Infection and Drug Resistance

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ORIGINAL RESEARCH

Prevalence and Molecular Characteristics Based on Whole Genome Sequencing of *Mycobacterium tuberculosis* Resistant to Four Anti-Tuberculosis Drugs from Southern Xinjiang, China

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Objective: Drug-resistant tuberculosis is a major public health problem, especially in the southern region of Xinjiang, China; however, there is little information regarding drug resistance profiles and mechanism of *Mycobacterium tuberculosis* in this area. The aim of this study was to determine the prevalence and molecular characteristics of *M. tuberculosis* resistant to four anti-tuberculosis drugs from this area.

Methods: Three hundred and forty-six isolates from the southern region of Xinjiang, China were included and used to perform phenotypic drug susceptibility testing and whole genome sequencing (WGS). Mutations in seven loci associated with drug resistance, including *rpoB* for rifampicin (RMP), *katG, inhA* promoter and *oxyR-ahpC* for isoniazid (INH), *rrs* 530 and 912 loops and *rpsL* for streptomycin (STR), and *embB* for ethambutol (EMB), were characterized.

Results: Among 346 isolates, 106, 60, 70 and 29 were resistant to INH, RMP, STR and EMB, respectively; 132 were resistant to at least one of the four anti-tuberculosis drugs and 51 were multi-drug resistant (MDR). Beijing genotype and retreated patients showed a significantly increased risk for developing MDR tuberculosis. Compared with the phenotypic data, the sensitivity and specificity for WGS to predict resistance were 96.7% and 98.6% for RMP, 75.5% and 97.1% for INH, 68.6% and 99.6% for STR, 93.1% and 93.7% for EMB, respectively. The most common mutations conferring RMP, INH, STR and EMB resistance were Ser450Leu (51.7%) in *rpoB*, Ser315Thr (44.3%) in *katG*, Lys43Arg (35.7%) in *rpsL* and Met306Val (24.1%) in *embB*.

Conclusion: This study provides the first information on the prevalence and molecular characters of drug resistant *M. tuberculosis* in the southern region of Xinjiang, China, which will be helpful for choosing early detection methods for drug resistance (ig, molecular methods) and subsequently initiation of proper therapy of tuberculosis in this area.

Keywords: *Mycobacterium tuberculosis*, whole-genome sequencing, resistance, prevalence, mutation, isoniazid, rifampicin, streptomycin, ethambutol

Introduction

Tuberculosis, caused by *Mycobacterium tuberculosis*, remains one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent (ranking above HIV/AIDS).[1] In 2020, World Health Organization (WHO) reported that 7.1 million people with tuberculosis were newly diagnosed and notified in 2019, up from 7.0 million in 2018 and a large increase from

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6.4 million in 2017.[2] China contributes 8.4% of the global incident tuberculosis cases, ranked third behind Indonesia (8.5%). A latest national tuberculosis epidemiology survey[3] (performed in 2010) reported that the prevalence of tuberculosis in western China is significantly higher than that in central or eastern China, and Xinjiang province in the northwestern China is one of the most serious tuberculosis endemic areas of China. The evolution and spread of rifampicin-/multidrug-resistant tuberculosis (RR-/MDR-TB) poses a major obstacle for controlling tuberculosis disease in the world and China. It is estimated that close to half a million people developed RR-TB, of which 78% had MDR-TB globally.[2] In a system review on studies from China, the prevalence of MDR-TB was 4.8% for new cases, 26.3% for retreatment cases in recent years,[4] meanwhile isoniazid (INH) and rifampicin (RMP) resistance in retreated cases was found to be the most common with prevalence of 40.0% and 33.3%, respectively. Molecular drug susceptibility testing (DST) methods have advantages on closing the gap between detection and treatment of drug-resistant tuberculosis. Understanding the drug resistance mechanism of *M. tuberculosis* in a certain area is helpful to choose an appropriate DST. However, little is known on the mechanism of drug-resistant *M. tuberculosis* in southern Xinjiang, China, stressing the urgency to investigate the prevalence and molecular mechanism of drug resistance in this area.

Previous studies indicated that the most common molecular mechanisms in *M. tuberculosis* have been associated with mutations in the *rpoB, katG, inhA* promoter, *rpsL, rrs* and *embB* genes.[5–9] Mutations in RMP resistant determined region (RRDR) of *rpoB* is the major cause for RMP resistance, with codons 450, 445 and 435 being the predominant sites.[10] INH resistance is mainly related to the mutations in *katG*, followed by that in the *inhA* promoter and *oxyR-ahpC* intergenic region.[11] Mutations in *rpoB* and *katG* were found attributed to 91.1–97.7% RMP resistance[5,12] and 65–88.5% INH resistance,[13,14] respectively. Resistance to streptomycin (STR) is mainly explained by nucleotide changes in *rpsL* and *rrs* genes particularly at codons *rpsL*43, *rpsL* 88 and in *rrs* 530 and 912 loops, accounting for 50–95% STR resistance.[6,15,16] Mutations in *embB* were identified to be responsible for 38–73% ethambutol (EMB) resistant strains.[15–17] Nevertheless, there is no recent data on the molecular nature of drug-resistant *M. tuberculosis* from southern region of Xinjiang, China.

Whole-genome sequencing (WGS) was recognized as a rapid and reliable method for determining drug resistance of *M. tuberculosis* and used as a routine investigation in a few high-income, low-tuberculosis burden countries such as England.[18] WGS allows simultaneous identification of all known resistance-associated loci, resulting in the decrease of missed diagnosis of drug resistance of more drugs than other molecular methods, such as line probe assay and GeneXpert, which both include limited numbers of loci and are incapable to differentiate silent mutations from those affecting drug efficacy, leading to false negative and false-positive results, respectively.[19,20]

In this study, we analyze the resistant characterization and the sequence polymorphisms in seven chosen genes or regions associated with RMP, INH, STR and EMB resistance based on WGS data of 346 *M. tuberculosis* isolates from southern region of Xinjiang, China and evaluate the ability of WGS to predict resistance and susceptibility compared with phenotypic DST. The sequences or regions were chosen on the basis of their demonstrated association with drug resistance according to previous literatures.[21–24] The results in the present study will be helpful to choose early detection methods of drug resistance (ig, molecular methods) and subsequent initiation of proper therapy of tuberculosis in southern region of Xinjiang, China.

Materials and Methods

Patients and Mycobacterial Isolates

This study was carried out between Sep 2017 and Dec 2018 in Xinjiang Uygur Autonomous Region Chest Hospital, Kashi Lung Hospital, Kuche County Infectious Disease Hospital and Wushi County Peoples Hospital, which all serve as the designated tuberculosis hospitals in southern region of Xinjiang. All of the pulmonary tuberculosis patients aged ≥16 years with positive cultures identified as *M. tuberculosis* complex (MTBC) and lived in southern Xinjiang, China for more than 10 years were interviewed and enrolled during the study period. Totally, 346 isolates were collected. Only one isolate per patient was collected and tested.

Drug Susceptibility Testing

The DST for all strains was performed in Xinjiang Uygur Autonomous Region Chest Hospital. Before DST, the strains were recovered on Löwenstein Jensen (L-J) medium for 4 weeks at 37°C. All positive cultures were tested

the growth status in the 7H9 broth contained specific drug concentrations of 9 drugs, which were prepared in 96-well plates from Encode Medical Engineering Co., Ltd, Zhuhai, China. The critical concentrations for the four studied drugs indicate resistance are defined by the laboratory of Xinjiang Uygur Autonomous Region Chest Hospital and shown as following: INH, 0.4 μg/mL; RMP, 4.0 μg/mL; STR, 4.0 μg/mL; EMB, 5.0 μg/mL.

