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Original Article

Genotypicdiversity of drug-resistant *Mycobacterium tuberculosis* isolates from Hebei, China

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Abstract: Drug-resistant tuberculosis (DR-TB), particularly multidrug-resistant tuberculosis (MDR-TB), has been identified as a major challenge for effective TB control. For rapid detection and proper treatment, molecular assays based on the identification of mutations in genes associated with drug resistance have been established to determine drug resistance. However, there is as yet little information about drug resistance-associated mutations of clinical *Mycobacterium tuberculosis* isolates from Hebei. In this study, four genetic loci *katG*, *inhA*, *oxyR-ahpC* intergenic region and *rpoB* were sequenced among 276 DR-TB isolates from Hebei to understand the association between specific mutations, drug resistance phenotypes and spoligotyping genotypes. Altogether, 83.8% of INH resistant isolates harbored at least one mutation of *katG*, *inhA,* and *oxyR-ahpC* intergenic region and 78.4% of RIF resistant isolates harbored one or more mutations in *rpoB*. The predominant mutation patterns of *rpoB* and *katG* in Hebei was Ser531Leu and Ser315Thr, respectively. Additionally, 91.2% of MDR isolates harbored at least one mutation in these four targeted fragments. Compared with the phenotypic data, the sensitivity and specificity of co-testing of *katG*, *inhA* promoter and *oxyR-ahpC* intergenic region for INH resistance were 83.8% and 96.8%, respectively and the *rpoB* exhibited a sensitivity of 78.4% and a specificity of 95.3% for RIF resistance. Furthermore, there was no association between drug resistance-conferring mutations and spoligotypes. This finding will be useful for the establishment of rapid molecular diagnostic methods in Hebei province, China.

Keywords: *Mycobacterium tuberculosis*, drug resistance, gene mutation

Introduction

Drug-resistant tuberculosis (DR-TB), particularly multidrug-resistant tuberculosis (MDR-TB), has been identified as a major challenge for effective TB control [1]. Globally, an estimated 3.3% of new TB cases and 20% of previously treated cases developedinto MDR-TB in 2014 [2]. China as one of the 22 high-burden countries, held 10% of the global TB cases. The latest data from the national survey of drug-resistant tuberculosis demonstrated that 5.7% of newly diagnosed TB patients and 25.6% of retreatments had MDR-TB in China [3].

Conventional drug susceptibility testing (DST) of cultured mycobacteria typically provides results within 1-3 months [4]. Even the up-to-date automated fluid culture method still takes an average of 14 days for TB identification and another 14 days are required to get the information on drug susceptibility pattern [5]. Therefore, TB patients often receive standard chemotherapy treatment, instead of a regimen designed according to the DST pattern, which may result in inadequate treatment and selection of drug-resistant strains. For rapid detection and proper treatment, molecular assays based on the identification of mutations in genes associated with drug resistance have been established to determine drug resistance [6]. However, there is a wide variation of circulating MTB strains worldwide, and the frequency, location and type of drug resistance-associated mutations vary in different geographical areasand different time periods [7-9].

Hebei province surrounds Beijing and Tianjin and its strategic position is increasingly significant due to the rapid development of economic

integration of the Beijing-Tianjin-Hebei region. The extensive and convenient traffic network makes Hebei a key transportation hub connecting Beijing with the entire country. The growing mobility of people and increasing morbidity of AIDS attribute to the widespread occurrence of TB in this area. So far, there is as yet little information about the drug resistance-associated mutations of clinical *Mycobacterium tuberculosis* isolates from Hebei. In the present study, we analyzed four genetic loci associated with INH and RIF, including *katG*, *inhA* promoter, *oxyR-ahpC* intergenic region and *rpoB*, among 276 DR-TB isolates from Hebei. Therefore, the association between specific mutations, drug resistance phenotypes and spoligotyping genotypes was clarified and the diagnostic potential of specific mutation for broad drug resistance was assessed.

