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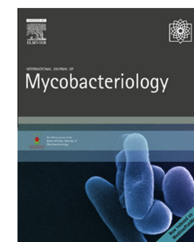

Correlating *rrs* and *eis* promoter mutations in clinical isolates of *Mycobacterium tuberculosis* with phenotypic susceptibility levels to the second-line injectables ☆

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Objective/background: The in vitro drug-susceptibility testing of *Mycobacterium tuberculosis* reports isolates as resistant or susceptible on the basis of single critical concentrations. It is evident that drug resistance in *M. tuberculosis* is quite heterogeneous, and involves low level, moderate level, and high level of drug-resistant phenotypes. Thus, the aim of our study was to correlate *rrs* (X52917) and *eis* (AF144099) promoter mutations, found in *M. tuberculosis* isolates, with corresponding minimum inhibitory concentrations of amikacin, kanamycin, and capreomycin.

Methods: Ninety *M. tuberculosis* clinical isolates were analyzed in this study. The minimum inhibitory concentrations were determined by MGIT 960 for 59 isolates with resistance-associated mutations in the *rrs* and *eis* promoter gene regions, and 31 isolates with wild-type sequences, as determined by the GenoType MTBDR*sl* (version 1) assay.

Results: The *rrs* A1401G mutation was identified in 48 isolates resistant to the second-line injectables. The *eis* promoter mutations C-14T ($n = 3$), G-10C ($n = 3$), G-10A ($n = 3$), and C-12T ($n = 2$) were found within 11 isolates with various resistance profiles to the second-line injectables. Thirty-one isolates had wild-type sequences for the *rrs* and *eis* promoter gene regions of interest, one of which was amikacin, kanamycin, and capreomycin resistant. The isolates with the *rrs* A1401G mutation had amikacin, kanamycin, and capreomycin minimum inhibitory concentrations of >40 mg/L, >20 mg/L, and 5–15 mg/L, respectively. The isolates with *eis* promoter mutations had amikacin, kanamycin, and capreomycin minimum inhibitory concentrations of 0.25–1.0 mg/L, 0.625–10 mg/L, and 0.625–2.5 mg/L, respectively.

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Conclusion: This study provides a preliminary basis for the prediction of phenotypic-resistance levels to the second-line injectables based upon the presence of genetic mutations associated with amikacin, kanamycin, and capreomycin resistance. The results suggest that isolates with *eis* promoter mutations have consistently lower resistance levels to amikacin, kanamycin, and capreomycin than isolates with the *rrs* A1401G mutation.

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Introduction

Drug-resistant tuberculosis (TB) poses a major threat to TB control efforts worldwide. There is an increasing global incidence of multidrug-resistant TB (MDR-TB) and extremely drug-resistant TB (XDR-TB), characterized by resistance to isoniazid, rifampin, a fluoroquinolone, and at least one of three second-line injectable (SLI) drugs: amikacin (AMK), kanamycin (KAN), and/or capreomycin (CAP). The effective treatment of MDR-TB, as well as the prevention of XDR-TB, is reliant upon the appropriate use of these SLIs. In order to establish appropriate treatment regimens, and to prevent the amplification of SLI resistance, it is critical that MDR-TB patients have full drug-susceptibility profiles established for their particular *Mycobacterium tuberculosis* infections prior to treatment [1].

The conventional diagnosis of drug-resistant *M. tuberculosis* relies upon the slow growth of mycobacterium cultures, and can take anywhere from several weeks to months to generate results [2]. While methods for the conventional diagnosis of MDR-TB are well established and generally effective, the culture-based detection of SLI resistance is more complicated and results are less reliable [3]. As such, rapid molecular diagnostic tests, detecting genetic mutations associated with drug resistance, are essential to the future diagnosis and management of M/XDR-TB.

