


Evaluation of MeltPro Assay in Identification of Second-Line Injectable Drug Resistance in Multidrug-Resistant Tuberculosis Isolates

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Objective: We compared the MeltPro assay to whole-genome sequencing (WGS) to investigate the molecular characterization of second-line injectable drug (SLID) resistance in multidrug-resistant tuberculosis (MDR-TB) isolates in Chongqing, China.

Methods: A total of 122 MDR-TB patient isolates were collected between March 2019 and June 2020 from Chongqing Municipality, China. Conventional drug-susceptibility testing was performed using the proportion method, followed to generate minimum inhibitory concentrations (MICs) of SLIDs determined by microplate alamarblue assay. All strains were subjected to both MeltPro and WGS assays.

Results: Among 122 MDR-TB isolates, 30 (24.6%), 22 (18.0%), and 14 (11.5%) were resistant to kanamycin (KM), amikacin (AM), and capreomycin (CM), respectively. Of the 31 SLID-resistant isolates, 24 (77.4%, 24/31) isolates harbored mutations in the *rrs* gene, with the most prevalent mutations in *rrs* A1401G (22/24, 91.7%). Mutation in *rrs* A1401G was associated with high levels of resistance to KM (MIC, ≥ 40 $\mu\text{g/mL}$) and AM (MIC, ≥ 64 $\mu\text{g/mL}$), but disparities in CM-resistance levels. Using phenotypic drug-susceptibility testing as gold standard, we found that the overall sensitivity of MeltPro and WGS was 87.1% and 90.32% and specificity 100% and 97.8%, respectively. Seven isolates had discordant results between phenotypic and genotypic resistance of SLIDs.

Conclusion: MeltPro is a promising diagnostic tool for accurate identification of SLID-resistant MTB isolates with mutations in the *rrs* and *eis* genes. There was a disparity between MeltPro with WGS results in the proportion of heterogeneous drug-resistant bacteria with *rrs* mutation and limited probes. Resistance mechanisms other than genetic mutations will affect the consistency of MeltPro and WGS with phenotypic drug-susceptibility results.

Keywords: MDR, MeltPro, resistance, genotype

Introduction

The prevalence of multidrug-resistant tuberculosis (MDR-TB), with an estimated of 0.41 million new cases in 2022, poses significant challenges to the prevention and control of TB disease globally.¹ MDR-TB patients are difficult to treat, and the treatment success rate was only 60% in 2021.¹ China has the second-greatest number of MDR-TB cases in the world, and the treatment success rate is only 54%.²

Effective anti-TB medications are essential for the treatment of MDR-TB. All-oral drugs are recommended in place of second-line injectable drugs (SLIDs), with the exception of amikacin due to several adverse events, for longer MDR-TB treatment in a 2018 guideline issued by the World Health Organization (WHO).³ Though the role of SLIDs has weakened, they should be considered as an alternative treatment when patients have limited access to all-oral drugs or have severe adverse reactions, such as with bedaquiline (BDQ). There is limited access to BDQ due to its high price in many countries, including China, and intolerance to BDQ in many patients.⁴ SLIDs are still emphasized as core drug treatment in standard short-course chemotherapy regimens, and they can prevent bacterial resistance to the core drug,

thus providing the most effective protection.^{5,6} Second-line injections have been used in China for many years and play a certain role in the treatment of MDR-TB, so they are still in mainstream use.

The diagnosis of drug-resistant TB by phenotypic drug-susceptibility testing (DST) is lengthy, laborious, and expensive.⁷ As such, reliable and automated molecular assays are recommended by the WHO for detecting resistance to first-line and second-line drugs.⁸ The MeltPro TB assay developed by Zeesan Biotech (Xiamen, China) is an innovative molecular test for detection of resistance to SLIDs. It is based on melting curve analysis with dual-labeled, self-quenched probes to detect multiple mutations in a single reaction, making it flexible and sensitive for use for clinical practice.⁹ MeltPro TB assays for detecting rifampin (RIF), isoniazid (INH), and fluoroquinolone resistance have been officially approved by the China Food and Drug Administration for diagnostic purposes in clinical settings. In addition, whole-genome sequencing (WGS) approaches have the potential to identify various genetic polymorphisms and predict drug-resistance profiles, and are used to evaluate sequencing technologies for routine genotypic DST.^{10,11} In this study, we compared the MeltPro assay with WGS to investigate the molecular characterization of SLID resistance in MDR-TB isolates in Chongqing, the largest municipality in southwest China and which has a high incidence of TB.

