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Molecular Characterization of Multidrug-Resistant *Mycobacterium tuberculosis* Isolates from China

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To investigate the molecular characterization of multidrug-resistant tuberculosis (MDR-TB) isolates from China and the association of specific mutations conferring drug resistance with strains of different genotypes, we performed spoligotyping and sequenced nine loci (*katG*, *inhA*, the *oxyR-ahpC* intergenic region, *rpoB*, *tlyA*, *eis*, *rrs*, *gyrA*, and *gyrB*) for 128 MDR-TB isolates. Our results showed that 108 isolates (84.4%) were Beijing family strains, 64 (59.3%) of which were identified as modern Beijing strains. Compared with the phenotypic data, the sensitivity and specificity of DNA sequencing were 89.1% and 100.0%, respectively, for isoniazid (INH) resistance, 93.8% and 100.0% for rifampin (RIF) resistance, 60.0% and 99.4% for capreomycin (CAP) resistance, 84.6% and 99.4% for kanamycin (KAN) resistance, and 90.0% and 100.0% for ofloxacin (OFX) resistance. The most prevalent mutations among the MDR-TB isolates were *katG315*, *inhA15*, *rpoB531*, *-526*, and *-516*, *rrs1401*, *eis-10*, and *gyrA94*, *-90*, and *-91*. Furthermore, there was no association between specific resistance-conferring mutations and the strain genotype. These findings will be helpful for the establishment of rapid molecular diagnostic methods to be implemented in China.

Tuberculosis (TB) remains a public threat to human health in China, with an incidence of 1.1 to 1.5 million cases per year (1). Owing to the implementation of the DOTS (directly observed treatment, short course) plan, the prevalence of TB used to be 30% lower (2). However, the increasing spread of multidrug-resistant TB (MDR-TB), which is resistant to at least the first-line antituberculosis agents (isoniazid [INH] and rifampin [RIF]), and the recent emergence of extensively drug-resistant tuberculosis (XDR-TB), with additional resistance to any fluoroquinolone (FQ) and at least one of the three second-line injectable drugs (capreomycin [CAP], kanamycin [KAN], and amikacin [AKM]), have become serious threats to effective TB control in China. According to the National Baseline Survey on Drug-Resistant Tuberculosis during 2007 to 2008 (3), approximately 8.32% of all notified TB cases were MDR-TB, and approximately 8% of the diagnosed MDR-TB cases were XDR-TB.

MDR- and XDR-TB is usually caused by inadequate anti-TB treatment or direct transmission of drug-resistant strains from one individual to another (4). The accurate and rapid detection of drug resistance is imperative for timely and adequate adjustments in treatment to interrupt the transmission of MDR- and XDR-TB (5). In this context, performing a molecular characterization of the drug target loci will contribute to the establishment of potential rapid molecular drug susceptibility testing (DST) as an alternative to traditional methods. However, previous reports have indicated that the frequency, location, and type of resistance-associated mutations vary in different geographical areas (6–8). Moreover, molecular epidemiological studies of TB have illustrated variations in the phylogeography of strains on a global scale (9, 10). Beijing family strains are most prevalent globally and are also associated with enhanced acquisition of drug resistance, but their resistance patterns vary regionally (11).

Although there have been several studies about molecular characterization of MDR- and XDR-TB isolates in China (12–17), only limited arrays of drug target loci in comparatively small numbers of strains were included in those analyses. In the present study, we analyzed nine genetic loci associated with drug resistance, including *katG*, *inhA*, the *oxyR-ahpC* intergenic region, *rpoB*, *tlyA*, *eis*, *rrs*, *gyrA*, and *gyrB*, among 128 MDR-TB isolates from China. In addition, the frequencies of resistance-conferring mutations were compared according to different TB strain genotypes.

MATERIALS AND METHODS

Ethical approval. We obtained approval for this study from the Ethics Committee of the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The patients with TB included in the present research were given a subject information sheet, and all gave written informed consent to participate in the study.

***Mycobacterium tuberculosis* isolates.** For this study, a total of 185 *M. tuberculosis* strains, isolated from 185 adult patients with pulmonary TB during 2009, were collected in six provincial tuberculosis hospitals in China, including hospitals in Fujian, Guizhou, Liaoning, Shaanxi, Shanghai, and Tibet. No epidemiological connection of these patients could be detected by standard investigation. Among the 185 isolates, 128 were identified as MDR-TB. The numbers isolated from each province were as follows: Fujian, 21 isolates; Guizhou, 20 isolates; Liaoning, 18 isolates; Shaanxi, 23 isolates; Shanghai, 22 isolates; and Tibet, 24 isolates. The

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TABLE 1 Distribution of different drug susceptibility patterns and genotypes among MDR-TB isolates from China

