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# Defining Multidrug-Resistant Tuberculosis: Correlating GenoType MTBDR*plus* Assay Results with Minimum Inhibitory Concentrations

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## Abstract

This study correlates Minimum Inhibitory Concentrations (MICs) of rifampicin (RIF) and isoniazid (INH) with GenoType MTBDR*plus* assay results for drug-resistant *Mycobacterium tuberculosis* (*MTB*) clinical isolates. MICs of RIF and INH were established for 84 and 90 isolates, respectively, testing six concentrations of each drug. Genotypic resistance to each drug was determined by GenoType MTBDR*plus* assay with 50 representative mutations confirmed by pyrosequencing, with mutations in the *rpoB* gene associated with RIF-resistance and mutations in the *katG* and/or *inhA* genes associated with INH-resistance. Based upon the correlation of MICs with specific genetic profiles, relative resistance levels were established for each isolate. Results indicate that *MTB* phenotypic resistance, currently based upon the testing of isolate susceptibility to a single drug concentration, may be more accurately profiled via quantitative MICs, and therefore the correlation of molecular diagnostic results with specific MICs may allow for more optimal treatment of infections.

### Keywords

Drug susceptibility testing; multidrug-resistant tuberculosis; level of resistance; mutations



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## 1. Introduction

The increase in prevalence of drug-resistant tuberculosis (DR-TB), including multi- and extensively drug-resistant tuberculosis (M/XDR-TB) (WHO, 2010), and the recent emergence of "totally drug-resistant TB" (Udwadia et al., 2012; Velayati et al., 2009) poses a significant threat to global TB control efforts (WHO, 2010; Shah et al., 2011; Gandhi et al., 2010; Jonsson et al., 2012; WHO, 2011).

*Mycobacterium tuberculosis* (*MTB*), the main agent of TB, is known to have heterogeneous phenotypic drug resistance profiles. TB phenotypic resistance can range from low- and moderate-resistance to drugs, resulting in infections that might still be effectively treated with increased dosing of the relevant TB medications, to high-level resistance, as seen in infections that cannot be treated with the drugs with which the isolate is resistant to (Springer et al., 2009). As such, estimating the Minimum Inhibitory Concentrations (MICs) of drugs against an individual patient's *MTB* infection can be an effective, if tedious, clinical diagnostic method (Heifets et al., 2009). However, in low-resource clinical settings with facility and cost limitations, these methods are beyond basic laboratory diagnostic capabilities.

Molecular diagnostics, including line probe assays (WHO, 2008a), such as the Hain GenoType MTBDR*plus* assay, and pyrosequencing assays, such as those utilizing the Qiagen PyroMark platform (Lin et al., 2014), have the potential to significantly improve MDR diagnosis and to facilitate real-time treatment decisions. However, these assays represent only indirect measures of phenotypic resistance, and the relationship between mutations in the *rpoB* and *katG*, *inhA*, and *ahpC* genes and rifampicin (RIF) - and isoniazid (INH)-MICs is not clearly defined. Additional data is necessary to fully exploit and interpret molecular diagnostics for the clinical evaluation of RIF and INH drug resistance in TB patients.

For RIF, mutations within 81bp region of the *rpoB* gene, encoding the *B* subunit of a DNA-dependent RNA polymerase, are responsible for conferring RIF resistance. Canonical mutations in this gene, including 516Val, 526Asp/Tyr and 531Leu, are well documented, whereas amino acid changes at codons 511, 515, 516, 518, 521, 522, and 533 have not been thoroughly evaluated for their association with phenotypic RIF-resistance. Notably, high-level RIF-resistance is reported more frequently than lower levels of resistance, supporting the analysis of these other, potentially RIF-resistance associated mutations for diagnostic purposes (Sirgel et al., 2013).

For INH, the accumulation of mutations in the *katG* gene and *inhA* promoter leads to development of resistance. Mutations within *katG* prevent the activation of the gene's respective prodrug, resulting in high-level INH resistance. Mutations in the *inhA* promoter gene are known to increase the level of protein expression, and are generally correlated with low-level INH resistance. (Santos et al., 2012).

