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Research article

Evaluation of genetic correlation with fluoroquinolones resistance in rifampicin-resistant Mycobacterium tuberculosis isolates

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ABSTRACT

Objective: To detect levofloxacin (LFX) and moxifloxacin (MFX) resistance among rifampicin-resistant tuberculosis (RR-TB) isolates, and predict the resistance level based on specific mutations in *gyrA* and *gyrB* genes.

Methods: A total of 686 RR-TB isolates were collected from Chinese Drug Resistance Surveillance Program from 2013 to 2020. The minimum inhibitory concentrations (MICs) of 12 anti-TB drugs were acquired using the broth microdilution method, followed by whole genome sequencing (WGS) analysis.

Results: Among the 686 RR isolates, the most prevalent resistance was to isoniazid (80.5 %) and ethambutol (28.4 %), followed by LFX (26.1 %) and MFX (21.9 %). The resistance rate of LFX (26.1%–99.4 %) was higher than that of MFX (21.9%–83.3 %) across various drug resistance patterns. Of the 180 fluoroquinolones (FQs) resistant isolates, 168 (93.3 %) had mutations in quinolone-resistant determining regions (QRDRs) with 21 mutation types, and Asp94Gly (32.7 %, 55/168) was the predominant mutation. Isolates with mutations in Asp94Asn and Asp94Gly were associated with high levels of resistance to LFX and MFX. Using broth microdilution method as gold standard, the sensitivities of WGS for LFX and MFX were 93.3 % and 98.0 %, and the specificities were 98.6 % and 95.0 %, respectively.

Conclusion: The resistance rate of LFX was higher than that of MFX among various drug resistance patterns in RR-TB isolates. The *gyrA* Asp94Gly was the predominant mutation type underlying

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FQs resistance. However, no significant difference was observed between mutation patterns in *gyrA* gene and resistance level of FQs.

1. Introduction

Drug-resistant tuberculosis (DR-TB) remains a threat to public health. In 2021, approximately 450,000 new rifampicin-resistant TB (RR-TB) cases were reported globally, with 78 % being multidrug-resistant tuberculosis (MDR-TB) [1]. The treatment of RR/MDR-TB remains difficult due to the high cost, limited effective drugs, prolonged treatment, and frequent side effects, leading to the treatment success rate of less than 60 % [2,3]. With the second largest number of RR/MDR-TB cases in the world, the treatment success rate in China was only 54 % below the global average, imposing a burden on health care resources [3].

Fluoroquinolones (FQs), one of the pivotal drugs in the treatment of MDR-TB, are classified as "group A″ drugs for MDR-TB treatment [4]. However, due to their widely prescribed in several infectious diseases, the FQs resistance is increased in *Mycobacterium tuberculosis*, resulted in poor treatment outcomes among MDR-TB patients [5,6]. Mutations in two short regions, known as quinolone-resistant determining regions (QRDRs) within the *gyrA* (codon 74–113) and *gyrB* (codon 500–538) genes, have been associated with FQs resistance in *Mycobacterium tuberculosis* [7,8]. Though detecting resistance-associated mutations in FQs can predict the presence and level of FQs resistance, the association remains debatable, which requires further exploration [9]. As a reliable, rapid and increasingly affordable technology, whole genome sequencing (WGS) can predict drug resistance profiles and monitor the acquisition of drug resistance, allowing prompt, appropriate initiation of treatment [10]. As levofloxacin (LFX) and moxifloxacin (MFX) are the two frequently prescribed to treat MDR-TB patients among FQs [11,12], we aimed to detect LFX and MFX resistance by WGS, and predict resistance level based on specific mutations within the *gyrA* and *gyrB* genes among RR-TB isolates, which will improve the diagnosis and treatment of RR-TB patients.

