Infection and Drug Resistance

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ORIGINAL RESEARCH

Drug-Resistant Profiles and Genetic Diversity of *Mycobacterium* Tuberculosis Revealed by Whole-Genome Sequencing in Hinggan League of Inner Mongolia, China

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Background: Tuberculosis remains a major public health concern in China, with varying prevalence and drug resistance profiles across regions. This study explores the genetic diversity and drug-resistant profiles of MTB strains in Hinggan League, a high TB burden in Inner Mongolia, China.

Methods: This population-based retrospective study, encompassing all culture-positive TB cases from Jun. 2021 to Jun. 2023 in Hinggan League. Drug resistant profiles and genetic diversity of MTB strains were assessed using phenotypic drug susceptibility testing and whole-genome sequencing. Risk factors associated with drug resistance were analyzed using univariate and multivariate logistic regression models.

Results: A total of 211 MTB strains were recovered successfully and included into final analysis. Lineage 2.2.1 (88.6%, 187/211) was the dominant sub-lineage, followed by lineage 4.5 (7.1%, 15/211) and lineage 4.4 (4.3%, 9/211). MTB strains exhibited the highest resistance rates to isoniazid (16.1%, 34/211), followed by rifampicin (10.0, 21/211). In addition, the MTB strains also showed relatively high rates of resistance against new and repurposed anti-TB drugs, with resistant rates of 2.4% (5/211) to delamanid and 1.9% (4/211) to bedaquiline. Overall, 25.6% (54/211) of MTB strains were DR-TB, and 14 MTB strains met the definition of MDR-TB, including 7 strains of simple-MDR-TB, 5 of pre-XDR-TB, and 2 of XDR-TB. Genetic analysis revealed that the dominant mutations of isoniazid-, rifampin-, ethambutol-, levofloxacin-/moxifloxacin-, and ethionamide- resistance were *katG*_Ser315Thr (46.4%), *rpoB*_Ser450Leu (47.4%), *embB*_Met306Val (25.0%), *gyrA*_Asp94Ala (40.0%), and *fabG1*_c15t (42.9%), respectively. Previously treated patients (AOR = 2.015, 95% CI: 1.052–4.210) and male patients (AOR = 3.858, 95% CI: 1.416–10.511) were identified as independent risk factors associated with DR-TB.

Conclusion: Our study offers crucial insights into the genetic diversity and drug-resistant profiles of TB strains circulating in Hinggan League. These findings are valuable for DR-TB surveillance and for guiding treatment regimens and public health interventions in the region.

Keywords: *Mycobacterium* tuberculosis, drug resistance, genetic diversity, whole-genome sequencing

Introduction

Tuberculosis (TB), caused by bacillus *Mycobacterium tuberculosis* (MTB), continues to be a major public health concern and was the second leading cause of death from a single infectious agent globally, after coronavirus disease (COVID-19).[1] Although TB is preventable and curable, the emergence and wide-spread of drug-resistant TB (DR-TB) poses a great

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challenge to effective TB control and prevention.[2] According to the World Health Organization (WHO), an estimated 410,000 individuals fell ill with rifampicin-resistant TB or multidrug-resistant TB (MDR/RR-TB) in 2022, of which only 40% received treatment.[1] Furthermore, untreated cases of DR-TB can act as a source of infection, leading to ongoing transmission of DR-TB and presenting a sustained threat to TB control.[3] Given the restricted therapy options, increased cost, and poorer prognosis associated with treating DR-TB, continuous vigilance is essential. Moreover, because early diagnosis and effective treatment can help prevent the development of drug resistance and TB-related deaths, understanding the drug resistance profile of TB strains and identifying patients with DR-TB is essential for guiding timely clinical interventions and enhancing cure rates.

Since the completion and publication of the full-genome sequence of *Mycobacterium tuberculosis* H37Rv in 1998,[4] whole-genome sequencing (WGS) has been extensively employed in different disciplines, including research, clinical settings, and routine surveillance.[5] WGS has proven valuable for predicting drug resistance, exploring genetic diversity, identifying transmission clusters, and elucidating the evolutionary dynamics of MTB strains.[6–8] To date, WGS has provided profound insights into the genomic drug resistance and diversity of MTB in various regions around the world.[9–11] In China, WGS of MTB has been applied to characterize the genotypic resistance in several province and cities.[2,12,13] However, the prevalent genotype and drug resistance patterns of MTB isolates exhibit significant variation among diverse regions,[14] highlighting the importance of conducting systematic research in areas where WGS has not yet been widely implemented, especially in remote regions.

Inner Mongolia, located northern China, is a vast multi-ethnic region and one of the provinces with a high TB burden in China.[15] Hinggan League is situated in the northeastern part of Inner Mongolia, and its reported TB incidence has consistently ranked first in the province, with an average annual reported incidence rate of 66.49/100 000.[16] However, information on the drug resistant patterns and dominant lineage of MTB strains circulating in this region is extremely limited, which largely hinders the development and implementation of effective prevention and control strategies.

In this study, we conducted a retrospective study of 281 MTB strains collected from 2021 to 2023 in Hinggan League. We investigated the drug resistance and genetic diversity of MTB strains using whole-genome sequencing and phenotypic drug susceptibility testing, which could help understand the strain diversity in the region and aid in the development of precise public health interventions.

