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Original article

Whole-genome sequencing for surveillance of tuberculosis drug resistance and determination of resistance level in China

Dongxin Liu [1, 2, †], Fei Huang [1, †], Guoliang Zhang [2, †], Wencong He [1, †], Xichao Ou [1, †], Ping He [1], Bing Zhao [1], Baoli Zhu [3], Fei Liu [3], Zhiyuan Li [3], Chunfa Liu [1], Hui Xia [1], Shengfen Wang [1], Yang Zhou [1], Timothy M. Walker [4], Lei Liu [2], Derrick W. Crook [4], Yanlin Zhao [1, *]

[1] *Chinese Centre for Disease Control and Prevention, Beijing, China*
[2] *National Clinical Research Centre for Infectious Diseases, Shenzhen Third People's Hospital, Shenzhen, Guangdong Province, China*
[3] *Institute of Microbiology, Chinese Academy of Sciences, Beijing, China*
[4] *Nuffield Department of Medicine, John Radcliffe Hospital, University of Oxford, Oxford, UK*

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ABSTRACT

Objectives: Phenotypic drug susceptibility testing for prediction of tuberculosis (TB) drug resistance is slow and unreliable, limiting individualized therapy and monitoring of national TB data. Our study evaluated whole-genome sequencing (WGS) for its predictive accuracy, use in TB drug-resistance surveillance and ability to quantify the effects of resistance-associated mutations on MICs of anti-TB drugs.
Methods: We used WGS to measure the susceptibility of 4880 isolates to ten anti-TB drugs; for pyrazinamide, we used BACTEC MGIT 960. We determined the accuracy of WGS by comparing the prevalence of drug resistance, measured by WGS, with the true prevalence, determined by phenotypic susceptibility testing. We used the Student–Newman–Keuls test to confirm MIC differences of mutations.
Results: Resistance to isoniazid, rifampin and ethambutol was highly accurately predicted with at least 92.92% (95% confidence interval [CI], 88.19–97.65) sensitivity, resistance to pyrazinamide with 50.52% (95% CI, 40.57–60.47) sensitivity, and resistance to six second-line drugs with 85.05% (95% CI, 80.27–89.83) to 96.01% (95% CI, 93.89–98.13) sensitivity. The *rpoB* S450L, *katG* S315T and *gyrA* D94G mutations always confer high-level resistance, while *rpoB* L430P, *rpoB* L452P, *fabG1* C-15T and *embB* G406S often confer low-level resistance or sub-epidemiological cutoff (ECOFF) MIC elevation.
Conclusion: WGS can predict phenotypic susceptibility with high accuracy and could be a valuable tool for drug-resistance surveillance and allow the detection of drug-resistance level; It can be an important approach in TB drug-resistance surveillance and for determining therapeutic schemes. **Dongxin Liu, Clin Microbiol Infect 2022;28:731.e9–731.e15**

Introduction

The vision of the End TB Strategy proposed by the World Health Organization (WHO) is a tuberculosis (TB) -free world by the year 2035 [1]. However, the emergence and wide spread of drug-resistant TB—especially multidrug-resistant TB, and extensively drug-resistant TB—is a major block to attaining this goal. In 2019, about 465 000 cases of rifampicin-resistant TB were estimated to have emerged, of which 78% were multidrug-resistant TB, but because of limited access to drug susceptibility testing (DST), only 38% of these patients received diagnoses and were treated [2]. WHO therefore urgently recommends universal implementation of

* Corresponding author. Yanlin Zhao, Chinese Centre for Disease Control and Prevention, Beijing, 102206, China.
E-mail address: zhaoyl@chinacdc.cn (Y. Zhao).
† Dongxin Liu, Fei Huang, Guoliang Zhang, Wencong He and Xichao Ou contributed equally to this work.

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DST to promoting individual therapy and regional surveillance, which is considered as a significant component of TB control programmes globally. Surveillance data analysis can bring to light national drug-resistance trends and be used to assess the burden of disease, devise diagnostic tools, assess the efficacy of TB control and preventive interventions, and formulate effective treatment protocols.