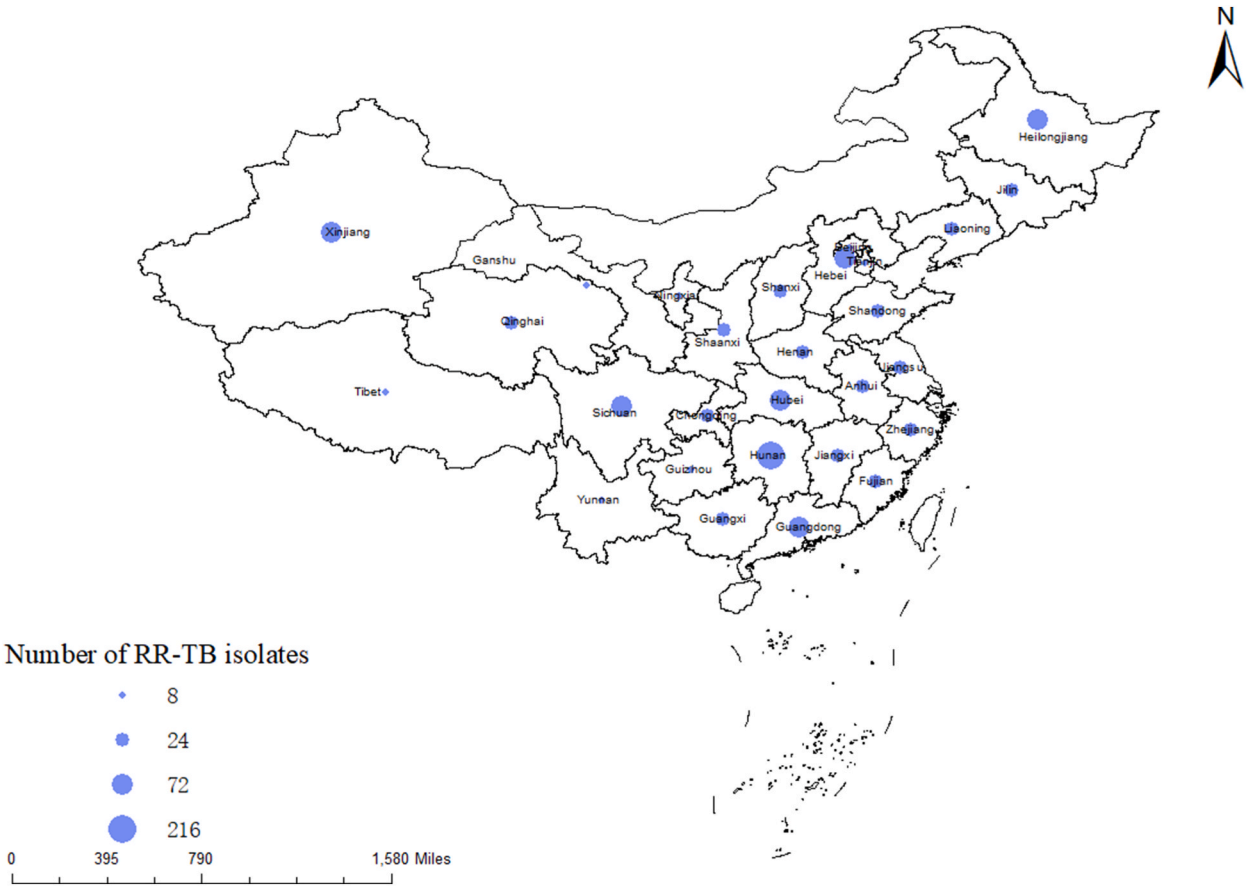


Fig. 1. The sampled RR-TB isolates from various geographic regions in China.

2. Materials and methods

2.1. Isolate selection

A total of 686 rifampicin-resistant TB (RR-TB) isolates, identified by 1 % proportion method on Löwenstein-Jensen (L-J) medium, were collected from 18 provinces, 3 municipalities and 4 autonomous regions included in the Chinese Drug Resistance Surveillance Program from 2013 to 2020 (Fig. 1) [13].

2.2. Broth microdilution DST

Phenotypic DST was performed using the broth microdilution method, which provided a minimal inhibitory concentration (MIC) (MYCOTB plate, Thermo Fisher Scientific Inc., USA), and a panel of 12 anti-TB drugs were tested including amikacin [AMK], ethambutol [EMB], ethionamide [ETO], isoniazid [INH], kanamycin [KAN], moxifloxacin [MFX], levofloxacin [LFX], and rifampicin [RIF]. MIC was defined as the lowest concentration without obvious visible bacterial growth. MTB H37Rv (ATCC 27294) strain was used to quality control (QC) all tests. Based on the recommendation by the World Health Organization (WHO) [14], the breakpoint concentration for LFX and MFX was1 μg/mL, and that of the other drugs were shown in supplementary table. Isolates with MIC value higher than the critical concentration were defined as resistance, and otherwise were sensitive. The high levels of LFX and MFX resistance were defined as MIC≥4 μg/mL and MIC≥2 μg/mL, and low levels of resistance were MIC < 4 μg/mL and MIC < 2 μg/mL, respectively [15]. The RR isolates were defined as resistance to RIF. The fluoroquinolones resistance was defined as resistance to LFX and/or MFX. MDR isolates were defined as resistance to at least INH and RIF, and pre-extensively drug resistant (pre-XDR) tuberculosis was defined as MDR/RR-TB isolates plus either FQ resistance.

