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# A LITERATURE ANALYSIS OF MOLECULAR DIAGNOSTIC TESTS AND MEDICATION SUSCEPTIBILITY TESTING FOR MDR-TB

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## Abstract

*MDR tuberculosis, which is characterized by resistance to the two most effective first-line medications, isoniazid and rifampin, is becoming more prevalent worldwide and is linked to high morbidity and death rates. The practical utility of these techniques is unclear despite the growing accessibility of innovative, quick diagnostic technologies for Mycobacterium tuberculosis (Mtb) drug susceptibility testing. Here, we go through the benefits, drawbacks, and workings of the existing Mtb DST tools as well as the mechanisms of action and resistance of Mtb to isoniazid and rifampin. We lay a special emphasis on molecular techniques with quick turnaround times, such as line probe assays, real-time polymerase chain reactions based on molecular beacons, and pyrosequencing. We come to the conclusion that both quick molecular drug testing and phenotypic approaches fall short in predicting MTB medication susceptibility and must be evaluated in the context of each patient's clinical history.*

***Keywords:*** *tuberculosis; multidrug-resistance, molecular diagnostic tools*

## INTRODUCTION

Globally, the prevalence of multidrug-resistant tuberculosis (MDR TB), which is indicated by resistance to the two antituberculous first-line medications, isoniazid and rifampin, is rising. The World Health Organization (WHO) stated that there were 3.6% of all incident TB cases globally in 2008, or an estimated 390,000-510,000 new cases of MDR TB. 2008 had a high rate of MDR TB mortality, with an estimated 150,000 attributable deaths1. XDR TB, which is defined as MDR plus resistance to a fluoroquinolone and an injectable second-line therapy, is defined as MDR plus resistance to both. The prognosis for drug-resistant TB is particularly poor in HIV-infected patients, with a recent South African study reporting one-year mortality of 71% for MDR and 83% for XDR TB; 40% of the MDR TB and 51% of the XDR TB HIV-coin The increasing incidence and death rates of MDR TB over the world need early DST and enhanced treatments.

We first discuss the mechanisms of resistance to isoniazid and rifampin to serve as a basis for our assessment of the existing diagnostic techniques for the diagnosis of MDR TB. The circular genome of the Mtb strain H37Rv, which has been the most thoroughly studied, was deciphered in 1998. It was found to have about 4,000 genes and more than 4 million base pairs. Since then, a substantial body of research has appeared that discusses the connection between certain gene mutations and DST.

### Isoniazid: Mechanism of action and resistance in Mycobacterium tuberculosis

Isoniazid enters the cell through passive diffusion and then has to be activated by the mycobacterial catalase peroxidase KatG. The two main enzymes, enoyl-acyl carrier protein reductase (inhA) and beta ketoacyl-acyl carrier protein synthase, that

are involved in the elongation cycle of fatty acid molecules are the targets of the activated isoniazid, which prevents the synthesis of the mycolic acids required for the mycobacterial cell wall. 5,6. In contrast to rifampin resistance, spontaneous mutations that cause isoniazid resistance are not localized in a single gene. The catalase's capacity to activate the prodrug of isoniazid7 is lost as a consequence of drug resistance mutations in the katG gene. H resistance may occur from mutations in the inhA gene or its promoters that change the active isoniazid binding site or boost InhA production. 8. While inhA mutations may result in both cross resistance to ethionamide and low-level isoniazid resistance, katG mutations may give high levels of isoniazid resistance. Although isoniazid mutations mostly affect the katG and inhA genes, they may also affect other genes that code for enzymes including ndh, ahpC, and furA. 5,9. The katG mutation (at codon 315) has been linked to between 31 to 97% of INH resistance, with larger frequency occurring in TB-endemic nations10. The incidence of inhA mutations was 11% in a recent research by Dalla Costa et al. on 224 INH-resistant Mtb isolates from Argentina, Brazil, and Peru. Eighty-six percent of the samples contained an inhA or katG mutation linked to INH resistance.

**Rifampin: Mechanism of action and resistance in *Mycobacterium tuberculosis***

Rifampin targets the subunit, one of the four subunits of the mycobacterial DNA-dependent RNA polymerase, which is encoded by the rpoB gene3,12 to block transcription and subsequently protein synthesis. Drug resistance is brought on by specific mutations in the rpoB gene that lower rifampin's affinity for the RNA polymerase. Since more than 90% of isolates resistant to rifampin are also resistant to isoniazid, rifampin resistance is regarded as a primary surrogate marker for MDR TB. Rifampin Resistance Determining Region (RRDR) of the rpoB gene has an 81-base pair core region (codon 507 to 533) where over 95% of mutations causing rifampin resistance occur12,17. Furthermore, codons 516, 526, or 531—which code for low-level resistance—have more than 92% of the alterations (which code for high-level resistance).

