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ORIGINAL ARTICLE

Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated from South-central in China

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Rifampicin (RIF) and isoniazid (INH) *Mycobacterium tuberculosis* isolates were characterized from South-central China and transmission patterns within the Beijing genotype were detected in multidrug-resistant isolates. Six genetic regions, including *rpoB* for RIF, and *katG*, *inhA*, *ahpC*, *mabA-inhA* promoter and *oxyR-ahpC* intergenic region for INH were analyzed by DNA sequencing in 60 multidrug-resistant isolates, including 7 extensively drug-resistant isolates. The genomic deletion RD105 was characterized by genotyping. The results showed that 91.7% of MDR isolates carried mutations in the *rpoB* gene and 85.0% of the MDR isolates had at least one mutation in the INH resistance-associated loci detected. In total, these six genetic regions are responsible for 95.0% of MDR isolates. Mutations in the XDR isolates were focused on *rpoB* 531 or *rpoB* 526, and *katG* 315, correlating to a higher frequency level of resistance to RIF MIC $\geqslant 8\,\mu g\,ml^{-1}$ and INH MIC $\geqslant 4\,\mu g\,m^{-1}$. Three novel *katG* mutants (G273S, I266T and P232S) and three new alleles (E458A, S509R and P535S) in the *rpoB* gene were identified. Among the 85 clinical isolates, 78 are Beijing genotypes and the other 7 are non-Beijing genotypes. The results present the identification of genetic markers in *M. tuberculosis* isolates, some of which may be unique to this particular geographic niche. An understanding of the mutations in these drug-resistant strains may aid in choosing the appropriate chemotherapy regimens on the pharmacogenetic properties of the mutations for the prevention and control of tuberculosis.
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Keywords: Beijing genotypes; extensively drug resistance; *M. tuberculosis*; multidrug-resistance; mutation

INTRODUCTION

Tuberculosis (TB) remains one of the major causes of death around the world, especially due to the emergence of multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB). There were approximately 440 000 cases of MDR-TB in 2008, which were resistant to both rifampicin (RIF) and isoniazid (INH). Almost 50.0% of MDR-TB cases worldwide are estimated to occur in China and India.[1] Approximately 8.0% of the patients with MDR-TB have XDR-TB, which is defined as resistance to at least RIF and INH, plus resistance to any fluoroquinolone and at least one of three injectable anti-TB drugs (capreomycin, kanamycin or amikacin).[2] Therefore, controlling tuberculosis caused by drug-resistant *M. tuberculosis* has become an urgent public health problem in many regions of the world, particularly in developing countries.

RIF and INH are the principal first-line drugs used in combination in tuberculosis chemotherapy. Therefore, it is important to understand the molecular basis for this resistance.[3] RIF, as a broad spectrum derivative of RIF, exerts its anti-tuberculosis role by binding to the bacterial RNA polymerase, thereby inhibiting RNA synthesis. Ninety-five percent of the resistance to RIF in *M. tuberculosis* is due to single mutation in an 81-bp core region, termed the RIF resistance-determining region (RRDR) of the *rpoB* gene, which encodes the beta subunit of DNA-dependent RNA polymerase.[4] INH is the first-line drug responsible for the initial dramatic decrease in actively metabolizing bacilli during treatment of TB, yet its mechanism of resistance remains only partially understood. Unlike RIF, INH resistance is associated with mutations in multiple loci, such as the catalase-peroxidase gene (*katG*), the enoyl-ACP reductase gene (*inhA*) and its promoter, the alkyl hydroperoxide reductase gene (*ahpC*), and the intergenic region between the *oxyR* and *ahpC* genes.[5,6]

Traditional culture-based methods of testing for drug resistance can take at least 4 weeks, as *M. tuberculosis* is a slow-growing organism, leading to higher mortality and the further spread of MDR infections. Thus, various molecular methods have been used to detect MDR-TB by identifying mutations associated with drug resistance.[7,8] Among these methods, DNA sequencing is the most direct and reliable, and can rapidly detect both known and novel mutations.