2.3. WGS analysis

Genomic DNA was prepared using the cetyltrimethylammonium bromide method, then qualified DNA samples were sent to the Annoroad Gene Technology (Beijing, China) for whole genome sequencing (WGS) using the Illumina Hiseq2500 platform. The overall quality of sequence reads was checked using FastQC (v0.11.8). Verified paired-end reads were filtered with Trimmomatic (v 0.38) using default values and a minimum Phred Quality score of 20. Then the reads of human reference genome GRCh38 are removed. Only filtered paired-end reads were kept for downstream analysis. Sequencing reads corresponding to *gyrA* and *gyrB* were aligned to those from the H37Rv reference genome (GenBank ID: NC_000962.3). SAMtools (v1.3.1) and GATK (v3.8.0) was used to call variants, including single nucleotide polymorphisms (SNPs) and insertion/deletions (indel).

2.4. Statistical analysis

The person chi-square test or Fisher exact test was used to compare proportions or resistant rates. Sensitivity and specificity were calculated at the 95 % confidence interval (*CI*), while concordance between two methods was performed with Kappa test. A P value $<$ 0.05 was considered statistically significant. All the statistical analyses were performed in the SPSS 20.0 (IBM Corp., Armonk, NY).

3. Results

3.1. Data description

Among the 686 RR isolates, the most prevalent resistance was to INH (80.5 %) and EMB (28.4 %), followed by LFX (26.1 %), MFX (21.9 %). The proportions of MDR and pre-XDR were 80.5 % and 26.2 %, respectively. Four MTB complex lineages were identified with 82.94 % lineage 2 (L2) (569/686), 15.74 % L4 (108/686), 1.17 % L3 (8/686) and 0.15 % L1 (1/686) (Table 1).

Table 1
Drug susceptibility patterns of 686 *Mycobacterium tuberculosis* isolates.

Resistance pattern	No. of strains (%)	Lineage1 No. (%)	Lineage2 No. (%)	Lineage3 No. (%)	Lineage4 No. (%)
RIF	686(100)	1(0.1)	569(82.9)	8(1.2)	108(15.7)
INH	552(80.5)	1(0.2)	467(84.6)	5(0.9)	79(14.3)
EMB	195(28.4)	1(0.5)	174(89.2)	0(0.0)	20(10.3)
LFX	179(26.1)	0(0.0)	153(85.5)	0(0.0)	26(14.5)
MFX	150(21.9)	0(0.0)	129(86.0)	0(0.0)	21(14.00)
ETO	89(13.0)	1(1.1)	77(86.5)	0(0.0)	11(12.4)
KAN	52(7.6)	1(1.9)	37(71.2)	0(0.0)	14(26.9)
AMK	39(5.7)	1(2.6)	36(92.3)	0(0.0)	2(5.1)
MDR	552(80.5)	1(0.2)	467(84.6)	5(0.9)	79(14.3)
pre-XDR	180(26.2)	0(0.0)	153(85.0)	0(0.0)	27(15.0)

RIF: rifampicin; INH: isoniazid; EMB: ethambutol; ETO: ethionamide; KAN: kanamycin; AMK: amikacin; MFX: moxifloxacin; LFX: levofloxacin.

3.2. FQs resistance across drug resistance patterns

As shown in Fig. 2, of the 686 RR-TB isolates, 21.7 % (149/686) were resistant to both LFX and MFX, and 26.2 % (180/686) were resistant to any FQs. For 552 MDR-TB isolates, 29.9 % and 25.7 % strains were resistant to LFX and MFX, respectively. For 180 pre-XDR isolates, 99.4 % and 88.3 % strains were resistant to LFX and MFX, respectively. The higher resistance rate to LFX and MFX was observed in KAN, ETO and AMK resistant isolates (50.0 %, 49.4 %, and 48.7 % for LFX, 40.4 %, 39.3 %, and 41.0 % for MFX, respectively) than that of RIF, INH and EMB resistant isolates (26.1 %, 29.9 %, and 41.5 % for LFX, 21.9 %, 25.7 %, and 37.9 % for MFX, respectively). The resistance rate of LFX was higher than that of MFX across various drug resistance patterns ($P < 0.001$).

