MUTATION IN THE *rpoB* GENE OF THE RIFAMPICIN RESISTANT *M. AVIUM* COMPLEX STRAINS FROM THAILAND

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Abstract. Forms of mutation never before described in the *rpoB* gene are reported for a sample of 20 rifampicin-resistant *Mycobacterium avium* Complex (MAC) strains isolated from AIDS patients in Thailand. All strains were analyzed by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and polymerase chain reaction-DNA sequencing (PCR-DNA sequencing). Sequence analysis of these strains revealed that only one strain (5%) has missense mutation at Lys–626 (Thr) and the rest of the strains had 15 different silent mutations within a 542 bp region of the *rpoB* gene. Five strains (25%) had a silent mutation at only one position, 7 (35%) at 2 positions, 7 (35%) at 3 positions, and 1 (5%) at 7 positions. The silent mutation at the Ala-630 codon occurred in the largest proportion of the strains (15 strains, 75%), followed by the Val-581 in 8 strains (40%), Tyr-578 and Thr-600 in 4 strains (20%), and Gly-597 in 3 strains (15%). This investigation demonstrates that mutation in the *rpoB* gene of MAC strains from Thailand are more varied than previously reported for RIF ^{r} MAC strains. PCR-SSCP screening clearly separated RIF ^{r} strains from rifampicin-susceptible (RIF ^{s}) strains.

INTRODUCTION

Human mycobacterial infections present a serious health problem in developing countries (Chuchottaworn *et al*, 1999; Gadelha *et al*, 2002) and developed countries (Blaxhult *et al*, 2002). Not only *Mycobacterium tuberculosis*, which causes human lung disease, but also *Mycobacterium avium* complex (MAC) disease prevalence have increased dramatically with the emergence of HIV-immunodeficiency. The treatment of infections, caused mostly by MAC isolates, is rather difficult due to primary resistance of the agent against most commonly used drugs (Dvorska *et al*, 2002).

Due to the increase in the AIDS pandemic, the prevalence of both *M. tuberculosis* and MAC species causing disseminated disease in AIDS patients is very high. Therefore, there is great pressure on clinical laboratories to rapidly and accurately detect and identify clinically important mycobacteria. Conventionally, mycobacteria grown in culture are identified by standard culture and biochemical tests (Wong *et al*, 2001). Conventional MAC cultures require 2-4 weeks, then another 2-4 weeks to match anti-mycobacterial drugs with susceptible bacteria. Culture time was significantly reduced about 2 weeks with the BACTEC system, which detects mycobacterial growth automatically, so that in just 4-6 days the drug susceptibility profile is known (Walters and Hanna, 1996). Recent molecular techniques have been applied to detect drug-resistant mycobacteria within 1-2 days. Such techniques include polymerase chain reaction (PCR)-DNA sequencing (Ramasoota *et al*, 2006), PCR-single-strand conformation (SSCP) (Lee *et al*, 1998), PCR-heteroduplex (Williams *et al*, 1994, 1998), line-probe assay (LIPA) (Hirano *et al*, 1999; Sintchenko *et al*, 1999) and PCR-reverse line blot hybridization (PLH) (Morcillo *et al*, 2002). Shortening the time between diagnosis and the onset of effective therapy can be one factor improving patient survival.

Rifampicin (RIF) is the main drug for the treatment of mycobacterial diseases like leprosy and tuberculosis, and the rifampicin derivative, rifabutin, is the main drug for treating MAC infection. However, widespread and improper use of rifampicin monotherapy has led to the emergence of the rifampicin-resistant (RIF ^{r}) strain, threatening the usefulness of this drug in treating mycobacterial diseases. Rifampicin acts by binding to bacterial RNA polymerase. The mechanism of RIF-resistant bacteria consists of mutation in the *rpoB* gene (encoding the RNA polymerase protein), which results in a different amino acid; then, rifampicin cannot bind to the RNA polymerase, leaving bacteria resistant instead of inactivated. *E. coli* was used for the first studies to elucidate the mechanism of RIF ^{r}. For instance, Jin and Gross (1988) mapped mutations

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leading to RIF^r in *E. coli* to 3 distinct loci near the center of the *rpoB* gene. The mechanism of RIF^r in *M. tuberculosis* was assumed to be similar to that in *E. coli*, and indeed, most mutations conferring RIF^r were found to occur in a small region of the *rpoB* gene, the so-called "mutation hot spot region" (Telenti *et al*, 1993; Williams *et al*, 1994; Musser, 1995).