Methods

Bacterial Strains

Simple randomized sampling was used to obtain a representative sample of patients with TB. A total of 122 MDR-TB patient isolates were collected between March 2019 and June 2020 from Chongqing Municipality, China. All isolates were subcultured on Löwenstein–Jensen medium for 4 weeks at 37°C. Demographic and clinical characteristics were obtained from the electronic recording and reporting system for TB in China.

Conventional Drug-Susceptibility Testing

Conventional DST using the 1% proportion method as per WHO guidelines¹² was performed with the following drug concentrations: RIF (40 µg/mL), INH (0.2 µg/mL), streptomycin (10 µg/mL), ethambutol (2 µg/mL), capreomycin (CM, 40 µg/mL), kanamycin (KM, 30 µg/mL), amikacin (AM, 30 µg/mL), levofloxacin (2 µg/mL), protionamide (40 µg/mL) and *p*-aminosalicylic acid (1 µg/mL). The MDR-TB was defined as resistance to at least INH and RIF. SLID resistance was defined as resistance to any of AM, CM, and KM.

Minimum Inhibitory Concentrations

The minimum inhibitory concentrations (MICs) of SLIDs were determined using broth microdilution in accordance with the guidelines of the Clinical and Laboratory Standards Institute.¹³ The breakpoint concentrations were defined as 1 µg/mL for AM and 2.5 µg/mL for KM and CM.¹⁴ *Mycobacterium tuberculosis* H37Rv (ATCC 27249) was used as the control strain. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth of mycobacteria.

MeltPro Assay

Genomic DNA was extracted from culture-positive *M. tuberculosis* isolates using the cetyltrimethylammonium bromide method, as previously reported.¹⁵ A MeltPro *M. tuberculosis* SLID-resistance mutation test kit (Zeesan, Xiamen, China) was used within the SLAN-96S system (Hongshi, Shanghai, China). The results were automatically exported by SLAN software (Zeesan). Mutation types, drug-resistance patterns, and the interpretation of results are shown in [Supplementary Table 1](#).

Whole-Genome Sequencing

Extracted genomic DNA was quantified using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific). Genomic DNA libraries were constructed using an Illumina Nextera kit according to the manufacturer's protocol. All whole-genome sequencing procedures were performed by Annoroad Gene Technology (Beijing, China) on an Illumina HiSeq 2500 (Illumina, San Diego, CA) sequencing platform with a mean depth of coverage >100×. The sequencing reads were aligned with the H37Rv reference genome (GenBank ID NC_000962.3) to analyze the variant calls for drug resistance.

Statistical Analysis

Pearson's χ^2 or Fisher's exact test was used to compare proportions or resistant rates. $P < 0.05$ was considered statistically significant. Sensitivity, specificity, positive predictive value, and negative predictive value were calculated at the 95% CI, while concordance between two methods was assessed with κ tests. All statistical analyses were performed in SPSS 20.0 (IBM, Armonk, NY).

Results

Demographic Characteristics of SLID-Resistant MDR Isolates

Of the 122 MDR patients, 68.9% (84/122) were male, 64.8% (79/122) were retreated cases, 38.5% (47/122) had lung cavitation, and 4.1% (5/122) had diabetes. The predominant genotype was lineage 2 (86.1%, 105/122). Of the 31 (25.4%) SLID-resistant isolates, cases with treatment history (77.4%) were more common than new cases (2.6%). The resistance rate of levofloxacin and *p*-aminosalicylic acid in patients resistant to SLIDs was higher than in patients susceptible to SLIDs (77.4% vs 35.2%, 25.8% vs 5.5%, respectively; $P < 0.05$; Table 1).

