

MUTATIONS IN THE *GYRA* AND *GYRB* GENES OF FLUOROQUINOLONE-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* FROM TB PATIENTS IN THAILAND

Pannamthip Pitaksajjakul¹, Waranya Wongwit¹, Wantanee Punprasit², Boonchuy Eampokalap³, Sharon Peacock⁴ and Pongrama Ramasoota¹

¹Faculty of Tropical Medicine, Mahidol University, Bangkok; ²Faculty of Public Health, Mahidol University, Bangkok; ³Bamrasnaradura Infectious Disease Institute, Ministry of Public Health, Nonthaburi; ⁴Wellcome Trust-Mahidol University-Oxford Tropical Medicine Research Program, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

Abstract. Among fluoroquinolone-resistant *Mycobacterium tuberculosis* (FQ^r-MTB) isolates, mutation at positions 90, 91, and 94 in *gyrA* gene and at positions 495, 516, and 533 in *gyrB* gene have been frequently reported. In this study, 35 isolates of FQ^r-MTB were collected from Siriraj Hospital and Chest Disease Institute. The quinolone-resistance-determining regions (QRDR) of *gyrA* and *gyrB* genes in all 35 FQ^r-MTB isolates and from the H37Ra MTB strain were amplified using polymerase chain reaction (PCR). DNA-sequencing and single-strand conformation polymorphism (SSCP) were further utilized for characterization of the mutations in the QRDR of *gyrA* and *gyrB* genes and mutation screening, respectively. From DNA-sequencing, 21 of 35 (60%) exhibited single-point mutations in different positions, at Ala90Val, Ser91Pro, and Asp94(Gly/Ala/His/Asn); and one novel mutation position at Gly88Cys in the *gyrA* gene and Asp495Asn in the *gyrB* gene. These positions were previously frequently reported to be responsible for FQ^r-MTB. The other 14 FQ^r-MTB isolates (40%) had no mutation. This study is the first report of mutation occurring only in the QRDR of the *gyrB* gene, without prior mutation in the *gyrA* QRDR among FQ^r-MTB isolates. By SSCP analysis for screening of the mutant FQ^r-MTB, the SSCP patterns of mutated FQ^r-MTB isolates were clearly differentiated from the SSCP patterns of FQ^s-MTB.

INTRODUCTION

Until recently, the association of TB with the AIDS pandemic has been leading to increased fatalities rates and the emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis* (Musser, 1995; Bifani *et al*, 1996; Fang *et al*, 1999; Hirano *et al*, 1999; Sintchenko *et al*, 1999). Fortunately, an increasing number of reports of MDR-TB alert the world that health and development will be jeopardized if action is not accelerated immediately. For these reasons and others, immediate prevention of MDR-TB strains is of utmost importance. These MDR-TB strains are very dangerous, not only because patients infected with these strains are difficult to treat and become increasingly difficult to treat the longer the clones are allowed to evolve within individuals, but also, in the community, the disease can spread to the other people the longer it remains undetected within individuals. To treat MDR-TB cases, second-line drugs have been used; fluoroquinolone (FQ) is one of them. The widespread use of this drug has led to the marked emergence of fluoroquinolone-resistant MTB (FQ^r-

MTB) in many countries (Alangaden *et al*, 1995; Xu *et al*, 1996; Siddiqi *et al*, 2002), including Thailand (Prachartam *et al*, 2001).

The mechanism of resistance to quinolone in *Mycobacterium* spp has been associated with mutations in the DNA gyrase, a tetrameric protein composed of two A and two B subunits. After Takiff *et al* (1994) first found mutations in the *gyrA* and *gyrB* genes of MTB, intensive attempts have been made to find sequence alterations in the *gyrA* and *gyrB* genes of MTB (Cambau *et al*, 1994; Alangaden *et al*, 1995; Kocagoz *et al*, 1996; Guillemain *et al*, 1998; Zhou *et al*, 2000; Onodera *et al*, 2001). The most frequently encountered mutations in quinolone-resistant *Mycobacterium* strains are concentrated in a conserved region of the *gyrA* and *gyrB* genes, referred to as the quinolone-resistance-determining region (QRDR) in the A and B subunits. For the A subunit, mutations were frequently found at amino acids positions (*M. tuberculosis* numbering system: Takiff *et al*, 1994): Ala90→Val, Ser91→Pro, and Asp94→His/Tyr. For the B subunit, mutations were frequently found at amino acids positions: Asp495→His/Asn, Arg516, and Asn533. For this reason, the detection of a missense mutation in the hot spot region at positions 90, 91, and 94 in *gyrA*; and at positions 495, 516, and 533 in *gyrB* is a rapid and efficient test for the molecular detection

Correspondence: Pongrama Ramasoota, Faculty of Tropical Medicine, Mahidol University, 420/6 Rajwithii Road, Bangkok 10400, Thailand.
E-mail: tmprpt@mahidol.ac.th

of FQ^r-MTB. These mutations can be detected, directly or indirectly, by different molecular techniques, such as polymerase chain reaction (PCR)-DNA sequencing, and PCR-single-strand conformation polymorphism (SSCP) (Sougakoff *et al.*, 1997).

