Use of Smear-Positive Samples To Assess the PCR-Based Genotype MTBDR Assay for Rapid, Direct Detection of the 
Mycobacterium tuberculosis Complex as Well as Its 
Resistance to Isoniazid and Rifampin

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Isoniazid (INH) and rifampin (RIF) are two of the most important antituberculosis drugs, and resistance to both of these drugs can often result in treatment failure and fatal clinical outcome. Resistance to these two first-line drugs is most often attributed to mutations in the katG, inhA, and rpoB genes. Historically, the identification and testing of the susceptibility of Mycobacterium tuberculosis complex (MTBC) strains takes weeks to complete. Rapid detection of resistance using the PCR-based Genotype MTBDR assay (Hain Lifescience GmbH, Nehren, Germany) has the potential to significantly shorten the turnaround time from specimen receipt to reporting of results of susceptibility testing. Therefore, the aim of the present study was to determine (i) the sensitivity and accuracy of the Genotype MTBDR assay for the detection of MTBC strains and (ii) the ability of the assay to detect the presence of INH and RIF resistance-associated mutations in katG and rpoB from samples taken directly from smear-positive clinical specimens. The results were compared with those obtained with the reference BACTEC 460TB system combined with standard DNA sequencing analysis methods for katG, inhA, and rpoB. A total of 92 drug-resistant and 51 pansusceptible smear-positive specimens were included in the study. The Genotype MTBDR assay accurately and rapidly detected MTBC strains in 94.4% of the 143 specimens and showed a sensitivity of 94.4% for katG and 90.9% for rpoB when used directly on smear-positive specimens. The assay correctly identified INH resistance in 48 (84.2%) of the 57 specimens containing strains with resistance to high levels of INH (0.4 μg/ml) and RIF resistance in 25 (96.2%) of the 26 specimens containing RIF-resistant strains.

The greatest concern of tuberculosis control programs is the appearance of multidrug-resistant (MDR) tuberculosis and extensively drug-resistant tuberculosis, which encodes resistance not only to isoniazid (INH) and rifampin (RIF) but also to at least three of the six main classes of second-line drugs (3). The cornerstones for the effective control of drug-resistant tuberculosis are the immediate isolation of patients and rapid detection of drug-resistant strains, followed by prompt implementation of an adequate antituberculosis therapy that is based on laboratory findings. Although the use of the liquid medium-based, semiautomated, radiometric BACTEC 460TB system (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD.) has significantly reduced the turnaround time for growth detection and testing of susceptibility of Mycobacterium tuberculosis complex (MTBC) strains, the determination of drug resistance in MTBC strains still requires the generation of a viable, pure culture (6). Recently, several nonradiometric, fully automated systems that are suitable alternatives to the reference radiometric system were introduced (18). However, despite the technical and safety advantages, these systems did not reduce the overall turnaround times (18). The clinical consequence of these extended assay times for susceptibility testing is that patients with drug-resistant tuberculosis may not be adequately treated and therefore could remain infectious for longer times than patients infected with susceptible strains.

Importantly, the increased understanding of the molecular basis of resistance to antituberculosis drugs, and the consequent optimization of molecular methods, has significantly shortened the turnaround time for the detection of the presence of MTBC strains and mutations encoding drug resistance directly in clinical specimens without the need for a viable and large biomass (4, 9, 14, 17, 20). INH and RIF are the most important antituberculosis drugs, and resistance to these drugs often results in treatment failure and fatal clinical outcome (5, 13). Collective observations have shown that a variety of mutations within the catalase peroxidase (katG) gene (in strains showing 60 to 90% resistance), the enoyl-acyl carrier protein reductase (inhA) gene (15 to 43%), or the RNA polymerase β-subunit (rpoB) gene (>96%) are found in INH- and RIF-resistant strains (17, 22, 24). The multiplex PCR-based solid-phase reverse hybridization Genotype MTBDR line probe assay (Hain Lifescience GmbH, Nehren, Germany) has been shown to be a rapid and accurate method to detect the most common mutations of katG and rpoB from MTBC growth-positive cultures, either liquid or solid, and it has the potential to shorten the overall turnaround time from specimen receipt to reporting of results of susceptibility testing (7, 11).