For MAC strain, the mechanism of RIF^r are still unclear. As report by Williams *et al* (1994) and 4 RIF^r *M. avium* strains studied for mutation in the *rpoB* gene; one strain had a Ser to Trp alteration in the homolog of amino acid 531, one had a Ser to Leu change at this position, and the other 2 had wild-type sequences in the *rpoB* region characterized. Guerrero *et al* (1994) studied sequence alteration in the *rpoB* gene of 31 MAC isolates with variable levels of RIF susceptibility; only one of the 31 organisms contained a missense mutation in the target region. Gomez *et al* (1995) found that none of the 20 MAC strains have the mutations associated with RIF^r in MTB, suggesting that unique mechanisms of drug resistance exist in MAC strains. The results of the last two studies were implied that alternative mechanisms may be responsible for the intrinsic RIF^r in MAC organisms. Additional potential mechanisms, including modifications of drug uptake or efflux, and the occurrence of a RIF-inactivating enzyme, have not yet been probed in detail.

Application of molecular techniques for detecting RIF^r MAC strain can dramatically reduce detection time (1-2 days) compared with the conventional method (2-4 weeks), and until now, no study has been conducted on mutation in the *rpoB* gene of RIF^r of MAC isolated from Thailand. In this study, molecular techniques, PCR-SSCP and PCR-DNA sequencing, were respectively applied for pre-screening and analyzing mutation in the *rpoB* gene of 20 RIF-resistant MAC strains obtained from AIDS patients in Thailand.

MATERIALS AND METHODS

Bacterial strains

The reference strain of *M. avium* ATCC (700898) used as control was obtained from Siriraj Hospital, Bangkok. The twenty clinical MAC strains were isolated from AIDS patients from the Chest Disease Institute and Bamrasnaradura Institute, Nonthaburi Province, Thailand, in 1997-2003. All 20 strains had been previously differentiated and identified by conventional culturing or biochemical testing, and by DNA hybridization assay (using commercial kits of Gen-Probe Inc, USA). To examine drug-resistance profiles (including resistance to rifampicin) (Table 1), the proportion method was conducted using Lowenstein-Jensen egg slant (WHO, 1998).

Preparation of chromosomal DNA from MAC strains

For each strain, one loopful of MAC culture was transferred to a 1.5 ml conical tube containing 0.5 ml sterile distilled water. The tube was boiled for 15 minutes. Then, 100 µl of lysis buffer were added to the solution, and incubated at 37°C overnight. Then, 100 µl of 5M NaCl were added, mixed thoroughly, and 80 µl of CTAB/NaCl solution were added, which had been pre-warmed at 65°C for 5 minutes. This was vigorously mixed by inverting the tube 20 times, until the liquid content became white (milky), then incubated at 65°C for 10 minutes. Eight hundred microliters of chloroform:isoamyl alcohol (24:1) were added and centrifuged at 13,000*g* for 5 minutes. The viscous upper phase was transferred to a new tube. Then, 800 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added and centrifuged at 13,000*g* for 5 minutes. To precipitate DNA, the upper aqueous phase was transferred to a new tube, and isopropanol was added, then incubated at -20°C for 2 hours. The tube was centrifuged at 13,000*g* for 15 minutes, and the supernatant discarded. The resulting DNA pellets were washed with 70% cold ethanol and centrifuged at 13,000*g* for 15 minutes; the supernatant was then discarded. The resulting purified DNA pellet was allowed to dry at room temperature and resuspended with 50 µl sterile distilled water. The completely dissolved DNA was diluted with sterile distilled water at a ratio of 1:200 and determined DNA concentration by UV spectroscopy at wavelength 260 nm [$1A_{260}$=50 µg/ml x 200 (dilution factor)]. The purity of the DNA was determined by of A_{260}/A_{280} ratio.

Primer design

In this study, the primers for amplification of MAC *rpoB* gene were designed by the program Primer Premier 3 and were synthesized by PROLIGO Primers & Probes (PROLIGO® Singapore Pty, Ltd). These three primers were designed to completely match the MAC *rpoB* gene sequence. The primers designated as MRB_1-for sequence 5´-TCA-ACA-TCC-GTC-CCG-TCG-3´/MRB_2-rev sequence 5´-CTC-GAG-GAA-CGG-GAT-CAT-3´ and MRB_1-for/MRB_3-rev sequence 5´-GGC-GGT-CAG-GTA-GTG-GAT-3´ were used to generate 2 amplified fragments, which would have expected sizes of 347-bp and 542-bp, respectively.