Materials and methods

Mycobacterium tuberculosis isolates

For this study, a total of 376 MTB isolates, each corresponding to an individual pulmonary TB patient, were collected fromlocal tuberculosis hospitals in Hebei province during 2014. The numbers isolated from each city were as follows: Shijiazhuang, 135 isolates; Baoding, 78 isolates; Cangzhou, 61 isolates; Qinhuangdao, 40 isolates; Xingtai 39 isolates; Handan, 8 isolates; Hengshui, 7 isolates; Tangshan, 4 isolates; Chengde, 2 isolates; Langfang, 2 isolates and none from Zhangjiakou. No epidemiological connection of these patients could be identified by standard investigation. Among the 376 isolates, 100 were identified as pan-sensitive strains and were therefore used as negative controls. *Mycobacterium tuberculosis* H37Rv (ATCC27294) was used as a reference.

Drug susceptibility testing

Drug susceptibility testing (DST) was performed using the Lowenstein-Jensen (L-J) proportion method at the critical concentration of 0.2, 40, 4 and 2 μg/ml for isoniazid (INH), rifampicin (RIF), streptomycin (STR) and ethambutol (EMB), respectively [10]. H37Rv was used as control for each batch of DST. Strains with resistance to one or more drugs were identified as DR-TB and strains resistant to at least INH and RIF were defined as MDR-TB.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from mycobacterial colonies grown on L-J medium using Mericon™ DNA Bacteria Kit (Qiagen, USA) according to the manufacturer's instructions. Expected fragments were amplified using the primers listed in Table S1. Each PCR reaction contained 15 μl 2×Taq MasterMix (CWBIO, China), 2 μl (10 μM) forward primer, 2 μl (10 μM) reverse primer, 40-60 ng of genomic DNA and double-distilled H_2O to bring the final volume to 30 μl. PCR was performed as follows: initial denaturation at 94°C for 5 min, and then 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. PCR products were then electrophoresed on 1.5% agarose gel and sized with 100 bp DNA ladder (CWBIO, China) to identify the quality of PCR products. H37Rv was used as positive control and double-distilled H_2O was used as negative control. Qualified PCR products were sent to TsingKe Biological Technology Company (Beijing, China) for sequencing using the same primers as in the PCR amplification. All sequence data were aligned with the corresponding sequences of H37Rv reference strain (GenBank accession number NC_000962) and analyzed by DNASTAR LasergeneMegAlign version 7.1.0.

Resolution of discrepant results

The agreement of DST and DNA sequencing varied for each drug and locus combination. When discrepancies occurred between DST and DNA sequencing, the repeated targeted gene sequencing has been conducted after growing in the BACTEC™ MGIT™ 960 liquid media. If the retesting result conflicted with the initial data, a third round of testing was performed, with the final value representing two out of the three cycles.

Spoligotyping

Spoligotyping was performed using the standard protocol described by Kamerbeek et al. [11]. First, the direct repeat (DR) region was amplified with primers of DRa (biotin-labeled) and DRb. PCR products were then hybridized to a membrane with a set of 43 different oligonu-

Table 1. Mutations of four drug resistance-associated loci among DT-TB isolates from Hebei

