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Characterisation of drug resistance-associated mutations among clinical multidrug-resistant *Mycobacterium tuberculosis* isolates from Hebei Province, China

Qianlin Li[a], Yuling Wang[b], Yanan Li[b], Huixia Gao[b], Zhi Zhang[b], Fumin Feng[a,1,*], Erhei Dai[b,1,*]

[a] Department of Epidemiology and Statistics, North China University of Science and Technology, Tangshan 063210, Hebei, China
[b] Department of Laboratory Medicine, The Fifth Affiliated Hospital of Shijiazhuang, North China University of Science and Technology, Shijiazhuang 050021, Hebei, China

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ABSTRACT

Objectives: Multidrug-resistant tuberculosis (MDR-TB) is a major public-health problem in China. However, there is little information on the molecular characterisation of clinical MDR-TB isolates in Hebei Province.

Methods: In this study, 123 MDR-TB isolates were identified in sputum cultures using traditional drug susceptibility testing. The isolates were analysed for mutations in seven genes associated with resistance to antituberculous four drugs: *katG* and *inhA* promoter for isoniazid (INH); *rpoB* for rifampicin (RIF); *gyrA* and *gyrB* for ofloxacin (OFLX); and *rrs* and *eis* promoter for kanamycin (KAN). All strains were genotyped by spoligotyping and 15-loci MIRU-VNTR analysis.

Results: A total of 39 distinct mutations were found at the seven loci in 114/123 (92.7%) MDR-TB isolates. Frequencies of INH, RIF, OFLX and KAN resistance-associated mutations were 82.1% (101/123), 83.7% (103/123), 92.1% (35/38) and 76.2% (16/21), respectively. The most prevalent mutations involved in resistance were: Ser315Thr in *katG* (70/123; 56.9%) and C(−15)T in *inhA* (15/123; 12.2%) for INH; Ser531Leu in *rpoB* (72/123; 58.5%) for RIF; Asp94Gly in *gyrA* (10/38; 26.3%) for OFLX; and A1401G in *rrs* (12/21; 57.1%) for KAN. Four novel *gyrB* mutants (Leu442Leu, Ser447Phe, Asn499Thr and Ala504Val) were identified. Mutations in *katG*, *rpoB* (or both) and the *inhA* promoter showed a sensitivity of 75.6% and specificity of 97.0% for detection of MDR-TB. DNA sequencing of the seven loci was 57.1% sensitive and 91.0% specific for prediction of XDR-TB isolates.

Conclusion: These results may be of value in rapid molecular detection of MDR- and XDR-TB isolates in clinical samples in Hebei Province.

1. Introduction

Tuberculosis (TB) is a severe chronic infectious disease caused by the bacterium *Mycobacterium tuberculosis* that usually affects the human respiratory system. The tubercle bacillus has been extensively investigated since its discovery in the 1880s [1] and, recently, diagnostic and therapeutic techniques dealing with TB have developed rapidly. Multidrug-resistant TB (MDR-TB), which is resistant to at least isoniazid (INH) and rifampicin (RIF), has emerged as a serious challenge to TB management and control [2]. In China, it is estimated that approximately 110 000 new MDR-TB cases occur each year, accounting for approximately one-quarter of new MDR-TB cases worldwide [3]. If no appropriate measures are taken, the prevalence of global TB will rise disastrously.

Hebei Province lies in the large Beijing–Tianjin region that plays a crucial role in the economy of Northern China. In this special location, population mobility and environmental conditions contribute to the spread of MDR-TB. According to the 2000 National Epidemiology Survey, there were 200 000 TB patients in Hebei Province, of whom 80 000 had active TB [4]. We previously analysed the genotypes of 1017 clinical *M. tuberculosis* isolates by spoligotyping and mycobacterial interspersed repetitive unit–variable number of tandem

* Corresponding authors.
E-mail addresses: fengfumin@ncst.edu.cn (F. Feng), daieh2008@126.com (E. Dai).
[1] These two authors contributed equally to this study.

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repeats (MIRU-VNTR) and explored the association between *embB*306 mutation patterns, first-line drug resistance profiles and genotypes [5,6]. However, little information is available on the molecular characterisation of clinical MDR-TB isolates from Hebei Province, China. The aim of this study was to investigate mutations in selected regions of seven genetic loci associated with antituberculous drug resistance, namely *katG*, *inhA* promoter, *rpoB*, *gyrA*, *gyrB*, *rrs* and *eis* promoter, among MDR-TB strains in order to examine the association between drug resistance, mutational profile, and clustering of lineages of MDR-TB isolates in Hebei Province.

2. Materials and methods

2.1. M. tuberculosis isolates

A total of 1017 *M. tuberculosis* clinical isolates were collected in 2014 from sputum specimens from individual pulmonary TB patients living in seven cities (Shijiazhuang, Handan, Xingtai, Baoding, Hengshui, Cangzhou and Qinhuangdao) of Hebei Province. Among the 1017 clinical isolates, 280 isolates were drug-resistant, of which 134 were MDR-TB based on first-line drug susceptibility testing (DST). A total of 123 MDR-TB isolates were successfully revived in Lowenstein–Jensen (L-J) culture medium after 3–4 weeks in a thermostat at 37 °C.

