The role of eis mutations in the development of kanamycin resistance in Mycobacterium tuberculosis isolates from the Moscow region

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Objectives: Kanamycin is an important second-line drug used to treat multidrug-resistant (MDR) tuberculosis (TB). Molecular analysis of the rrs gene seems to be not enough to identify every case of kanamycin resistance. In the present study we evaluated the incidence of eis mutations in kanamycin-resistant Mycobacterium tuberculosis isolates.

Methods: We analysed 70 MDR M. tuberculosis clinical isolates. All isolates were screened for rrs and eis mutations using single-strand conformation polymorphism and sequencing. Phenotypic drug susceptibility testing was performed using Bactec MGIT 960 and the absolute concentration method on Lowenstein–Jensen medium.

Results: eis mutations were found in 10 isolates. The most prevalent mutations were the A1401G substitution in the rrs gene and the C14T substitution in the eis promoter region.

Conclusions: Our study shows that the eis promoter region is a useful molecular marker of kanamycin resistance in the Moscow region. Complex analysis of rrs and eis mutations will significantly reduce the time to diagnose kanamycin resistance in TB patients, compared with phenotypic drug resistance testing.

Keywords: XDR-TB, second-line injectable drugs, drug-susceptibility testing, molecular assays

Introduction

According to the WHO report, in 2010 there were an estimated 8.8 million incident cases of tuberculosis (TB) globally. In Russia, the epidemiological situation is complicated by a high prevalence of multidrug-resistant (MDR) TB, showing resistance to at least isoniazid and rifampicin. Furthermore, extensively drug-resistant (XDR) strains of Mycobacterium tuberculosis, defined as MDR with additional resistance to any of the fluoroquinolones and any of the second-line injectable drugs (kanamycin, amikacin or capreomycin), emerge as a serious challenge to TB management and control. MDR-TB and XDR-TB require more expensive, long-term and less effective treatment courses than pan-susceptible TB. As the choice of chemotherapeutic agents is limited, rapid and accurate drug susceptibility testing (DST), especially for second-line injectable drugs, is crucial.

Second-line injectable drugs include the aminoglycosides kanamycin and amikacin, and a polypeptide antibiotic—capreomycin. Kanamycin and capreomycin are the most commonly used second-line injectable drugs in Russia. The mechanism of action of the second-line injectable drugs consists of binding to the 16S rRNA in the 30S ribosomal subunit, thus arresting protein synthesis in the bacterial cell. Mutations in the 16S rRNA gene, rrs, cause high-level resistance to kanamycin and cross-resistance to amikacin and sometimes capreomycin. In a recent paper, Zaunbrecher et al. reported the impact of mutations in the eis promoter region on M. tuberculosis kanamycin susceptibility. eis encodes an aminoglycoside acetyltransferase specific to kanamycin. Mutations in the eis promoter region lead to an increased synthesis of the enzyme and inactivation of the drug.

The aim of the present study was to define the role of eis mutations in the development of kanamycin resistance in patients from antituberculosis clinics in Moscow. Few studies have investigated this to date and, to our knowledge, none in Russia.

Material and methods

Bacterial isolates

For the present study we chose 70 M. tuberculosis clinical isolates. Isolates were obtained from sputum provided by patients previously treated at the Moscow Scientific and Clinical Antituberculosis Center and two specialized antituberculosis clinics in Moscow. All isolates were identified as MDR on the basis of bacteriological culture in Mycobacteria...
Growth Indicator Tube (MGIT) medium] identification and DST. First-line DST for rifampicin and isoniazid was performed using Bactec MGIT 960 according to the manufacturer’s instructions. The absolute concentration method on Lowenstein–Jensen slants, recommended by the Russian Ministry of Health, was used to perform second-line DST with the following concentrations for both kanamycin and capreomycin: 30 and 50 mg/L. This was part of the Center’s mycobacteriological laboratory routine.

Thirty-two MDR isolates were additionally resistant to second-line injectable drugs according to phenotypic DST. Twenty-five isolates were resistant to both kanamycin and capreomycin; 7 were resistant to only kanamycin.