Materials and Methods

Study Population

This retrospective investigation was based on drug resistant tuberculosis surveillance work in Hinggan League, Inner Mongolia, China. The study population included all MTB strains from suspected pulmonary TB cases with sputum smear-positive results, collected from local designated hospitals in the six counties of Hinggan League. Between June 2021 and June 2023, a total of 218 MTB strains were collected: 5 from Arxan, 16 from Tuchuan County, 23 from Horqin Right Middle Banner, 26 from Horqin Right Front Banner, 32 from Ulanhot, and 116 from Jalaid Banner, as shown in Figure 1. Demographic information (including sex, age, residence, occupation, and nationality) and medical records (including complications and previous TB treatment history) of these TB patients were extracted and matched from the surveillance database. The data were collected and stored electronically by local medical staff during patient visits, following the acquisition of informed consent from the patients. The study received ethical approval on 2023, which complies with the Declaration of Helsinki, from the Institutional Review Board of China CDC (202336).

Drug Susceptibility Testing (DST)

Drug susceptibility testing was carried out using UKMYC6 plates (Thermo Fisher Scientific, USA), designed by the CRyPTIC Consortium. UKMYC6 exhibits the capability to quantitatively assess resistance levels to various anti-TB drugs with reliable reproducibility.[17] All steps were performed by trained and specialized personnel, strictly adhering to the prescribed protocols.[2] Plates inoculated with the bacterial solution were securely sealed with adhesive membranes and then placed in incubation at 37°C in 5% CO_2 for a duration of 14 days. The minimum inhibitory concentration (MIC) was determined as the lowest concentration without evident visible bacterial growth in comparison to the positive

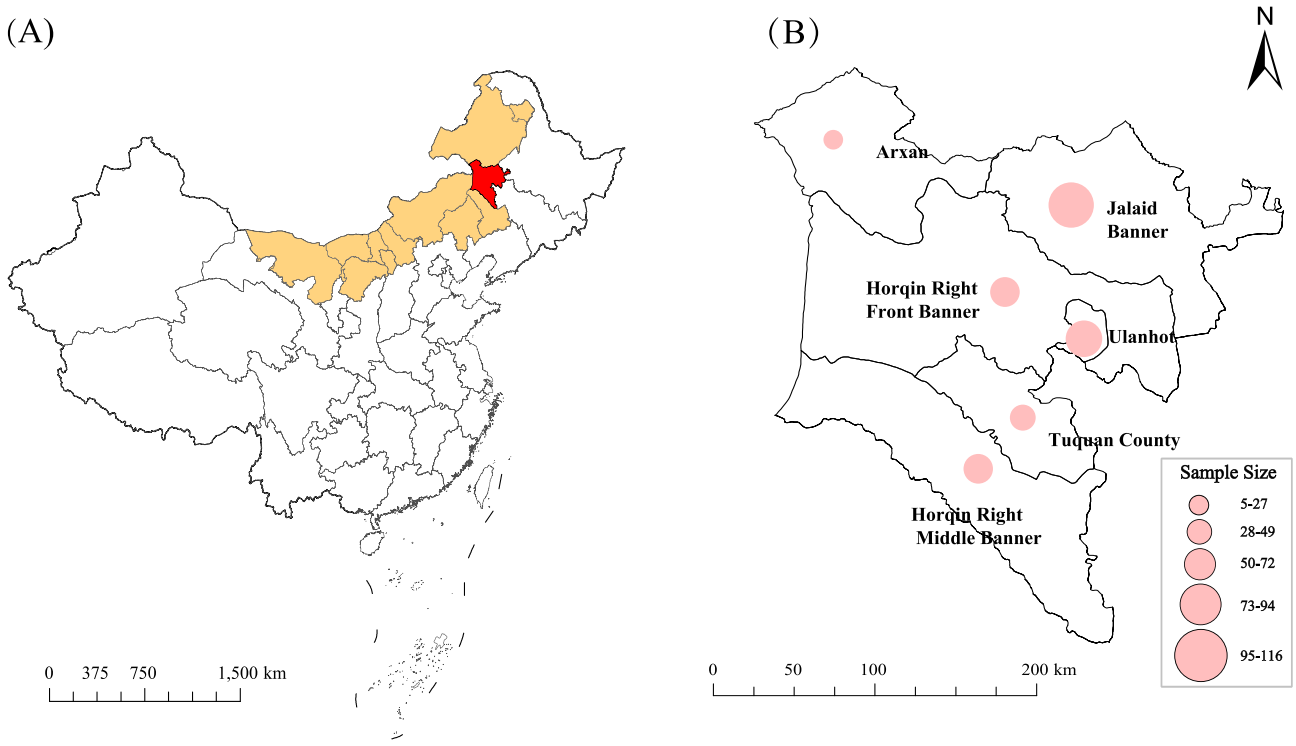

Figure 1 Distribution of study sites in Hinggan League, Inner Mongolia, China. (**A**) The location of Hinggan League and Inner Mongolia in China. Orange indicates Inner Mongolia, red indicates Hinggan League. (**B**) The distribution of six sites in Hinggan League and the sample size of MTB strains collected in each sub-region.

control. The concentration range and breakpoint concentration for each drug on the UKMYC6 plate can be found in Table S1. MTB strains were categorized as resistant (R) to a particular drug if the MIC value surpassed the breakpoint concentration.[18]

DNA Extraction and Whole Genome Sequencing

All MTB strains were scraped from L-J slant, and genomic DNA were extracted using the cetyltrimethylammonium bromide (CTAB) method.[19] Genomic DNA quality and concentration were evaluated using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and Qubit 2.0 fluorometer (Invitrogen, Thermo Fisher Scientific, USA), respectively. All whole genome sequencing procedures were conducted by Annoroad Gene Technology company (Beijing, China) using Illumina HiSeq X10 (Illumina, Inc.) with 2 × 150 paired-end (PE) strategies.