2.2. Drug susceptibility testing

DST was performed using the proportion method as recommended by the World Health Organization (WHO) [7]. The critical concentrations were 0.2 μg/mL for INH, 40 μg/mL for RIF, 2 μg/mL for ethambutol (EMB), 4 μg/mL for streptomycin (STR), 2 μg/mL for ofloxacin (OFLX) and 30 μg/mL for kanamycin (KAN). All *M. tuberculosis* isolates were inoculated on an inclined surface of drug-containing and drug-free medium in a 37 °C thermostat for 4 weeks. An isolate was regarded as resistant if the rate of growth on drug-containing medium was ≥1% of that observed in drug-free medium. A random selection of 100 pansusceptible isolates were utilised from the 1017 *M. tuberculosis* strains as negative controls. *M. tuberculosis* H37Rv (ATCC 27294) was used as a reference strain for quality control.

2.3. DNA extraction and fragment amplification

Genomic DNA from *M. tuberculosis* isolates was extracted from L-J cultures using a mericon™ DNA Bacteria Kit (QIAGEN, Valencia, CA). The target fragments of *katG* (including codon 315), *inhA* promoter, *rpoB* (including codons 507–533), *gyrA* (including codons 88–94), *gyrB* (including codons 500–538), *rrs* (including position 1401) and *eis* promoter were amplified using the primers listed in Table 1. Each 20 μL of PCR mixture contained 10 μL of 2× *Taq* MasterMix (CWBOIO, Beijing, China),1 μL of each of the forward and reverse primers (10 μM), 1 μL of genomic DNA and 7 μL of distilled H_2O. The PCR conditions consisted of a pre-denaturation step at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s; followed by a final extension at 72 °C for 10 min.

2.4. Sequencing of DNA fragments

PCR products were analysed by electrophoresis in 2% agarose and were sized using a 100-bp DNA ladder (CWBOIO). Successful amplification products were sent to TsingKe Biological Technology Co. (Beijing, China) for sequencing. Sequencing data were compared with the respective wild-type alleles of strain H37Rv using multiple sequence alignment (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Resolution of discrepant results

When discrepant results occurred between DST and DNA sequencing, repeat testing was performed using both methods. If the repeated result conflicted with the original data, a third round of testing was conducted, with the two concordant results accepted as the final value.

2.6. Spoligotyping

Spoligotyping was performed as described previously [10]. Briefly, this method adopted primers DRa (5'-GGTTTTGGGTCTGACGAC-3') and DRb (5'-CCGAGAGGGGACGGAAAC-3') to first amplify the direct repeat (DR) region of *M. tuberculosis*. The PCR product was then hybridised to a membrane on which 43 spacer oligonucleotide probes were immobilised. Identification results were entered into a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA) and were matched with the database SpolDB4.0 (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp). Beijing genotype strains are divided into two predominant sublineages (atypical and typical) according to the absence or presence of the spacer region between 35 and 43.

2.7. Mycobacterial interspersed repetitive unit–variable number of tandem repeats (MIRU-VNTR) typing

MIRU-VNTR typing was used to characterise the genotypes as previously described by Supply et al. [11]. The 15-loci MIRU-VNTR used the following markers: Mtub04; ETRC; ETRD; MIRU40;

Table 1
Primers used for PCR and sequencing.

Gene	Primer sequence (5' → 3')	Product size (bp)	Reference
katG	F, GATCGTCGGCGGTCACACTT	731	This study
katG	R, CGTTGACCTCCCACCCGACT		
inhA	F, TGCCCAGAAAGGGATCCGTCATG	455	This study
inhA	R, ATGAGGAATGCGTCCGCGGA		
rpoB	F, GAGCCCCCGACCAAAGA	629	This study
rpoB	R, ATGTTGGGCCCCTCAGG		
gyrA	F, TCGACTATGCGATGAGCGTG	772	This study
gyrA	R, CGGGATATTGGTTGCCATGC		
gyrB	F, GCGCTGACGTCGGTGGTGAA	485	[8]
gyrB	R, ATTCCGGGTCACTGCGCTGC		
rrs	F, TCGTGTCGTGAGATGTTGGG	685	This study
rrs	R, TGCTCGCAACCACTATCCAG		
eis	F, GCGTAACGTCACGGCGAAATT	567	[9]
eis	R, GTCAGCTCATGCAAGGTG		

MIRU10; MIRU16; Mtub21; QUB11b; ETRA; Mtub30; MIRU26; ETRE; Mtub39; QUB26; and QUB4156. An unweighted pair-group method with arithmetic mean (UPGMA) tree was constructed using MIRU-VNTR*plus* (http://www.miru-vntrplus.org).

2.8. Data analysis and definitions

Statistical data management and analysis were carried out using IBM SPSS Statistics v.22.0 (IBM Corp., Armonk, NY). A *P*-value of <0.05 was considered statistically significant. Simple MDR-TB was defined as isolates resistant only to INH and RIF; pre-extensively drug-resistant TB (pre-XDR-TB) was defined as MDR-TB isolates with resistance to either OFLX or KAN; and extensively drug-resistant TB (XDR-TB) was defined as MDR-TB isolates with resistance to both OFLX and KAN.