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It has been reported that some mutations have a geographical distribution.[8–10] However, little data have been available on the molecular characterization of multidrug-resistant clinical isolates of *M. tuberculosis* from South-central region in China. In this study, the type and frequency of mutations in the *rpoB*, *katG*, *mabA-inhA* regulatory region, the structural gene, *ahpC*, and the *oxyR-ahpC* intervening region were investigated. The MICs of RIF and INH for MDR strains were determined. These studies identified genetic markers of MDR *M. tuberculosis* isolates that are unique to this geographical region and will further the understanding of the molecular mechanism of drug resistance, particularly multi-drug resistance in *M. tuberculosis*. Currently, the mechanism of multidrug resistance in *M. tuberculosis* still needs to be elucided. Based on the analysis of the relationship between the gene typing and MIC, the findings of present study further reveal the molecular mechanism of MDR-TB strains in China. Also, the results help to supplement and optimize the current molecular detection techniques, which assist in the personalized treatment for the MDR-TB patients. In addition, the molecular characterization identified recent transmission patterns of multidrug-resistant isolates of the Beijing genotype in South-central China.

Large numbers of reports have confirmed that the Beijing genotype strains had a deletion in RD105, which was a unique characteristics of Beijing genotypes isolates. Therefore, the deletion of RD105 sequence can serve as a simple and rapid method to identify Beijing genotypes strains. Analyzing the distribution and transmission of MTB in this area can provide a theoretical basis for controlling and preventing infectious diseases in Hubei Province.[11–12]

MATERIALS AND METHODS

Clinical isolates and drug susceptibility testing (DST)

A total of 1733 clinical isolates of *M. tuberculosis* was obtained from 5299 patients with pulmonary tuberculosis in the Wuhan Medical Treatment Center, Hubei, South-central China, from August 2009 to July 2010. All isolates were routinely cultured with egg-based Löwenstein-Jensen medium at 37 °C and were evaluated by proportion method for DST.[13]

Proportion method was performed on DSTs use the following drug concentrations in the modified Löwenstein-Jensen medium: RIF, 40.0 μg ml^{-1}; INH, 0.2 μg ml^{-1}; ethambutol, 2.0 μg ml^{-1}; streptomycin, 4 μg ml^{-1}. In addition, some second-line drugs, widely used in China, were also performed on DSTs with the following concentrations: kanamycin, 30.0 μg ml^{-1}; paramycin, 1.0 μg ml^{-1}; protionamide (Pto), 40.0 μg ml^{-1}; ofloxacin, 2.0 μg ml^{-1}; thiophen-2-carboxylic acid hydrazine (TCH), 5.0 μg ml^{-1}; para-nitrobenzoic acid (PNB), 500 μg ml^{-1}.

The sodium 2,3-bi(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT) colorimetric method[14] was performed in 7H9-S medium containing Middlebrook 7H9 broth, 10% oleic acid albumin dextrose catalase and 0.5% glycero and 0.1% Casitone. By using 96-well plates, the MIC was determined with 7H9-S medium containing twofold dilution series of the RIF ranging from 0.125 to 8 μg ml^{-1}, the INH ranging from 0.063 to 4 μg ml^{-1}.

Sixty-five clinically resistant isolates, including 60 MDR strains and 7 extensively drug resistant (XDR) strains, were selected from 664 (38.3%, 664/1733) drug-resistant strains, and 20 pan-susceptible stains were also randomly chosen as control (Table 1).