Table 1 Characteristics of SLIDs^R and SLIDs^S MDR isolates (n=122)

	n (%)	n (%)			
		SLIDs ^R (n=31)	SLIDs ^S (n=91)	OR(95% CI)	P
Sex					
Female	38(31.1)	12(38.7)	26(28.6)	Ref.	
Male	84(68.9)	19(61.3)	65(71.4)	0.63(0.27–1.49)	0.29
Age (years)					
≤40	41(33.6)	13(41.9)	28(30.8)	Ref.	
41–59	60(49.2)	15(48.4)	45(49.5)	0.72(0.30–1.73)	0.46
≥60	21(17.2)	3(9.7)	18(19.8)	0.36(0.09–1.44)	0.14
Lineage					
4	17(13.9)	4(12.9)	13(14.3)	Ref.	
2	105(86.1)	27(87.1)	78(85.7)	1.13(0.34–3.75)	1.00
Treatment history					
New cases	43(35.2)	7(22.6)	36(39.6)	Ref.	
Retreated	79(64.8)	24(77.4)	55(60.4)	2.24(0.88–5.75)	0.09
Lung cavitation					
No	75(61.5)	19(61.3)	56(61.5)	Ref.	
Yes	47(38.5)	12(38.7)	35(38.5)	1.01(0.44–2.33)	0.98
Diabetes mellitus					
No	117(95.9)	30(96.8)	87(95.6)	Ref.	
Yes	5(4.1)	1(3.2)	4(4.4)	0.73(0.08–6.74)	1.00
Previous exposure					
None	8(6.6)	1(3.2)	7(7.7)	Ref.	
FL drugs	70(57.4)	11(35.5)	59(64.8)	1.31(0.15–11.68)	1.00
FL and SL drugs	44(36.1)	19(61.3)	25(27.5)	5.32(0.60–46.99)	0.21
Resistance to					
SM	90(73.8)	21(67.7)	69(75.8)	0.67(0.27–1.64)	0.38
EMB	49(40.2)	14(45.2)	35(38.5)	1.32(0.58–3.00)	0.51
LFX	56(45.9)	24(77.4)	32(35.2)	6.32(2.46–16.27)	0.00
PTO	13(10.7)	6(19.4)	7(7.7)	2.88(0.89–9.36)	0.14
PAS	13(10.7)	8(25.8)	5(5.5)	5.98(1.79–20.03)	0.01

Abbreviations: R, resistant; S, susceptible; FL, first-line; SL, second-line; SM, streptomycin; EMB, ethambutol; LFX, levofloxacin; PTO, protionamide; PAS, *p*-aminosalicylic acid.

Second-Line Injectable Drug–Resistance Profile

Phenotypic DST showed that among 122 MDR isolates, 30 (24.6%), 22 (18.0%), and 14 (11.5%) were resistant to KM, AM, and CM, respectively. Of the 31 (25.5%) SLID-resistant isolates, 13 (41.9%) isolates showed cross-resistance to KM, AM, and CM, with 12 (92.3%) isolates harboring the *rrs* A1401G mutation and one (7.7%) *rrs* G1484T + *rrs* A1401G on WGS. Nine (29.0%) isolates were cross-resistant to AM and KM and carried the *rrs* A1401G mutation. Only one (3.2%) isolate showed resistance to CM (Figure 1).

Of the 31 SLID-resistant isolates, 24 (77.4%, 24/31) isolates harbored mutations in the *rrs* gene, with the most prevalent mutations being *rrs* A1401G (22/24, 91.7%), followed by *rrs* G1484T (1/24, 4.2%) and *rrs* A1401G + *rrs* G1484T (1/24, 4.2%), while 95.5% (21/22) and 78.6% (11/14) of isolates with the *rrs* A1401G mutation were observed in AM- and CM-resistant isolates, respectively. The one isolate (3.2%) of *rrs* A1401G + *rrs* G1484T was associated with KM, AM, and CM resistance, and one isolate (3.2%) with *rrs* G1484T showed resistance only to KM (Figure 2).

