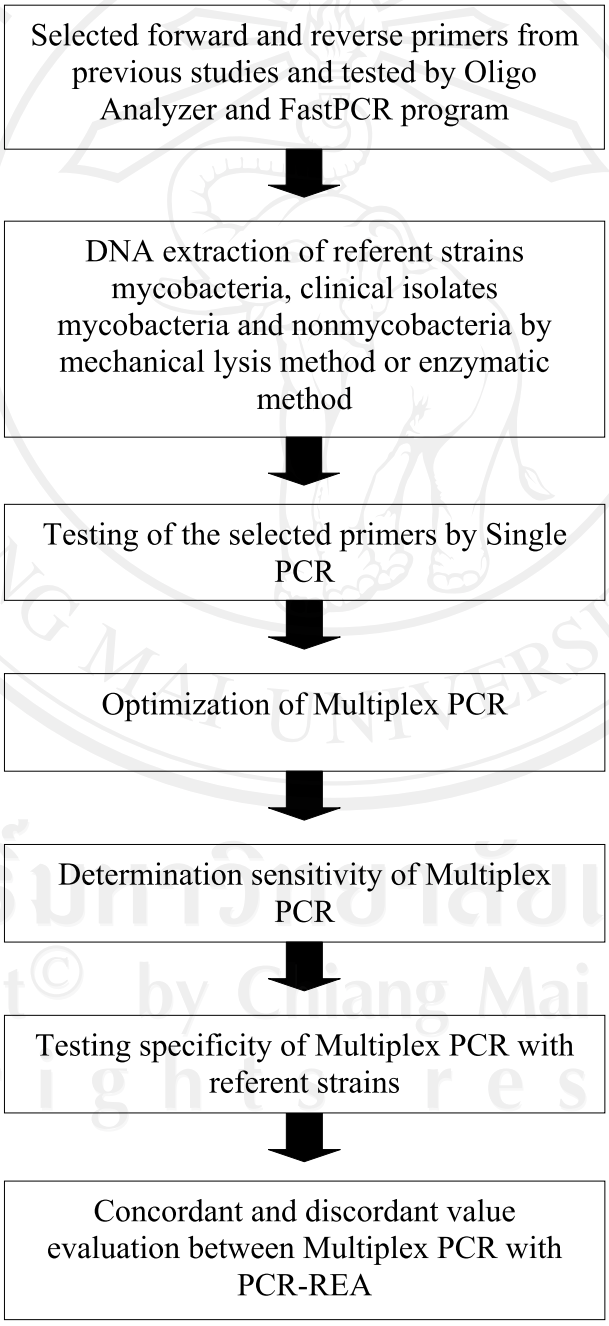
CHAPTER III

MATERIALS AND METHODS

RESEARCH DESIGN



MATERIALS and METHODS

1. Bacteria Strains

All bacterial strains used in this study were shown in Table 2 and 3. There were 172 bacterial strains.

1.1 Referent strains of mycobacteria

The 21 mycobacterial referent strains were kindly provided by Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiangmai University and Mr. Sakarin, TB center 10 Chiangmai.

1.2 Clinical mycobacteria

A total of 100 mycobacterial clinical isolates were obtained from the following sources; 51 isolates from Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiangmai University, 13 isolates from Faculty of Veterinary Medicine, Chiangmai University, 3 isolates from Maharaj Nakorn Chiangmai Hospital and 33 isolates from Lampang Hospital.

1.3 Referent strains of nonmycobacteria

The 3 nonmycobacterial referent strains were obtained from Division of Microbiology, Department of Clinical Pathology, Lampang Hospital.

Clinical nonmycobacteria

A total 48 nonmycobactria clinical isolates used in this study were obtained from Division of Clinical Microbiology, Department of Medical Technology, Faculty of Associated Medical Science, Chiangmai University (n=6), Maharaj Nakorn Chiangmai Hospital (n=4) and Lampang Hospital (n=38).

2. Culture media

All of mycobacteria used in this study were subculture on Lowenstein-Jensen egg medium. Gram-positive bacteria were subculture on blood agar. Gram-negative bacteria were subculture on MacConkey agar.

