Correlation of *gyrA/B* mutations with level of susceptibility to fluoroquinolone of *Mycobacterium tuberculosis* isolates

Nuttaporn Nakkerd, Siriporn O-thong, Rattapha Chinli, Popchai Ngamskulrungroj, Piriyaporn Chongtrakool and Suporn Foongladda*

Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University

*Corresponding author. E-mail address: suporn.foo@mahidol.ac.th

Abstract

Since fluoroquinolones (FQ), such as ofloxacin (OFX) and moxifloxacin (MXF) have been widely used for tuberculosis treatment. Resistance to FQ has emerged and leading to cases of untreatable tuberculosis. In mycobacteria, FQ bind to DNA gyrase and inhibit DNA replication leading to bacterial cell death. DNA gyrase encoded by *gyrA* and *gyrB*. Mutations in the two short regions known as quinolone-resistant determining regions (QRDRs) have been associated with FQ resistance in *M. tuberculosis* (MTB). Therefore, the aim of this study was to determine the level of FQ susceptibility with mutation of *gyrA* and *gyrB* genes from 116 multidrug-resistant tuberculosis (MDR-TB) clinical isolates (including six XDR-TB) in Siriraj hospital, Thailand. The *gyrA* and *gyrB* sequence performed on MTB clinical isolates were compared with FQ susceptibility by the standard agar proportion method on Middlebrook 7H10 (APM) and minimal inhibitory concentration (MIC) by microbroth dilution method. Our data revealed that 23.3% of MDR-TB was resistant to FQ. The most common substitution in *gyrA* occurred at position D94, resulted in D94G (n=14, 51.9%), D94Y (n=3, 11.1%) and D94H (n=1, 3.7%). The following common substitution occurred at position A90V (n=5, 18.5%) and S91P (n=4, 14.8%). In addition, double mutations in both *gyrA/B*, S91P/E501D were found in one isolate (3.7%). There were linked to moderate to high level OFX and MXF resistance ranged from 4 to ≥32 μg/ml and 2 to ≥8 μg/ml, respectively. All of these mutations were occurred exclusively in FQ-resistant isolates. A combination of these 6 mutations exhibited the 100 % of sensitivity and 100 %, specificity for detection of OFX, and 100 % of sensitivity and 98.88 % specificity for MXF resistances in MDR-TB.

Keywords: tuberculosis, fluoroquinolones, MDR-TB

Introduction

Tuberculosis (TB) is the main cause of worldwide death infectious disease. Of the 480,000 cases of multidrug-resistant TB (MDR-TB), resistance to at least rifampicin and isoniazid, estimated to have occurred in 2014. The incidence rate of TB cases in Thailand in 2014 was 22/100,000 with a mortality rate of 6.6/100,000. Estimation of MDR-TB occurred at a frequency of 2% and 19% among new TB cases and retreatment TB cases (WHO, 2015).

The most efficient drugs for TB treatment are isoniazid (INH) and rifampicin (RIF). *M. tuberculosis* strains which are resistant to at least isoniazid and rifampicin, called multidrug-resistant tuberculosis (MDR-TB) (CLSI, 2011). Therefore, MDR-TB has become the main threat for tuberculosis control strategies. While extensively drug-resistant TB (XDR-TB) is MDR-TB isolates resistant to fluoroquinolones (FQ) and one of the second-line injectable drugs such as, kanamycin (KAN), amikacin (AMK) and capreomycin (CAP) (Caminero, Sotgiu, Zumla, & Migliori, 2010) However, FQ have been widely used for TB treatment and served routinely as monotherapy for the empirical treatment of numerous respiratory infections. The main target of FQ in *M. tuberculosis*

is the DNA gyrase, encoded by *gyrA* and *gyrB* (Yin & Yu, 2010). Mutations in the two short regions known as quinolone-resistant determining regions (QRDRs) have been associated with FQ resistance in *M. tuberculosis* (Pitaksajjakul et al., 2005). To date, a variety of molecular base techniques are used to determine FQ resistant. Therefore, we determined the mutations in *gyrA* and *gyrB* genes of *M. tuberculosis* clinical isolates with the level of ofloxacin and moxifloxacin susceptibility by detection of minimum inhibitory concentration (MIC) and the standard agar proportion method. Our data provide prominence to combined genes sequencing in the investigation of drugs susceptibility in *M. tuberculosis* and challenge the validity of the current phenotypic approach as the diagnostic gold standard for determining drugs resistance.