3.3. Molecular characteristic of FQs-resistant MTB isolates

Of 180 FQs resistant isolates, 168 (93.3 %) had mutations in QRDRs and 12 (6.7 %) possessed no mutations in *gyrA* and *gyrB* genes. Of the168 FQs-resistant isolates, 7 isolates (4.2 %, 7/168) carried double mutations in the *gyrA* gene, and 8 isolates (4.8 %, 8/168) harbored mutations in both *gyrA* and *gyrB* gene. And 21 mutation types were identified, with Asp94Gly being the most common (32.7 %, 55/168), followed by Ala90Val (23.2 %, 39/168) and Asp94Tyr (13.1 %, 22/168). Of the 6 FQs-sensitive isolates, 5 isolates (83.3 %, 5/6) carried mutations in *gyrB*, and one (16.7 %, 1/6) harbored mutation in *gyrA* gene (Table 2).

3.4. Correlation of gyrA mutations with FQs MICs

The MICs of isolates with predominant *gyrA* mutations were shown in Fig. 3. All isolates with mutations in Asp94Asn and Asp94Gly were associated with high levels of resistance to LFX (MIC≥4 μg/mL) and MFX (MIC≥2 μg/mL). The 89.7 % Ala90Val (35/39), 83.3 % Ser91Pro (5/6), 68.8 % Asp94Ala (11/16), and 72.7 % Asp94Tyr (16/22) were associated with high levels of resistance to LFX (MIC≥4 μg/mL). While 71.8 % Ala90Val (28/39), 100 % Ser91Pro (6/6), 75 % Asp94Ala (12/16), and 81.8 % Asp94Tyr (18/22) were associated with high levels of resistance to MFX (MIC≥2 μg/mL). Isolates with double mutations in *gyrA* gene were associated with high levels of resistance to both LFX and MFX (Table 2), but they were not shown in Fig. 3 due to the small number.

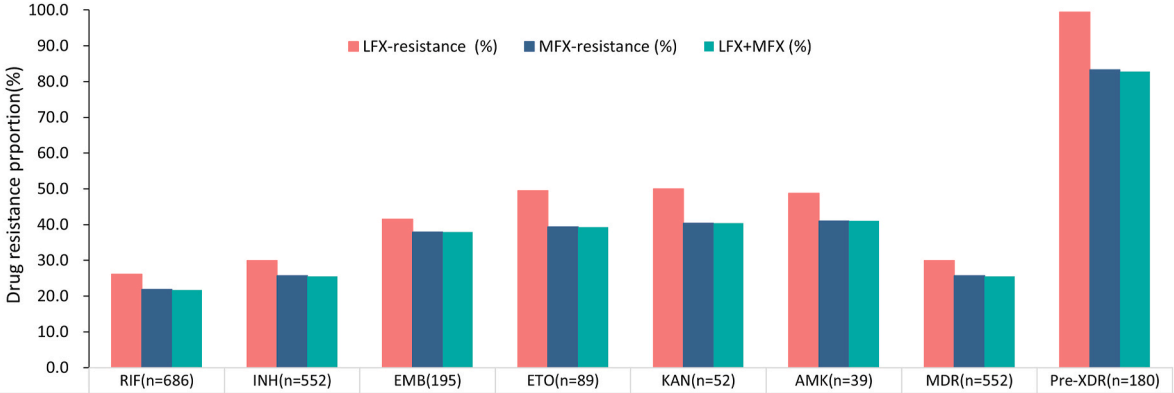
3.5. Performance of WGS referring to phenotypic DST

Using broth microdilution method of phenotypic DST as gold standard, the sensitivities of WGS for LFX and MFX were 93.3 % (95 % *CI*, 88.6 to 96.5) and 98.0 % (95 % *CI*, 94.3 to 99.6), and the specificities were 98.6 % (95 % *CI*, 97.2 to 99.4) and 95.0 % (95 % *CI*, 92.8 to 96.7), respectively (Table 3). The concordance between WGS and phenotypic DST was high for both LFX ($\kappa = 0.928$) and MFX ($\kappa = 0.879$) (Table 3).