Genotypic Mutation and Phylogenomic Analysis

The quality of paired-end reads was assessed using FastQC (v0.11.9) and subsequently trimmed using Trimmomatic (v0.39) as described previously. Qualified reads were mapped to the reference H37Rv genome (GenBank ID: NC_000962.3) using the BWA-MEM algorithm (v0.7.16). Variant calling from the sorted mapped sequences was performed using Samtools (v1.13) and bcftools (v1.13). Filtering of variants was conducted based on specified criteria, including a mapping quality greater than 30 and a minimum read depth of 10. Annotation of the filtered variant SNPs was performed using SnpEff (v4.3). Drug-resistant mutations were detected using TBProfiler (v2.8.12).

Phylogenetic analysis was conducted using the snippy pipeline (v4.3.6) for the alignment of core SNPs (https://github.com/tseemann/snippy). Positions of SNPs within PE/PPE/PGRs genes were excluded. Subsequently, the remaining SNPs in each isolate within the alignment was filtered using Gubbins and were integrated into a sequence alignment. The maximum-likelihood phylogenetic tree was constructed using RAxML based on core SNPs, with 1000 bootstrap iterations and the general time reversible (GTR+G) model. The lineage and sub-lineage information of *M. tuberculosis*

isolates was detected using fast-lineage-caller v1.0 (https://github.com/farhat-lab/fast-lineagecaller). And the phylogenetic tree was visualized by tvBOT (https://www.chiplot.online/tvbot.html).[20]

Statistical Analysis

Comparisons of categorical data were analyzed using the Chi-square test or Fisher's exact test. Univariate and multivariate logistic regression model were used to analyze risk factors related to drug resistance. All variables with $P < 0.2$ at univariate analysis were included in multivariate logistic regression model. The statistical analysis was conducted using SPSS version 18.0 software (SPSS Inc., Chicago, Illinois). $P < 0.05$ was considered statistically significant.

Definitions

Pan-susceptible TB was characterized as the MTB strain exhibiting sensitivity to all anti-TB drugs (isoniazid, rifampicin, rifabutin, ethambutol, amikacin, kanamycin, levofloxacin, moxifloxacin, ethionamide, delamanid, bedaquiline, linezolid, clofazimine) assessed in this study and confirmed through in vitro DST. DR-TB was defined as MTB resistant to at least one of the anti-TB drugs tested in this study. Mono-DR-TB was characterized as MTB strains resistant to only one of the anti-TB drugs assessed in this study. Poly-DR-TB was defined as MTB resistance to at least two or more anti-TB drugs but excluding concurrent resistance to rifampicin and isoniazid. MDR-TB was designated as MTB resistance to at least isoniazid and rifampicin. Simple MDR-TB was defined as MDR-TB strains that do not exhibit resistance to fluoroquinolones (levofloxacin or moxifloxacin) or second-line injectable anti-TB drugs (amikacin or kanamycin). Pre-XDR-TB was characterized as MDR/RR-TB with additional resistance to any fluoroquinolone drugs. XDR-TB was defined as MDR/RR-TB with additional resistance any fluoroquinolone and at least one additional Group A drug (bedaquiline or linezolid).

Results

Demographic and Clinical Characteristics

In total, 218 MTB isolates from unique TB patients were collected in Hinggan League between Jun. 2021 and Jun. 2023. All MTB strains were thawed and re-cultured for phenotypic drug susceptibility testing and whole genome sequencing, while 7 were excluded due to failure of re-culture, contamination, or missing demographic information. Thus, 211 MTB strains were included for further analysis. Out of the 211 MTB strains, 77.3% (163/211) were obtained from male patients. The median age of these patients was 53 years (interquartile range [IQR], 34–65). The majority of patients lived in rural regions (73.0%, 154/211) and their occupation was predominantly farmer (72.0%, 152/211). In total, 8.1% (17/211) of patients exhibited extrapulmonary TB, and 13.7% (29/211) had diabetes. Almost all patients had no concurrent hepatitis B virus (HBV) or human immunodeficiency virus (HIV) infections. The majority of TB cases (73.9%, 156/211) were newly diagnosed, while 26.1% (55/211) of cases had been previously treated. Detailed socio-demographic and clinical information about the study population is presented in Table 1

Population Structure of MTB Strains

A total of 211 MTB strains were phylogenetically analyzed and two main lineages were observed. The most prevalent lineage was lineage 2 (East Asian) (88.6%, 187/211), characterized exclusively by sub-lineage 2.2.1 (modern Beijing) (100.0%, 187/187). Lineage 4 (Euro-American) was represented by 24 MTB strains, constituting 11.4% (24/211) of the total. Among these, 9 isolates belonged to lineage 4.4 (37.5%, 9/24), and 15 belonged to lineage 4.5 (62.5%, 15/24) (Figure 2).

