Emergence of Ofloxacin Resistance in *Mycobacterium tuberculosis* Clinical Isolates from China: gyrA Mutation Analysis by Denaturing HPLC and DNA Sequencing

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A high rate of double point mutations in gyrA gene (56% of 87 ofloxacin-resistant *M. tuberculosis* clinical isolates) indicates the emergence of fluoroquinolone resistance. This is the first report to describe denaturing HPLC (DHPLC) analysis of mutations in gyrA gene of *M. tuberculosis* in a large number of clinical isolates.

At present, fluoroquinolones have been studied as a first-line treatment for tuberculosis (9). However, fluoroquinolone resistance among *M. tuberculosis* strains is emerging, with important implications for treatment (4). Fluoroquinolones have been widely used for tuberculosis treatment in China for more than 10 years, and have been given routinely as monotherapy for the empirical treatment of numerous outpatient infections. Thus, China may be one of the countries with the highest rate of fluoroquinolone abuse and resistance in the world. This work is to determine quinolone resistance determining regions (QRDRs) of gyrA gene in ofloxacin-resistant isolates from China by DHPLC and DNA sequencing methods.

The 109 clinical isolates (87 shown to be ofloxacin-resistant and 22 susceptible by a routine proportional method) were collected from different patients with pulmonary...
tuberculosis (65 males and 44 females, aged 17 to 73 years, with 2 to 6 months of fluoroquinolone treatment) over a period of 2 years (2002 to 2003) at Beijing Tuberculosis and Lung Tumor Research Institute, Tongzhou, China. MICs of ofloxacin were detected by an absolute concentration method in L-J medium, and the concentration ranges were 0.125, 0.25, 1, 2, 4, 8, 10, 16, 20, and 32 µg/ml. DHPLC analysis: *M. tbc* H37Rv (ATCC25618) and *M. tbc* Erdman (ATCC35801) were used as reference strains. DHPLC was performed with WAVE DNA fragment analysis system (Transgenomic Inc.). Melting temperature for gyrA analysis was 67.7°C. The conditions for DNA hybridization and DHPLC analysis have been described in detail elsewhere (10). DNA sequencing: a 227-bp DNA fragment corresponding to the quinolone resistance-determining region (QRDR) was generated by PCR with the primer set: forward 5′–GACCGCAGCCACGCCAAG–3′ and reverse 5′–AGCATCACCATCGCCAACG–3′. After purification, PCR product (5 ng) was used as a template for TaqCycle Sequencing using ABI Prism Big Dye Terminator sequencing kits (Applied Biosystems). Cycle sequencing products were subsequently analyzed on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

GyrA mutations were found to occur predominantly at codons 90, 91 and 94, and in 4 types of codon 94 mutation (94Asp->Gly, Ala, Tyr, and Asn)(FIG1), largely confirming the findings of other researchers (1, 2, 7, 11, 12). The previously reported mutation involving codon 88 was not found (5). All of the 109 clinical isolates had codon 95 ACC natural polymorphism, which paralleled the results for 138 other isolates from China (2). However, two new findings were unexpected. One was that 49 of the 87 ofloxacin-resistant isolates (56%) carried double point mutations, and the other was that among these double-mutated isolates, 20% (10/49) harbored Ala74Ser mutation (FIG2), which has not been reported previously in *M. tuberculosis*. Double point mutation of gyrA is relatively rare (2, 5, 12) and is generally thought to be uncommon in clinical isolates. Ala74Ser mutation has been reported only in other bacteria (8, 16). This indicates the already emergence of fluoroquinolone resistance in China.

DHPLC analysis: First, *M. tbc* H37Rv was routinely applied as a reference strain, and this revealed that all 109 isolates carried mutation (aberrant peak patterns in FIG. 3 ABCD). DNA sequencing showed that all the strains possessed codon 95 AGC→ACC (Ser→Thr) natural polymorphism, which did not have a significant impact on fluoroquinolone susceptibility (4). To improve the DHPLC detection capacity, the other reference strain was selected from H37Ra, *M. tbc* Kuruno strain, *M. tbc* Erdman and BCG Pasteur (Data not shown). We found that *M. tbc* Erdman (fluoroquinolone-susceptible, with codon 95 ACC in gyrA QRDRs) was the best as the second reference strain in this study. Those isolates only with codon 95 AGC→ACC showed a normal peak (FIG3, D1). Thus, the influence from this codon 95 AGC-ACC natural polymorphism was successfully avoided. When *M. tbc* H37Rv and *M. tbc* Erdman reference strains were used, a wild-type peak pattern appeared, indicating no point mutation in gyrA QRDRs. Of course, if an isolate carries any point mutation at a codon except codon 95, an aberrant peak pattern would appear. One interesting thing is that most of the isolates with the same mutation showed the same
DHPLC patterns. The peak profiles of each mutation are shown in FIG. 4. Asp$^{94}$Gly and Asp$^{94}$Asn changes revealed nearly similar patterns that were difficult to distinguish from each other. Other mutations had their own peak patterns. Therefore, it is thought that, to some extent, specific DHPLC patterns may predict the types of resistance.

DHPLC is a relatively new technique utilizing heteroduplex formation between wild-type and mutated DNA strands to identify mutations and was predicted to be a potentially useful genotypic screening method for gene mutations conferring drug resistance in *M. tuberculosis* (3,8,10). In this study, by introducing *M. tbc* H37Rv and *M. tbc* Erdman as two reference strains, the interference from codon 95 AGC:ACC natural polymorphism was successfully evaded. Since no other polymorphism has been found in *gyrA* QRDRs except for codon 95, and all the point mutations in codons 74, 88, 90, and 91 correlate with fluoroquinolone resistance, the DHPLC method devised in this study can be regarded as a useful and powerful tool for analysis of *gyrA* mutation in tuberculosis. (Acknowledgments: Dr. Ruiru Shi is a recipient of The Japan-China Medical Association Fellowship sponsored by Sasagawa Memorial Foundation. Most of this study was presented at 41st US-Japan Cooperative Medical Science Program Tuberculosis and Leprosy Research Conference at Kagoshima, Japan, in July, 2006)

REFERENCES


Figure legends

FIG. 1 Nucleotide sequence and missense mutations within the QRDRs of gyrA. All the isolates contain a naturally occurring polymorphism 95 AGC to ACC. Seventy-three (84%) of the 87 ofloxacin-resistant clinical isolates were found to carry codon 94 mutation, and 49 (56%) were found to harbor a double point mutation.

FIG. 2 Ofloxacin MIC relative to the gyrA QRDRs allele spectrum. Ofloxacin MICs are indicated above each panel. The number (n) in each MIC group is shown in each panel. Bars indicate the percentage represented by each allele.

FIG. 3 DHPLC patterns of gyrA genes. DHPLC patterns of the 109 clinical isolates, when M.tb H37Rv was used as a reference strain. Patterns A, B, and C are shown as examples (details are shown in FIG.4). W means H37Rv wild type. When M.tb Erdman was used as a reference strain, A, B, and C above were changed to A1, B1, C1, respectively. W1 indicates the M.tb Erdman wild type. Isolates (MIC less than 2 µg/ml) that harbored only 95 ACC natural polymorphism with no other mutation in gyrA QRDRs showed the wild type pattern C1.

FIG. 4. Specific DHPLC pattern of each gyrA QRDR mutation type.