Materials and methods

Mycobacterium tuberculosis clinical isolates

In total, 116 MDR-TB clinical isolates (including 6 XDR) had been obtained from the Mycobacteriology Service Unit, Department of Microbiology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand during 2012-2015. In addition, *M. tuberculosis* H37Rv (ATCC 27294) was used as an internal control, since it was susceptible to all tested drugs. All MTB were cultured on Lowenstein-Jensen (LJ) medium at 37°C for 3 weeks before the following studies.

Standard agar proportion method

Drug susceptibility against ofloxacin (OFX) and moxifloxacin (MXF) of the isolates were determined using standard agar proportion method (APM) according to the Clinical and Laboratory Standards Institute (CLSI, 2011) methods. Stock solutions of drugs were prepared from reference powders and then used for preparation of the critical concentrations. OFX at 2 μg/ml and MXF at 0.5 μg/ml. Middlebrook 7H10 (BD Diagnostic Systems, Sparks, MD), supplemented with 10% OADC (Difco, Livonia, MI) were prepared. For preparation of bacterial inoculum, a 1.0 McFarland standard isolate suspension was serially diluted 10-fold, from 10^{-1} to 10^{-4}, in sterile distilled water. Several dilutions of inoculums (10^{-2} and 10^{-4}) were dropped onto both control (without drugs) and drug containing media, M7H10 with 10% OADC and incubated at 37°C. Results were read at 21 days and up to 28 days, depending on control growth. All isolates were considered as resistant to a given drug when growth of 1% or more above the control was observed in drug-containing media. Quality was controlled by using *M. tuberculosis* H37Rv as a reference strains for each set of APM tested.

Minimum inhibitory concentration detection

MIC of OFX and MXF were detected by broth dilution microtiter method using TREK Sensititre® MYCOTB MIC plate (TREK Diagnostic Systems, USA) as described by manufactured instruction. Several colonies were selected from LJ medium using a sterile loop and inoculated into a test tube containing saline-Tween and glass beads (TREK Diagnostics). After being vortex for 30 to 60 s, the inoculum was allowed to settle for 15 min and adjusted to a 0.5 McFarland standard equivalent using a nephelometer. The numbers of colony forming unit (CFU) were determined for each isolate tested to verify that the inoculum was within a specific targeted amount ($\sim 10^5$ CFU/ml). One hundred microliters of the inoculum was transferred to 11 ml of Middlebrook 7H9 broth containing OADC (TREK Diagnostics) and vortex for 20 s. One hundred microliters was transferred to the 96 well MYCOTB plate containing the preparedness of 10-fold diluted antibiotics. MYCOTB plates were

covered with plastic seals provided by the manufacturer. Plates were incubated at 37°C and MICs were examined at 10 and 21 days using a manual read aided by a mirrored viewer. The MIC is recorded as the lowest antibiotic concentration that reduces visible growth.

DNA sequencing

Genomic DNA was extracted from freshly cultured bacteria. A loopful of *M. tuberculosis* culture was suspended in microcentrifuge tube containing 500 µl of sterilized water and then heated in a 95°C water bath for 30 min. followed by centrifugation at 13,500 rpm for 2 min. The supernatant was transferred to another microcentrifuge tube. Template DNA was prepared by QIAGEN QIAmp DNA mini kit (QIAGEN GmbH, Hilden, Germany) and preserved at −20°C until use.

The primers for PCR amplification of *gyrA* and *gyrB* were designed with reference to gene sequences (GenBank accession number NC_000962.3) by the Primer 3 program. *GyrA* was amplified with the use of the *gyrA* F (5'-CCTGCGTTC GATTGCAAAC-3') and *gyrA* R (5'- CTTCGGTGTACCTCATCGCC-3') primers. *GyrB* was amplified with the use of the *gyrB* F(5'-CAAATCGTTTGTGCAGAAGG-3') and *gyrB* R (5'-ATGGTGGTCTCCCACAACTC-3') primers. The PCR products of *gyrA* and *gyrB* were 423 and 738 bps in size, respectively, as shown in Figure1.