Furthermore, we classified the strains of lineage 2 according to Shitikov's schemes,[21] which divided the L2 strains into 11 genotypes based on genetic markers. In this study, 86 of L2 isolates were divided into five genotypes. To be specific, 88.37% (76/86) of the L2 strains is asian_african_2, following the asia_ancestral_3 (5/86), asian_african_3 (2/86), and pacific_RD150 (2/86). And one strain belongs to asia_ancestral_2.

Phenotypic Drug-Resistant Profile

The phenotypic drug resistant profile of 211 MTB strains included in this study was determined by 3 first-line anti-TB drugs (RIF, INH, and EMB), 6 second-line anti-TB drugs (RFB, KAN, AMI, MXF, LFX, and ETH) and 4 new and repurposed

Table 1 Socio-Demographic Characteristics of TB Patients in This Study

Characteristics	New Cases (n=156) n (%)	Retreated Cases (n=55) n (%)	Total (n=211) n (%)
Sex			
Male	119 (76.3)	44 (80.0)	163 (77.3)
Female	37 (23.7)	11 (20.0)	48 (22.7)
Age (years)			
<30	14 (9.0)	1 (1.8)	15 (7.1)
30–44	29 (18.6)	13 (23.6)	42 (19.9)
45–59	60 (38.5)	19 (34.5)	79 (37.4)
≥60	53 (34.0)	22 (40.0)	75 (35.5)
Residence			
Rural	110 (70.5)	44 (80.0)	154 (73.0)
Urban	46 (29.5)	11 (20.0)	57 (27.0)
Occupation			
Farmer	110 (70.5)	42 (76.4)	152 (72.0)
Non-farmer	46 (29.5)	13 (23.6)	59 (28.0)
Nationality			
Han	72 (46.2)	29 (52.7)	101 (47.9)
Meng	79 (50.6)	24 (43.6)	103 (48.8)
Others	5 (3.2)	2 (3.6)	7 (3.3)
Extrapulmonary TB			
Yes	13 (8.3)	4 (7.3)	17 (8.1)
No	141 (90.4)	50 (90.9)	191 (90.5)
Unknown	2 (1.3)	1 (1.8)	3 (1.4)
Diabetes			
Yes	24 (15.4)	5 (9.1)	29 (13.7)
No	130 (83.3)	49 (89.1)	179 (84.8)
Unknown	2 (1.3)	1 (1.8)	3 (1.4)
Hepatitis B			
Yes	0 (0.0)	0 (0.0)	0 (0.0)
No	154 (98.7)	54 (98.2)	208 (98.6)
Unknown	2 (1.3)	1 (1.8)	3 (1.4)
HIV			
Yes	1 (0.6)	0 (0.0)	1 (0.5)
No	153 (98.1)	55 (100.0)	208 (98.6)
Unknown	2 (1.3)	0 (0.0)	2 (0.9)

anti-TB drugs (DLM, BDQ, LZD, and CFZ). As presented in Table 2, MTB strains exhibited the highest resistance rates to isoniazid (16.1%, 34/211), followed by rifampicin (10.0, 21/211), rifabutin (10.0%, 20/211), ethionamide (7.1%, 15/211), ofloxacin (5.7%, 12/211), moxifloxacin (5.7%, 12/211), and ethambutol (5.7%, 12/211). MTB strains showed a relatively low resistance to kanamycin and amikacin, with rates of 1.9% and 1.4%, respectively. Worryingly, the results indicate that MTB strains tested in this study had a relatively high rates of resistance against new and repurposed anti-TB drugs, with resistant rates of 2.4% (5/211) to delamanid and 1.9% (4/211) to bedaquiline. Concerning drug resistant patterns, 54 MTB strains (25.6%, 54/211) demonstrated resistance to at least one anti-TB drugs tested in this study. Among them, 24 strains (11.4%, 24/211) were classified as mono-DR-TB, 16 strains (7.6%, 16/211) were classified as poly-DR-TB. A total of 14 MTB strains met the definition of MDR-TB, including 7 strains of simple-MDR-TB, 5 of pre-XDR-TB, and 2 of XDR-TB (Table 2, Figure 2). Remarkably, no statistically significant difference was observed in resistant rates against various drugs and drug-resistant patterns between lineage 2 and lineage 4 (Table 2).

