Rapid genotypic assays to identify drug-resistant *Mycobacterium tuberculosis* in South Africa

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**Objectives**: Molecular assays to detect drug resistance in *Mycobacterium tuberculosis* are more rapid than standard drug susceptibility testing. To evaluate the efficacy of such assays in this setting, the GenoType® MTBDRplus assay (HAIN Lifescience) and multiplex allele-specific PCR assays were carried out.

**Methods**: The GenoType® MTBDRplus assay was evaluated for the detection of rifampicin and isoniazid resistance in 223 *M. tuberculosis* isolates of known phenotypic drug sensitivity. The presence of KatG S315T and *inhA* C–15T mutations that confer isoniazid resistance was determined using multiplex allele-specific PCR assays. The relationship between isolate lineage and resistance determinant was investigated by spoligotyping and mycobacterial interspersed repetitive unit–variable number tandem repeat analysis.

**Results**: The GenoType® MTBDRplus assay detected multidrug-resistant, isoniazid-monoresistant and rifampicin-monoresistant isolates with sensitivities of 91.5%, 56.1% and 70%, respectively. Multiplex allele-specific PCR detected isoniazid resistance in 91.5% of the MDR isolates and 53.7% of the isoniazid-monoresistant isolates. The W-Beijing lineage was overrepresented in the MDR subgroup of strains (odds ratio, 3.29; 95% confidence interval, 1.76–6.16).

**Conclusions**: A proportion of isoniazid resistance, particularly in isoniazid-monoresistant isolates of lineage X3, is due to resistance determinants other than KatG S315T and *inhA* C–15T. The fact that these isolates will be indicated as drug susceptible highlights the need for determining local patterns of resistance mutations to provide users with information regarding the capabilities of rapid genotypic assays.

Keywords: isoniazid, rifampicin, resistance, epidemiology

**Introduction**

The rapid emergence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB), defined as resistance to rifampicin and isoniazid,¹ poses a serious threat to the treatment of tuberculosis (TB) worldwide. Treatment of MDR isolates requires the use of more costly and more toxic second-line drugs.² A delay in the diagnosis of MDR-TB associated with standard drug susceptibility testing (DST) methods is likely to contribute to the transmission of resistant isolates. Line probe assays, direct DNA sequencing, molecular beacon analysis and biprobe analysis rapidly detect drug resistance accurately, but these methods are usually expensive and require the training of specialized personnel. Recently, the WHO has recommended the use of line probe assays for the rapid diagnosis of MDR-TB worldwide, and the South African National Department of Health has indicated plans to roll out the GenoType® MTBDRplus assay nationwide.

As rifampicin monoresistance is relatively rare, detection of rifampicin resistance determinants is a good indicator of MDR-TB. Point mutations in *rpoB*, encoding the β-subunit of DNA-dependent RNA polymerase, have been shown to account for the majority of rifampicin resistance worldwide.³ More specifically, 95% of these rifampicin resistance-causing mutations are located within an 81 bp hotspot region of *rpoB*.
spanning codons 507–533, known as the rifampicin resistance-determining region (RRDR). Mutations in codons 516, 526 and 531 of RpoB are most commonly associated with high-level rifampicin resistance, but the frequency with which these mutations are observed varies by geographic location.

Although isoniazid resistance in *M. tuberculosis* is more complex in that a number of genes are implicated, up to 95% of isoniazid resistance may be due to mutations in *katG*. The most frequently observed alteration in *katG* is a serine-to-threonine substitution at codon 315 (S315T), located within the active site of the catalase moiety of *katG*. The S315T alteration in this proposed binding site of isoniazid prevents *katG*-mediated activation of isoniazid. Additionally, mutations in the promoter region of *inhA* account for 8% to 20% of isoniazid resistance in *M. tuberculosis*. A C-to-T substitution at nucleotide –15 results in the overexpression of InhA, an NADH-dependent enoyl-acyl reductase involved in mycolic acid synthesis, and isoniazid resistance arises as a result of drug titration.

The GenoType® MTBDRplus assay is a reverse hybridization-based technique that can rapidly detect mutations most frequently associated with MDR-TB. Multiplex allele-specific (MAS) PCR assays designed to detect isoniazid resistance due to *katG* S315T and *inhA* C–15T were carried out, as described previously. A mismatch between a primer and the targeted mutation enabled the detection of the mutation using these assays. In the presence of a *katG* S315T mutation, an amplicon of 435 bp was obtained, while the presence of wild-type *katG* resulted in the amplification of a 293 bp product. The *inhA* MAS-PCR assay resulted in two amplification products (451 and 119 bp) in wild-type isolates. In the presence of a C–15T mutation in *inhA*, only the larger 451 bp product was observed. Using sequencing, the presence or absence of *katG* S315T and *C–15T inhA* was confirmed in wild-type and mutant isolates, and these isolates were used as controls in subsequent MAS-PCR assays.