In this study, mutation in the QRDR of the *gyrA* and *gyrB* genes of FQ^r-MTB isolates from Thailand were studied using PCR-DNA sequencing and simple mutation detection technique of PCR-SSCP, respectively.

MATERIALS AND METHOD

Fluoroquinolone-resistant *M. tuberculosis* isolates

A total of 35 FQ^r-MTB isolates were collected from Siriraj Hospital (16 isolates) and the Chest Disease Institute (19 isolates). All isolates were previously tested for drug susceptibility (including ofloxacin) by proportion and absolute concentration methods.

Preparation of chromosomal DNA from *M. tuberculosis* isolates

For each strain, one loopful of bacteria, cultured on Lowenstein-Jensen medium, was transferred to a 2.0 ml conical tube containing 0.5 ml of sterile water. Each tube was then vortexed for one minute and frozen at -80°C for 30 minutes before being incubated at 95°C for 5 minutes. Freezing and thawing was repeated three times. One hundred µl of lysis buffer [0.5% SDS, 20 mg/ml Proteinase-K, and Tris-EDTA (pH 8)] was added to the solution that was later incubated overnight at 37°C. One hundred µl of 5M NaCl was added and mixed thoroughly, followed by the addition of 80 µl of CTAB/NaCl solution that had been prewarmed at 65°C for 5 minutes. The tubes were inverted until the liquid content became white (milky). They were then incubated at 65°C for 10 minutes. Eight hundred µl of chloroform/isoamyl alcohol (24:1) was added to each tube and mixed well, followed by centrifugation at 13,000 rpm for 5 minutes. The viscous upper phase was then removed and transferred to a new tube. Eight hundred µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added, mixed well, and spun for 5 minutes. To precipitate the DNA, the upper aqueous phase was transferred to new tubes, and 450 µl of isopropanol was added to each. The solution was then incubated at -20°C for three hours. After that, the tubes were centrifuged at 13,000 rpm for 15 minutes, and the supernatant was discarded. The resulting DNA pellets were then washed with 1 ml of cold 70% ethanol to remove residual CTAB. The tubes were centrifuged at 13,000 rpm for 5 minutes, the supernatant was discarded, and the pellets were dried. Afterwards, each resulting purified DNA pellet was dissolved in 50 µl

of sterile distilled water for further analysis. The purity and concentration of DNA were then measured by spectrophotometer (Beckman, model DU 7500) at 260 nm and 280 nm, respectively.

PCR procedures for gyrase gene amplification

Aliquots of purified *M. tuberculosis* DNA were added (Kocagöz *et al.*, 1996) to a PCR reagent, and to amplify a 320 bps region of *gyrA*, primers GyrA-f (5'-CAGCTACATCGACTATGCGA-3') and GyrA-r (5'-GGCTTCGGTGTACCTCAT-3') were used, and, to amplify a 429 bps fragment of *gyrB*, primers GyrB-f (5'-CCACCGACATCGGTGATT-3') and GyrB-r (5'-CTGCCACTTGAGTTTGTACA-3') were used. The 50 µl of PCR mixture contained 12.5 mM KCl, 12.5 mM (NH₄)₂SO₄, 25 mM Tris-HCl, 2.5 mM MgSO₄, 0.4 mM (each) deoxynucleoside triphosphate, 0.5 µM (each) primer, 200 ng of DNA lysate, and 2 U Taq DNA polymerase (NEB, New England). The reaction mixtures were then put in the thermal cycler (Perkin-Elmer 2400, Applied Biosystem, USA) that carried out the following PCR programs: (1) for the *gyrA* primer, 95°C for 3 minutes, 95°C, 58°C and 72°C (1 minute each), for 2 cycles, 95°C, 57°C and 72°C (1 minute each), for 2 cycles, 95°C, 56°C and 72°C (1 minute each), for 2 cycles, 95°C, 55°C and 72°C (1 minute each), for 25 cycles with a final 5 minutes extension step at 72°C; and (2) for the *gyrB* primer, 95°C for 3 minutes, 95°C, 69°C and 72°C (1 minute each) for 2 cycles, 95°C, 68°C and 72°C (1 minute each) for 2 cycles, 95°C, 67°C and 72°C (1 minute each) for 2 cycles, 95°C, 66°C and 72°C (1 minute each) for 2 cycles, 95°C, 65°C and 72°C (1 minute each), for 30 cycles, with a final 5 minutes extension step at 72°C.

The amplified PCR products were stained with GelStar [4:96/ Gel Star (BMA, Rockland, ME, USA): DMSO] for 15 minutes and mixed with a loading dye (0.09% bromophenol blue, 0.09% xylene cyanol FF, 60% glycerol, and 60 mM EDTA) before being loaded into 1.5% agarose gel (SeaKem[®] LE agarose, Rockland, ME USA). The electrophoresis was performed in 0.5% Tris-borate-EDTA buffer (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA-disodium) for 40 minutes at 100 V at room temperature. The gels were visualized on a Dark Reader Transilluminator (Model DR-45M, BMA, Rockland, ME, USA).