For the drug-susceptible isolates diagnosed by Xinjiang Uygur Autonomous Region Chest Hospital carrying gene mutations, the susceptibility of these isolates was repeated by proportional method using L-J slants in the National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention. The critical concentrations for each drug indicating resistance are shown as following: INH, 0.2 μg/mL; RMP, 40.0 μg/mL; STR, 4.0 μg/mL; EMB, 2.0 μg/mL.[25]

DNA Isolation

Genomic DNA was extracted from *M. tuberculosis* colonies on L-J medium using the cetyltrimethylammonium bromide (CTAB) method.[26] Briefly, after the strains were killed at 80°C for 30 min, 100 μL of 10% sodium dodecyl sulfate (SDS) and 5 μL proteinase K (10 mg/mL) was added, and samples were vortexed for a few seconds and incubated for 10 min at 65°C. Then, the samples were added with 100 μL solution of CTAB-NaCl (4.1% NaCl and 10% CTAB) and then mixed with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]; 700 μL) and centrifuged for 15 min at 13,000 × g in an Eppendorf centrifuge. The aqueous phase (650 μL) was then separated and mixed with an equal volume of isopropanol. The samples were left at −20°C for 30 min and then centrifuged for 15 min at 13,000 × g. The DNA pellet was washed once with 70% ethanol, air dried, and resuspended in a final volume of 100 μL Tris-EDTA (TE, pH8.0).

Genome Sequencing

DNA libraries were prepared with genomic DNA using kits as instructed by the manufacturer. DNA libraries were then selected to perform cluster growth and 150 bp paired-end sequencing on DNB SEQ-2000 instrument (Beijing Genomics Institute, China). The raw FASTQ sequence reads were filtered by removing the adapter sequences, duplicate reads, low-quality reads that had a quality score below 20 in more than 30% of the bases. The clean reads were mapped to the genome of H37Rv (GenBank accession number, NC_000962.2) using in-house softwares Bowtie2 (Version 2.3.4.1) and samtools (Version 1.7). VarScan (Version 2.4.4) was used for single-nucleotide polymorphisms (SNPs) finding. All the genome wide SNPs were identified by the VarScan software by parsing the mapping genome sequence data, and then the SNPs related to phylogeny or located in PE/PPE gene family regions were filtered out. An average of 15.4 million sequence reads were acquired per genome at a depth of 500× and with coverage of 98.0%.

Mycobacterium Species Identification

A fast K-mer algorithm method (KmerFinder) was used to predict bacterial species in this study. After the Software KmerFinder 3.2[27] was downloaded, the local Strain Type library was used to identify the species. The Query coverage ≥85% was considered as MTBC species. All isolates in the present study were identified as MTBC.

Identify Mutations in Drug Resistance–Associated Genes or Regions

Identification of resistance-causing SNPs from genome-wide sequence is challenging. We chose 7 known resistance genes and regions on the basis of their demonstrated association with drug resistance and according to previous literatures[21–24] (Table 1). All mutations in these genes and regions were compared with the pan-susceptible reference genome (H37Rv, accession number: NC_000962.2) at the level of SNPs in promoter regions or intergenic regions, amino acids in genes, or insertions and deletions. The phenotypic and genotypic results were compared to determine the specificity and sensitivity for each gene with WGS to predict resistance.

Spoligotyping

All isolates were analyzed by spoligotyping, performed according to a standard protocol.[28] Simply, the DNA was amplified with primers DRa (5′- Biotin - GGTTTTGGGTCTGACGAC −3′) and DRb (5′- GGTTTTGGGTCTGACGAC −3′). Then, the amplified products were hybridized with PALL Biodyne membrane prepared with 43 spacer oligonucleotide probes. After washing with 2×SSPE solution (360 mM NaCl, 20 mM NaH_2PO_4, 2 mM EDTA [pH 7.2]) supplemented with 0.5% SDS, the membrane was hybridized with streptavidin-peroxidase conjugate. The final image was detected with a chemiluminescence system, including the ECL

Table 1 Mutations Within Seven Drug Resistance-Associated Loci in Drug Resistant *Mycobacterium tuberculosis* Isolates

Drug (n*)	Gene/Region	Codon Change(s)	Amino Acid/Nucleotide Changes	No. of Mutated Isolates
RMP (60)	*rpoB*[a]	450(TCG-TTG)	Ser450Leu	27
		450(TCG-TTG)+150(GAC-GGC)	Ser450Leu+Asp150Gly	1
		450(TCG-TTG)+534(GTG-ATG)	Ser450Leu+Val534Met	1
		450(TCG-TTG)+545(GAC-GAG)	Ser450Leu+Asp545Glu	1
		450(TCG-TTG)+571(GAC-TAC)	Ser450Leu+Asp571Tyr	1
		450(TCG-ATG)	Ser450Met	4
		450(TCG-TGG)	Ser450Trp	1
		450(TCG-TGG)+432(CAA-GAA)	Ser450Trp+Gln432Glu	1
		430(CTG-CCG)	Leu430Pro	6
		430(CTG-CCG)+445(CAC-CAG)	Leu430Pro+His445Gln	1
		430(CTG-CCG)+491(ATC-CTC)	Leu430Pro+Ile491Leu	1
		445(CAC-TAC)	His445Tyr	3
		445(CAC-GAC)	His445Asp	1
		445(CAC-GAC)+435(GAC-GGC)	His445Asp+Asp435Gly	1
		445(CAC-GAC)+512(AAG-GAG)	His445Asp+Lys512Glu	1
		445(CAC-AAC)	His445Asn	2
		445(CAC-AAC)+435(GAC-GGC)	His445Asn+Asp435Gly	1
		445(CAC-GGC)	His445Gly	1
		435(GAC-TAC)	Asp435Tyr	1
		435(GAC-TTC)	Asp435Phe	1
		441(TCG-TTG)	Ser441Leu	1
INH (106)	*katG*	315(AGC-ACC)	Ser315Thr	38
		315(AGC-ACC)	Ser315Thr	2[b]
		315(AGC-ACC)+590(AAG-GAG)	Ser315Thr+Lys590Glu	4
		315(AGC-AAC)	Ser315Asn	2
		315(AGC-CGC)	Ser315Arg	1
		98(TAC-TCC)	Tyr98Ser[$]	1
		105(ATG-AAG)	Met105Lys[$]	1
		138(AAC-AGC)	Asn138Ser	1
		138(AAC-CAC)	Asn138His	1
		139(GCC-CCC)	Ala139Pro	1
		139(GCC-CCC)+140(AGC-AAC)	Ala139Pro+Ser140Asn	1
		145(CGC-CCC)	Arg145Pro[$]	1
		151(GTC-TTC)	Val151Phe[$]	1
		183(TTC-CTC)	Phe183Leu[$]	1
		189(GAC-GGC)	Asp189Gly	1
		191(TGG-GGG)	Trp191Gly	2
		234(GGG-GAG)	Gly234Glu[$]	1
		249(CGC-CAC)	Arg249His[$]	1[c]
		298(TTG-TCG)	Leu298Ser[$]	1
		382(CTC-CGC)	Leu382Arg[$]	1
		394(ACG-GCG)	Thr394Ala	1[b]
		481(TCG-TTG)	Ser481Leu[$]	1[b]
		498(CGC-CAC)	Arg498His[$]	1[b]
		619(CTC-CGC)	Leu619Arg[$]	1
		630(GGC-GTC)	Gly630Val[$]	1[b]
		632(CGC-CAC)	Arg632His[$]	1[b]
		711(TAT-GAT)	Tyr711Asp[$]	2
		728(TGG-CGG)	Trp728Arg[$]	1[b]

(*Continued*)

Table 1 (Continued).