**DST**

Kanamycin and capreomycin MICs for all isolates were determined using the Bactec MGIT 960 automated system. For both antibiotics, 2.5 mg/L was chosen as the breakpoint concentration. If 32 mg/L of the drug did not inhibit the \textit{M. tuberculosis} growth, the exact MIC for such an isolate was not determined and was considered 32 mg/L.

**DNA extraction**

Cultures were washed with PBS and then incubated with lysis buffer at 99°C for 20 min. DNA samples were stored at −20°C.

**PCR amplification**

A 300 bp fragment of the 3’ part of the \textit{rrs} gene and a 250 bp DNA fragment containing the \textit{eis} promoter region were amplified by PCR. An aliquot of 30 μL of reaction medium contained 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.5% Tween 20, 5.0% formamide, 2.5% MgCl₂, 2.5 U of Taq polymerase, 200 mM of each dNTP, 0.5 mM of each appropriate primer and 3 μL of DNA sample. The cycling conditions were as follows: denaturation at 95°C for 5 min; then 30 cycles of denaturation at 95°C for 20 s, annealing at 55°C (\textit{rrs}) or 59°C (\textit{eis}) for 30 s and extension at 72°C for 20 s; and, lastly, a final extension step at 72°C for 5 min.

**Single-strand conformation polymorphism (SSCP)**

SSCP was run in 8% polyacrylamide gel at 400 V for ~3.5 h. The kanamycin- and capreomycin-susceptible \textit{M. tuberculosis} H37Rv strain was used as the control. Gels were silver stained. The gel documentation system Epi Chemi II Darkroom (UV) and Image Scope Lite software were used to analyse gels.

**DNA sequencing**

All DNA samples were sequenced to confirm SSCP results and to characterize \textit{rrs} and \textit{eis} mutations. Sequence reactions were carried out in the GS Junior system (Roche Applied Science) and all sequences were compared with the M. tuberculosis H37Rv strain.

**Results**

**Determination of MICs using Bactec MGIT 960**

Kanamycin and capreomycin MICs for all the isolates were determined using Bactec MGIT 960. All isolates resistant to the drugs on Lowenstein–Jensen medium had MICs ranging from 5 to 32 mg/L for kanamycin and from 5 to 10 mg/L for capreomycin. Two isolates that were kanamycin susceptible on Lowenstein–Jensen medium were found to be resistant using Bactec MGIT 960 (MIC = 5 mg/L).

**Detection of \textit{rrs} and \textit{eis} mutations**

All \textit{M. tuberculosis} strains were screened for \textit{rrs} and \textit{eis} mutations using both SSCP and DNA sequencing. On the basis of the SSCP results, 24 \textit{M. tuberculosis} strains displayed band patterns different from H37Rv in \textit{rrs} and 9 in the \textit{eis} promoter region. One strain carried mutations in both DNA fragments. Sequence analysis revealed an A1401G substitution in all \textit{rrs} mutants and four different \textit{eis} mutations, the most common being the C14T substitution, which was present in four samples. Mutation G10A occurred in three samples and mutations G37T and C12T each occurred once. The sample carrying mutations in both DNA fragments was identified as an A1401G, G10A genotype.

**Correlation of phenotypic DST with molecular assays**

Molecular assay results were compared with the results of phenotypic DST and the data are shown in Table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>\textit{rrs}</th>
<th>\textit{eis}</th>
<th>KAN</th>
<th>CAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1401G</td>
<td>wt</td>
<td>R/R</td>
<td>R/R</td>
<td>16 to &gt;32</td>
</tr>
<tr>
<td>A1401G</td>
<td>G10A</td>
<td>R/R</td>
<td>R/S</td>
<td>8</td>
</tr>
<tr>
<td>A1401G</td>
<td>C14T</td>
<td>R/R</td>
<td>S/S</td>
<td>16, 16 and 32</td>
</tr>
<tr>
<td>A1401G</td>
<td>C14T</td>
<td>R/S</td>
<td>S/S</td>
<td>16</td>
</tr>
<tr>
<td>A1401G</td>
<td>G10A</td>
<td>R/R</td>
<td>S/S</td>
<td>10 and 10</td>
</tr>
<tr>
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<td>G10A</td>
<td>S/S</td>
<td>S/S</td>
<td>5</td>
</tr>
<tr>
<td>A1401G</td>
<td>C12T</td>
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<td>S/S</td>
<td>5</td>
</tr>
<tr>
<td>A1401G</td>
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<td>S/S</td>
<td>&lt;2.5</td>
</tr>
<tr>
<td>A1401G</td>
<td>wt</td>
<td>S/S</td>
<td>S/S</td>
<td>&lt;2.5</td>
</tr>
</tbody>
</table>