DNA extraction and isolates identified

Genomic DNA of *M. tuberculosis* isolates was extracted as previously described.[15] *M. tuberculosis* culture isolates previously identified by conventional biochemical methods were confirmed by 16S rRNA gene sequencing.[16]

PCR amplification and sequencing

Oligonucleotide primers were designed from the *M. tuberculosis* H37Rv genome sequence[17] using Primer Premier 5.0 (PREMIER Biosoft, Palo Alto, CA, USA) and Oligo 6 software (Molecular Biology Insights, Inc., Cascade, CO, USA). The uniqueness of the sequences of the primers designed was analyzed with the Blast search (http://www.ncbi.nlm.nih.gov). The nucleotide sequences for the mutant genes obtained in the present study were deposited in the GenBank database (Table 2). Primers were synthesized by Invitrogen Bio Co. (Shanghai, China). Six fragments were amplified and sequenced: *rpoB* (including the 81-bp RRDR), *katG* (including codon 315), *inhA*, *ahpC* gene, *mabA-inhA* regulatory region and the *oxyR-ahpC* intergenic region. Sequence data were assembled and analyzed by CLUSTAL W software (European Bioinformatics Institute). PCR amplification was performed with the EDC-810 model thermal cycler (Eastwin Life Sciences, Inc., Beijing, China), and the *Pfu Taq* DNA polymerase with high fidelity was used to avoid false priming. The amplicons were purified with the E.Z.N.A. MicroElute Cycle-Pure Kit (Omega, Lilburn, GA, USA). Sequence analysis was determined on both strands by direct sequencing of the PCR products on an automated model 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) with fluorescence-labeled dideoxynucleotide terminators (ABI Prism Big Dye terminator cycle sequencing ready reaction kit; Sangaon Biotech Co., Ltd, Shanghai, China).

Table 1 Prevalence of drug-resistance phenotypes among clinical *M. tuberculosis isolates* ($n = 85$)

		No. of isolates	
Resistance phenotypes	*No. of isolates*	*Beijing* (n = *78*)	*Non-Beijing* (n = *7*)
Fully susceptible	20	18	2
MDR	60	56	4
XDR	8		
Other phenotypes	5		
S	1	1	
H	1	1	
HS	2	2	
HLP	1		1

Abbreviations: H, isonicotinic acid hydrazide; L, levofloxacin; MDR, multidrug resistance; P, pyrazinamide; S, streptomycin; XDR, extensively drug resistance.

Table 2 Primers used in this study and resulting PCR products

Drug	*Accession no. (Genbank)*	*Primer*	*Sequence (5′ → 3′)*	*Product (bp)*
Rifampicin	L27989	*rpoB-F*	CGCTTGCACGAGGGTCAGA	428
		rpoB-R	GTTTCGATCGGGCACATCC	
Isoniazid	X68081	*katG-F*	TCGGCGATGAGCGTTACAG	459
		katG-R	CGTCCTTGGCGGTGTATTG	
	BX842579	*ahpC-F*	ACCTTTGCCTGACAGCGACT	667
		ahpC-R	TAGGCCGAAGCCTTGAGGAG	
	CP003248	*inhA-F*	CGGCATGGGTATGGGCCACT	751
		inhA-R	CGGCGTCGCATCCTTCATGT	
	Z81451	*oxyR-ahpC-F*	CGCAACGTCGACTGGCTCATA	359
		oxyR-ahpC-R	GCCTGGGTGTTCGTCACTGGT	
	U41388	*mabA-inhA -F*	CCTCGCTGCCCAGAAAGGGA	248
		mabA-inhA -R	ATCCCCCGGTTTCCTCCGGT	

Genotyping method

The identification of Beijing genotypes strains was performed using the RD105 deletion-targeted amplified by PCR.[18] Primers were designed flanking this 3467 base pair (bp) deletion polymorphism P1 and P2 to detect the presence or absence of this deletion in the *M. tuberculosis* genome. A third primer P3, which is internal to deletion RD105, was also utilized. A χ^2 test was performed with SPSS17.0 software (SPSS China, Shanghai, China) to analyze the distribution characteristics of Beijing genotypes.

RESULTS

All 85 strains chosen for analysis were confirmed as *M. tuberculosis* by 16S rRNA gene sequencing. Specific resistant genes or regulatory regions were sequenced, and the sequencing results were compared with the DST results. No mutation was found in the pan-susceptible isolates.