China has always had a high prevalence of drug-resistant TB, and culture-based (phenotypic) drug-resistance monitoring for TB has been ongoing for many years [3]; however, important limitations of culture-based surveying make it difficult to establish continuous surveillance of drug resistance at regular intervals, especially for high disease burden or resource-limited areas. These limitations include the need for timely refrigerated transportation to maintain the microbial viability of samples, a long incubation time for results, a requirement for stringent laboratory conditions, and huge workload for the reference laboratories. Rapid diagnostic tools, such as GeneXpert MTB/RIF Ultra and GenoType MTBDR*plus* assays [4,5], have been widely adopted, as they are faster and cheaper than traditional culture-based DST, yet the emergence of drug-resistant strains that escape detection by such assays reveals the importance of developing more comprehensive technologies that include a wider range of resistance determinants [6]. Whole-genome sequencing (WGS) could identify the known drug-resistance conferring mutations and, recently, WGS-based detection methods have proved capable of replacing culture-based DST for first-line drugs [7]. However, implementation of this technology is not yet feasible in countries with a high TB burden because of cost and technical expertise constraints. As a result, little evidence was acquired on the relevance between WGS prediction and phenotypic testing results under the background of population-based cross-sectional surveillance.

Additionally, most current phenotypic and genetics-based DST approaches generate a binary result—'resistant' or 'susceptible'—so may miss the strains that elevate the MIC value but not enough to be labelled 'resistant'. Resistance level has a significant impact on the formulation of treatment regimens, as both the use of informed high-dose regimens (trialled to extend the clinical utility of relatively less toxic and more widely available drugs such as rifampicin and isoniazid [8]) and strains below the ECOFF can also lead to a bad clinical outcome (incurable or relapse) [9]. Previous efforts have attempted to determine the drug-resistance levels of resistance variants, but they have been limited by small sample sizes and few kinds of drugs [10].

To resolve these issues, we performed WGS and determined the MICs of ten drugs for 4880 *Mycobacterium tuberculosis* isolates selected from patient samples across China.

Materials and methods

Sample collection and drug susceptibility testing

The study obtained 5053 isolates from the Chinese national TB drug-resistance surveillance program from 70 counties in all 31 provinces. Sampling method and bacterial isolation referred to the first national survey of drug-resistant tuberculosis in China [3]. Of these, 4922 had both WGS and phenotypic DST results available. The national TB reference laboratory had performed DST for 13 anti-TB drugs using the MIC method (Sensititre™ MYCOTB plate) for 12 of them (isoniazid, rifampicin, ethambutol, streptomycin, ofloxacin, moxifloxacin, kanamycin, amikacin, rifabutin, cycloserine, ethionamide and *p*-aminosalicylic acid) and the proportion method (BACTEC MGIT 960) for pyrazinamide [11,12]. Cycloserine and *p*-aminosalicylic acid were excluded from our analysis because phenotypic DST results for these drugs are unreliable [13,14]. Of the fluoroquinolones, only moxifloxacin was representative of its class. The ECOFF for each drug is shown in the Supplementary material (Table S1). This study was approved by the Ethics Review Committee of China CDC, and informed consent was obtained from each participant.

Whole-genome sequencing and prediction of susceptibility

For WGS of isolates, we prepared the DNA using the cetyltrimethylammonium bromide extraction method of DNA purification [15]. Genomic DNA was then sequenced using the Illumina Hiseq 2000 platform. Paired-end reads were mapped with Stampy [16] (version 1.0.17) to the H37Rv (GenBank NC000962.3) reference genome. SAMtools mpileup [17] (version 0.1.18) was used for calling variants based on at least five reads and both strands were mapped by the reads. When minority alleles accounted for more than 10% of the read depth, mixed calls were assigned. Cortex (version 1.0.5.21) was used to identify insertions and deletions [18].

