RESEARCH NOTE

GYRA AND *GYRB* MUTATIONS IN OFLOXACIN-RESISTANT *MYCOBACTERIUM TUBERCULOSIS* CLINICAL ISOLATES IN THAILAND

Pannamthip Pitaksajjakul[1, 2], Suwalee Worakhunpiset[1], Angkana Chaiprasert[3], Jirakarn Boonyasopun[4] and Pongrama Ramasoota[1, 2]

[1]Center of Excellence for Antibody Research (CEAR), [2]Department of Social and Environmental Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok; [3]Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok; [4]Chest Disease Institute, Ministry of Public Health, Nonthaburi, Thailand

Abstract. In order to identify mutations in *gyrA* and *gyrB* genes in 92 ofloxacin-resistant *Mycobacterium tuberculosis* (OFX^r-MTB) clinical isolates collected from Siriraj Hospital, Mahidol University and Chest Disease Institute, Thailand. The quinolone resistance-determining regions (QRDR) of *gyrA* and *gyrB* in all 92 OFX^r-MTB isolates were amplified using polymerase chain reaction and sequenced. There were 70 isolates with point mutations associated with ofloxacin resistance. In *gyrA* QRDR, 69 isolates had mutations in *gyrA* Gly88 (Ala/(75), Ala90 (Val), Ser91 (Pro) and Asp94 (Gly/Ala/His/Asn), the latter being the most common (42%). Only one isolate was found with mutation at position Asp495 (Asn). The other 22 isolates had no mutations in both *gyrA* and *gyrB* QRDR. Thus, point mutations in *gyrA* and *gyrB* QRDR were responsible for OFX^r-MTB clinical isolates in Thailand.

Keywords: *Mycobacterium tuberculosis*, ofloxacin-resistant, mutation, *gyrA*, *gyrB*

INTRODUCTION

Tuberculosis (TB) is the cause of death worldwide, especially when it associated with AIDS pandemic, and the emergence of multidrug-resistant (MDR) strains of *Mycobacterium tuberculosis*, has led to increased fatalities rates (Bifani *et al*, 1996; Dye *et al*, 1999; Gandhi *et al*, 2006). Patients infected with these MDR-TB strains are difficult to treat, and this becomes increasingly more difficult the longer the strains 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. For these reasons, an immediate prevention of the spread of MDR-TB strains is of utmost important.

To treat MDR-TB cases, fluoroquinolone drugs, including ofloxacin and ciprofloxacin have been currently used (Cambau *et al*, 1994). Fluoroquinolone

Correspondence: Pongrama Ramasoota, Center of Excellence for Antibody Research (CEAR), Faculty of Tropical Medicine, Mahidol University, 420/6 Ratchawithi Road, Bangkok 10400, Thailand.
E-mail: tmprt@mahidol.ac.th

drugs are also being widely used to treat other bacterial diseases. The widespread use of these drugs has led to the marked emergence of fluoroquinolone resistant MTB (FQ^r-MTB) in many countries (Fang *et al*, 1999; Espinal *et al*, 2001) and also in Thailand (Prachaktam *et al*, 2001).

The resistance mechanism to quinolone drug of *Mycobacterium* spp has been associated with mutations in DNA gyrase, a tetrameric protein composed of two A and two B subunits, encoded by *gyrA* and *gyrB*, respectively (Cambau and Gutmann, 1993). Missense mutations within quinolone resistance-determining region (QRDR) in *gyrA* and *gyrB* subunits have been identified as being associated with fluoroquinolone resistance. For A subunit, the most common mutations involved a substitution of Asp 94→His/Tyr, Ala 90→Val, and Ser 91→Pro. For B subunit, mutations are frequently found at Asp 495→His/Asn, Arg 516 and Asn 533 (Kocagöz *et al*, 1996; Siddiqi *et al*, 2002). Detection of mutations in these hot spot regions at positions 90, 91, and 94 in *gyrA* and positions 495, 516, and 533 in *gyrB* is a rapid and efficient test for molecular biology detection of FQ^r-MTB. These mutations can be detected, directly or indirectly, by different molecular biology techniques, such as polymerase chain reaction (PCR)-DNA sequencing, and PCR-single strand conformation polymorphism (SSCP) (Sougakoff *et al*, 1997).

This study identified mutations in the QRDRs of both *gyrA* and *gyrB* genes in ofloxacin-resistance *Mycobacterium tuberculosis* (OFX^r-MTB) clinical isolates from Thailand using direct PCR-DNA sequencing.