Mutation patterns in MDR and XDR isolates

Forty-nine of the sixty MDR were found to have a mutation in the six characterized genetic regions. Forty-one MDR isolates (68.3%,41/60) were found to carry mutations in the *rpoB* gene and *katG* gene, and 21 isolates (35.0%, 21/60) contained mutations in both *rpoB*531 and *katG*315. Sequencing of three MDR isolates failed to identify any mutations in the six regions investigated. Therefore, these six genetic regions account for 95.0% (57/60) of the MDR isolates characterized.

Of the 49 MDR isolates, 44 (89.8%) were Beijing genotypes and the other 5 were non-Beijing genotypes. Among the 36 non-MDR, 34 (94.4%) were Beijing genotypes and 2 isolates were non-Beijing genotypes ($\chi^2 = 0.593$, $P = 0.441$).

Among the seven XDR isolates, four were found to possess alterations in *rpoB* 531 and *katG* 315, and one of them had a third mutation in the *mabA-inhA* regulatory region (C-15T; Table 3). Two of the remaining three XDR isolates carried mutations in three fragments, one had mutations in *rpoB* H526L (CAC→CTC), *katG* S315T (AGC→ACC) and *inhA* A190S (GCC→TCC); and the other isolate had a mutation in *rpoB* S531L (TCG→TTG) with a seven amino acids (GKTHGAG) insertion mutation in *katG* 279-280, plus base substitutions C-15T in *mabA-inhA* regulatory region. The last isolate harbored mutations in both *rpoB* Q517(CAG deletion) and *mabA-inhA* regulatory region C-15T. All of the seven XDR were non-Beijing genotypes.

RIF resistances and *rpoB* gene

Of the 60 RIF resistance isolates, 55 (91.7%, 55/60) carried mutations in RRDR of the *rpoB* gene, 45 isolates had a single-point mutation, 8 had double mutations (13.3%, 8/60), 2 had a deletion that was the same in both isolates and 5 have no mutation (8.3%, 5/60, MIC> 2–8 μg ml^{-1}; Table 4). The most frequently mutated *rpoB* codons were codon 531, 526 and 516, with mutation frequencies of 51.7% (31/60, MIC⩾2–8 μg ml^{-1}), 18.3% (11/60, MIC>2–8 μg ml^{-1}) and 10.0% (6/60, MIC>2–8 μg ml^{-1}), respectively. Consistent with previous reports, the mutation S531L was the most common mutation in *rpoB* gene. Codon 526 harbored the most variable mutations. Six isolates exhibited codon 516 mutations, including D516V and D516G. Eight isolates with seven different types of double mutations were identified. In addition, one deletion was detected at codon 517 (CGC, MIC⩾4 μg ml^{-1}) in two stains. Of these mutations, three have not previously been described, E458A (GCG→GAG; GenBank: JF812083), S509R (AGC→AGA) and P535S (CCC→TCC). Five isolates (8.3%) had no mutation in the RRDR of *rpoB* gene. There are 55 high-level (MIC⩾4–8 μg ml^{-1}) and 5 low-level (MIC⩽0.5–4 μg ml^{-1}) RIF resistance strains among 60 MDRs.

Of the 55 RIF mutant strains, 51 (92.7%) were Beijing genotypes and 4 (7.3%) were non-Beijing genotypes. Besides, among the 30 RIF-susceptible strains, 27 (90.0%) were Beijing genotypes and 3 (10.0%) isolates were non-Beijing genotypes ($\chi^2 = 0.191$, $P = 0.662$).

INH resistances and *katG, inhA, ahpC mabA-inhA* and *oxyR-ahpC*

In 84.4% (54/64) of the INH resistances strains, at least one mutation was found in the *katG, inhA, ahpC, mabA-inhA* regulatory region and *oxyR-ahpC* intergenic region, which included mutations at *katG* codon 315 in isolates. And no mutation was found in 10 MDR isolates (15.6%, MIC⩾2–4 μg ml^{-1}; Table 5). Of the 64 INH resistances, 70.3% (45/64) had genetic alterations in *katG* nucleotide fragment. Mutations at codon 315 in the *katG* region contributed the majority of INH resistance, with high frequency of 62.5% (40/64, MIC>1–4 μg ml^{-1}) isolates, and the common base substitution mutation S315T (AGC→ACC) was the most frequently alteration. Three isolates carried other exchanges in codon 315. In addition, we found three novel types of mutation in *katG* gene, G273S (GGT→AGT, MIC>4 μg ml^{-1}; GenBank: HQ913569), I266T (ATC→ACC, MIC=4 μg ml^{-1}; GenBank: HQ913567) and P232S (CCG→TCG,

