CHAPTER V

RESULTS

1. Optimization of multiplex PCR

A multiplex PCR with primers as described by Wilton and Cousins [37] and Kulski [38] was performed with modification. Primers MYCGEN-F and MYCGEN-R [37, 38] used for amplification of genus *Mycobacterium* were replaced by primers AFB-F and AFB-R described by Han et al. [108]. Amplification was performed in 50 µl mixture containing 1x buffer, 2 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates (dNTPs; dATP, dCTP, dGTP, and dTTP), 1.0 U of *Taq* polymerase, primers (50 pmole each of AFB-F, AFB-R and MYCINT-F, 6.5 pmole of MYCAV-R, and 5 pmole of each 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].

The optimal concentration of AFB-F, AFB-R, MYCINT-F, MYCAV-R, TB1-F, and TB1-R was determined by varying the concentration of primers at 50, 37.5, 25, 12.5, 10, 7.5, and 5 pmole. It was found that the optimal concentration of AFB-F, AFB-R, MYCINT-F, MYCAV-R, TB1-F, and TB1-R was 25, 25, 25, 12.5, 12.5, and 12.5, respectively. After the optimal concentration of primers was known, the optimal concentration of Taq polymerase was determined by varying the amount of Taq polymerase (0.5, 1.0, 1.25, 1.50, and 2.0 unit per 50 µl volume of the reaction mixture) in the PCR reaction mixture. The lowest amount of Taq polymerase which gave a satisfactory result was 1.25 unit per 50 µl volume of the reaction mixture.

2. Sensitivity of multiplex PCR

The sensitivity of multiplex PCR was tested with serial 10-fold diluted purified DNAs from reference strains of *Mycobacterium* species listed in Table 8. The PCR products were electrophoresed on 2% agarose gel containing ethidium bromide. Figure 7 showed that with *M. tuberculosis* H37Rv, the detection limit was 10 pg of DNA, which amounts to 2,000 mycobacteria. The detection limits were the same for other *M. tuberculosis* complex such as *M. tuberculosis* H37Ra, *M. bovis* ATCC 19210, *M. bovis* BCG KK 12-02, and *M. africanum* KK 13-02 (results not shown).

The detection limit for *M. avium* ATCC 25291 and *M. intracellulare* ATCC 13950 was found to be 10 pg (Figure 8) and 1 ng (Figure 9) of DNA, respectively. For other mycobacterial species listed in Table 8, the detection limit was found to be 10 pg of DNA. Only the result from *M. kansasii* ATCC 12478 was shown in Figure 10.

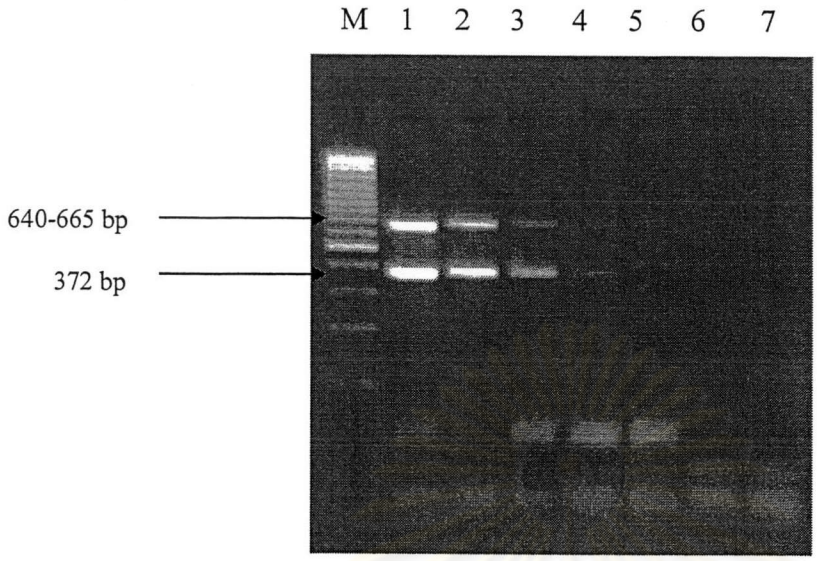

Figure 7. Sensitivity of the detection of *M. tuberculosis* H37Rv KK 11-20 using multiplex PCR. Lane: M, 100 bp-DNA ladder; 1, *M. tuberculosis* H37Rv 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, 100 fg; 7, negative control

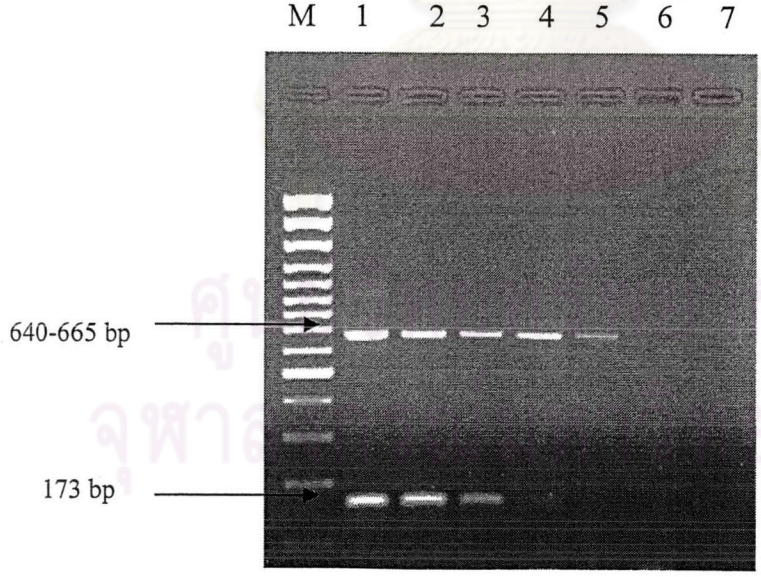

Figure 8. Sensitivity of the detection of *M. avium* ATCC 25291 using multiplex PCR. Lane: M, 100 bp-DNA ladder; 1, *M. avium* 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg; 6, negative control

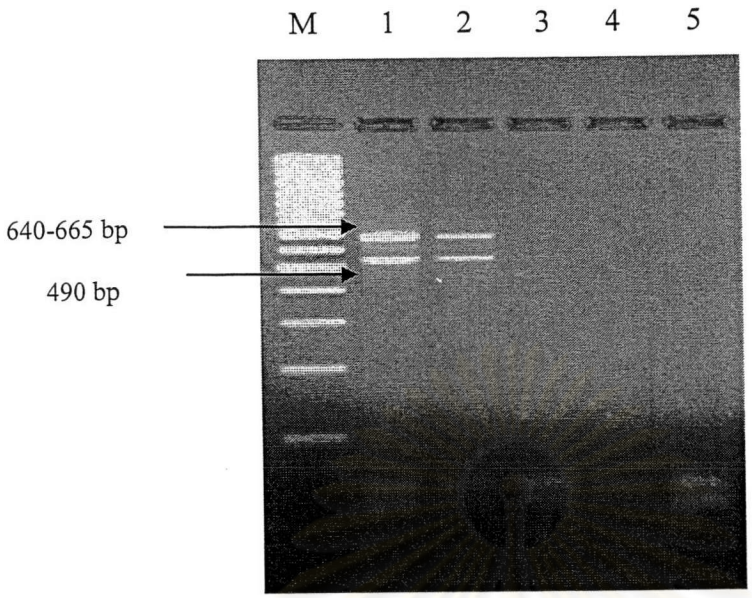

Figure 9. Sensitivity of the detection of *M. intracellulare* using multiplex PCR. Lane: M, 100 bp-DNA ladder; 1, *M. intracellulare* ATCC 13950 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, negative control