3. Results

3.1. Demographic characteristics

A total of 123 MDR-TB patients were included in the analysis (82 male and 41 female; median age 32 years, range 14–83 years). There was a significant difference in the distribution of MDR-TB isolates among patients aged <40 years, 41–59 years and ≥60 years (P = 0.034). Patients who worked as farmers were the most numerous of all four occupation groups among farmers, students, workers and other. The proportion of patients with newly-treated disease was 38.2% (47/123), whereas the remaining patients (76/123; 61.8%) were re-treated patients. In addition, 12.2% of patients (15/123) were diabetic and 87.8% (108/123) were non-diabetic. The demographic information is summarised in Table 2.

3.2. Analysis of drug susceptibility testing results

Of the 123 available MDR-TB isolates, 52 (42.3%) were resistant to EMB, 99 (80.5%) to STR, 38 (30.9%) to OFLX and 21 (17.1%) to KAN. A total of 78 isolates (63.4%) were resistant to both INH and RIF only and were referred to as simple MDR-TB, 31 (25.2%) were additionally resistant to either OFLX (19.5%; 24/123) or KAN (5.7%; 7/123) and were referred to as pre-XDR-TB, and 14 (11.4%) were additionally resistant to both OFLX and KAN and were referred to as XDR-TB.

3.3. Detection of mutations associated with multidrug-resistant tuberculosis

A total of 39 distinct mutations in the seven genetic loci were detected in 114 clinical isolates, accounting for 92.7% of the 123 MDR-TB isolates tested. Of the 123 MDR-TB isolates, 112 (91.1%) were found to carry mutations in the *katG* gene and/or the *inhA* promoter and *rpoB* gene. Of the 38 OFLX-resistant isolates examined, 33 (86.8%) were found to possess mutations in *gyrA*, whereas 4 (10.5%) had mutations in *gyrB*. Of the 21 KAN-resistant isolates,13 (61.9%) had mutations in the *rrs* gene and 3 (14.3%) had mutations in the *eis* promoter. Detailed characterisation of the mutations is shown in Table 3.

3.4. Mutations in the katG gene and inhA promoter conferring isoniazid resistance

Six types of mutation causing INH resistance were identified at the *katG* gene in 72.4% (89/123) of the MDR-TB isolates. Codon 315 of *katG* was affected in 86 isolates (69.9%). The most frequently occurring mutation was G → C at nucleotide position 944 resulting in a Ser315Thr amino acid substitution in 70 (56.9%) of the isolates. Sixteen isolates had a G → A change at the same position (944), resulting in a Ser315Asn replacement. Double mutations at codons 308 (Ile → Thr) and 374 (Ser → Tyr) were observed in one isolate. Mutations in codon 379 (Ala → Val) and codon 394 (Thr → Ala) were each identified in one isolate. None of the pansusceptible isolates had mutations in the *katG* gene.

Mutations in the *inhA* promoter were detected in 17 (13.8%) of the MDR-TB isolates and 1 pansusceptible isolate. There were three types of mutation in the *inhA* promoter. Twelve isolates had a single nucleotide change at position −15 (C → T). One isolate each had a mutation at position −8 (T → A) and another at −8 (T → G), coupled with a mutation at *katG*315. The remaining three isolates had a C(−15)T mutation co-existing with mutations at codon 315 (Ser → Thr) or codon 379 (Ala → Val) in the *katG* gene.

Table 2
Demographic characteristics of different drug resistance groups.[a]

Characteristic	Total no. of isolates	Simple MDR-TB (n = 78)		Pre-XDR-TB (n = 31)		XDR-TB (n = 14)	
		n (%)	P-value	n (%)	P-value	n (%)	P-value
Sex							
Male	82	52 (63.4)	0.999	20 (24.4)	0.769	10 (12.2)	0.688
Female	41	26 (63.4)		11 (26.8)		4 (9.8)	
Age group							
<40 years	77	55 (71.4)	0.034	16 (20.8)	0.222	6 (7.8)	0.259
41–59 years	33	15 (45.5)		12 (36.4)		6 (18.2)	
≥60 years	13	8 (61.5)		3 (23.1)		2 (15.4)	
Occupation							
Farmer	66	44 (66.7)	0.195	16 (24.2)	0.658	6 (9.1)	0.104
Student	21	16 (76.2)		4 (19.0)		1 (4.8)	
Worker	13	6 (46.2)		3 (23.1)		4 (30.8)	
Other[b]	23	12 (52.2)		8 (34.8)		3 (13.0)	
Diabetes mellitus							
Yes	15	8 (53.3)	0.387	4 (26.7)	0.889	3 (20.0)	0.262
No	108	70 (64.8)		27 (25.0)		11 (10.2)	
Treatment							
New cases	47	33 (70.2)	0.218	10 (21.3)	0.430	4 (8.5)	0.430
Re-treated cases	76	45 (59.2)		21 (27.6)		10 (13.2)	

[a] Simple MDR-TB includes isolates resistant only to isoniazid and rifampicin; pre-XDR-TB includes MDR-TB isolates additionally resistant to either ofloxacin (OFLX) or kanamycin (KAN); and XDR-TB includes MDR-TB isolates additionally resistant to both OFLX and KAN.
[b] Includes unemployed, self-employed, institution staff and service staff.

Table 3
Distribution of genetic mutations among clinical multidrug-resistant tuberculosis isolates from Hebei Province, China.