DNA sequencing

The PCR products for direct DNA sequencing were purified to separate the unincorporated nucleic acid and primers from the amplified DNA by filtration with a Nucleospin extract kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). Sequencing reactions, with a DNA sequencing kit (dye terminator cycle

sequencing ready reaction; Applied Biosystem, Foster City, CA, USA), were performed with 5 µl of purified PCR product and 3.2 pmol of the GyrA-for and GyrB-for primer. 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 a loading buffer, heat-denatured for 2 minutes at 90°C, and immediately loaded onto a 4% acrylamide gel in an automated DNA sequencer (Applied Biosystems Prism 377; Perkin Elmer Corp).

Screening of mutation by single strand conformation polymorphism (SSCP)

SSCP analysis was performed according to the method described by Bassam *et al* (1991), with a few modifications.

Polyacrylamide gel preparation. A polyacrylamide gel for SSCP was prepared by diluting the stock solution, 30% acrylamide solution (29:1(w/w) of acrylamide to N,N'-methylene-bisacrylamide), to 12% acrylamide solution with a 10X TBE buffer containing 50 % glycerol. To set a SSCP gel (7 cm x 8 cm x 0.75 mm in size), 8 ml of 12% acrylamide solution was added, with 67 µl of freshly prepared 10 % ammonium persulfate and 20 µl of N,N,N',N'-tetramethylethylenediamine (TEMED). The gel solution mixture was immediately poured between glass plates by 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 4°C for at least 5 hours before loading the samples.

Electrophoresis. Before loading the samples, the gel was pre-electrophoresed for at least 1 hour at 100 volts in order to remove gel polymerization reactant. After that, 8 µl 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 95°C for 15 minutes and immediately cooled on ice before loading onto the gel. Electrophoresis was then carried out using a Mini-Vertical Unit (Model TV100, Scie-Plas, England) in 0.5 X TBE buffer at 40 mA, and at 4°C for 8-10 hours, depend 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 the AmpliFLP™ D1S80 PCR Amplification Kit (Perkin-Elmer), with a few modifications. After electrophoresis, the gel was fixed

with 10% ethanol for 10 minutes and was then soaked in 160 mM HNO₃ for 5 minutes. Subsequently, the gel was rinsed twice with distilled water before soaking in a staining solution containing 0.2% silver nitrate (AgNO₃) for 20 minutes. After staining, the gel was rinsed with distilled water, and a 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 a stop solution (10% glacial acetic acid) directly to the developer solution, and the gel was incubated for 5 minutes. The gel was rinsed twice with distilled water and dried between two sheets of cellophane film for a permanent record.

RESULTS

Amplification of *gyrA* and *gyrB* QRDR using PCR

Using PCR with touch-down programs and primers that were described earlier, all of 35 fluoroquinolone-resistant *Mycobacterium tuberculosis* (FQ^r MTB) isolates and one fluoroquinolone susceptible strain (*M. tuberculosis* H37Ra standard strain ATCC 25177) produced a strong single band of 320 bps PCR products of *gyrA* gene, and 429 bps PCR products of *gyrB* gene (Fig 1). These amplicons included quinolone-resistance-determining regions (QRDRs) of both *gyrA* and *gyrB* gene, respectively. Among all 35 FQ^r MTB isolates, both PCR products were further used as a template for DNA-sequencing and single strand conformation polymorphism (SSCP), respectively.

Mutation in *gyrA* and *gyrB* QRDR by DNA-sequencing

Thirty-five FQ^r MTB isolates were analyzed. The QRDRs of *gyrA* and *gyrB* genes were sequenced after amplification by PCR to analyze the mutations associated with fluoroquinolone resistant (FQ^r). The sequence analysis of the QRDRs of *gyrA* and *gyrB* genes in FQ^r MTB isolates revealed that the mutation positions were the same as those of previous reports (Siddiqi *et al*, 1998, 2002; Kocagöz *et al*, 1996). These altered codons, at 88, 90, 91, and 94, have been found within the hotspot region of *gyrA*, as shown in Table 2. Twenty-one of 35 isolates (60%) have mutations in the *gyrA* hotspot region, and 14 isolates (40%) were found to have no mutation, as shown in Tables 1 and 3.

All 35 FQ^r MTB isolates were also checked for mutations in the *gyrB* locus that is associated with low levels of resistance. Only one FQ^r MTB isolate (2.9 %) contained a mutation within the 429 bps *gyrB* PCR product tested that is the substitution of Asp (495) by Asn (Tables 2 and 3).

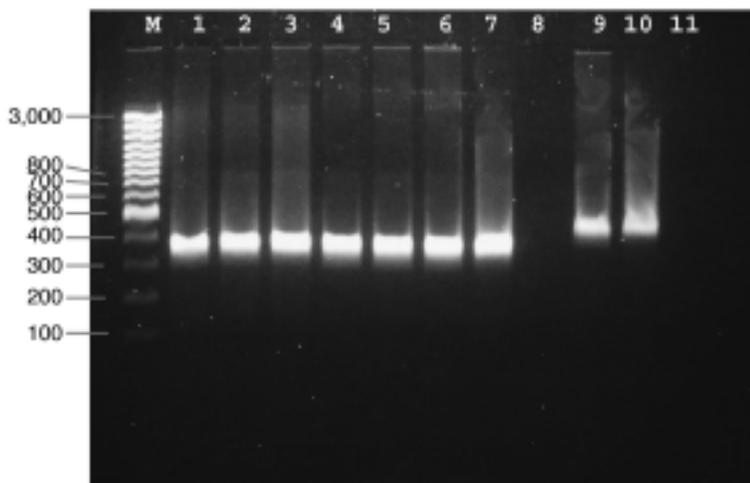


Fig 1- PCR amplification of 320 bps of *gyrA* gene and 429 bps of *gyrB* gene. Lane M, molecular sized marker (100 bp DNA ladder plus); lane 1-6, 320 bps *gyrA* PCR product from FQ^R-MTB; lane 7, 320 bps *gyrA* positive control; lane 8, *gyrA* negative control; lane 9, 429 bps *gyrB* PCR product from FQ^R-MTB; lane 10, *gyrB* positive control; lane 11, *gyrB* negative control.