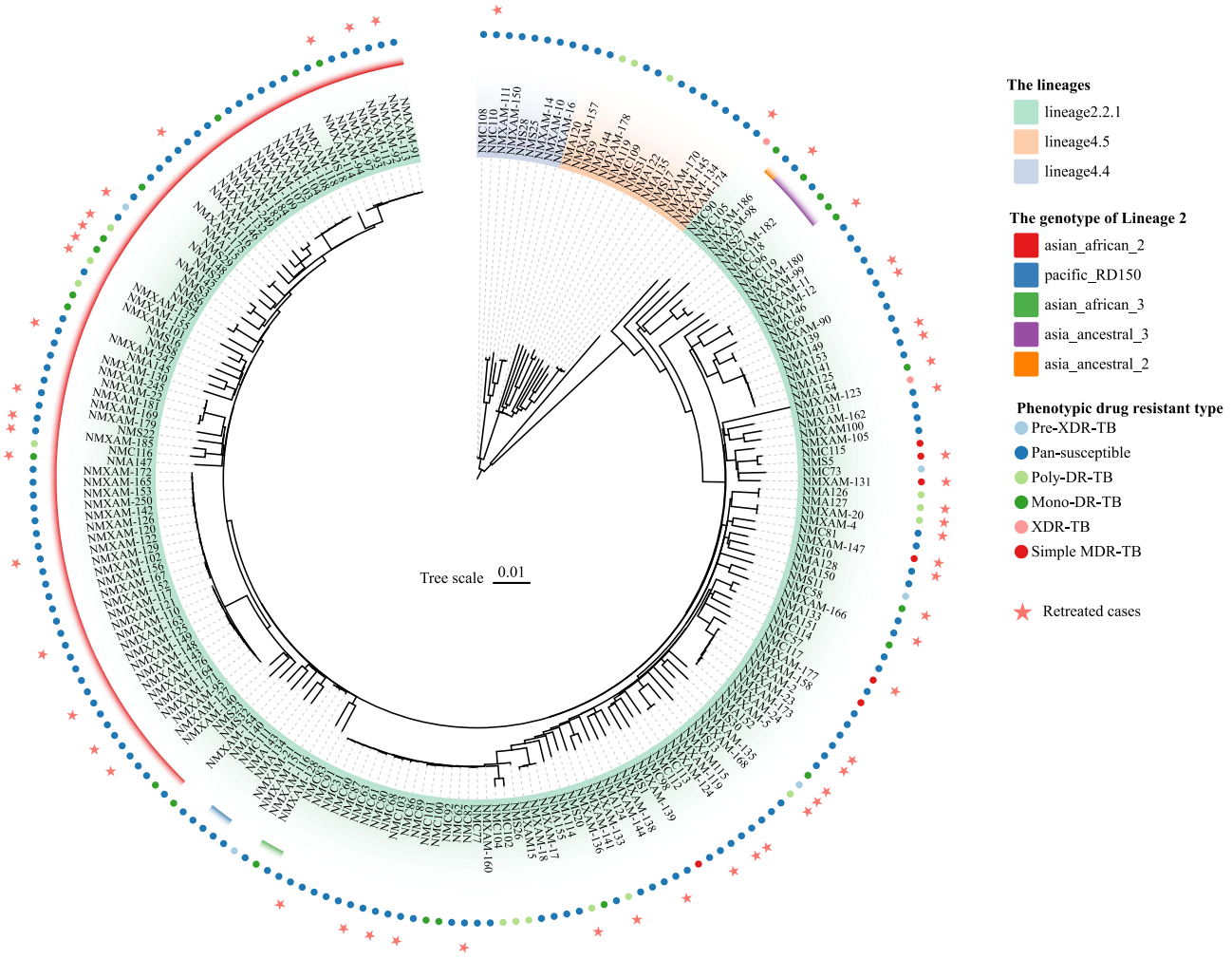

Figure 2 Phylogenetic tree of 211 MTB strains and annotated with drug resistant information. The maximum likelihood phylogenetic tree was generated using RAxML, with 1000 bootstraps iterations to enhance robustness. The lineage and phenotypic drug-resistant types are represented by colored blocks, aligning with the legend displayed on the right. The Stars on the tree highlight patients with previously treatment.

Genotypic Drug Resistant Profile

The genotypic drug resistant mutations based on WGS were determined and summarized in Table 3. A total of 19 strains (9.0%, 19/211) were identified as harboring rifampicin-resistant gene mutations, all located in the rifampicin-resistance determining region (RRDR) of the *rpoB* gene. The most prevalent mutation type was *rpoB*_Ser450Leu, observed in 47.4% (9/19) of rifampicin genotypically resistant strains. The strains carried mutation *rpoB*_Leu430Pro and *rpoB*_Leu452Pro were phenotypically sensitive to rifampicin. Additionally, two strains (10.6%, 2/19) had concurrent mutations in the *rpoC* gene. INH-resistant gene mutations were detected in 28 MTB isolates (13.3%, 28/211), with the most frequent mutation being *katG*_Ser315Thr, observed in 46.4% (13/28) of INH-resistant strains. The four strains carrying the *fabG1*_c.-15C>T or *ahpC*_c.-48G>A mutations exhibited phenotypic sensitivity to isoniazid in our results. Additionally, eight strains (28.6%, 8/28) displayed combined mutations at two or more sites, primarily involving mutations in *katG* and *fabG1*/*ahpC* promoter. Mutations associated with EMB-resistance were detected in 12 MTB strains (5.7%, 12/211), predominantly related to mutations in *embB* gene. Notably, among these mutations, *embB*_Met306Val (25.0%, 3/12) and *embB*_Met306Ile (16.7%, 2/12) emerged as the most common mutations identified in this study. Ten MTB strains (4.7%, 10/211) were detected with gene mutations associated with fluoroquinolones (FQs), and all mutations were solely located in the quinolone-resistance-determining region (QRDR) for FQs. The predominant mutation types observed in the *gyrA* gene were Asp94Ala (40.0%, 4/10) and Asp94Gly (40.0%, 4/10). For