Table 3 Mutations in genes associated with INH and RIF of the XDR-TB clinical isolates ($n = 7$)

		Genetic alteration					
Isolates	*Drug resistance*	*rpoB*	*katG*	*ahpC*	*inhA*	*oxyR-ahpC*	*mabA-inhA*
WH40	IRSKL	S531L TCG→TTG	S315T AGC→ACC	WT	WT	WT	WT
WH50	IRSELK	S531L TCG→TTG	S315T AGC→ACA	WT	WT	WT	WT
WH72	IRSELK	S531L TCG→TTG	S315T AGC→ACC	WT	WT	WT	C-15T
WH88	IRSELPK	H526L CAC→CTC	S315T AGC→ACC	WT	A190S GCC→TCC	WT	WT
WH90	IRSEPLK	S531L TCG→TTG	GKTHGAG insert in 279-280	WT	WT	WT	C-15T
WH93	IRSELK	Q517(CAG deletion)	WT	WT	WT	WT	C-15T
WH100	IRSELK	S531L TCG→TTG	S315T AGC→ACC	WT	WT	WT	WT

Abbreviations: E, ethambutol; I, isoniazid (INH); K, kanamycin; L, levofloxacin; P, p-aminosalicylic acid; R, rifampin (RIF); S, streptomycin; WT, wild type; XDR-TB, extensively drug-resistant

tuberculosis.

Table 4 Distrbution of mutations in the *rpoB* gene among MDR-TB clinical isolates ($n=60$)

				No. of isolates		
Locus	*Mutations*[a]	*Nucleotide change*	*No. of isolates (%)*	*Beijing (n=56)*	*non-Beijing (n=4)*	*MIC (μg ml^{-1})*
531	S531L	TCG→TTG	31 (51.7)	26	3	⩾8
				1		4
				1		2
526	H526Y	CAC→TAC	2 (3.3)	2		>8
	H526L	CAC→CTC	3 (5.0)	2		4
				1		2
	H526R	CAC→CGC	1 (1.7)	1		None[b]
	H526P	CAC→CCC	1 (1.7)	1		>8
	H526C	CAC→TGC	1 (1.7)	1		2
526 and 535	H526T	CAC→ACC	1 (1.7)	1		>8
	P535S	CCC→TCC				
526 and 509	H526R	CAC→CGC	1 (1.7)	1		⩾4
	S509R	AGC→AGA				
526 and 511	H526N	CAC→AAC	1 (1.7)	1		⩾4
	H511P	CTG→CCG				
516	D516V	GAC→GTC	3 (5.0)	3		⩾2–4
516 and 511	D516G	GAC→GGC	2 (3.3)	1	1	⩾8
	H511P	CTG→CCG				
	D516V	GAC→GTC	1 (1.7)	1		
	H511P	CTG→CCG				
533	L533P	CTG→CCG	1 (1.7)	1		0.5
	L533L	CTG→TTG	1 (1.7)	1		4
533 and 511	L533P	CTG→CCG	1 (1.7)	1		4
	H511P	CTG→CCG				
513 and 458	Q513P	CAA→CCA	1 (1.7)	1		>8
	E458A	GAG→GCG				
517	Q517del[b]	CAG→del	2 (3.3)	2		⩾4
527	K527Q	AAG→CAG	1 (1.7)	1		>8
None	Wild type	Wild type	5 (8.3)⩾0.125	4		>8
				1		2

Abbreviations: del, deletion; MDR-TB, multidrug-resistant tuberculosis.
[a]Mutations in the *rpoB* gene are denoted as follows: first, the amino-acid residue in the wild-type sequence; second, the codon position and third, the amino-acid residue in the mutated sequence.
[b]Bacterial strain has died.