Association of MICs and Genetic Mutations

Three isolates (10%) with *eis* C–10T were associated with a low level of resistance (MIC <10 µg/mL) to KM, and 22 (73.3%) isolates harboring the *rrs* A1401G mutation showed high levels of resistance (MIC ≥40 µg/mL). The *rrs* A1401G mutation was associated with high levels of resistance to AM (MIC ≥64 µg/mL), but there were disparities in CM resistance. The one isolate with *rrs* A1401G + *rrs* G1484T showed a high level of resistance to KM and AM, but a low level of resistance to CM. The one isolate with *tlyA* 135 ins_G (MIC=5 µg/mL) was resistant only to CM, but at a low level (MIC ≤10 µg/mL; Figure 2).

Comparing MeltPro and WGS in Phenotypic DST

Using phenotypic DST as gold standard, of the 31 SLIDs-resistant isolates, 27 were correctly identified by MeltPro with sensitivity of 87.1%, and 28 isolates were correctly identified by WGS with sensitivity of 90.32%. The overall specificity of MeltPro and WGS was 100% and 97.8%, respectively. There was excellent agreement between these two methods and phenotypic DST ($\kappa \geq 0.85$; Table 2). In terms of SLID resistance detected by MeltPro and WGS, two isolates with phenotypic susceptibility to SLIDs were identified on MeltPro, and two mutations within the *tlyA* gene were detected on WGS: one 590 del_T (35.37%) and the other 394 ins_A (95.38%). Five isolates showing phenotypic resistance were identified as SLID-sensitive by MeltPro or WGS. Two isolates identified as SLID-susceptible by MeltPro showed phenotypic resistance, and WGS analysis indicated that one isolate harbored the *rrs* A1401G mutation (24.65%) and the other *tlyA* 135 ins_G

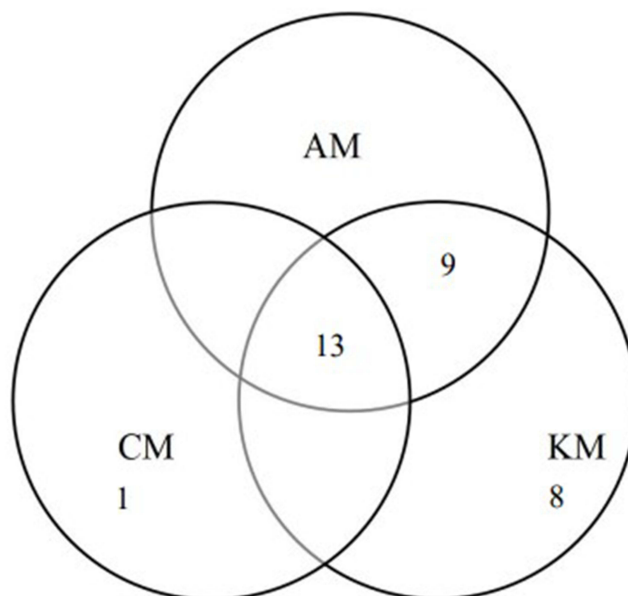


Figure 1 Cross-resistance of MDR against SLIDs.