Table 2 Drug Resistant Profiles of MTB Strains

Drugs/ Drug Resistant Patterns	Total Strains (n=211) no. (%)	Lineage 2 (n=187) no. (%)	Lineage 4 (n=24) no. (%)	*P*
First-Line Drugs				
Rifampicin	21 (10.0)	20 (10.7)	1 (4.2)	0.520
Isoniazid	34 (16.1)	32 (17.1)	2 (8.3)	0.420
Ethambutol	12 (5.7)	12 (6.4)	0 (0.0)	0.368*
Second-Line Drugs				
Rifabutin	20 (9.5)	20 (10.7)	0 (0.0)	0.138*
Kanamycin	4 (1.9)	4 (2.1)	0 (0.0)	>0.999*
Amikacin	3 (1.4)	3 (1.6)	0 (0.0)	>0.999*
Moxifloxacin	12 (5.7)	12 (6.4)	0 (0.0)	0.368*
Levofloxacin	12 (5.7)	12 (6.4)	0 (0.0)	0.368*
Ethionamide	15 (7.1)	13 (7.0)	2 (8.3)	>0.999
New and Repurposed Drugs				
Delamanid	5 (2.4)	5 (2.7)	0 (0.0)	>0.999
Bedaquiline	4 (1.9)	3 (1.6)	1 (4.2)	0.385*
Linezolid	5 (2.4)	5 (2.7)	0 (0.0)	>0.999*
Clofazimine	2 (0.9)	2 (1.1)	0 (0.0)	>0.999*
Drug Resistant Patterns				
DR-TB	54 (25.6)	51 (27.3)	3 (12.5)	0.118
Mono-DR-TB	24 (11.4)	24 (12.8)	0 (0.0)	0.084*
Poly-DR-TB	16 (7.6)	13 (7.0)	3 (12.5)	0.577
MDR-TB	14 (6.6)	14 (7.5)	0 (0.0)	0.377*
Simple MDR-TB	7 (3.3)	7 (3.7)	0 (0.0)	>0.999*
Pre-XDR-TB	5 (2.4)	7 (2.7)	0 (0.0)	>0.999*
XDR-TB	2 (0.9)	2 (1.1)	0 (0.0)	>0.999*

Note: * indicates *P* value was calculated by Fisher exact test.

aminoglycosides, only one strain was identified as harboring gene mutations related to AMI/KAN, specifically with *rrs*_a1401g. Mutations conferring resistance to ETH primarily targeted the *fabG1* c-15t gene (42.9%, 3/7), but one strain carried *fabG1*_c.-8T>C exhibited phenotypic sensitivity to ETH. Combined mutations were detected in two ETH-resistant strains (28.6%, 2/7), and one strain displayed an insertion in the *ethA* gene (14.3%, 1/7). No known gene mutations related to new and repurposed anti-TB drugs (DLM, BDQ, CFZ, LZD) in our study (Table 3).

Table 3 Genotypic Drug Resistant Spectrum of 211 MTB Strains

Drug	Phenotypic Drug Resistant	Genotypic Drug Resistant	No. of Strains
Rifampicin (RIF)	R	*rpoB*_Ser450Leu	9
	R	*rpoB*_His445Tyr	3
	R	*rpoB*_Ser450Asn	1
	R	*rpoB*_Ser441Leu	1
	R	*rpoB*_His445Asn	1
	R	*rpoB*_Ser450Leu + *rpoC*_Leu527Val	1
	R	*rpoB*_Ser450Leu + *rpoC*_Ile491Thr	1
	WT	*rpoB*_Leu430Pro	1
	WT	*rpoB*_Leu452Pro	1
	R	NA	4

(*Continued*)

Table 3 (Continued).

Drug	Phenotypic Drug Resistant	Genotypic Drug Resistant	No. of Strains
Isoniazid (INH)	R	*katG*_Ser315Thr	13
	R	*katG*_Ser315Asn	2
	R	*katG*_Ser140Asn	1
	R	*katG*_Ile317Leu + Ile335Thr	1
	R	*katG*_Ser315Thr + *fabG1*_c.-8T>A	1
	R	*katG* _Tyr155Ser + *fabG1*_c.-15C>T	1
	R	*katG*_Tyr155Ser + *fabG1*_c.-8T>A	1
	R	*katG*_g.2154868_2156147del + *ahpC*_c.-48G>A	1
	R	*fabG1*_c.-8T>A + *ahpC*_c.-48G>A	1
	R	*fabG1*_c.-8T>C + *inhA* _Ser94Ala	1
	R	*fabG1*_c.-17G>T + *inhA* _Ser94Ala	1
	WT	*fabG1*_c.-15C>T	2
	WT	*ahpC* _c.-48G>A	2
	R	*NA*	10
Ethambutol (EMB)	R	*embB* _Met306Val	3
	R	*embB* _Met306Ile	2
	R	*embB* _Gly406Ala	1
	R	*embB* _Gln497Arg	1
	R	*embB* _His1002Arg	1
	R	*embB* _His312Arg + Asn399Asp	1
	R	*embB* _Met306Val + Gly406Ala	1
	R	*embC*_Ile297Leu	2
	WT	NA	1
Moxifloxacin/ Levofloxacin (MXF/LFX)	R	*gyrA* _Asp94Ala	4
	R	*gyrA* _Asp94Gly	4
	R	*gyrA* _Ser91Ala	2
	R	NA	2
Amikacin/ Kanamycin (AMI/KAN)	R	*rrs*_r.1401a>g	1
	R	NA	2
Ethionamide (ETH)	R	*fabG1*_c.-15C>T	3
	R	*ethA* _c.672_673insG	1
	R	*fabG1*_c.-17G>T + *inhA* _Ser94Ala	1
	R	*fabG1*_c.-8T>C + *inhA*_Ser94Ala	1
	R	NA	10
	WT	*fabG1*_c.-8T>C	1

Note: WT indicates wild type, R indicates resistant.