MIC = 4 μg ml^{-1}; GenBank: HQ913568). Interestingly, a 10.8-kb fragment deletion in WH56 isolate comprising the *katG* with seven upstream genes and one downstream gene was found in one MDR isolate (Figure 1). In addition, one novel insert mutation was identified in the *katG* gene, resulting in a seven-amino-acid, GKTHGAG, insert between codons 279 and 280 (MIC⩾4 μg ml^{-1}).

Mutations in *inhA* and *ahpC* with their noncoding regions were identified in 21.9% (14/64) isolates. No alteration was found in the *inhA* structural gene, whereas one mutation was detected in *ahpC* structural gene: A190S (GCC→TCC). In contrast, 12.5% (8/64) of the INH-resistant isolates harbored base substitutions C-15T in the *mabA-inhA* regulatory region. Three of them had an additional mutation in the *katG* gene. 9.4% (6/64) isolates had nucleotide substitutions at loci of −46, −52 and -54 in the *oxyR-ahpC* intergenic region, and two of isolates had an additional mutation in *katG* gene. There are 56 high-level (MIC⩾2–4 μg ml^{-1}) and 8 low-level (MIC⩽0.25–2 μg ml^{-1}) INH resistance strains among 64 MDRs.

Of the 64 INH mutant strains, 59 (92.2%) were Beijing genotypes and 5 (7.8%) were non-Beijing genotypes. Among the 30 INH-susceptible strains, 19 (90.5%) were Beijing genotypes and 2 (9.5%) isolates were non-Beijing genotypes ($\chi^2=0.061$, $P=0.804$).

Genotyping of the Beijing genotypes strains

PCR was performed on the 85 clinical isolates of *M. tuberculosis* to identify the Beijing genotype. We obtained a 1466-bp PCR amplified product for the non-Beijing and H37Rv, and a 761-bp for the Beijing genotype (Figure 2). PCR on 78 (91.8%) obtained a 761-bp product, identifying a Beijing isolate and 7 strains (8.2%) did not generate the expected PCR products, identifying a non-Beijing isolate. The H37Rv strain was identified as a non-Beijing isolate (Table 1).

DISCUSSION

It has been reported that the frequency of *rpoB* mutations range from 76.0% (19/25)[19] to 99.0% (102/103),[20] suggesting that mutations in the *rpoB* gene are possible molecular markers for MDR-TB. In this study, the mutation frequency of *rpoB* gene was 91.7% (55/60) consistent with the observed resistance prevalence around the world.[8,21–23] The most frequently mutated *rpoB* codons were 531, 526 and 516 (MIC⩾2–8 μg ml^{-1}), which have been reported elsewhere,[10,20,21] indicating that there is no geographic difference in the main mutation types and mutation rates of *rpoB* gene in South-central China. Double mutations were observed in 8/60 (13.3%) isolates in this study, which is higher than reported in other studies from Shanghai, 7.4% (18/242),[23] and Germany, 2.0%,[20] but lower

Table 5 Mutations in the genes of *katG, inhA, ahpC mabA-inhA* and *oxyR-ahpC* INH-resistant isolates ($n=64$)