Table2. Mycobacteria strains used in this study

Referent strains of mycobacteria	Clinical isolates of mycobacteria
M. austroafricanum ATCC 33464	*M. avium* (39)
M. avium ATCC 25291	*M. chelonae* (2)
M. bovis BCG	*M .fortuitum* (1)
M .fortuitum ATCC 6841	*M. gordonae* (2)
M. fortuitum TBD 012-01	*M. kansasii* (1)
M. gordonae ATCC 14470	*M. scrofulaceum* (1)
M. gordonae TBD 071-05	*M. tuberculosis* complex (51)
M. intracellulare ATCC 13950	Rapid mycobacteria (3)
M. kansasii ATCC 12478	
M. kansasii TBD 111-03	
M. neoaurum ATCC 25795	
M. nonchromogenicum ATCC 19530	
M. phlei ATCC 23024	
M. scrofulaceum ATCC 19982	
M. scrofulaceum TBD 192-03	
M. szulgai ATCC 3201	
M. terrae ATCC 15778	
M. terrae TBD 201-02	
M. tuberculosis ATCC 27294	
M. xenopi ATCC 13250	
M. xenopi TBD 231-02	

( ) numbers in the brackets represent number of sample

Table3. Nonmycobacteria strains used in this study

Referent strains of nonmycobacteria	Clinical isolates of nonmycobacteria
E. coli ATCC 25922	*Acinetobacter baumanii* (2)
P. aeruginosa ATCC 27853	*Burkholderia pseudomallei* (1)
S. aureus ATCC 25923	*Candida albican* (2)
	Citrobacter freundii (2)
	Enterobacter aerogenes (2)
	Enterobacter cloacae (2)
	Enterococcus faecium (2)
	Enterococcus faecalis (1)
	Escherichia coli (2)
	Klebsiella pneumoniae (2)
	Nocadia spp.(7)
	Proteus mirabilis (2)
	Proteus vugaris (2)
	Pseudomonas. aeruginosa (2)
	Rhodococcus spp. (3)
	Salmonella spp. (2)
	Serratia marcescens (2)
	Shigella flexneri (1)
	Staphyrococcus aureus (2)
	*Streptococcus gr.*A (1)
	Streptococcus pneumoniae (2)
	Streptococcus viridans (2)
	Vibrio parahaemolyticus (2)

( ) numbers in the brackets represent number of sample

3. Selection and testing of primers

All 10 primers were selected or modified from primers and probes using in the studies of Heekyung Park *et al.* in 2000 and Likuan Aiong *et al* in 2006. In order to identify the 6 most common human infectious mycobacteria by Multiplex PCR, primers specific for each mycobacteria were searched from previous published study. They were specific to the 16S-23S rDNA gene of mycobacteria. Six primers as ITS-F1, MYCOM-2, MACF, FORF, SCOR and TBR were selected from the study of Heekyung Park *et al* [5, 158]. Three primers, INT, KAN1 and KAN2 were modified from probes used in Likuan Aiong *et al* study [27]. The last one primer mKAN was used in this study. The mKAN was modified from KAN1 and KAN2 for detection of all 3 types of *M. kansasii*. One set of amplification primers ITS-F1 and MYCOM-2, were used to detect *Mycobacterium* spp. *M. avium* complex were detected by MACF and MYCOM-2. *M. fortuitum* were detected by FORF and MYCOM-2. *M. scrofulaceum* were detected by ITS-F1 and SCOR. *M. tuberculosis* complex were detected by ITS-F1 and TBR. *M. intracellulare* were detected by INT and MYCOM-2. *M. kansasii* were detected by KAN1/KAN2/mKAN and MYCOM-2. The nucleotide sequences and characteristics of ten selected primers were shown in Table 4 and Figure 1. All primers were checked by Fast PCR computerized program, which were analyzed product size and specificity to 36 sequences of mycobacteria searching from Genbank as shown in Table 5. In additional, these primers were determined for primer dimers, primer-primer compatibility and designed suitable primers pairing for multiplex PCR by Oligonucleotide Analyzer program, which was considered from delta G (dG) value.

Table4. Nucleotide sequences and characteristics of ten selected primers using in this study

Primer	Sequence 5' to 3'	Tm (^{o}C)	Target species	Position (s)
ITS-F1	CGA AGC CAG TGG CCT AAC CC	66	Panmycobacteria	*16S rDNA*
MYCOM-2	ATG CTC GCA ACC ACT ATC CA	60		*ITS*
MACF	CCC TGA GAC AAC ACT CGG TC	64	MAC	117-136
FORF	CCG TGA GGA ACC GGT TGC CT	66	*M. fortuitum*	45-64
INT	GGT CGA TCC GTG TGG AGT CC	64	*M. intracellulare*	133-152
KAN1	GGG TGC GCA ACT GTA AAT GA	60	*M. kansasii*	150-169
KAN2	AAA AGT GCC CCA ATT GGT GG	60		
mKAN	AAA AGC ACC CCA ATA GGT GG	60		
SCOR	TTC GGC CAC GCA TCC GTT TA	62	*M. scrofulaceum*	210-229
TBR	ACC TGG AAC AAG TCC GAG TG	62	MTB complex	129-148