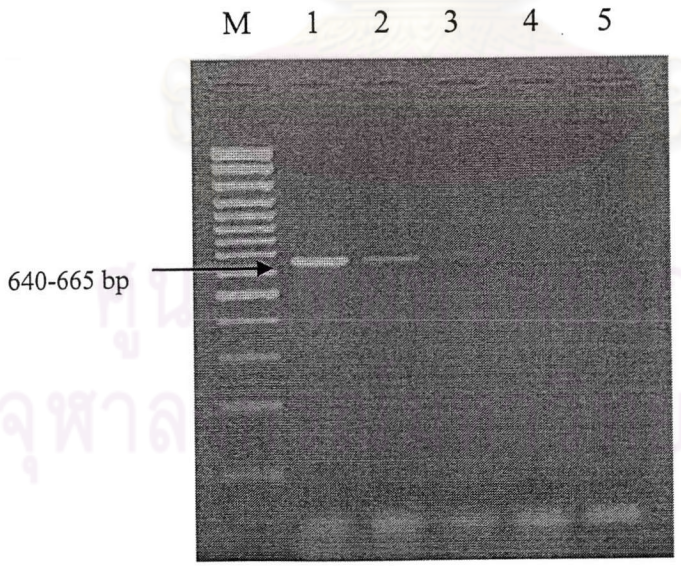

Figure 10. Sensitivity of the detection of *M. kansasii* using multiplex PCR. Lane: M, 100 bp-DNA ladder; 1, *M. kansasii* ATCC 12478 1 ng; 2, 100 pg; 3, 10 pg; 4, 1 pg; 5, negative control

3. Specificity of multiplex PCR

The specificity of multiplex PCR was tested with 10 ng of purified chromosomal DNAs from 10 nonmycobacterial strains listed in Table 8. Only *Nocardia asteroides* and *Nocardia brasiliensis* which belong to genus closely related to *Mycobacterium*, could be amplified as faint bands shown in Figure 11 (lanes12 and 13).

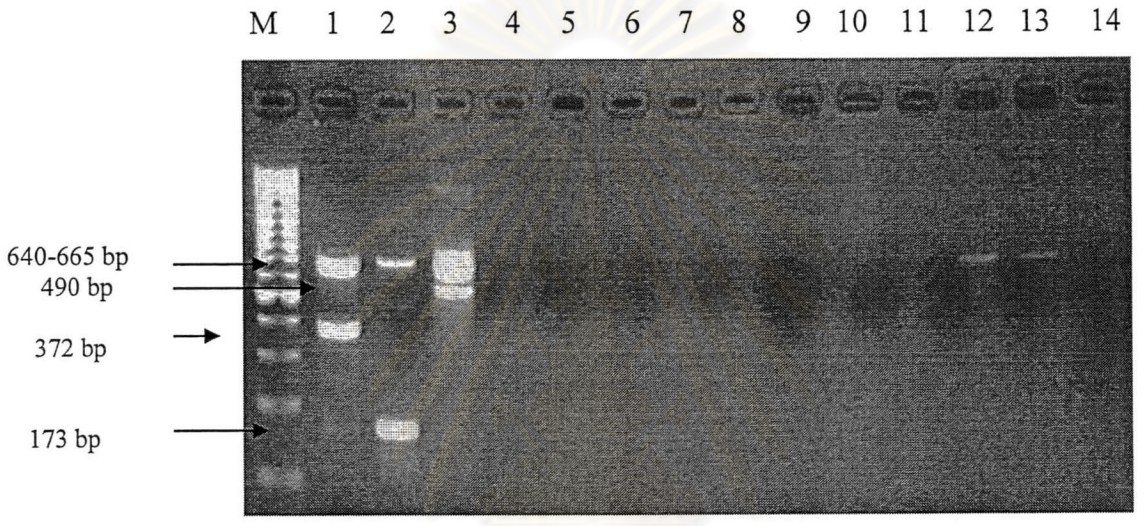

Figure 11. Electrophoresis separation of PCR products obtained by multiplex PCR of genomic DNAs of the *M. tuberculosis*, *M. avium, M. intracellulare* and the other microorganisms in Table 8 . Lanes M, 100 bp-DNA ladder; 1, *M. tuberculosis* H37Rv (positive control); 2, *M. avium* ATCC 25291 (positive control); 3, *M. intracellulare* ATCC 13950 (positive control); 4, *Escherichia coli*; 5, *Klebsiella pneumoniae*; 6, *Haemophilus influenzae*; 7 , *Pseudomonas aeruginosa*; 8, *Pseudomonas pseudomallei*; 9, *Staphylococcus aureus*; 10, *Streptococcus pneumoniae*; 11, *Candida albicans*; 12, *Nocardia aeteroides*; 13, *Nocardia brasiliensis*; 14, negative control

4. Sensitivity of the reverse dot blot hybridization

Biodyne C membranes were spotted with amino-linked probes as shown in Table 10. The sensitivity of the reverse dot blot hybridization assay with biotinylated 16S rDNA amplified fragments was tested with 20 µl of the same PCR products used for agarose gel electrophoresis. It was found that, the lowest template hybridized with specific oligonucleotide probes was amplified from 10 pg of *M. tuberculosis* (Figure 12), 10 pg of *M. avium*, 1 ng of *M. intracellulare*, and 10 pg of other mycobacterial species. Representative result of *M. xenopi* was shown in Figure 13.

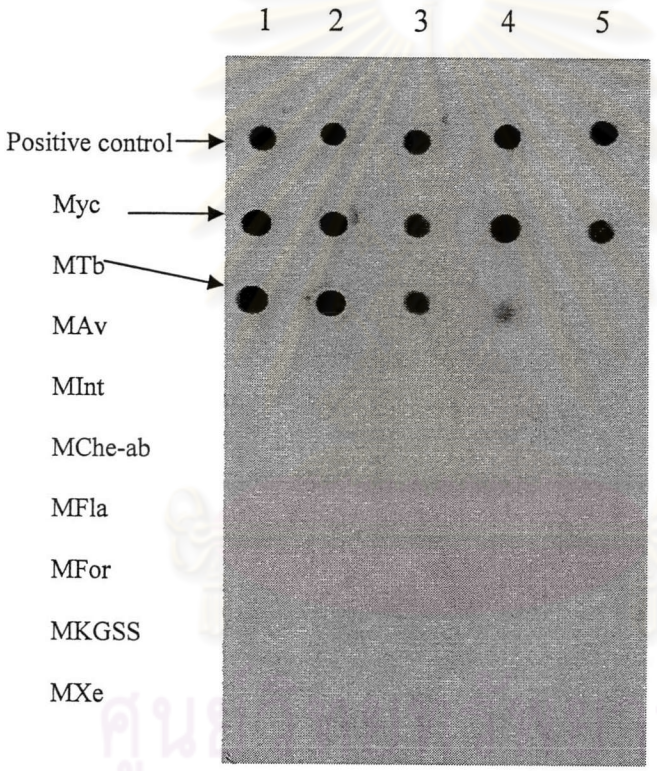

Figure 12. Determination of the sensitivity of PCR by the reverse dot blot hybridization asaay. Lane: 1, *M. tuberculosis* H37Rv 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg

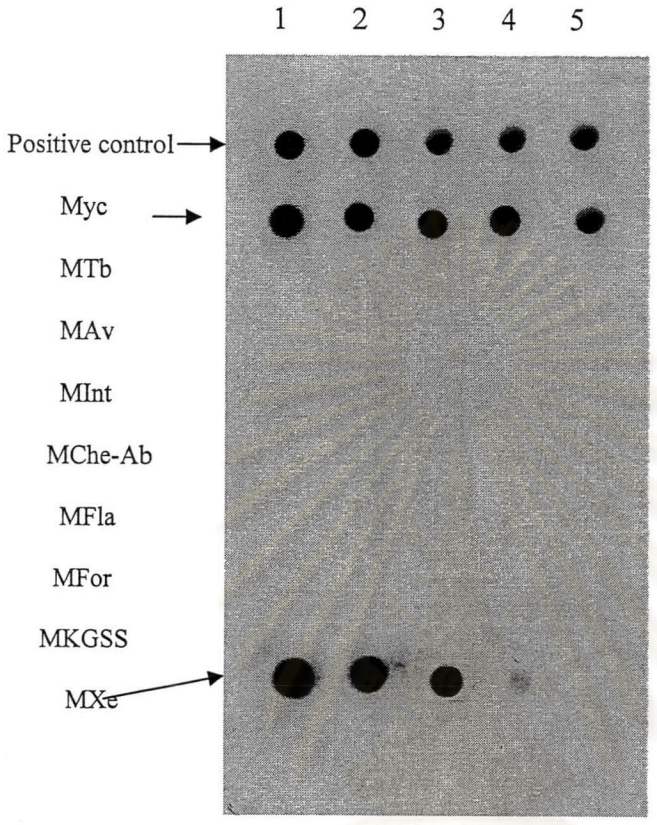

Figure 13. Determination of the sensitivity of PCR by the reverse dot blot hybridization asaay. Lane: 1, *M. xenopi* ATCC 19250 10 ng; 2, 1 ng; 3, 100 pg; 4, 10 pg; 5, 1 pg

5. Specificity of the reverse dot blot hybridization

The specificity of the reverse dot blot hybridization assay with biotinylated 16S rDNA amplified fragments was tested with 20 µl of PCR products amplified from 10 ng of purified DNAs of 20 mycobacterial species listed in Table 8 and *Nocardia asteroides*, and *Nocardia brasiliensis*. Table 11 showed the result of the hybridization of the probes with PCR products from all the strains tested. Mismatches between the species-specific probes and the corresponding regions of other mycobacterial species were detailed in Table 12. All PCR product from the mycobacterial strains hybridized with the *Mycobacterium*-specific probe (Myc). Although PCR products from *N. asteroids* and *N. brasiliensis* hybridized with genus-specific probes but they did not hybridize with species-specific probes. Reference strains of *M. tuberculosis, M. bovis,* and *M. africanum,* hybridized correctly with the probe for the *M. tuberculosis* complex, while *M. avium, M. intracellulare, M. gordonae, M. flavescens, M. fortuitum*, and *M. xenopi*, reacted with their specific probes. *M. chelonae* and *M. abscessus* hybridized correctly with the probe for MChe-ab. *M. kansasii,* and *M. scrofulaceum* hybridized correctly with the probe for MKGSS. A representative blot was shown in Figure 14.

Table 11. Results of hybridization of mycobacterial and nonmycobacterial 16S rDNA PCR products with specific oligonucleotide probes

Species (n)[a]	Result of hybridization with probes									
	MTb	MAv	MInt[b]	MChe-Ab[c]	MFla[d]	MFor[e]	MGor	MKGSS	MXe[f]	Myc
M. tuberculosis (2)	+	-	-	-	-	-	-	-	-	+
M. bovis (2)	+	-	-	-	-	-	-	-	-	+
M. africanum (1)	+	-	-	-	-	-	-	-	-	+
M. asiaticum (1)	-	-	-	-	-	-	-	-	-	+
M. avium (1)	-	+	-	-	-	-	-	-	-	+
M. intracellulare (1)	-	-	+	-	-	-	-	-	-	+
M. abscessus (1)	-	-	-	+	-	-	-	-	-	+
M. fllavescens (1)	-	-	-	-	+	-	-	-	-	+
M. fortuitum (1)	-	-	-	-	-	+	-	-	-	+
M. gordonae (1)	-	-	-	-	-	-	+	-	-	+
M. kansasii (1)	-	-	-	-	-	-	-	+	-	+
M. scrofulaceum (1)	-	-	-	-	-	-	-	+	-	+
M. marinum (1)	-	-	-	-	-	-	-	-	-	+
M. nonchromogenicum (1)	-	-	-	-	-	-	-	-	-	+
M. xenopi (1)	-	-	-	-	-	-	-	-	+	+
M. terrae (1)	-	-	-	-	-	-	-	-	-	+
M. vaccae (1)	-	-	-	-	-	-	-	-	-	+
M. szulgai (1)	-	-	-	-	-	-	-	-	-	+
N. asteroids (1)	-	-	-	-	-	-	-	-	-	+
N. brasiliensis (1)	-	-	-	-	-	-	-	-	-	+

[a] n, number of strains

[b] pInt probes used were pInt1 and pInt2

[c] pChe-ab probes used were pChe-Ab1 and pChe-Ab2

[d] pFla probes used were pFla1 and pFla2

[e] pFor probes were used pFor1, pFor2, and pFor3

[f] pXe probes were used pXe1 and pXe2

Table 12 Mismatches between species-specific probes and the corresponding regions in the 16S rDNA of other mycobacterial species

Target sequence*	No. of mismatches with probe:**														
	MTb	MAv	MInt1	MInt2	MChe-Ab 1	MChe-Ab2	MFla1	MFla2	MFor1	MFor2	MFor3	MGor	MKGSS	MXe1	MXe2
M. tuberculosis complex	0	4	5	3	5	7	7	8	5	7	7	5	5	7	8
M. avium	4	0	5	2	7	10	7	8	9	8	9	3	4	6	5
M. intracellulare I[a]	3	2	0	2	7	5	9	10	7	9	5	5	3	8	4
M. intracellulare II[a]	5	4	2	0	8	7	10	8	9	7	7	3	4	9	5
M. chelonae-M. abscessus I[a]	7	6	10	9	0	5	5	4	3	4	3	5	5	5	6
M. chelonae-M. abscessus II[a]	9	9	10	9	5	0	5	5	3	4	3	7	4	5	6
M. flavescens I[a]	9	10	10	8	9	9	0	1	11	12	10	6	6	7	6
M. flavescens II[a]	9	10	11	8	9	9	1	0	11	12	11	7	6	7	6
M. fortuitum I[a]	6	5	8	5	2	2	4	5	0	2	2	5	6	5	6
M. fortuitum II[a]	8	7	9	6	3	4	5	4	2	0	2	7	6	5	6
M. fortuitum III[a]	8	8	8	5	3	4	6	5	2	2	0	7	6	5	6
M. gordonae	4	8	7	7	6	9	16	15	8	9	8	0	4	5	6
M. kansasii	5	4	5	4	9	9	8	7	8	9	8	4	0	4	5
M. gastri	5	4	5	4	9	9	8	7	8	9	8	4	0	4	5
M. scrofulaceum	5	4	5	4	9	9	8	7	8	9	8	4	0	4	5
M. xenopi I[a]	9	5	7	7	9	9	7	8	9	7	9	6	3	0	1
M. xenopi II[a]	9	5	7	7	6	9	7	8	9	7	9	6	3	1	0

* Sequences data were retrieved from GenBank.

** Details of the probes were given in Table 10.

[a] Different 16S rDNA sequences were retrieved from GenBank.