Drug	Locus	Nucleotide/codon change	Nucleotide/amino acid change	No. (%) of isolates	Collaborative mutations
INH-resistant (123)	*katG*	AGC → ACC	Ser315Thr	66 (53.7)	
		AGC → ACC	Ser315Thr	2 (1.6)	C(−15)T in *inhA*
		AGC → ACC	Ser315Thr	1 (0.8)	T(−8)G in *inhA*
		AGC → ACC	Ser315Thr	1 (0.8)	T(−8)A in *inhA*
		AGC → AAC	Ser315Asn	16 (13.0)	
		ATC → ACC/TCC → TAC	Ile308Thr/Ser374Tyr	1 (0.8)	
		ACG → GCG	Thr394Ala	1 (0.8)	
		GCC → GTC	Ala379Val	1 (0.8)	C(−15)T in *inhA*
	Total			89 (72.4)	
	inhA	C → T	C(−15)T	12 (9.8)	
		C → T	C(−15)T	2 (1.6)	Ser315Thr in *katG*
		C → T	C(−15)T	1 (0.8)	Ala379Val in *katG*
		T → A	T(−8)A	1 (0.8)	Ser315Thr in *katG*
		T → G	T(−8)G	1 (0.8)	Ser315Thr in *katG*
	Total			17 (13.8)	
RIF-resistant (123)	*rpoB*	TCG → TTG	Ser531Leu	71 (57.7)	
		CAG → CGG/TCG → TTG	Gln482Arg/Ser531Leu	1 (0.8)	
		CAC → CGC	His526Arg	6 (4.9)	
		CAC → GAC	His526Asp	5 (4.1)	
		ACC → CCC/CAC → GAC	Thr508Pro/His526Asp	1 (0.8)	
		GAC → GTC	Asp516Val	3 (2.4)	
		CTG → CCG	Leu533Pro	2 (1.6)	
		CTG → CCG	Leu511Pro	1 (0.8)	
		CTG → CCG/GAC → AAC	Leu511Pro/Asp516Asn	1 (0.8)	
		CTG → CCG/GAC → TAC	Leu511Pro/Asp516Tyr	1 (0.8)	
		CTG → CCG/GAC → GGC	Leu511Pro/Asp516Gly	1 (0.8)	
		CAC → TAC	His526Tyr	3 (2.4)	
		CAC → GCC	His526Ala	1 (0.8)	
		CAC → AAC	His526Asn	1 (0.8)	
		CAC → CTC	His526Leu	2 (1.6)	
		CAA → CCA	Gln513Pro	1 (0.8)	
		TCG → CAG	Ser522Gln	1 (0.8)	
		GAC → GGC	Asp516Gly	1 (0.8)	
	Total			103 (83.7)	
OFLX-resistant (38)	*gyrA*	GCG → GTG	Ala90Val	8 (21.1)	
		GCG → GTG	Ala90Val	1 (2.6)	Asn499Thr in *gyrB*
		GAC → GGC	Asp94Gly	9 (23.7)	
		GAC → GGC	Asp94Gly	1 (2.6)	Leu442Leu in *gyrB*
		GAC → GCC	Asp94Ala	5 (13.2)	
		GAC → AAC	Asp94Asn	6 (15.8)	
		GAC → TAC	Asp94Tyr	3 (7.9)	
	Total			33 (86.8)	
	gyrB	GCG → GTG	Ala504Val	1 (2.6)	
		TCC → TTC	Ser447Phe	1 (2.6)	
		CTG → TTG	Leu442Leu	1 (2.6)	Asp94Gly in *gyrA*
		AAC → ACC	Asn499Thr	1 (2.6)	Ala90Val in *gyrA*
	Total			4 (10.5)	
KAN-resistant (21)	*rrs*	A → G	A1401G	12 (57.1)	
		T → C	T1491C	1 (4.8)	
	Total			13 (61.9)	
	eis	G → A	G(−10)A	2 (9.5)	
		C → T	C(−14)T	1 (4.8)	
	Total			3 (14.3)	
Pansusceptible (100)	*inhA*	C → T	C(−15)T	1 (1.0)	
	rpoB	CTG → CCG	Leu511Pro	1 (1.0)	
		TCG → TTG	Ser531Leu	1 (1.0)	
	gyrA	GCG→GTG	Ala90Val	3 (3.0)	
		GAC → GCC	Asp94Ala	2 (2.0)	
	gyrB	GGT → GTT	Gly436Val	1 (1.0)	
	Total			9 (9.0)	

INH, isoniazid; RIF, rifampicin; OFLX, ofloxacin; KAN, kanamycin.

3.5. Mutations in rpoB conferring resistance to rifampicin

A total of 18 mutational profiles were distributed in the *rpoB* gene within the RIF resistance-determining region (RRDR). One type of mutation at codon 531 (Ser → Leu) predominated and was observed in 72 isolates (58.5%). The most variable mutation was at codon 526: there were 12 isolates (9.8%) with His → Arg/Asp replacements, 3 isolates (2.4%) with His → Tyr replacement, 2 isolates (1.6%) with His → Leu replacement and 2 isolates (1.6%) with His → Ala/Asn replacements. Five isolates with eight different types of double mutation were identified. In addition, mutations at codons 511 (Leu → Pro), 513 (Gln → Pro), 516 (Asp → Val/Gly), 522 (Ser → Gln) and 533 (Leu→Pro) were found in nine isolates. Two pansusceptible isolates contained *rpoB* mutations (one at codon 511 and another at codon 531).