Drug (n*)	Gene/Region	Codon Change(s)	Amino Acid/Nucleotide Changes	No. of Mutated Isolates
	inhA promoter	– – –	C-15T T-8A C-93T	6[d] 1[e] 1
	oxyR-ahpc intergenic region	–	C-52T	1[e]
		–	C-54T	4[f]
		–	C-57T	2[e]
		–	C-72T	1[e]
		–	C-73A	1
		–	T-77G	1[e]
STR (70)	*rrs 530* and *912* loops	– – – –	A514C C517T A908C T950C	8 1 4 1
	rpsL	43(AAG-AGG) 88(AAG-AGG)	Lys43Arg Lys88Arg	25 9
EMB (29)	*embB*	306(ATG-CTG)+983(CCG-CGG) 306(ATG-ATA) 306(ATG-GTG) 306(ATG-ATC) 306(ATG-ATT)+655(CCG-GCG)+1024 (GAC-AAC) 406(GGC-GCC) 406(GGC-GAC) 406(GGC-AGC) 246(GGC-CGC) 328(GAT-GGT) 328(GAT-GGT)+354(GAC-GCC) 328(GAT-GGT)+1024(GAC-AAC) 354(GAC-GCC) 495(GCC-ACC) 575(ATG-ATA)	Met306Leu+Pro983Arg Met306Ile Met306Val Met306Ile Met306Ile+Pro655Ala +Asp1024Asn Gly406Ala Gly406Asp Gly406Ser Gly246Arg Asp328Gly Asp328Gly+Asp354Ala Asp328Gly+Asp1024Asn Asp354Ala Ala495Thr Met575Ile	1 4 7 3 1 2 1 1 1 1 1 1 1 1 1

Notes: [a]The amino acid number is based on *M. tuberculosis* H37Rv codon number; [b]Means that combined mutations in the *inhA* promoter or *oxyR-ahpC* intergenic region; [c]Means that combined mutations in *inhA* promoter and *oxyR-ahpC* intergenic region; [d]Means that 1 out of 6 isolates carried mutations in *katG* and 1 out of 6 isolates carried mutations in *katG and oxyR-ahpC* intergenic region; [e]Combined with mutations in *katG*; [f]Means 1 out of 4 isolates carried mutations in *katG* and 1 out of 4 isolates carried mutations in *katG* and *inhA* promoter; *n means the No. of corresponding drug resistant isolates; [$]Novel mutations in *katG*.
Abbreviations: RMP, rifampicin; INH, isoniazid; STR, streptomycin; EMB, ethambutol.

detection liquid (Amersham, Buckinghamshire, United Kingdom) and ECL-Hyperfilm (Kodak, Rochester, NY).

Statistical Analysis

The spoligotyping results were entered into an Excel spreadsheet in binary format and compared with the spoligotyping database SpolDB4 (http://www.pasteur-guadeloupe.fr:8081/SITVIT2), the results were also analyzed using BioNumerics software (Version 5. 0, Applied Maths, Sint-Martens-Latem, Belgium). Cluster analysis was performed and a dendrogram was generated in Bionumerics using the Dice similarity coefficient and UPGMA coefficient. In addition, SPSS 16.0 (SPSS Inc., Chicago, IL, United States) was used to perform chi-square test and logistic regression analysis. A *P* value less than 0.05 was defined as significant. The extent of association was shown as an odds ratio (OR) and 95% confidence interval (95% CI).

Results

Demographic Information

A total of 346 patients diagnosed with pulmonary tuberculosis were enrolled with a mean age of 50.4 (±19.3) years. Of these, 48.3% (167/346) were males and 51.7% (179/346) were females; 53.5% (185/346) were new cases and 46.5% (161/346) were retreated cases.

Drug Resistance Profiles

According to the DST results acquired from Xinjiang Uygur Autonomous Region Chest Hospital, a total of 78 drug-susceptible isolates were found carried mutations in the 7 genes or regions which maybe attributed to the higher critical drug concentrations defined by Xinjiang Uygur Autonomous Region Chest Hospital, so DSTs on these isolates were repeated in National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention and then the susceptibility patterns of these isolates was adopted in the present study.

Phenotypic DST results against the four anti-tuberculosis drugs showed that among 346 isolates, 214 were fully drug susceptible, 132 (38.2%; 95% CI, 33.0–43.3%) were resistant to at least one drug; the isolates resistant to INH, RMP, STR and EMB were 106 (30.6%; 95% CI, 25.8–35.5%), 60 (17.3%; 95% CI, 13.3–21.4%), 70 (20.2%; 95% CI, 16.0–24.5%) and 29 (8.4%; 95% CI, 5.4–11.3%), respectively. In total, 51 isolates (14.7%; 95% CI, 11.0–18.5%) were identified as MDR. Thirty-eight out of 51 (74.5%) MDR isolates were from retreated patients. A full susceptibility profile for all strains is shown in Table 2.

Table 2 Drug Susceptibility Patterns of 346 Clinical *Mycobacterium tuberculosis* Isolates

Susceptibility or Resistance	Number of Strains
Fully susceptible*	214
H-monoresistant	37
R-monoresistant	4
S-monoresistant	16
E-monoresistant	1
Over all poly-resistant	23
HS	17
RS	3
HSE	1
RSE	2
MDR	51
HR	15
HRS	11
HRE	5
HRSE	20

Note: *Means the isolates were simultaneously susceptible to isoniazid, rifampicin, streptomycin, and ethambutol.
Abbreviations: H, isoniazid; R, rifampicin; S, streptomycin; E, ethambutol; MDR, multi-drug resistant.

Genotype Distribution of the *M. tuberculosis* Isolates

Among the *M. tuberculosis* isolates for WGS, 202 (58.4%) belonged to the Beijing genotype, while 144 (41.6%) were non-Beijing family, which included the CAS1-Delhi family (47), Ural-2 family (31), T1 family (12), EAI family (11), T2 family (5), T3 family (3), T family (2), H3 family (2), LAM9 family (2), H1 family (1), Ural-1 family (1), and un-defined genotypes (27).

Among the 346 isolates, a total of 53 spoligotypes were identified. Of these, 33 spoligotypes were previously represented as Shared International Types (SITs) according to SpolDB4.0, while the other 34 were reported for the first time (Table 3). After clustering with BioNumerics software, 312 (90.2%) isolates were classified into 19 clusters containing 2 or more strains. Additionally, 34 (9.8%) strains did not form clusters (Figure 1, Table 3). Of the 19 clusters, about 76.3% (264/346) of all isolates were contained in 6 predominant clusters, including SIT 1 (Beijing, 181 isolates), SIT 127 (Ural-2, 30 isolates), SIT 25 (CAS1-Delhi, 16 isolates), SIT 26 (CAS1-Delhi, 13 isolates), SIT 357 (CAS1-Delhi, 13 isolates) and SIT 27 (EAI, 11 isolates).