The aims of the present retrospective study were to determine the sensitivity and accuracy of the Genotype MTBDR
assay when it is used directly on smear-positive clinical specimens and to compare the results with those obtained by the reference BACTEC 460TB system and direct DNA sequencing analysis of katG, inhA, and rpoB. The additional analysis of inhA by DNA sequencing allowed the determination of whether the inclusion of this gene in a modified Genotype MTBDR assay would further improve the performance of the test.

MATERIALS AND METHODS

Clinical specimens. One hundred forty-three acid-fast bacillus smear-positive sputum specimens were analyzed. These specimens included clinical specimens that had been received for routine mycobacterial testing between January 2000 and October 2005 and that had been shown to be MTBC positive by Amplified Mycobacterium Tuberculosis Direct tests (Gen-Probe Incorporated, San Diego, CA). Only one specimen per patient was analyzed. The specimens were initially digested and decontaminated by using a modified Petroff's NaOH method (23). After decontamination, the concentrated sediment was suspended in 3.0 ml sterile phosphate-buffered saline (pH 6.6), and smear preparations were prepared by adding 0.1 ml of sediment using the Ziehl-Neelsen acid-fast staining method (10). After incubation for growth detection, the leftover sediment was stored at −80°C. This leftover sediment was later thawed and used for Genotype MTBDR testing.

Growth detection and conventional susceptibility testing. Routine media including a BACTEC 12B vial, a Lowenstein-Jensen slant, and a Middlebrook 7H10/T/H1 selective biplate were also inoculated, incubated at 37°C, and monitored for 8 weeks before being reported as negative. When growth was detected, the mycobacteria were identified by the DNA AccuProbe (Gen-Probe Incorporated, San Diego, CA) and conventional methods, with susceptibility testing performed using the BACTEC 460TB system as reported previously (8, 10, 21).

For INH and RIF susceptibility, all strains were tested at two concentrations: 0.1 and 0.4 μg/ml for INH and 0.5 and 2.0 μg/ml for RIF. If MTBC strains were identified, final identification to the species level was made using PCR-based deletion analysis (15). When the strain was found to be drug resistant, the BACTEC susceptibility results were confirmed by the proportion method using Middlebrook 7H10 agar as described elsewhere previously (10).

Genotype MTBDR assay. The Genotype MTBDR line probe assay (Hain Lifescience GmbH, Nehren, Germany) was carried out according to the manufacturer's instructions and as described previously with the use of a modified amplification protocol (7). Briefly, 1 ml of the decontaminated and concentrated specimens was centrifuged at 10,000 × g for 15 min, the supernatant was discarded, and the pellet was resuspended in 300 μl sterile distilled water. The specimen was then heat killed at 95°C for 20 min in a heat block. This was followed by a 15-min sonication step. After sonication, 5 μl of the supernatant was used immediately for amplification, while the remainder was stored at −20°C. Amplification was done according to the following modified amplification protocol: denaturation at 95°C for 15 min; 10 cycles of denaturation at 95°C for 30 s and elongation at 58°C for 120 s; an additional 30 cycles of denaturation at 95°C for 25 s, annealing at 53°C for 40 s, and elongation at 70°C for 40 s; and a final extension step at 70°C for 8 min.

The biotin-labeled PCR product was denatured and hybridized to a strip with specific oligonucleotide probes. One probe is complementary with an MTBC-specific gene region of the 23S rRNA gene (Tub), and one probe is specific for the rpoB gene (rpoB-Uni) and should be always positive for all MTBC strains, while five wild-type (WT) probes (WT1 to WT5) encompass the region of the rpoB gene encoding amino acids 509 to 534. Four other probes are specific for the most common mutations: D516V, H526Y, H526D, and S531L. For one MTBC strain, the Genotype MTBDR assay would further improve the performance of the test.