The other set of primers was assigned to amplify 16S rRNA of *M. avium* strain. The primer was designed as Mygen-for sequence 5´-AGA-GTT-TGA-TCC-TGG-CTC-AG-3´ and Mycav-rev sequence 5´-ACC-

Table 1
Drug resistance pattern of MAC strains from Bamrasnaradura Institute and Chest Disease Institute.

No	Institute	Drug resistance pattern			
		INH	RIF	STM	EMB
1	Bamrasnaradura	R	R	R	R
2	Bamrasnaradura	R	R	R	R
3	Bamrasnaradura	R	R	R	R
4	Bamrasnaradura	R	R	R	R
5	Bamrasnaradura	R	R	R	R
6	Bamrasnaradura	R	R	R	R
7	Bamrasnaradura	R	R	R	R
8	Bamrasnaradura	R	R	R	R
9	Chest Disease	R	R	R	R
10	Chest Disease	R	R	R	R
11	Chest Disease	R	R	R	R
12	Chest Disease	R	R	R	R
13	Chest Disease	R	R	R	R
14	Chest Disease	R	R	R	S
15	Chest Disease	R	R	R	R
16	Chest Disease	R	R	R	S
17	Chest Disease	R	R	R	S
18	Chest Disease	R	R	R	R
19	Chest Disease	R	R	R	S
20	Chest Disease	R	R	R	R

INH = Isoniazid; RIF = Rifampicin; STM = Streptomycin; EMB = Ethambutal

AGA-AGA-CAT-GCG-TCT-TG-3´. These primers produced 180 bp product of 16S rRNA of *M. avium* strain.

PCR procedure for 16S rRNA and *rpoB* gene amplification

Reaction mixtures contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 1.5 mM each deoxynucleoside triphosphate, 20 pmol each primer and approximately 1.0 U of *Taq* polymerase. This procedure produced a 542-bp or 347-bp product of the *rpoB* gene. To each mix, 5 μl of template DNA, prepared as described above, were added. Amplification involved an initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisting of a 45 seconds denaturation at 95°C, 45 second annealing at 60°C, and 45 second primer extension at 72°C, followed by a final extension of 5 minutes at 72°C. Products were maintained at 4°C until analyzed by electrophoresis. One reference *M. avium* strain, ATCC 700898, was incorporated as a positive control and the water blank was incorporated as a negative control for each PCR mixture to assess reproducibility and consistency.

Detection of PCR products

The PCR products (amplicons) were detected using agarose gel electrophoresis at 100 volts for 45 minutes. One point five percent agarose gel was used to analyze the PCR products after amplification. The gel was prepared by completely dissolving agarose powder (Seakam® LE, Rockland, USA) upon heating in 0.5X TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA, pH 8.0); it was allowed to cool to around 50ºC before pouring into an electrophoresis tray with comb inserted. Eight microliters of PCR product mixture were stained with GelStar for 15 minutes and mixed with 1/3 volume of loading dye (25% glycerol, 60 mM EDTA, 0.25% bromophenol blue) and loaded into the gel slots in the submarine condition. Electrophoresis was performed at 100 volts for 45 minutes. After electrophoresis, DNA patterns were visualized using a Dark Reader transilluminator (Clare Chemical Research Model DR-45M, Dolores, USA). Photographs were digitalized and stored in JPG file format.

Screening of mutation by single strand conformation polymorphism (SSCP)

SSCP analysis was performed according to the method described by Bannai and co-workers (1996) with a few modifications.

Polyacrylamide gel preparation. Polyacrylamide gel for SSCP was prepared by dilution of the stock solution, 30% acrylamide solution (29:1(w/w) of acrylamide to N,N´-methylene-bisacrylamide) to 10% acrylamide solution with 10X TBE buffer containing 50% glycerol. To set a SSCP gel (7 cm x 8 cm x 0.75 mm in size), 5 ml of 12% acrylamide solution was added with 67 μl of freshly prepared 10% ammonium persulfate and 10 μl of N,N,N´N´-tetramethyl-ethylenediamine (TEMED). The gel solution mixture was immediately poured between the glass plates, using a syringe until the glass plate sandwich was full; the comb was placed into the assembled gel sandwich. The gel was left to polymerize for at least 45 minutes at room temperature. After the gel was cast, it was left at 4ºC for at least 5 hours before loading samples.