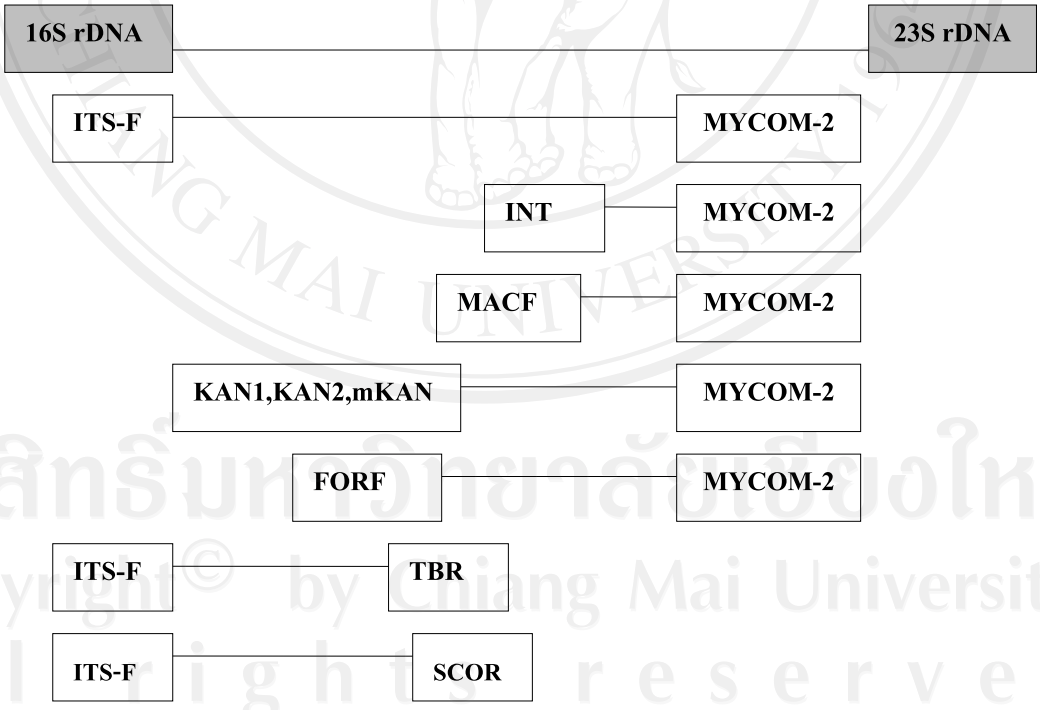


Figure1. Schematic diagram of primers pairing each other

Table5. Genbank accession numbers of *16S-23S rDNA* of 36 mycobacteria using for Fast PCR computerized program testing.

Organism	Accession No.	Organism	Accession No.
M. abcessus	AJ314869	*M. intracellulare*	AJ536036
M. asiaticum	AF191087	*M. kansasii*	L42263, L42262, L42264
M. avuim	X52918	*M. lentiflavum*	AF318174
M .chelonae	AJ291584	*M. leprae*	X56657
M. conspicuum	X92668	*M. lufu*	X74055
M. diernhoferi	AF186463	*M. malmoense*	Y14184
M. farcinogenes	Y10384	*M. marinum*	Y14185
M. flavescens	AJ291586	*M. paratuberculosis*	X74495
M. fortuitum	X52933	*M. phlei*	X74493
M. gastri	X97633	*M. scrofulaceum*	AJ536034
M. genavense	Y14183	*M. senegalense*	Y10385
M. gilvum	AJ314876	*M. shimoidei*	X99219
M. gordonae	AJ 315574	*M. simiae*	X75599
M. habana	X74056	*M. smegmatis*	X76257
M. hemophilum	AY579400	*M. szulgai*	X99220
M. tuberculosis	L15623	*M. terrae*	Z46427
M. ulcerans	X99217	*M. triplex*	Y14189
M. xenopi	Y14192	*M. triviale*	X99221

4. Extraction of genomic DNA

4.1 Crude extraction of genomic DNA

The genomic DNA of bacteria was prepared by mechanical lysis method as previous described by Sansila *et al* (1998) [158]. Briefly, a loop of bacteria grown on culture media was suspended to 1.5 ml microcentrifuge tube containing 0.5 ml of distilled water. Then, the tube was vortexed vigorously for 1 minute then boiled at 100^{o}C for 20 minutes. After boiling, the tube was vortexed vigorously for 1 minute. The suspension was centrifuged at 3000 rpm for 15 minutes. The aqueous phase was used for PCR reaction or stored at -20^{o}C until used.