Analysis of Risk Factors Related to Any DR-TB

To identify risk factors related to any drug-resistant tuberculosis, demographic information and clinical characteristics of patients, as well as genetic background of MTB strains were included into univariate and multivariate logistic regression model. As shown in Table 4, any DR-TB occurs at a higher rate in male patients than in female patients (30.1% vs 10.4%, $P = 0.009$). Additionally, the occurrence of DR-TB was more prevalent among previously treated TB cases compared to new cases (38.2% vs 21.2%, $P = 0.035$). Lineage 2 had a higher rate of any drug-resistance than Lineage 4, but the difference was not statistically significant (27.3% vs 12.5%, $P = 0.131$). After multifactorial correction, the analysis revealed that the risk of developing DR-TB was almost 4 times higher in male patients compared to female (AOR = 3.858, 95% CI:1.416–10.511). Furthermore, individuals with retreatment history faced a two-fold higher risk of developing DR-TB compared to new patients (AOR = 2.105, 95% CI: 1.052–4.210). None of the other factors had a statistically significant effect on the development of DR-TB in this study (Table 4).

Table 4 Univariate and Multivariate Analysis of Risk Factors Related to DR-TB

Variables	Pan-Susceptible (n=157, rate [%])	Any DR-TB (n=54, rate [%])	COR (95% CI)	*P*	AOR (95% CI)	*P*
Sex						
Male	114 (69.9)	49 (30.1)	3.696 (1.381–9.896)	0.009	3.858 (1.416–10.511)	0.008
Female	43 (89.6)	5 (10.4)	Ref		Ref	
Age (years)						
<30	13 (86.7)	2 (13.3)	0.487 (0.100–2.366)	0.372		
30–44	30 (71.4)	12 (28.6)	1.267 (0.539–2.975)	0.587		
45–59	57 (72.2)	22 (27.8)	1.222 (0.593–2.519)	0.586		
≥60	57 (76.0)	18 (24.0)	Ref			
Residence						
Rural	114 (74.0)	40 (26.0)	1.078 (0.534–2.176)	0.835		
Urban	43 (75.4)	14 (24.6)	Ref			
Occupation						
Farmer	109 (72.2)	42 (27.8)	1.541 (0.746–3.185)	0.243		
Non-farmer	48 (80.0)	12 (20.0)	Ref			
Nationality						
Han	70 (69.3)	31 (30.7)	1.675 (0.897–3.128)	0.105	1.695 (0.885–3.246)	0.112
Others	87 (79.1)	23 (20.9)	Ref		Ref	
Extrapulmonary TB						
Yes	11 (64.7)	6 (35.3)	1.719 (0.603–4.906)	0.311		
No	145 (75.9)	46 (24.1)	Ref			
Unknown*	1 (33.3)	2 (66.7)				
Diabetes						
Yes	20 (69.0)	9 (31.0)	1.423 (0.603–3.357)	0.420		
No	136 (76.0)	43 (24.0)	Ref			
Unknown*	1 (33.3)	2 (66.7)				
Treatment history						
Yes	34 (61.8)	21 (38.2)	2.302 (1.183–4.481)	0.014	2.105 (1.052–4.210)	0.035
No	123 (78.8)	33 (21.2)	Ref		Ref	
Lineages						
L2	136 (72.7)	51 (27.3)	2.625 (0.751–9.179)	0.131	2.210 (0.614–7.950)	0.225
L4	21 (87.5)	3 (12.5)	Ref		Ref	

Note: * were not included in univariate and multivariate logistic regression model; Variables with *P*<0.2 at univariate analysis were included in multivariate logistic regression model.

Abbreviations: COR, Crude Odds Ratio; AOR, Adjusted Odds Ratio; CI, Confidence Interval.

Discussion

To the best of our knowledge, this is the first in-depth study to offer a comprehensive understanding of the molecular epidemiology of TB in Hinggan League, China, through the integration of phenotypic DST and WGS to identify genetic diversity and drug-resistant profiles of MTB strains. As expected, lineage 2.2.1 (modern Beijing) was the dominant genotype of MTB strains circulating in Hinggan League. The widespread distribution of the Beijing genotype can be attributed to factors such as high transmission efficiency, heightened virulence, and an increased risk of drug resistance.[22–24] Some studies have suggested that Beijing genotype strains are more likely to develop DR-TB.[2,25] However, the results of the present study revealed no correlation between the Beijing genotype and drug resistance, which may be due to the small sample size in this study or other factors such as different treatment regimens,[26] and further analysis with a larger sample size is needed.