Electrophoresis. Before loading samples, the gel was pre-electrophoresed for at least 1 hour at 100 volts to remove gel polymerization reactant. Then, an aliquot (3-4μl) of each PCR product was

mixed thoroughly with 2 volumes of formamide loading dye (95% formamide, 0.5 M EDTA, 0.05% bromophenol blue and 0.05% xylene-cyanol FF). The mixtures were denatured at 95ºC for 10 minutes, and immediately cooled on ice before loading onto the gel. Electrophoresis was then carried out using a Mini-PROTEAN II electrophoresis cell (BIO-RAD), in 0.5 X TBE buffer at 200 volts, 4ºC for 8-10 hours depending on the size of the DNA fragment being analyzed. The SSCP patterns were visualized by silver staining.

Silver staining. Silver staining was performed according to the protocol of AmpliFLP™ D1S80 PCR Amplification Kit (Perkin-Elmer) with a few modifications. After electrophoresis, the gel was fixed with 10% ethanol for 10 minutes, and then soaked in 160 mM HNO_3 for 5 minutes. Subsequently, the gel was rinsed twice with distilled water before soaking in staining solution containing 0.2% silver nitrate ($AgNO_3$) for 20 minutes. After staining, the gel was rinsed with distilled water and the chilled developer solution (3% sodium carbonate, 0.05% formaldehyde) was added. The gel was agitated well until all bands became visible. The developing reaction was terminated by adding stop solution (10% glacial acetic acid) directly to the developer solution. Then, the gel was incubated for 5 minutes and rinsed twice with distilled water, and dried between two sheets of cellophane film for permanent record.

DNA sequencing

The 542-bp PCR product of the *rpoB* gene containing the sequence of the 347-bp *rpoB* gene was used as the template for DNA sequencing. The PCR products for direct DNA sequencing were purified to separate the unincorporated nucleic acid and primers from amplified DNA by filtration with the QIA quick purification kit (Qiagen, USA). Cycle sequencing reaction was done using a BigDye™ terminator cycle sequencing ready reaction kit according to the protocol provided by the manufacturer, with some modifications. The reaction in a total volume of 10 μl was composed of 4 μl of terminator ready mix, 300-500 ng double-stranded DNA template, 10 pmole of sequencing primer. The reaction was denatured at 96ºC for 3 minutes and processed for 25 cycles, as follows: 96ºC for 10 seconds of denaturation, 50ºC for 5 seconds of annealing, and 50ºC for 4 minmutes of extension. After cycle sequencing, the Centri-sep spin columns (Applied Biosystems) were used to separate the unincorporated dye terminators and primers from the extension products. The products were dried in a vacuum centrifuge, resuspended in loading buffer, heat-denatured for 2 minutes at 90ºC, and immediately loaded onto 4% acrylamide gel in an automated DNA sequencer (Applied Biosystems Prism 377; Perkin Elmer Corp).

RESULTS

PCR amplification of the 16S rRNA of MAC strains

All 20 *Mycobacterium avium* complex (MAC) strains were confirmed by 16S rRNA amplification using the primer Mygen-for and Mycav-rev. The PCR product produced a single band with the expected size at 189 bps.

PCR amplification and sequence analysis of the *rpoB* gene of MAC strains

All 20 MAC strains produced a strong single-band 542-bp PCR product of the *rpoB* gene, starting at the Leu-499 codon and ending at the Ser-667 codon, which was used as the DNA sequencing template. By increasing the chance of finding new sequence-altered codon positions, this longer PCR allowed us to find unreported point mutations at positions outside the regions of the *rpoB* gene. Specifically, we found unreported point mutation at position Lys-626 (Thr) and 15 different silent mutations at positions Leu-546, Val-550, Gly-556, Glu-565, Gly-566, Pro-567, Ser-574, Tyr-578, Aeg-580, Val-581, Gly-585, Gly-597, Thr-600, Ala-630, and Ala-667 (Table 2).

Our DNA sequence analysis of the 20 RIF^r MAC strains revealed that 5 of the 20 strains (25%) had a silent mutation at only one position, 7 (35%) at 2 positions, 7 (35%) at 3 positions, and 1 (5%) at 6 positions.