4.2 Enzymatic extraction of genomic DNA of mycobacteria

The genomic DNA of mycobacteria was prepared by enzymatic method, which described previously by Van Soolingen *et al* (1998) [159]. Briefly, a looful of cells was transferred to a microcentrifuge tube which contained 400 µl TE buffer, and then boiled at 80^{o}C for 20 minutes. Fifty microlitres of 10 mg/ml lysozyme was added. The suspension was vortexed and incubated at 37 ^{o}C for overnight. After adding 75 µl of 10% SDS/proteinase K mixture, the suspension was incubated at 65 ^{o}C for 10 minutes. One hundred microliter of 5 M NaCl and 100 µl of CTAB/NaCl solution (which was prewarm at 65 ^{o}C) were added. The mixture was vortexed until the liquid became white (milky) and then incubated at 65 ^{o}C for 10 minutes. For protein separation, 750 µl of chloroform/isoamyl alcohol was added, vortexed for at least 10 second and then centrifuge at 12,000xg for 5 minutes. 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 RT 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 the TE buffer. DNA solution was stored at -20^{o}C until used.

The concentration of the DNA was determined by using the spectrophotometry method. The DNA was diluted in TE buffer at an appropriate dilution and the optical density (O.D.) was then measured for nucleic acid and protein at a wavelength of 260 and 280 nm, respectively with UV spectrophotometer. The quantity of DNA was calculated by equation:

Quantity of DNA (ng/µl) = $O.D._{260}$ x dilution factor x 50*

* 1 $O.D._{260}$ = 50 µg/ ml of double stranded DNA concentration

The purifity of DNA preparation could be estimated by the ratio between O.D. 260 and 280. The ratio of 1.8 or more indicated acceptable purity of DNA. If the value had been lower, the preparation would have contained some contaminant (e.g., protein or phenol).

5. Single PCR for detection of each species of mycobacteria

The single PCR reaction for each mycobacteria, was performed by using ITS-F1 as forward primer and MYCOM -2 as reverse primer for mycobacteria detection. The MACF as forward primer and MYCOM-2 as reverse primer were used for *M. avium* complex detection. The FORF as forward primer and MYCOM-2 as reverse primer were used for *M. fortuitum* detection. The INT as forward primer and MYCOM-2 as reverse primer were used for *M. intracellulare* detection. The KAN1, KAN2, mKAN as forward primers and MYCOM-2 as reverse primer were used for *M. kansasii* detection. The ITS-F1 as forward primer and SCOR as reverse primer were used for *M. scrofulaceum* detection. The ITS-F1 as forward primer and TBR as reverse primer were used *M. tuberculosis* complex detection. The total volume of master mix was 50 µl. The component of each reaction was 5 µl of 10X buffer (500 mmol/L KCl, 100 mmol/L Tris HCl (pH 9.0)), 3 µl of 25 mM $MgCl_2$ (1.5 mM), 1 µl of 10 mM each dNTPs (dATP, dGTP, dTTP and dCTP, 0.2 mM), 0.2 µl of 5 U *Taq* DNA polymerase (*Taq* Faststart, Roche, Germany), 31 µl of distilled water and 5 µl of genomic DNA. The PCR reaction was carried out in a Thermalcycle PCT 200 (MJ Research, Walthman, MA, USA) with the condition of initial denaturation at 94^0C for 5 min; followed by 30 cycles of 94^0C for 1 min, annealing temperature at 60^0C for 1 min, and extension of the nucleotide at 72^0C for 1 min. The complete extension was performed by final extension of 72^0C for 10 min [158]. The PCR product was determined by 2.5% agarose gel with 100 voltage potension for 40 minutes, and visualized under UV light after ethidium bromide staining.