**Materials and methods**

**Bacterial isolates and susceptibility testing**

*M. tuberculosis* isolates obtained from patients at Groote Schuur Hospital (GSH), Cape Town, in 2006 were identified by Ziehl–Neelsen staining and *M. tuberculosis*-specific PCR. DST for rifampicin and isoniazid was carried out on 652 isolates in the diagnostic laboratory at GSH using the BACTEC MGIT 960 system (MGIT 960; Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Standard critical concentrations of 1 mg/L for rifampicin and isoniazid was carried out on 652 isolates in the diagnostic laboratory at GSH using the BACTEC MGIT 960 system (MGIT 960; Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Standard critical concentrations of 1 mg/L for rifampicin and 0.1 mg/L for isoniazid were used.

**Genomic DNA preparation**

Colonies from *M. tuberculosis* isolates grown on Lowenstein–Jensen-slanted agar were resuspended in 500 μL of distilled H₂O and heat-inactivated at 80°C for 1 h. Genomic DNA was extracted as described previously and resuspended in 55 μL of distilled H₂O. Alternatively, genomic DNA was obtained from cells in 1 mL of MGIT culture by centrifugation of samples and resuspension in 500 μL of distilled H₂O before heat-killing at 80°C for 1 h.

**GenoType® MTBDRplus assays**

The GenoType® MTBDRplus assay (HAIN Lifescience) was carried out to detect rifampicin and isoniazid resistance with laboratory staff blind to phenotypic drug resistance results. Following multiplex PCR and reverse hybridization, the mutations most frequently associated with MDR were detected. Amplification reactions consisted of 35 μL primer nucleotide mix (GenoType® MTBDRplus), 3 mM MgCl₂, 1 U of HotStartplus Taq DNA polymerase (Qiagen) and 5–25 ng of genomic DNA. Hybridization and stringency washes were carried out according to the manufacturer’s instructions using a TwinCubator (HAIN Lifescience).

**MAS-PCR assays**

MAS-PCR assays for the detection of *KatG* S315T and *inhA* C–15T were carried out, as described previously. A mismatch between a primer and the targeted mutation enabled the detection of the mutation using these assays. In the presence of a *KatG* S315T mutation, an amplicon of 435 bp was obtained, while the presence of wild-type *KatG* resulted in the amplification of a 293 bp product. The *inhA* MAS-PCR assay resulted in two amplification products (451 and 119 bp) in wild-type isolates. In the presence of a C–15T mutation in *inhA*, only the larger 451 bp product was observed. Using sequencing, the presence or absence of *KatG* S315T and C–15T *inhA* was confirmed in wild-type and mutant isolates, and these isolates were used as controls in subsequent MAS-PCR assays.

**DNA sequencing and analysis**

Following agarose gel electrophoresis, PCR products of interest were excised and purified from gels using the MinElute Gel Extraction Kit (Qiagen). Automated DNA sequencing of these products was carried out at the Stellenbosch University DNA Sequencing Facility, Cape Town, using an ABI 3130 Genetic Analyser. DNA sequences were analysed using DNAMAN (version 4.0, Lynnon Biosoft). Nucleotide and amino acid sequences obtained were compared with existing sequences in the database using BLAST.

**Genotyping**

Spoligotyping was carried out using genomic DNA from all isolates. The hybridization patterns obtained were analysed using the SPOTCLUST SpolDB3-based model (http://cgi2.cs.rpi.edu/~bennek/SPOTCLUST.html) to assign isolates to specific genotype families. The genotype of the isolates was further differentiated using 12-locus mycobacterial interspersed repetitive unit (MIRU)–variable number tandem repeat (VNTR) analysis with modifications for use on an ABI 3100 Analyser (Applied Biosystems). The BURST algorithm (http://eburst.mlst.net/) was used to describe the relationship between MIRU–VNTR genotypes.