DST	Locus	Codon/nucleotide	Codon/nucleotide change	Amino acid/nucleotide change	n (%)	Other mutations
INH^S	Wild type				79	
	katG	297	GGC→GTC	Gly→Val	1	
		302	AGC→AGG	Ser→Arg	1	
		315	AGC→ACC	Ser→Thr	1	
		394	ACG→GCG	Thr→Ala	1	
	inhA	-15	C→T	C→T	1	
	oxyR-ahpC	-81	C→T	C→T	1	
INH^R	Wild type				31	
	katG	308/374	ACC→CCC/TCC→TAC	Thr→Pro/Ser→Tyr	1	*oxyR-ahpC C(-52)T*
		315	AGC→AAC	Ser→Asn	15	
			AGC→ACC	Ser→Thr	98	
			AGC→ACC	Ser→Thr	2	*inhA* C(-15)T
		315/317	AGC→AAC/ATC→GTC	Ser→Asn/Ile→Val	1	
			AGC→AAC/ATC→GTC	Ser→Asn/Ile→Val	3	*oxyR-ahpC* C(-52)T
		318	GAC→AAG	Glu→Lys	1	*oxyR-ahpC* C(-52)T
		337	ATC→GTC	Tyr→Asp	1	
		379	GCC→GTC	Ala→Val	1	
		394	AGC→GCG	Thr→Ala	1	*inhA* C(-15)T
	inhA	-8	T→A	T→A	1	*katG* S315T
			T→C	T→C	1	*katG* S315T
			T→G	T→G	1	*katG* S315T
		-15	C→T	C→T	19	
			C→T	C→T	2	*katG* S315T
			C→T	C→T	1	*katG* T394A
			C→T	C→T	1	*oxyR-ahpC* G(-51)A
			C→T	C→T	2	*oxyR-ahpC* C(-52)T
			C→T	C→T	2	*oxyR-ahpC* C(-81)T
		-17	C→T	C→T	1	
	oxyR-ahpC	-41	C→A	C→A	1	
		-48	G→A	G→A	1	
		-51	G→A	G→A	3	1 with *inhA C*(-15)T
		-52	C→T	C→T	1	
			C→T	C→T	2	*inhA* C(-15)T
			C→T	C→T	1	*katG* S315T
			C→T	C→T	1	*katG* E318K
			C→T	C→T	1	*katG* S315T/I317V
			C→T	C→T	1	*katG* T308P/S374Y
		-54	C→T	C→T	1	
		-57	C→T	C→T	1	
		-72	C→T	C→T	2	
		-74	G→A	G→A	2	
		-81	C→T	C→T	2	*inhA* C(-15)T
RIF^S	Wild type				103	
	rpoB	511	CTG→CCG	Leu→Pro	2	
		516	GAC→GTC	Asp→Val	1	
		526	CAC→AAC	Asp→Asn	1	
			CAC→CTC	Asp→Leu	1	
			CAC→TAC	Asp→Tyr	1	
			CAC→TCC	Asp→Ser	1	
		531	TCG→TTG	Ser→Leu	3	
		533	CTG→CCG	Leu→Pro	1	

RIF^R	Wild type				35
	rpoB	508/526	ACC→CCC/CAC→CGC	Thr→Pro/His→Arg	1
		511	CTG→CCG	Leu→Pro	2
		511/516	CTG→CCG	Leu→Pro/ Asp→Tyr	1
			CTG→CCG	Leu→Pro/ Asp→Asn	1
			CTG→CCG	Leu→Pro/ Asp→Gly	1
		511/526	CTG→CCG/CAC→AAC	Leu→Pro/His→Asn	1
		513	CAA→CCA	Gln→Pro	1
		516	GAC→GTC	Asp→Val	4
		522	TCG→CAG	Ser→Gln	1
			TCG→TTG	Ser→Leu	1
		526	CAC→CGC	His→Arg	6
			CAC→AAC	His→Asn	1
			CAC→CTC	His→Leu	2
			CAC→GCC	His→Ala	1
			CAC→TAC	His→Tyr	5
			CAC→GAC	His→Asp	9
		526/531	CAC→GAC/TCG→TTG	His→Asp/Ser→Trp	1
		531	TCG→TTG	Ser→Trp	1
			TCG→TTC	Ser→Phe	1
			TCG→TTG	Ser→Leu	84
		533	CTG→CCG	Leu→Pro	2

Table 2. Evaluation of four drug resistance-associated loci and DST phenotypes of DR-TB isolates and pan-sensitive controls

Drug	Locus	Resistant isolates[a]		Sensitive isolates[b]		Pan-sensitive controls[c]		Sensitivity[d] (%)	Specificity[e] (%)
		M	NM	M	NM	M	NM		
INH	*katG*	124	67	4	81	0	100	64.9%	97.8%
	inhA	31	160	1	84	0	100	16.2%	99.5%
	oxyR-ahpC	21	170	1	84	0	100	11.0%	99.5%
	katG and/or inhA	149	42	5	80	0	100	78.0%	97.3%
	katG and/or inhA and/or oxyR-ahpC	160	31	6	79	0	100	83.8%	96.8%
RIF	*rpoB*	127	35	11	103	0	100	78.4%	95.3%

[a]Isolates with resistance to INH or RIF; M, mutation; NM, no mutation. [b]Isolates sensitive to INH or RIF, but resistant to other drugs. [c]Isolates sensitive to all four drugs. [d]Number of resistant isolates with mutation/total number of resistant isolates. [e]Number of sensitive isolates without mutation and number of pan-sensitive controls without mutation/total number of sensitive isolates and pan-sensitive controls.

cleotide probes covalently bound to its surface. The results in binary format were compared against the international spoligotype database SpolDB4.0 (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/) to obtain the spoligotyping pattern [12].