Type	DST result	No. (%) of isolates	No. (%) of isolates of indicated genotype					
			Beijing	Modern Beijing	T	H	MANU2	Orphan
Simple MDR-TB	INH+RIF	65 (50.8)	57 (52.8)	30 (46.9)	4 (44.5)	1 (25.0)	0 (0.0)	3 (60.0)
Pre-XDR-TB	INH+RIF+CAP	1 (0.8)	0 (0.0)	0 (0.0)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)
	INH+RIF+CAP+KAN	2 (1.6)	2 (1.9)	2 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	INH+RIF+OFX	47 (36.7)	37 (34.2)	24 (37.5)	3 (33.3)	3 (75.0)	2 (100.0)	2 (40.0)
XDR-TB	INH+RIF+OFX+CAP	2 (1.6)	2 (1.9)	2 (3.1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	INH+RIF+OFX+KAN	6 (4.6)	5 (4.6)	1 (1.6)	1 (11.1)	0 (0.0)	0 (0.0)	0 (0.0)
	INH+RIF+CAP+KAN+OFX	5 (3.9)	5 (4.6)	5 (7.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Total		128 (100.0)	108 (100.0)	64 (100.0)	9 (100.0)	4 (100.0)	2 (100.0)	5 (100.0)

remaining 57 isolates (Fujian, 9 isolates; Guizhou, 8 isolates; Liaoning, 9 isolates; Shaanxi, 10 isolates; Shanghai, 10 isolates; and Tibet, 11 isolates) were pan-sensitive and were therefore used as negative controls. H37Rv (ATCC 27294) was used as a reference.

Drug susceptibility testing. Drug susceptibility testing was performed using the Lowenstein-Jensen (L-J) proportion method (PM) (18) at the National Tuberculosis Reference Laboratory, which participates in the annual proficiency testing of DST organized by the Hong Kong Supranational Tuberculosis Reference Laboratory and has passed each testing since 2003. The critical concentrations for the PM were 0.2 μg/ml for INH, 40 μg/ml for RIF, 40 μg/ml for CAP, 30 μg/ml for KAN, and 2.0 μg/ml for ofloxacin (OFX). Results were read 28 days after inoculation of the medium. H37Rv was used as a control with each batch of strains used for drug susceptibility testing. Strains resistant to INH and RIF but not to CAP, KAN, or OFX were defined as simple MDR-TB strains. Pre-XDR was defined as resistance to INH and RIF and either OFX or one of the other two drugs (CAP and KAN). XDR-TB strains were defined as isolates resistant to INH and RIF as well as to OFX and at least one of the other two drugs (CAP and KAN).

DNA extraction. Colonies of *M. tuberculosis* isolates on L-J slants were scraped, resuspended in 500 μl Tris-EDTA (TE) buffer (pH 8.0), and then heat inactivated at 80°C for 1 h. Genomic DNA was extracted by a conventional method (19) and stored at −20°C for further use.

Spoligotyping and data analysis. Spoligotyping was performed using 43 covalently bound oligonucleotides derived from the spacer sequences of *M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG P3 as previously described by Kamerbeek et al. (20). The results were entered into an Excel spreadsheet in binary format and compared with the spoligotyping database SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp).

Detection of IS*6110* in the NTF region. All Beijing family strains identified by spoligotyping were amplified by PCR to detect the presence or absence of IS*6110* in the NTF region (21), using the following primers: F-6110, 5′-CCAGATATCGGGTGTGTCGAC-3′; and R-6110, 5′-TGCCGTTGTCGAAATCTAAACCC-3′. Strains with the insert yielded an amplified product of ≈1,800 bp, while those without the insert yielded a PCR product of ≈700 bp.

PCR amplification and sequencing. Expected fragments were amplified using the primers listed in Table S1 in the supplemental material. Each 30-μl PCR mixture contained 15 μl 2× *Taq* master mix (TaKaRa), 1 μl (each) of the forward and reverse primers (5 μM), 12 μl distilled H_2O, and 1 μl of genomic DNA. The reaction conditions consisted of a denaturation step of 5 min at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C, with a final extension step of 5 min at 72°C. PCR products were sent for sequencing. All sequence data were manipulated with BioEdit, version 7.05.3, and were compared to published sequences (GenBank accession number NC_000962).

Resolution of discrepant results. In cases of discrepant results between the DST and DNA sequencing, retesting was performed twice using both methods. If the retesting results were in conflict with the original data, a third round of testing was completed, with the final value representing two of the three cycles of testing.

Statistical analysis. The chi-square test or Fisher's exact test was used to compare the proportions of drug resistance-conferring mutations in genetic loci between Beijing genotype and non-Beijing genotype MDR-TB isolates. All statistical analyses were performed in SPSS 16.0 (SPSS Inc.).