Table 1
Point mutations in *gyrA* QRDR (from codons Ser27 to Gly132) of 22 ofloxacin resistant *M. tuberculosis* isolates from TB patients in Thailand.

Codon	Nucleotide mutation	Type	Amino acid change	Mutation frequency (isolates)/(%)
No mutation	-	-	-	14 (40)
88	GGC→TGC	Substitution	Gly→Cys	1 (2.8)
90	GCG→GTG	Substitution	Ala→Val	5 (14.3)
91	TCG→CCG	Substitution	Ser→Pro	2 (5.7)
94	GAC→GGC	Substitution	Asp→Gly	7 (20)
94	GAC→GCC	Substitution	Asp→Ala	2 (5.7)
94	GAC→CAC	Substitution	Asp→His	2 (5.7)
94	GAC→AAC	Substitution	Asp→Asn	2 (5.7)

Table 2
Point mutation in *gyrB* QRDR (from codons Thr467 to Leu 608) of 22 ofloxacin resistant *M. tuberculosis* isolates from TB patients in Thailand.

Codon	Nucleotide mutation	Type	Amino acid change	Mutation frequency (isolates)/(%)
No mutation	-	-	-	34 (97.1)
495	GAC→ AAC	Substitution	Asp→Asn	1 (2.9)

Considering the mutation positions of all mutant isolates, 13 isolates (61.9%) had mutations at position Asp 94 (7 isolates changed to glycine, 2 isolates

changed to alanine, 2 isolates changed to His, and the other 2 isolates changed to Asn); and 5 isolates (23.8%) contained mutations in codon 90, resulting in amino

Table 3

The mutation patterns in *gyrA* and *gyrB* QRDR of ofloxacin resistant *M. tuberculosis* isolates from TB patients in Thailand.

Isolates ^a	Drug resisted ^b	IS6110 ^c pattern	Gyrase subunit mutation	
			<i>GyrA</i>	<i>GyrB</i>
1(T2-CCI-0003)	RSO	NB	Asp94Gly(GAC→GGC)	-
2(T2-CCI-0016)	HO	H	-	-
3(T2-CCI-0021)	O	BJ	-	-
4(T2-CCI-0042)	O	SB	-	-
5(T2-CCI-0056)	HRO	SB	Ala90Val (GCG→GTG)	-
6(T2-SIMI-0072)	HRSO	BJ	-	-
7(T2-SIMI-0119)	HRSO	BJ	-	-
8(T2-SIMI-0186)	HRSEO	BJ	-	Asp495Asn(GAC→AAC)
9(T2-SIMI-0201)	HRSEO	BJ	-	-
10(T2-SIMI-0202)	HRSEO	BJ	-	-
11(T2-SIMI-0240)	HRSEO	SB	-	-
12(T2-SIMI-0333)	REO	BJ	-	-
13(T2-SIMI-0353)	HRSEO	SB	Asp94Gly(GAC→GGC)	-
14(T2-SIMI-0502)	HO	SB	-	-
15(T2-SIMI-0569)	HRSEO	FB	Asp94Gly(GAC→GGC)	-
16(T2-SIMI-0596)	HREO	SB	Asp94Gly(GAC→GGC)	-
17(CCI-1)	HRSKO	NA	Asp94Ala(GAC→GCC)	-
18(CCI-2)	SO	NA	Ser91Pro(TCG→CCG)	-
19(CCI-6)	HRSO	NA	Asg94Ala(GAC→GCC)	-
20(CCI-11)	HRSO	NA	Ala90Val (GCG→GTG)	-
21(CCI-13)	HRSO	NA	-	-
22(CDI-14)	HRSO	NA	Asp94Gly (GAC→GGC)	-
23(CDI-15)	HRSEO	NA	Asp94His (GAC→CAC)	-
24(CDI-16)	HRSKO	NA	Asp94Gly (GAC→GGC)	-
25(CDI-17)	HRO	NA	-	-
26(CDI-18)	HRSKO	NA	Ala90Val(GCG→GTG)	-
27(CDI-19)	HRO	NA	Asp94Gly(GAC→GGC)	-
28(CDI-20)	HRSEO	NA	Asp94Asn(GAC→AAC)	-
29(CDI-21)	HRSO	NA	Asp94Gly(GAC→GGC)	-
30(CDI-22)	HRSO	NA	Gly88Cys(GGC→TGC)	-
31(CDI-23)	HRSEO	NA	Ala90Val(GCG→GTG)	-
32(CDI-24)	HRSO	NA	Ser91Pro(TCG→CCG)	-
33(CDI-25)	HRO	NA	Asp94His(GAC→CAC)	-
34(CDI-26)	HSO	NA	Ala90Val(GCG→GTG)	-
35(CDI-67)	RO	NA	-	-