For the RIF^r MAC strains that carried a mutation, the silent mutation at the Ala-630 codon occurred in the largest proportion of the strains (15 strains, 75%), followed by the Val-581 in 8 strains (40%), Tyr-578 and Thr-600 in 4 strains (20%), and Gly-597 in 3 strains (15%) (Table 2).

SSCP for screening of the mutation in the *rpoB* gene of MAC strains

The 372 bp PCR products with *rpoB* gene mutation were amplified using MRB1-for and MRB2-rev and expressed a sharp, single band. Then, the 372 bp amplicon was analyzed by PCR-SSCP. A representative SSCP analysis is illustrated in Fig 1. The relative differences in single-strand DNA migration allowed for the discrimination of control strain (lanes 3 and 7) and the rifampicin-resistant strains (lanes 1, 2, 4, 5, 6, 8, 9), and as expected, the effect of each particular mutation type yielded different SSCP patterns. The PCR product from RIF^r MAC strain with silent mutation also showed migration shift by SSCP. For example, RIF^r

Table 2
Codon positions of mutations in 20 *Mycobacterium avium* complex RIF r strain from AIDS patients in Thailand.

No	Codon #	Nucleotide Mutation	Type	Amino acid changes	Mutation frequency (%)
1	546	GAg --> GAa	Silent	Leu --> Leu	2 (10)
2	550	GTg --> GTa	Silent	Val --> Val	1 (5)
3	556	GGc --> GGg	Silent	Gly --> Gly	2 (10)
4	565	GAg --> GAa	Silent	Glu --> Glu	1 (5)
5	566	GGt --> GGc	Silent	Gly --> Gly	1 (5)
6	567	CCc --> CCg	Silent	Pro --> Pro	1 (5)
7	574	TCg -->TCa	Silent	Ser -->Ser	2 (10)
8	578	TAt --> TAc	Silent	Tyr --> Tyr	4 (20)
9	580	cGG -->aGG	Silent	Arg --> Arg	1 (5)
10	581	GTc --> GTg	Silent	Val --> Val	8 (40)
11	585	GGg --> GGc	Silent	Gly --> Gly	1 (5)
12	597	GGc --> GGa	Silent	Gly --> Gly	3 (15)
13	600	ACC --> ACt	Silent	Thr --> Thr	4 (20)
14	626	AaG --> AcG	Substitution	Lys --> Thr	1 (5)
15	630	GCg --> GCc	Silent	Ala --> Ala	15 (75)
16	667	GCg --> GCc	Silent	Ala --> Ala	1 (5)

E. coli numbering system for the β-subunit of the RNA polymerase (Ovchinnikov *et al*, 1981).

MAC strains with silent mutation at codon Ala-630 (GCg to GCc) (lane 2) produced a migration shift compared with that of control strain (wild type).

From our result using PCR-SSCP, when applied to the 572 bp PCR product, it could not differentiate between the SSCP pattern of RIF r MAC strain with *rpoB* gene mutation and the control strain (wild type).

DISCUSSION

Rifampicin resistance has been shown to result from mutation in the *rpoB* gene, which encodes the beta-subunit of RNA polymerase (Telenti *et al*, 1993). More than 95% of RIF r strains are associated with mutations within the 81-bp region (codon 507-533), namely, the rifampicin-resistant determining region (RRDR) (Herrera *et al*, 2003).

To study the relationship between RIF r phenotype and *rpoB* gene mutation, PCR-DNA sequencing and PCR-SSCP techniques were utilized by amplifying part of the *rpoB* gene using PCR; then, the PCR product was sequenced and compared with that of the reference strain (wild type).

The patterns of frequencies of *rpoB* mutations at the different positions were different from those reported in 3 previous studies of RIF r MAC strains (Guerrero *et al*, 1994; Williams *et al*, 1994; Gomez

