Performance of MTBDR plus for detecting high/low levels of Mycobacterium tuberculosis resistance to isoniazid

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OBJECTIVE: To evaluate the performance of MTBDR plus over MTBDR.

RESULTS: In 113 isolates, MTBDR plus detected all 76 RMP-resistant (RMP-R) strains and all 64 INH-resistant (INH-R) strains with KatG-315 mutations, 59 of which displayed a high level of INH resistance. It also identified 18 strains undetectable by MTBDR, without mutation in KatG-315 but with a −15 C→T mutation in the regulatory region of inhA, of which 15 displayed a low level of INH resistance. Thirteen INH-R strains, which mainly harboured mutations in KatG at positions other than 315, were undetected by MTBDR plus.

CONCLUSION: MTBDR plus retains the accuracy shown by MTBDR in detecting RMP resistance and is more sensitive in detecting INH resistance (86% vs. 67%), particularly at low levels (minimum inhibitory concentration <1 mg/l, 69% vs. 17%). The negative predictive value of the test (the probability of a strain with a wild-type test being susceptible to INH) is >98% when the rate of INH is <10%, as it is in France.

KEY WORDS: tuberculosis; drug resistance; isoniazid; molecular diagnosis; negative predictive value

THE RAPID IDENTIFICATION of drug resistance, particularly of multidrug resistance (MDR), in clinical isolates of Mycobacterium tuberculosis is an important challenge to ensure rapid and adequate chemotherapy of tuberculosis (TB) and to prevent the spread of resistant strains. Rifampicin (RMP) and isoniazid (INH) are the two main drugs used for the treatment of TB. More than 95% of RMP-resistant M. tuberculosis strains have a mutation in the hotspot region (codons 505 to 533) of the rpoB gene coding for the RNA polymerase subunit β.1–3 The mutations causing INH resistance, on the other hand, are located in several genes. The main mutation is located at codon 315 of the katG gene coding for the catalase-peroxidase KatG. The second most frequent mutations, −15 C→T and −8 T→G or T→A in the fabG-inhA regulatory region, affect the level of expression of the target InhA, the enoyl-ACP-reductase.3–13 Finally, several mutations have also been reported in the InhA protein, particularly S94A.14 Besides these frequent mutations, more than 150 distinct mutations scattered throughout the katG gene at positions other than codon 315 have been reported in the literature.15 Mutations in genes other than katG and inhA have also been reported, such as ndb, oxyR-aphC, kasa and furA (see the review by Vilchèze and Jacobs),16 but the possible role of these mutations in INH resistance has not been clearly established.

Several molecular methods have been developed to predict drug resistance in M. tuberculosis. We recently evaluated the first version of a commercial DNA strip assay, Genotype® MTBDR (Hain LifeScience GmbH, Nehren, Germany), which detects resistance to RMP and INH.17 The test is based on reverse hybridisation of rpoB and katG amplicons to immobilised membrane-bound probes, allowing the detection of the most frequent mutations in rpoB (Ser531Leu, His526Tyr, His526Asp and Asp516Val) and in katG (Ser315Thr).17–24 Our results showed that the test detected 100% of the strains resistant to RMP but only 67% of those resistant to INH; 89% of the strains with a high level of resistance to INH and 17% with a low level of resistance.17

The aim of the present study was to evaluate the performance of the Genotype® MTBDR plus assay, an upgraded version of Genotype® MTBDR in the
detection of RMP and INH resistance, and to assess its advantages over the earlier version of the test.

**MATERIALS AND METHODS**

**Strains**

We included 113 *M. tuberculosis* strains: 94 clinical strains collected between 2003 and 2004 at the National Reference Centre for Mycobacteria, isolated from TB cases diagnosed in France, and 19 strains provided by the World Health Organization (WHO), all of which had already been used for the evaluation of GenoType® MTBDR.\(^{17}\) Drug susceptibility testing (DST) was performed using the proportion method on Löwenstein-Jensen medium,\(^{25}\) using concentrations of 40 mg/l for RMP and 0.1, 0.2, 1 and 10 mg/l for INH. A low level of resistance to INH was defined as resistance to INH concentrations >0.1 but <1 mg/l of INH (H0.1), while a high level of resistance was defined as resistance to ≥1 mg/l. In the present study, among those strains showing resistance to INH ≥1 mg/l, we distinguished those resistant to ≥1 mg/l but <10 mg/l (H1) from those with a very high level of resistance (≥10 mg/l, H10).

Among the 113 strains, 76 were resistant to RMP and 95 were resistant to INH; 72 were resistant to both (MDR).