**PHENOTYPIC ASSAYS FOR DETECTION OF MDR TB**

Despite its acknowledged shortcomings, traditional phenotypic DST is still the gold standard for MTB DST. In resource-rich nations, liquid media are utilized for DST more often than solid media (Table 1). Traditional solid media DST use the agar percentage, absolute concentration, or resistance ratio approach using Lowenstein-Jensen (LJ) or Middlebrook 7H10/11 (MB7H10/MB7H10/MB7H10/MB7H10/ MB7H10/MB7H10/MB7H10/MB7H10/MB7H10/ The agar percentage technique compares the number of colonies growing on a medium containing a medication at a specified critical concentration to the number of colonies growing on a medium devoid of the drug 19. Critical concentrations for first-line medicines in MB7H10 medium are: H 0.2 g/mL (low-level resistance) and 1 g/mL (high-level resistance), R 1g/mL, and E 10 g/mL19. When liquid media replaced solid medium for DST in the United States, the lengthy turnaround time (TAT) for culture from specimen collection was greatly reduced (from 8–12 weeks to 3–7 weeks). The BACTEC 460 TB was the first machine that used liquid media for DST. It was

replaced by the BACTEC MGIT 960 system, which employed fluorescent light emission to detect tuberculosis development and was constrained by concerns with radioactive waste disposal and handling. The BACTEC MGIT 960 has a sensitivity and specificity of above 95%20,21 for detecting isoniazid and rifampin resistance. On the MGIT 960, the critical drug concentrations for first-line medicines are as follows: H 0.1 g/mL (low-level resistance) and 0.4 g/Ml (high-level resistance), R 2g/mL, and E 5 g/mL19. The Versa TREK system is another automated broth-based approach that can simultaneously identify mycobacterial growth and undertake phenotypic DST to first-line medicines by measuring oxygen consumption. Other innovative quick phenotypic approaches include colorimetric methods that use the color change of a chemical dye (e.g., tetrazolium bromide and resazurin) for culture and DST, microscopic observation of drug susceptibility, and nitrate reduction tests. Limitations of quick phenotypic approaches include the unreliability of traditional breakpoints, lower accuracy in cultures including other mycobacteria, and the likelihood of decreased fitness and development of mutant organisms, which may need a greater inoculum to maximize test sensitivity.

## GENOTYPIC METHODS FOR DETECTING ISONIAZID AND RIFAMPIN RESISTANCE

### DNA Sequencing

However, some research and public health laboratories, as well as the CDC Molecular Detection of Drug Resistance (MDDR) service, do offer conventional DNA sequencing for the detection of mutations associated with Mtb resistance. This is because it is expensive, requires specialized knowledge, and takes a lot of time. A "chain-termination approach" is used in conventional DNA sequencing to order DNA fragments. To be more precise, it first attaches a primer to a single strand of denatured DNA. A DNA polymerase then starts extending DNA at the primer location. It ultimately comes to an end when the phosphodiesterase connection between two successive nucleotides is broken by a dye-labeled dideoxynucleotide. DNA fragments of varying lengths are produced by this cycle, which may then be sequenced after being separated by electrophoresis. Conventional DNA sequencing is still the industry standard for DNA sequencing since it is very accurate and has the benefit of reading bigger volumes of DNA. It serves as the cornerstone for the development of several quick molecular tests, such as line probe assays, molecular beacon-based RT-PCR, and pyrosequencing (Table 2). These tests can both recognize Mtb isolates and check for rifampin and isoniazid medication resistance.

### Line probe assays (PCR-based)

In line probe tests, DNA is extracted, a gene area known to be linked with resistance is amplified, and the PCR products are then reverse-hybridized with immobilized, industry-standard probes to detect resistance-related gene mutations22. As an example, the INNO-LipA® Rif.TB test includes "S" probes that are wild-type in addition to "R" probes that look for resistance mutations in the RRDR of the rpoB region. One of the "S" probes not being detected suggests rifampin resistance23. The INNO-LipA® Rif.TB (Innogenetics, Belgium), Genotype® MTBDR, and second-generation Genotype® MTBDRplus are currently the

only three commercially available line probe tests for the detection of first-line treatment resistance of Mtb (Hain Life Science GmbH, Germany). All three tests can identify the rpoB gene changes that cause rifampin resistance, but only Genotype® MTBDR can also detect katG mutations, and only Genotype® MTBDRplus can detect both katG and inhA mutations24. In clinical specimens and laboratory isolates, two recent meta-analyses examining the precision of the line probe tests showed sensitivity to identify rifampin resistance mutations to be 94-100% and specificity to be 99-100%22,25. Using 15 investigations and 1,738 specimens from various nations and body locations, the first metaanalysis assessed the precision of the INNO-LipA® Rif.TB tests in comparison to susceptibility data derived from either the BACTEC 460 or the agar percentage technique. Although the sensitivity on the cultured isolates was more than 95%, the assay's direct clinical specimens showed significant variability (range 80%–100%). Similar to the first meta-analysis, the second examined 3,349 laboratory isolates and clinical samples from different regions and assessed the precision of the Genotype® MTBDR test (by comparison to the agar proportion technique, BACTEC 460, and/or BACTEC MGIT 960). The Genotype® MTBDR assay's combined sensitivity and specificity for identifying INH resistance were only 84.3% (76.6%-89.8%) and 99.5% (97.5%-99.9%), respectively. The line probe tests have a TAT of one to two days. In a few investigations, the Genotype® MTBDR and INNO-LipA® Rif.TB were compared head to head in terms of how well they could identify MDR TB. One such research examined 52 Mtb clinical isolates from Finland and Russia and compared the two line probe tests with DST and traditional sequencing. Each test identified 51/52 (98.1%) of the rifampin resistance discovered by DST, resulting in a 100% concordance rate in the detection of rifampin resistance. The rpoB mutations discovered by DNA sequencing were identified by the Genotype®MTBDR and INNO-LipA® Rif.TB with detection rates of 92.3% and 96.2%, respectively. Given that the mutations causing resistance reside located in the RRDR of the rpoB gene, as they are in more than 95% of rifampin-resistant strains, line probe assays are quick and reliable techniques for detecting rifampin resistance, despite not being FDA-approved. Due to the small number of mutations that induce INH resistance that are reflected in the test, they are less useful clinically in diagnosing isoniazid resistance. Because they do not depend on DNA sequencing technology, the line probe assays also have other significant drawbacks, such as their insensitivity to identifying new mutations and their inability to distinguish between changes that produce resistance and those that do not. The variation in the frequency of rifampin and INH resistance mutations by region16 may help to explain some of the variation in test sensitivity.