**Results**

**DST**

DST was carried out on 652 *M. tuberculosis* isolates obtained from patients at GSH in 2006. A total of 95 isolates were identified as MDR, 67 isolates as isoniazid-monoresistant and 11 as rifampicin-monoresistant. Of these, 82 MDR, 41 isoniazid-resistant and 10 rifampicin-monoresistant isolates were included (Table 1), as the remaining isolates were non-viable when the study commenced. A total of 90 randomly selected isolates susceptible to both rifampicin and isoniazid were included as controls in both genotypic assays and spoligotyping. The genotypic assays were evaluated against the gold standard DST results; sequence analysis was carried out on isolates with discrepancies.
Use of GenoType MTBDRplus assays for detection of isoniazid and rifampicin resistance

The GenoType MTBDRplus assay was evaluated for determining the isoniazid and rifampicin susceptibilities of all 223 M. tuberculosis isolates included in this study. The assay indicated isoniazid resistance in 75 of 82 (91.5%) MDR isolates and in 26 of 41 (63.4%) isoniazid-monoresistant isolates (Table 1). Both KatG S315T and inhA C-15T mutations were detected in 10 of 82 MDR isolates, while no isoniazid-monoresistant isolates harboured both mutations. One isolate produced no M. tuberculosis complex (TUB) hybridization signal, indicating that it is not M. tuberculosis. The isolate was confirmed as M. tuberculosis using the GenoType Mycobacterium CM assay (HAIN Lifescience). In one isolate, complete absence of hybridization to any katG probes was noted, suggesting a katG deletion in this isolate. Both wild-type and mutant inhA hybridization signals were obtained in two isoniazid-monoresistant isolates, suggesting heteroresistance, and were therefore interpreted as isoniazid-resistant.

Resistance to rifampicin was indicated in 80 of 82 (97.6%) MDR isolates (Table 1). Of these, a serine-to-leucine amino acid substitution at codon 516 (S531L) in RpoB was identified in 71 isolates. One isolate carried an H526D substitution and two isolates carried a D516V substitution. Rifampicin resistance could be determined in five isolates due to the absence of wild-type signals corresponding to mutations at codons 516 (1), 526 (2) and either 531 or 533 (2); however, the specific mutation could not be detected as no corresponding mutant probe was indicated in the assay. One isolate had all eight rpoB wild-type probes and the MUT3 probe binding, implying either a mixed infection or the presence of a heterogeneous isolate that is only partially resistant to rifampicin. Rifampicin resistance detected (70%) in the rifampicin-monoresistant isolates was due to S531L substitution (3), a mutation at codons 518 (1), 526 (1), either 531 or 533 (1) and either 514 or 515 (1). Three of 41 isoniazid-monoresistant isolates were indicated as rifampicin-resistant; two through the absence of signal from RpoB wild-type probes 3 and 4, suggesting mutations at codons 515/516, and one through the absence of a signal from wild-type probe 7, suggesting a mutation at codon 526.

Of the 90 susceptible isolates included, 89 (98.9%) were indicated as susceptible to both antibiotics using the GenoType MTBDRplus assay (Table 1). In one susceptible isolate, a signal was obtained from both the inhA wild-type and the MUT1 probes. This may be indicative of isolate heterogeneity or a mixed infection.

Use of MAS-PCR assays for detection of KatG S315T and inhA C-15T

MAS-PCR assays designed to detect the S315T KatG and inhA C-15T mutations were carried out on all isolates. Products corresponding to the wild-type genotype were obtained from the katG and inhA wild-type controls, and products indicating a KatG S315T substitution or an inhA C-15T mutation were obtained from either of the two mutant control isolates. Amplicons representative of wild-type isolates were obtained from all 25 isoniazid-susceptible control isolates included in both assays (Table 2). Following MAS-PCR analysis of 82 MDR isolates, 75 (91.5%) were identified as harbouring KatG...
S315T (53.7%), inha C−15T (25.6%) or both (12.2%) mutations, while neither mutation was detected in 7 isolates (Table 2). Of the 41 isoniazid-monoresistant isolates, 12 (29.3%) had the KatG S315T substitution, 10 (24.4%) had the inha C−15T mutation and no isolates had both mutations. Neither mutation was detected in 16 of 41 (39.0%) isoniazid-monoresistant isolates. Sequence analysis confirmed the presence of KatG S315T in one isolate and inha C−15T in another isolate, as indicated by the GenoType MTBDRplus assay (Table 2). Two isolates produced no amplicons in either assay. From the same isolate that lacked hybridization signal from all katG probes in the GenoType MTBDRplus assay, amplicons corresponding to both internal and full-length katG were not obtained (data not shown), suggesting that katG may be deleted in this isolate. Amplicons corresponding to wild-type inha were obtained from this isolate.