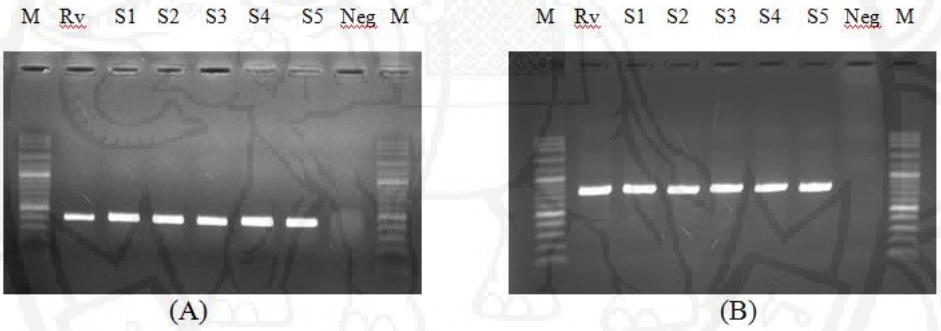

Figure 1 The PCR products of *gyrA* (A) and *gyrB* (B) were 423 and 738 bp in size, respectively. M; marker, Rv; *M. tuberculosis* H37Rv, S1-S5 show that the PCR product was generated from the MTB clinical isolates and Neg; negative control.

After amplification PCR products were purified to remove unincorporated nucleotides and primers with QIAquick PCR Purification Kit (Qiagen, Germany) and used as the template for DNA sequencing. PCR products were sequenced with a forward primer by 1st BASE Sequencing INT, Malaysia. Mutations in *gyrA* and *gyrB* were identified by comparison with *M. tuberculosis* H37Rv GenBank accession number NC 000962 by ClustalX program.

Statistical analysis

SPSS software (SPSS Inc. - Chicago, IL, USA) was used for the statistical analysis. Test results were based on comparison of MICs derived from the MYCOTB plate to critical concentrations found using the APM as reference method. For each drug, receiver operating characteristic (ROC) analysis was performed to estimate MYCOTB cutoff values. Sensitivity and specificity were calculated by using two by two table analyses.

Results

Distribution of *gyrA* and *gyrB* mutations among FQ-resistant and susceptible MTB isolates

FQ susceptibility testing by APM revealed that 27 of 116 (23.3%) isolates were resistant to both OFX and MXF at 2 and 0.5 μg/ml, respectively. Mutations were observed in QRDR of *gyrA* and *gyrB* genes among FQ-resistant isolates. Amino acid position S95T mutation in *gyrA*, which was a natural polymorphism was occurred in both FQ-resistant and FQ-susceptible isolates. The single amino acid mutation sites were in codons A90V, S91P, D94G, D94H and D94Y of *gyrA* gene, the relative frequencies of these codons were 5 (18.5%), 3 (11.1%), 14 (51.9%), 1 (3.7%) and 3 (11.1%), respectively (Table 1). One isolate had mutations in both *gyrA/B* genes, S91P/E501D (3.7%). These mutations were not observed among 89 (76.7%) FQ-susceptible isolates. The mutation in T4R, T5E and T4E occurred in 2 (2.2%) of FQ-susceptible isolates, implied that these mutation do not relate to FQ resistance in MTB.

Table 1 Substitutions in *gyrA* and *gyrB* among FQ-resistant and FQ-susceptible MTB clinical isolates

Substitutions		No. of isolates		% of total
gyrA	*gyrB*	FQ-R (n=27)	FQ-S (n=89)	substitutions
A90V		5		18.5
S91P	E501D	1		3.7
S91P		3		11.1
D94G		14		51.9
D94H		1		3.7
D94Y		3		11.1
T4R/T5E			1	1.1
T4E			1	1.1
Total		27	2	100

Level of MIC with mutation of *gyrA* and *gyrB*

The different mutations of *gyrA/B* and OFX, MXF MICs of the total MTB clinical isolates are shown in Table2. The most common substitution conferring FQ resistance among MDR-TB occurred at position D94. The second most common substitution occurred at position A90V and S91P. These linked to moderate to high level OFX and MXF resistance ranged from 4 to ≥32 μg/ml and 2 to ≥8 μg/ml, respectively. While FQ-susceptible isolates with and without mutations, the OFX MICs were ≤0.25 to 2 μg/ml and MXF MICs were ≤0.06 to 1 μg/ml (Table 2).