WGS predictions of phenotypic susceptibility were based on the mutations in or upstream of drug-resistance-related genes, detailed resistance-related mutations of drugs are shown in the Supplementary material (Table S2). Isolates with resistance-conferring mutations were considered to be phenotypic resistant, whereas isolates containing only wild-type genes were predicted to be phenotypic susceptible. Laboratory error was assumed in instances where susceptible phenotypes were recorded despite the occurrence of the high-confidence resistance conferring mutation *katG* S315T for isoniazid and *rpoB* S450L for rifampin [19].

We also determined the mutations detected by rapid molecular diagnostic methods (see Supplementary material Table S3).

Data analysis

Analysis was performed using Stata 13.1 (Stata Corp, College Station, TX, USA). MICs were consistent with the characteristics of the genomic data. Log_2MIC was assumed to be a normally distributed variable and compared between groups using one-way analysis of variance. If the analysis of variance showed that there were statistical differences between different mutations, the Student–Newman–Keuls test was further used to compare the log_2MIC.

Results

Strain data description

Of the 4922 isolates that had both WGS and DST results available, high-confidence resistance-conferring mutations were detected in 42 phenotypic susceptible isolates: 25 containing the *katG* S315T mutation, nine with the *rpoB* S450L mutation and eight with both of the mutations. All 42 of these isolates (0.85% of the total samples) were excluded from further analysis because of likely laboratory mislabelling. Of the 4880 isolates that remained, full phenotypic profiles were available for all ten drugs except pyrazinamide, for which 2346 were available. The coverage of selected mutations for the 4880 sequenced isolates is provided in the Supplementary material (Table S4). Four major lineages were observed, with 3645 isolates from the L2 strain, 1206 from the L4 strain, 26 from the L1 strain and three from the L3 strain.

Prediction efficiency of WGS

For the first-line drugs isoniazid, rifampin, ethambutol and pyrazinamide, the sensitivity of the WGS prediction was 94.22%, 96.69%, 92.92% and 50.52%, respectively, and the specificity was

Table 1
Prediction of phenotypes of resistance to ten drugs and multidrug resistance

Drug	Resistant phenotype			Susceptible phenotype			Sensitivity (95% CI)	Specificity (95% CI)
	R	S	Total	R	S	Total		
Isoniazid	668	41	709	44	4127	4171	94.22 (92.50–95.94)	98.95 (98.64–99.26)
Rifampicin	350	12	362	52	4466	4518	96.69 (94.84–98.53)	98.85 (98.54–99.16)
Ethambutol	105	8	113	90	4677	4767	92.92 (88.19–97.65)	98.11 (97.73–98.50)
Pyrazinamide	49	46	97	19	2230	2249	50.52 (40.57–60.47)	99.16 (98.78–99.53)
Moxifloxacin	182	32	214	44	4622	4666	85.05 (80.27–89.83)	99.06 (98.78–99.34)
Rifabutin	313	13	326	44	4510	4554	96.01 (93.89–98.13)	99.03 (98.75–99.31)
Amikacin	27	3	30	3	4847	4850	90.00 (79.26–100.00)	99.94 (99.87–100.00)
Kanamycin	35	2	37	4	4839	4843	94.59 (87.30–100.00)	99.92 (99.88–100.00)
Streptomycin	550	80	630	38	4212	4250	87.30 (84.70–89.90)	99.11 (98.82–99.39)
Ethionamide	75	8	83	100	4697	4797	90.36 (84.01–96.71)	97.91 (96.36–98.34)
Multidrug resistance	291	15	306	28	4546	4574	95.10 (92.68–97.52)	99.39 (99.16–99.61)

Abbreviations: R, resistant; S, susceptible.

98.95%, 98.85%, 98.11% and 99.16%, respectively. For the second-line drugs, the sensitivity varied from 85.05% to 96.01% (Table 1).