In this study, we determined the MICs of RIF and INH for *MTB* clinical isolates and categorized the isolates by phenotypic levels of resistance based on MIC values. Ultimately,

we correlated these phenotypic resistance levels with GenoType MTBDR*plus* assay results, with 50 representative mutations confirmed by pyrosequencing.

## 2. Materials and Methods

### 2.1 Setting

The study was performed at the Mycobacteriology Laboratory of P. D. Hinduja National Hospital (PDHNH) and Medical Research Centre (MRC), a tertiary care hospital in Mumbai, India that has a referral bias towards nonresponders.

### 2.2 Ethical approval

The study was approved by the Institutional Review Board (IRB) of Hinduja Hospital. Written consent was waived as the study was carried out on 120 consecutively archived *MTB* isolates for which GenoType MTBDR*plus* assay, as well as MGIT 960 drug susceptibility testing (DST) for RIF and INH, had been previously performed.

### 2.3 Phenotypic MGIT Drug Susceptibility Testing

All isolates were subjected to conventional phenotypic DST for RIF and INH by MGIT 960 (Becton Dickinson Diagnostic System, Sparks, MD) with Epicenter software, using critical concentrations recommended by the World Health Organization (WHO, 2008b). MGIT DST quality control was ensured daily using a known (genotypically confirmed) resistant isolate as well as the H37Ra pansusceptible strain.

**Preparation of Drugs:** A stock solution of RIF (Becton Dickinson Diagnostic System, Sparks, MD Catalogue 245126) was prepared by unfiltered dissolution of the drug in dimethyl sulfoxide (DMSO) and further dilution with distilled water. INH (Becton Dickinson Diagnostic System, Sparks, MD and catalogue 245126) was prepared by dissolution in distilled water, filtration, and then further dilution with distilled water. Both drugs were stored at −80°C for up to 6 months. Preparation of the inoculum, inoculation and incubation was performed as per MGIT 960 manufacturer instructions (Becton Dickinson Diagnostic System, Sparks, MD) (Siddiqi et al., 2012).

**2.3.1 MIC using MGIT 960—**MICs were performed via MGIT 960 using a macrodilution method where different concentrations of the drugs were prepared and injected into different MGIT tubes. The subsequent inoculation and incubation was performed as for phenotypic DST determination.

**Selection of drug concentrations for MICs:** MIC testing levels were selected in order that the seven drug concentrations would define the phenotypical resistance levels of the majority of mutations. The WHO has established a critical concentration of 1μg/ml for RIF and 0.1μg/ml for INH (WHO, 2008b). For RIF and INH, concentrations above and below the critical concentration were tested as follows: For estimation of RIF MICs we used two concentrations below the critical concentration (0.5 and 0.25μg/ml), and four concentrations above the critical concentration (2.0, 4.0, 10.0 and 20.0μg/ml). For estimation of INH MICs

we used two concentrations below the critical concentration (0.05 and 0.025μg/ml), and four concentrations above the critical concentration (0.5, 1.0, 3.0 and 10.0μg/ml).

### 2.4 Genotypic Methods

**GenoType MTBDR*plus* assay—**120 consecutive archived isolates were selected for which both GenoType MTBDR*plus* assay and DST (at the WHO approved critical concentration) had been performed previously. The GenoType MTBDR*plus* assay (Hain Lifescience, Nehren, Germany) procedure was comprised of 3 steps. First, a GenoLyse kit was used for DNA extraction from decontaminated patient samples/bacterial cultures. Multiplex polymerase chain reaction (PCR) amplification with biotinylated primers was then performed in a thermal cycler (Eppendorf) under the following cycling conditions: denaturation 95°C/15 min, initial denaturation 95°C/30 sec, annealing 58°C/2 min (10 cycles), denaturation 95°C/25 sec, annealing 53°C/40 sec, extension 70°C/40 sec (30 cycles), final extension 70°C/8 min. Finally, reverse hybridization was performed as per manufacturer instructions.

A strict, unidirectional workflow was adhered to for all experiments. Quality control was ensured by testing a pansusceptible *MTB* strain, H37Ra, along with a strain confirmed to carry resistance-associated mutations, during each MGIT 960 run. In order to confirm that no cross contamination occurred, a negative control was also run with each batch (Ajbani et al., 2012).