MATERIALS AND METHODS

A total of 92 OFX^r *M. tuberculosis* clinical isolates (16 isolates from Siriraj Hospital, Mahidol University, Bangkok and 76 isolates were from Chest Disease Institute, Bangkok) were tested for drug susceptibility by absolute or proportion method (Prachaktam *et al*, 2001). MICs of ofloxacin were detected at 2 g/ml of drug concentration.

Mutations in *gyrA* and *gyrB* QRDRs from all isolates were analyzed by DNA-sequencing as described by Pitaksajjakul *et al* (2005). In brief, genomic DNA was purified by CTAB/NaCl and phenol/chloroform extraction methods and purified by 70% ethanol. For *gyrA* and *gyrB* QRDR amplification, primers GyrA-f (5′-CAGCTACATCGACTATGCGA-3′), and GyrA-r (5′GGCTTCGGTGTACCTCAT-3′) and primers GyrB-f (5′-CCACCGACATCGGTGGATT-3′) and GyrB-r (5′-CTGCCACTTGAGTTTGTACA-3′) were used to generate amplicons of the 320 bps and 429 bps of *gyrA* and *gyrB*, respectively. Amplicons were sent for DNA sequencing by a commercial sequencing service (BSU, Thailand). Mutations in each isolates were identified by comparing with standard H37Ra 25177 and ofloxacin-sensitive *M. tuberculosis* strains using BLAST program.

RESULTS

Out of 92 isolates, there were 70 (76%) isolates with single missense mutation. No double point mutation was found. In *gyrA* QRDR, 69 of 92 (75%) isolates have mutations in *gyrA* hot spot region (Table 1). Four codons (Gly88, Ala90, Ser91, and Asp94) were identified as being affected, resulting in 8 amino acids changes. Among these codons, Asp94 mutation (Gly/Ala/His/Asn) was the most common. In *gyrB* QRDR, only one isolate (1%) had mutation at Asp495Asn (Table 2).

Table 1
Point mutations in *gyrA* QRDR of 92 ofloxacin-resistant *M. tuberculosis* isolates from TB patients in Thailand.

Codon	Mutation	Amino acid change	No. of isolates/(%)
88	GGC → TGC	Gly → Cys	1 (1)
88	GGC → GCG	Gly → Ala	1 (1)
90	GCG → GTG	Ala → Val	19 (20)
91	TCG → CCG	Ser → Pro	9 (10)
94	GAC → GGC	Asp → Gly	22 (24)
94	GAC → GCC	Asp → Ala	6 (6)
94	GAC → CAC	Asp → His	7 (8)
94	GAC → AAC	Asp → Asn	4 (4)

Table 2
Point mutation in *gyrB* QRDR of 92 ofloxacin-resistant *M. tuberculosis* isolates from TB patients in Thailand.

Codon	Mutation	Amino acid change	No. of isolates/(%)
495	GAC → AAC	Asp → Asn	1 (1)

DISCUSSION

As fluoroquinolone resistance is mainly due to mutations in DNA gyrase, encoded by *gyrA* and *gyrB* in the QRDR region (Cambau and Gutmann, 1993; Cambau *et al*, 1994; Takiff *et al*, 1994; Alangaden *et al*, 1995; Xu *et al*, 1996; Siddiqi *et al*, 1998), direct DNA sequencing was used in this study.

The majority (76%) of OFX^r-MTB clinical isolates revealed mutations in *gyrA* QRDR at Asp94, Ala90, Ser91, and Gly88, which have been frequently found to be associated with fluoroquinolone resistance (Cambau *et al*, 1994; Kogacöz *et al*, 1996; Xu *et al*, 1996; Siddiqi *et al*, 2002). In addition, mutation at Asp94 (Gly/Ala/His/Asn) has been reported to result in the higher level of fluoroquinolone resistance and considered to be high-level resistance mutation position (Alangaden *et al*, 1995; Siddiqi *et al*, 1998), and is the most common mutation found in this study.

For mutations occurred in *gyrB* QRDR, we found only one isolate with mutation at position Asp495 in *gyr*B QRDR. This mutation was also found in laboratory strains of *M. tuberculosis* by Kocagöz *et al* (1996) and in clinical isolates reported by Zhou *et al* (2000).