Resistance mutations and level for first-line drugs

In China, the most frequently occurring mutation, *rpoB* S450L, seemed exclusively associated with high MICs, whereas the least frequently occurring mutations, *rpoB* L430P and *rpoB* L452P, also elevated the MICs but were mostly at or below the resistance threshold. Eight strains had mutations outside the rifampicin resistance determining region (RRDR) of *rpoB* gene is from *rpoB* L430 to *rpoB* L452, the positions V170 and I491 were outside this region, with *rpoB* V170F showing as high-level resistant and *rpoB* I491F showing as low-level resistant or sub-ECOFF. Mutations in

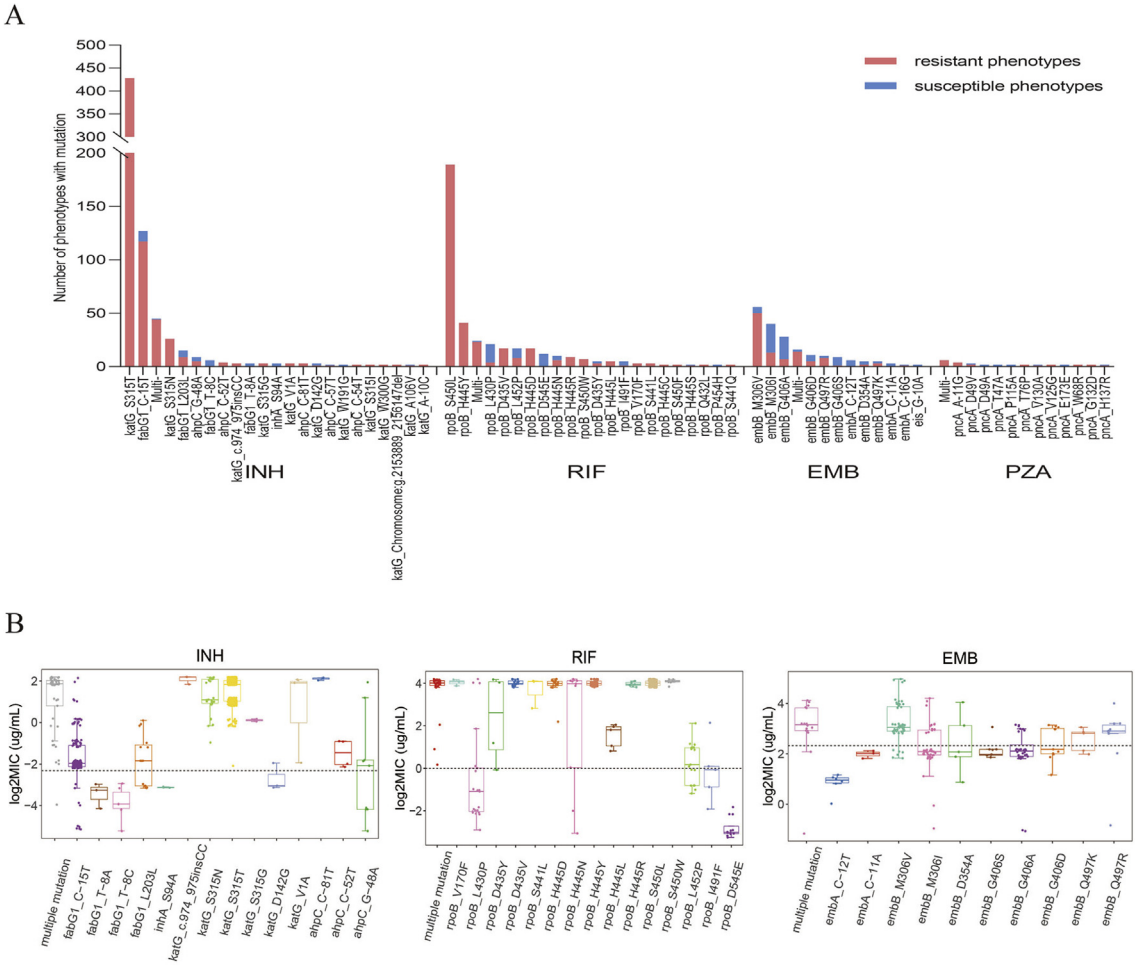

Fig. 1. Distribution and log_2MIC of different mutations of first-line drugs. (a) Resistance mutations distribution in *Mycobacterium tuberculosis* isolates for first-line drugs. Mutations that were only noted once are not shown, multi: more than one drug-resistant mutation occurred in one isolate. Detailed numbers are shown in the Supplementary material (Table S7). (b) Log_2MIC of different mutations. Mutations that were noted fewer than three time are not shown, multi: more than one drug-resistant mutation occurred in one isolate. Epidemiological cut-off is denoted by dotted line. Proportional method used for PZA drug susceptibility testing and no MIC results were acquired. Abbreviations: INH, isoniazid; RIF, rifampin; EMB, ethambutol; PZA, pyrazinamide.