Nucleotide sequence accession numbers. All new sequences were deposited in the GenBank database under accession numbers KJ410762 for the *katG* Gly299Ser gene mutants and KJ410759, KJ410761, KJ410758, and KJ410760 for the *rpoB* Gly457Val, Asp472Gly, Ser522Met, and Ile572Thr gene mutants, respectively.

RESULTS

Drug susceptibility patterns of MDR-TB isolates. Drug susceptibility testing of MDR-TB isolates showed that 46.9% (60/128 isolates) of isolates were resistant to OFX, 7.8% (10/128 isolates) were resistant to CAP, 10.2% (13/128 isolates) were resistant to KAN, 10.2% (13/128 isolates) were XDR-TB isolates, 3.9% (5/128 isolates) were resistant to all of the second-line anti-TB drugs tested, and the remaining 50.8% (65/128 isolates) were susceptible to all of the second-line anti-TB drugs tested (Table 1).

Spoligotyping and detection of IS*6110* in the NTF region. Spoligotyping results for the MDR-TB isolates showed that a total of 108 isolates (84.4%) belonged to the Beijing family, while 20 isolates (15.6%) belonged to non-Beijing families, which included the T family (9 isolates), H family (4 isolates), MANU2 family (2 isolates), and orphans (5 isolates) (Table 1). Among the 108 Beijing family strains, 64 (59.3%) were confirmed to be modern Beijing strains based on the presence of IS*6110* upstream of the NTF region. The distribution of genotypes in simple MDR-TB, pre-XDR-TB, and XDR-TB isolates is also shown in Table 1.

INH resistance and mutations of *katG*, *inhA*, and the *oxyR-ahpC* intergenic region. Fragments of the hot regions of *katG*, the *inhA* promoter, and the *oxyR-ahpC* intergenic region, which are known to harbor the majority of resistance-associated mutations, were examined. Among 128 INH-resistant isolates, 114 (89.1%) had mutations in one or more of these three regions (Table 2). Eighty-two isolates (64.1%) had only *katG* mutations, 8 isolates (6.3%) had only *inhA* mutations, and 10 isolates (7.8%) had only *oxyR-ahpC* intergenic region mutations, while 14 isolates (10.9%) harbored two or three mutations in these three fragments. No mutations were detected in 14 isolates. The most frequent mutations were *katG315* (70.3%) and the *inhA* promoter 15 mutation (8.6%) (Table 2). A novel mutation, Gly299Ser, was detected in

