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eis Promoter C14G and C15G Mutations Do Not Confer Kanamycin Resistance in *Mycobacterium tuberculosis*

Suporn Pholwat,[a] Suzanne Stroup,[a] Scott Heysell,[a] Oleg Ogarkov,[b,c] Svetlana Zhdanova,[b] Girija Ramakrishnan,[a] Eric Houpt[a]

Division of Infectious Diseases and International Health, Department of Medicine, University of Virginia, Charlottesville, Virginia, USA[a]; Scientific Centre for Family Health and Human Reproduction Problems, Irkutsk, Russian Federation[b]; Regional TB-Prevention Dispensary, Irkutsk, Russian Federation[c]

We studied the significance of particular *eis* mutations on *Mycobacterium tuberculosis* drug resistance using a specialized transduction strategy. Recombinant strains harboring *eis* promoter mutations C14T, C12T, and G10A exhibited kanamycin resistance with MICs of 40, 10, and 20 μg/ml, respectively, while recombinant strains harboring C14G and C15G mutations were kanamycin susceptible (MIC, 2.5 to 5 μg/ml). Each of the *eis* mutants tested remained amikacin susceptible (MIC, 0.5 to 4 μg/ml). The identification of specific *eis* mutations is needed for accurate genotypic susceptibility testing for kanamycin.

The emergence of multidrug-resistant (MDR) tuberculosis (TB) and extensively drug-resistant (XDR) TB has slowed efforts to eliminate global TB. Optimal antibiotic selection requires drug susceptibility results. Conventional drug susceptibility testing relies on mycobacterial culture and requires weeks to months to complete, while molecular detection of genetic mutations enables faster results (1). A limitation to molecular diagnostics for TB drug susceptibility testing is the uncertainty of which mutations confer resistance. This problem has expanded as sequencing methods of TB strains have proliferated, leading to the identification of large numbers of mutations of unknown significance. To definitively elucidate the contribution of any individual mutation to the drug-resistant phenotype, a demonstration of *in vitro* resistance following direct bacterial genetic manipulation to generate the mutation of interest is required.

Kanamycin (KAN) is an important second-line injectable drug used worldwide to treat MDR TB, but it is also associated with considerable toxicity (2). Thus, rapid and accurate drug susceptibility testing for KAN is valuable. Mutations in the 16S rRNA gene (*rrs*) can cause high-level (MIC, >80 μg/ml) resistance to KAN, as well as to amikacin (AMK) and capreomycin (3). However, molecular analysis of the common A1401G *rrs* mutation explains only ~56% of KAN resistance, while the mutations G10A and C14T in the promoter of *eis*, encoding an aminoglycoside acetyltransferase, explain another 33% of KAN resistance as estimated by one review (4).

Mutations in *eis* promoter appear particularly common among MDR-TB isolates from regions of the Russian Federation (5). In the course of characterizing TB isolates from a drug-resistant TB referral hospital in the Irkutsk, Siberia region of the Russian Federation (6), we identified two MDR isolates with an *eis* promoter C14G mutation, one of which was reported to be resistant to KAN (30 μg/ml) by the Lowenstein-Jensen proportion phenotypic method and had no other mutations in the relevant *eis* or *rrs* region by Sanger sequencing. We also found two isolates with a C15G mutation, both of which were KAN susceptible; however, an isolate with this mutation was reported by others to have KAN and AMK resistance (4). Zaunbrecher et al. previously found that the *eis* promoter mutation C14T conferred KAN resistance, while G10A, G37T, and C12T mutations conferred lower level resistance (7). We wished to confirm these results and also determine if C14G or C15G mutations directly influence the resistance phenotype. Mutations in the −10 to −14 region of *eis* are particularly important to understand, since this region is interrogated by the Hain GenoType MTBDRsl assay v2.0 (8).