rpoB D545 and *rpoB* P454I resulted in no elevation in MIC. For isoniazid, high-level resistance was caused by the canonical S315T mutation (60.37%, 428/709), followed by *fabG1* C-15T (16.50%, 117/709), which seemed to be associated with low-level resistance. Non-canonical isoniazid resistance-associated variants were identified in *aphC*, *inhA* and *ndh*. Ethambutol resistance mutations occurred majorly in *embB* M306, with *embB* M306V as the most frequent mutation (44.25%, 50/113) in resistant strains, whereas *embB* M306I and variants in *embB* G406 also elevated the MIC but mostly at sub-threshold levels; furthermore, mutations in the *embA* promoter had less elevation effect on MIC than those in *embB*. Statistic differences of the mean (log_2MIC) between different mutations for first-line drugs are shown in the Supplementary material (Table S5). As is typical for pyrazinamide, the mutations were scattered throughout the *pncA* gene and promoter, comprising numbers of single nucleotide polymorphisms and indels; the most frequent resistance-conferring mutation was *pncA* A-11G (Fig. 1a,b).

Resistance determinants and level for second-line drugs

The mechanism of resistance to fluoroquinolones was mutation in either subunit of DNA gyrase (*gyrA* or *gyrB*), where the most frequently occurring variant was *gyrA* D94. Among these mutations, *gyrA* D94G, *gyrA* D94H and *gyrA* D94N seemed to be associated with high-level resistance, whereas *gyrA* D94A and *gyrA* D94Y conferred low MIC value. Variants in *gyrB* had less elevation effect than *gyrA* mutations. Rifabutin is a structural analogue to