TABLE 2 Drug resistance-associated mutations among MDR-TB isolates from China

Drug (no. of resistant isolates)	Locus	Codon/nucleotide change(s)	Amino acid/nucleotide change(s)	No. (%) of isolates	Other mutation(s)
INH (128)	*katG*	NM[a]	NM	34 (26.6)	
		GGC-AGC	Gly299Ser[c]	1 (0.8)	
		AGC-CGC	Ser302Arg	1 (0.8)	
		AGC-ACA	Ser315Thr	1 (0.8)	
		AGC-ACC	Ser315Thr	72 (56.3)	
		AGC-ACC	Ser315Thr	4 (3.1)	T(−8)C of *inhA*
				1 (0.8)	T(−8)A of *inhA*
				1 (0.8)	C(−15)T of *inhA*
				1 (0.8)	C(−34)T of *inhA*
				1 (0.8)	G(−6)A of *oxyR-ahpC* intergenic region
				2 (1.6)	C(−10)T of *oxyR-ahpC* intergenic region
		AGC-AAC	Ser315Asn	6 (4.7)	
		AGC-AAC/GCC-ACC	Ser315Asn/Ala379Thr	1 (0.8)	
		CTG-CCG	Leu378Pro	1 (0.8)	C(−30)T of *oxyR-ahpC* intergenic region
		ACT-ATT	Thr380Ile	1 (0.8)	C(−15)T of *inhA* and C(−12)T of *oxyR-ahpC* intergenic region
	inhA	NM	NM	110 (85.9)	
		T-A	T(−8)A	1 (0.8)	
				1 (0.8)	Ser315Thr of *katG*
		T-C	T(−8)C	4 (3.1)	Ser315Thr of *katG*
		C-T	C(−15)T	7 (5.5)	
				1 (0.8)	Ser315Thr of *katG*
				1 (0.8)	G(−6)A of *oxyR-ahpC* intergenic region
				1 (0.8)	C(−30)T of *oxyR-ahpC* intergenic region
				1 (0.8)	Thr380Ile of *katG* and C(−12)T of *oxyR-ahpC* intergenic region
		C-T	C(−34)T	1 (0.8)	Ser315Thr of *katG*
	oxyR-ahpC intergenic region	NM	NM	111 (86.7)	
		G-A	G(−6)A	3 (2.3)	
				1 (0.8)	Ser315Thr of *katG*
				1 (0.8)	C(−15)T of *inhA*
		C-T	C(−10)T	1 (0.8)	
				2 (1.6)	Ser315Thr of *katG*
			C(−12)T	1 (0.8)	
				1 (0.8)	Thr380Ile of *katG* and C(−15)T of *inhA*
			C(−30)T	3 (2.3)	
				1 (0.8)	C(−15)T of *inhA*
				1 (0.8)	Leu378Pro of *katG*
			C(−39)T	2 (1.6)	
RIF (128)	*rpoB*[b]	NM	NM	8 (6.3)	
		GGC-GTC/TCG-TTC	Gly457Val[c]/Ser531Leu	1 (0.8)	
		CTG-CGG/CAC-TAC	Leu459Arg/His526Tyr	1 (0.8)	
		GAG-GGG/CAC-TAC	Asp472Gly[c]/His526Tyr	1 (0.8)	
		TTC-CTC/CTG-CCG	Phe505Leu/Leu511Pro	1 (0.8)	
		ACC-CCC/CTG-CCG	Thr508Pro/Leu511Pro	1 (0.8)	
		CAG-CAC/CAC-TAC	Gln510His/His526Tyr	1 (0.8)	
		CTG-CCG	Leu511Pro	1 (0.8)	
		CTG-CCG/AGC-GGC	Leu511Pro/Ser512Gly	1 (0.8)	
		CTG-CCG/GAC-GGC	Leu511Pro/Asp516Gly	2 (1.6)	
		GAC-GGC	Asp516Gly	2 (1.6)	
		GAC-GTC	Asp516Val	3 (2.3)	
		GAC-TAC	Asp516Tyr	2 (1.6)	
		GAC-TAC/CAC-GAC	Asp516Tyr/His526Asp	1 (0.8)	
		GAC-GCC/CTG-CCG	Asp516Ala/Leu533Pro	1 (0.8)	
		GAC-GGC/CTG-CCG	Asp516Gly/Leu533Pro	2 (1.6)	
		TCG-ATG	Ser522Met[c]	1 (0.8)	
		CAC-GAC	His526Asp	12 (9.4)	
		CAC-AAC	His526Asn	1 (0.8)	
		CAC-CGC	His526Arg	3 (2.3)	
		CAC-TAC	His526Tyr	7 (5.4)	
		CAC-TTC	His526Phe	1 (0.8)	
		CAC-TAC/GAG-GGG	His526Tyr/Glu541Gly	1 (0.8)	
		TCG-TTG	Ser531Leu	67 (52.3)	
		TCG-TTG/ATC-ACC	Ser531Leu/Ile561Thr	1 (0.8)	
		TCG-TTT	Ser531Phe	1 (0.8)	
		CTG-CCG	Leu533Pro	2 (1.6)	
		CTG-CCG/GAA-GCA	Leu533Pro/Glu562Ala	1 (1.6)	
		ATC-ACC	Ile572Thr[c]	1 (0.8)	
CAP (10)	*tlyA*	NM	NM	10 (100.0)	
	rrs	NM	NM	4 (40.0)	
		A-G	A(1401)G	6 (60.0)	
KAN (13)	*eis*	NM	NM	9 (69.2)	
		G-A	G(−10)A	3 (23.1)	
		C-T	C(−14)T	1 (7.7)	
	rrs	NM	NM	6 (46.2)	
		A-G	A(1401)G	7 (53.8)	

(Continued on following page)

TABLE 2 (Continued)

Drug (no. of resistant isolates)	Locus	Codon/nucleotide change(s)	Amino acid/nucleotide change(s)	No. (%) of isolates	Other mutation(s)
CAP and KAN (7)	*tlyA*	NM	NM	7 (100.0)	
	eis	NM	NM	7 (100.0)	
	rrs	NM	NM	1 (14.3)	
		A-G	A(1401)G	6 (85.7)	
OFX (60)	*gyrA*	NM	NM	7 (11.7)	
		GCC-TCC/GAC-GGC	Ala74Ser/Asp94Gly	1 (1.7)	
		GAC-GGC	Asp89Gly	1 (1.7)	
		GCG-GTG	Ala90Val	11 (18.3)	
		GCG-GTG/GAC-GCC	Ala90Val/Asp94Ala	1 (1.7)	
		TCG-CCG	Ser91Pro	4 (6.7)	
		GAC-AAC	Asp94Asn	4 (6.7)	
		GAC-CAC	Asp94His	2 (3.3)	
		GAC-GCC	Asp94Ala	7 (11.7)	
		GAC-GCC	Asp94Ala	1 (1.7)	Thr500Pro of *gyrB*
		GAC-GGC	Asp94Gly	15 (25.0)	
		GAC-GGC	Asp94Gly	1 (1.7)	Ala504Val of *gyrB*
		GAC-TAC	Asp94Tyr	5 (8.3)	
	gyrB	NM	NM	57 (94.9)	
		GAC-AAC	Asp461Asn	1 (1.7)	
		ACC-CCC	Thr500Pro	1 (1.7)	Asp94Ala of *gyrA*
		GCG-GTG	Ala504Val	1 (1.7)	Asp94Gly of *gyrA*