3.6. Mutations in gyrA and gyrB conferring ofloxacin resistance

Among the 123 MDR-TB strains, 38 (30.9%) were resistant to OFLX, of which 33 (86.8%) carried mutations in *gyrA*. The most

frequent mutation was at codon 94 (24/38; 63.2%), where four distinct mutations (Asp → Gly, Asp → Ala, Asp → Asn and Asp → Tyr) were noted. Another mutation at codon 90 (Ala → Val) was found in 9 (23.7%) of the 38 resistant isolates. Two missense mutations in *gyrA* (Ala90Val and Asp94Ala) were found in five pansusceptible isolates.

Four new types of mutation at codons 442 (Leu → Leu), 447 (Ser → Phe), 499 (Asn → Thr) and 504 (Ala → Val) were identified in *gyrB* in 4 (10.5%) of the 38 OFLX-resistant isolates. Double substitutions in *gyrB* + *gyrA* were observed in two isolates. Only one mutation (Gly436Val) was found in the *gyrB* gene of a pansusceptible isolate.

3.7. Mutations in rrs and eis promoter conferring kanamycin resistance

Of the 21 KAN resistance isolates, mutations in the *rrs* gene and the *eis* promoter were identified in 16 isolates (76.2%), but none were found in the pansusceptible isolates. Thirteen isolates (61.9%) had mutations in the *rrs* locus. Transitions at nucleotide positions 1401 (A → G) and 1491 (T → C) in the *rrs* sequence were observed in 12 (57.1%) and 1 (4.8%) isolate, respectively. Two nucleotide changes in the *eis* promoter region were present in three isolates; these occurred at nucleotide position −10 (G → A) in 2 isolates (9.5%) and −14 (C → T) in 1 isolate (4.8%).

3.8. Predicting multidrug-resistant and extensively drug-resistant tuberculosis by DNA sequencing

Compared with the phenotypic data, the sensitivities for detecting INH, RIF, OFLX and KAN resistance based on analysis of resistance-associated genes were 82.1%, 83.7%, 92.1% and 76.1%, respectively. The specificities of DNA sequencing results associated with genes for the prediction of INH, RIF, OFLX and KAN resistance were 99.0%, 98.0%, 94.0% and 100.0%, respectively. Detection of MDR-TB with mutations in the *katG*, *rpoB* and/or *inhA* promoter exhibited a sensitivity of 75.6% and a specificity of 97.0%, respectively. DNA sequencing of the seven loci in the study was 57.1% sensitive and 91.0% specific for the detection of XDR-TB isolates (Table 4).

3.9. Spoligotyping

According to comparison of the spoligotyping results with spoligotype international types (SITs), the typical Beijing genotype (SIT1) represented 89.4% (110/123) of the MDR-TB isolates, and 7 isolates (5.7%) belonged to atypical Beijing genotype (SITs 190, 260, 265 and 2101). The 6 remaining isolates belonged to clades T1 (SIT291 and 53) (3/123; 2.4%), T2 (SIT52) (1/123; 0.8%), LAM1 (SIT961) (1/123; 0.8%) and U (SIT602) (1/123; 0.8%). Among the 117 Beijing genotype isolates, 73 (62.4%) were simple MDR-TB, 30 (25.6%) were pre-XDR-TB and 14 (12.0%) were XDR-TB.

3.10. MIRU-VNTR

Genotyping of the MDR-TB isolates using 15-loci MIRU-VNTR revealed that the isolates were divided into 107 genotypes, of which 99 isolates were unique and 24 formed cluster patterns (Fig. 1). Eight small clusters ranging from two to five isolates were distinguished. The cumulative clustering rate and recent transmission rate determined by the MIRU-VNTR typing were 19.5% and 6.5%, respectively. Furthermore, the C1–C8 clusters, which contained 24 MDR-TB isolates, were also resistant to EMB or STR (or both) in addition to having mutations in *katG*, *inhA* promoter and/or *rpoB*. The C1–C3 and C6 clusters with 5 isolates had additional drug resistance to OFLX coupled with *gyrA* mutations. The C2 and C4 clusters with two XDR-TB isolates had identical drug resistance and associated mutations. All of the clustered strains belonged to the Beijing family.

4. Discussion

Although several studies have recently been published on the prevalence of mutations causing multidrug resistance in *M. tuberculosis*, this is the first study to provide a description of mutations at seven genetic loci associated (or speculated to be associated) with the development of MDR-, pre-XDR and XDR-TB in Hebei Province, China. The proportion of XDR-TB among patients infected with MDR-TB strains was 11.4% (14/123), i.e. within the range of 6.3–18.7% reported by others [8,12,13]. In addition, among

Table 4
Comparison of phenotypic drug susceptibility testing with sequence analysis of selected regions in seven drug resistance-associated loci.