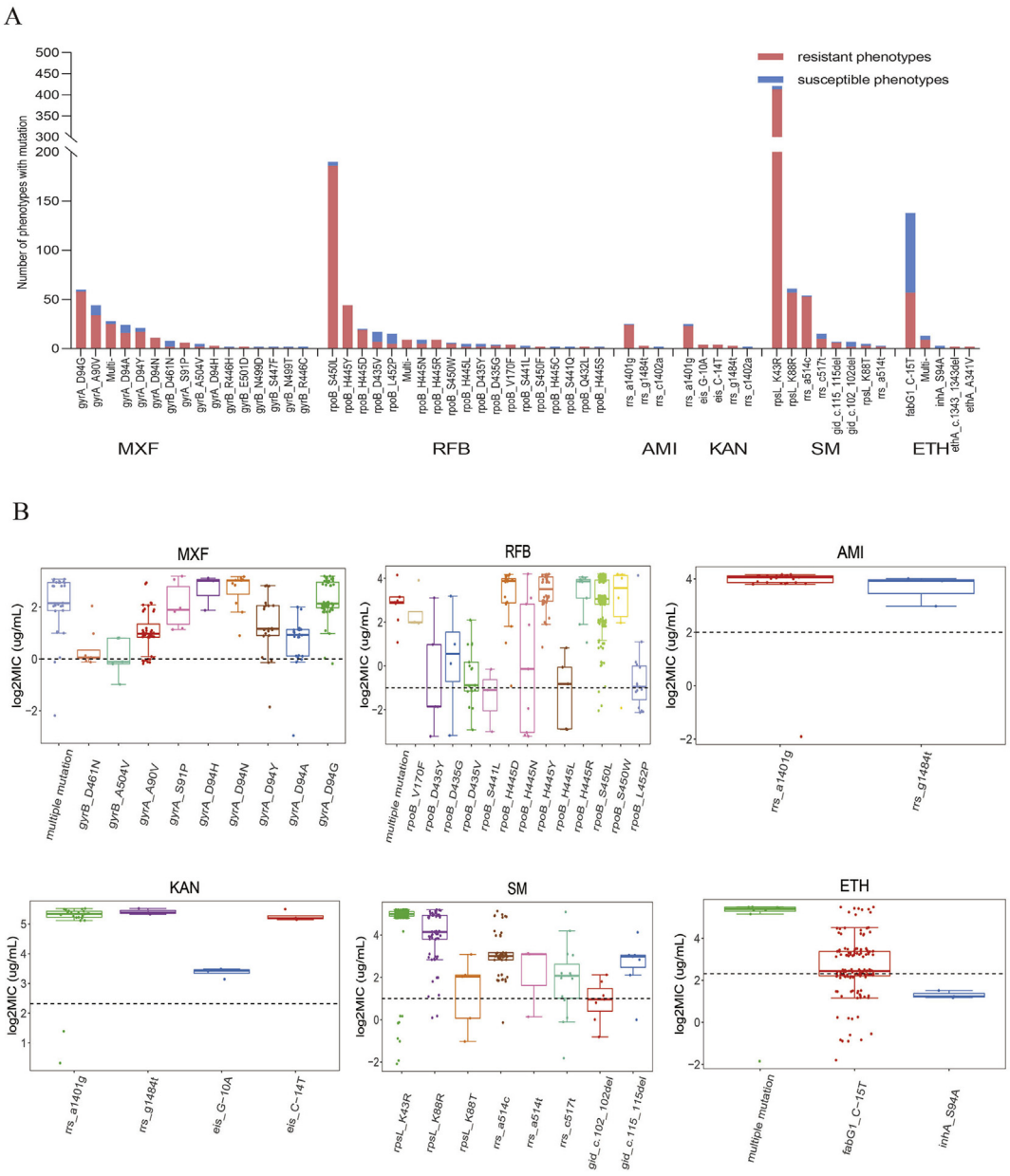

Fig. 2. Distribution and log_2MIC of different resistance determinants for second-line drugs. (a) Resistance mutations distribution in *Mycobacterium tuberculosis* isolates for second-line drugs. Mutations that were only noted once are not shown, multi: more than one drug-resistant mutation occurred in one isolate. Detailed numbers are shown in the Supplementary material (Table S7). (b) Log_2MIC of different mutations. Mutations that were noted fewer than three times are not shown, multi: more than one drug-resistant mutation occurred in one isolate. Epidemiological cutoff is denoted by dotted line. Abbreviations: MXF, moxifloxacin; RFB, rifabutin; AMI, amikacin; SM, streptomycin; ETH, ethionamide.

rifampicin; mutations in *rpoB* were associated with lower elevations in rifabutin MIC compared with rifampicin. Both amikacin and kanamycin high-level resistance-conferring mutations were detected in *rrs* a1401g and *rrs* g1408t, but we detected *rrs* c1402a in only two isolates, with no elevating effect on MIC. Promoter mutations in *eis*, which are associated with kanamycin resistance, were also detected, of which only *eis* C-14T was associated with high-level resistance. For streptomycin, variants in *rpsL K43* and *rpsL K88* accounted for 75.08% (473/630); *rpsL K43R* and *rpsL K88R* were associated with high-level resistance, but mutations in *rrs* seemed to have less effect on MIC elevating than *rpsL*. Ethionamide is a structural analogue of isoniazid and is activated by the monooxygenase ethA. In our data, variants in the *fabG1* promoter (*fabG1* C-15T) were found more often in ethionamide-resistant isolates than mutations in *ethA*, and *fabG1* C-15T was associated with high-level elevation in MIC (Fig. 2a,b). Statistical differences of the mean (log_2MIC) between different mutations for second-line drugs are shown in the Supplementary material (Table S6).