^a CCI = Central Chest Institute, Ministry of Public Health, SIMI = Dept Microbiology, Faculty of Science, Mahidol University

^b H = Isoniazid, R = Rifampicin, S = Streptomycin, E = Ethambutol, K = Kanamycin, O = Ofloxacin

^c BJ = Beijing, FB = Few Band, H = Heterogeneous, NB = Nonthaburi, SB = Single Band, NA= Did not test for the IS6110 pattern

acid substitution from alanine to valine. Two isolates (9.5%) had mutations at position Ser91 that were replaced by proline (Table 4). One (4.3%) novel mutation position was found in this study at Gly88 that change to Cys (Tables 1, and 3), and one isolate (4.5%) for *gyrB* mutation at Asp495.

Screening of mutant FQ^r MTB isolates by SSCP analysis

In this study, SSCP was used for the screening of mutations in *gyrA* and *gyrB* amplicons, from 35 FQ^r MTB isolates, and one FQ^s- MTB strain. By using 320 bps *gyrA* amplicons, five different SSCP patterns

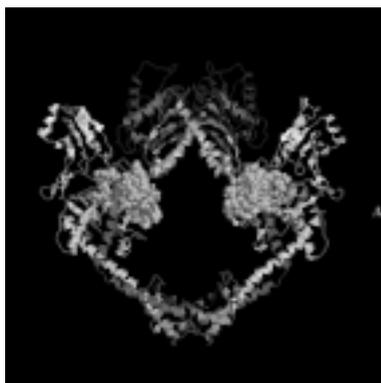


Fig 2- Crystal structure of the 92-kDa fragment of yeast topoisomerase II (rasmol software). A, region corresponding to GyrA; B, region corresponding to GyrB. Residues corresponding to quinolone resistant determining regions (QRDRs) on GyrA are in space-filling format. (adapted from Heddle and Maxwell, 2002).

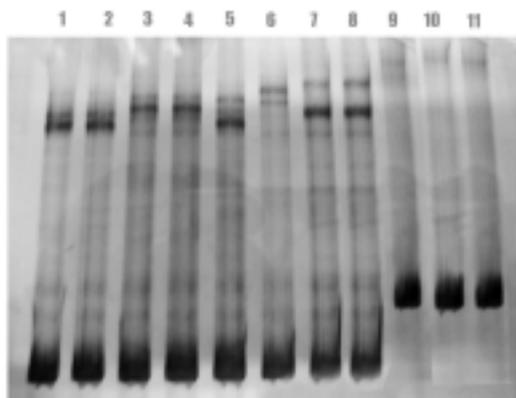


Fig 3- PCR-SSCP of 320 bps of *gyrA* and 429 bps of *gyrB* in 11 isolates of FQ^r-MTB and wild type. Lanes 1-2, mutation with Asp94Gly; lanes 3-4, mutation with Asp94Ala; lane 5, mutation with Ala90Val; lane 6, mutation with Ser91Pro; lane 7, FQ^r-MTB without mutation; lane 8, wild type of *gyrA* amplicons; lane 9, mutation with Asp495Asn; lane 10, FQ^r-MTB without mutation; lane 11, wild type of *gyrB* amplicons.

were obtained (Fig 3). The SSCP patterns of *gyrA* FQ^r MTB isolates were clearly differentiated from that of the control strain, based on the migration pattern of a single strand of DNA through the polyacrylamide gel. Among the *gyrA* amplicons with different mutation positions, at codons 90, 91, and 94, different SSCP patterns were identified. Moreover, among the *gyrA* amplicons with mutations at codon 94 that had different amino acid substitutions (2 isolates changed to Gly

and 2 isolates changed to Ala), the SSCP patterns were also different. Additionally, mutation screening in *gyrB* amplicons was also observed. However, by using 429 bps *gyrB* amplicons, the SSCP pattern among *gyrB* mutant (FQ^r MTB) isolates and fluoroquinolone susceptible (FQ^s) strain were not different. For each FQ^r MTB isolate with a mutation and FQ^s MTB strain, the SSCP patterns were indistinguishable in repeated analyses (data not shown).

DISCUSSION

As mentioned by several researchers (Sanders, 1988; Kaatz *et al*, 1991; Piddock and Zhu, 1991; Cambau and Gutmann, 1993; Denis and Moreau, 1993), the alteration of DNA gyrase and decreased levels of drug accumulation have been identified as two principal mechanisms of fluoroquinolone resistance in several species of bacteria. The results in this study indicated that similar mechanisms might be present in the FQ^r-MTB isolates that were tested in our study. Therefore, fluoroquinolone resistance is mainly due to alterations in the DNA gyrase, the unique type II topoisomerase of mycobacteria (Takiff *et al*, 1994; Alangaden *et al*, 1995; Kocagöz *et al*, 1996). These alterations are substitutions in the quinolone-resistance-determining region (QRDR) of the *gyrA* gene (regions 74 to 113 by the *M. tuberculosis* numbering system) and in regions 495 to 533 of the *gyrB* gene (*M. tuberculosis* numbering system) (Dauendorffer *et al*, 2003). The PCR primers used in this study were designed to flank the QRDR of the *gyrA* and *gyrB* genes, respectively (Kocagöz *et al*, 1996).