Overall, more than 25% of MTB strains exhibited resistance at least one anti-TB drugs tested in this study, highlighting a relatively severe drug-resistant situation Hinggan League. The result showed that 6.6% of the strains belong to MDR-TB, which is similar to the national level in China (7.42%),[27] but higher than the level of Anhui

(4.20%)[28] and lower than the level of Guangxi (7.1%).[12] Notably, our data showed a high percentage of pre-XDR-TB and XDR-TB strains, with 35.7% and 14.3% of the MDR isolates belong to pre-XDR-TB and XDR-TB, respectively, and this high proportion was mediated by fluoroquinolone resistant strains. Given that fluoroquinolones remain a key component of MDR-TB treatment in China, our results emphasize the urgent need for diagnosing fluoroquinolone resistance prior to treatment regimen development.[18] Previous study reported that over 90% of RR-TB cases are concurrently resistant to INH, and RR-TB is often considered an alternative marker for MDR-TB.[29] However, our study revealed that 33.3% (7/21) of RR-TB cases remained sensitive to INH, aligning with the findings of a previously reported meta-analysis.[30] This suggests that rifampicin resistance may not be an entirely reliable indicator of MDR-TB in this region. Concurrently, our results emphasize the importance of not excluding INH from the treatment regimen for patients with initial RR-TB. More importantly, we observed a relatively higher resistant rate against DLM and BDQ, which highlight the importance of universal drug susceptibility testing for TB patients.[31]

With regard to the molecular mechanism of resistance to anti-TB drugs, gene mutations were identified by screening whole-genome data using a reliable online tool called TB Profiler.[32] Consistent with previous studies, mutations in the RRDR of *rpoB* and the *katG*_315 codon clearly dominated in rifampicin- and isoniazid-resistant mutant strains.[2,12,18,33] The significance of *embB* gene mutations, especially at codon 306, has been controversial due to their presence in both EMB-resistant and EMB-sensitive strains. In our study, the most prevalent *embB* gene mutations were Met306Val, (25.0%) and Met306Ile (16.7%), whereas Met306Ile was the most common mutation type in Hainan strains.[34] In line with prior studies, the dominant fluoroquinolone-resistance gene identified was *gyrA* mutation.[12,34] In this study, the mutation rate within the QRDR reached 100.0%, predominantly characterized by Asp94Ala (40.0%) and Asp94Gly (40.0%) in the *gyrA* gene. No known gene mutations were detected in TB resistant to DLM, suggesting that further study is urgently required to reveal the underlying genetic basis. In this study, a notable disparity is evident between the observed phenotypic resistance and the genotypic resistance to new and repurposed drugs. This gap is primarily attributed to a limited understanding of the mechanisms of drug resistance, which urgently needs to be addressed.[31] Notably, our results show that some strains with genetic mutations associated with resistance do not exhibit phenotypic resistance, which may be due to some genetic mutations belonging to borderline mutations, such as *rpoB*_Leu430Pro and *rpoB*_Leu452Pro and *fabG1*_ C-15T.[35] Borderline mutations are typically associated with low levels of resistance and slightly elevated MICs that remain below the critical concentration of current DST systems.[35–37] Additionally, we also observed some strains displaying phenotypic resistance without detectable known mutations, indicating alternative mechanisms, such as drug efflux pumps and reduced permeability of the cell wall to the drug, may also contribute to the development of drug resistance in MTBC.[2,38]

Exploring the relevant risk factors of DR-TB can help identify vulnerable patients,[39] and timely intervention and management of these people are crucial for optimally and effectively controlling the DR-TB as well as TB epidemic. It is well established that a previous treatment history is a risk factor for the development of DR-TB.[39] Our results revealed that the risk of DR-TB in patients with previously treatment history was two times higher than that in new patients. Poor patient compliance and unregulated drug use are the primary reasons for the development of drug resistance.[40,41] Another identified risk factor was gender, with the findings of the present study illustrating that males had a higher risk of developing drug-resistant tuberculosis (DR-TB) compared to females. This aligns with expectations, as men are prone to numerous risk factors, including smoking and alcohol abuse, compared to women, potentially elevating their risk of contracting DR-TB.[42] Given the independent risk factors associated with DR-TB identified in this study, namely a history of retreatment and male patients, it is imperative to emphasize the management of patients with these characteristics in our future efforts to reduce the occurrence of drug resistance.

The main limitation of our study is that the MTB strains examined here were exclusively from pulmonary TB cases with sputum smear-positive. However, it is worth noting that some studies suggest that around 17% of drug-resistant TB patients and 20% of multidrug-resistant TB cases may obtain from smear-negative TB patients.[3] Furthermore, given the limited number of strains resistant to specific drugs such as amikacin or kanamycin, the corresponding molecular characteristics of resistance identified in this study may lack representativeness. Thirdly, due to the limited sample size, phenotypically and genotypically heterogeneous drug-resistant strains were not analyzed in-depth in this study. The sample size will be expanded subsequently to further elucidate potential new mechanisms of resistance or the effect of epistatic genes.

In conclusion, our study offers crucial insights into the genetic diversity, phenotypic, and genotypic drug-resistant profiles of TB strains circulating in Hinggan League. We found that 25.6% of MTB strains were DR-TB and highest resistance rates to isoniazid (16.1%), with a relatively severe drug-resistant situation. Notley, the MTB strains had a relatively high rates of resistance against new and repurposed anti-TB drugs in this study. Previously treated patients and male patients were identified as independent risk factors associated with DR-TB. Overall, these findings serve as a significant reference for DR-TB surveillance, guiding the design of treatment regimens, and tailoring public interventions to address the specific challenges in the region.

Data Sharing Statement

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive in National Genomics Data Center with accession number CRA017087.

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Disclosure

The authors have declared that there were no competing interests in this work.

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