Statistical analysis

The statistical analysis was done by SPSS16.0 software. The Chi-square test or Fisher's exact test was usedto compare the proportion of different groups. A two-sided *P*-value of less than 0.05 was considered statistically significant.

Results

Study population

Among the 276 isolates, 192 (69.6%) were from male patients and 84 (30.4%) from female patients. The ages of these patients ranged from 14 to 83 years old (mean ± standard error [SE], 43.8±1.0). All of these patients were

Table 3. Mutation patterns of *katG*315 in different INH phenotypes

DST	Total	Mutant n (%)	Wild type n (%)	*P*-value for trend
INH sensitive	85	11 (12.9)	74 (87.1)	<0.0001
INH mono-resistance	34	13 (38.2)	21 (61.8)	
INH + STR/INH + RIF	52	37 (71.2)	15 (28.8)	
INH + STR + RIF/INH + RIF + EMB/INH + STR + EMB	59	36 (61.0)	23 (39.0)	
INH + STR + RIF + EMB	46	33 (71.7)	13 (28.3)	

Hebei residents, and 55.8% (154/276) were newly diagnosed TB cases.

Drug susceptibility phenotypes

In this study, 100 isolates were pan-sensitive (P^S) strains and 276 were DR-TB, of which 191 were resistant to INH (INH^R), 85 sensitive to INH (INH^S), 162 resistant to RIF (RIF^R) and 114 sensitive to RIF (RIF^S), including 125 MDR-TB. The detailed DST patterns of 276 DR-TB arelisted in Table S2.

INH resistance-associated mutations

Fragments of the hot regions of *katG*, *inhA*promoter and *oxyR-ahpC* intergenic region were examined in this study. None of the 100 P^S isolates carried any mutations in these three targeted fragments. In the INH^R group, 160 (83.8%) harbored mutations in one or more of these three regions, of which 124 (64.9%) harbored mutations in *katG*, 31 (16.2%) harbored mutations at *inhA* and 21 (11.0%) harbored mutations at *oxyR-ahpC* intergenic region. The novel mutations were observed in *katG*315 (62.3%) and *inhA* promoter 15 (14.1%). However, no mutations were detected in the remaining 31 isolates. In the INH^S group, 6 (7.1%) isolates were mutant strains, of which 4 (4.7%) carried only *katG* mutations, 1 (1.2%) carried only *inhA* mutations and 1 (1.2%) carried only *oxyR-ahpC* intergenic region mutations (**Table 1**). Compared with the DST phenotypes, the sensitivity and specificity of cotesting of *katG*, *inhA* promoter and *oxyR-ahpC* intergenic region were 83.8% and 96.8%, respectively. The cotesting result was better than those obtained using a single locus (**Table 2**).

Since *katG*315 was the most common mutation site in INH resistant isolates, the mutation rate of this codon in different INH phenotype groups was analyzed. Data showed that the mutation rate of *katG*315 increased as the rising numbers of drug resistance (*P*-value for trend <0.0001) (**Table 3**).

RIF resistance-associated mutations

To investigate the mutations associated with RIF, a 629-bp region of *rpoB*, including 81-bp RIF resistance-determining region (RRDR), it was sequenced. Altogether, 78.4% (127/162) of the RIF^R isolates harbored at least one mutation within *rpoB*, while the other 35 isolates lacked such a mutation. All of the *rpoB* mutations were detected within the RRDR region. One hundred and twenty three isolates (75.9%) carried a single mutation, and 4 isolates had double mutations. The most common mutations were codon 531 and 526, with the mutation rate of 53.7% (87/162) and 16.7% (27/162), respectively. The mutations at codon 508, 511, 516, 522, and 533 were also detected (**Table 1**).