**Molecular beacon-based real time-polymerase chain reaction (RT-PCR)**

The GeneXpert® MTB/Rif TB test is another widely available hybridization technique for the detection of MDR TB (Cepheid, CA). The molecular beacons and probes for hybridization to various target segments within a region of the gene of interest are used in this real-time PCR test. The beacons generate fluorescent

signals when there is perfect nucleotide concordance between the probe and target sequence. Signaling's absence signals a mutation in the relevant examined area segment. Both high and low MDR TB prevalence nations have evaluated molecular beacon-based RT-PCR techniques[27–33]. Rifampin resistance may be detected in clinical specimens with a sensitivity of 86-100% and a specificity of 95-100%, but sensitivity is greater in smear-positive cases[33]. The detection of isoniazid resistance in clinical isolates has a sensitivity of 76%-94% and a specificity of 100%, respectively. Poor-quality sputum samples, smear-negative mycobacterial populations, and the presence of mixed and drug-resistant mycobacterial populations outside of the studied area are all common causes of reduced sensitivity. High sensitivity and specificity in detecting MDR TB, quick TAT (less than 2 hours), hands-free processing, near-patient technology, and high throughput are only a few of the major benefits of molecular beacon-based RT-PCR assays[29,33]. Due to the fact that amplification, hybridization, and analysis take place within of a single closed well, cross-contamination is almost eliminated31. These assays may also find previously undetected mutations in a particular location, therefore they are not only restricted to the detection of predetermined changes. The expense of the equipment, the difficulty to identify mutations that cause resistance outside of the designated target area, and the discovery of silent mutations that are mistakenly perceived as imparting resistance are the main drawbacks to their usage.

## PYROSEQUENCING

Recently, M. tuberculosis mutations linked to treatment resistance have been found by using the quick, automated DNA sequencing method known as pyrosequencing18,34-38. In its "sequencing by synthesis" approach, a DNA polymerase is used to synthesize a strand of DNA that is complementary to the DNA segment of interest. ATP is produced and used to power the luciferase reaction36 when the DNA polymerase incorporates a nucleotide complementary to a base pair on the research strand template. Pyrosequencing, like traditional sequencing, may provide precise DNA sequences, allowing for the detection of both known and unknown mutations. Pyrosequencing has a number of benefits over traditional DNA sequencing, including lower costs, quick and easy processing, simple interpretation, and relatively high throughput. Pyrosequencing's inability to accurately read continuous, lengthy sequences (i.e., those longer than 50 nucleotides) is a significant drawback35. It is quite useful for identifying mutations linked to rifampin resistance (sensitivity 92–100%, specificity 92–100% in clinical and laboratory data). Because many INH-resistance mutations are still undiscovered and are found outside of the frequently investigated areas of the katG and inhA genes18,34-39, its value in identifying isoniazid resistance is lower (sensitivity 64%-81%, specificity 100% among clinical and laboratory isolates).

## CONCLUSIONS

Rapid DST is required because to the increasing incidence of MDR TB globally and the related global morbidity and death. In low-income, MDR TB-endemic nations,

diagnostic tools must not only be very accurate in identifying rifampin and isoniazid resistance, quick TATs, and high throughput, but also reasonably priced. Rapid molecular diagnostics are an excellent supplement to traditional phenotypic testing and are ideally suited for the confirmation of suspected MDR TB. But as of right now, neither phenotypic nor fast molecular drug testing are 100% accurate in predicting MTB drug susceptibility[40,41]. Even though quick molecular testing does not predict resistance, clinicians should still consider treating patients who are at high risk for developing MDR until findings of phenotypic susceptibility are available. Only suspected drug-resistant MTB isolates that show inconsistent findings from fast molecular testing and phenotypic susceptibility should be subjected to DNA sequencing. Finally, future recommendations should include a diagnostic methodology to help doctors and clinical labs monitor and find MDR TB.

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