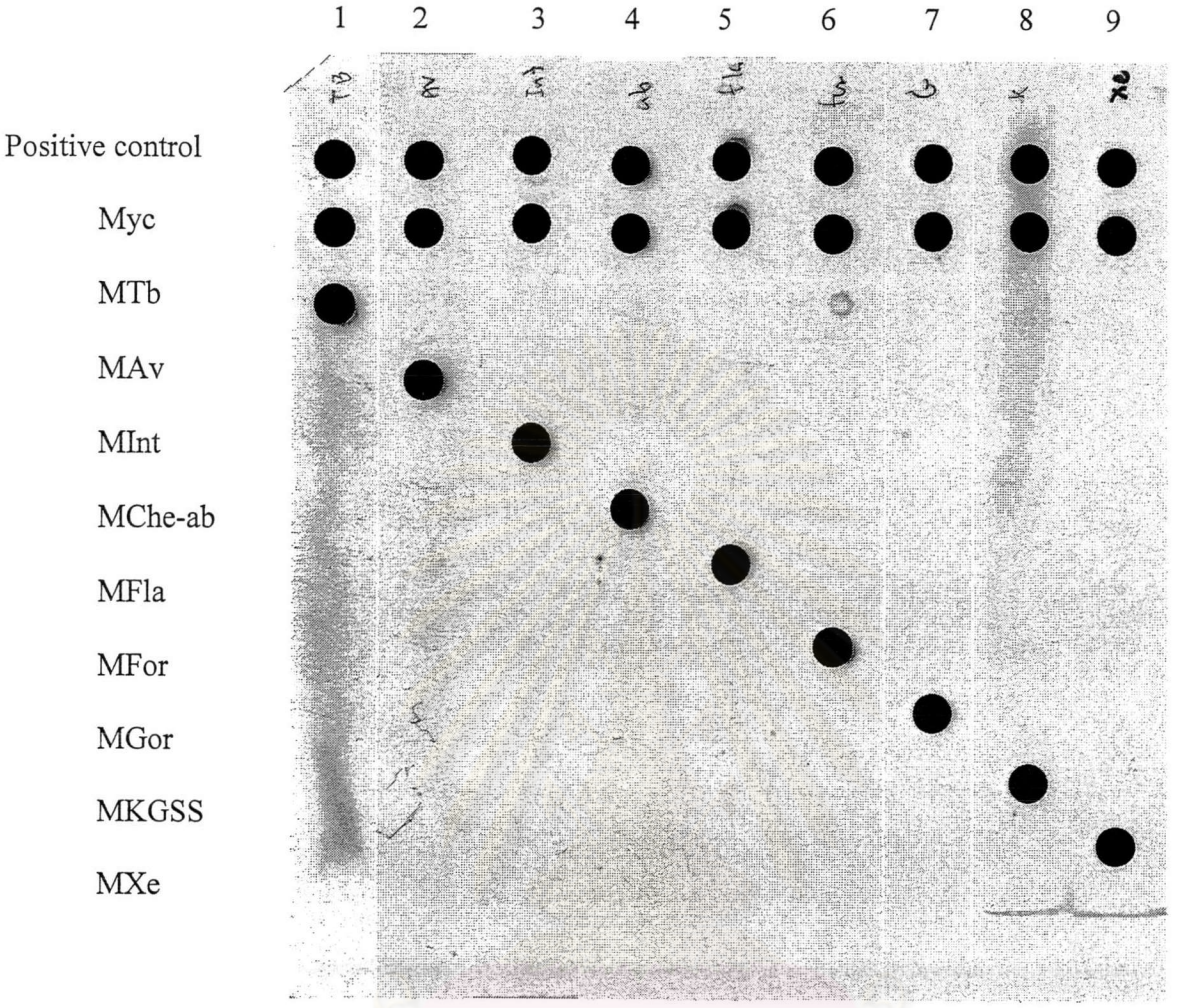

Figure 14. Representative example of the specificity of the reverse dot blot hybridization assay. The oligonucleotide probes were dot-blotted onto a Biodyne C membrane in a strip. Lane: 1, *M. tuberculosis* H37Rv; 2, *M. avium*; 3, *M. intracellulare*; 4, *M. abscessus*; 5, *M. flavescens*; 6, *M. fortuitum*; 7, *M. gordonae*; 8, *M. kansasii*; 9, *M. xenopi*

6. Comparison of two DNA extraction methods from colonies of clinical isolates

A comparison of two DNA extraction methods was performed in 10 clinical isolates of *M. avium*. DNAs extracted by heat lysis method yielded a single band of species-specific (173 bp) PCR product in all tested samples but yielded a genus-specific (640-665 bp) band only in 7 of 10 tested samples. Alkaline wash and heat lysis method gave better DNA preparation as bands of genus-specific and species specific PCR product were detected in all 10 tested samples. From this result, alkali wash and heat lysis method was then chosen for amplification of mycobacterial DNA from colonies. Representative result was shown in Figure 15.

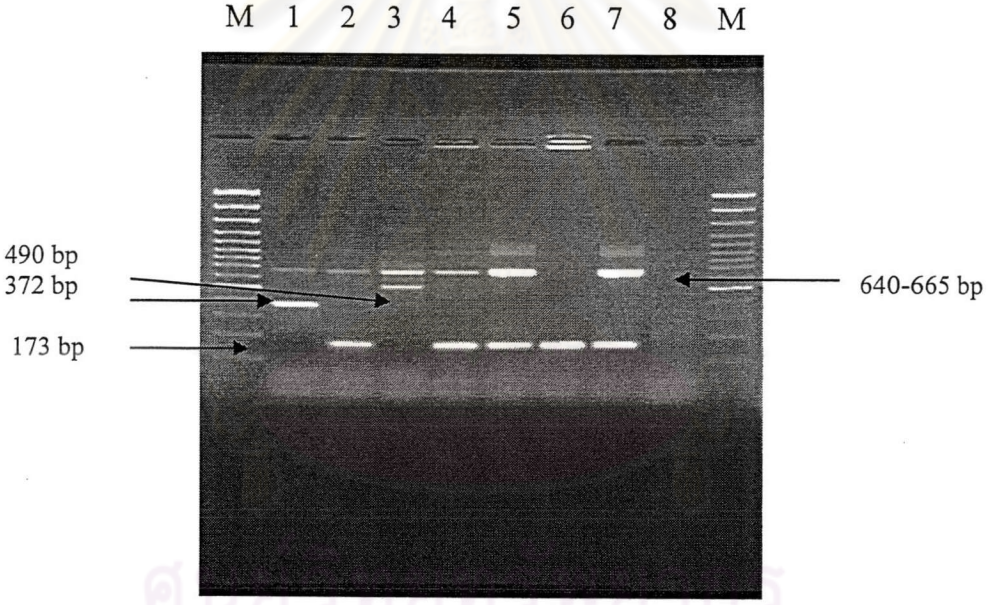

Figure 15. Amplification of mycobacterial DNA from clinical isolates by two extraction methods. Lanes M, 100 bp-DNA ladder; 1, *M. tuberculosis* H37Rv (positive control); 2, *M. avium* ATCC 25291 (positive control); 3, *M. intracellulare* ATCC 13950 (positive control); 4, clinical isolate 1 (heat lysis); 5, clinical isolate 1 (alkaline wash and heat lysis); 6, clinical isolate 2 (heat lysis); 7, clinical isolate 2 (alkaline wash and heat lysis); 8, negative control

7. Results of multiplex PCR and reverse hybridization tested with of *Mycobacterium* clinical isolates

In order to evaluate multiplex PCR and reverse hybridization for detection and identification of the members of genus *Mycobacterium*, clinical isolates which have been identified were tested first. Clinical isolates as listed in Table 9 were subjected to DNA extraction by alkaline wash and heat lysis method and the extracted DNAs were amplified by multiplex PCR. Expected DNA bands were seen from all clinical isolates as shown in Table 13 and each PCR product hybridized with its-specific probe. Representative result of multiplex PCR and reverse hybridization was shown in Figure 16 and Figure17, respectively.

Table 13. Results of multiplex PCR and reverse hybridization tested with of *Mycobacterium* clinical isolates

Species	Number of isolates	No. of isolates identified by multiplex PCR as:				Number of samples identified by reverse hybridization	Reverse hybridization assay probe*	Reverse hybridization interpretation
		Mycobacterium genus (640-665 bp)	*M. avium* (173 bp)	*M. intracellulare* (490 bp)	*M. tuberculosis* complex (372bp)			
*M. tuberculosis***	50	50	-	-	50	2	MTb	*M. tuberculosis* complex
*M. avium***	24	24	24	-	-	2	MAv	*M. avium*
M. intracellulare	2	2	-	2	-	2	MInt	*M. intracellulare*
M. abscessus	2	2	-	-	-	2	MChe-Ab	*M. chelonae* or *M. abscessus*
M. chelonae	2	2	-	-	-	2	MChe-Ab	*M. chelonae* or *M. abscessus*
M. scrofulaceum	2	2	-	-	-	2	MKGSS	*M. kansasii* , *M. gastri*, *M. scrofulaceum* or *M. simiae*
M. flavescens	1	1	-	-	-	1	MFla	*M. flavescens*
M. kansasii	1	1	-	-	-	1	MKGSS	*M. kansasii* , *M. gastri*, *M. scrofulaceum* or *M. simiae*
M. fortuitum	1	1	-	-	-	1	MFor	*M. fortuitum*
Total	**85**	**85**	**24**	**2**	**50**	**15**		

* Details of the probes were given in Table 10.

