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Multiplex allele-specific PCR combined with PCR-RFLP analysis for rapid detection of *gyrA* gene fluoroquinolone resistance mutations in *Mycobacterium tuberculosis*

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ABSTRACT

A combined use of MAS-PCR (multiplex allele-specific PCR) and PCR-RFLP (PCR restriction fragment length polymorphism), was established to detect mutations in codons 90, 91 and 94 of the *gyrA* gene in *Mycobacterium tuberculosis* (*M. tuberculosis*). With conventional phenotypic drug susceptibility testing as a reference standard, the sensitivity, specificity and accuracy of the modified method for *gyrA* gene mutation detection were 70.8%, 100% and 84.8% respectively.

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As a broad-spectrum antibiotic, fluoroquinolone was introduced into clinical practice in China nearly 20 years ago and has been widely used in the treatment of numerous common bacterial infections, including tuberculosis (TB) cases with resistance to first-line TB drugs and TB patients with severe adverse reaction to the first-line agents (Yew et al., 1995; Gillespie and Kennedy, 1998; Berning, 2001; Wang et al., 2006). Recent studies showed that previous exposure to fluoroquinolone was correlated with fluoroquinolone resistance in *M. tuberculosis* (Ginsburg et al., 2003b; Wang et al., 2007). Therefore, it is especially important to maintain information about the fluoroquinolone susceptibility in different TB patient population to guide selection of the most appropriate treatment.

However, conventional phenotypic drug susceptibility testing on Lowenstein–Jensen (L–J) media requires 28 to 42 days after the primary culture has been isolated. The rapid method on liquid media such as BACTEC MGIT960 also requires 4–13 days (Ardito et al., 2001; Adjers-Koskela and Katila, 2003). The molecular methods to detect fluoroquinolone resistance in *M. tuberculosis* provide an attractive alternative. They could identify resistant strains as soon as possible to allow for timely and adequate adjustments in treatment and to minimize the transmission of drug-resistant strains.

As a molecular method detecting the incidence of fluoroquinolone resistance, MAS-PCR assay was applied in the previously reported (Evans and Segal 2010). However, this method only detected the Gyr A D94G mutation (Evans and Segal, 2010), which only accounts for a minority of fluoroquinolone resistance. Since mutations in codons 90, 91 and 94 of GyrA are the most frequently observed in the fluoroquinolone resistant *M. tuberculosis* (Guillemin, Jarlier et al., 1998; Cheng et al., 2004; Shi et al., 2006), we developed a combined use of MAS-PCR and PCR-RFLP to detect GyrA A90V, S91P, D94A and D94G mutations which most frequently occur in fluoroquinolone resistance.

For this research, 65 clinical isolates identified as fluoroquinolone resistant *M. tuberculosis*, 60 clinical isolates identified as fluoroquinolone sensitive *M. tuberculosis* and H37Rv (ATCC 27294) were included. These isolates obtained from different patients with TB in Fujian, Liaoning, Tibet and Sichuan of China. Fluoroquinolone susceptibility testing with ofloxacin (2 mg/l) was performed with L–J medium by the proportional method, in accordance with World Health Organization (WHO) guidelines (WHO, 2008).

Colonies of *M. tuberculosis* isolates on L–J slants were scraped and resuspended in 500 µl distilled physiological saline solution and heated inactivated at 80 °C for 1 h. Genomic DNA was extracted as conventional method (van Embden et al., 1993).

To verify the efficiency of the combined use of MAS-PCR and PCR-RFLP, DNA sequencing of *gyrA* was performed. For sequencing, a DNA fragment of 858 bp, corresponding to the fluoroquinolone resistance determining region (QRDR), was generated by PCR with the primer pair 5'-TCGACTATGCGATGAGCGTG and 5'-CGATGCGTAAACCGACCC.

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The reaction conditions consisted of a denaturation step of 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 58.5 °C, 40 s at 72 °C, and a final extension step of 3 min at 72 °C. PCR products were sent for sequencing. All sequence data were manipulated with BioEdit version 7.0.5.3 and were compared with the published sequences for the *gyrA* gene (GenBank accession number NC_000962).