We generated point mutations by allelic exchange of C14G and C15G along with the previously reported mutations G10A, C12T, and C14T to understand the relationship between each mutation and the phenotypic susceptibility. We generated recombinant strains using a transduction strategy described by Jain et al. (9) with modifications as described in the supplemental methods. The parental *Mycobacterium tuberculosis* H37Rv and its recombinant derivative were subjected to drug susceptibility testing against amikacin (AMK) and kanamycin (KAN) using liquid (Trek Sensititre MYCOTB) and solid (agar proportion) media as described previously (10). The *M. tuberculosis* H37Rv and its recombinant strain (*eis* wild type) were susceptible to AMK (MIC, 0.5 to 1 μg/ml) and KAN (MIC, 2.5 μg/ml). The recombinant strains that harbored the *eis* promoter G10A mutation were KAN resistant (MIC, 20 μg/ml). Strains harboring the C12T mutation were KAN resistant with a lower MIC (10 μg/ml), while strains with the C14T mutation and the clinical strain harboring C14T had the highest MIC (40 μg/ml). The novel C14G and C15G mutant strains remained susceptible to KAN with MICs of 2.5 to 5 μg/ml. Each of the recombinant strains harboring *eis* promoter mutations was susceptible to AMK with an MIC of 0.5 to 4 μg/ml (Table 1).

The value of this work is in the direct testing of the contribution of individual mutations to TB drug resistance so that molecular diagnostic labs can interpret sequence data. We used direct allelic exchange as was used in a previous study to examine the C14T mutation (7); this is preferable to the complementation

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Address correspondence to Eric Houpt, erh6k@virginia.edu.

G.R. and E.H. contributed equally to this work.

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TABLE 1 Aminoglycoside resistance of specific *eis* promoter mutants

Strain	*eis* mutation	Trek[a] MIC (μg/ml)		Agar proportion MIC (μg/ml)	
		KAN	AMK	KAN	AMK
Reference					
H37Rv	WT[b]	2.5	0.5	2.5	1
Clinical					
O16	C14T	40	2	40	4
Recombinant					
R001	WT	2.5	0.5	2.5	1
R101	G10A	20	2	20	2
R102	G10A	20	2	20	2
R103	G10A	20	2	20	2
R201	C12T	10	0.5	10	2
R202	C12T	10	0.5	10	2
R203	C12T	10	0.5	10	2
R301	C14G	2.5	0.5	2.5	1
R302	C14G	2.5	0.5	2.5	1
R303	C14G	2.5	0.5	2.5	1
R401	C14T	40	2	40	4
R402	C14T	40	2	40	4
R403	C14T	40	2	40	4
R501	C15G	5	0.5	5	1
R502	C15G	5	0.5	5	1

[a] Trek, Sensititre MYCO TB; KAN, kanamycin; AMK, amikacin.
[b] WT, wild type.

strategy previously used for several other mutations, which could potentially impact transcript levels and confound the analysis (7). In agreement with the previous study (7), we found that the *eis* promoter mutations C14T and G10A did indeed confer KAN resistance and should be considered resistance mutations. However, the C12T mutation led to a modest increase in the KAN MIC and should be viewed with suspicion after molecular detection. Others have found the C12T mutation in sensitive strains (4, 11), probably because the MIC is near the critical concentration. The clinical impact of a KAN MIC of 10 μg/ml is unknown and difficult to elucidate in a multidrug regimen, but we would advocate choosing AMK with such a mutation, if available. Finally, we demonstrated that C14G and C15G mutations should not be considered causal resistance mutations, and the resistance observed in such strains is presumably due to some other mutation or mechanism. Whether these promoter mutants, regardless of KAN MIC change, lead to overexpression of *eis* and an enhanced monocyte survivor phenotype, as previously suggested (7), remains untested.

The Hain GenoType MTBDRsl assay v2.0 includes a mutation probe targeting the C14T change, such that detection of this band, with loss of the wild-type C14, C12, and G10 ("WT2") bands, denotes resistance (8). This result is straightforward and accurate. Inclusion of a G10A mutation band in the assay would be particularly useful, since this is a resistance-conferring mutation and appears to be even more prevalent (4). Until such time, the presence of a G10A, C12T, C14G, or C15G mutation will lead to a confusing loss of the WT2 band without further information; our results indicate the molecular interpretation of these mutations should be resistant, intermediate, susceptible, and susceptible, respectively. Indeed, this single loss of the WT2 band was the most prevalent abnormality in a recent evaluation of the kit (11). For optimal genotypic susceptibility testing for kanamycin, the identification of specific *eis* mutations in the −10 to −15 region is needed.

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