RESULTS

BACTEC 460TB susceptibility results. Following growth detection of the 143 smear-positive specimens, a total of 92 specimens showed resistance to INH and/or RIF. Of these 92 specimens, 26 were MDR, including 24 specimens resistant to a high level (0.4 μg/ml) and 2 specimens resistant to a low level (0.1 μg/ml) of INH; 33 were resistant to a high level of INH; and 33 were resistant to only a low level of INH. Fifty-one specimens were pansusceptible according to the BACTEC 460TB system.

Sensitivity of the Genotype MTBDR assay for MTBC katG and rpoB. Of the 143 MTBC-containing specimens, 135 (94.4%) were correctly identified by the MTBC-specific TUB capture probe. With respect to the assay for the resistance-associated genes, amplification of katG provided conclusive results, i.e., successful amplification for 135 (94.4%) of the 143 specimens, and amplification of rpoB provided conclusive results for 130 (90.9%) of the 143 specimens. Although amplification of katG was successful in a total of 142 strains, katG results could not be validated for seven specimens due to the lack of a positive hybridization signal with the MTB-specific probe.

Performance of the Genotype MTBDR assay on specimens with strains resistant to high (0.4 μg/ml) and low (0.1 μg/ml) levels of INH and with INH-susceptible strains. A total of 57 (40.1%) specimens contained strains that were resistant to high (0.4 μg/ml) levels of INH by the BACTEC 460TB assay. Twenty-four (42.1%) of the 57 strains were MDR. Results of the Genotype MTBDR assay for MDR strains are summarized in Table 1. In 48 (84.2%) of the 57 specimens, the Genotype MTBDR assay results were in agreement with results from BACTEC 460TB susceptibility testing and the DNA sequencing results (Table 1). For one (1.8%) of these 48 specimens, the Genotype MTBDR assay indicated the presence of strains that were both resistant and susceptible to INH (weak katG WT with strong katG MUT1 mutation-specific bands) (Table 1). DNA sequencing confirmed an S315T mutation in this strain. In one (1.8%) specimen, DNA sequencing revealed a rare S315I mutation of katG that was missed by the Genotype MTBDR test. Although an oligonucleotide probe specific for this mutation is not present in the Genotype MTBDR test, the positive hybridization signal with the katG WT probe falsely indicated susceptibility to INH. For 7 (12.3%) of the 57 specimens, the Genotype test indicated INH susceptibility (no detectable mutation at codon S315), while amplification was unsuccessful for three genes (MTBC-specific 23S rRNA, katG, and rpoB) in one (1.8%) specimen by the assay (Table 1).

However, DNA sequencing was also unable to find any S315 katG mutations in these eight (14%) strains. DNA sequencing revealed mutations in inhA in only 2 (3.5%) of the 57 strains (both MDR; a T-to-G and a T-to-A point mutation at position −8 upstream of the translation start site of inhA) (Table 1).
A total of 35 (24.6%) specimens contained strains that were resistant to low levels of INH (0.1 μg/ml), two (5.7%) strains of which were MDR (Table 1). Amplification was unsuccessful for two genes (MTBC-specific 23S rRNA and rpoB) in three (8.6%) specimens (Table 1). Although amplification of katG was successful in all three specimens, because of an unsuccessful amplification for the MTBC, these results could not be validated. DNA sequencing did not identify katG mutations in these strains (Table 1). In 2 (5.7%) of the 35 strains, the Genotype MTBDR assay indicated the presence of a katG mutation (katG positive but negative for WT, MUT1, and MUT2 probes) (Table 1). DNA sequencing revealed two rare katG mutations in these strains. Neither the Genotype MTBDR test nor DNA sequencing was able to detect any katG mutations in 30 (85.7%) of the 35 specimens (Table 1). Interestingly, analysis of DNA sequences of inhA showed mutations in 18 (51.4%) of the 35 strains that were resistant to low levels of INH (a C-to-G point mutation at position −15 in 16 strains, a T-to-C point mutation at position −8 in 1 MDR strain, and a double mutation of a C-to-G point mutation at position −15 plus a T-to-C mutation at position −8 in 1 strain).