In this study, we have presented molecular genetic analysis for detecting FQ^r-MTB isolates from TB patients in Thailand, using PCR- DNA sequencing and PCR-SSCP. From the study of the occurring mutations in QRDR of *gyrA* in all of 35 FQ^r-MTB isolates by DNA-sequencing, the results revealed that the mutations at codons Ala90, Ser91, and Asp94 of the *gyrA* gene found in this study were also the most common position that have been frequently found and associated with FQ^r-MTB (Takiff *et al*, 1994; Alangaden *et al*, 1995; Kocagöz *et al*, 1996; Siddiqi *et al*, 1998, 2002). Among these mutation positions, from the mutant isolates, Asp94 was the most common mutation (13 of 21 isolates, 61.9%). The same most-common mutation position was also reported earlier by other researchers (Siddiqi *et al*, 2002; Cheng *et al*, 2004). A mutation position at Asp94 (Gly/Ala) has been reported, resulting in a higher level of fluoroquinolone resistance than that of a Ala90 Val mutation (Alangaden *et al*, 1995; Xu *et al*, 1996).

Moreover, one novel mutation at Gly88Cys was found in this study.

Mutations in the QRDR of the *gyrB* gene were also identified by DNA sequencing. One (2.9%) FQ^r-MTB isolate found mutation in this region, at codon Asp495Asn, that was the same position reported earlier (Kocagöz *et al*, 1996).

When comparing our finding mutation with that of the previous studies, the mutation positions in *gyrA* and *gyrB* QRDR of MTB in this study corroborated those of previous studies (Alangaden *et al*, 1995; Kocagöz *et al*, 1996; Siddiqi *et al*, 2002) in that most of the mutations occurred at the position Ala90, Ser91, and Asp94 of the *gyrA* gene, and at the position Asp495 of the *gyrB* gene (Tables 5 and 6).

Guillemin *et al* (1998) have reported that *gyrB* QRDR mutations at position Asp495, Arg516, and

Asn533 (*M. tuberculosis* numbering system) are positions conferring to FQ^r-MTB. However, from a previous study of FQ^r-MTB isolates, mutation in *gyrA* gene was considered to be the first step for clinically important levels of resistance, and mutation in *gyrB* gene was found to occur later, as the second step mutation with prior mutation in *gyrA* gene (Kocagöz *et al*, 1996). In contrast, in this study, one FQ^r-MTB isolate was found to have mutation only in *gyrB* QRDR at codon Asp495Asn, without prior mutation in *gyrA* gene. This was an unusual mutation pattern found in FQ^r-MTB, however, this finding corroborated a previous report (Heddle and Maxwell, 2002) of FQ^r *E. coli* in that FQ^r mutant isolates with *gyrB* mutation alone could yield two sets of fluoroquinolone resistant features: first, they show altered enzyme activity, and second, they show altered quinolone binding sensitivities. Heddle and Maxwell (2002) also reported that the QRDR of *gyrB*

Table 4
Point mutations in *gyrA* and *gyrB* QRDR among 22 ofloxacin resistant *M. tuberculosis* mutant isolates.

No	<i>GyrA</i> mutation	<i>gyrB</i> mutation	Mutation frequency strain (%)
1	Gly88Cys	-	1 (4.5%)
2	Ala90Val	-	5 (22.7%)
3	Ser91Pro	-	2 (9.1%)
4	Asp94Gly, Ala, His, Asn	-	13 (59.1%)
5	-	Asp495Asn	1 (4.5%)

Table 5
Comparison of *gyrA* QRDR mutations of ofloxacin resistant *M. tuberculosis* strains from three reports: Siddiqi *et al*, 2002; Kocagöz *et al*, 1996; Alangaden, 1995, and this study.

<i>gyrA</i> mutation	Frequency of codon substitution [no. (%) of isolation]			
	Siddiqi <i>et al</i> , 2002 (69strains)	Kocagöz <i>et al</i> , 1996 (13 strains)	Alangaden, 1995 (5 strains)	This study (35 strains)
No mutation	61 (88.48)	1 (7.69)	-	15 (42.8%)
Gly88Cys	-	-	-	1(2.8%)
Ala90Val	3 (4.34)	5 (38.46)	1 (20)	4 (11.4%)
Ser91Pro	1(1.44)	1 (7.69)	-	2(5.7%)
Asp94 Gly	4 (5.8)	-	1 (20)	7(20%)
Asp94Ala	-	-	-	2(5.7%)
Asp94His	-	1 (7.69)	-	2(5.7%)
Asp94Tyr	-	1 (7.69)	-	-
Asp94Asn	-	-	3 (60)	2(5.7%)
Ala90Val, Ser91Pro	-	1 (7.69)	-	-
Ala90Val, Asp94Gly	-	3 (23.1)	-	-

Table 6
Comparison of *gyrB* mutations of quinolone resistant *M. tuberculosis* strains from two reports (Siddiqi *et al*, 2002; Kocagoz *et al*, 1996), and this study.