A high level of cross-resistance has been observed between the aminoglycosides AMK and KAN [4,5]. CAP, a cyclic polypeptide, can be used as a substitute, although it is structurally dissimilar to the aminoglycosides [4,6]. For patients with MDR-TB, if CAP resistance additionally occurs, an increased incidence of treatment failure and patient mortality is observed [7]. Furthermore, studies conducted in clinical isolates and laboratory-generated *M. tuberculosis* mutants have noted the additional occurrence of cross-resistance between AMK/KAN and CAP, limiting the treatment options for these infections [4,5,8]. SLI resistance is mainly conveyed by mutations in the *rrs* and *eis* promoter gene regions of *M. tuberculosis* isolates. AMK/KAN and CAP are known to effect protein synthesis in *M. tuberculosis*, and resistance to these compounds is primarily conveyed by changes in the 16S ribosomal RNA (encoded by the *rrs* gene) [4,5,9]. A high-level AMK/KAN resistance and a lower-level CAP resistance can be caused by mutation in *rrs* A1401G [4]. The transfer of an acetyl group from acetyl coenzyme A to an amine group of aminoglycoside is catalyzed by *eis* (aminoglycoside acetyltransferase); a multiacetylation capability at the 2′-, 3-, or 6′ positions of aminoglycoside antibiotics is shown by the *eis* of *M. tuberculosis* [10]. Although these resistance mechanisms have been well defined, it is critical to additionally correlate specific genetic mutations with levels of SLI resistance in order to guide M/XDR-TB treatment regimens and improve patient outcomes. Herein, we establish the minimum inhibitory concentrations (MICs) for 90 clinical *M. tuberculosis* isolates with specific *rrs* and *eis* promoter mutations.

Materials and methods

Setting and ethical approval

A written consent was waived for all participants, as the study was carried out on 90 archived isolates for which pyrosequencing (PSQ), GenoType MTBDR*sl* assay (version 1) and MGIT 960 drug-susceptibility testing (DST) (utilizing the World Health Organization-approved critical concentrations), was performed previously. Sample collection, MGIT culture, GenoType MTBDR*sl* assay, and PSQ were performed at the Mycobacteriology Laboratory of the P. D. Hinduja Hospital & Medical Research Centre, a tertiary-care hospital in Mumbai, India with a referral bias toward TB treatment nonresponders. This study was approved by the Institutional Review Board of P. D. Hinduja Hospital & Medical Research Centre.

Phenotypic MGIT DST

We performed a standardized DST to KAN using the BACTEC MGIT 960 System (Becton, Dickinson and Company Diagnostic Systems, Sparks, MD, USA) with the EpiCenter software. The manufacturer's protocol was strictly followed for preparation of the inocula, inoculation, and incubation. For KAN, a breakpoint concentration of 2.5 mg/L was utilized based upon previous findings [11]. The critical concentrations of AMK and CAP were 1.0 mg/L and 2.5 mg/L, respectively. The stock solutions of AMK, KAN, and CAP (Sigma–Aldrich, St. Louis, Missouri, USA) were prepared by dissolving the compounds in distilled water. The drugs were filtered, further diluted with distilled water, and stored at −80 °C for up to 6 months. A genotypically confirmed resistant strain and a pan-susceptible strain, H37Rv, were tested weekly by the MGIT 960 DST as a quality control.

Quantitative DST

Six concentrations of KAN and seven concentrations of AMK and CAP were used to establish the MICs for the *M. tuberculosis* isolates included in this study. Two concentrations below the critical concentration (0.25 mg/L and 0.5 mg/L) and three concentrations above the critical concentration (4.0 mg/L, 20.0 mg/L, and 40.0 mg/L) were used to establish the AMK

MICs; two concentrations below the critical concentration (0.625 mg/L and 1.25 mg/L) and three concentrations above the critical concentration (5.0 mg/L, 10.0 mg/L, and 20.0 mg/L) were used for KAN; and two concentrations below the critical concentration (0.625 mg/L and 1.25 mg/L) and four concentrations above the critical concentration (5.0 mg/L, 10.0 mg/L, 15.0 mg/L, and 30.0 mg/L) were used for CAP.