** Only two clinical isolates were tested.

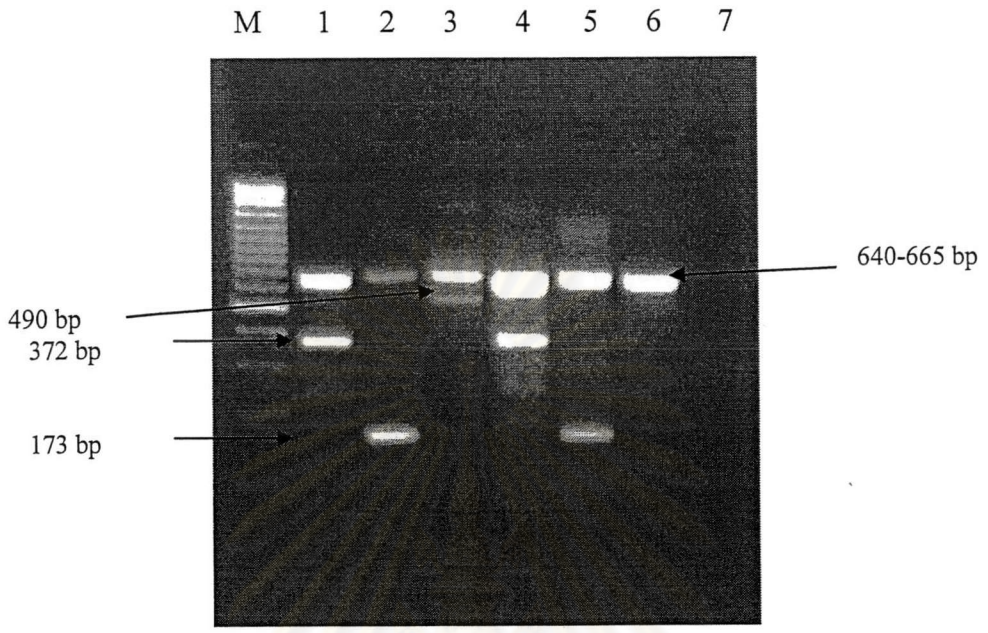

Figure 16. Electrophoresis separation of PCR products obtained by multiplex PCR. genomic DNAs of the *M. tuberculosis*, *M. avium, M. intracellulare* and clinical isolates. Lanes M, 100 bp-DNA ladder; 1, *M. tuberculosis* H37Rv (positive control); 2, *M. avium* ATCC 25291 (positive control); 3, *M. intracellulare* ATCC 13950 (positive control); 4, clinical isolates 4 (*M. tuberculosis*); 5, clinical isolates 5 (*M. avium*); 6, clinical isolates 10 (*M. abscessus*); 7, Negative control

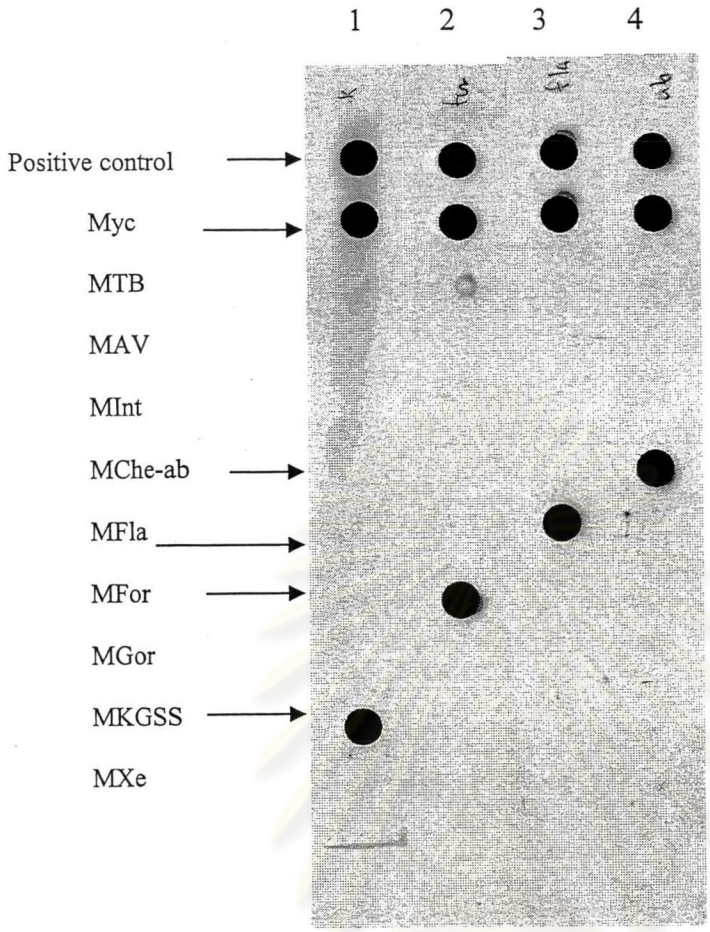

Figure 17. Representative example of the specificity of the reverse dot blot hybridization assay from clinical isolates. The oligonucleotide probes were dot-blotted onto a Biodyne C membrane in a strip. Lane: 1, *M. kansasii*; 2, *M. fortuitum*; 3, *M. flavescens*; 4, *M. abscessus*

8. Results of multiplex PCR and reverse hybridization assay tested in hemoculture samples and clinical specimens compared with results of conventional methods

In order to evaluate multiplex PCR and reverse hybridization for detection and identification of the members of genus *Mycobacterium* in routine laboratory, fifty signal-positive hemoculturee and fifty AFB-positive clinical specimens were tested. The extracted DNAs were amplified by multiplex PCR. Table 14 showed that multiplex PCR detected *M. tuberculosis*, *M. avium*, and *Mycobacteruium* genus from 6, 41, and 3 signal-positive hemoculture samples, respectively. Reverse hybridization assay could identify *Mycobacterium* genus in these three samples as *M. chelonae* and *M. abscessus* by probe MChe-Ab. Of these three *Mycobacterium* genus, two were identified as *M. abscessus* and one as *M. chelonae* by conventional methods. Representative result of multiplex PCR of hemoculture samples and clinical specimens was shown in Figure 18 and Figure19, respectively. Representative result of reverse hybridization of hemoculture samples and clinical specimens was shown in Figure 20.

Table 15 showed that multiplex PCR detected *M. tuberculosis*, *M. avium*, and *Mycobacteruium* genus from 36, 7, and 7 AFB-positive clinical specimens, respectively. Reverse hybridization assay could identify *Mycobacterium* sp. in these seven samples as *M. fortuitum* (two samples) by probe MFor, *M. flavescens* (one sample) by probe MFla, *M. chelonae* and *M. abscessus* (two samples) by probe MChe-Ab and *M. kansasii, M. gastri, M. scrofulaceum,* or *M. simiae* (two samples) by probe MKGSS. Identification of culture obtained from these samples by conventional method confimed the correct identification of reverse dot blot hybridization, i.e. reverse dot blot hybridization could identification *Mycobacterium* genus in pus as *M. chelonae* or *M. abscessus* by probe MChe-Ab and the culture obtained was identified to be *M. abscessus*.