**Pyrosequencing—**Fifty representative isolates of the 120 *MTB* isolates utilized in this study were sequence-confirmed by pyrosequencing (PSQ). A total of 30 isolates had the *rpoB* gene region pyrosequenced to confirm the genotypic RIF-resistance, based upon the presence or absence of known resistance-associated *rpoB* mutations. PSQ results confirmed GenoType MTBDR*plus* assay findings, in that a PSQ-resistant result was marked by the absence of wildtype probes and the presence of the corresponding mutation probe (MUT1, MUT2A, MUT2B and/or MUT3), or, as in the case of 14 isolates, with absence of wildtype probes and the absence of the corresponding mutation probe in the GenoType assay. Similarly, 20 representative isolates had the relevant *katG* and *inhA* gene regions pyrosequenced to confirm GenoType INH-resistance results. PSQ data was collected for six isolates showing GenoType mutations in both the *katG* and *inhA* genes, six showing only *katG* mutations, and four showing only *inhA* mutations. Four isolates determined to be INH- and RIF-wildtype via the GenoType assay were also pyrosequenced.

PSQ consisted of PCR amplification followed by PSQ reaction on the the PyroMark Q96 ID system (Qiagen, Valencia, CA). The HotStarTaq kit and deoxynucleoside triphosphate (dNTP) mixtures (Qiagen, Valencia, CA) were used in the PCR master mix. The PCR mixture contained 2.5μl of DNA and 22.5μl of a PCR master mix, which included 1× supplied PCR buffer, 2.5mM $MgCl_2$, 0.96mM dNTP mixture, 1× Q-solution, 0.5μM primer, and 1U of HotStarTaq. Amplification involved initial activation of the HotStarTaq at 95°C for 15 min, 50 cycles of amplification at 94°C for 15 s, 60°C for 30 s, and 72°C for 20 s, and final extension at 72°C for 5 min. PyroMark Q96 (5 by 96) reagents were used for PSQ with the sequence analysis mode of the PyroMark Q96 ID system utilized for result interpretation

(Lin et al., 2014). The molecular marker IS6110 was utilized to confirm the presence of *MTB*. The targeted loci for detection of DR-TB were the RIF resistance-determining region (RRDR) of *rpoB* for RIF resistance and regions of the *katG* gene and *inhA* and *ahpC* promoters for INH resistance (Lin et al., 2014).

## 3. Results

### 3.1 Phenotypic Results

Phenotypic MGIT DST results found 18/120 isolates to be MDR (resistant to INH and RIF) but susceptible to fluoroquinolones (FQs) and to injectables (amikacin, kanamycin and capreomycin), while 53/120 were MDR with additional resistance to FQs, 9/120 were XDR with resistance to injectables and FQs, and 7/120 were INH-monoresistant. Three isolates were found to be susceptible to RIF and to injectables, but resistant to all other drugs tested. A total of 30/120 isolates were phenotypically susceptible to all drugs tested.

**3.1.1 MIC results—**We determined the MICs of RIF and INH for the *MTB* isolates and assessed their correlation with known genetic mutations. The genotypic and phenotypic findings of the 120 *MTB* isolates, with regards to RIF and INH, are summarized in Table 1. Resistance levels were established for the various mutations with respect to their MIC range. The correlation of specific mutations with phenotypic resistance levels is summarized in Tables 2 and 3.

### 3.2 Genotypic results

Mutations in the *rpoB* gene were observed in 84 *MTB* isolates; *katG* and i*nhA* mutations were observed in 90 *MTB* isolates, with 24 of these isolates having mutations in both genes. Thirty-six isolates evaluated for RIF-resistance were wildtype, or lacking resistance-associated mutations in the *rpoB* RRDR, while 30 isolates tested for INH-resistance were wildtype for the associated gene regions of interest. 16.6% of isolates evaluated for RIF-resistance via GenoType assay showed the absence of wildtype probles, but also had an absence of the corresponding mutation probe. Agreement between phenotypic MGIT DST and genotypic MTBDR*plus* assay for INH-resistance was 100% and for RIF-resistance it was 95%. PSQ results of 30 *MTB* isolates evaluated for RIF-resistance and 20 isolates evaluated for INH-resistance are summarized in Tables 4 and 5.