GenoType MTBDRsl assay

The procedure for the GenoType MTBDR*sl* assay was as follows:

1. DNA extraction: DNA was extracted from all decontaminated patient samples using a GenoLyse kit (Hain Lifescience, Nehren, Germany).
2. Polymerase chain reaction (PCR): Multiplex PCR amplification was conducted for the *rrs* gene of interest utilizing biotinylated primers. PCR was performed with the following cycling conditions: initial denaturation 95 °C/15 min; (denaturation 95 °C/30 s, annealing 58 °C/2 min [10 cycles]); (denaturation 95 °C/25 s, annealing 53 °C/40 s, extension 70 °C/40 s [30 cycles]); and final extension 70 °C/8 min.
3. Hybridization: Reverse hybridization/GenoType MTBDR*sl* assay was performed as per manufacturer's instructions (Hain Lifescience) [12].
4. Analysis: The GenoType MTBDR*sl* assay evaluates the *rrs* gene for mutations at positions 1401/1402 and 1484. The absence of the test wild-type (WT) marker, WT1, and the presence of the mutation (MUT) probe, MUT1, correspond to the A1401G mutation, while the absence of the test WT2 marker and the presence of the probe MUT2 correspond to the G1484T mutation.

PSQ

A total of 20 *M. tuberculosis* isolates were additionally *rrs* sequence confirmed and evaluated for the presence of the *eis* promoter mutations by PSQ. Reagents from the HotStarTaq kit and deoxynucleotide-triphosphate mixtures (Qiagen, Valencia, CA, USA) were used in the PCR master mix. PSQ was performed as follows:

1. DNA extraction: The PSQ assay utilized crude DNA, extracted by a simple heating procedure (95 °C, 25 min).
2. PCR solutions: Each PCR reaction contained 2.5 μL of extracted isolate DNA and 22.5 μL of the PCR master mix (1× PCR buffer, 2.5 mM $MgCl_2$, 0.96 mM deoxynucleotide-triphosphate mixture, 1× Q-Solution, 0.5 μM *rrs* or *eis* promoter primers, and 1 U of HotStarTaq).
3. PCR steps: The initial activation was done with HotstarTaq polymerase at 95 °C for 15 min, followed by 50 cycles of amplification (94 °C for 15 s, 60 °C for 30 s, and 72 °C for 20 s), and a final extension at 72 °C for 5 min.
4. PSQ reaction: PSQ was conducted for the −6 to −47 regions of the *eis* promoter, and the 1401/1402 region of the *rrs* gene. PSQ was performed with PyroMark Q96 reagents, utilizing the sequence-analysis mode of the PyroMark Q96 ID system (Qiagen) [13].

Results

Phenotypic DST and MIC results

Sixty phenotypically XDR-TB strains were included in this study. Eleven (found to have *eis* promoter mutations) were resistant to KAN at the critical concentration, but sensitive to AMK and CAP. Forty-eight isolates (determined to have the *rrs* A1401G mutation) were found to be resistant to KAN, AMK, and CAP. One isolate (with WT sequences for both the *rrs* and *eis* promoter) was also resistant to KAN, AMK, and CAP at the critical concentration. Thirty isolates were pan susceptible to all drugs tested. The MICs of AMK, KAN, and CAP for all isolates are shown in Table 1.

Genotypic results

The agreement between the GenoType MTBDR*sl* assay results (based solely upon the presence of resistance-associated *rrs* mutations) and the phenotypic DST was 97% for AMK, 96% for KAN, and 86% for CAP. The sequencing and DST results for the 20 isolates subjected to PSQ are summarized in Table 2. One isolate, found to be phenotypically resistant to the three SLIs, had *rrs* and *eis* promoter WT sequences by both the GenoType MTBDR*sl* and PSQ assays.

Correlation of KAN MICs with resistance-associated mutations

The isolates harboring the *rrs* A1401G mutation had AMK, KAN, and CAP MICs of >40 mg/L, >20 mg/L, and 5–15 mg/L, respectively, whereas the isolates with *eis* promoter mutations were found to have AMK, KAN, and CAP MICs ranging from 0.25 mg/L to 1.0 mg/L, 5.0 mg/L to 10 mg/L, and 0.625 mg/L to 2.5 mg/L, respectively (Table 1). As such, the *rrs* A1401G mutation correlated with high-level AMK and KAN resistance, and moderate-level CAP resistance. The *eis* promoter mutations correlated with low-level AMK and CAP resistance, and moderate-level KAN resistance.