MAS-PCR assays were designed to detect 90A and 94D of *GyrA* mutations by identifying a mismatch between the primers and the targeted mutations. The primers used for 90A mutation were F-*gyrA*90: 5'-ACCACCCGCACGGCGATGC-3' and R-*gyrA*: 5'-GATATTGGTTGCCATGCC-3'. For 94D mutation, the primers were F-*gyrA*: 5'-TATGCGATGAGCGT-GAT-3' and R-*gyrA*94: 5'-GCCATGCGCACCAGTTGT-3' (Fig. 1A). Each PCR assays had a final volume of 30 µl and contained 1 × *easytaq* PCR Buffer (TRANS), 1.25 U *easytaq* DNA polymerase (TRANS), 40–100 ng genomic DNA as template, 0.125 mM dNTP, 3 pmol of F-*gyrA* and R-*gyrA*, 12 pmol of F-*gyrA*90 and 5 pmol of R-*gyrA*94. The reaction conditions consisted of a denaturation step at 95 °C for 5 min followed by 5 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 40 s, 5 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, and 20 cycles of 95 °C for 30 s, 53 °C for 30 s, and 72 °C for 40 s, followed by a final elongation step at 72 °C for 3 min. The amplified fragments (5 µl) were electrophoresed in 2% agarose gels and visualized under UV light.

For PCR-RFLP analysis, the PCR products approximately 260 bp were amplified with primers F-91: CGATTCCGGCTTCCGCCCGACCGC and R-91: ATTTCCCTCAGCATCTCCATCGCCA (Fig. 2A). The amplification conditions were 95 °C for 5 min followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, DNA extension at 72 °C for 30 s and a final extension at 72 °C for 5 min. Then these products were isolated by using the Cycle pure kit (Axygen), and resulting product were further digested with the restriction enzyme *Taq* I (Takara). A 100 ng DNA was digested with 10 U of *Taq* I at 65 °C for 60 min. The resulting restriction fragments were electrophoresed in 2% agarose gels and visualized under UV light. The *GyrA* codon 91 mutation TCG → CCG could eliminate the *Taq* I site (TTCGA) in wild-type *GyrA* and could be

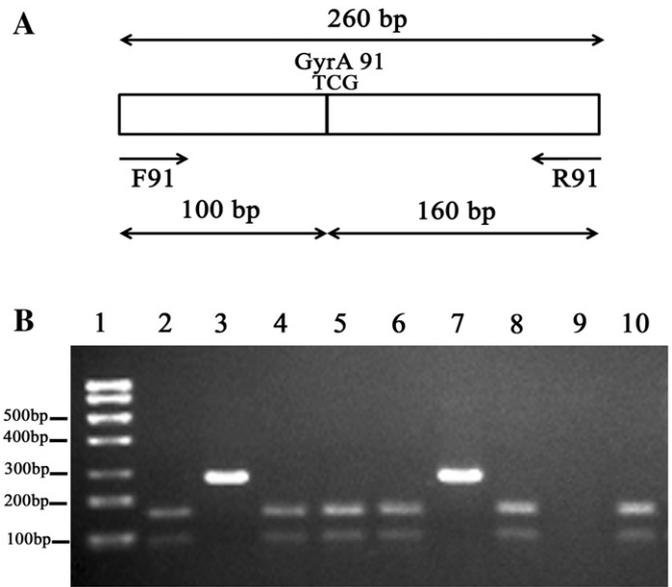


Fig. 2. Schematic view and 2% agarose gel electrophoresis of PCR-RFLP results. A: The vertical line represents *Taq* I recognition site, short arrows indicate the primers used for PCR-RFLP, long double-headed arrow depicts the resulting PCR fragment, and short double-headed arrow represents the two enzyme fragments. A unique *Taq* I recognition site was found in 91 codon from fluoroquinolone-susceptible strains, but not in 91 codon mutated strains. 100-bp band and 160-bp band represent fluoroquinolone-susceptible isolates that had no mutation at 91 codon, 260-bp band, 91 codon mutated. B: Lane 1, DNA ladder; lane 2, 90 codon GCG → GTG; lane 3, 91 codon TCG → CCG; lane 4, 94 codon GAC → GGC; lane 5, 94 codon GAC → GCC; lane 6, 90 codon and 94 codon GCG → GTG and GAC → GGC; lane 7, 91 codon and 94 codon TCG → CCG and GAC → GGC; lane 8, fluoroquinolone-susceptible strain; lane 9, the negative control; lane 10, H37Rv.

detected by cleavage with the restriction enzyme reaction. As a result, the size of the digest was 260 bp for the 91 codon mutated strains.