**DNA sequencing of the −15 regulatory region of the fabG-inhA operon**

We previously reported the DNA sequencing results of katG, inhA and the fabG-inhA regulatory region for strains with no S315T mutation in KatG.\(^{17}\) We also determined the sequences of inhA and the fabG-inhA regulatory region in the 62 strains with a S315T mutation in the KatG protein, using the sequencing protocol previously described.\(^{17}\)

**MTBDR plus test**

MTBDR plus is an upgraded version of the MTBDR test that presents as a strip coated with 27 probes (Figure). The absence of hybridisation with the wild-type probes indicates the presence of mutations that could potentially lead to RMP and INH resistance in *M. tuberculosis*. For INH resistance, the presence of the most frequently observed mutations is confirmed by positive hybridisation with six mutant probes covering katG 315 and the positions -16, -13 and -8 in the regulatory region of fabG-inhA (Figure).\(^{26}\) For RMP resistance, three additional rpoB wild type probes have been added to the MTBDR plus test, which now covers codons 506 to 533.

The amplification reactions and the Genotype® MTBDR plus test were performed as recommended by the manufacturer.

**RESULTS**

Typical hybridisation patterns obtained with the MTBDR plus DNA strips are shown in the Figure.

The results obtained for rpoB in the 76 RMP-resistant strains were in total agreement with the sequencing results: all of the 76 RMP-resistant strains were carrying rpoB mutations that were correctly identified by the test, and no mutations were found among the 37 RMP-susceptible strains. As expected, mutation S531L was predominant (47%). One of the RMP-resistant strains found by sequencing to carry three mutations, F505L, L511P and S531C, yielded negative hybridisation signals with the wild-type probes WT1 (covering position 505), WT2 (position 511) and WT8 (position 531).

Regarding the results obtained with MTBDR plus for the katG and fabG-inhA regulatory region probes, we observed that the corresponding hybridisation intensities were faint compared to those observed at the level of the rpoB probes (Figure), but there was no ambiguity in the reading of the hybridisation signals. Among the 95 INH-resistant strains tested, all the
64 strains (67%) that exhibited an amino acid substitution at position 315 of the KatG protein after sequencing were identified by MTBDR 

**Table 1** Sequencing data and drug resistance phenotypes for the 95 INH-resistant *M. tuberculosis* isolates analysed by the MTBDRplus assay

<table>
<thead>
<tr>
<th>Sequencing data for <em>katG</em> (S315), <em>inhA</em> and the <em>fabG-inhA</em> regulatory region</th>
<th>Strains exhibiting INH resistance phenotype</th>
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</thead>
<tbody>
<tr>
<td><strong>katG</strong> (S315)</td>
<td><strong>inhA</strong></td>
</tr>
<tr>
<td>S315T</td>
<td>wt</td>
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<td>wt</td>
<td>S94A</td>
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<td>wt</td>
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</tbody>
</table>

*H1, resistance to \( \geq 1 \) but <10 mg/l; H10, resistance to \( \geq 10 \) mg/l.

1Strains with other mutations in KatG (n = 3; L141F, E553K and F658V) or no identifiable mutation (n = 3).

2Strain with a KatG mutation G494D.

3Strains with mutations R595STOP, G494D and W341G in KatG.

INH = isoniazid; wt = wild-type.

64 strains (67%) that exhibited an amino acid substitution at position 315 of the KatG protein after sequencing were identified by MTBDR **plus** as carrying such a mutation (Table 1). Two of these 64 strains, which displayed a low level of resistance to INH (H0.1), had an S315N mutation and were detected by the test through negative hybridisation signal with the wild-type 315 probe. The 62 other strains displayed the S315T mutation, 49 of which had no other mutation in *inhA* and the *fabG-inhA* regulatory region. Almost all 49 strains were resistant to H1 (42/49, 86%) or to H10 (4/49, 8%), whereas only three were resistant to H0.1 (Table 1). Among the 62 strains carrying S315T, 10 were shown by both MTBDR **plus** and DNA sequencing to cumulate a −15 C→T substitution in the *fabG-inhA* regulatory region, the majority of which (7/10) were resistant to H10 (Table 1). An additional S315T strain, which was resistant to H1, was found by the test to cumulate a −8 T→C substitution in the *fabG-inhA* regulatory region (Table 1). Finally, DNA sequencing of the promoter region indicated that the last two S315T strains, which were resistant to H1, had a −102 G→A and a −47 G→A mutation, respectively, mutations that were not detected by the test.