## 4. Discussion

The various mutations our study identified within the *katG*, *inhA* and *rpoB* genes correlated with INH and RIF MICs, establishing specific drug-susceptibility levels for 120 *MTB* isolates (see Table 1). Clear differences were observed between the drug susceptibility levels of genotypically wildtype isolates and those harboring resistance-associated mutations. In total, mutations associated with RIF-resistance were found within the *rpoB* gene of 84 isolates and mutations associated INH-resistance were found within the *katG* and *inhA* genes of 90 of the 123 *MTB* isolates evaluated in this study.

Among the 84 RIF-resistant isolates, 70 canonical mutations [Asp516Val (n=3), His526Tyr/Asp (n=10), and Ser531Leu (n=57)] and 14 disputed mutations

[Asp516Tyr/Phe/Ala (n=5), His526Cys/Asn/Arg (n=5), and Leu533Pro (n=4)] were observed. We found the MIC of isolates with the Asp516Phe/Ala/Val mutation (5.9%, or 5/84 of RIF-resistant isolates) to be 10μg/ml. These isolates were defined as having a moderate level of resistance. This finding is in line with an earlier study reporting 85% (29/34) of isolates with Asp516Val mutations having a MIC range of 10–15μg/ml (Sirgel et al., 2013). Within our study, 11% (10/84) of isolates with His526Tyr/Asp mutations had MICs >20 μg/ml which is similar to a previously published study wherein 15% (3/19) of isolates with His526Tyr/Asp mutations had MICs >50μg/ml (Springer et al., 2008). RIF MICs >20μg/ml were confirmed for 67% (57/84) of isolates with Ser531Leu mutations in our study, a result comparable to another study's report of 78% (15/19) of isolates with a Ser531Leu mutation having a MIC >20μg/ml (Springer et al., 2008). Isolates with Asp516Tyr mutations, and some with 526 mutations, showed discordant MGIT RIF MIC results. This is not a novel finding, as recent studies have shown that phenotypic RIF-resistance testing is not binary for all *rpoB* mutations, and MGIT 960 culture methods are known to incorrectly evaluate some RIF-resistance conferring mutations (Rigouts et al., 2013). Silent mutations, such as TTC514TTT and GGC507GGT, although possible sources of GenoType MTBDR*plus* wildtype probe absent and mutation probe absent results, were not encountered in the isolates evaluated in our study (Gracelin et al., 2014)

Out of 90 INH-resistant isolates, we observed the following mutations: Ser315Thr1/2 in the *katG* gene (n=50), C-15T within the *inhA* promoter (n=16), and *katG* with *inhA* (n=24) at Ser315Thr1/2 with C-15T or T-8C. Of the 90 isolates found to be resistant to INH at 0.1μg/ml, 14 were found to be susceptible to the drug at 0.5μg/ml (Table 1). 88% (14/16) of isolates with an *inhA* promoter mutation at C-15T, alone, showed low-level INH-resistance. This type of low-level resistance may potentially be overcome in TB treatment with increase in INH dosing, particularly in areas of low endemicity (Springer et al., 2008; Guo et al., 2006). In our study, 88% (21/24) of isolates having concurrent mutations in the *katG* and *inhA* gene regions evaluated i.e. Ser315Thr1 with C-15T or T-8C were determined to have MICs >10μg/ml. Finally, 100% (50/50) of isolates with the Ser315Thr1/2 mutation in *katG* were determined to have MICs of 3– ≥10μg/ml. Our findings, in this respect, confirm previously published results of MIC values associated with mutations at Ser315 (Springer et al., 2008).

Notably, both RIF and INH testing yielded low level MIC results in some instances, despite the presence of resistance-associated mutations in the RRDR of the *rpoB* gene, and the *inhA* promoter, respectively.