Comparison of prevalence determined by three methods

There is a large overlap between resistance determined by WGS and the phenotypic prevalence for most drugs. For nearly all drugs the resistance rate detected by WGS was higher than that of phenotypic DST (Fig. 3). One reason is that ECOFF and sub-ECOFF resistance mutations can be missed by phenotyping. Conversely, the prevalence of drug resistance detected with rapid molecular diagnostic methods was generally lower than the prevalence from phenotypic testing (Fig. 3) because these tools only target high-frequency mutations.

Discussion

Here we have presented the results of a cross-sectional surveillance study of 4880 individuals with TB from all provinces of China to assess the efficiency of WGS in confirming resistance to the most commonly used first-line and second-line anti-TB agents compared with phenotypic susceptibility testing. Using WGS combined with quantitative MIC measurements, our results showed that WGS is highly accurate at predicting phenotypic resistance. For ethambutol, the resistance rate detected by WGS was much higher than with phenotypic testing. We found this was because a large number of ethambutol resistance-associated mutations elevated the MIC to a sub-ECOFF level. These sub-threshold or threshold elevations in MIC may nevertheless be clinically meaningful, as elevated MICs predispose *M. tuberculosis* strains to develop higher-level resistance and risk treatment failure [20,21]. WGS resistance rates were slightly higher than phenotypic resistance rates for other drugs for the same reason—sub-threshold or threshold elevations. Pyrazinamide was the one drug for which the resistance rate predicted by WGS was much lower than the phenotypic resistance rate. This is no surprise, as the sole methodology recommended by WHO for pyrazinamide susceptibility testing (BACTEC MGIT 960 liquid culture) often produces false-positive results [22]. In addition, the phenotypic testing for pyrazinamide has low reproducibility, making it a weak testing method with which to make comparisons [23]. Ethionamide resistance-associated variants were found to be higher than phenotypic resistance strains, which were also caused by sub-ECOFF or threshold elevation mutations. Overall, according to our data, using phenotyping for surveillance can underestimate the problem of drug resistance because phenotyping is not always reliable. WGS has the ability to accurately predict drug resistance and give us a more accurate picture of national TB drug resistance.

In addition to highlighting variants associated with sub-ECOFF elevations in MICs that may be missed using traditional binary methods, this work also highlighted variants that result in different resistance levels. High-dose isoniazid and rifampin have been proven effective in treating certain resistant strains [24,25]. This will be meaningful for choosing therapeutic schedules in settings that lack new anti-TB drugs or second-line drugs. To further improve our understanding of drug-resistance-conferring variants, phenotypic and genotypic results data should be considered against the background of clinical outcome data, in considering the suboptimal reliability and reproducibility of phenotypic