125 *M. tuberculosis* clinical isolates (including 65 fluoroquinolone-resistant and 60 fluoroquinolone-susceptible *M. tuberculosis*) from TB cases and H37Rv control strain were studied for identification of mutation in the *gyrA* gene by DNA sequencing as well as the combined use of MAS-PCR and PCR-RFLP. The results of nucleotide sequencing showed that the AGC → ACC mutation at codon 95 was observed in all clinical isolates except for H37Rv. 52 of the 65 (80%) fluoroquinolone resistant *M. tuberculosis* carried additional *gyrA* mutations apart from the codon 95 mutations. Of these 52 *gyrA* mutation isolates, point mutation types were clustered within codon 90, 91 and 94 mutations (Table 1).

Twelve isolates carried a mutation at codon 90, with a resulting Ala → Val change at this position. Nine isolates contained a mutation at codon 91, causing a Ser → Pro change. Codon 94 was the most frequently mutated site. Thirty-three of the 65 fluoroquinolone-resistant

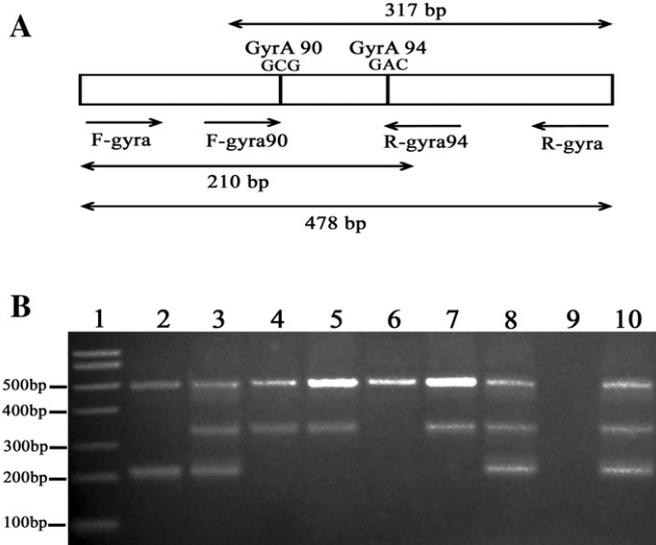


Fig. 1. Schematic presentation and 2% agarose gel electrophoresis of MAS-PCR assays. A: The vertical line represents the position of each mutation, short arrows indicate the primers used for MAS-PCR, and long double-headed arrows depict the resulting PCR fragments. 210-bp, 317-bp and 478-bp bands represent fluoroquinolone-susceptible isolates that had no mutation at any of the 90 and 94 codon; 210-bp and 478-bp bands, 90 codon mutated; 317-bp and 478-bp bands, 94 codon mutated; only 478-bp band, both 90 and 94 codon mutate at the same time. B: Lane 1, DNA ladder; lane 2, 90 codon GCG → GTG; lane 3, 91 codon TCG → CCG; lane 4, 94 codon GAC → GGC; lane 5, 94 codon GAC → GCC; lane 6, 90 codon and 94 codon GCG → GTG and GAC → GGC; lane 7, 91 codon and 94 codon TCG → CCG and GAC → GGC; lane 8, fluoroquinolone-susceptible strain; lane 9, the negative control; lane 10, H37Rv.

Table 1

Results of *gyrA* sequencing of fluoroquinolone resistant isolates.

Codon H37Rv sequence/ amino acid	Mutations sequencing/ amino acid	Mutation frequency (isolates)/(%)
90 GCG/Ala	GTG/Val	11 (16.9)
91 TCG/Ser	CCG/Pro	8 (12.3)
94 GAC/Asp	AAC/Asn	1 (1.5)
94 GAC/Asp	TAC/Tyr	4 (6.2)
94 GAC/Asp	CAC/His	1 (1.5)
94 GAC/Asp	GCC/Gly	12 (18.5)
94 GAC/Asp	GCC/Ala	13 (20.0)
90 GCG/Ala + 94 GAC/Asp	GTG/Val + GGC/Gly	1 (1.5)
91 TCG/Ser + 94 GAC/Asp	CCG/Pro + GCC/Ala	1 (1.5)
	No mutation	13 (20.0)

Table 2

The evaluation of the drug susceptibility testing and the combined use of MAS-PCR and PCR-RFLP among 125 clinical isolates.