[a] NM, no mutation.
[b] Amino acid numbers are based on homologous mutations in *Escherichia coli*.
[c] The mutation was not previously reported.

katG. However, no mutations in the three target fragments were detected in the 57 susceptible isolates. When the genotypic data for the combination of *katG*, *inhA*, and the *oxyR-ahpC* intergenic region were compared to the phenotypic data, the sensitivity and specificity were 89.1% (114/128 isolates) and 100% (57/57 isolates), respectively, which are better results than those obtained using genotypic data for either gene alone (Table 3).

RIF resistance and *rpoB* mutations. To investigate the mutations associated with RIF resistance, a 543-bp region of *rpoB*, including the 81-bp RIF resistance-determining region (RRDR), was sequenced. Altogether, 93.8% (120/128 isolates) of the RIF-resistant isolates harbored at least one mutation within *rpoB*, while the other 8 isolates lacked such a mutation (Table 2). One hundred four isolates (81.3%) had a single mutation, and 16 (12.5%) had two mutations each. When all of the mutations were considered, regardless of whether they were single or double mutations, a total of 28 genotype patterns were identified. The most frequently mutated codons were codons 531, 526, and 516, with mutation frequencies of 58.3% (70/120 isolates), 24.2% (29/120 isolates), and 10.8% (13/120 isolates), respectively (Table 2). The

TABLE 3 Evaluation of sequence analysis of specific regions of nine drug resistance-associated loci and phenotypic drug susceptibility testing[a]

		No. of isolates					
		Resistant		Susceptible			
Drug	Locus	With mutation	Without mutation	With mutation	Without mutation	Sensitivity[b] (%)	Specificity[c] (%)
INH	*katG*	94	34	0	57	73.4	100.0
	inhA	18	110	0	57	14.1	100.0
	oxyR-ahpC intergenic region	17	111	0	57	13.3	100.0
	katG and/or *inhA*	104	24	0	57	81.3	100.0
	katG and/or *inhA* and/or *oxyR-ahpC* intergenic region	114	14	0	57	89.1	100.0
RIF	*rpoB*	120[d]	8	0	57	93.8	100.0
CAP	*rrs*	6	4	1	174	60.0	99.4
	tlyA	0	10	0	175	NA[e]	NA
KAN	*rrs*	7	6	0	172	53.8	100.0
	eis	4	9	1	171	30.8	99.4
	rrs or *eis*	11	2	1	171	84.6	99.4
OFX	*gyrA*	53	7	0	125	88.3	100.0
	gyrB	3	57	0	125	5.0	100.0
	gyrA and/or *gyrB*	54	6	0	125	90.0	100.0

[a] The diagnostic performance of DNA sequencing in comparison with DST was determined after the resolution of natural polymorphisms and silent mutations.
[b] Number of resistant isolates with mutation/total number of resistant isolates. NA, not applicable.
[c] Number of susceptible isolates without mutation/total number of susceptible isolates. NA, not applicable.
[d] Includes 16 isolates with two mutations.

following four novel mutations were detected in *rpoB*: Gly457Val, Asp472Gly, Ser522Met, and Ile572Thr. In contrast, none of the 57 susceptible isolates possessed a mutation within the target fragment of the *rpoB* gene. Detection of mutations in the *rpoB* region showed a sensitivity of 93.8% (120/128 isolates) and a specificity of 100% (57/57 isolates) (Table 3).

CAP resistance and *tlyA* and *rrs* mutations. Since resistance to CAP has been attributed to mutations within a 516-bp fragment of *rrs* and the entire open reading frame of *tlyA*, both fragments were analyzed in the present study. Among the 128 isolates, 10 were resistant to CAP, including 7 that were also resistant to KAN. Within the *tlyA* fragment, the CTA → CTG mutation at codon 11 was observed in all clinical TB isolates except for H37Rv. Besides that mutation, there was no other mutation within *tlyA* among all the clinical isolates. Screening of the *rrs* gene revealed the A1401G mutation to be the only observed mutation, and it was present in 6 CAP-resistant isolates and 1 CAP-susceptible isolate (Table 2). The remaining clinical isolates had no mutation in the *rrs* region. Thus, the identification of CAP resistance due to the *rrs* mutation exhibited a sensitivity and specificity of 60.0% (6/10 isolates) and 99.4% (174/175 isolates), respectively (Table 3).

