Novel multiplex allele-specific PCR assays for the detection of resistance to second-line drugs in *Mycobacterium tuberculosis*

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Objectives: The use of rapid molecular assays for the detection of resistance to second-line drugs would significantly decrease the time delay in diagnosing drug-resistant tuberculosis (TB) that is associated with conventional phenotypic drug susceptibility testing. In this study, multiplex allele-specific (MAS)-PCR assays designed to detect the GyrA D94G and *rrs* A1401G mutations were evaluated for detection of ofloxacin and kanamycin resistance.

Methods: GyrA D94G and *rrs* A1401G MAS-PCR assays were carried out on 288 *Mycobacterium tuberculosis* isolates. Phenotypic drug susceptibility testing of ofloxacin and kanamycin was performed on selected multidrug-resistant TB isolates using the indirect proportions method.

Results: MAS-PCR assays detected GyrA D94G and *rrs* A1401G mutations in phenotypically resistant isolates with clinical sensitivities of 54.5% (6 of 11) and 90.0% (9 of 10), respectively, and specificities of 100% were obtained for both assays. A GyrA A90V mutation was identified in 4 of 11 (36.4%) ofloxacin-resistant isolates that did not carry a D94G substitution.

Conclusions: Rapid genotypic assays designed to detect GyrA D94G and A90V mutations and *rrs* A1401G mutations could detect up to 90.0% of extensively drug-resistant (XDR)-TB in the Western Cape region. The use of these assays in the clinical setting would significantly reduce the time to diagnosis of XDR-TB, enabling the administration of appropriate treatment regimens at the outset of therapy.

Keywords: XDR-TB, ofloxacin, kanamycin

Introduction

The emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* isolates, reported as >490 000 cases per annum, severely compromises the treatment of tuberculosis (TB). The use of costly, toxic second-line drugs (SLDs) for the treatment of MDR-TB may contribute to the emergence of extensively drug-resistant (XDR)-TB, defined as MDR-TB with additional resistance to a fluoroquinolone and at least one of the injectable SLDs kanamycin, amikacin or capreomycin. XDR-TB is particularly prevalent in countries with a high HIV burden, such as South Africa, and is associated with a high mortality rate.

The slow generation time of *M. tuberculosis* contributes to the significant time delay associated with standard phenotypic drug susceptibility testing (DST), for which there are currently no standardized guidelines. Rapid molecular assays for detection of drug resistance in *M. tuberculosis* provide an attractive alternative.

Limited data available suggest that up to 35% of ofloxacin and up to 87% of kanamycin resistance worldwide can be attributed to a D94G amino acid substitution within the quinolone resistance-determining region (QRDR) of GyrA and an A1401G nucleotide substitution in *rrs*, respectively. The efficacy of multiplex allele-specific (MAS)-PCR assays targeting GyrA D94G and *rrs* A1401G for the identification of resistance to ofloxacin and kanamycin was evaluated.

Materials and methods

**Bacterial isolates and susceptibility testing**

*M. tuberculosis* isolates obtained from patients at Groote Schuur Hospital, Cape Town, in 2006 and 2009 were identified and subjected to DST for rifampicin and isoniazid as previously described. Of 136 isolates identified as MDR (Table 1), 54 viable isolates were further tested for resistance to ofloxacin and kanamycin using the indirect proportions method on Middlebrook 7H11 agar containing ofloxacin (Sigma-Aldrich) at 2 mg/L and kanamycin (Sigma-Aldrich) at 6 mg/L, in accordance with WHO guidelines.
Genomic DNA preparation

Colonies from M. tuberculosis isolates grown on Lowenstein–Jensen (LJ) slanted agar, or pelleted cells from 1 mL of MGIT (mycobacteria growth indicator tube) culture, were resuspended in 500 μL of distilled H₂O (dH₂O) and heat inactivated at 80 °C for 1 h. Genomic DNA was extracted as previously described.4

