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# Rapid Detection of Genetic Mutations Associated with Drug-resistant *Mycobacterium tuberculosis*

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Public Health (Epidemiology)

by

Marva Lynn Seifert

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2015

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The Dissertation of Marva Lynn Seifert is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2015

DEDICATION

*To Lars, whose support and encouragement made this possible,*

*and to Aléa and Solei who bring such joy and sunshine to my life*

EPIGRAPH

TB is like living with a bomb in your lungs.

You just lie around very quietly hoping it won't go off.

*Sylvia Plath*

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LIST OF ACRONYMS

| | |
|---|---|
| AFB | Acid fast bacilli |
| AMK | Amikacin |
| CAP | Capreomycin |
| CFU/mL | Colony forming units per milliliter |
| EMB | Ethambutol |
| FQ | Flouroquinolone |
| GAT | Gatifloxacin |
| GCDD | Global Consortium for Drug-resistant TB Diagnostics |
| INH | Isoniazid |
| IRB | Institutional Review Board |
| KAN | Kanamycin |
| LAMP | Loop-mediated Isothermal Amplification |
| LVX | Levofloxacin |
| MDR | Multidrug-resistant |
| MGIT | Mycobacteria Growth Indicator Tube |
| MODS | Microscopic Observations of Drug Susceptibility |
| MOX | Moxifloxacin |
| *Mtb* | *Mycobacterium tuberculosis* |
| MUT | Mutation |
| OFX | Ofloxacin |
| PSQ | Pyrosequencing |
| PZA | Pyrazinamide |
| RIF | Rifampicin |
| SLID | Second-line Injectable Drugs |

| | |
|---|---|
| STR | Streptomycin |
| TB | Tuberculosis |
| WHO | World Health Organization |
| WT | Wild Type |
| XDR | Extensively Drug-resistant |

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ABSTRACT OF THE DISSERTATION

Rapid Detection of Genetic Mutations Associated with
Drug-resistant *Mycobacterium tuberculosis*

by

Marva Lynn Seifert

Doctor of Philosophy in Public Health (Epidemiology)

University of California, San Diego, 2015
San Diego State University, 2015

Professor Stephanie Brodine, Chair
Professor Timothy Rodwell, Co-Chair

Background: Drug resistance continues to threaten recent global progress toward tuberculosis (TB) control. Rapid and accurate detection of resistance is imperative for successful treatment outcomes and reduction in transmission of resistant strains.

Methods: Global frequency and distribution of mutations associated with isoniazid resistance were characterized through an exhaustive systematic literature review. The diagnostic performance of a newly redesigned line probe assay (LPA) among both Acid Fast Bacilli (AFB) smear positive and negative specimens was evaluated using cross-sectional data from a large multisite diagnostic study. Additionally, the association of clinical characteristics and valid test results

(susceptible or resistant) was assessed using logistic regression. Individual probe results from two LPAs were compared to both sequencing data and phenotypic susceptibility results, and the relative contribution of absent wild type probe hybridization to the detection of phenotypic resistance was determined by comparing the areas under the receiver operating characteristic curves.

Results: Globally 64% of isoniazid resistant specimens harbor the katG315 mutation and 19% harbor the inhA-15 mutation, together these two mutations are associated with 80% of isoniazid resistance. Sensitivity and specificity of the LPA for multidrug-resistant TB detection were 95.1% (95%CI 92.6, 96.9) and 99.1% (95%CI 97.1, 100.0) respectively, among both AFB smear positive and negative specimens. Sensitivity for isoniazid resistance was significantly lower among AFB smear negative specimens at 81.6% (95%CI 65.1, 91.7) compared to 94.9% (95%CI 92.4, 96.6) among AFB smear positive specimens. Additionally, AFB smear gradation was significantly associated with valid test results after controlling for HIV status, diabetic status, body mass index, and history of previous treatment. The inclusion of absent wild type probe hybridization in resistance classification for LPAs significantly improved detection of both rifampicin and flouroquinolone phenotypic resistance ($P<0.001$).

Conclusions: Variation in mutation frequency justifies local monitoring of resistance associated mutations. The newly redesigned LPA performed well among both AFB smear positive and negative specimens, however, bacillary load influenced both the probability of obtaining an interpretable result and test sensitivity. The evaluation of individual LPA probe results demonstrated that current LPA resistance classification algorithms successfully differentiate between phenotypically susceptible and resistant specimens.