KAN resistance and *eis* and *rrs* mutations. Since resistance to KAN is caused by mutations in *rrs* or the promoter of *eis*, a 516-bp region of the open reading frame of *rrs* and the promoter of *eis* were investigated. Among all 128 MDR-TB isolates, 13 isolates were resistant to KAN, and 11 of these (84.6%) contained mutations within the *rrs* or *eis* region of interest (Table 2). None of the isolates contained mutations within both loci. The most frequent *rrs* region mutation was A1401G, which was observed in 7 isolates (53.8%), as a single mutation (Table 2). Mutations within the promoter region of *eis* included the G(−10)A (3 isolates [23.1%]) and G(−14)T (1 isolate [7.7%]) mutations. We also found a KAN-susceptible MDR isolate harboring a C(−12)T mutation. All other KAN-susceptible isolates were determined to be wild type for the target fragment of the *rrs* or *eis* region. Hence, sequence analyses of *rrs* and *eis* had a sensitivity and specificity of 84.6% (11/13 isolates) and 99.4% (171/172 isolates), respectively (Table 3), for the detection of KAN resistance among the strains analyzed.

OFX resistance and *gyrA* and *gyrB* mutations. Because known mechanisms of FQ resistance are caused by mutations in the quinolone resistance-determining region (QRDR) of the gene encoding subunit A or B of DNA gyrase (*gyrA* or *gyrB*), fragments comprising these regions were analyzed in this study. The results revealed that all the clinical isolates had an AGC-to-ACC polymorphism at codon 95. Among 128 MDR-TB isolates, 60 were resistant to OFX. Of them, 53 isolates (88.3%) contained mutations within *gyrA* (Table 2). The most predominant mutations occurred at codon 94 (37 isolates [61.7%]), where the Asp codon was replaced with a Gly (17 isolates [28.3%]), Ala (9 isolates [15.0%]), Tyr (5 isolates [8.3%]), Asn (4 isolates [6.7%]), or His (2 isolates [3.3%]) codon. The Asp94 mutations were also found in two combinations: once with an Ala74Ser mutation and once with an Ala90Val mutation. The Ala90Val mutation was the next most predominant mutation (12 isolates [20.0%]). Other mutations within *gyrA* included Ser91Pro ($n = 4$), Ala74Ser ($n = 1$), and Asp89Gly ($n = 1$). Mutations within *gyrB* were observed in three isolates (5.0%), with Asp461Asn, Thr500Pro, and Ala504Val mutations. Double substitutions in *gyrA* and *gyrB* were detected in two isolates (Table 2). All OFX-susceptible isolates did not display mutations within these two target fragments. The sensitivity and specificity of DNA sequencing of *gyrA* and *gyrB* for the detection of OFX resistance were 90.0% (54/60 isolates) and 100.0% (125/125 isolates), respectively (Table 3).

Association of *M. tuberculosis* genotype with resistance-conferring mutations. The distribution of resistance-conferring mutations according to *M. tuberculosis* genotype was summarized in Table S2 in the supplemental material. Statistical analysis indicated that there was no significant difference in the occurrence of resistance-conferring mutations of *katG*, *inhA*, the *oxyR-ahpC* intergenic region, *rpoB*, *rrs*, *gyrA*, and *gyrB* between Beijing and non-Beijing strains (see Table S2).

DISCUSSION

Despite China having the second highest prevalence of TB and MDR-TB, information regarding the molecular characterization of drug-resistant strains still remains unclear for many regions of China. This study is the first to include MDR-TB, pre-XDR-TB, and XDR-TB isolates to determine molecular mutations conferring drug resistance in six provinces of China. Furthermore, more drug resistance-associated loci were analyzed in our research.

The reported percentage of XDR-TB strains among MDR-TB strains varies between provinces in China, ranging from 6.3% to 18.7% (13, 15, 16, 22–24). Our results showed that the proportion of XDR-TB isolates in this study was 10.2% (13/128 isolates). We also identified that a significant proportion of MDR-TB isolates (39.1% [50/128 isolates]) were pre-XDR-TB and that the majority of pre-XDR-TB isolates were resistant to OFX (94.0% [47/50 isolates]). Modeling studies support the notion that unless evolution of MDR-TB into XDR-TB is slowed, the number of XDR-TB cases could increase exponentially (25). Therefore, some appropriate strategies must be implemented to identify and cure patients with pre-XDR-TB before they develop XDR-TB.

Genotyping of the isolates pointed out that the Beijing family was the predominant genotype (84.4%) among 128 MDR-TB isolates. This is similar to the data from some provinces of China (16, 26–28). The modern Beijing genotype, which is known for a wider spectrum of resistance patterns (29), constituted the majority (59.3% [64/108 isolates]) of the Beijing family strains.