Table 3 Numbers and Frequencies of *Mycobacterium tuberculosis* Isolates Clustered by Spoligotyping

Parameter	Value
No. of isolates studied	346
No. of clusters	19
No. of new found spoligotypes	30
Mean no. of isolates per cluster	16.2
No.(%) of clustered isolates	312 (90.2%)
No.(%) of unclustered isolates	34 (9.8%)

Factors Linked to Drug-Resistant Tuberculosis

Risk factors for MDR and drug-resistant (but not MDR) tuberculosis were also analyzed in the present study. As shown in Table 4, the Beijing genotype infected with and

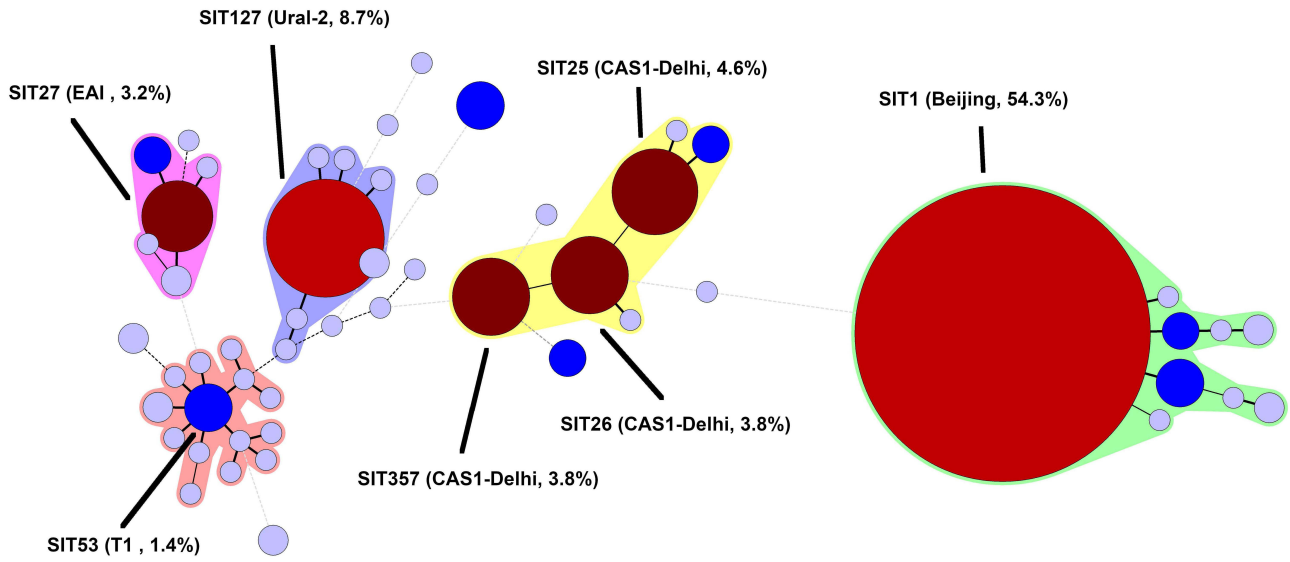

Figure 1 Minimum spanning tree generated with spoligotypes of *Mycobacterium tuberculosis*.
Notes: Each circle represents a particular spoligotype, and the size of circle is relative to the number of strains with that spoligotype. The percentage represents the proportion of each spoligotype. The annotations in the figure were the seven most frequent spoligotypes.

retreated patients presented significantly higher risks for developing MDR-TB than pan-susceptible (simultaneously susceptible to INH, RMP, STR and EMB) tuberculosis with ORs of 3.05 (95% CI, 1.4 to 6.6; $P< 0.01$) and 6.46 (95% CI, 3.1 to 13.6; $P< 0.01$), respectively.

RMP and *rpoB*

RMP resistance-conferring mutations in *rpoB* in *M. tuberculosis* isolates were identified by WGS. A total of 21 genotype patterns in *rpoB* were identified. A 96.7% (58/60) RMP-resistant *M. tuberculosis* isolates carried mutations in the RRDR of *rpoB*. The most frequently mutated codons were 450, 445, 430 and 435 with mutation frequencies of 61.7% (37/60 isolates), 18.3% (11/60 isolates), 13.3% (8/60 isolates), and 6.7% (4/60 isolates) (Table 1). Two novel mutations Asp150Gly and Asp571Tyr were only found in RMP-resistant isolates and both combined with mutations in the *rpoB* RRDR region (Table 1). Four out of 286 (1.4%) RMP-susceptible isolates carried nonsynonymous mutations within the whole sequence of the *rpoB* gene (Supplemental Table 1), of which 1 isolate carried Gln432Glu, which is in the RRDR of *rpoB*. The detection of mutations in *rpoB* by WGS resulted in 96.7% sensitivity and 98.6% specificity compared with phenotypic DST (Table 5).

INH and *katG, inhA* Promoter and *oxyR-Ahpc* Intergenic Region

According to the WGS results, among 106 INH-resistant and 240 INH-susceptible isolates, 72 and 5 carried mutations in *katG,* respectively, whilst 8 and 1 in the *inhA* promoter and 10 and 1 in the *oxyR-ahpC* intergenic region, respectively (Table 1 and Supplemental Table 1). The *katG* Ser315Thr was the most dominant mutation found in 44.3% (47/106) phenotypic INH resistant strains; among the isolates carried this mutation, only two combined mutations in the *inhA* promoter and/or the *oxyR-ahpC* intergenic region. Among 25 INH-resistant isolates, which carried mutations in *katG* non-315, seven combined mutations in the *inhA* promoter and/or the *oxyR-ahpC* intergenic region. Among 34 INH-resistant isolates, which carried the wild-type of *katG*, eight carried mutations in the *inhA* promoter or the *oxyR-ahpC* intergenic region. Five isolates only carried mutation in the *inhA* promoter, whilst three only carried mutations in the *oxyR-ahpC* intergenic region (Table 1 and Supplemental Table 2). There were still 26 INH-resistant strains with the wild-type of three sequenced gene and regions.

Of 24 additional mutations except *katG*315, 23 were found only in phenotypic INH-resistant isolates (Table 1, Supplemental Table 1). Eight of the 23 combined mutations in *katG, inhA* promoter and/or *oxyR-ahpC* intergenic region (Supplemental Table 2) and 16 of the 23 were novel mutations in *katG* (Table 1). A total of 7 INH susceptible isolates were also found mutations in *katG, inhA* promoter and/or *oxyR-ahpC* intergenic region (Supplemental Table 1).

Compared with the phenotypic DST, WGS predicts INH resistance based on mutations in *katG* combined with *inhA* promoter and *oxyR-ahpC* intergenic region with higher sensitivity of 75.5% (VS 67.9%, 9.4% and 7.5%) than that based

Table 4 Factors Associated with Drug-Resistant Tuberculosis

Factor	No.(%) of Isolates			Drug-Resistant but Not MDR TB vs Pan-Susceptible[&] TB		MDR TB vs Pan-Susceptible[&] TB	
	Susceptible TB	Drug-Resistant but Not MDR TB	MDR- TB	Odds Ratio (95% CI)	*P* value	Odds Ratio (95% CI)	*P* value
Sex							
Male	102	39	26	Reference		Reference	
Female	112	42	25	1.05(0.63–1.77)	0.85	0.85(0.43–1.68)	0.64
Age group							
<30 yrs	47	14	15	Reference		Reference	
30–59 yrs	77	30	21	0.62(0.28–1.35)	0.23	2.44(0.92–6.41)	0.07
≧60 yrs	90	37	15	0.94(0.53–1.67)	0.83	1.95(0.87–4.39)	0.11
Occupation							
Others	75	48	22	Reference		Reference	
Farmer	139	33	29	0.79(0.41–1.54)	0.49	0.75(0.34–1.71)	0.49
Residence area							
Rural	162	58	41	Reference		Reference	
Urban	52	23	10	1.11(0.56–2.20)	0.76	0.48(0.19–1.22)	0.12
Treatment							
New cases	137	35	13	Reference		Reference	
Retreated cases	77	46	38	1.23(0.72–2.10)	0.45	6.46(3.07–13.60)	<0.01*
Genotypes							
Non-Beijing	100	33	11	Reference		Reference	
Beijing	114	48	40	1.32(0.77–2.24)	0.31	3.05(1.40–6.64)	<0.01*

Notes: *P<0.05 (significant); [&]Pan-susceptible means that the *Mycobacterium tuberculosis* isolates were simultaneously susceptible to isoniazid, rifampicin, streptomycin and ethambutol.