## 5. Conclusions

In order to effectively curb the rise and spread of MDR-TB throughout the globe, there is an urgent need to quickly and accurately determine clinical drug susceptiblity profiles of drug-resistant isolates. The correlation of genotypic test results, including line probe assays and pyrosequencing, with a specific range of MIC values for each drug, presents an invaluable tool for quickly guiding treatment decisions in clinical laboratories worldwide. Of particular interest, this study finds the majority of *MTB* isolates with the canonical *rpoB* mutations His526Asp/Tyr and Ser531Leu to have a high-level of resistance to RIF, whereas the

Asp516Val mutation was associated with a moderate level of resistance to RIF, as seen via MIC testing. As each mutation within the *rpoB* gene does not confer the same level of phenotypic RIF-resistance, there is a need for additional MIC and genetic testing to confirm correlations between genotypic mutations and phenotypic resistance levels. We recommend additional clinical outcome studies to assess whether disputed mutations, with MICs around RIF-resistance critical concentration cutoffs, are associated with treatment failure. For example, the C-15T *inhA* mutation, found to correlate with low-level INH-resistance, is a point of clinical relevance, as isolates with this mutation might be treated with higher doses of INH. In conclusion, since effective MDR-TB treatment relies upon our knowledge of quantitative susceptibility testing results, the correlation of genotypic test results with phenotypic resistance levels is a critical component of TB diagnosis that should be further evaluated in order to prevent the spread of drug resistance and promote the optimum use of the few drugs available for TB treatment.

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## Highlights

- *Mycobacterium tuberculosis* resistance is heterogeneous i.e. involves Low, Moderate, High level of resistance.
- MIC can predict this level of resistance.
- Low level of resistance is a point of clinical relevance

**Table 1**

MIC results of RIF and INH:

| Mutations | | GenoType MTBDR*plus*assay | | Total no. of isolates | No. of isolates | MIC range of INH | MIC range of RIF |
|---|---|---|---|---|---|---|---|
| *katG* with *inhA* | *rpoB* | *katG* with *inhA* | *rpoB* | | n=24 | | |
| Ser315Thr1 C15T | Ser531Leu | MUT1 MUT1 | MUT3 | 18 | 18 | >10 | >20 |
| Ser315Thr1 T8C | Ser531Leu | MUT1 MUT3A | MUT3 | 1 | 1 | 10 | >20 |
| Ser315Thr1 T8C | Ser531Leu | MUT1 MUT3A | MUT3 | 1 | 1 | >10 | >20 |
| Ser315Thr2 T8C | Ser531Leu | MUT2 MUT3A | MUT3 | 1 | 1 | 10 | >20 |
| Ser315Thr1 C15T | Asp516Tyr | MUT1 MUT1 | Absence of WT3/4 | 1 | 1 | >10 | 4 |
| Ser315Thr1 C15T | His526Asp | MUT1 MUT1 | MUT2B | 1 | 1 | >10 | >20 |
| Ser315Thr1 T8C | His526Asp | MUT1 MUT3A | MUT2B | 1 | 1 | 10 | >20 |
| *katG* | *rpoB* | *katG* | *rpoB* | | n=50 | | |
| Ser315Thr1 | His526Cys | MUT1 | Absence of WT7 | 1 | 1 | 10 | 2 |
| Ser315Thr1 | His526Asn | MUT1 | Absence of WT7 | 3 | 2 | 10 | 0.25 |
| | | | | | 1 | 10 | 0.5 |
| Ser315Thr1 | Leu533Pro | MUT1 | Absence of WT8 | 4 | 1 | 10 | 2 |
| | | | | | 2 | 10 | 4 |
| | | | | | 1 | >10 | 4 |
| Ser315Thr1 | His526Tyr | MUT1 | MUT2A | 2 | 2 | 3.0 | >20 |
| Ser315Thr1 | Asp516Val | MUT1 | MUT1 | 3 | 3 | 10 | 10 |
| Ser315Thr1 | Asp516Tyr | MUT1 | Absence of WT3,4 | 2 | 1 | 10 | 10 |
| | | | | | 1 | 10 | 0.25 |
| Ser315Thr1 | Asp516Phe | MUT1 | Absence of WT 3/4 | 1 | 1 | 10 | 10 |
| Ser315Thr1 | Asp516Ala | MUT1 | Absence of WT 3+8 | 1 | 1 | 3.0 | 10 |
| Ser315Thr1 | Ser531Leu | MUT1 | MUT3 | 25 | 7 | 3.0 | >20 |
| | | | | | 16 | 10 | >20 |
| | | | | | 2 | >10 | >20 |
| Ser315Thr2 | Ser531Leu | MUT2 | MUT3 | 2 | 2 | 10 | >20 |
| Ser315Thr1 | His526Asp | MUT1 | MUT2B | 5 | 2 | >10 | >20 |
| | | | | | 3 | 10 | >20 |