	Mutations[a,b]				*No. of isolates*		
Genes	*Locus*	*Nucleotides*	*Amino acids*	*No. of isolates (%)*	*Beijing ($n=59$)*	*non-Beijing ($n=59$)*	*MIC ($\mu g\,ml^{-1}$)*
katG	*katG* 315	AGC→ACC	S→T	33 (51.6)	26	2	⩾4
					4	1	⩽1–2
		AGC→AAC	S→N	1 (1.6)	1		⩾4
		AGC→ACA	S→T	1 (1.6)	1		4
		AGC→GGC	S→G	1 (1.6)	1		⩾4
	katG 232	CCG→TCG	P→S	1 (1.6)	1		4
	katG 266	ATC→ACC	I→T	1 (1.6)	1		4
	katG 279 -280	7 Amino acids GKTHGAG insertion		1 (1.6)	1		⩾4
katG and *oxyR-ahpC*	*katG*	10.8 kb Deletion		1 (1.6)	1		None[c]
	oxyR-ahpC -52	C→T					
	katG 273	GGT→AGT	G→S	1 (1.6)	1		>4
	oxyR-ahpC -52	C→T					
katG and *mabA-inhA*	*katG* 315	AGC→ACC	S→T	3 (4.7)	3		
							4
	mabA-inhA -15	C→T					
katG and *inhA*	*katG* 315	AGC→ACC	S→T	1 (1.6)	1		4
	ahpC 190	GCC→TCC	A→S				
oxyR-ahpC	−46	Insert one nucleotides T		1 (1.6)	1		0.5
	−52	C→A		1 (1.6)	1		>2
	−54	C→T		2 (3.1)	2		⩾2
ahpC	none	None		0 (0.0)	0		
mabA-inhA	−15	C→T		5 (7.8)	2		<0.25–1
					3		⩾2
None	None	Wild type		10 (15.6)	8	2	⩾2–4

Abbreviation: INH, isoniazid.
[a]Mutations in the *katG, inhA* and *ahpC* genes are denoted as follows: first, the amino-acid residue in the wild-type sequence; second, the codon position and third, the amino-acid residue in the mutated sequence.
[b]Mutations in the *oxyR-ahpC* intergenic region and *mabA-inhA* regulatory region are denoted as follows: the nucleotide change is indicated relative to the start of the following open reading frame for mutations at the *mabA-inhA* regulatory region.
[c]Bacterial strain is died.

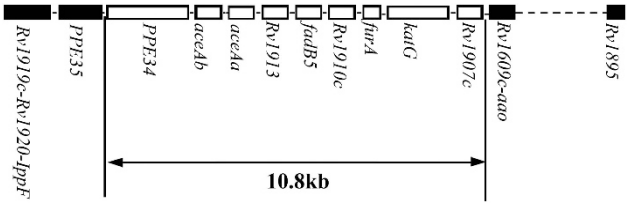

Figure 1 A 10.8-kb gene deletion fragment in WH56 isolates. White box represents for the genes deleted.

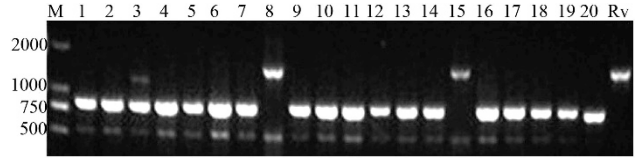

Figure 2 RD105 amplified by PCR. Lane M: DL 2000 Marker; Lane 1–20: 20 clinical stains; Rv: H37Rv (non-Beijing family strain).

than the 76.1% reported from isolates in Afghanistan.[24] Nucleotide changes in codon 531: TCG→TTG, 51.7% (31/60), were more predominantly observed among isolates, and correlating to a higher frequency level of resistance to RIF MIC⩾8 μg ml^{-1} in our study (Table 4). It is unclear whether novel mutation types E458A (GAG→GCG, MIC>8 μg ml^{-1}), P535S (CCC→TCC, MIC>8 μg ml^{-1}) and S509R (AGC→AGA, MIC⩾4 μg ml^{-1}) are associated with RIF resistance because these isolates also carried mutations commonly associated with RIF resistance.[25] Five isolates (8.3%, MIC>2–8 μg ml^{-1}) had no mutation in the *rpoB* gene, in accordance with the previous reports,[20] which suggest that the mutations may be elsewhere or that there are unknown mechanisms of drug resistance.