Abbreviations: CI, confidence interval; TB, tuberculosis; MDR, multi-drug resistant.

Table 5 The Ability of Whole-Genome Sequencing Analysis for Drug Resistance Prediction in Comparison with the Phenotypic Drug Susceptibility Testing

Drugs	Genes	No. of Resistant Isolates (%)		No. of Susceptible Isolates (%)		χ^2	*P*	Sensitivity (%)	Specificity (%)
		With Mutations	Without Mutations	With Mutations	Without Mutations				
RMP	*rpoB*	58	2	4	282	306.0	<0.01	96.7	98.6
INH	*katG*	72	34	5	235	184.2	<0.01	67.9	97.9
	inhA promoter	8	98	1	239	12.1	<0.01	7.5	99.6
	oxyR-ahpC intergenic region	10	96	1	239	16.6	<0.01	9.4	99.6
	katG and *inhA* promoter	77	29	6	234	198.4	<0.01	72.6	97.5
	katG, inhA promoter and *oxyR-ahpC* intergenic region	80	26	7	233	205.6	<0.01	75.5	97.1
STR	*rrs 530 and 912 loops*	14	56	1	275	47.3	<0.01	20.0	99.6
	rpsL	34	36	0	276	148.7	<0.01	48.6	100.0
	rrs 530 and 912 loops or *rpsL*	48	22	1	275	208.4	<0.01	68.6	99.6
EMB	*embB*	27	2	19	298	166.4	<0.01	93.1	94.0

Abbreviations: RMP, rifampicin; INH, isoniazid; STR, streptomycin; EMB, ethambutol.

on the mutations from a single gene, however with a lower specificity of 97.1% (VS 99.6% and 97.9%) (Table 5).

STR and *rrs* 530 and 912 Loops and *rpsL*

The WGS analysis on mutations in *rrs* 530 and 912 loops and *rpsL* associated with STR resistance showed that, among 70 STR-resistant isolates, 14 carried mutations in *rrs* 530 and 912 loops, 34 in *rpsL*. As shown in Table 1, for *rrs*, the most frequent mutations were A514C and A908C, which occurred in 11.4% (8/70) and 5.7% (4/70) phenotypic STR-resistant isolates. For *rpsL*, the most frequent codons were 43 and 88, which were observed in 35.7% (25/70) and 12.9% (9/70) phenotypic STR-resistant isolates. Mutations in *rrs* and *rpsL* genes accounted for 68.6% of the STR-resistant isolates. We also found that, among 276 STR-susceptible isolates, only one carried mutation C517T in *rrs* (Supplemental Table 1). The sensitivity and specificity of WGS to predict STR resistance according to mutations in *rpsL* combined with *rrs* were 68.6% and 99.6% (Table 5).

EMB and *embB*

The whole *embB* sequence of 346 *M. tuberculosis* isolates was analyzed in this study. Among the 29 isolates resistant to EMB, 27 isolates (93.1%) carried mutations in *embB*. The most prevalent codon was Met306 (16, 55.2%), where the codon ATG (Met) was replaced with GTG (Val, 7, 24.1%), ATA (Ile, 4, 13.8%), ATC (Ile, 3, 10.3%), ATT (Ile, 1, 3.4%) and CTG (Leu, 1, 3.4%), respectively. Four of EMB-resistant isolates carried Gly406 codon mutations, where the codon GGC (Gly) was replaced with GCC (Ala, 2, 6.9%), GAC (Asp, 1, 3.4%) and AGC (Ser, 1, 3.4%) (Table 1). Other mutations at codon 328 (n =3), codon 354 (n = 2, one was combined with condon 328), codon 246 (n = 1), codon 495 (n = 1) and codon 575 (n = 1) were found in EMB resistant isolates, as listed in Table 1. Among the EMB-susceptible isolates, mutations in Met306 and Gly406 were also the most predominant in *embB* gene, furthermore, 12 mutations except Met306 and Gly406 were found (shown in Supplemental Table 1). WGS analysis on *embB* had a sensitivity and specificity of 93.1% and 94.0%, respectively (Table 5).

Discussion

The prevalence of RR-TB and MDR-TB patients was 17.3% (60/346) and 14.7% (51/346) in the southern Xinjiang, China, in the present study, both comparable to a previous study from Xinjiang (19.4% and 13.2%),[29] however, both higher than the data from a national survey of drug-resistant tuberculosis in 2007 in China (5.1% and 10.2%),[30] indicating a serious epidemic of drug-resistant tuberculosis in the southern Xinjiang, China. Yet the epidemic of drug-resistant tuberculosis in southern Xinjiang, China maybe overestimated due to that the isolates of this study were collected from tuberculosis designated hospitals rather than random survey.

The Beijing genotype family is the dominant lineage in southern Xinjiang, accounting for 58.4% of strains in the present study, which is in line with Yuan et al's report from Xinjiang, China[31] and the data from the south of China (53.2%),[32] but lower than that from the north of China (76.5%).[32] According to subtype analysis with SITs, the most predominant clusters were SIT 1 (Beijing), SIT 127 (Ural-2), SIT 25 (CAS1-Delhi), SIT 26 (CAS1-Delhi), SIT 357 (CAS1-Delhi) and SIT 27 (EAI). Three subtypes SIT 357, 25 and 26 of CAS-Delhi family were evenly distributed in this area (all about 3%, Figure 1). A previous study from Xinjiang province has demonstrated that Beijing genotype strains might be correlated with INH and EMB resistance.[33] Another epidemiological study reported that the Beijing genotype showed greater correlation with RMP and ofloxacin resistance and MDR phenotypes in China.[34] We also observed that the Beijing genotype exhibited a significantly higher risk for developing MDR-TB compared to non-Beijing genotype, suggesting that Beijing genotype be responsible for the spread and emergence of MDR-TB in this region. As reported in previous studies,[35,36] retreated patients in this study were also found with high risk for developing MDR-TB, stressing the importance of early diagnosis, and timely, long-term and standardized medication, especially among patients treated within the hospital system in this region.

Our results present the first WGS-based molecular characterization of *M. tuberculosis* from southern region of Xinjiang in China. We focused on genes known to confer resistance to four anti-tuberculosis drugs in *M. tuberculosis*. WGS could offer a rapid and comprehensive SNP identifications, which help to understand the drug resistance mechanisms and choose suitable molecular DSTs suitable for this region leading to quicker and more appropriate treatment.

Previous reports show that mutations in *rpoB* are the main cause of RMP resistance.[14,37] Our results showed a sensitivity of 96.7% based on *rpoB* mutations for predicting RMP resistance by WGS, consistent with the data

from Jiangxi province, China[14] and France.[37] The most common mutations are in the *rpoB* RRDR region, particularly in codons 450, 445, 430 and 435,[14,22,37] which are in line with the results that 86.7% of RMP resistant isolates had at least one of these four codon mutations in the present study. Two novel mutations Asp150Gly and Asp571Tyr combined with mutations in the *rpoB* RRDR and only existed in RMP-resistant isolates. It is unclear if these mutations play a direct role in RMP resistance; therefore, their possible functions should be verified in further studies.