Drug	Locus	No. of resistant isolates		No. of susceptible isolates		Validity (%)		Security (%)	
		MT	WT	MT	WT	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
INH	*katG*	89[a]	34	0	100	72.3 (63.4–79.9)	100.0 (95.4–100.0)	100.0 (94.8–100.0)	74.6 (66.2–81.6)
	inhA	17	106	1	99	13.8 (8.5–21.5)	99.0 (93.8–99.9)	94.4 (70.6–99.7)	48.3 (41.3–55.3)
	katG and/or *inhA*	101[a]	22	1	99	82.1 (74.0–88.2)	99.0 (93.8–99.9)	99.0 (93.9–99.9)	81.8 (73.5–88.0)
RIF	*rpoB*	103[b]	20	2	98	83.7 (75.8–89.5)	98.0 (92.3–99.6)	98.1 (92.6–99.7)	83.1 (74.8–89.1)
OFLX	*gyrA*	33	5	5	95	86.8 (71.1–95.1)	95.0 (88.2–98.1)	86.8 (71.1–95.1)	95.0 (88.2–98.1)
	gyrB	4	34	1	99	10.5 (3.4–25.7)	99.0 (93.5–99.9)	80.0 (29.9–98.9)	74.4 (65.0–80.8)
	gyrA and/or *gyrB*	35	3	6	94	92.1 (77.5–97.9)	94.0 (86.9–97.5)	85.4 (70.1–93.9)	96.9 (90.6–99.2)
KAN	*rrs*	13	8	0	100	61.9 (38.7–81.0)	100.0 (95.4–100.0)	100.0 (71.7–100.0)	92.6 (85.5–96.5)
	eis	3	18	0	100	14.3 (3.8–37.4)	100.0 (95.4–100.0)	100.0 (31.0–100.0)	84.7 (76.7–90.5)
	rrs and *eis*	16	5	0	100	76.1 (52.5–90.9)	100.0 (95.4–100.0)	100.0 (75.9–100.0)	95.2 (88.7–98.2)
MDR-TB	*katG* and/or *rpoB*	111[c]	12	2	98	90.2 (83.2–94.6)	98.0 (92.3–99.7)	98.2 (93.1–99.7)	89.1 (81.4–94.0)
	inhA and/or *rpoB*	104[b]	19	3	97	84.6 (76.7–90.2)	97.0 (90.8–99.2)	97.2 (91.4–99.3)	83.6 (75.3–89.6)
	katG and/or *inhA*, *rpoB*[d]	93[c]	30	3	97	75.6 (66.9–82.7)	97.0 (90.8–99.2)	96.9 (90.5–99.2)	76.4 (67.9–83.3)
XDR-TB	*katG*, *inhA*, *rpoB*, *gyrA*, *gyrB*, *rrs*, *eis*[e]	8	6	9	91	57.1 (29.6–81.1)	91.0 (83.2–95.5)	47.1 (23.9–71.5)	93.8 (86.5–97.5)

MT, mutant type; WT, wide-type; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; INH, isoniazid; RIF, rifampicin; OFLX, ofloxacin; KAN, kanamycin; MDR-TB, multidrug-resistant tuberculosis; XDR-TB, extensively drug-resistant tuberculosis.

[a] Included one isolates with two mutations.

[b] Included five isolates with two mutations.

[c] Included six isolates with two mutations.

[d] Specific resistance-related genes corresponding to MDR-TB were *katG* and/or *inhA* (INH) and *rpoB* (RIF).

[e] Specific resistance-related genes corresponding to XDR-TB were *katG* and/or *inhA* (INH), *rpoB* (RIF), *gyrA* and/or *gyrB* (OFLX) and *rrs* and *eis* (KAN).