Table 2 Substitutions in *gyrA/B* found in 116 MDR-TB clinical isolates with the MIC of MXF/OFX and standard agar proportion method (APM).

Substitutions		APM	No. of isolates by MICs to drugs (μg/ml)																Total (%)
			MXF									OFX									
gyrA	*gyrB*		≤0.06	0.12	0.25	0.5	1	2	4	8	>8	≤0.25	0.5	1	2	4	8	16	32	>32	
A90V	WT	R						3	2							2	3				5(4.3)
		S																			
S91P	E501D	R									1									1	1 (0.9)
		S																			
S91P	WT	R							1		2					1	1	1			3 (2.6)
		S																			

Table 2 (Cont.)

Substitutions		APM	No. of isolates by MICs to drugs (μg/ml)																	Total (%)
			MXF									OFX								
gyrA	*gyrB*		≤0.06	0.12	0.25	0.5	1	2	4	8	>8	≤0.25	0.5	1	2	4	8	16	32	>32	
D94G	WT	R						4	6	3	1					1	5	6	1	1	14 (12.1)
		S																			
D94H	WT	R								1								1			1 (0.9)
		S																			
D94Y	WT	R							1	2							1	1	1		3 (2.6)
		S																			
T4R/T5E	WT	R																			
		S			1									1							1 (0.9)
T4E	WT	R																			
		S		1									1								1 (0.9)
WT	WT	R																			
		S	3	8	47	25	3	1				3	38	40	6						87 (75.0)
Total			3	9	48	25	3	8	10	6	4	3	39	41	6	4	10	9	2	2	116

WT= wild type, R=resistant, S=susceptible

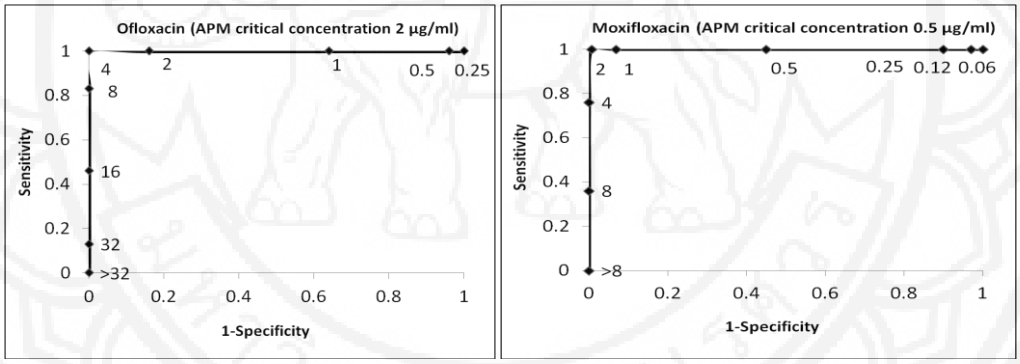

Figure 2 Receiver operating characteristic (ROC) curves for each tested drug.
Number in bold are MYCOTB plate minimum inhibitory concentrations.

Correlation of MIC and AMP results

The range of agreement between MYCOTB results and APM results was considered in ROC curve results (Figure 2). OFX showed the higher agreement, with a large area under the curve (AUC) of 1.00, while MXF had an AUC of 0.99. The MIC cutoff values were defined as 4 and 2 μg/ml for OFX and MXF resistance, respectively. MYCOTB plate sensitivity and specificity, using APM results as the reference comparator are shown in Table 3. OFX at 2 μg/ml exhibited the sensitivity of 100% and specificity of 100%, MXF at 0.5 μg/ml exhibited the sensitivity of 100% and specificity of 98.88%.