None of the P^S isolates conferred a mutation within *rpoB*. In the RIF^S group, the majority (90.4%, 103/114) showed no mutations within the target fragment of *rpoB*. The mutations at codon 511, 516, 526, 531, and 533 were detected in 2, 1, 4, 3, and 1 isolate, respectively. In particular, all of the 11 RRDR mutants were resistant to INH, of which 4 also showed resistance to STR and 1 to EMB. Statistical analysis demonstrated that the RIF^R group had a significantly higher RRDR mutation rate than the RIF^S and P^S group, and the RIF^S group had a higher RRDR mutation rate than the P^S group, indicating that RRDR was a potential biomarker for broad drug resistance, not only for RIF resistance. However, no significant difference was observed between initial RIF resistance isolates and acquired RIF resistance isolates (**Table 4**). Additionally, *rpoB* exhibited a sensitivity of 78.4% and a specificity of 95.3% for detecting RIF resistant phenotypes (**Table 2**).

Table 4. RRDR mutation rates of different RIF phenotype groups

DST[a]	Total	Wild type n (%)	RRDR mutation n (%)	*P*-value
Pan-sensitive	100	100 (100)	0	
RIF^S	114	103 (90.4)	11 (9.6)	<0.0001[b]
RIF^R	162	35 (21.6)	127 (78.4)	<0.0001[c]
Initial RIF resistance	72	18 (25.0)	54 (75.0)	
Acquired RIF resistance	90	17 (18.9)	73 (81.1)	0.3477[d]

[a]RIF^S: isolates sensitive to RIF but resistant to other first-line anti-TB drugs; RIF^R: isolates resistant to RIF. [b]Comparison of gene mutations between pan-sensitive and RIF^S isolates. [c]Comparison of gene mutations between RIF^S and RIF^R isolates. [d]Comparison of gene mutations between initial RIF resistance and acquired RIF resistance isolates.

Table 5. Association between four drug resistance-associated loci and different spoligotypes

Gene	Mutation	Beijing family				Non-Beijing family	*P*-value[b]
		Typical	Atypical	*P*-value[a]	Total		
katG	Mutant	123	8	0.5724	131	7	0.2452
	Wild type	120	5		125	13	
inhA	Mutant	35	3	0.4176	38	2	0.7482
	Wild type	208	10		218	18	
oxyR-ahpC	Mutant	19	2	0.2893	21	1	1.0000
	Wild type	224	11		235	19	
rpoB	Mutant	123	9	0.2575	132	6	0.1021
	Wild type	120	4		124	14	

[a]Comparison of gene mutations between the typical Beijing family and the atypical Beijing family. [b]Comparison of gene mutations between the Beijing family and the non-Beijing family.

Mutations of MDR isolates

To investigate the diagnostic value of gene mutations for MDR, which are at least resistant to INH and RIF, the gene mutation patterns of *katG*, *inhA* promoter, *oxyR-ahpC* intergenic region and *rpoB* in MDR isolates were analyzed. Among the 125 MDR isolates, 114 (91.2%) harbored at least one mutation in these four targeted fragments, while 11 (8.8%) had no such mutations. In total, 82.4% (103/125) of MDR isolates carried mutations in *rpoB* and 88.0% (110/125) had at least one mutation in the INH resistance-associated loci detected, of which 71.2% (89/125) carried mutations in *katG*, 13.6% (17/125) in *inhA* promoter and 12.8% (16/125) in *oxyR-ahpC*. The most commonly combined mutations were *katG* and *rpoB* (64.8%, 81/125), with *katG*-315 and *rpoB*531 being the predominant mutation sites, which were detected in 55 MDR isolates (Table S3).

Spoligotyping

In total, 93.0% (93/100) of the P^S isolates belonged to the Beijing family and 7.0% (7/100) belonged to the non-Beijing family, including 6 isolates from T lineage and 1 from the undefined lineage. Among the DR-TB isolates, 92.8% (256/276) were from the Beijing family, of which 243 isolates were identified as the typical Beijing family characterized by the presence of spacers 35 to 43 and 13 isolates were the atypical Beijing family or Beijing-like family, with the absence of one or more oligonucleotides at spacers 35 to 43. The remaining 20 isolates (7.2%) were from the non-Beijing family, including 12 isolates of T lineage, 2 of H lineage, 1 of LAM lineage, 1 of U lineage and 3 of undefined lineage. Statistical analysis indicated that there were no significant difference in the mutation rates of *katG*, *inhA* promoter, *oxyR-ahpC* intergenic region and *rpoB* between the typical and atypical Beijing isolates, and the Beijing and non-Beijing isolates (**Table 5**).