| Mutations | | GenoType MTBDR*plus* assay | | Total no. of isolates | No. of isolates | MIC range of INH | MIC range of RIF |
|---|---|---|---|---|---|---|---|
| Ser315Thr1 | His526Arg | MUT1 | Absence of WT7 | 1 | 1 | 10 | >20 |
| *inhA* | *rpoB* | *inhA* | *rpoB* | | n=16 | | |
| C15T | WT | MUT1 | WT | 6 | 6 | 0.5 | 0.25 |
| C15T | Ser531Leu | MUT1 | MUT3 | 9 | 6 | 0.5 | >20 |
| | | | | | 3 | 1 | >20 |
| C15T | His526Tyr | MUT1 | MUT2A | 1 | 1 | 1 | >20 |
| WT | | | | 30 | 25 | 0.1 | 0.25 |
| | | | | | 5 | 0.05 | 0.25 |

**Table 2**

MIC of RIF and correlation of mutations with level of resistances:

| Mutations/ Level of resistance | Asp516Ala | Asp516Phe | Asp516Tyr | Asp516Val | His526Cys | His526Asn | His526Tyr | His526Asp | His526Arg | Ser531Leu | Leu533Pro |
|---|---|---|---|---|---|---|---|---|---|---|---|
| ≤4 (Low) | | | 2 | | 1 | 3 | | | | | 4 |
| 10 (Moderate) | 1 | 1 | 1 | 3 | | | | | | | |
| >20 (High) | | | | | | | 3 | 7 | 1 | 57 | |

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**Table 3**

MIC of INH- Correlation of mutations with level of resistance:

| Mutations / Level of Resistance | *katG* with *inhA* | *katG* | *inhA* |
|---|---|---|---|
| | Ser315Thr1withC15T Ser315Thr1withT8C, Ser315Thr2withT8C | Ser315Thr1 Ser315Thr2 | C15T |
| 0.5–1.0 (Low) | 0 | 0 | 16 |
| 3.0–10.0 (Moderate) | 3 | 45 | 0 |
| >10.0 (High) | 21 | 5 | 0 |

**Table 4**

PSQ results for *rpoB* (n=30):

| No. of isolates | GenoType MTBDR*plus* assay | PSQ |
|---|---|---|
| 1 | Absence of WT3 | 516GAC-GCC |
| 1 | Absence of WT3/4 | 516GAC-TTC |
| 3 | Absence of WT3/4/8 | 516GAC-TAC |
| 1 | Absence of WT7 | 526CAC-TGC |
| 1 | Absence of WT7 | 526CAC-CGC |
| 3 | Absence of WT7 | 526CAC-AAC |
| 3 | Absence of WT3/4 & presence of MUT1 | 516GAC-GTC |
| 3 | Absence of WT7 & presence of MUT2A | 526CAC-TAC |
| 3 | Absence of WT7/4 & presence of MUT2B | 526CAC-GAC |
| 3 | Absence of WT8 & presence of MUT3 | 531TGC-TTG |
| 4 | Absence of WT8 | 533CTG-CCG |
| 4 | WT | WT |

**Table 5**

PSQ results for *katG* and *inhA* (n=20):

| No. of isolates | GenoType MTBDR*plus* assay | | PSQ | |
|---|---|---|---|---|
| | ***katG*** | ***inhA*** | ***katG*** | ***inhA*** |
| 4 | MUT1 | MUT1 | 315AGC-ACC | -15T |
| 1 | MUT1 | MUT3A | 315AGC-ACC | -8C |
| 1 | MUT2 | MUT3A | 315AGC-ACA | -8C |
| 4 | MUT1 | WT | 315AGC-ACC | WT |
| 2 | MUT2 | WT | 315AGC-ACA | WT |
| 4 | WT | MUT1 | WT | -15T |
| 4 | WT | WT | WT | WT |