Mutations in the *katG* gene are often considered to be the major contributors for INH resistance, followed by that in the *inhA* promoter and *oxyR-ahpC* intergenic region.[12] Accordingly, our study indicated that mutations in *katG* accounted for 67.9% INH resistance, lower than results from Hunan province, China,[36] America[38] and Kyrgyz Republic.[39] The differences can be attributed to geographical variations. In the present study, 23 additional mutations except *katG*315 were found in INH resistant isolates, of which 16 were novel mutations, and only eight novel mutations combined with *katG*315 or *inhA* promoter or *oxyR-ahpC* intergenic region mutations. All of these novel mutations were found only in phenotypic INH-resistant isolates, suggesting that these mutations were resistance-associated but needed to be further verified by in vitro mutagenesis experiments. In addition, 8 out of 34 INH-resistant isolates possessed wild-type *katG* carried *inhA* C (−15) T, T (−8) A and C-93T and/or mutations in the region of *oxyR-ahpC* from −77 to −52. So, the *inhA* (−15), T (−8) A and *oxyR-ahpC* −77 to −52 combined with *katG*315 can make a preferable set for INH-resistance diagnoses. The mutation of *inhA* C (−93) T is first reported by us, its role on INH resistance needs to be further confirmed. The combination of mutations in *katG, inhA*, and the *oxyR-ahpC* intergenic region were found in 75.5% of INH-resistant isolates, which were far lower than 92.7% reported in another study from China.[6] There may be alternative mechanisms involved in the INH resistance of these isolates, and possibly the mutations occur in other structural genes or other loci. A review about mechanisms of isoniazid resistance suggested that mutations in *kasA* and *ndh* genes were the tertiary cause for INH resistance, followed by *iniABC, fadE* and *furA*.[11] Besides, several recent studies pointed out the contribution of efflux pump to INH resistance, and this could explain why the remaining 20–30% of phenotypically resistant isolates that do not contain any genotypic mutation.[40,41] Seven INH susceptible isolates carried mutations in *katG, inhA* promoter or *oxyR-ahpC* intergenic region, which may be conferred with low-level INH resistance and easily lead to misdiagnosis or not be associated with INH resistance.

Resistance to STR is due to alterations within *rpsL, rrs* (530 loop and the 912 loops).[42] Two mutations *rpsL* Lys43Arg and Lys88Arg were the most common in STR resistant isolates in the present study, which were similar to the data from other areas in China[17] and Pakistan.[23] The most frequent mutation in *rrs* in STR resistant isolates was A514C, followed by C517T and A908C, in line with other reports.[6,42] There was no isolate carried *rrs* mutation combined with *rpsL* mutation in this study, suggesting that mutations in *rrs* and *rpsL* genes were mutually exclusive. Mutations in *rrs* and *rpsL* genes accounted for 68.6% STR resistance, higher than the data from the tertiary care tuberculosis hospital in China[35] and Mexico,[43] but lower than that from other regions,[44,45] indicating that there were regional differences in the mutations associated with STR resistance and additional resistance mechanisms correlated with STR resistance, such as *gidB* which was reported to contribute 2.4% to 37.5% STR resistance.[42,44,46–48]

EMB resistance was mostly associated with mutations within the *embB* gene (codon 306 as the dominant).[50] In the present study, *embB* mutations were found in 93.1% EMB resistant isolates, showed a high mutation prevalence compared to previous reports, ranged from 20% to 90.9%.[21,49,50] The most prevalent mutation at locus 306 was detected, which was also the most predominant codon in EMB-susceptible isolates. The phenomenon was also reported in previous studies.[8,51] Overall 6.0% of EMB-susceptible isolates also carried mutations in *embB*, which is close to the data from a previous study (6.5%),[52] which maybe attributed to the narrow range of EMB critical concentrations for differentiating resistant and susceptible strains, and the possible presence of microcolonies that are difficult to detect visually.[53] A previous study shows that, using the EMB concentration with 1.6 μg/mL instead of 2.0 μg/mL in L-J slants by the proportional method, more than 90% EMB-susceptible isolates that carried *embB*306 mutations could be successfully recognized as EMB-resistant isolates, while the EMB susceptible isolates with wild-type *embB* were not changed.[54]

Studies on EMB resistance showed that mutations outside *embB* codon 306 and 406, do occur but are quite rare.[51,55] However, substitutions at other codons were identified in 12 EMB-susceptible and eight EMB-resistant isolates. Two novel mutations *embB* Ala495Thr and Met575Ile were found only in EMB resistant isolates. The role of these mutant types remained unknown, therefore required further exploration.

Two out of 29 EMB resistant isolates were not found mutations in *embB*. Mutations in *embC* and *embA*, which were shown to be involved in EMB resistance development,[15,49] were not included in the present study. However, Wan et al[6] found that mutations in *embC* or *embA* always combined with mutations in *embB*, and both EMB resistant and susceptible isolates had the same low frequencies of mutations in *embC* or *embA*.

A systematic review about WGS in *M. tuberculosis* for detection of drug resistance pointed out that the sensitivities of WGS compared with phenotypic DST for the following anti-tuberculosis drugs were high but also varied: RMP (89.2% to 100%), INH (90% to 100%), EMB (71.4% to 95.8%), STR (57.1% to 89.9%).[56] In the present study, the sensitivities between the WGS and phenotypic DST for the four anti-tuberculosis drugs were similar to previous studies, ranging from 68.6% to 96.7%.

Conclusions

Comprehensively identifying mutations by WGS could provide a comprehensive understanding on the drug resistance mechanism caused by chromosome mutations. The data in the present study raises our understanding of the prevalence and molecular characteristics of *M. tuberculosis* resistant to INH, RMP, EMB and STR in southern region of Xinjiang China, which would be benefited for choosing early detection methods of drug resistance and subsequently initiation of proper therapy of tuberculosis in southern region of Xinjiang, China.

Abbreviations

CI, confidence interval; CTAB, cetyltrimethylammonium bromide; DST, drug susceptibility testing; EMB, ethambutol; INH, isoniazid; L-J, Löwenstein Jensen; MTBC, *M. tuberculosis* complex; OR, odds ratio; RMP, rifampicin; RRDR, rifampicin resistant determined region; RR-/MDR-TB, rifampicin-/multidrug-resistant tuberculosis; SDS, sodium dodecyl sulfate; SITs, Shared International Types; SNPs, single-nucleotide polymorphisms; STR, streptomycin; TE, Tris-EDTA; WGS, whole-genome sequencing; WHO, World Health Organization.

Data Sharing Statement

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical Approval and Consent to Participate

This study obtained approval from the Ethics Committee of Xinjiang Uygur Autonomous Region Chest Hospital and was conducted in accordance with the Declaration of Helsinki. The patients with tuberculosis were included in the present research only after we received informed written consent from themselves or from their parents/guardians if they were children (≤18 years of age).

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Disclosure

The authors declare that they have no competing interests.