Table 14. Results of multiplex PCR and reverse hybridization assay tested in hemoculture samples compared with results of conventional methods

Number of hemoculture samples	No. of sample detected by multiplex PCR as:				Number of samples identified by reverse hybridization	Reverse hybridization assay probe*	Reverse hybridization interpretation	Species identification by conventional methods** (n)
	Mycobacterium genus (640-665 bp)	*M. avium* (173 bp)	*M. intracellulare* (490 bp)	*M. tuberculosis* complex (372bp)				
6	6	-	-	6	NT	NT	NT	*M. tuberculosis*
41	41	41	-	-	NT	NT	NT	*M. avium*
3	3	-	-	-	3	MChe-Ab	*M. chelonae* or *M. abscessus*	*M. abscessus* (2) *M. chelonae* (1)
Total	**50**	**41**	**-**	**6**	**3**			

n, number of strains

NT, not tested

* Details of the probes were given in Table 10.

** Biochemical methods and AccuProbe

Table 15. Results of multiplex PCR and reverse hybridization assay tested in clinical specimens compared with results of conventional methods

Type of specimens	Kinyoun result	Number of clinical samples	No. of specimens detected by multiplex PCR as:				Number of specimens identified by reverse hybridization	Reverse hybridization assay probe* (n)	Reverse hybridization interpretation	Species identification by methods** (n)
			Mycobacterium sp. (640-665 bp)	*M. avium* (173 bp)	*M. intracellulare* (490 bp)	*M. tuberculosis* complex (372bp)				
Sputum	+3	2	2	-	-	2	NT	NT	NT	*M. tuberculosis*
	+2	1	1	-	-	1	NT	NT	NT	*M. tuberculosis*
	+1	31	31	-	-	31	NT	NT	NT	*M. tuberculosis*
	+1	7	7	7	-	-	NT	NT	NT	*M. avium*
	+1	6	6	-	-	-	6	MFor (2)	*M. fortuitum*	*M. fortuitum* (2)
								MKGSS (2)	*M. kansasii* , *M. gastri*, *M. scrofulaceum* or *M. simiae*	*M. kansasii* (1) *M. scrofulaceum* (1)
								MFla (1)	*ns*	*M. flavescens* (1)
								MChe-Ab (1)	*M. chelonae* or *M. abscessus*	*M. chelonae* (1)
Pus	+1	1	1	-	-	1		NT	NT	*M. tuberculosis*
	+1	1	1	-	-	-	NT	MChe-Ab (1)	*M. chelonae* or *M. abscessus*	*M. abscessus* (1)
Body fluid	+1	1	1	-	-	1	1	NT	NT	*M. tuberculosis*
Total		**50**	**50**	**7**	-	**36**	NT **7**			

n, number of strains

** Biochemical methods and AccuProbe

NT, not tested

* Details of the probes were given in Table 10.

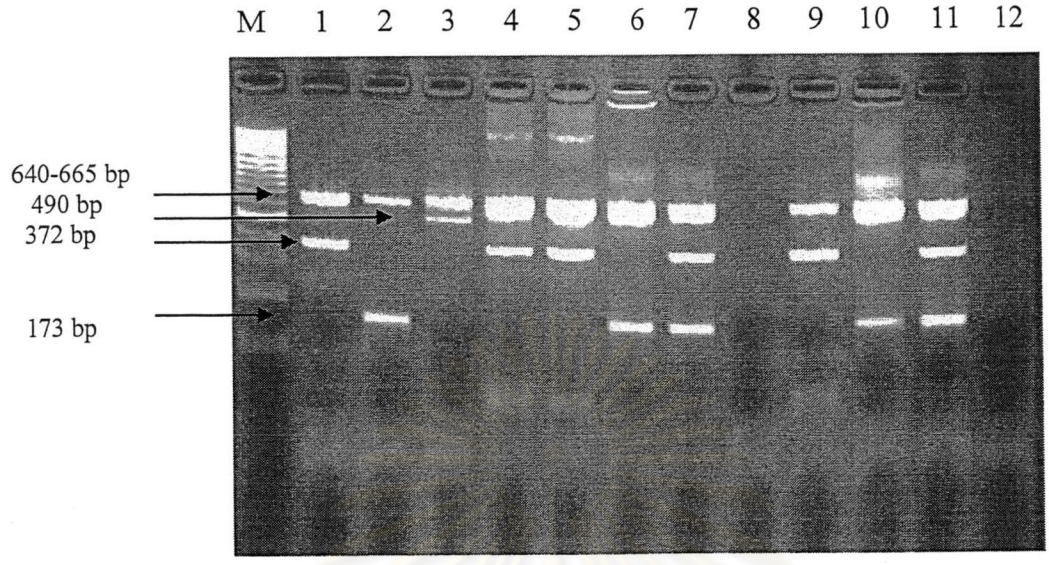

Figure 18. Electrophoresis separation of PCR products obtained from AFB-positive hemoculture samples. Lanes M, 100 bp-DNA ladder; 1, *M. tuberculosis* H37Rv (positive control); 2, *M. avium* ATCC 25291 (positive control); 3, *M. intracellulare* ATCC 13950 (positive control); 4, sample MB 1 ; 5, sample MB 1(spiked with 1 ng *M. tuberculosis*); 6, sample MB 2 ; 7, sample MB 2 (spiked with 1 ng *M. tuberculosis*); 8, sample MB 53 (signal-positive hemoculture but AFB negative) ; 9, sample MB 53 (spiked with 1 ng *M. tuberculosis*); 10, sample MB 3 ; 11, sample MB 3 (spiked with 1 ng *M. tuberculosis*); 12, negative control

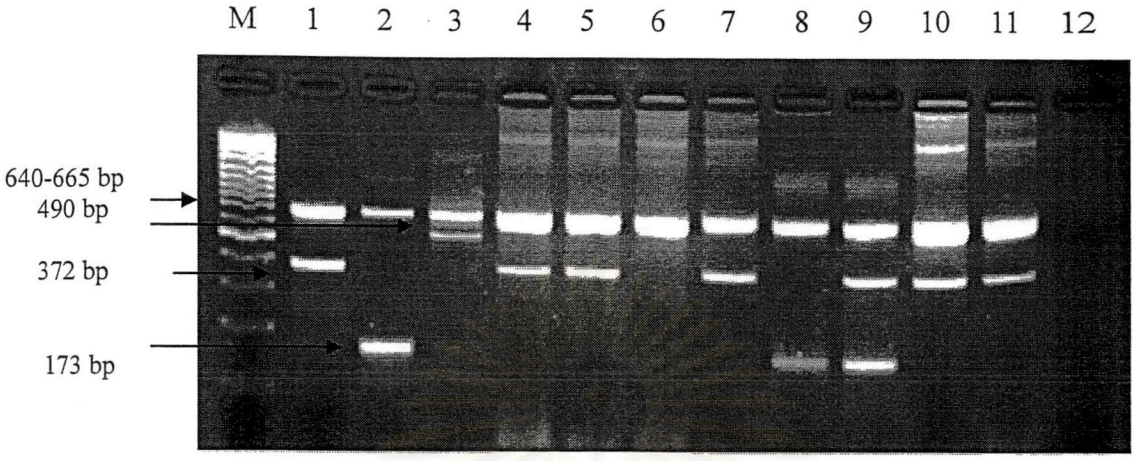

Figure 19. Electrophoresis separation of PCR products obtained from AFB-positive clinical specimens. Lanes M, 100 bp-DNA ladder; 1, *M. tuberculosis* H37Rv (positive control); 2, *M. avium* ATCC 25291 (positive control); 3, *M. intracellulare* ATCC 13950 (positive control); 4, specimen S1; 5, specimen S1 (spiked with 1 ng *M. tuberculosis*); 6, specimen S2; 7, specimen S2 (spiked with 1 ng *M. tuberculosis*); 8, specimen S3; 9, specimen S3 (spiked with 1 ng *M. tuberculosis*); 10, specimen S4; 11, specimen S4 (spiked with 1 ng *M. tuberculosis*); 12, negative control