GyrB mutation position	Frequency of codon substitution (no. (%) of isolation)		
	Siddiqi <i>et al</i> (2002)	Kocagöz <i>et al</i> (1996)	This study
No mutation	all	10(76.9)	34(97.1)
Asp495Asn	-	1(7.69)	1(2.9)
Asp495His	-	2(15.38)	-

gene has been described to involve the region of *gyrA* QRDR for complete quinolone-binding pocket formation as shown in Fig 3.

Among 35 FQ^r-MTB isolates tested, 13 isolates (37.14%) with a fluoroquinolone-resistant phenotype showed no mutations in both 320 bps region of *gyrA* and 429 bps region of *gyrB* gene. Which is similar to the study of Siddiqi *et al* (2002), as their results showed that 88.5% (61 of 69 strains) of FQ^r-MTB isolates lacked mutation in these regions. This can be explained in that the mutation in other areas outside the 320 bps region of *gyrA* and 429 bps region of *gyrB* gene might be responsible for FQ^r-MTB isolates in this study, as found in study of FQ^r *E coli* (Friedman *et al*, 2001). Moreover, the other mechanisms for a decreasing level of fluoroquinolone accumulation in the cell, as a result of either a decreased influx by cell wall permeability or a increased efflux by multidrug-mediate pump, may also be associated (Alangaden *et al*, 1995; Kocagöz *et al*, 1996).

Mycobacteria are intrinsically resistant to most common antimicrobial drugs, including fluoroquinolone. It may be due to their extremely low cell wall fluidity and permeability, as they contain a very complex lipid bilayer composed of mycolic acid residues (Liu *et al*, 1996). This kind of outer membrane limits the penetration of many hydrophilic antimicrobial agents, including ofloxacin (one kind of fluoroquinolone used in this study). Not only the low level of cell wall permeability, but also efflux-mediated resistance and efflux pumps, confer resistance to one or several compounds, as has also been described in mycobacteria (Liu *et al*, 1996; Takiff *et al*, 1996; Silva *et al*, 2001).

To identify the presenting mutations among FQ^r-MTB isolates, SSCP analysis was used. By using 320 bps *gyrA* amplicons, five different SSCP patterns found in this study were correlated with the specific mutation presented in FQ^r-MTB isolates and compared with the results from DNA-sequencing. The SSCP patterns between *gyrA* amplicons of FQ^r-MTB isolates and the

control strain were different, and also between FQ^r-MTB isolates with different mutation positions. Additionally, there were no differences in SSCP patterns between FQ^r-MTB isolates without mutations and the control strain. However, by using 429 bps *gyrB* amplicons, it failed to differentiate between the mutant isolates and control strain. This limitation may be due to our 429 bps *gyrB* amplicons that were larger than the optimal amplicon size recommended for this technique. To achieve high sensitivity of this technique, PCR product size is one of the most important factors. In general, the smaller the product, the higher the sensitivity, with the optimum being less than 400 bps (Markoff *et al*, 1997). For the longer PCR product, restriction enzymes can be used to yield a series of bands that can be assayed simultaneously. Moreover, the other factors, such as temperature during electrophoresis, concentration of acrylamide/bis-acrylamide, denaturant, glycerol concentration, and buffer concentration have all been shown to alter considerably ssDNA mobility (Chen *et al*, 1995). Therefore, further analysis for optimization of both the optimal ssDNA fragment size and other factors may provide better results and higher sensitivity for *gyrB* mutation screening by this technique. For its rapidity, simplicity, and high sensitivity of SSCP, it can be concluded that SSCP is a useful mutation screening technique. However, confirmation of the precise mutation position by DNA-sequencing is required.

ACKNOWLEDGEMENTS

This work was supported by the Thailand Tropical Disease Research Programme (T2) (Grant ID number: 02-2-TUB-02-031). We would also like to thank the BIOTEC for the student scholarship throughout the research.

REFERENCES

Alangaden GJ, Manavathu EK, Vakulenko SB, Zvonok NM, Lerner SA. Characterization of fluoro-