References

1. MacNeil AGP, Sismanidis C, Date A, Maloney S, Floyd K, Floyd K. Global epidemiology of tuberculosis and progress toward meeting global targets — worldwide, 2018. *MMWR Morb Mortal Wkly Rep*. 2020;69:281–285. doi:10.15585/mmwr.mm6911a2
2. World Health Organization. *Global Tuberculosis Report 2020*. Geneva: World Health Organization, Licence: CC BY-NC-SA 30 IGO; 2020.
3. Wang L, Chen Sh, Zhao Y, et al. The fifth China national epidemiology of tuberculosis survey 2010. *Chin J Antituberculos*. 2012;34(8):485–508.
4. Duan Q, Chen Z, Chen C, et al. The prevalence of drug-resistant tuberculosis in Mainland China: an updated systematic review and meta-analysis. *PLoS One*. 2016;11(2):e0148041. doi:10.1371/journal.pone.0148041
5. Wan L, Guo Q, Wei JH, et al. Accuracy of a reverse dot blot hybridization assay for simultaneous detection of the resistance of four anti-tuberculosis drugs in *Mycobacterium tuberculosis* isolated from China. *Infect Dis Poverty*. 2020;9(1):38. doi:10.1186/s40249-020-00652-z
6. Wan L, Liu H, Li M, et al. Genomic analysis identifies mutations concerning drug-resistance and Beijing genotype in multidrug-resistant *Mycobacterium tuberculosis* isolated from China. *Front Microbiol*. 2020;11:1444. doi:10.3389/fmicb.2020.01444

7. Rabahi MF, Conceição EC, de Paiva LO, et al. Characterization of *Mycobacterium tuberculosis* var. africanum isolated from a patient with pulmonary tuberculosis in Brazil. *Infect Gene Evol*. 2020;85:104550. doi:10.1016/j.meegid.2020.104550
8. Li MC, Chen R, Lin SQ, et al. Detecting ethambutol resistance in *Mycobacterium tuberculosis* isolates in China: a comparison between phenotypic drug susceptibility testing methods and DNA sequencing of embAB. *Front Microbiol*. 2020;11:781. doi:10.3389/fmicb.2020.00781
9. Li G, Guo Q, Liu H, et al. Detection of resistance to fluoroquinolones and second-line injectable drugs among *Mycobacterium tuberculosis* by a reverse dot blot hybridization assay. *Infect Drug Resist*. 2020;13:4091–4104. doi:10.2147/IDR.S270209
10. Eddabra R, Neffa M. Mutations associated with rifampicin resistance in *Mycobacterium tuberculosis* Isolates from Moroccan patients: systematic review. *Interdiscip Perspect Infect Dis*. 2020;2020:5185896. doi:10.1155/2020/5185896
11. Unissa AN, Subbian S, Hanna LE, Selvakumar N. Overview on mechanisms of isoniazid action and resistance in *Mycobacterium tuberculosis*. *Infect Genet Evol*. 2016;45:474–492. doi:10.1016/j.meegid.2016.09.004
12. Chen Y, Zhao B, Liu HC, et al. Prevalence of mutations conferring resistance among multi- and extensively drug-resistant *Mycobacterium tuberculosis* isolates in China. *J Antibiot (Tokyo)*. 2016;69(3):149–152. doi:10.1038/ja.2015.106
13. Zhou A, Nawaz M, Duan Y, et al. Molecular characterization of isoniazid-resistant *Mycobacterium tuberculosis* isolates from Xi'an, China. *Microb Drug Resist*. 2011;17(2):275–281. doi:10.1089/mdr.2010.0135
14. Luo D, Chen Q, Xiong G, et al. Prevalence and molecular characterization of multidrug-resistant *M. tuberculosis* in Jiangxi province, China. *Sci Rep*. 2019;9(1):7315. doi:10.1038/s41598-019-43547-2
15. Farhat MR, Sultana R, Iartchouk O, et al. Genetic determinants of drug resistance in Mycobacterium tuberculosis and their diagnostic value. *Am J Respir Crit Care Med*. 2016;194(5):621–630. doi:10.1164/rccm.201510-2091OC
16. Miotto P, Tessema B, Tagliani E, et al. A standardised method for interpreting the association between mutations and phenotypic drug resistance in *Mycobacterium tuberculosis*. *Eur Respir J*. 2017;50(6):1701354. doi:10.1183/13993003.01354-2017
17. Li GL, Zhao DF, Xie T, et al. Molecular characterization of drug-resistant Beijing family isolates of *Mycobacterium tuberculosis* from Tianjin, China. *Biomed Environ Sci*. 2010;23(3):188–193. doi:10.1016/S0895-3988(10)60051-7
18. Satta G, Lipman M, Smith GP, Arnold C, Kon OM, McHugh TD. *Mycobacterium tuberculosis* and whole-genome sequencing: how close are we to unleashing its full potential? *Clin Microbiol Infect*. 2018;24(6):604–609. doi:10.1016/j.cmi.2017.10.030
19. Coll F, McNerney R, Preston MD, et al. Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. *Genome Med*. 2015;7(1):51. doi:10.1186/s13073-015-0164-0
20. Aubry A, Sougakoff W, Bodzongo P, et al. First evaluation of drug-resistant *Mycobacterium tuberculosis* clinical isolates from Congo revealed misdetection of fluoroquinolone resistance by line probe assay due to a double substitution T80A-A90G in GyrA. *PLoS One*. 2014;9(4):e95083. doi:10.1371/journal.pone.0095083
21. CLSI. Susceptibiliy testing of mycbacteria, Nocardia spp., and other aerobic actinomycetes. 3rd ed. In: *CLSI Standard M24*. Wayne, PA: Cinical and Laboratory Standards Institute; 2018.
22. Mvelase NR, Pillay M, Sibanda W, Ngozo JN, Brust JCM, Mlisana KP. *rpoB* mutations causing discordant rifampicin susceptibility in *Mycobacterium tuberculosis*: retrospective analysis of prevalence, phenotypic, genotypic, and treatment outcomes. *Open Forum Infect Dis*. 2019;6(4):ofz065. doi:10.1093/ofid/ofz065
23. Ali A, Hasan Z, McNerney R, et al. Whole genome sequencing based characterization of extensively drug-resistant *Mycobacterium tuberculosis* isolates from Pakistan. *PLoS One*. 2015;10(2):e0117771. doi:10.1371/journal.pone.0117771
24. Kardan-Yamchi J, Kazemian H, Battaglia S, et al. Whole genome sequencing results associated with minimum inhibitory concentrations of 14 anti-tuberculosis drugs among rifampicin-resistant isolates of *Mycobacterium tuberculosis* from Iran. *J Clin Med*. 2020;9(2):465. doi:10.3390/jcm9020465
25. World health organization. *Updated Interim Critical Concentrations for First-Line and Second-Line DST (As for May 2012)*. World Health Organization; 2012.
26. Honoré-Bouakline S, Vincensini JP, Giacuzzo V, Lagrange PH, Herrmann JL. Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction. *J Clin Microbiol*. 2003;41(6):2323–2329. doi:10.1128/JCM.41.6.2323-2329.2003
27. Hasman H, Saputra D, Sicheritz-Ponten T, et al. Rapid whole-genome sequencing for detection and characterization of microorganisms directly from clinical samples. *J Clin Microbiol*. 2014;52(1):139–146. doi:10.1128/JCM.02452-13
28. Kamerbeek J, Schouls L, Kolk A, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol*. 1997;35(4):907–914. doi:10.1128/jcm.35.4.907-914.1997
29. Qi YC, Ma MJ, Li DJ, et al. Multidrug-resistant and extensively drug-resistant tuberculosis in multi-ethnic region, Xinjiang Uygur Autonomous Region, China. *PLoS One*. 2012;7:e32103.
30. Zhao Y, Xu S, Wang L, et al. National survey of drug-resistant tuberculosis in China. *N Engl J Med*. 2012;366(23):2161–2170. doi:10.1056/NEJMoa1108789
31. Yuan L, Mi L, Li Y, Hui Z, Li Z, Li Z. Genotypic characteristics of *Mycobacterium tuberculosis* circulating in Xinjiang, China. *Infect Dis*. 2016;48(2):108–115. doi:10.3109/23744235.2015.1087649
32. Pang Y, Zhou Y, Bing Z, et al. Spoligotyping and drug resistance analysis of *Mycobacterium tuberculosis* strains from national survey in China. *PLoS One*. 2012;7(3):e32976. doi:10.1371/journal.pone.0032976
33. Wang XH, Ma AG, Han XX, et al. Correlations between drug resistance of Beijing/W lineage clinical isolates of *Mycobacterium tuberculosis* and sublineages: a 2009–2013 prospective study in Xinjiang province, China. *Med Sci Monit*. 2015;21:1313–1318. doi:10.12659/MSM.892951
34. Pang Y, Zhou Y, Zhao B, et al. Spoligotyping and drug resistance analysis of *Mycobacterium tuberculosis* strains from national survey in China. *PLoS One*. 2012;7(3):e32976.
35. Zhao LL, Huang MX, Xiao TY, et al. Prevalence, risk and genetic characteristics of drug-resistant tuberculosis in a tertiary care tuberculosis hospital in China. *Infect Drug Resist*. 2019;12:2457–2465. doi:10.2147/IDR.S209971
36. Zhao LL, Chen Y, Chen ZN, et al. Prevalence and molecular characteristics of drug-resistant *Mycobacterium tuberculosis* in Hunan, China. *Antimicrob Agents Chemother*. 2014;58(6):3475–3480. doi:10.1128/AAC.02426-14
37. Genestet C, Hodille E, Berland JL, et al. Whole-genome sequencing in drug susceptibility testing of *Mycobacterium tuberculosis* in routine practice in Lyon, France. *Int J Antimicrob Agents*. 2020;55(4):105912. doi:10.1016/j.ijantimicag.2020.105912
38. Campbell PJ, Morlock GP, Sikes RD, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2011;55(5):2032–2041. doi:10.1128/AAC.01550-10
39. Isakova J, Sovkhozova N, Vinnikov D, et al. Mutations of *rpoB, katG, inhA* and *ahp* genes in rifampicin and isoniazid-resistant *Mycobacterium tuberculosis* in Kyrgyz Republic. *BMC Microbiol*. 2018;18(1):22. doi:10.1186/s12866-018-1168-x
40. Machado D, Couto I, Perdigão J, et al. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. *PLoS One*. 2012;7(4):e34538. doi:10.1371/journal.pone.0034538