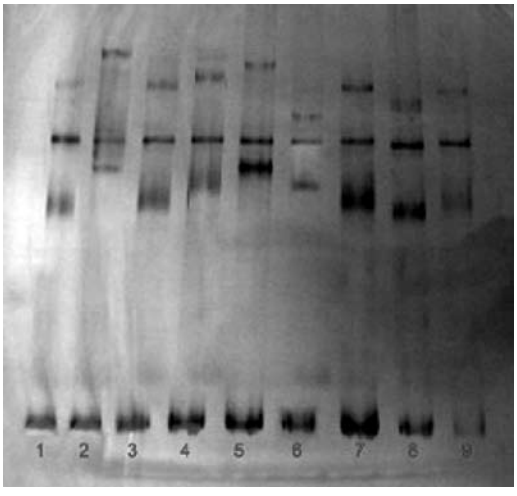

Fig 1- Photography of SSCP pattern among selected RIF r *Mycobacterium* strains, with *rpoB* gene mutation compared with control strain (wild type). Lane 1: the RIF r strain has silent mutation at Gly-556, Tyr-578; lane 2: the RIF r strain has silent mutation at Thr-600; lane 3: control strain (wild type); lane 4: the RIF r strain has silent mutation at Tyr-578; lane 5: the RIF r strain has silent mutation at Ser-574, Gly-597; lane 6: the RIF r strain has silent mutation at Val-550, Gly-556, Arg-580; lane 7: control strain (wild type); lane 8: the RIF r strain has silent mutation at Val-581; lane 9: the RIF r strain has silent mutation at Glu-565, Gly-566, Pro-567, Val-581.

Table 3
Comparison of mutations in *Mycobacterium avium* complex RIF^r strains in four reports and this study.

rpoB mutation position (based on *rpoB* gene of *E.coli* numbering system)	Frequency of codon substitution or silent mutation			
	Guerrero *et al*, 1994 (n=15)	Williams *et al*, 1994 (n=4)	Gomez *et al*, 1995 (n=20)	This study (n=20)
Pro-497 (silent)	4 (26.8)	-		-
Val-581 (silent)	-	-		3 (15)
Thr-600 (silent)	-	-		2 (10)
Ser-531 (Trp/Leu)	-	2 (50)		-
Glu-546, Val-581 (silent)	-	-		2 (10)
Ser-574, Gly-597 (silent)	-	-		2 (10)
Tyr-578, Ala-630 (silent)	-	-		3 (15)
Val-581, Gly-585 (silent)	-	-		1 (5)
Val-581, Gly-597 (silent)	-	-		1 (5)
Thr-600, Ala-630 (silent)	-	-		1 (5)
Gly-566, Tyr-578, Ala-630 (silent)	-	-		1 (5)
Val-550, Gly-556, Arg-580, Ala-630 (silent)	-	-		1 (5)
Ala-500, Leu-511, Gly-523, Leu-524, Arg-540 (silent)	-	-		1 (5)
Glu-565, Gly-566, Pro-567, Val-581, Ala-667 (silent) Lys-626 (Thr)	-	-		1 (5)
Arg-496, Pro-497, Val-499, Ala-500, Ser-512, Ser-522, Gly-523, Leu-524, Leu-530, Pro-535, Gly-536, Arg-540(silent)	4 (26.7)	-		-
Arg-496, Pro-497, Val-499, Ala-500, Ser-512, Ser-522, Leu-524, Arg-528, Arg-529, Leu-530, Pro-535, Arg-540(silent)	2 (13.35)		-	-
Arg-496, Pro-497, Val-499, Ala-500, Ser-512, Ser-522,Leu-524, Arg-528, Arg-529, Gly-534, Pro-535, Arg-540(silent) Asn-494 (Ser)	1 (6.67)	-		-
No mutation	3 (20)	2 (50)	20 (100)	1 (5)

E. coli numbering system for the β-subunit of the RNA polymerase (Ovchinnikov *et al*, 1981).

et al, 1995), which found that the *rpoB* mutations in RIF^r MAC strains were single amino acid substitutions (single point), at position Asn-494 (Ser), Ser-531 (Trp/Leu) and silent mutation at Arg-496, Pro-497, Val-499, Ala-500, Leu-511, Ser-512, Ser-522, Gly-523, Leu-524, Arg-528, Arg-529, Leu-530, Gly-534, Pro-535, Gly-536, and Arg-540 (Guerrero *et al*, 1994; Williams *et al*, 1994) (Table 3).

All 20 (100%) of the RIF^r MAC strains had nucleotide change outside the 81-bp core region (RRDR) of the *rpoB* gene, which contradicts the results of the previous studies of RIF^r MAC strains (Guerrero *et al*, 1994; Williams *et al*, 1994), where all strains had nucleotide change inside the 81-bp core region (RRDR) of the *rpoB* gene.