Table 3 Resistance pattern of 116 MDR-TB clinical isolates using the MYCOTB MIC plate and agar proportion method.

Drug	MYCOTB plate result	No. of isolates with each APM		Sensitivity (%)	Specificity (%)
		Resistance	Susceptible		
Ofloxacin	Resistance	27	0	100	100
	Susceptible	0	89		
Moxifloxacin	Resistance	27	1	100	98.88
	Susceptible	0	88		

Discussion

Fluoroquinolones (FQ) are the most effective antimicrobial agents used as the major drugs for treatment of MDR-TB. The poorly controlled use of FQ can contribute to the emergence of FQ resistance in *M. tuberculosis* (MTB), which may influence the clinical outcome of MDR tuberculosis patients. DNA gyrase are the target of FQ in *M. tuberculosisis*, consisting of two A and two B subunits encoded by the *gyrA* and *gyrB* genes. The mutations within the QRDR, including a conserved region of *gyrA* (codons 88 to 94) (Maruri et al., 2012) and *gyrB* (codons 500 to 538) (Von Groll et al., 2009), have been identified as the primary mechanism conferring FQ resistance (Aubry, Pan, Fisher, Jarlier, & Cambau, 2004).

Frequency of mutation in this study was observed in *gyrA* position 94 (66.7%), 90 (18.5%) and follow by 91 (13.33%) as previous reports from China and Russian Federation that 64.5-75.6% of OFX-resistant isolates carried mutation *gyrA* positions 94 and 90 (Zhang, Lu, Wang, Pang, & Zhao, 2014). They also reported high-level FQ resistance, with six different amino acid substitutions D94H, Y, N, A, G or C which accounted for 32.6% of FQ resistant in MTB. Whereas, we found high level MIC of MFX (>16 mg/liter) in S91P and D94G in 4 isolates (13.33%). Few single mutation in *gyrB* gene have been reported in 2.9-15.5% of OFX-resistant strains, including R485H, D500N, and D500A (Zhang et al., 2014). However, we found only one isolates at E501D. We also found double substitutions in both *gyrA/gyrB* (S91P/E501D) with high level of MIC in one isolates. These have been reported such as A90V/S486Y, D94N/D538T and D94A/D538T with high-level FQ resistance ($\geq$16 mg/liter) (Zhang et al., 2014).

In this study, *gyrA/B* mutations were classified into 6 types, with A90V, S91P, S91P/E501D, D94G, D94H and D94Y, respectively. All of these were occurred exclusively in FQ-resistant isolates. Analysis of these mutations exhibited the sensitivity 100%, specificity 100% and accuracy 100%, respectively, for detection of FQ resistance in MTB. The most common substitution conferring FQ resistance among MDR-TB occurred at position D94. The second most common substitution occurred at position 90 and 91. The MIC cutoff values are the averages of two consecutive test values, were defined as 4 and 2 μg/ml for OFX and MXF resistance, respectively. These mutations were linked to moderate to high level OFX and MXF resistance ranged from 4 to $\geq$32 μg/ml and 2 to $\geq$8 μg/ml, respectively. While FQ-susceptible isolates with and without mutations, the OFX MICs were ranged from

≤0.25 to 2 μg/ml and MXF MICs were ranged from ≤0.06 to 1 μg/ml.

Although the mutation in *gyrB* that related to FQ resistance were infrequent, E501D was observed in one FQ-resistant isolate consistent with findings from Vietnam, reported to 12 μg/ml for *gyrB* with the E540D mutation (difference numbering system) (An et al., 2009). The present data show that FQ-resistant MTB isolates with mutation in both *gyrA* and *gyrB* did not differ from FQ-resistant MTB isolates with only single mutation in *gyrA*. Recent study performed in the Russian Federation reported mutations a novel mutation 1457 C⟶T was found in the *gyrB* gene (Kontsevaya et al., 2011). Founded on the current study, more than 30% of FQ-resistant strains did not harbor the mutations in the QRDRs of *gyrA* and *gyrB*, suggesting that another mechanism, including an active drug efflux pump and decreased cell wall permeability to the drug, may also confer the FQ resistance of these strains.