Relationship between genotype and resistance phenotype

The W-Beijing lineage was overrepresented in the MDR subgroup of strains (59.8%) (odds ratio, 3.29; 95% confidence interval, 1.76–6.16) (Figure 1), which is in accordance with previous observations. A high proportion of these MDR W-Beijing strains (85.7%) harbour S531L mutations in RpoB. In contrast, 10% (1 of 10) of rifampicin-monoresistant isolates, 26.8% (11 of 41) of isoniazid-monoresistant isolates and 31.1% (28 of 90) of isolates susceptible to rifampicin and isoniazid were W-Beijing isolates. A total of 11% (9 of MDR isolates were of the LAM3/F11 family, compared with 9.8% (4) of isoniazid-monoresistant isolates, 18.9% (17) of susceptible isolates and, interestingly, 70% (7 of 10) of rifampicin-monoresistant isolates. Within the isoniazid-monoresistant group of isolates, the X3 lineage (24.4%) was most prevalent. The remaining isolates were T1, T3, T4, T5, LAM4, LAM9, EA1, H1, H3, S, X1, X2, X3, CAS, Family 36 or H37Rv. Unique profiles were obtained for 3 MDR isolates, 3 isoniazid-monoresistant isolates and 15 susceptible control isolates.

Further discrimination of the 49 W-Beijing MDR isolates and the 7 LAM3/F11 rifampicin-monoresistant isolates was carried out by MIRU–VNTR. MDR W-Beijing isolates harbouring the inha C−15T isoniazid resistance determinant were found to cluster together (Figure 2a). These isolates were also resistant to rifampicin due to an S531L mutation in RpoB. All LAM3/F11 rifampicin-monoresistant isolates cluster within the same

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**Table 2.** Evaluation of MAS-PCR assays for the detection of isoniazid resistance in M. tuberculosis

<table>
<thead>
<tr>
<th>Isolates (n)</th>
<th>KatG S315T</th>
<th>inha C−15T</th>
<th>both</th>
<th>neither</th>
<th>ND</th>
<th>R detected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR (82)</td>
<td>44</td>
<td>21</td>
<td>10</td>
<td>7</td>
<td>0</td>
<td>91.5</td>
</tr>
<tr>
<td>INHR (41)</td>
<td>12</td>
<td>10</td>
<td>0</td>
<td>16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>53.7</td>
</tr>
<tr>
<td>RIFR (10)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RIFS INHS (25)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

INH, isoniazid; R, rifampicin; R, resistance; ND, not determined; MDR, multidrug-resistant; <sup>a</sup>, resistant; <sup>b</sup>, susceptible.

<sup>a</sup>Sequence analysis confirmed KatG S315T (1) and inha C−15T (1) mutations, detected by GenoType assay, in two isolates.

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**Figure 1.** Relationship between resistance phenotype and genotype of M. tuberculosis isolates as determined by spoligotyping. MDR, multidrug-resistant; INH, isoniazid-monoresistant; RIF, rifampicin-monoresistant; S, susceptible. Other isolate lineages include T1, T3, T4, T5, LAM4, LAM9, EA1, H1, H3, S, X1, X2, X3, CAS, Family 36 or H37Rv.

**Figure 2.** Genetic clustering of W-Beijing MDR isolates (a) and rifampicin-monoresistant LAM3/F11 isolates (b) as determined by MIRU–VNTR analysis. Each isolate is represented by a shape. Isolates connected by a straight line differ from one another at a single allele. Outliers differ from all other isolates at two or more alleles.
Identification of drug resistance in *M. tuberculosis*

MIRU–VNTR group (Figure 2b). The MIRU–VNTR digital profiles may be found in Table S1, available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

**Discussion**

The emergence of drug-resistant isolates of *M. tuberculosis* poses a serious threat to global TB control. Assays for the rapid detection of resistance, such as the GenoType™ MTBDRplus assay and MAS-PCR, enable earlier detection of resistance and thereby tailoring of treatment regimens. In this study, overall MDR, isoniazid monoresistance and rifampicin monoresistance were detected by the GenoType™ MTBDRplus assay, with sensitivities of 91.5%, 56.1% and 70%, respectively. This is in contrast to a large-scale evaluation of the GenoType™ MTBDRplus assay recently undertaken in the Western Cape, in which sensitivities of 97.7%, 96.5% and 72.7% for MDR, isoniazid monoresistance and rifampicin monoresistance, respectively, were reported. However, specificities of 98.9%, 98.9% and 100.0% for MDR, isoniazid resistance and rifampicin resistance detection, respectively, are similar to those described by Barnard et al.