All 20 strains (100%) of the RIF^r MAC strains had

silent mutation, which was similar to the reports from a previous study of 70 RIF ^{r} *M. tuberculosis* strains from Thailand (Ramasoota *et al*, 2006), where 85.7% of the strains had silent mutations at Gln-517 position; moreover, one RIF^{r} strain had silent mutations at 29 positions. Silent mutation at position Gln-517 was also reported from the study of RIF ^{r} *M. tuberculosis* strain from Australia, silent mutation may have a role in low-frequency genetic alteration in the *rpoB* gene of RIF ^{r} *M. tuberculosis* (Sintchenko *et al*, 1999).

Even though silent mutations do not change amino acid encoding, they can create a "codon bias" effect; most of the 20 amino acids are encoded by more than one codon, and normally the highly expressed genes have more frequently used codons. But silent mutation is a low-frequency used codon; therefore, in a highly expressed gene, changing from a frequently used codon to a "low-frequency used codon" can result in phenotypic changes, which can happen under selection pressure, such as antibiotic use (http://bioweb.usc.edu/course/2000-spring/documents/bisc313-ch18.pdf).

Moreover the mechanism related to RIF ^{r} in MAC strain may derive from other possible factors, not only the mutation in the identified *rpoB* region, at the positions reported. First there may be other areas of the *rpoB* gene in which mutations may confer resistance; mutations in other subunits of the RNA polymerase may contribute to rifampicin resistance (Hetherington *et al*, 1995). Second, other possible mechanisms of drug resistance, such as decreased antibiotic entry into the bacterial cell or the exclusion of the antibiotic due to impermeability of the cell wall (Lambert, 2002), drug efflux or enzymatic degradation, or modification of rifampicin such as ribosylative inactivation (Quan *et al*, 1997), may be involved.

PCR-SSCP is a useful technique for detecting single nucleotide substitution in genetic disease. Several investigators have reported on the utility of the PCR-SSCP method for screening mutation in the *rpoB* gene of *M. tuberculosis* associated with rifampicin resistance (Telenti *et al*, 1993; Felmlee *et al*, 1995).

When the 372 bp amplicon with *rpoB* gene mutation was analyzed by PCR-SSCP, all kinds of mutation could be detected, by presenting the mobility shift for all strains with nucleotide substitution (silent mutation) compared with the control strain.

When a silent mutation and a missense mutation occur and causes rifampicin resistance, then the PCR-SSCP results presenting a mutation correctly predict rifampicin resistance. But, if any isolated rifampicin-susceptible strain had a silent mutation, it would cause false positive PCR-SSCP results. As reported by Kim and colleagues (1997), 2 phenotypically susceptible strains with silent mutation at Gln513 and deletion mutation of 508 and Ser 509 showed different PCR-SSCP patterns from the reference strain.

When the 542 bp amplicon with *rpoB* gene mutation was analyzed by PCR-SSCP, the PCR-SSCP pattern did not differ from the control strain. Although PCR-SSCP has proven to be a simple and effective method for detecting single base substitutions, it has limitations in detection from a large DNA product. The sensitivity of PCR-SSCP tends to decrease as DNA product length increases. The originator of the method suggested a maximum analyzable size of 430 bp (Orita *et al*, 1989). For optimal results, DNA fragment size should be < 400 bp (Markoff *et al*, 1997). A sequence longer than 400 bp should be divided into shorter segments before analysis by SSCP. This can be done by generating overlapping sub separately or by amplifying the intact fragment and then digesting it with restriction enzyme (Markoff *et al*, 1997).

PCR-SSCP detects only the presence, not the type, of mutation. In this regard, PCR-SSCP analysis should be supported by DNA sequencing, and cumulative data concerning the relationship between the phenotype and types of mutations are required.

Further work with the *rpoB* gene can help to explain more precisely the mechanism of RIF^{r} in MAC strain. For this we need more sequence data and more understanding of the detail of the structure-function relationships for the RNA polymerase β-sub-unit, which are possible using X-ray crystallographic and other techniques, such as DNA micro-array. This entails further investigation of the RIF^{r} MAC strain with larger sample sizes, and with samples taken from different geographic regions.

ACKNOWLEDGEMENTS

We acknowledge, with thanks, financial support from the Thailand Tropical Diseases Research Program (T2) and Faculty of Tropical Medicine, Mahidol University, Thailand.

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