In conclusions, the MIC data purpose the cutoff information at 4 μg/ml for OFX and 2 μg/ml for MXF resistance. This MIC data could provide clinical impact for FQ resistant detection within 10 to 14 days. Mutation in *gyrA* S91P, D94G and *gyrA* together with *gyrB* were associated with high-level resistance to FQ among the MDR-TB isolates circulating in Thailand.

Acknowledgments

We thank the microbiology laboratory technicians and Mycobacteriology Service Unit, Department of Microbiology, Faculty of Medicine, Siriraj hospital, Mahidol university, Bangkok Thailand. This work was supported by National Institutes of Health grant R01 AI093358.

References

An, D. D., Duyen, N. T. H., Lan, N. T. N., Ha, D. T. M., Kiet, V. S., Chau, N. V. V., & Caws, M. (2009). Beijing genotype of Mycobacterium tuberculosis is significantly associated with high-level fluoroquinolone resistance in Vietnam. *Antimicrobial agents and chemotherapy*, *53*(11), 4835-4839.

Aubry, A., Pan, X. S., Fisher, L. M., Jarlier, V., & Cambau, E. (2004). Mycobacterium tuberculosis DNA gyrase: interaction with quinolones and correlation with antimycobacterial drug activity. *Antimicrobial agents and chemotherapy*, *48*(4), 1281-1288.

Caminero, J. A., Sotgiu, G., Zumla, A., & Migliori, G. B. (2010). Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis. *The Lancet infectious diseases*, *10*(9), 621-629.

Clinical Laboratory Standards Institute. (2011). Susceptibility testing of mycobacteria, nocardia, and other aerobic actinomycetes: approved standard. *Clinical Laboratory Standards Institute*, *31*(5), M24-A2.

Kontsevaya, I., Mironova, S., Nikolayevskyy, V., Balabanova, Y., Mitchell, S., & Drobniewski, F. (2011). Evaluation of two molecular assays for rapid detection of Mycobacterium tuberculosis resistance to fluoroquinolones in high-tuberculosis and-multidrug-resistance settings. *Journal of clinical microbiology*, *49*(8), 2832-2837.

Maruri, F., Sterling, T. R., Kaiga, A. W., Blackman, A., van der Heijden, Y. F., Mayer, C., & Aubry, A. (2012). A systematic review of gyrase mutations

associated with fluoroquinolone-resistant Mycobacterium tuberculosis and a proposed gyrase numbering system. *Journal of antimicrobial chemotherapy*, *67*(4), 819-831.

World Health Organization. (2015). Global tuberculosis report 2015. WHO/HTM/TB/2015.22. Geneva, Switzerland: World Health Organization. Retrieved from http://apps.who.int/iris/bitstream/10665/191102/1/9789241565059_eng.pdf

Pitaksajjakul, P., Wongwit, W., Punprasit, W., Eampokalap, B., Peacock, S., & Ramasoota, P. (2005). Mutations in the gyrA and gyrB genes of fluoroquinolone-resistant Mycobacterium tuberculosis from TB patients in Thailand. *Southeast Asian journal of tropical medicine and public health*, *36*, 228-37.

Von Groll, A., Martin, A., Jureen, P., Hoffner, S., Vandamme, P., Portaels, F., & da Silva, P. A. (2009). Fluoroquinolone resistance in Mycobacterium tuberculosis and mutations in gyrA and gyrB. *Antimicrobial agents and chemotherapy*, *53*(10), 4498-4500.

Yin, X., & Yu, Z. (2010). Mutation characterization of gyrA and gyrB genes in levofloxacin-resistant Mycobacterium tuberculosis clinical isolates from Guangdong Province in China. *Journal of Infection*, *61*(2), 150-154.

Zhang, Z., Lu, J., Wang, Y., Pang, Y., & Zhao, Y. (2014). Prevalence and molecular characterization of fluoroquinolone-resistant Mycobacterium tuberculosis isolates in China. *Antimicrobial agents and chemotherapy*, *58*(1), 364-369.