41. Rodrigues L, Villellas C, Bailo R, Viveiros M, Aínsa JA. Role of the Mmr efflux pump in drug resistance in *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother*. 2013;57(2):751–757. doi:10.1128/AAC.01482-12
42. Islam MM, Tan Y, Hameed H, Chhotaray C, Zhang T. Phenotypic and genotypic characterization of streptomycin-resistant multidrug-resistant *Mycobacterium tuberculosis* clinical isolates in Southern China. *Microb Drug Resist*. 2020;26(7):766–775. doi:10.1089/mdr.2019.0245
43. Madrazo-Moya CF, Cancino-Muñoz I, Cuevas-Córdoba B, et al. Whole genomic sequencing as a tool for diagnosis of drug and multidrug-resistance tuberculosis in an endemic region in Mexico. *PLoS One*. 2019;14(6):e0213046. doi:10.1371/journal.pone.0213046
44. Wu X, Gao R, Shen X, et al. Use of whole-genome sequencing to predict *Mycobacterium tuberculosis* drug resistance in Shanghai, China. *Int J Infect Dis*. 2020;96:48–53. doi:10.1016/j.ijid.2020.04.039
45. Al-Mutairi NM, Ahmad S, Mokaddas EM. Correction to: molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) isolates identifies local transmission of infection in Kuwait, a country with a low incidence of TB and MDR-TB. *Eur J Med Res*. 2020;25(1):14. doi:10.1186/s40001-020-00412-7
46. Chen X, He G, Wang S, Lin S, Chen J, Zhang W. Evaluation of whole-genome sequence method to diagnose resistance of 13 anti-tuberculosis drugs and characterize resistance genes in clinical multi-drug resistance *Mycobacterium tuberculosis* isolates from China. *Front Microbiol*. 2019;10:1741. doi:10.3389/fmicb.2019.01741
47. Zhao LL, Liu HC, Sun Q, et al. Identification of mutations conferring streptomycin resistance in multidrug-resistant tuberculosis of China. *Diagn Microbiol Infect Dis*. 2015;83(2):150–153. doi:10.1016/j.diagmicrobio.2015.06.020
48. Jagielski T, Ignatowska H, Bakuła Z, et al. Screening for streptomycin resistance-conferring mutations in *Mycobacterium tuberculosis* clinical isolates from Poland. *PLoS One*. 2014;9(6):e100078. doi:10.1371/journal.pone.0100078
49. Zhao LL, Sun Q, Liu HC, et al. Analysis of *embCAB* mutations associated with ethambutol resistance in multidrug-resistant *Mycobacterium tuberculosis* isolates from China. *Antimicrob Agents Chemother*. 2015;59(4):2045–2050. doi:10.1128/AAC.04933-14
50. Khosravi AD, Sirous M, Abdi M, Ahmadkhosravi N. Characterization of the most common *embCAB* gene mutations associated with ethambutol resistance in *Mycobacterium tuberculosis* isolates from Iran. *Infect Drug Resist*. 2019;12:579–584. doi:10.2147/IDR.S196800
51. Mohammadi B, Ramazanzadeh R, Nouri B, Rouhi S. Frequency of codon 306 mutations in *embB* gene of *Mycobacterium tuberculosis* resistant to ethambutol: a systematic review and meta-analysis. *Int J Prev Med*. 2020;11:112. doi:10.4103/ijpvm.IJPVM_114_19
52. Allix-Béguec C, Arandjelovic I, Bi L, et al. Prediction of susceptibility to first-line tuberculosis drugs by DNA sequencing. *N Engl J Med*. 2018;379(15):1403–1415.
53. Madison B, Robinson-Dunn B, George I, et al. Multicenter evaluation of ethambutol susceptibility testing of *Mycobacterium tuberculosis* by agar proportion and radiometric methods. *J Clin Microbiol*. 2002;40(11):3976–3979. doi:10.1128/JCM.40.11.3976-3979.2002
54. Zhang N, Zhao XQ, Wan KL. Preliminary study on the appropriate concentrations of drug used in the drug-susceptibility test to detect the Ethambutol-resistant isolates of *Mycobacterium tuberculosis*. *Chin J Zoonoses*. 2009;25:1049–1053.
55. Bakuła Z, Napiórkowska A, Bielecki J, Augustynowicz-Kopeć E, Zwolska Z, Jagielski T. Mutations in the *embB* gene and their association with ethambutol resistance in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from Poland. *Biomed Res Int*. 2013;2013:167954. doi:10.1155/2013/167954
56. Papaventsis D, Casali N, Kontsevaya I, Drobniewski F, Cirillo DM, Nikolayevskyy V. Whole genome sequencing of *Mycobacterium tuberculosis* for detection of drug resistance: a systematic review. *Clin Microbiol Infect*. 2017;23(2):61–68. doi:10.1016/j.cmi.2016.09.008