MAS-PCR assays

MAS-PCR assays were designed to identify GyrA D94G and rrs A1401G mutations by detecting a mismatch between the primer and the targeted mutation. Both PCR assays had a final volume of 50 μL and contained 1× GoTaq PCR buffer (Promega), 1.25 U of GoTaq® DNA polymerase (Promega) and 50–200 ng of genomic DNA as template. When amplifying gyrA, 0.15 mM of each dNTP (Fermentas), 10 pmol of GYRAF (5’-CCGGATCGAACCCTGGAC-3’), 30 pmol of GYRAR1 (5’-CCATGGCACCAGCTGT-3’), 20 pmol of GYRAR2 (5’-CCATGGGATGAAATCGACTG-3’) and 3 mM Mg²⁺ (Promega) were used. The reaction conditions consisted of an initial denaturation step at 95 °C for 5 min followed by 5 cycles of 95 °C for 15 s, 68 °C for 5 s and 72 °C for 20 s, 5 cycles of 95 °C for 15 s, 64 °C for 5 s and 72 °C for 20 s, and 25 cycles of 94 °C for 15 s, 62 °C for 5 s and 72 °C for 20 s, followed by a final elongation step at 72 °C for 5 min. For amplification of rrs, 0.2 mM of each dNTP (Fermentas), 20 pmol of RRSF (5’-GTGAGATGTTGGGTTAAGTCC-3’), 5 pmol of RRSR1 (5’-GTTACCGACTTTCATGACGT-3’), 40 pmol of RRSR (5’-TGTTGCCTCCCTTAGAAAGGAG-3’) and 1.5 mM Mg²⁺ (Promega) were used. The cycling conditions for rrs MAS-PCR were as for gyrA except that primer annealing was carried out at 66 °C for the first 5 cycles, at 64 °C for the next 5 cycles and at 62 °C for the remaining 25 cycles. Amplification of 427 and 260 bp products following gyrA MAS-PCR and 481 and 353 bp following rrs MAS-PCR (Figure 1) indicates the presence of wild-type sequence. The absence of the smaller product in both assays indicates detection of the GyrA D94G and rrs A1401G mutations. Sequence analysis of the products from wild-type and mutant isolates confirmed the absence or presence of the mutations, and these isolates were used as controls in all subsequent MAS-PCR assays.

Results

Drug susceptibility testing

Phenotypic DST of 54 MDR-TB isolates by the indirect proportions method indicated 3 (5.6%) as resistant to ofloxacin alone and 2 (3.7%) as resistant to kanamycin alone, defined as pre-XDR.6 A further eight (14.8%) isolates were resistant to both ofloxacin and kanamycin, or XDR.

Detection of GyrA D94G and rrs A1401G using MAS-PCR assays

MAS-PCR assays designed to detect GyrA D94G and rrs A1401G were carried out on 288 isolates (Table 1). The GyrA D94G mutation was indicated in 15 of 136 (11.0%) MDR-TB isolates, and in 1 of 10 (10.0%) rifampicin monoresistant isolates, with the remaining 272 isolates indicated as wild-type at this locus (Table 1). Similarly, the rrs A1401G nucleotide substitution was indicated in 32 of 136 (23.5%) MDR-TB isolates, 2 of 41 (4.9%) isoniazid monoresistant isolates and 17 of 101 (16.8%) isolates susceptible to rifampicin and isoniazid (Table 1). The remaining 237 isolates produced amplicons indicative of wild-type rrs at this locus.

Figure 1. Schematic representation and agarose gel electrophoresis of MAS-PCR assays for the identification of (a) GyrA D94G and (b) rrs A1401G resistance determinants. The position of each mutation is represented by a vertical line, arrows indicate positions of the primers used for PCR amplification and horizontal lines represent the expected amplicons with sizes indicated in bp. MW, Hyperladder IV (Bioline); WT, wild-type isolates; M, mutant isolates.
Second-line drug resistance in M. tuberculosis

**Correlation of phenotypic DST of ofloxacin and kanamycin with MAS-PCR assays**

Phenotypic DST identified 11 of 54 (20.4%) MDR-TB isolates as ofloxacin resistant, and a GyrA D94G mutation was indicated by MAS-PCR in 6 (54.5%) of these. Sequence analysis of the QRDR of the remaining five isolates identified a GyrA A90V mutation in four, whilst the remaining isolate had no identifiable mutation within this region. Of the 10 MDR isolates phenotypically resistant to kanamycin, 9 (90.0%) had an *rrs* A1401G nucleotide substitution indicated by MAS-PCR. Of the eight isolates phenotypically identified as XDR, four contained both a GyrA D94G substitution and an *rrs* A1401G mutation. The remaining four isolates contained a GyrA A90V substitution, with three of these isolates containing *rrs* A1401G.