In 4 (7.8%) of the 51 INH-susceptible, as well as pansusceptible, specimens, amplification was unsuccessful for the MTBC and rpoB. Although the assay successfully amplified katG and correctly indicated susceptibility to INH in these specimens, these results could not be validated because of the inability of the assay to detect the presence of the MTBC. The Genotype MTBDR assay predicted susceptibility to INH in all the remaining 47 (92.2%) control specimens.

### Performance of the Genotype MTBDR assay for specimens with RIF-resistant and -susceptible strains

A total of 26 (18.3%) specimens contained RIF-resistant strains according to the BACTEC 460TB assay. All strains were MDR. Results with the Genotype MTBDR assay for MDR strains are summarized in Table 2. RIF resistance was correctly predicted by the Genotype MTBDR test in 25 (96.2%) specimens, while amplification was unsuccessful by the assay for the rpoB gene in one (3.8%) specimen (Table 2). For 3 (11.5%) of these 26 specimens, the Genotype MTBDR assay indicated the presence of both RIF-resistant and -susceptible strains (two strains with MUT H526Y plus rpoB WT probes, and one strain with MUT S531L and rpoB WT probes). Of the 26 RIF-resistant strains, 13 (44.8%) carried mutation H526Y, 12 (41.4%) carried mutation S531L, and 1 (3.4%) carried mutation Del526.

The Genotype MTBDR test correctly indicated RIF susceptibility in 102 (87.2%) of the RIF-susceptible strains (43.6% of strains were pansusceptible, 28.2% of strains were resistant to high levels of INH, and 28.2% of strains were resistant to low levels of INH) (Table 2). However, in 1 of these 102 specimens, the test failed to detect the presence of a silent (no amino acid change) R528R rpoB mutation (all rpoB WT probes were positive). In addition, amplification was unsuccessful for three genes (M. tuberculosis complex-specific 23S rRNA, katG, and rpoB).

### Table 1. Performance of the Genotype MTBDR assay with isoniazid-resistant and -susceptible strains

<table>
<thead>
<tr>
<th>Resistance or susceptibility by BACTEC 460TB</th>
<th>Genotype MTBDR</th>
<th>inhA sequencing</th>
<th>katG315 sequencing</th>
<th>Mutation +</th>
<th>Mutation −</th>
<th>Unsuccessful amplification</th>
<th>Mutation +</th>
<th>Mutation −</th>
<th>Mutation +</th>
<th>Mutation −</th>
</tr>
</thead>
<tbody>
<tr>
<td>High-level INH resistant (0.4 μg/ml) (n = 57)</td>
<td>48 (84.2)</td>
<td>8 (14)</td>
<td>1 (1.8)</td>
<td>49 (86)</td>
<td>8 (14)</td>
<td>2 (3.5)</td>
<td>55 (96.5)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Low-level INH resistant (0.1 μg/ml) (n = 35)</td>
<td>2 (5.7)</td>
<td>30 (85.7)</td>
<td>3 (8.6)</td>
<td>2 (5.7)</td>
<td>33 (94.3)</td>
<td>18 (51.4)</td>
<td>17 (48.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>INH susceptible (n = 51)</td>
<td>47 (92.2)</td>
<td>4 (7.8)</td>
<td>0</td>
<td>51 (100)</td>
<td>0</td>
<td>51 (100)</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

- A total of 42% of these strains were multidrug resistant.
- A total of 5.7% of these strains were multidrug resistant.
- Amplification was unsuccessful for three genes (M. tuberculosis complex-specific 23S rRNA, katG, and rpoB).
- Amplification was unsuccessful for two genes (M. tuberculosis complex-specific 23S rRNA and rpoB).