- quinolone-resistant mutant strains of *Mycobacterium tuberculosis* selected in the laboratory and isolated from patients. *Antimicrob Agents Chemother* 1995;39:1700-3.
- Bassam BJ, Caetano-Anolles G, Gresshoff PM. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal Biochem* 1991; 198: 217. Erratum in: *Anal Biochem* 1991;198:217.
- Bifani PJ, Plikaytis BB, Kapur V, *et al.* Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. *JAMA* 1996;275:452-7.
- Cambau E, Gutmann L. Mechanisms of resistance to quinolones. *Drugs* 1993;45 (suppl 3):15-23.
- Cambau E, Sougakoff W, Besson M, Truffot-Pernet C, Grosset J, Jarlier V. Selection of *gyrA* mutant of *Mycobacterium tuberculosis* resistant to fluoroquinolones during treatment with ofloxacin. *J Infect Dis* 1994;170:1351.
- Chen X, Baumstark T, Steger G, Riesner D. High resolution SSCP by optimizing of the temperature by transverse TGGE. *Nucleic Acids Res* 1995;23:4524-5.
- Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC. Multiplex PCR amplicon conformation analysis for rapid detection of *gyrA* mutations in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. *Antimicrob Agents Chemother* 2004;48:596-601.
- Dauendorffer JN, Guillemin I, Aubry A, *et al.* Identification of mycobacterial species by PCR sequencing of quinolone resistance-determining regions of DNA gyrase genes. *J Clin Microbiol* 2003;41:1311-5.
- Denis A, Moreau NJ. Mechanisms of quinolone resistance in clinical isolates: accumulation of sparfloxacin and of fluoroquinolones of various hydrophobicity, and analysis of membrane composition. *J Antimicrob Chemother* 1993; 32:379-92.
- Fang Z, Doig C, Rayner A, Kenna DT, Watt B, Forbes KJ. Molecular evidence for heterogeneity of the multiple-drug-resistant *Mycobacterium tuberculosis* population in Scotland (1990 to 1997). *J Clin Microbiol* 1999;37:998-1003.
- Friedman SM, Lu T, Drlica K. Mutation in the DNA gyrase A gene of *Escherichia coli* that expands the quinolone resistance-determining region. *Antimicrob Agents Chemother* 2001;45:2378-80.
- Guillemin I, Jarlier V, Cambau E. Correlation between quinolone susceptibility patterns and sequences in the A and B subunits of DNA gyrase in mycobacteria. *Antimicrob Agents Chemother* 1998;42:2084-8.
- Heddle J, Maxwell A. Quinolone-binding pocket of DNA gyrase: role of GyrB. *Antimicrob Agents Chemother* 2002;46:1805-15.
- Hirano K, Abe C, Takahashi M. Mutations in the *rpoB* gene of rifampicin-resistant *Mycobacterium tuberculosis* strains isolated mostly in Asian countries and their rapid detection by line probe assay. *J Clin Microbiol* 1999;37:2663-6.
- Kaatz GW, Seo SM, Ruble CA. Mechanisms of fluoroquinolone resistance in *Staphylococcus aureus*. *J Infect Dis* 1991;163:1080-6.
- Kocagöz T, Hackbarth CJ, Ünsal I, Rosenberg EY, Nikaido H, Chambers HF. Gyrase mutations in laboratory-selected, fluoroquinolone-resistant mutants of *Mycobacterium tuberculosis* H37Ra. *Antimicrob Agents Chemother* 1996;40:1768-74.
- Liu J, Takiff HE, Nikaido H. Active efflux of fluoroquinolones in *Mycobacterium smegmatis* mediated by LfrA, a multidrug efflux pump. *J Bacteriol* 1996;178:3791-5.
- Markoff A, Savov A, Vladimirov V, Bogdanova N, Kremensky I, Ganev V. Optimization of single-strand conformation polymorphism analysis in the presence of polyethylene glycol. *Clin Chem* 1997; 43:30-3. Erratum in: *Clin Chem* 1997;43:692.
- Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. *Clin Microbiol Rev* 1995;8:496-514.
- Onodera Y, Tanaka M, Sato K. Inhibitory activity of quinolones against DNA gyrase of *Mycobacterium tuberculosis*. *J Antimicrob Chemother* 2001; 47:447-50.
- Piddock LJV, Zhu M. Mechanisms of action of sparfloxacin against and mechanism of resistance in gram-negative and gram-positive bacteria. *Antimicrob Agents Chemother* 1991;35:2423-7.
- Prachartam R, Angkananukool K, Vibhagool A. In vitro susceptibility testing of levofloxacin and ofloxacin by microtiter plate Alamar blue against multidrug and non multidrug resistant *Mycobacterium tuberculosis* in Thailand. *J Med Assoc Thai* 2001;84:1241-5.
- Sanders CC. Ciprofloxacin: in vitro activity,

- mechanism of action, and resistance. *Rev Infect Dis* 1988;10:516-27.
- Silva PEA, Bigi F, Santangelo MP, *et al.* Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2001;45:800-4.
- Sintchenko V, Chew WK, Jelfs PJ, Gilbert GL. Mutations in *rpoB* gene and rifabutin susceptibility of multidrug-resistant *Mycobacterium tuberculosis* strains isolated in Australia. *Pathology* 1999; 31:257-60.
- Siddiqi N, Shamim M, Jain NK, *et al.* Molecular genetic analysis of multi-drug resistance in Indian isolates of *Mycobacterium tuberculosis*. *Mem Inst Oswaldo Cruz* 1998;93:589-94.
- Siddiqi N, Shamim M, Hussain S, *et al.* Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India. *Antimicrob Agents Chemother* 2002;46: 443-50.
- Sougakoff W, Lemaitre N, Cambau E, Szpytma M, Revel V, Jarlier V. Nonradioactive single-strand conformation polymorphism analysis for detection of fluoroquinolone resistance in mycobacteria. *Eur J Clin Microbiol Infect Dis* 1997;16:395-8.
- Takiff HE, Salazar L, Guerrero C, *et al.* Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. *Antimicrob Agents Chemother* 1994;38:773-80.
- Xu C, Kreiswirth BN, Sreevatsan S, Musser JM, Drlica K. Fluoroquinolone resistance associated with specific gyrase mutations in clinical isolates of multidrug-resistant *Mycobacterium tuberculosis*. *J Infect Dis* 1996;174:1127-30.
- Zhou J, Dong Y, Zhao X, *et al.* Selection of antibiotic-resistant bacterial mutants: allelic diversity among fluoroquinolone-resistant mutations. *J Infect Dis* 2000;182:517-25.