6. Single-tube Multiplex PCR for detection of mycobacteria

The Multiplex PCR was performed in one tube. The constituent of the 50 µl component reagents were as follows: 3 µl of 10X buffer, 5 µl of 20 µM each primer (ITS-F1, MYCOM-2, MACF, FORF, INT, KAN1, KAN2, mKAN, SCOR, TBR), 0.2 µl of 5 U *Taq* DNA polymerase (*Taq* Faststart, Roche, Germany) and 5 µl genomic DNA. The amount of $MgCl_2$ (varied from 1.0 to 2.5 mM) and dNTPs (varied from 100 to 300 µM) in the reaction must be optimized to ensure the sufficiency of amplified products. The PCR was carried out by denaturation at 94^0C for 5 min, 30 cycles of amplification at 94^0C for 1 min, at annealing temperature (varied from 55^0C

to 66^{0}C) for1 min, at 72^{0}C for 1 min and final extension at 72^{0}C for 10 min with Thermal cycler system PCT 100.

6.1 Optimization of annealing temperature (T_m)

The Multiplex PCR was carried out by the concentration of 1.5 mM $MgCl_2$, 200 µM each dNTPs [158]. The annealing temperature was varied from 55-66^{0}C for the detection of genus and 6 species of mycobacteria by using gradient PCR program of Thermalcycle PCT 200 machine. The optimal annealing temperature for Single-tube Multiplex PCR was evaluated from the PCR products on 2.5% agarose gel electrophoresis.

6.2 Optimization of $MgCl_2$ concentration

The optimization of $MgCl_2$ concentration in Multiplex PCR was carried out by varying the concentration of $MgCl_2$ at 1.0, 1.5, 2.0 and 2.5 mM. The Multiplex PCR reation was running at the optimal annealing temperature by fixing the concentration of dNTPs concentrations at 200 µM. The optimal concentration of $MgCl_2$ in Single-tube Multiplex PCR was evaluated from the PCR product of 6 *Mycobacterium* spp.

6.3 Optimization of dNTPs concentration

The optimization of dNTPs concentration in Multiplex PCR was carried out by varying the concentration of dNTPs at 100, 200 and 300 µM. The Multiplex PCR reaction was running at optimal annealing tempetature and optimal $MgCl_2$ concentration. The optimal concentration of dNTPs in Single-tube Multiplex PCR was evaluated from PCR products of *Mycobacterium* spp.

7. Determination of Sensitivity or lower detection limit of Single-tube Multiplex PCR in detection and identification of the mycobacteria

To determine the sensitivity or lower detection limit of the mycobacterial DNA concentration were evaluated by Single-tube Multiplex PCR. The purified DNA of 6 *Mycobacterium* spp. was 10-fold serially diluted in TE buffer from 100 pg/ µl to 1 fg/µl. The minimum concentration of mycobacterial DNA that could be observed in the agarose gel was determined as the lower detection limit of the assay.

8. The efficiency of Single-tube Multiplex PCR method for detection and identification.

The 15 referent strains of mycobacteria were subcultured on LJ medium. The cultured colonies of mycobacteria were extracted for DNA by crude extraction. Each DNA sample was performed by Single-tube Multiplex PCR for mycobacterial detection and identification.

9. Comparison of the identification results by Single-tube Multiplex PCR with PCR –REA and PNB screening test.

One hundred isolates of clinical mycobacteria were used for identification by Single-tube Multiplex PCR. They comprised of 64 isolates which were identified by PCR-REA technique from Division of Clinical Microbiology, Department of Medical Technology, and Faculty of Associated Medical Sciences. Thirty-three isolates were collected from TB center 10, Chiangmai. All of 33 isolates were only screened for MTB and NTM by PNB screening test. Another 3 isolates from Maharaj Nakorn Chiangmai Hospital were tested by INNoLiPA. The identification results were compared to each other and the percent of concordance or discordance was calculated.

The calculation was:

$$\text{Percent concordance} = \frac{\text{Numbers of both correct}}{\text{Total numbers tested}} \times 100$$

$$\text{Percent discordance} = \frac{\text{Numbers of both incorrect}}{\text{Total numbers tested}} \times 100$$

10. Specificity of Single tube Multiplex PCR for detection of mycobacteria.

Fifty-one isolates of nonmycobacteria were studied. There were 3 isolates of referent strain: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and 48 isolates of clinical isolated bacteria. All of these bacterias DNA were extracted by lysis technique. A loopful of bacterial cells was suspended in 0.5 ml water and boiled for 20 minutes gaining the crude extracted DNA. Five microlite of crude DNA were used in Single-tube Multiplex PCR parallel with the mycobacterial DNA sample. The detection result of this experiment was determined by positive band of amplified product