Discussion

Conventional culture-based DST method cannot satisfy the clinical need for rapid diagnosis due to the slow growth of *M. tuberculosis* and the diagnostic delay remains an obstacle to effective TB care. Consequently, molecular assays based on detection of resistance-conferring mutations are established for rapid diagnosis of DR-TB. Several studies on molecular characterization of MTB strainsin China have been reported. However, the frequency and distribution of drug resistance-associated mutations vary in different geographical areas and different time periods [9, 13]. Despite the special geographic location of Hebei province, information regarding the genetic diversity of DR-TB isolates is still unavailable in this area. In this study, a comprehensive analysis of resistance-associated mutations of INH and RIF in DR-TB clinical isolates from Hebeiwas conducted.

INH resistance is considered to be associated with mutations in several genes, particularly in *katG* and *inhA* promoter. Notably, 64.9% and 16.2% of INH^R isolates in the current study carried point mutations in *katG* and *inhA* promoter, respectively. The most frequent mutation sites were *katG*315 and *inhA* promoter 15. The *oxyR-ahpC* intergenic regionwas also detected, but it was only identified in a low proportion (11.0%) of INH^R isolates in our study. The combination of *katG*, *inhA* and *oxyR-ahpC* intergenic region could detect 83.8% of INH resistance isolates from Hebei province, similar to previous data from China [14, 15].

Approximately 90.2-98.0% of RIF resistant isolates around the world harbored mutations in the RRDR of *rpoB* gene, with codon 531, 526, and 516 being the most prevalent mutation sites [8, 14, 16-18]. However, only 78.4% of RIF resistant isolates possessed at least one mutation in RRDR in the current study, lower than the reported data, which was partially because the enrolled RIF resistant strains included both MDR isolates and RIF mono-resistant isolates. Furthermore, we found that history of previous chemotherapy for tuberculosis was not associated with gene mutations conferring resistance to RIF, contradictory withan earlier report [5, 19], which may result from the different DST strain population enrolled in different studies.

Early detection of MDR-TB is essential to prevent its transmission in the community and initiate effective anti-TB treatment regimen. Therefore, the association between the gene mutations detected and MDR phenotypes was analyzed. Altogether, 82.4% of MDR isolates carried mutations in *rpoB* and 88.0% had at least one mutation in the INH resistance-associated loci detected. The predominant mutation patterns of *rpoB* and *katG* in Hebei was Ser531Leu and Ser315Thr, respectively, which was consistent with a report from Jiangxi, Hunan and South-central China [14, 19, 20].

The apparent global success of the Beijing lineage suggested that it might have selective advantages (higher virulence or transmissibility) over other MTB genotypes. However, no significant difference in occurrence of resistance-conferring mutations of *katG*, *inhA*, the *oxyR-ahpC* intergenic region and *rpoB* between Beijing and non-Beijing isolates was observed, consistent with previous result [15].

Additionally, the current study confirmed the absence of mutations in the detected genes in drug resistant isolates and this phenomenon has been also reported in several other studies [21, 22]. The discrepancy between DST phenotypes and gene mutation genotypes may be attributed to mutations that occur outside the amplified regions or that are located in other genes. For example, mutations in *kasA* and *ndh* have been detected in INH resistant isolates [23, 24].

This study has some potential limitations. First, the number of strains analyzed is relatively small, even though this is probably the first comprehensive study of *Mycobacterium tuberculosis* in Hebei. Second, the current study was limited to an evaluation of the hospital setting only, and transmission that may have occurred in households or communities was not determined. Finally, only four target genes were sequenced in our study and mutations in other location sites will be analyzed in the following study.

Conclusions

In summary, 83.8% of INH resistant isolates harbored at least one mutation of *katG*, *inhA* and *oxyR-ahpC* intergenic region and 78.4% of RIF resistant isolates harbored one or more mutations in *rpoB*. The predominant mutation patterns of *rpoB* and *katG* in Hebei was Ser-531Leu and Ser315Thr, respectively. Additionally, 91.2% of MDR isolates harbored at least one mutation in these four targeted fragments. Compared with the phenotypic data, the sensitivity and specificity of co-testing of *katG*, *inhA* promoter and *oxyR-ahpC* intergenic region for INH resistance were 83.8% and 96.8%, respectively and the *rpoB* exhibited a sensitivity of 78.4% and a specificity of 95.3% for RIF resistance. Furthermore, there was no association between drug resistance-conferring mutations and spoligotypes. This finding will be useful for the establishment of rapid molecular diagnostic methods in Hebei province, China.