Methods	Results	Drug susceptibility testing results		Sensitivity (%)	Specificity (%)	Accuracy (%)
		Resistant	Susceptible			
MAS-PCR and PCR-RFLP	Mutated	46	0	70.8	100	84.8
	Not mutated	19	60			

isolates had mutations at this site, resulting in a total of five different types of amino acid changes (Asp → Ala, Asp → Gly, Asp → Tyr, Asp → His, Asp → Asn). Of the 33 isolates with 94 codon mutation, two were found to have another mutation at 90 or 91 codon. The great majority of isolates that possessed a *gyrA* mutation exhibited a characteristic MAS-PCR or PCR-RFLP pattern and readily distinguished by visual inspection from those of the wild-type sequence. Fig. 1B shows the MAS-PCR generated distinct banding patterns for different mutation profiles. Amplification of 210, 317 and 478 bp products indicated the presence of wild-type sequence of fluoroquinolone susceptible strains and H37Rv. The absence of 317 or 210 bp products indicated detection of the GyrA 90A or 94D mutations. The appearance of the product only 478 bp showed that both 90A and 94D of GyrA mutation at the same time. The results of DNA sequencing demonstrated all 11 isolates carrying 90 codon mutation and 27 isolates carrying 94 codon mutation (including 2 isolates having two mutations) were successfully detected by the MAS-PCR.

Fig. 2B shows the PCR-RFLP analysis that was performed to differentiate 91S mutated strains from non-91S mutated strains. As expected, the smaller fragments of 100 bp and 160 bp were detected from non-91S mutated strains digested with *Taq* I, whereas the 91S mutation strains remained undigested. DNA sequencing also proved that PCR-RFLP analysis could be applied for the characterized 91S mutated strains from non-91S mutated *M. tuberculosis*.

Correlation between the drug susceptibility testing and the combined use of MAS-PCR and PCR-RFLP assay were evaluated the effects of determining mutations in clinical isolates. Using the results of phenotypic drug susceptibility testing as the reference standard, the sensitivity, specificity and accuracy of the DNA sequencing and the combined use of MAS-PCR and PCR-RFLP for detecting fluoroquinolone resistance were assessed, respectively (Table 2).

Of the 65 phenotypic DST proven fluoroquinolone resistant isolates, 46 were identified as ofloxacin resistance by MAS-PCR and PCR-RFLP; all 60 ofloxacin sensitive isolates defined by phenotypic DST were classified as fluoroquinolone sensitive isolates by MAS-PCR and PCR-RFLP. The sensitivity, specificity and accuracy of MAS-PCR and PCR-RFLP were 70.8%, 100% and 84.8% respectively.

The use of the MAS-PCR assays to investigate the incidence of fluoroquinolone resistance was shown in a previously reported study (Evans and Segal, 2010). However, their method only detected the GyrA D94G mutation (Evans and Segal, 2010), which only accounted for 18.5% (12/65) of fluoroquinolone resistance in our research. The MAS-PCR assay we used could detect GyrA A90V, D94G and D94A mutations which accounted for 58.5% (38/65) mutations of fluoroquinolone resistance. We had tried to design the many primers for the MAS-PCR assay detecting the mutation at codon 91. The results were not satisfactory. Since the codon 91 of the wild type Gyr A containing the *Taq* I site (T↑CGA), we used the ordinary PCR and RFLP to detect the mutation at codon 91. Our results showed that PCR-RFLP assay could identify all strains with a mutation at codon 91 (8/65). Thus, the combined use of MAS-PCR and PCR-RFLP could successfully detect 70.8% (46/65) of fluoroquinolone resistance. Moreover, our method based exclusively PCR and ordinary agarose gel electrophoresis without any supplementary sequence analysis.

In this study, amino acids at positions 90, 91 and 94 of GyrA are the most frequently substituted sites among fluoroquinolone resistant clinical isolates, largely verifying of some previous studies (Ginsburg et al., 2003a;

Ginsburg et al., 2003c; Shi, Zhang, et al., 2006). Previous reported mutations involving codon 88 (Pitaksajjakul et al., 2005) were not found in our strains. Our method was based on the codon 90, 91 and 94 mutations of *gyrA*. This led to a limitation that fluoroquinolone resistant isolates without *gyrA* mutation (19/65) were not identified by this method. However, such resistance was usually low-level phenotypic resistance (ofloxacin MIC around 2 mg/l) (Cheng et al., 2004) which might be related to mutations outside the QRDR of *gyrA* (Mokrousov et al., 2008), changes in outer membrane permeability or an overexpression of efflux pump exporting fluoroquinolones (Kocagoz et al., 1996; Escribano et al., 2007). Furthermore, this method only identified the mutation occurring on the second base of codon 94 in *gyrA*, which was the most frequent mutation for the codon 94 (27/33). Six with mutation occurring on the first base of codon 94 could not be detected by this method. Therefore, the combined use of MAS-PCR and PCR-RFLP could not replace phenotypic drug susceptibility testing. It may provide an essential initial diagnosis and further optimization of this method remains an important focus of future studies.

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