INH resistance is often considered to be caused by mutations in several genes (30), most commonly *katG*, particularly at codon 315, and the promoter region of *inhA* (8, 31, 32). Accordingly, we observed that 73.4% and 14.1% of MDR-TB isolates had point mutations in *katG* and *inhA*, respectively (Table 2), and the frequencies were similar to the data from Jiangxi (16) and Shanghai (14), China. We also identified a novel mutation of *katG*: Gly299Ser. Further studies are required to assess its specific effects on *katG* function. In addition, mutations in the *oxyR-ahpC* intergenic region were detected among 13.3% (17/128 isolates) of MDR-TB isolates, 58.8% (10/17 isolates) of which had a mutation only in the *oxyR-ahpC* intergenic region. Thus, the combination of mutations in *katG*, *inhA*, and the *oxyR-ahpC* intergenic region could detect 89.1% of INH-resistant isolates among the MDR-TB isolates. Fourteen MDR-TB isolates had no resistance-associated mutations in the three targets analyzed, suggesting that resistance in these isolates could be attributed to mutations outside the sequenced area or in other genes, such as *kasA*, *mshA*, and *ndh* (8, 33, 34).

Previous reports indicated that 95% or more of RIF-resistant *M. tuberculosis* strains worldwide have mutations within the RRDR region of the *rpoB* gene. We found mutations in this region

in 93.8% of MDR-TB isolates, consistent with other reports from China (12, 14, 17, 27). The most common mutations of *rpoB* were in codons 531 (58.3%), 526 (24.2%), and 516 (10.8%) (Table 2), correlating with data from Nepal (35) and other regions (36). Moreover, four novel mutations (Gly457Val, Asp472Gly, Ser522Met, and Ile572Thr) were identified in the present study, and three of them fell outside the RRDR region of the *rpoB* gene. It is uncertain if these mutations are involved in RIF resistance. Among them, two mutations (Gly457Val and Asp472Gly) were accompanied by another mutation within the RRDR region. It is unknown if these represent compensatory mutations or play a direct function in RIF resistance. Further research is therefore required to characterize their roles.

Cross-resistance between CAP and KAN is well documented (13, 32, 37–39) and was also supported by this study. Among the 10 CAP-resistant isolates, only 3 (30.0%) were CAP monoresistant; the remaining 7 (70.0%) were cross-resistant to both CAP and KAN, indicating a high level of cross-resistance between CAP and KAN. Substantial proportions (51 to 96%) of CAP-resistant isolates have been shown to carry the A1401G mutation (13, 32, 38–41). Our study showed that 60% of the CAP-resistant isolates carried the A1401G mutation. These mutated strains were resistant to both CAP and KAN. This result supported the existing data showing that the A1401G mutation in *rrs* actually mediated resistance to both CAP and KAN (13, 32, 37–39). The other four isolates resistant to CAP, including three CAP-monoresistant isolates, harbored no mutation in the *rrs* region. One additional isolate was susceptible to CAP but resistant to KAN and displayed the A1401G mutation in *rrs*, implying that the A1401G mutation does not always confer cross-resistance to KAN and CAP. *tlyA* is another gene associated with CAP resistance (32, 42). Mutations in *tlyA* among CAP-resistant isolates occur at different rates (1.0 to 13.0%) in different regions (13, 32, 39, 41, 43). However, there was no mutation conferring CAP resistance found within the *tlyA* gene in this study. Although retesting was performed using DST and DNA sequencing, the retesting results remained the same. Hence, mutation within *tlyA* was not observed, probably due to the limited numbers of CAP-resistant isolates.

Common *rrs* mutations associated with KAN resistance include the A1401G, C1402T, and G1484T mutations (32, 38). In our research, the A1401G mutation was the only mutation observed in the *rrs* gene (Table 2). In addition to *rrs*, mutations in the promoter region of the *eis* gene confer low-level resistance to KAN (44). The most commonly reported resistance-related *eis* mutations include the G(−10)A and C(−14)T mutations (44). Our study supports these findings, as 4 isolates were found to have *eis* mutations, including the G(−10)A (3 isolates) and C(−14)T (1 isolate) mutations. Thus, among 13 KAN-resistant isolates, 11 isolates (84.6%) contained mutations in either *rrs* or *eis*, with no overlap between the two genes. One KAN-susceptible MDR isolate also carried a C(−12)T mutation in *eis*, which has been reported to be associated with susceptibility to KAN (44).