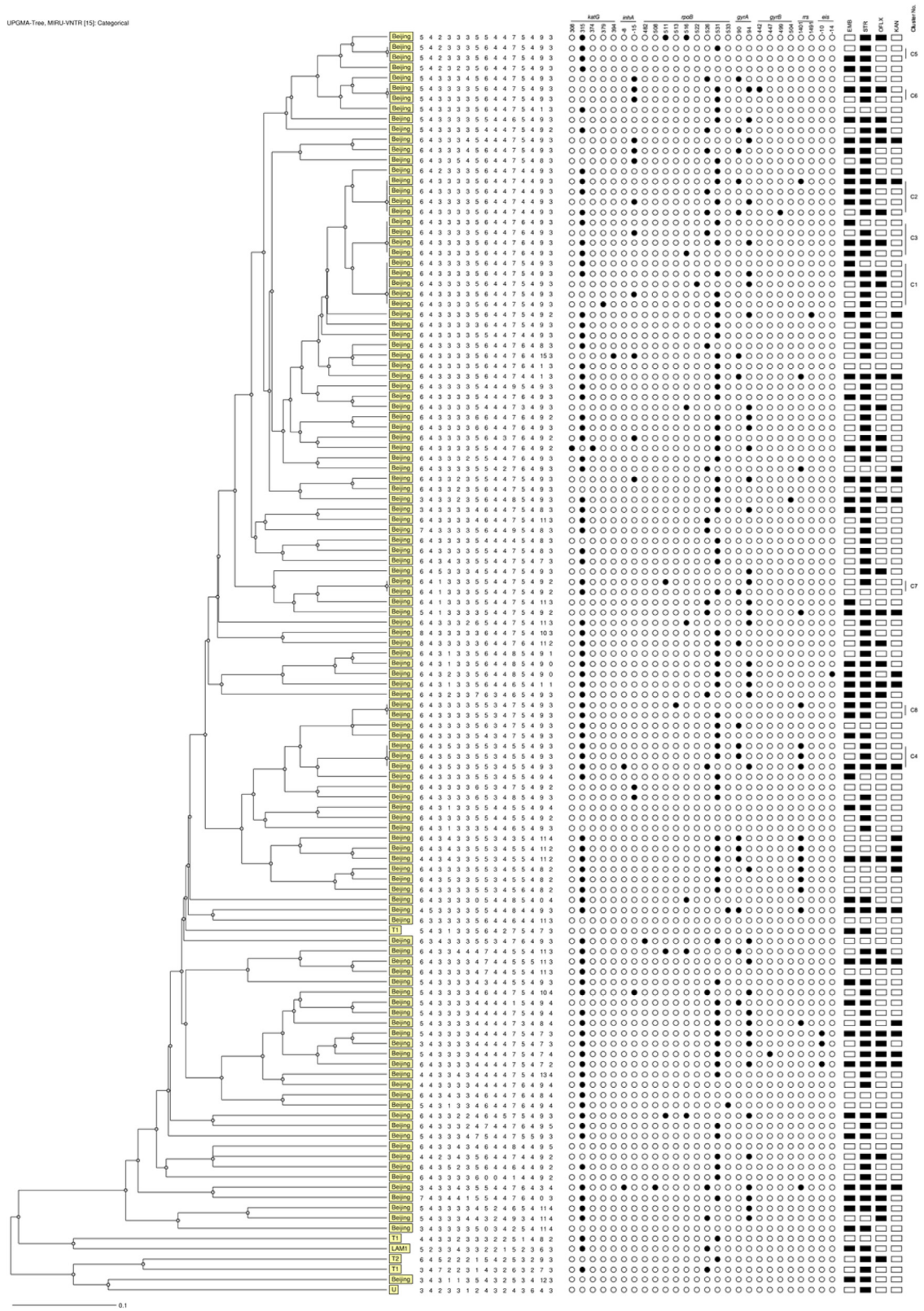

Fig. 1. A UPGMA tree based on 15-loci MIRU-VNTR of 123 clinical multidrug-resistant *Mycobacterium tuberculosis* isolates. From left to right: lineage; 15-loci MIRU-VNTR profile; mutational pattern; and drug resistance pattern. UPGMA, unweighted pair-group method with arithmetic mean; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number of tandem repeats; EMB, ethambutol; STR, streptomycin; OFLX, ofloxacin; KAN, kanamycin.

patients with MDR-TB, 25.2% (31/123) were pre-XDR-TB, placing them only one step away from developing XDR-TB. Therefore, appropriate therapy options should be advocated to prevent the spread of MDR-TB.

Globally, INH resistance has frequently been associated with mutations in the *katG* and *inhA* promoter, especially of codon 315 in *katG* and –15 in the *inhA* promoter. Of the 123 MDR-TB clinical isolates examined, 86.2% contained mutations in the *katG* region

and/or *inhA* promoter, consistent with reports from Europe and the USA [14,15]. The majority of mutations occurred at codon 315 in *katG*, accounting for 69.9% (86/123) of the MDR-TB isolates, which is similar to mutation rates reported in Poland (66.0%) and Southern Turkey (76.7%) [16,17]. Such a high mutation rate may be due to codon 315 itself conferring a selective advantage by reducing the ability of KatG to activate INH, while keeping catalase peroxidase activity to protect INH-resistant TB from oxidative stress [18]. Interestingly, two isolates in which an C(−15)T mutation in the *inhA* promoter co-occurred with a Ser315Thr substitution in *katG* indicated that the mechanism exhibited by these strains to achieve high-level resistance was a multistep one where the *inhA* mutation was likely acquired in the first place, followed by a high-level resistance mutation in Ser315Thr. The minimum inhibitory concentrations (MICs) will have to be further examined to confirm this speculation. Furthermore, 34 MDR-TB isolates had no resistance-associated mutations in the two targets analysed, suggesting that resistance in these isolates could be attributed to other genes outside the sequenced DNA, such as the structural regions of *inhA*, *kasA*, *mshA* and *ndh* [19].

Previous reports indicated that ≥95% of RIF-resistant isolates had mutations in the RRDR region of the *rpoB* gene [15,20,21]. In the current study, mutations in *rpoB* were found in 83.7% (103/123) of the MDR-TB isolates, which is lower than in previous reports [9,12,22], suggesting that the molecular mechanism of RIF resistance is not fully understood. The most frequently observed mutation occurred at codon 531 (58.5%), followed by codon 526 (15.4%) and codon 516 (5.7%), consistent with earlier investigations [23,24]. Different *rpoB* mutations are associated with different levels of RIF resistance. A previous study has shown that most strains with mutations at codons 531 and 526 display high-level resistance to RIF and are also resistant to INH [25]. Consequently, detection of mutations in the *rpoB* gene alone can be used to initially screen for MDR-TB.

OFLX plays an important role in the treatment of MDR-TB. Mutations in *gyrA* and *gyrB* are known to be a source of OFLX resistance. The current study showed that 86.8% (33/38) of the OFLX-resistant isolates had mutations in the *gyrA* gene, whilst only 10.5% (4/38) contained mutation in the *gyrB* gene, which is in agreement with previous observations that *gyrB* mutations are less common than *gyrA* mutations [26,27]. Mutations in codons 94 and 90 in general were the most commonly observed mutations [28,29], suggesting that these mutations may be used as a molecular marker for detecting resistance to OFLX. Four new mutations at codons 442 (Leu → Leu), 447 (Ser → Phe), 499 (Asn → Thr) and 504 (Ala → Val) in the *gyrB* locus were found in the current study; however, it will be necessary to explore the correlation between these novel mutations and OFLX resistance in further studies.