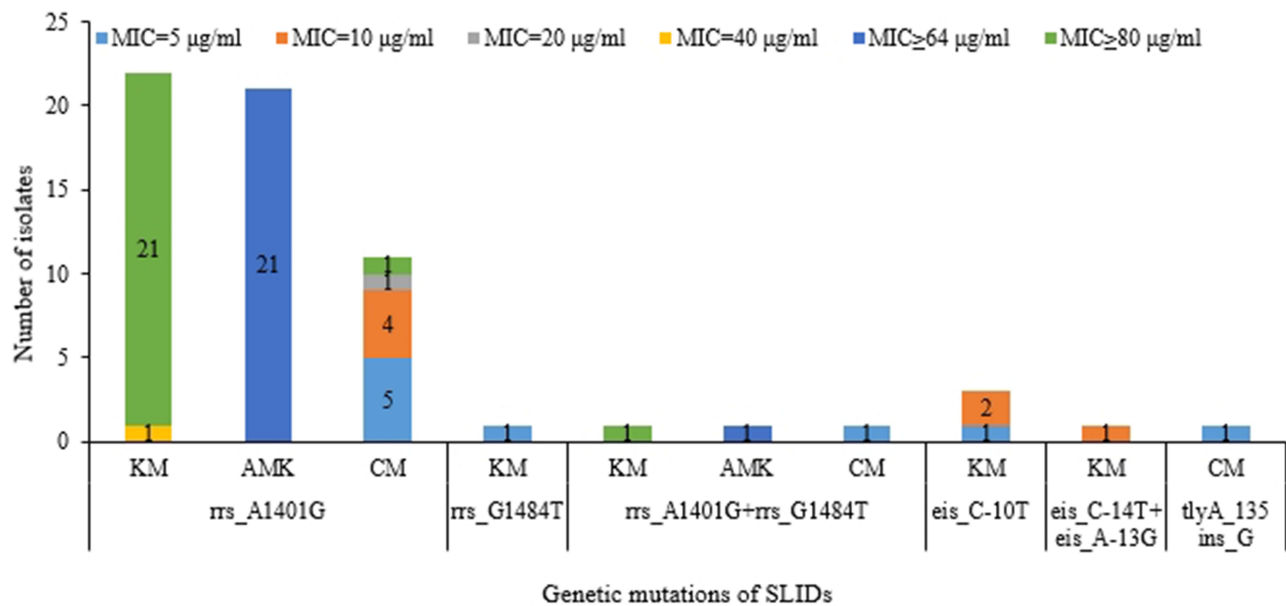


Figure 2 Association of MICs and genetic mutations of SLIDs (KM, kanamycin; AM, amikacin; CM, capreomycin) resistance in MDR isolates.

(94.76%). One phenotypically SLID-resistant isolate was predicted to be resistant by MeltPro with *eis* C-14T/*eis* A-13G/*eis* G-10A mutations, but was identified as sensitive by WGS. Two phenotypically SLID-resistant isolates were identified as sensitive by MeltPro and WGS (Table 3).

Table 2 Comparison of MeltPro and WGS for Detecting SLIDs Resistance

	Phenotypic DST		Total	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	P	κ
	R (n=31)	S (n=91)							
Resistant	MeltPro	0	27	87.10 (69.24–95.78)	100 (94.95–100)	100 (84.5–100)	95.79 (88.96–98.64)	<0.001	0.910
	27	0							
Susceptible	MeltPro	91	95	90.32 (73.10–97.47)	97.80 (91.53–99.62)	93.33 (76.49–98.84)	96.74 (90.09–99.15)	<0.001	0.891
	4	91							
Resistant	WGS	2	30	90.32 (73.10–97.47)	97.80 (91.53–99.62)	93.33 (76.49–98.84)	96.74 (90.09–99.15)	<0.001	0.891
	28	2							
Susceptible	WGS	89	92	90.32 (73.10–97.47)	97.80 (91.53–99.62)	93.33 (76.49–98.84)	96.74 (90.09–99.15)	<0.001	0.891
	3	89							

Abbreviations: DST, drug-susceptibility testing; NPV, negative predictive value; PPV, positive predictive value; WGS, whole-genome sequencing.

Table 3 Analysis on Discordant results between phenotypic and genotypic methods for SLID resistance

	Strain	Phenotypic DST			MIC, µg/mL			MeltPro	WGS
		KM	AM	CM	KM	AM	CM		
Phenotypic susceptibility/genotypic resistance	22A011	S	S	S	0.63	0.125	1.25	WT	<i>tlyA</i> 590 del_T (35.37%)
	22A012	S	S	S	1.25	0.125	1.25	WT	<i>tlyA</i> 394 ins_A (95.38%)
Phenotypic resistance/genotypic susceptibility	22A049	R	R	S	≥80	>64	2.5	WT	<i>rrs</i> A1401G (24.65%)
	22A083	S	S	R	2.5	0.5	5	WT	<i>tlyA</i> 135 ins_G (94.76%)
	22A037	R	S	S	10	1	1.25	<i>eis</i> -14C>T/ <i>eis</i> -13A>G/ <i>eis</i> -10G>A	WT
	22A097	R	R	R	40	32	5	WT	WT
	22A148	R	S	S	20	0.5	2.5	WT	WT

Abbreviations: DST, drug-susceptibility testing; MIC, minimum inhibitory concentration; R, resistant; S, susceptible; CM, capreomycin; KM, kanamycin; AM, amikacin; WGS, whole-genome sequencing; WT, wild type.