### Table 2. Performance of the Genotype MTBDR assay with rifampin-resistant and -susceptible strains

<table>
<thead>
<tr>
<th>Resistance or susceptibility by BACTEC 460TB</th>
<th>Genotype MTBDR</th>
<th>rpoB sequencing</th>
<th>Mutation +</th>
<th>Mutation −</th>
<th>Unsuccessful amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIF-resistant (n = 26)</td>
<td>25 (96.2)</td>
<td>0</td>
<td>1 (3.8)</td>
<td>26 (100)</td>
<td></td>
</tr>
<tr>
<td>RIF-susceptible (n = 117)</td>
<td>102 (87.2)</td>
<td>12 (10.2)</td>
<td>114 (97.4)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- All strains were multidrug resistant.
- Fifty-one (43.6%) strains were pansusceptible, 33 (28.2%) strains were resistant to a high level (0.4 μg/ml) of isoniazid, and 33 (28.2%) strains were resistant to a low level (0.1 μg/ml) of isoniazid.
- Three strains showed a wild-type and mutation probe-positive hybridization pattern together.
- These strains showed susceptibility to RIF at concentrations of 2.0 μg/ml (reportable concentration) and resistance at concentrations of 0.5 μg/ml by the BACTEC 460TB system.
- Amplification was unsuccessful for one gene (rpoB).
- Amplification was unsuccessful for three genes (M. tuberculosis complex-specific 23S rRNA, katG, and rpoB) in one strain, for two genes (M. tuberculosis complex-specific 23S rRNA and rpoB) in seven strains, and for one gene (rpoB) in four strains.
can be identified. However, the rapid diagnostic predictions of growth-based assays until all INH resistance-related mutations have been identified. Thus, there is a need to continue testing via the katG gene to determine INH resistance to high levels of INH, and for a majority of the strains encoding resistance to rifampin (RIF-resistant strains). For seven of the strains encoding resistance, the Amplified Mycobacterium Tuberculosis Direct detection of rifampicin resistance in sputum and biopsy specimens from tuberculosis patients by the Genotype MTBDR test did not show any association with the length of time of freezing.

**Age of frozen specimens.** Eighteen (19.6%) of the 92 resistant strains were received in 2000, 17 (18.5%) were received in 2001, 14 (15.2%) were received in 2002, 15 (16.3%) were received in 2003, 23 (25%) were received in 2004, and 5 (5.4%) were received in 2005. Of the 51 susceptible strains, 3 (5.9%) were received in 2000, 17 (33.3%) were received in 2003, 23 (25%) were received in 2004, and 17 (33.3%) were received in 2005. Unsuccessful amplification with the Genotype MTBDR test did not show any association with the length of time of freezing.

**DISCUSSION**

If we are to meet the goal of controlling the spread of drug-resistant tuberculosis, the time frame of many weeks required for detection, identification, and drug susceptibility testing of MTBC strains by growth detection must be shortened. This is best achieved by the introduction of modern molecular methods that can be applied directly to clinical specimens. The findings of the present study show that the recently developed Genotype MTBDR multiplex PCR assay is a suitable molecular method for this purpose. The assay offers a simple protocol that is compatible with routine workflow and can be completed within 24 h.

The results of the present study also indicate that the Genotype MTBDR assay has an overall good performance and sensitivity (successful amplification for all strains) for the rapid detection of MTBC strains by growth detection must be shortened. This is best achieved by the introduction of modern molecular methods that can be applied directly to clinical specimens. The findings of the present study show that the recently developed Genotype MTBDR multiplex PCR assay is a suitable molecular method for this purpose. The assay offers a simple protocol that is compatible with routine workflow and can be completed within 24 h.

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