16S rRNA is encoded by the *rrs* gene and its mutation is now considered the primary mechanism of KAN resistance, particularly A1401G, a specific molecular marker for high-level KAN resistance [28]. As expected, A1401G was the predominant mutation in *rrs*. Moreover, mutations in the *eis* promoter region, another target of KAN, represent the second most common mechanism of low-level resistance to this drug (5 μg/mL < MIC < 80 μg/mL) [30]. In the current study, only three isolates harboured mutations in the promoter region of *eis*, namely G(−10)A and C(−14)T, mutation types that have been reported previously [31,32]. Among the 21 KAN-resistant isolates, 16 contained mutations in either the *rrs* or *eis* promoter, with no overlap between the two genes. A combination of *rrs* and *eis* could increase KAN resistance detection sensitivity without decreasing specificity, indicating that the two genes can serve as a good sensitivity marker for screening KAN.

The sensitivities for predicting INH, OFLX and KAN resistance from DNA sequencing data compared with phenotypic data in this study were 82.1%, 92.1, and 76.1%, respectively, which is in accordance with previous results [33–35]. The sensitivity of sequence analysis for RIF resistance was 83.7%, which is lower than values obtained in other studies [36,37]. Regional variation may explain this difference. Using a combination of three loci (*katG*, *rpoB* and/or *inhA* promoter), the DNA sequencing assay was able to identify 75.6% of MDR-TB isolates, similar to results reported in Jiangxi Province [13]. Upon combination of the data sets for seven loci (*katG*, *inhA* promoter, *rpoB*, *gyrA*, *gyrB*, *rrs* and *eis* promoter), the sensitivity and specificity for predicting XDR-TB was 57.1% and 91.0%, respectively, a value also found by Zhao et al. [22].

After acquiring drug resistance, the Beijing genotype retained a certain degree of adaptability and so continued to spread in the environment [38,39]. This means that drug-resistant strains with the Beijing genotype have a strong potential for virulence. In this study, the proportion of Beijing genotype in different DST patterns was higher than in other studies. The degree of drug resistance and the types of mutation demonstrated no association with the Beijing genotype, which is inconsistent with previous reports [40–43]. We speculate that this discrepancy maybe due to regional diversity.

To better understand the transmission of MDR-TB strains, we attempted to establish a phylogenetic tree using 15-loci MIRU-VNTR with detailed genetic and phenotypic profiles. The 123 MDR-TB strains could be classified into eight small clusters containing 24 isolates, whereas the remaining 99 isolates exhibited unique patterns. Approximately 79.8% of the isolates from unique patterns indicated reactivation events during this study period. The low clustering rate (19.5%) and small cluster size (ranging from two to five per cluster) suggested that the MDR-TB isolates may have appeared under specific conditions, especially in areas of frequent human migrations. The very different genotypic patterns and drug resistance profiles suggest that acquired resistance is an important factor for the emergence of MDR-TB in Hebei Province.

The present study has some weaknesses. First, pansusceptible isolates were used as negative controls, which could result in an overestimation of the specificity for DNA sequencing. Hence, further research is needed both with drug-resistant and drug-susceptible isolates as controls in order to evaluate the validity of the DNA sequencing assay. Second, some mutations causing drug resistance may have been missed. Whole-genome sequencing technology is required to discover new molecular mechanisms to compensate for this deficiency. Finally, DST was performed only at a critical concentration, but the MICs were not established; hence, the full breadth of phenotypic–genotypic relationships was not evaluated.

In summary, this is the first report on the genotypic analysis of seven loci (*katG*, *inhA* promoter, *rpoB*, *gyrA*, *gyrB*, *rrs* and *eis* promoter) in clinical *M. tuberculosis* isolates from Hebei Province. A total of 39 distinct mutations at the seven loci were detected in 114 clinical isolates, accounting for 92.7% of the 123 MDR strains tested. The frequency of INH, RIF, OFLX and KAN resistance-associated mutations in MDR-TB isolates was 82.1% (101/123), 83.7% (103/123), 92.1% (35/38) and 76.2% (16/21), respectively. The most prevalent mutations involved in INH, RIF, OFLX and KAN resistance were Ser315Thr in *katG* (70/123; 56.9%), C(−15)T in *inhA* (15/123; 12.2%), Ser531Leu in *rpoB* (72/123; 58.5%), Asp94Gly in *gyrA* (10/38; 26.3%) and A1401G in *rrs* (12/21; 57.1%), respectively. Four novel *gyrB* mutants (Leu442Leu, Ser447Phe, Asn499Thr and Ala504Val) were identified. Detection of MDR-TB isolates with mutations in *katG*, *rpoB* and/or *inhA* promoter showed a sensitivity of 75.6% and a specificity of 97.0%, respectively. DNA sequencing of the seven loci in the study was 57.1% sensitive and 91.0% specific for detection of XDR-TB isolates. This accuracy for the above loci as predictive markers of MDR- and XDR-TB suggests that these loci

may form the basis of an optimised molecular diagnostic method for detecting MDR-TB.

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Competing interests

None declared.

Ethical approval

Informed consent was received from all patients involved in this study. Ethical approval was obtained from the Institutional Review Board of the Fifth Hospital of Shijiazhuang.

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