Discussion

As it will take time to transition to all-oral regimens, SLIDs remain in use,¹⁶ so there will be a period with the injection and oral regimen coexistence. Molecular tests for rapid TB drug-resistance detection have become increasingly popular. In the present study, we assessed the diagnostic accuracy of MeltPro with WGS assays for SLID resistance among MDR-TB patients.

This study showed that 25.4% MDR-TB patients had resistance to at least one SLID, slightly higher than another report (17.3%) in Chongqing,¹⁷ which may be attributed to medication history and the diversity in TB epidemiology. In the present study, the most frequent mutation was *rrs* A1401G (77.4%) in SLID-resistant isolates, which was higher than Chinese Center for Disease Control and Prevention (40.7%)¹⁸ and Hebei (33.3%) figures.¹⁹ This phenomenon was principally due to the differences in sequencing methods, as WGS showed an obvious advantage of improved sensitivity over Sanger sequencing. We found that the A1401G mutation was present in 73.3% of KM-resistant, 78.6% of CM resistant and 95.5% of AM-resistant isolates, and the mutation-prevalence trend was consistent with a previous report.^{18,20} In accordance with previous research,^{21,22} the *rrs* A1401G mutation was associated with a high level of resistance to KM and AM, while CM showed different levels of resistance. In our study, 92.3% of isolates carried the A1401G mutation, contributing to cross-resistance and indicating that *rrs* might serve as a marker to predict cross-resistance, which was consistent with a previous study.²³ Mutations in the *eis* promoter were found only in KM-resistant isolates with low-level resistance, implying that it does not always confer cross-resistance among SLIDs, which was concordant with previous studies.^{17,24,25}

The excellent sensitivity of both MeltPro and WGS was associated with a high frequency of A1401G (91.7%) in *rrs* mutations circulating in Chongqing. Excellent agreement with phenotypic DST indicated that MeltPro and WGS assays provided an accurate option for the diagnosis of SLID resistance. Discordance occurred in four SLID isolates identified as sensitive by MeltPro but resistant by WGS, including three isolates with mutations in the *thyA* gene, which was not covered by MeltPro, leading to inconsistent results. The other isolate, with 24.65% *rrs* A1401G heteroresistance, was identified by WGS assay but missed by MeltPro, which was principally attributed to the different limits of detection of various molecular diagnostics. The one phenotypically SLID-resistant isolate was predicted to be resistant by MeltPro with mutations in *eis* C-14T/*eis* A-13G/*eis* G-10A, but sensitive by WGS, which may be attributed to the incomplete WGS database of mutation sites. In addition, the design principle of the MeltPro kit, with these three mutation sites in the ROX fluorescence channel, resulted in differences in ΔT_m values and uninterpretable specific mutation type.

Though it has been shown to be accurate and reliable compared to conventional phenotypic diagnostics,⁷ there is a potential barrier to WGS popularization in resource-limited settings, due to its cumbersome nature, cost, and technical accessibility. Recent work demonstrated that a high-resolution melting curve outperformed conventional PCR-based assays on identifying heteroresistant samples,^{26,27} indicating that MeltPro was a better solution for accurate heteroresistance. Two isolates showing phenotypic resistance were identified as SLID-sensitive by MeltPro and WGS, which may be attributed to other resistance mechanisms, such as drug efflux and decreased permeability of the cell wall.

In conclusion, MeltPro is a promising diagnostic tool for accurate identification of SLID-resistant MTB isolates with mutations in the *rrs* and *eis* genes. The proportion of heterogeneous drug-resistant bacteria with *rrs* mutations and limited probes in MeltPro resulted in disparity with WGS results. Resistance mechanisms other than genetic mutations will affect the consistency of MeltPro and WGS in phenotypic drug-susceptibility results. Though SLIDs have been removed as a mainstream treatment of drug-resistant TB, developing molecular assays with other drugs presently used for treatment is important.

Data Sharing

Data can be made available through contact with the corresponding author.

Ethical Approval

This study was approved by the Ethics Committee of Chongqing Tuberculosis Control Institute (KY201801), and written informed consent was obtained from all participants. This study was conducted in accordance with the Declaration of Helsinki.

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Disclosure

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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