KAN, kanamycin; CAP, capreomycin; wt, wild-type; R, resistant; S, susceptible.
All A1401G mutants were resistant to both kanamycin and capreomycin. MICs ranged from 16 to >32 mg/L for kanamycin-resistant isolates and from 5 to 10 mg/L for capreomycin-resistant isolates.

The eis mutants were resistant only to kanamycin, with MICs ranging from 5 to 32 mg/L. Interestingly, one isolate carrying a G10A substitution and one isolate with a C12T mutation were kanamycin susceptible according to the absolute concentration method, but kanamycin resistant using Bactec MGIT 960 (MIC = 5 mg/L).

**Discussion**

Data acquired with molecular assays and DST using Bactec MGIT 960 were concordant in all cases. Nevertheless, some inconsistency was observed between these two methods and the absolute concentration method. Two isolates that were kanamycin susceptible on Lowenstein–Jensen medium harboured eis mutations and had MICs of 5 mg/L. Zaunbrecher et al. suggest that mutations in the eis promoter region lead only to a low level of resistance to kanamycin, possibly implying that these two isolates might be resistant to concentrations of <30 mg/L kanamycin on Lowenstein–Jensen medium. However, a technical mistake during routine DST with the absolute concentration method cannot be completely excluded.

Discordant data exist about the association of the C12T substitution in the eis promoter region with kanamycin resistance. In a previous work, C12T mutants had increased MICs but remained susceptible to kanamycin, although isolates were considered drug resistant at an MIC of 5 mg/L. In another study, C12T mutants were found in kanamycin-resistant isolates. In our study, isolates were considered drug resistant at an MIC of 2.5 mg/L; the only isolate carrying the C12T substitution had an MIC of 5 mg/L and was therefore reported as kanamycin resistant.

All capreomycin-resistant isolates carried only the A1401G substitution in the rrs gene. The isolate with an A1401G, G10A genotype was resistant to both drugs. We observed absolute cross-resistance between kanamycin and capreomycin in isolates harbouring the A1401G mutation. In contrast, there was no cross-resistance at all between kanamycin and capreomycin in clinical isolates carrying eis mutations. eis encodes an acetyltransferase specific to kanamycin, and thus the lack of cross-resistance between the two drugs seems perfectly logical and is in agreement with previously published data.

We found no significant difference between the level of kanamycin resistance in isolates containing rs and eis mutations in general. Nevertheless, substitutions A1401G and C14T seemed to cause the highest level of kanamycin resistance (MICs 16 to >32 mg/L). The lowest level of kanamycin resistance might be attributed to mutations C12T and G37T, although these mutations were each found only once and there are insufficient data to draw conclusions. Surprisingly, the isolate with both substitutions A1401G and G10A had a relatively low kanamycin MIC (8 mg/L) compared with other isolates with the A1401G mutation.

Molecular analysis of the eis promoter region allowed the identification of 9 (26%) isolates of *M. tuberculosis* resistant to kanamycin in addition to 25 (74%) isolates with the rrs mutation. Further studies should be carried out to establish whether all eis mutations are clinically significant. At present, the eis promoter region appears to be a valuable molecular marker of kanamycin resistance and molecular analysis of rrs alone clearly fails to detect a large proportion of kanamycin-resistant isolates in the Moscow region.

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**Transparency declarations**

None to declare.

**References**