**Table 1. Identification of M. tuberculosis isolates carrying GyrA D94G and *rrs* A1401G mutations using MAS-PCR assays**

<table>
<thead>
<tr>
<th>M. tuberculosis isolates (n)</th>
<th>GyrA D94G</th>
<th><em>rrs</em> A1401G</th>
<th>GyrA D94G and <em>rrs</em> A1401G</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR (136)</td>
<td>4</td>
<td>21</td>
<td>11</td>
</tr>
<tr>
<td>INH&lt;sup&gt;r&lt;/sup&gt; (41)</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>RIF&lt;sup&gt;r&lt;/sup&gt; (10)</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RIF&lt;sup&gt;r&lt;/sup&gt;INH&lt;sup&gt;r&lt;/sup&gt; (101)</td>
<td>0</td>
<td>17</td>
<td>0</td>
</tr>
</tbody>
</table>

MDR, multidrug resistant; INH, isoniazid; RIF, rifampicin; <sup>r</sup>, resistant; <sup>s</sup>, susceptible.

**Discussion**

The rapid implementation of effective drugs at the outset of MDR-TB treatment could contribute significantly to decreasing the mortality rate associated with HIV/TB co-infection. Molecular methods to detect drug resistance more rapidly could be of great value in aiding the timely administration of appropriate antibiotics to TB patients infected with drug-resistant strains.

The use of MAS-PCR assays to investigate the incidence of additional resistance to ofloxacin and/or kanamycin in MDR isolates from the Western Cape indicated a total of 25 of 136 (18.4%) as pre-XDR. A further 11 (8.1%) isolates were additionally resistant to both antibiotics.

Validation of the MAS-PCR assays by phenotypic DST of 54 MDR-TB isolates indicated clinical sensitivities of 54.5% and 90% for the GyrA D94G and *rrs* A1401G assays, respectively, and specificities of 100% for both assays. Though GyrA D94G is most frequently observed, it only accounts for up to 35% of global ofloxacin resistance. Another frequently observed GyrA mutation, A90V, was identified in this study in four of five ofloxacin-resistant isolates that did not carry the D94G substitution. In the remaining isolate, a mutation outside the QRDR of GyrA or within the QRDR of GyrB, or active efflux may account for ofloxacin resistance. Similarly, kanamycin resistance in the one isolate that did not carry an *rrs* A1401G mutation suggests the role of an alternative kanamycin resistance mechanism, such as an alternative mutation in *rrs* or increased expression of the gene encoding the aminoglycoside acetyltransferase Eis.

MAS-PCR analysis of the non-MDR isolates identified none that carried both GyrA D94G and *rrs* A1401G mutations. The fact that 2 (4.9%) isoniazid monoresistant and 17 (16.8%) susceptible isolates carried an *rrs* A1401G mutation alone, and that 21 (15.4%) of the MDR isolates carried *rrs* A1401G alone and only 4 (2.9%) harboured GyrA D94G alone, may suggest that resistance to kanamycin is more readily acquired than resistance to ofloxacin. Additionally, the fact that 11 of 15 (73.3%) ofloxacin-resistant MDR isolates also carry an *rrs* A1401G mutation suggests that ofloxacin resistance in the absence of kanamycin resistance is rare. Identification of kanamycin resistance may therefore serve as a marker for the detection of pre-XDR isolates with the potential to progress to XDR.

The finding that 30 of 36 (83.3%) MDR-TB isolates with additional resistance to ofloxacin and/or kanamycin carry a KatG S315T mutation (data not shown) suggests an association between this mutation and acquisition of additional SLD resistance determinants. The KatG S315T mutation has little effect on enzymatic activity, and therefore confers minimal fitness cost, which may enable isolates carrying this mutation to more readily acquire and maintain additional resistance determinants.

In this study, up to 54.5% of ofloxacin resistance may be detected if due to a GyrA D94G substitution, and this may be increased to 90.9% if GyrA A90V detection is included in the MAS-PCR assay. Though the distribution of resistance determinants may vary geographically, this study indicates that rapid assays for the detection of GyrA D94G and A90V and *rrs* A1401G could identify up to 90% of XDR-TB cases in the Western Cape. Detection of XDR-TB may be enhanced by screening *tlyA* for capreomycin-resistance determinants. While molecular assays may not replace phenotypic DST, they may provide an essential initial diagnosis that can inform individualized treatment regimens in patients harbouring lethal, drug-resistant strains of *M. tuberculosis*.

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