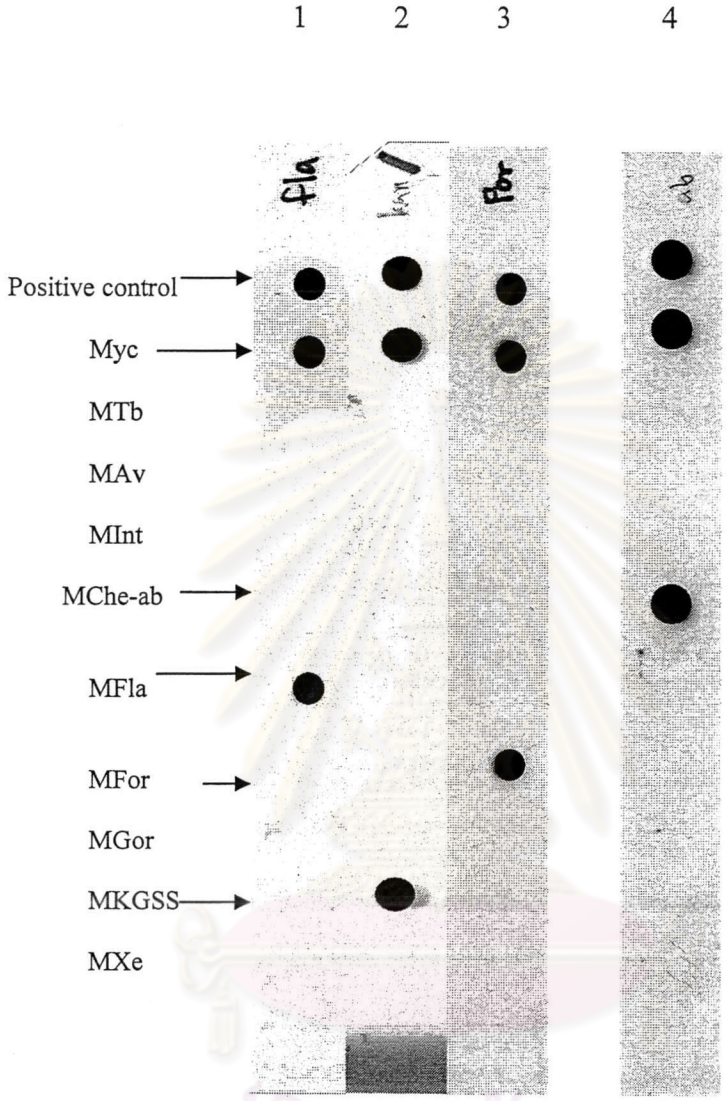

Figure 20. Representative result of the specificity of the reverse dot blot hybridization assay tested in hemoculture samples and clinical specimens. The oligonucleotide probes were dot-blotted onto a Biodyne C membrane in a strip. Lane: 1, sputum sample (S 23); 2, sputum sample (S 40); 3, sputum sample (S 46); 4, Hemoculture sample (MB 13)

9. 16S rDNA sequencing

Ten samples (3 hemoculture samples and 7 clinical specimens) in which *Mycobacterium* genus was detected by multiplex PCR and reverse dot blot hybridization, were subjected to PCR-sequencing of 16S rDNA region A. Sequencingt gave concordant results with those obtained by conventional methods. Representative result of 16S rDNA sequencing of *M. abscessus, M. fortuitum, M. kansasii, M. scrofulaceum* and *M. flavescens* was shown in Figure 21, Figure 22, Figure 23, Figure 24, and Figure 25, respectively.