Discussion

This study correlates specific *rrs* and *eis* promoter mutations identified in *M. tuberculosis* clinical isolates with AMK, KAN, and CAP phenotypic-resistance levels. Overall, the MIC ranges for *M. tuberculosis* isolates with WT *rrs* and *eis* gene sequences were different from the MIC ranges for the isolates harboring resistance-associated mutations in these gene regions. The *rrs* and *eis* promoter mutations associated with SLI resistance were found in 59 of the 90 *M. tuberculosis* isolates evaluated in this study. These two genes, alone, appeared to be highly sensitive markers for SLI resistance, as only one of the 60 SLI-resistant isolates evaluated in the study was determined to have WT sequences for the gene regions evaluated. This isolate may have had SLI resistance-associated mutations in other genes, such as *tlyA* and *gidB*, that were not assessed in this study. Overall, our results provide a preliminary basis for the prediction of SLI phenotypic-resistance levels based upon the presence or absence of specific resistance-

Table 1 – Mutations found within *Mycobacterium tuberculosis* clinical isolates and their associated minimum inhibitory concentrations against the second-line injectables.

Mutation	LPA[a]	Total no. of isolates	No. of isolates	MIC (mg/L)		
				AMK	KAN	CAP
			rrs			
A1401G	MUT1	48	25	>40	>20	5.0
			20	>40	>20	10
			3	>40	>20	15
			eis			
G-10C	WT	3	1	0.5	2.5	0.625
			1	0.25	5.0	0.625
			1	0.5	5.0	0.625
G-10A	WT	3	1	1.0	5.0	2.5
			1	1.0	2.5	0.125
			1	0.5	10	0.625
C-12T	WT	2	1	0.5	5.0	0.625
			1	0.25	5.0	0.625
C-14T	WT	3	1	0.25	10	0.625
			1	1.0	10	1.25
			1	0.25	0.625	0.625
			WT			
WT[b]	1	1	4.0	10	5.0	
WT	30	25	0.25	0.625	0.625	
		5	0.5	0.625	1.25	

Note: AMK = amikacin; CAP = capreomycin; KAN = kanamycin; WT = wild type.

a Line probe assay.

b One isolate, characterized as WT, but resistant to AMK, KAN, and CAP at their respective break points, could contain a mutation in gene regions not assessed by MTBDR*sl* or pyrosequencing.

Table 2 – Pyrosequencing results for 20 *Mycobacterium tuberculosis* clinical isolates with wild-type MTBDR*sl* assay results.

No. of isolates	GenoType MTBDR*sl* assay	DST (critical concentration)			PSQ
		AMK	KAN	CAP	
8	WT	Susceptible	Resistant	Susceptible	*eis* mutation
3	WT	Susceptible	Susceptible	Susceptible	*eis* mutation
4	WT	Susceptible	Susceptible	Susceptible	WT
1	WT	Resistant	Resistant	Resistant	WT
4	*rrs* MUT1	Resistant	Resistant	Resistant	A1401G

Note. AMK = amikacin; CAP = capreomycin; DST = drug-susceptibility testing; KAN = kanamycin; PSQ = pyrosequencing; WT = wild type.

associated mutations in both the *rrs* and *eis* promoter gene regions of *M. tuberculosis* clinical isolates.

The peak serum concentrations for KAN, AMK, and CAP have been previously reported at 22.5 mg/L, 20–40 mg/L, and 10–30 mg/L, respectively [14,15]. Taking these concentrations into account, M. tuberculosis resistance levels to each drug may be categorized as low, moderate, or high. As noted in Table 1, the rrs A1401G mutation appears to consistently correspond to a high level of AMK and KAN resistance (MICs >40 mg/L, >20 mg/L). The KAN MICs for isolates with the A1401G mutation have been previously documented to be as high as >80 mg/L, and clinical studies have confirmed that this particular rrs mutation confers KAN resistance even at these high concentrations, with additional cross-resistance to AMK with MICs of >64 mg/L [5] and, to a lesser extent, CAP with MICs of 5–10 mg/L [16]. Conversely, isolates with eis promoter mutations have been documented to be resistant to KAN, but susceptible to AMK and CAP at their respective break points [4,16–18]. Our study identified 11 isolates with eis promoter mutations. These isolates had low- to moderate-level KAN resistance, and a low level of resistance to AMK and CAP. These eis promoter mutations, C-14T, G-10C, G-10A, and C-12T, have been shown to result in a significant increase in eis transcript levels, as well as in a corresponding increase in the expression of an enzyme that acetylates KAN, inactivating the drug [18,19].