4. Discussion

The widely prescribed of FQs to treat several infectious diseases increases the resistance rate in *M. tuberculosis*, impairing effectiveness of treatment in RR/MDR-TB patients [6,16]. Therefore, we investigated the prevalence of later-generation FQs (LFX and MFX) resistance, and identified the potential relationship with genotypes among 686 RR-TB isolates.

In this study, the resistance rate to LFX (26.1 %) and MFX (21.9 %) in RR/MDR-TB isolates was slightly higher than the global rate (20.0 %) [3]. According to previous report, the resistance rate of FQs among MDR isolates varied from 28.6 % to 43.6 % across regions in China [17,18], which may be attributed to the widely used in the clinical treatment of bacterial infections [19]. Therefore,



	RIF(n=686)	INH(n=552)	EMB(195)	ETO(n=89)	KAN(n=52)	AMK(n=39)	MDR(n=552)	Pre-XDR(n=180)
LFX-resistance (%)	26.1	29.9	41.5	49.4	50.0	48.7	29.9	99.4
MFX-resistance (%)	21.9	25.7	37.9	39.3	40.4	41.0	25.7	83.3
LFX+MFX (%)	21.7	25.5	37.9	39.3	40.4	41.0	25.5	82.8

Fig. 2. Resistance rate of Mycobacterium Tuberculosis isolates against FQs across various resistance patterns.

Table 2
Genetic analysis of the FQs-resistant MTB isolates.

Resistance isolates (No.)	Mutation of gyrA	Mutation of gyrB	No. of isolates (%)	MIC range (μg/mL)	
				LFX	MFX
FQs-resistant isolates (168)	Asp94Gly		55(32.7)	4–32	2–8
	Ala90Val	–	39(23.2)	2–16	0.5–8
	Asp94Tyr	–	22(13.1)	2–16	1–8
	Asp94Ala	–	15(8.9)	1–32	1–4
	Asp94Asn	–	8(4.8)	4–32	4–8
	Ser91Pro	–	6(3.6)	2–16	2–8
	Asp94Gly	Asp461Asn	3(1.8)	16	4
	Gly88Ala	–	2(1.2)	32	8
	Asp89Asn	–	2(1.2)	2–16	2–8
	Ala90Val	Thr500Asn	2(1.2)	2–32	1–8
	Ala90Val/Ser91Pro	–	2(1.2)	4–8	4
	Ala90Val/Asp94Gly		2(1.2)	4	2–4
	Asp94His	–	2(1.2)	4	2
	Asp94Ala	Asn499Thr	1(0.6)	16	8
	Asp94Gly	Thr500Asn	1(0.6)	4	0.5
	Asp94Tyr	Glu501Asp	1(0.6)	32	8
	Ala74Ser	–	1(0.6)	2	0.5
	Ala90Val/Asp94Ala	–	1(0.6)	8	4
	Ala90Val/Asp94Asn		1(0.6)	16	8
	Ser91Pro/Asp94Gly		1(0.6)	16	8
	–	Arg446Leu	1(0.6)	1	2
FQs-sensitive isolates (6)	–	Arg446His	2(33.3)	0.5–1	0.5
	–	Arg446Cys	1(16.7)	1	1
	Asp94Ala	–	1(16.7)	1	1
	–	Asp461Asn	1(16.7)	0.5	0.5
	–	Ala504Val	1(16.7)	0.5	0.5