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Disclosure of conflict of interest

None.

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Table S1. Primers for PCR amplification and DNA sequencing

Gene	Sequence (5'-3')	Position	Product size (bp)
rpoB	F)GAGCCCCCGACCAAAGA	760644-761272	629
	R)ATGTTGGGCCCCTCAGG		
katG	F)GATCGTCGGCGGTCACACTT	2154683-2155413	731
	R)CGTTGACCTCCCACCCGACT		
inhA promoter	F)TGCCCAGAAAGGGATCCGTCATG	1673278-1673732	455
	R)ATGAGGAATGCGTCCGCGGA		
oxyR-ahpC	F)GCTTGATGTCCGAGAGCATCG	2725809-2726509	700
Intergenic region	R)GGTCGCGTAGGCAGTGCCCC		

Table S2. Drug resistance patterns of DR-TB isolates

DST	n (%)
Mono resistance	92 (33.3)
STR	42 (15.2)
INH	13 (4.7)
RIF	34 (12.3)
EMB	3 (1.1)
Multi-resistance	184 (66.7)
STR + RIF	18 (6.5)
STR + INH	31 (11.2)
STR + EMB	3 (1.1)
RIF + INH	21 (7.6)
RIF + EMB	2 (0.7)
STR + RIF + INH	55 (19.9)
STR + RIF + EMB	4 (1.4)
STR + INH + EMB	1 (0.4)
RIF + INH + EMB	3 (1.1)
STR + INH + RIF + EMB	46 (16.7)

Table S3. Mutation patterns of multi-drug resistance clinical isolates from Hebei, China (N=125)

Locus	Codon/nucleotide changes	Amino acid/nucleotide changes	n (%)	Other mutations
NM*			11 (8.8)	
katG + *rpoB*	AGC-ACC, CAG-CGG/TCG-TTG	Ser315Thr, Gln482Arg/Ser531Leu	1 (0.8)	
	AGC-ACC, ACC-CCC/CAC-CGC	Ser315Thr, Thr508Pro/His526Arg	1 (0.8)	*inhA*T(-8)A
	AGC-ACC, CTG-CCG	Ser315Thr, Leu511Pro	1 (0.8)	
	AGC-ACC, CTG-CCG/GAC-TAC	Ser315Thr, Leu511Pro/Asp516Tyr	1 (0.8)	
	AGC-ACC, CTG-CCG/GAC-AAC	Ser315Thr, Leu511Pro/Asp516Asn	1 (0.8)	
	AGC-ACC, CTG-CCG/GAC-GGC	Ser315Thr, Leu511Pro/Asp516Gly	1 (0.8)	
	AGC-ACC, CAA-CCA	Ser315Thr, Gln513Pro	1 (0.8)	
	AGC-ACC, GAC-GTC	Ser315Thr, Asp516Val	1 (0.8)	
	AGC-ACC, CAC-TAC	Ser315Thr, His526Tyr	1 (0.8)	*inhA* T(-8)G
	AGC-ACC, CAC-CGC	Ser315Thr, His526Arg	4 (3.2)	
	AGC-ACC, CAC-AAC	Ser315Thr, His526Asn	1 (0.8)	
	AGC-ACC, CAC-CTC	Ser315Thr, His526Leu	1 (0.8)	*inhA*C(-15)T
	AGC-ACC, CAC-CTC	Ser315Thr, His526Leu	1 (0.8)	
	AGC-ACC, CAC-GAC	Ser315Thr, His526Asp	4 (3.2)	