Among the 31/95 INH-resistant strains (33%) that had no mutation at codon 315 in *katG*, the test correctly identified all of the 18 strains (58%) that were shown by sequencing to carry a −15 C→T substitution in the *fabG-inhA* regulatory region (Table 1); the majority of them (15/18, 83%) displayed a low level of resistance to INH (H0.1), and only three were resistant to H1 (Table 1). Five of these 18 strains cumulated either an S94A mutation in InhA (n = 4, resistant to H0.1) or a −102 G→A substitution (n = 1, resistant to H1).

The last 13 INH-resistant strains, which were shown by both DNA sequencing and by MTBDR **plus** to have no mutation at codon 315 in KatG, were found to have no mutation in the *fabG-inhA* regulatory region by both genetic approaches (Table 1). Three of these 13 strains were shown by DNA sequencing to have an S94A mutation in InhA, while seven displayed amino acid substitutions in KatG at positions other than 315. As expected, these 10 strains were not detected by MTBDR **plus**. The last three strains had no detectable mutations by DNA sequencing in either KatG or InhA (see Table 1), and were not detected by the test.

**DISCUSSION**

In this study the new MTBDR **plus** test detected 86% of the 95 INH-resistant strains isolated in France, compared to 67% with the earlier version of the test. It is of note that the new test was able to detect mutations in 94% of the INH-resistant strains with a high level of resistance (minimum inhibitory concentration [MIC] \( \geq 1 \) mg/l), and 69% of the strains with a low level of INH resistance (>0.1 but <1 mg/l), while the first version of the test detected 89% of the highly resistant strains, but only 17% of the strains with a low level of resistance. Our results therefore demonstrate that the advantage of the new MTBDR **plus** test over the first version of the test (+19%) lies mainly in its ability to detect strains with a low level of INH resistance (+52%), with a slight improvement in its efficiency (+5%) in detecting strains with high-level resistance. In the subset of 72 strains that were also resistant to RMP (MDR), MTBDR **plus** detected RMP resistance in all and multiresistance (RMP + INH resistance) in 64 (89%).

In the panel of strains included in the present study, S315T in KatG was found to be the most frequent mutation in INH-resistant strains (65%), and was confirmed to confer alone a high level of resistance to INH, as 42/49 (85%) strains that had a S315T mutation but no mutation in the *fabG-inhA* regulatory region were found to be resistant to H1 and 4/49 to H10. Seven of the 10 strains that were found to cumulate mutations S315T in KatG and −15 C→T in the *fabG-inhA* regulatory region displayed a very high level of resistance (to H10), suggesting that the two mutations act synergistically.

The −15 C→T mutation in the *fabG-inhA* regulatory region screened by the MTBDR **plus** test was found to be relatively frequent (n = 28, 29%) in our set of strains, and the majority (n = 18, 19%) did not cumulate a S315T mutation in KatG. −15 C→T alone conferred a low level of INH resistance (MIC >0.1 but <1 mg/l) in 15 of these 18 strains, confirming previous reports by Guo et al. and Gali et al.27,28 By contrast, the −8 T→C mutation was detected in only
one strain resistant to H1, cumulating an S315T mutation in KatG (Table 1), while we did not find the T→A mutation at the same position. This finding confirms the rarity of these two mutational events previously reported to confer a very low level of INH resistance (MIC 0.25 mg/l, when present alone).27-29

Mutations at position −8 might therefore have a limited impact and may not be systematically associated with resistance to INH. Two other mutations (−102 G→A and −47 G→C) in the fabG-inhA regulatory region, not screened by the MTBDR plus test, were detected by DNA sequencing in association with either S315T or −15 C→T in three of the strains resistant to H1 (see Table 1). Apart from positions −16, −15 and −8, several nucleotide substitutions in the fabG-inhA regulatory region have been reported previously in INH-resistant strains, but neither −102 nor −47 have been observed previously and their role in INH resistance remains unclear.

Finally, 13 INH-resistant strains were not detected by the MTBDR plus test (14%). They exhibited either a high level (n = 4) or a low level (n = 9) of INH resistance. Seven had mutations in KatG at positions different from 315, three had an S94A mutation in InhA, and no mutation was detectable in the three remaining strains.

Miotto et al. recently reported the performance of the MTBDR plus assay in the detection of INH resistance in 173 INH-resistant strains isolated in Italy that had previously been analysed with the earlier test.32 They reported that, compared to the earlier version, MTBDR plus correctly identified an additional 12% of INH-resistant strains, leading to a final rate of detection of 79% of INH-resistant strains. In the study, in which the level of INH resistance of the strains was not specified, the strains with mutation in the regulatory region of inhA but no KatG-315 mutation represented 12% of the total number of INH-resistant strains,32 a rate slightly lower than in our study. In another study, by Hillemann et al., MTBDRplus detected 92% of 75 INH-resistant strains (all MDR strains isolated in Germany), compared to 88% detected using the earlier version of the test.26 In this study, which does not report the level of INH resistance, the small difference between the two versions of the test was explained by the fact that only 4% of the strains had a −15 C→T mutation alone. Overall, in the two studies previously published on the MTBDR plus test and in the present study, INH resistance detection ranged between 79% and 92%. Conversely, in these three studies, 8–21% of the INH-resistant strains were missed by MTBDR plus, mainly due to mutations in KatG at positions other than 315.