It has been reported that *M. tuberculosis* resistance to INH was the first step in the evolution of a MDR phenotype.[26] 71.7% (43/60) MDR isolates in our study were observed to have mutations in the *katG* fragments, consistent with the reports from many different geographical regions.[10,23,27] The most common mutation in the *katG* gene was at codon 315, 62.5% (40/64, MIC⩾4 μg ml^{-1}), similar to the mutation rates previously reported as 72.7% in Shanghai, China,[23] 68.4% in Cairo, Egypt,[16] and 68.7% in California, USA.[10] However, this is lower than the mutation rates reported in the Russian Federation (93.6%) and in Brazil (87.1%).[23] It appears that mutation in *katG* 315 can show geographical differences in frequency and may not be a suitable independent marker for INH resistance. In this study, nucleotide changes in codon 315: AGC→ACC, 62.5%(40/64), were more predominantly observed among isolates, and correlating to a higher frequency level of resistance to INH MIC⩾4 μg ml^{-1} (Table 5). However, it remains to be

determined if the novel mutations G273S (MIC > 4 μg ml^{-1}), I266T (MIC = 4 μg ml^{-1}), P232S (MIC = 4 μg ml^{-1}) and the 10.8-kb deletion affect susceptibility to INH.

We also sequenced the structure genes of *inhA* and *ahpC*, the *mabA-inhA* regulatory region and *oxyR-ahpC* intergenic region. No mutation was detected in the structure gene of *inhA* in all 85 isolates. However, 12.5% (8/64) of the INH-resistant isolates harbored base substitutions C-15T in the *mabA-inhA* regulatory region, consistent with the prior investigation.[28,29]

In this study, only one mutation, A190S, in the *inhA* structural gene was found, coupled with S315T mutation in the *katG* gene, a mutation frequency much lower than previous reports.[30] Among all mutations in *oxyR-ahpC* intergenic region, one isolate also had a G273S (GGT→AGT) novel mutation in *katG* gene. An association between *oxyR-ahpC* intergenic region mutations and mutations in *katG* other than at codon 315 has been reported previously.[31] Our results implied that the relationship between mutations of *ahpC* structural gene and *oxyR-ahpC* intergenic region, and INH resistance still is not very clear, and is worthy of further investigation.

Of particular importance, in this study, were seven XDR isolates, accounting for 11.7% (7/60) of the identified MDR isolates, which is much higher than previous reports.[32,33] Mutations in the XDR isolates were focused on *rpoB* 531 or *rpoB* 526, and *katG* 315. Recently, however, Müller *et al.* reported that the percentage of isolates exhibiting *mabA-inhA* regulatory region C-15T mutations increased significantly from 48.4% and 62.4%, respectively, in MDR isolates to 85.5% and 91.9% in XDR isolates.[34] The results of this study showed that only 42.9% (3/7) of XDR isolates were detected to have C-15T mutations, and these isolates also had an accompanying mutation in *katG* gene. Hence, more studies need to be performed to investigate if the *mabA-inhA* regulatory region was associated with the XDR isolates.

At the genetic level, gene alterations can change the structure of a target protein via mutations in the coding region or the amount of the protein expressed by modulating gene regulation, both of which ultimately cause the anti-drug resistance in *M. tuberculosis*.

In this study, the Beijing genotypes (91.8%) were the major epidemic genotypes among the 85 clinical isolates. There are no visible differences between the Beijing genotype and drug resistance. Beijing genotypes account for 91.8% of all isolates and non-Beijing genotype account for 7.2%. As the isolates in different regions had different characteristics, it is necessary to carry out studies on the relationship between Beijing genotype and drug resistance, pathogenicity and the ability to transmit.

In conclusion, this is the first report on the simultaneous genotypic analysis of the *rpoB*, *katG*, *inhA*, *ahpC*, *mabA-inhA* regulatory region and *oxyR-ahpC* intergenic region in South-central China *M. tuberculosis* isolates. These six gene regions account for 95.0% (57/60) of the MDR *M. tuberculosis* isolates. These information illustrate the need for further investigations to develop a more rapid and specific assay for the detection of MDR *M. tuberculosis* to be used as a screening method in areas. These observations also suggested that the drug resistance mechanisms of these first-line antituberculosis drugs were not completely clear and still need further research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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