Sequencing of the QRDR regions of *gyrA* and *gyrB* revealed that 88.3% of the OFX-resistant isolates were identified by mutations in the *gyrA* gene, while only 5.0% of these isolates were identified by mutations in the *gyrB* gene. This observation is in agreement with reports that *gyrB* mutations are less common than *gyrA* mutations (16, 45). The majority of isolates displayed mutations in *gyrA* at codons 94 and 90, with a low frequency of mutation at codon 91, similar to data from India (46), Pakistan (47), Germany (45), the United States (33), and Jiangxi, China (16). A mutation at codon 95 which is not related to OFX resistance and instead serves as a natural polymorphism and a marker for evolutionary genetics was found in all clinical isolates (48). Three isolates with different mutations in *gyrB* (Asp461Asn, Thr500Pro, and Ala504Val) were also detected in this study. The isolate with the Asp461Asn mutation exclusively carried the *gyrB* mutation and had no additional mutation in *gyrA*. Although mutations in *gyrB* have been described to occur together with a *gyrA* mutation, leading to high-level resistance (48), single mutations in *gyrB* were recently reported (45, 49, 50). Consequently, mutations in *gyrB* might have an impact on the development of FQ resistance. Only 6 OFX-resistant strains in our study had no mutation in the QRDR of either *gyrA* or *gyrB*. To explore the possibility of TB cultures comprising a mixed population of resistant and susceptible bacteria and the inability of ordinary PCR-based DNA sequencing to detect the small proportion of drug-resistant TB bacteria among the predominant wild-type TB bacteria, we recultured these 6 strains on L-J medium containing OFX and reanalyzed the QRDR of *gyrA* and *gyrB*. The new DNA sequencing results displayed that four of six strains carried mutations in codon 94 of *gyrA*. Therefore, new PCR typing methods increasing the sensitivity of mutation analysis by selectively amplifying mutant alleles in a wild-type background should be applied for the detection of low-level drug resistance mutations in TB bacteria (51, 52). There were still two isolates without mutation in the QRDR of either *gyrA* or *gyrB*, suggesting that these strains probably harbored mutations outside the QRDR or that the resistance may have been caused by other mechanisms, such as enhanced drug efflux (53).

Compared to the phenotypic data, the sensitivities for detecting INH, RIF, CAP, KAN, and OFX resistance by DNA sequencing in our study were 89.1%, 93.8%, 60.0%, 84.6%, and 90.0%, respectively, which were similar to the findings from other investigations (14, 17, 32, 35). The specificities of DNA sequencing for diagnosing INH, RIF, CAP, KAN, and OFX resistance were 100%, 100%, 99.4%, 99.4%, and 100%, respectively, consistent with the findings from some regions (13, 14, 16, 17, 45) but higher than the data from the United States (32). One possible reason is that a limited number of susceptible strains were included in this study. Furthermore, the mutation characteristics of clinical isolates from different areas might have some geographical differences.

Studies in Russia (54) and Ukraine (55) found that *rpoB531* was highly prevalent in strains of the Beijing genotype. In contrast, we found no significant association between the genotype and any resistance-conferring mutation, including *rpoB531*. Similar results were also found in reports from eastern Asian countries and regions (56, 57). This suggested that the association of particular mutations and the Beijing genotype could be a regional evolutionary phenomenon.

There are several limitations of this study. First, although a large number of clinical MDR-TB isolates were tested ($n = 128$), the number of second-line drug-resistant isolates, especially CAP ($n = 10$)- and KAN ($n = 13$)-resistant isolates, was relatively small. This might have limited the detection of the variety of gene variations. Second, pan-sensitive strains were used as negative controls, and the number of strains in the negative-control group was limited, which could have overestimated the specificity of DNA sequencing. Therefore, additional studies containing a substantial panel of drug-resistant or -sensitive isolates will be required to calculate the accuracy and predictive values of sequencing-

based assays. Furthermore, there was still some drug resistance that could not be explained in our study. Whole-genome sequencing might be needed to find more loci associated with drug resistance and to improve the performance of the sequencing-based assay.

In conclusion, our data indicate that the majority of MDR-TB strains in China belong to the modern Beijing genotype. Compared with the phenotypic data, sequence analyses of hot regions of *katG*, *inhA*, the *oxyR-ahpC* intergenic region (for resistance to INH), *rpoB* (RIF resistance), *tlyA* (CAP resistance), *eis* (KAN resistance), *rrs* (CAP or KAN resistance), *gyrA*, and *gyrB* (OFX resistance) showed a high sensitivity and specificity for the detection of drug resistance in China. This allows the potential development of rapid molecular diagnostic methods to be implemented in China. The most frequent mutations in the *katG*, *inhA*, *rpoB*, *rrs*, *eis*, and *gyrA* genes were consistent with those reported from other regions of the world (8, 32, 45). There was no association between a specific resistance-conferring mutation and the strain genotype. We also identified one novel mutation in *katG* (Gly299Ser) and four new alleles in *rpoB* (Gly457Val, Asp472Gly, Ser522Met, and Ile572Thr), which will broaden current knowledge of the molecular mechanisms of drug resistance of *M. tuberculosis*. Further efforts are required to elucidate their actual roles in the drug resistance of *M. tuberculosis*.

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All authors declare that they have no competing interests.

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