Based on global sensitivity values of the test ranging between 79% and 92%, it is possible to estimate the capacity of the test to predict INH susceptibility (i.e., its negative predictive value) as well as the number of detected and missed INH-resistant cases, which depends on the lack of sensitivity of the MTBDR plus test and on the prevalence of INH resistance in settings where it is used. Table 2 shows the estimated results for different examples of settings covering a wide range of prevalence of INH resistance. The calculations indicate that the MTBDR plus assay would miss at most two INH-resistant patients among 100 tested TB patients if the rate of INH resistance is <10%, which is the situation in France for new TB cases in foreign-born patients, and at most one patient in the case of a rate <5%, which is the situation for new TB cases in patients born in France. However, where INH resistance is high, particularly >20%, MTBDR plus could miss 2–14 INH-resistant patients among 100 tested TB patients.

### CONCLUSIONS

The plus version of the MTBDR kit is an easy and rapid tool for genotypic detection of RMP and INH resistance in M. tuberculosis, as published by Khuê et al.33 and by the WHO.34

### Table 2

Estimated performance of the MTBDR plus test for detecting resistance to INH in France and other epidemiological settings, according to prevalence of INH to resistance in M. tuberculosis, as published by Khuê et al.33 and by the WHO.34

<table>
<thead>
<tr>
<th>Prevalence of INH resistance in defined settings</th>
<th>Estimated negative predictive value of the test*</th>
<th>Estimated number of INH-resistant patients among 100 tested patients*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3% (France: new cases, patients born in France)</td>
<td>99.4–99.7</td>
<td>2–3</td>
</tr>
<tr>
<td>8% (France: new cases, foreign-born patients)</td>
<td>98.3–99.1</td>
<td>6–7</td>
</tr>
<tr>
<td>9% (France: previously treated patients, patients born in France)</td>
<td>98.1–99.0</td>
<td>7–8</td>
</tr>
<tr>
<td>19% (France: previously treated patients, foreign-born patients)</td>
<td>95.5–97.7</td>
<td>15–17</td>
</tr>
<tr>
<td>5–8% (e.g., new cases, Canada, Italy, Germany)</td>
<td>98.3–99.4</td>
<td>4–7</td>
</tr>
<tr>
<td>9–13% (e.g., new cases, ivory Coast, Bolivia, Peru, Philippines, Beijing China, Guandong China)</td>
<td>95.5–98.5</td>
<td>7–12</td>
</tr>
<tr>
<td>20–25% (e.g., new cases, Estonia, Georgia, Orel-Tomsk Russian Federation, Tamil Nadu India, Liaoning China, Vietnam)</td>
<td>97.7–97.6</td>
<td>16–22</td>
</tr>
<tr>
<td>40–45% (e.g., new cases, Uzbekistan, Azerbaijan, Kazakhstan)</td>
<td>87.8–93.7</td>
<td>32–40</td>
</tr>
<tr>
<td>60–70% (e.g., previously treated patients, Ukraine, Estonia, Lithuania, Kazakhstan)</td>
<td>83.3–86.9</td>
<td>48–63</td>
</tr>
</tbody>
</table>

*Simulation of the negative predictive values and numbers of detected and missed INH-resistant cases is based on global sensitivity values of the test reported in references 27 and 24 and in the present study, and ranging between 79% and 92%.

INH = isoniazid; WHO = World Health Organization.
resistance in *M. tuberculosis*. It is very efficient for detecting mutations causing RMP resistance, a surrogate for multidrug resistance, and significantly improves the detection of strains displaying a low level of INH resistance (i.e., those with −15 C→T), on which INH has some activity and can potentially be used for treatment. As −15 C→T is systematically associated with co-resistance to the second-line drug ethionamide, detection of this mutation by MTBDR plus is also of interest to orient the therapeutic decision in cases with multidrug-resistant TB. Finally, because the limit of detection of INH-resistant strains by MTBDR plus ranges from 79% to 92% (86% in the present study), it is safely applicable in predicting INH susceptibility in settings with a low prevalence of INH resistance, but should be used with caution in settings with a high prevalence of INH resistance.

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**References**