Zhou et al (2000) found that fluoroquinolone drug concentration is mainly responsible for the mutation selection pattern of FQ^r-MTB. At low levels of drug, the resistant mutant is usually recovered in non-gyraseA target, whereas many distinct mutations were observed at moderate drug concentrations, at Gly88, Ala90, Ser91, Asp94 in *gyrA*. By increasing drug concentration, the mutant selection occurs predominantly at the high-level resistance mutation position, Asp94. Similarly our

study with MDR-TB clinical isolates with ofloxacin resistance (C8-H fluoroquinolone) at 2 g/ml (moderate concentration) showed that most of mutations occurred at Asp94Gly/Ala/Asn/His.

Zhou *et al* (2000) also found that the distinct mutation position occurred with moxifloxacin at the lower concentration tested (0.5 g/ml) In Thailand, moxifloxacin or other more effective drugs in this group are not yet clinically used for MDR-TB treatment due to their high toxic side effects. Accordingly, to reduce the chance of relapse, emergence of drug resistance and successful treatment, a shorter protocol with more effective drugs is recommended.

We found only one isolate has *gyrB* mutation of Asp495Asn and 22 isolates with fluoroquinolone resistance phenotype showed no mutation in both *gyrA* and of *gyrB* QRDR gene. Perhaps the drug concentration used (2 g/ml) was not high enough for these latter isolates to select mutation in the primary drug target (*gyrA* gene), or in *gyr*B QRDR. Mutation in other positions outside these regions may involved other mechanisms, such as decreased influx or increased efflux by multidrug-mediate pump encoded by *lfrA*, may be involved, as reported in *Mycobacterium smegmatis* (Denis and Moreau, 1993; Sander *et al*, 2000; Silva *et al*, 2001).

ACKNOWLEDGEMENTS

This work was supported by Thailand Tropical Disease Research Programme (T2) (Grant ID number: 02-2-TUB-02-031). We also thank BIOTEC/NSTDA for a graduate student scholarship for this research.

REFERENCES

Alangaden GJ, Manavathu EK, Vakulenko SB, Zvonok NM, Lerner SA. Characterization of fluoroquinolone-resistant mutant strains of *Mycobacterium tuberculosis* selected in the laboratory and isolated from patients. *Antimicrob Agents Chemother* 1995; 39: 1700-3.

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. Machanisms of resistance to quinolones. *Drugs* 1993; 45 (suppl 3): 15-23.

Cambau E, Sougakoff W, Besson M, Truffot-Pernot C, Grosset J, Jarlier V. Selection of *gyrA* mutant of *Mycobacterium tuberculosis* resistant to fluoroquinolones during treatment with ofloxacin. *J Infect Dis* 1994; 170: 479-83.

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.

Dye C, Scheele S, Dolin P, Pathania V, Raviglione M C. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. *JAMA* 1999; 273: 220-6.

Espinal M A, Laszlo A, Simonsen L, *et al*. Global trends in resistance to antituberculosis drugs 2001; 344: 1294-303.

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.

Gandhi N, Moll A, Sturm A, *et al*. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet* 2006; 368: 1575-80.

Kocagöz T, Hackbarth CJ, Ünsal I, Rosenberg EY, Nikaido H, Chambers HF. Gyrase mutations in laboratory-selected, fluoro-

quinolone-resistant mutants of *Mycobacterium tuberculosis* H37Ra. *Antimicrob Agents Chemother* 1996; 40: 1768-74.

Pitaksajjakul P, Wongwit W, Punprasit W, Eampokalap B, Peacock S, Ramasoota P. Mutations in the *gyrA* and *gyrB* genes of fluoroquinolone-resistant *Mycobacterium tuberculosis* from TB patients in Thailand. *Southeast Asian J Trop Med Public Health* 2005; 36 (suppl 4): 228-37.

Pracharktam 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.

Sander P, De Rossi E, Boddinghaus B, *et al*. Contribution of the multidrug efflux pump LfrA to innate mycobacterial drug resistance. *FEMS Microbiol Lett* 2000; 193: 19-23.

Siddiqi N, Shamim Md, Jain N K, *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.

Silva PE, Bigi F, de la Paz Santangelo M, *et al*. Characterization of P55, a multidrug efflux pump in *Mycobacterium bovis* and *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2001; 45: 800-4.

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 H E, 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*. [Published erratum appears in *J Infect Dis* 1997; 175: 1027]. *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.