Comparable results for the detection of isoniazid resistance were obtained using the GenoType™ MTBDRplus and MAS-PCR assays. Both assays indicated the same genetic basis of isoniazid resistance in 75 of 82 (91.5%) MDR isolates and in 34 of 41 (82.9%) isoniazid-monoresistant isolates. The katG S315T mutation accounts for isoniazid resistance in 53.7% of isoniazid-monoresistant isolates. The katG S315T mutation was observed in 25.6% of MDR isolates and in 24.4% of isoniazid-monoresistant isolates. Providing further evidence for an association between katG S315T and MDR-TB, a C→15T mutation in the promoter region of *inha* was observed in 25.6% of MDR isolates and in 24.4% of isoniazid-monoresistant isolates. The MDR W-Beijing isolates that harbour an *inha* C→15T mutation cluster together following MIRU–VNTR analysis, suggesting dissemination of a clonal lineage. Isoniazid resistance could not be determined in seven MDR isolates that would have been interpreted as isoniazid-susceptible by both genotypic assays. Although both assays suggested deletion of *katG*, and hence isoniazid resistance, in one isolate, neither assay could confirm resistance due to the failure to indicate a specific resistance determinant.

The fact that 36.6% and 46.3% of isoniazid resistance in isoniazid-monoresistant isolates was not detected by either GenoType™ MTBDRplus assay or MAS-PCR, respectively, is of particular concern. This high prevalence of isoniazid-resistant isolates harbouring neither KatG S315T nor *inha* C→15T contrasts with reports attributing isoniazid resistance in the majority of isolates to either or both of these mutations. It may be that less prevalent mutations in *katG*, *inha*, *ndh* and *oxyR-ahpc*, or in a novel marker, play a role in isoniazid resistance in these isolates. Since 7 of the 13 isoniazid-monoresistant isolates that were indicated as susceptible in these assays are of the X3 lineage, this may represent dissemination of a dominant isoniazid-monoresistant clone with a unique isoniazid resistance determinant in the Western Cape.

The GenoType™ MTBDRplus assay was effective at identifying rifampicin resistance in the MDR isolates, with a sensitivity of 97.6%. Rifampicin resistance in the two isolates indicated as rifampicin-susceptible is more than likely due to mutations outside the RRDR of *rpoB* or possibly in an as yet unidentified locus. *rpoB* S531L accounted for rifampicin resistance in 80.4% of MDR and rifampicin-monoresistant isolates, somewhat higher than reported worldwide, but comparable to the 70.5% reported in this geographical area. In contrast, the proportion of mutations at codons 526 (4.3%) and 516 (3.3%) of *rpoB* is lower than that observed elsewhere. The low fitness cost associated with *rpoB* S531L may account for the high frequency with which it is observed.

A caveat in the interpretation of the GenoType™ MTBDRplus assay with respect to rifampicin detection is that resistance may be indicated by the absence of a wild-type hybridization signal alone, without confirmation by a mutant probe signal. As a wild-type probe may not hybridize due to a mutation in the RRDR that is not associated with a resistance phenotype, such rifampicin-susceptible isolates would be called resistant and may lead to the unnecessary removal of rifampicin from treatment regimens. In addition, isolates indicated as resistant due to a mutation at codon 533 may be susceptible, and similar caution should be observed. Of greater concern, GenoType™ MTBDRplus failed to detect rifampicin monoresistance in 3 of 10 (30%) cases. Treatment of these cases with standard TB regimens may hasten the emergence of MDR isolates in these patients. Furthermore, 7 of 10 rifampicin-monoresistant *LAM3/F11* isolates fall within the same MIRU–VNTR cluster, suggesting expansion of a rifampicin-monoresistant *LAM3/F11* strain.

In conclusion, the results of this study illustrate that the geographical distribution of mutations resulting in drug resistance in *M. tuberculosis* in this region is different from that reported elsewhere. This may have important implications for the roll-out of rapid genotypic tests to identify drug-resistant *M. tuberculosis*, which may impact the progression of *M. tuberculosis* to MDR- and, ultimately, XDR-TB. In particular, we have identified a high proportion of what may be clonally related isoniazid- and rifampicin-monoresistant strains that are not identified as resistant by the GenoType™ MTBDRplus assay. If rapid genotypic assays for the detection of drug resistance are to be widely used, there is a need to continually monitor local patterns of drug-resistance mutations to ensure that if clonal groups of *M. tuberculosis* do emerge, they are properly diagnosed as drug-resistant.

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**Transparency declarations**

None to declare.

**Supplementary data**

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).
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References