Importantly, although the Eis enzyme was found to have some cross-reactivity with AMK, the activity was limited, and *eis* mutants are found to be largely susceptible to this other aminoglycoside, as reflected by current testing and treatment guidelines [17,18]. As Eis utilizes KAN as a substrate up to three times more efficiently than AMK, the noted lack of cross-resistance is unsurprising. This preferential substrate utilization by Eis is likely a result of aminoglycoside structural differences; AMK sterically delays acetylation, as it contains an L-hydroxyaminobuteroyl amide group substitution in the

N1 position of the deoxystreptamine ring [18]. It is notable that, even though *rrs* mutations confer cross-resistance to KAN and AMK, KAN-resistant isolates with *eis* promoter mutations may still be sensitive to AMK, and thus, both KAN and AMK should be considered individually when determining phenotypic drug-susceptibility profiles [17]. Indeed, the *eis* promoter mutations were found only within AMK- and CAP-susceptible isolates in our study, suggesting that these drugs could potentially be used to treat strains housing *eis* promoter mutations.

Our observation that different *eis* promoter mutations correspond to varying levels of phenotypic resistance to KAN, AMK, and CAP is also notable. The *eis* promoter mutations C-14T and G-10A corresponded to low- or moderate-level resistance to KAN, with KAN MICs generally ranging from 2.5 μg/mL to 10 μg/mL, and MICs corresponding with low-level resistance to AMK and CAP (0.25–1.0 mg/L and 0.625–2.5 mg/L), comparable with values reported by previous studies [16,18]. The association of the C-12T mutation with KAN resistance, however, has been a point of contention. In one study, C-12T mutants were shown to have slightly increased KAN MICs, although they retained sensitivity to KAN and AMK [18]. Another study found isolates with the C-12T mutation to have KAN resistance at an MIC of 5.0 mg/L, although the CAP MICs were <2.5 mg/L [16]. Overall, all studies found aminoglycoside MICs to be lower for *eis* promoter mutants compared to *rrs* mutants [10,16,18]. This supports our characterization of *eis* promoter mutants as "low- to moderate-level" KAN resistant with MICs ranging from 0.625 mg/L to 10 mg/L, "low-level" AMK resistant with MICs from 0.25 mg/L to 1.0 mg/L, and "low-level" CAP resistant with MICs from 0.625 mg/L to 2.5 mg/L. However, the overlap seen for KAN MIC ranges for the various mutants (i.e., MICs for -14T mutants were not very different than -10A or -12T mutants) suggests the need for further studies to clearly define associated phenotypic-resistance levels.

The sequencing of additional gene regions not evaluated in this study, such as *gidB* and *tlyA*, might additionally contribute to our understanding of the genetic basis of the observed SLI phenotypic-resistance levels of *M. tuberculosis* clinical isolates. These initial results, however, confirm that specific mutations in the *rrs* and *eis* promoter are promising markers for improved diagnostic assay sensitivity and specificity for the detection of injectable-drug resistance. Furthermore, our findings suggest that each drug should be considered independently when considering the results of molecular diagnostic assays, in order to determine the most effective treatment regimens for M/XDR-TB cases.

Conclusion

These results suggest that the various mutations associated with SLI resistance may be correlated with specific SLI phenotypic-resistance levels. The *rrs* canonical mutation, A1401G, was found to confer a high level of AMK and KAN resistance to *M. tuberculosis* isolates, but a moderate level of CAP resistance. The *eis* promoter mutations were generally determined to confer lower levels of AMK and CAP resistance, and low to moderate levels of KAN resistance. Thus, together, the *rrs* and *eis* promoter mutations appear to have utility as molecular markers for aminoglycoside-resistance levels, directing optimal treatment regimens for M/XDR-TB cases.

Conflicts of interest

The authors declared no conflicts of interest.

Acknowledgments

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