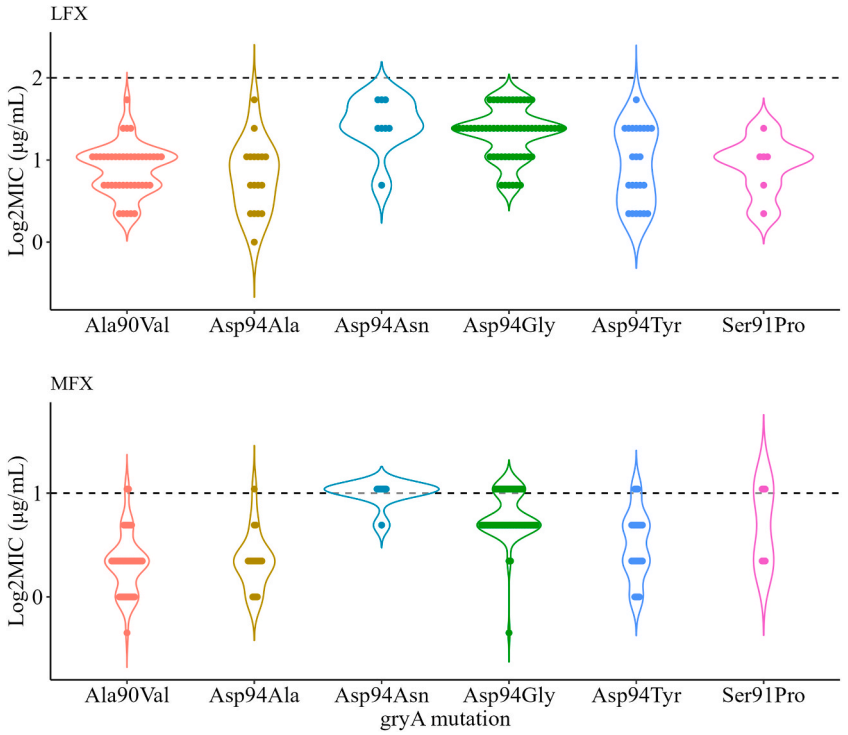


Fig. 3. Distribution and log_2MIC of different mutations of FQs. The Log_2MIC of frequently occurring resistance mutations distribution in RR-TB isolates for Levofloxacin. Mutations that were fewer than four times are not shown. The cut-off level of resistance is denoted by a dashed line.

Table 3
Efficiency of WGS for detecting FQs resistance.

Pattern	Drug	Resistance phenotype		Susceptible phenotype		Sensitivity % (95 % *CI*)	Specificity % (95 % *CI*)	κ
		Genotypic R	Genotypic S	Genotypic R	Genotypic S			
Total	LFX	167	12	7	500	93.3 (88.6–96.5)	98.6 (97.2–99.4)	0.928
	MFX	147	3	27	509	98.0 (94.3–99.6)	95.0 (92.8–96.7)	0.879

Note: R resistance; S Susceptible.

susceptibility test of FQs was of great significant to initiate the treatment of RR/MDR-TB. However, the choice between LFX and MFX was controversial. In this study, we found the resistance rate of LFX was higher than that of MFX across various drug resistance patterns. Consistent with previous research and animal studies, MFX had lower MIC and better bactericidal activity than LFX [20,21]. However, another study revealed that there was no difference in final treatment outcomes between LFX and MFX in FQs sensitive MDR-TB patients [22]. This phenomenon may be attributed to the discrepancies between in vitro drug sensitivity results and in vivo treatment effects, or the difference in the genotype of epidemic strains. So it is crucial to monitor the clinical outcomes and prevent the increase of resistance to FQs.

Detecting of genetic mutations in the QRDRs of *gyrA* and *gyrB* genes is crucial to identify FQs resistance. In the present study, Asp94Gly (32.7 %, 55/168) in *gyrA* was the most common mutation. However, the mutation sites and frequencies of *gyrA* varied across studies [18,23–25], which may be attributed to the difference in detection techniques, breakpoint concentrations, epidemic strains, research population and medical history. Besides, only Asp461Asn and Glu501Asp were classified as interim resistance markers for *gyrB* according to the catalogue of mutations [14], which was in agreement with previous studies that *gyrB* mutations were rare in FQs resistant isolates [26,27]. Furthermore, 12 FQs resistant strains possessed no *gyrA* and *gyrB* genes mutations, suggesting that other mechanisms may account for FQs resistance, such as drug efflux pumps and decreased cell-wall permeability. For the two FQs sensitive isolates with resistance mutations of Asp94Ala in *gyrA* and Asp461Asn in *gyrB*, the MIC levels were nearly at critical concentration, and the drug sensitivity results may be related to some errors or incorrect interpretation. So monitoring the resistant mutations during treatment is essential to prevent the spread of drug-resistant strains.