	AGC-AAC, CAC-GCC	Ser315Asn, His526Ala	1 (0.8)	
	AGC-ACC, TCG-TTG	Ser315Thr, Ser531Leu	37 (29.6)	
	AGC-ACC, TCG-TTG	Ser315Thr, Ser531Leu	1 (0.8)	*inhA* C(-15)T
	AGC-ACC, TCG-TTC	Ser315Thr,Ser531Phe	1 (0.8)	
	AGC-ACC, TCG-TGG	Ser315Thr, Ser531Trp	1 (0.8)	
	AGC-AAC, TCG-TTG	Ser315Asn, Ser531Leu	12 (9.6)	
	AGC-AAC/ATC-GTC, TCG-TTG	Ser315Asn/Ile317Val, Ser531Leu	2 (1.6)	
	AGC-ACC, CTG-CCG	Ser315Thr, Leu533Pro	2 (1.6)	
	ACC-CCC/TCC-TAC, TCG-TTG	Thr308Pro/Ser374Tyr, Ser531Leu	1 (0.8)	*oxyR-ahpC* C(-52)T
	GAG-AAG, TCG-TTG	Glu318Lys, Ser531Leu	1 (0.8)	*oxyR-ahpC* C(-52)T
	GCC-GTC, TCG-TTG	Ala379Val, Ser531Leu	1 (0.8)	
	AGC-GCG, TCG-TTG	Thr394Ala, Ser531Leu	1 (0.8)	*inh*AC(-15)T
inhA + rpoB	C-T, CAC-CGC	C(-15)T, His526Arg	2 (1.6)	*oxyR-ahpC* C(-81)T
	C-T, CAC-CTC	C(-15)T, His526Leu	1 (0.8)	*katG* Ser315Thr
	C-T, CAC-TAC	C(-15)T, His526Tyr	1 (0.8)	*oxyR-ahpC* C(-52)T
	C-T, TCG-TTG	C(-15)T, Ser531Leu	7 (5.6)	
	C-T, TCG-TTG	C(-15)T, Ser531Leu	1 (0.8)	*katG*Ser315Thr
	C-T, TCG-TTG	C(-15)T, Ser531Leu	1 (0.8)	*katG* Thr394Ala
	C-T, TCG-TTG	C(-15)T, Ser531Leu	1 (0.8)	*oxyR-ahpC*G(-51)A
	T-A, ACC-CCC/CAC-CGC	T(-8)A, Thr508Pro/His526Arg	1 (0.8)	*katG*Ser315Thr
	T-G, CAC-TAC	T(-8)G, His526Tyr	1 (0.8)	*katG*Ser315Thr
oxyR-ahpC + rpoB	C-A, CAC-TAC	C(-41)A, His526Tyr	1 (0.8)	
	G-A, TCG-CAG	G(-48)A, Ser522Gln	1 (0.8)	
	G-A, TCG-TTG	G(-51)A, Ser531Leu	1 (0.8)	*inh*AC(-15)T
	G-A, TCG-TTG	G(-51)A, Ser531Leu	1 (0.8)	
	C-T, CAC-TAC	C(-52)T, His526Tyr	1 (0.8)	
	C-T, TCG-TTG	C(-52)T, Ser531Leu	1 (0.8)	*katG* Glu318Lys
	C-T, TCG-TTG	C(-52)T, Ser531Leu	1 (0.8)	*katG*Thr308Pro/Ser374Tyr
	C-T, TCG-TTG	C(-52)T, Ser531Leu	1 (0.8)	*inh*AC(-15)T
	C-T, TCG-TTG	C(-57)T, Ser531Leu	1 (0.8)	
	C-T, CAC-TAC	C(-72)T, His526Tyr	1 (0.8)	
	C-T, CAC-GAC	C(-72)T, His526Asp	1 (0.8)	
	C-T, CAC-CGC	C(-81)T, His526Arg	2 (1.6)	*inh*AC(-15)T
katG	AGC-ACC	Ser315Thr	7 (5.6)	
	AGC-ACC/ATC-CTC	Ser315Thr/Ile317Val	1 (0.8)	*oxyR-ahpC* C(-52)T
inhA	C-T	C(-15)T	1 (0.8)	
oxyR-ahpC	G-A	G(-51)A	1 (0.8)	
	C-T	C(-52)T	1 (0.8)	*katG* Ser315Thr/Ile317Val
	G-A	G(-74)A	1 (0.8)	
rpoB	TCG-TTG	Ser531Leu	3 (2.4)	
	GAC-GTC	Asp516Val	1 (0.8)	

*NM, no mutation.