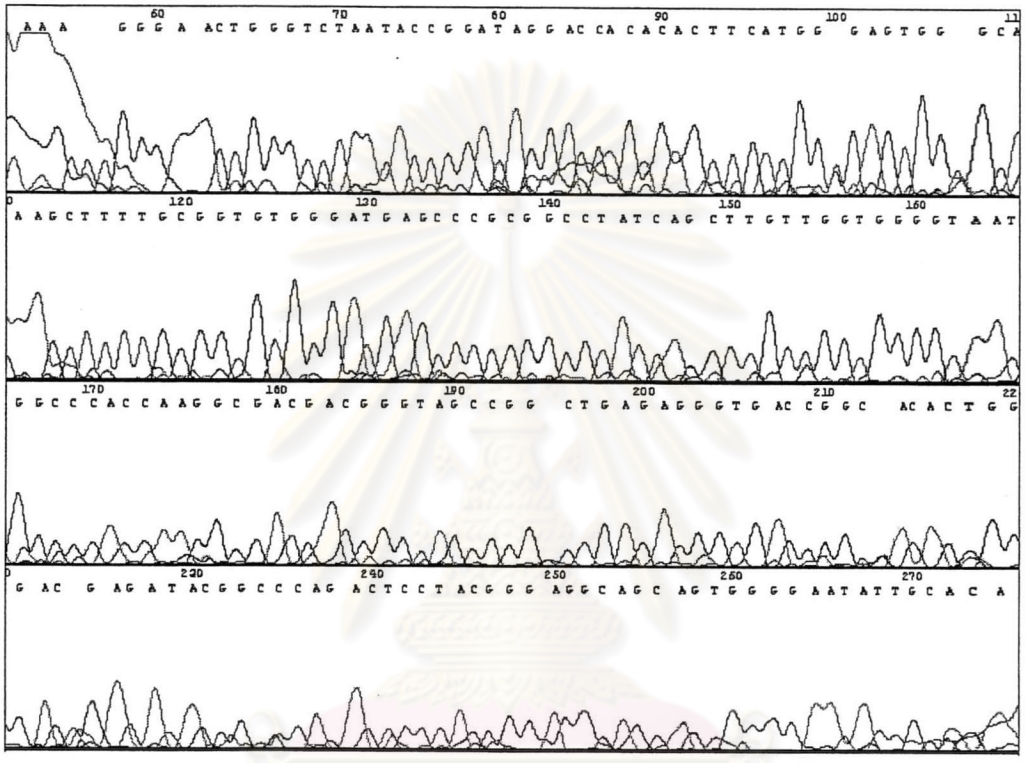

Figure 21. Chromatogram of sequencing by automate sequencer of *M. abscessus*

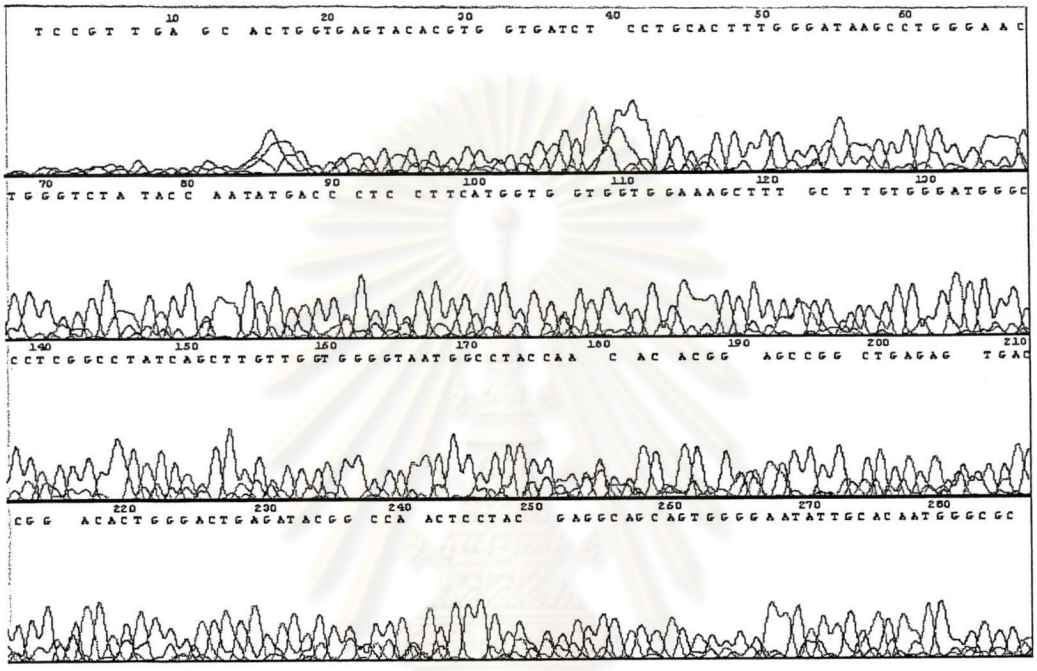

Figure 22. Chromatogram of sequencing by automate sequencer of *M. fortuitum*

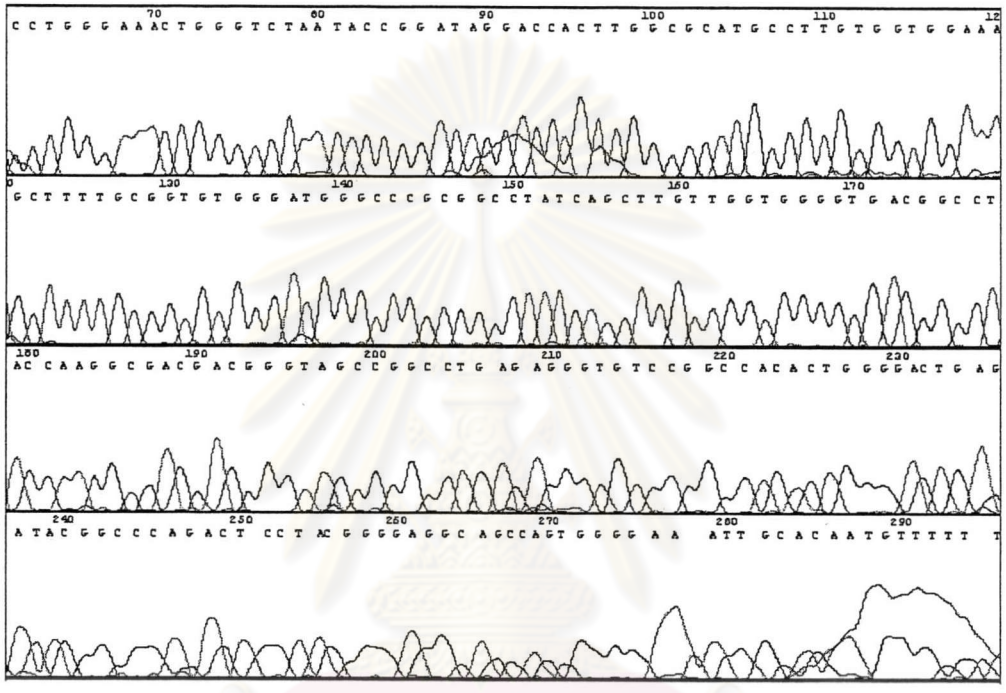

Figure 23. Chromatogram of sequencing by automate sequencer of *M. kansasii*

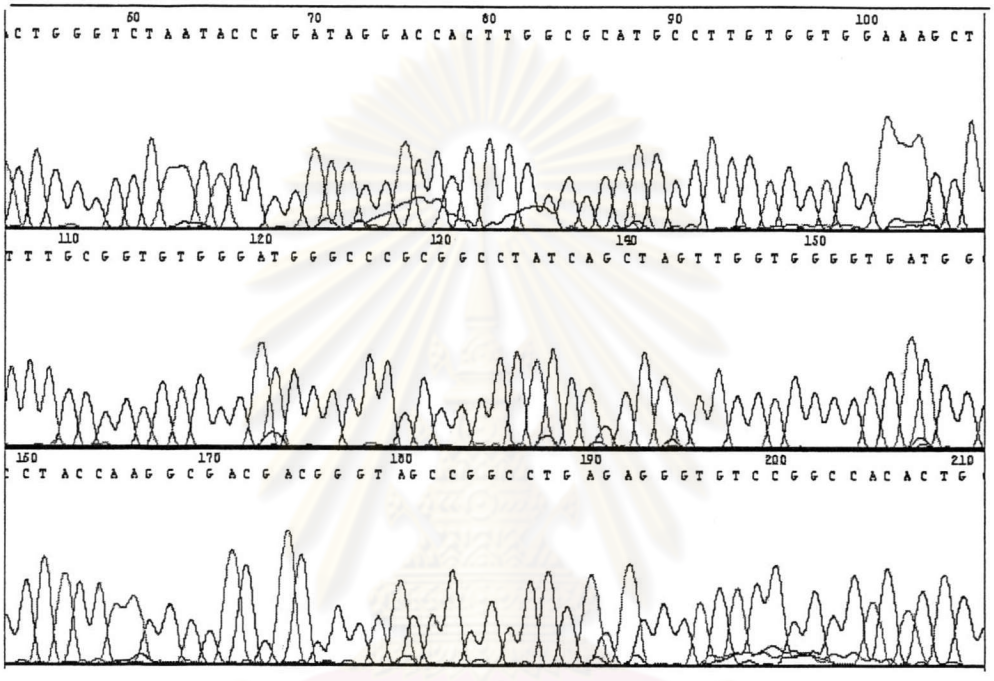

Figure 24. Chromatogram of sequencing by automate sequencer of *M. scrofulaceum*

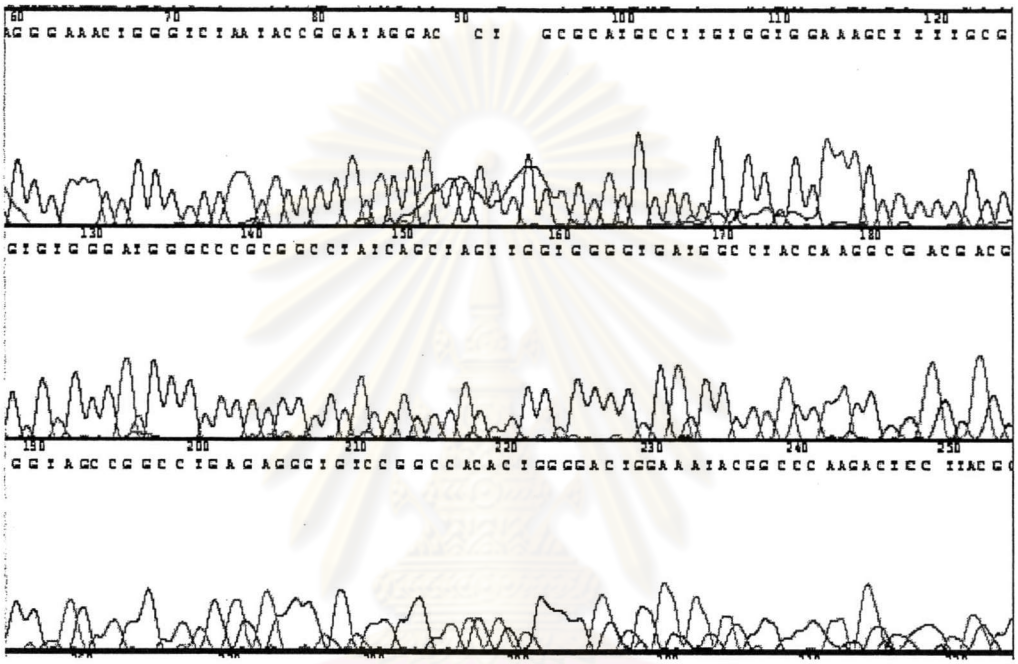

Figure 25. Chromatogram of sequencing by automate sequencer of *M. flavescens*