Previous studies have shown that levels of FQs resistance were associated with mutations in the QRDR of *gyrA* gene, and double mutations were related to high-level resistance [28,29]. However, no significant difference was observed between mutation patterns in *gyrA* gene and the level of FQs resistance, which was consistent with that from India and East China [30,31]. These disparities may be attributed to the inclusion of MTB isolates with various drug-resistance profiles and geographic diversity in the prevalence of *gyrA* mutations [28]. In present study, we also observed that isolates with double mutations in *gyrA* gene were associated with high levels of resistance to both LFX and MFX. Data from Hu et al. revealed that combined *gyrB* Asp461Asn mutation could significantly increase MICs in FQs resistance isolates with Ala90Val mutation [28]. Though isolates combined *gyrB* Asp461Asn and Glu501Asp mutation had high-level resistance of LFX and MFX in this study, the MIC levels of FQs resistance isolates with *gyrA* mutations were not significantly increased. Whether the presence of a *gyrB* mutation impaired the interaction of FQ with the DNA gyrase complex harboring the *gyrA* mutation, or was associated with a fitness cost need further to be verified. So understanding of genetic correlation is crucial to prevent high-level resistance, inform treatment strategies and develop new therapeutic interventions.

Whole genome sequencing, a powerful tool to accurately reveal gene mutations, has been used to predict of anti-TB drug resistance [32]. In present study, WGS achieved above the 93 % level of sensitivity and specificity values in predicting resistance to LFX and MFX. A study conducted by Finci et al. found that WGS can achieve 94.8 % sensitivity and 97.1 % specificity for predicting resistance to LFX [33]. Another study in Europe revealed that the predictive rates of WGS for LFX and MFX was 83.3 % [34]. The disparity may be attributed to the genetic diversity of investigated strains, or highly dependent on mutation sites in drug-resistance prediction analysis [35]. We found the concordance between WGS and phenotypic DST was high for both LFX and MFX, which was consistent with a study in Shanghai with the concordance of 95.3 % for MFX and 93 % for LFX [36], indicating that WGS provided a promising option for accurate identification of FQs resistant MTB.

Our study has several limitations. First, the association between lineage of each isolate and genetic polymorphisms was not analyzed in this study. Second, data on clinical information was not available, whether the resistance to FQs developed from previous exposure cannot be determined. Finally, the lack of clinical outcome was another limitation, hampering the analysis of correlation of drug sensitivity results with the treatment effects.

5. Conclusion

The resistance rate of LFX was higher than that of MFX among various drug resistance patterns of RR-TB isolates. The *gyrA* Asp94Gly was the predominant mutation underlying FQs resistance. However, no significant difference was observed between mutation patterns in *gyrA* gene with the level of FQs resistance.

Declarations

Author contribution statement

Xichao Ou and Yanlin Zhao designed the study and revised the manuscript. Chong Teng, Hui Li, Ling Li, Dan Su, Hui Teng, and Xiaolong Cao contributed in study design, data collection, and analysis. Bing Zhao, Hui Xia, Yuanyuan Song, and Yang Zheng conducted laboratory testing. Huiwen Zheng and Xichao Ou conducted in manuscript writing. All authors have read and approved the manuscript.

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Ethical approval

The study was approved by the Institutional Review Board of China CDC (202336). Patient consent was waived due to all isolates used in this study were from previous drug-resistance surveillance (DRS) work, and no additional data and specimens were collected. Each patient signed an informed consent form during the routine DRS.

Data availability statement

Data included in article/supplementary material/Accession numbers: PRJCA025898, National Genomics Data Center (https://ngdc.cncb.ac.cn/).

CRediT authorship contribution statement

Chong Teng: Visualization, Methodology, Formal analysis, Conceptualization. **Ling Li:** Methodology, Investigation, Formal analysis. **Dan Su:** Methodology, Investigation, Formal analysis. **Hui Li:** Methodology, Investigation, Formal analysis. **Bing Zhao:** Resources, Investigation. **Hui Xia:** Resources, Investigation. **Hui Teng:** Methodology, Investigation, Formal analysis. **Yuanyuan Song:** Resources, Investigation. **Yang Zheng:** Resources, Investigation. **Xiaolong Cao:** Investigation, Formal analysis. **Huiwen Zheng:** Writing – original draft, Funding acquisition, Conceptualization. **Yanlin Zhao:** Writing – review & editing, Funding acquisition, Conceptualization. **Xichao Ou:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31959.

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