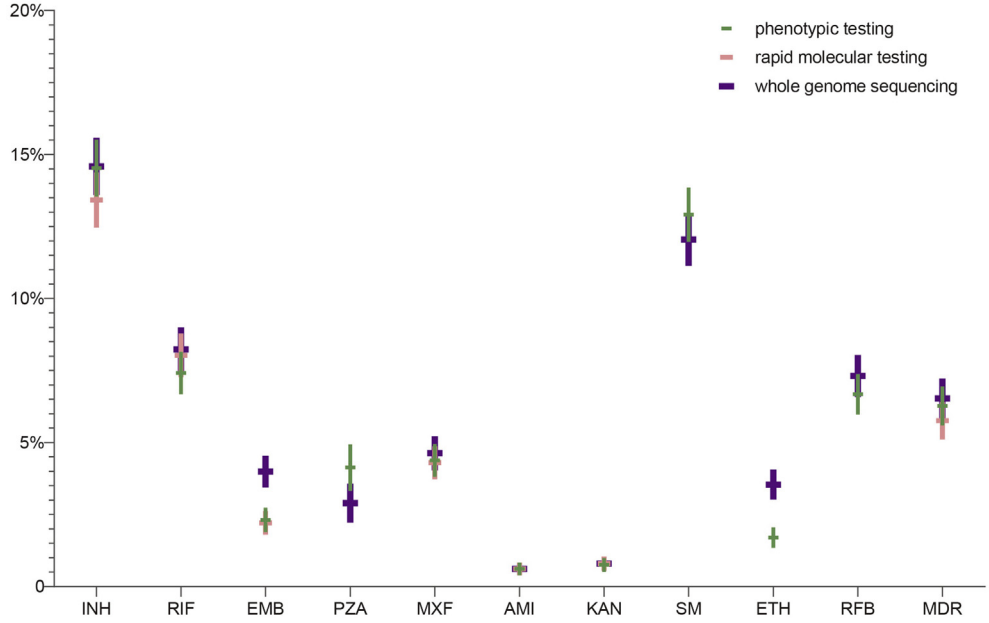

Fig. 3. Prevalence of drug resistance, estimated through whole-genome sequencing, phenotyping and rapid molecular method. Absolute numbers are shown in the Supplementary material (Table S8), the graph shows the resistance rate and its standard deviation. No data were acquired from pyrazinamide, ethionamide, streptomycin and rifabutin by rapid molecular method.

susceptibility testing for some anti-TB agents and the uncertainty of the most appropriate critical concentrations.

Rapid, evidence-based triage of patients with drug-resistant TB infections to prompt appropriate TB treatment regimens can be achieved in a timely manner using molecular DST methods. However, the current rapid molecular methods endorsed by WHO (i.e. GeneXpert MTB/RIF and line probe assays) remain insufficient for providing comprehensive drug-resistance information, without which effective treatment and drug-resistance surveillance cannot be achieved. Therefore, we advise WGS-based drug-resistance prediction, after rapid molecular diagnosis, to spot the emergence of mutations that escape other diagnostic methods.

The cost of WGS is already lower than phenotypic testing and is progressively decreasing; WGS is also easy to operate and is less instrument dependent than phenotypic testing. For these reasons, it could be particularly valuable in settings with low laboratory capacity and a shortage of skilled people. In addition, the reproducibility and robustness of sequencing data make it possible to re-analyse to discover uncharted resistance-conferring mutations, to predict resistance to new anti-TB drugs.

Our study had some limitations. First, we did not include new drugs (bedaquiline and delamanid) or repurposed drugs (linezolid and clofazimine) in our analysis, although WHO has endorsed these drugs for the treatment of multidrug-resistant TB [26]. An increasing number of patients use these drugs for treatment, so resistance-related mutations for these drugs are attracting increasing attention. Second, some isolates contained confirmed drug-resistance-conferring mutations, and although phenotypically susceptible, no repeat experiments were conducted to verify this. Third, we found that multiple mutations seemed to confer high-level resistance, but because of the limited numbers of strains, we did not categorize and analyse them. Fourth, lineage may also affect the prediction of resistance by WGS, but we did not take lineage into account in this study.

In conclusion, the fundamental purpose of drug-resistance surveillance is to estimate the disease burden, monitor trends and enable a prompt and effective response to public health problems. Our findings prove that WGS can have an important role in surveillance and diagnosis of drug-resistant TB, especially in consideration of the unreliability of phenotypic testing methods and the fact that available rapid molecular diagnostic tools can only detect a few resistance mutations at a time. From this study, we have a baseline of drug-resistance mutations in China, and we can now spot emerging clones and escape mutations when our next surveillance programme is conducted. We can then adjust our targeted rapid molecular tests as needed to improve individual clinical care. Additionally, according to the change in drug-resistance trends in different regions, targeted strategies for drug-resistant TB can be formulated. To accelerate the transition from a reliance on phenotypic results to genotypic results for drug resistance and resistance-level prediction in China and other countries with a high TB burden, it is crucial that vast amounts of accurate phenotypic, genotypic and clinical outcome data become available.

Transparency declaration

All authors report no conflicts of interest relevant to this article. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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Author contributions

YZhao, DL, FH, TW, DC, LL and BZhu contributed to the study design, DL, WH, PH, CL, XO, SW, XH and BZ collected the data and carried out the laboratory testing, FL, ZL, YZ, XR and TL helped with the bioinformatics analysis, HF, GZ and DL performed the data analysis and prepared the report for publication.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2021.09.014.

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