# INTRODUCTION

Tuberculosis (TB) has been one of the leading causes of death by an infectious agent throughout human history. If left untreated, more than half of those infected with active disease eventually succumb. The development of antibiotics, and their subsequent use for anti-tuberculosis therapy in the 1940s, resulted in significant declines in both incidence and mortality. In fact, combined-drug regimens were so effective, that by the 1970s there was optimism about the potential of worldwide TB eradication. Funding for TB control and research waned correspondingly. But by the late 1980s, alarming increases in TB incidence rates were observed in countries with high rates of human immunodeficiency virus (HIV) infection. Focus on TB control was subsequently renewed; and access to TB care and treatment was expanded. By the early 2000s the dual epidemics of HIV and TB appeared to peak and incidence rates of TB again began to decline. However, in spite of this decline in TB incidence, the proportion of drug resistant cases has continued to slowly increase, further threatening control efforts.

Treatment outcomes for those infected with drug-resistant disease are significantly poorer than for those with susceptible disease. Failure to rapidly identify and treat patients with drug resistant TB increases the likelihood of transmission and reduces the effectiveness of anti-tuberculosis drugs in controlling infection. Timely and targeted therapy however can significantly improve the potential for treatment success. Rapid and accurate detection of specific drug resistance, therefore, is critical for drug resistant TB control.

Conventional culture based drug susceptibility testing requires considerable resources and technical skill. Additionally, culture-based methods can take weeks to months to yield results due to the slow growing nature of the *Mycobacterium*

*tuberculosis* (*Mtb*), the organism that causes TB. Renewed interest in rapid diagnostics for detecting drug resistance over the past decade has resulted in an exponential increase in the number of diagnostic tests both currently in use and under development. The majority of these newer diagnostics use molecular based methods to detect drug resistance, classifying strains as resistant or susceptible based on the detection of genetic mutations associated with resistance. Underlying mechanisms of drug resistance are, however, not completely understood, and the mutations associated with resistance have not yet been exhaustively characterized. The ability of these rapid tests to classify resistance is generally characterized as good; but problems associated with incorrectly classified results remain. False positive and false negative results both have significant clinical care implications for individual patients and for overall resistance detection.

The aim of this dissertation study is to further the understanding of genetic mutations associated with drug resistant TB and their association with rapid diagnostic performance. This aim will be accomplished through the following three studies. 1) A systematic literature review will be conducted to characterize the global frequency and distribution of mutations associated with isoniazid resistance, one of two first-line anti-tuberculosis drugs. Results will not only contribute to a better understanding of mutation patterns and geographic variability associated with isoniazid resistance, but also influence the design of future molecular diagnostics and provide a context for interpretation of current molecular based test results. 2) An evaluation of the performance of a newly redesigned line probe assay—one of several molecular based diagnostic tests endorsed by the World Health Organization for the detection of first-line drug resistance—will be performed among both smear positive and negative specimens. Additionally the association of clinical and

demographic characteristics with the likelihood of obtaining a valid test result will be assessed. Study results will contribute to the body of evidence assessing the performance of line probe assays. 3) Line probe assay results will be compared to genetic sequencing to determine individual probe performance for both wild type and mutation containing sequences. A separate examination of line probe assay results to culture based phenotypic testing will also be conducted to determine if absent wild type sequence hybridization contributes significantly to the detection of resistance. Results will contribute to the understanding of the nuance of the line probe assay design and its ability to detect true resistance.

Both line probe assay studies will draw on data collected through the Global Consortium for Drug-resistant TB Diagnostics (GCDD), an international collaborative based at the University of California, San Diego. A total of 1,128 participants were enrolled between 2012 and 2013 at study sites in India, Moldova, and South Africa. Participants presenting with suspected drug resistant TB provided sputum specimens which were tested using multiple culture based and molecular based methods. The study was approved by the University of California, San Diego internal review board and the review boards associated with each respective study site.

# CHAPTER 1

# LITERATURE REVIEW

## Drug-resistant Tuberculosis

Robert Koch identified the infectious agent, *Mycobacterium tuberculosis* (*Mtb*), as the cause of tuberculosis (TB) disease in 1882; however, it was not until 1944 that the first antibiotic was used to control TB.(1, 2) Following the introduction and widespread use of streptomycin mono-therapy, evidence of drug resistance and relapse began to emerge.(3-5) Concurrent use of multiple drugs was found to minimize the development of drug resistance; and by the 1960s, isoniazid (INH) and rifampicin (RIF)—the backbone of first-line anti-tuberculosis drugs, were administered in combination with significant success.(3, 4)

## Evolution and Classification of Drug-resistance

*Mycobacterium tuberculosis* (*Mtb*) is a clonal bacterium with a relatively static genome.(6, 7) Drug resistance develops when, during chromosomal replication, errors or mutations occur in either the genes that encode drug targets themselves, or in genes that encode enzymes that activate anti-tuberculosis drugs. Spontaneous mutations occur at a frequency of $10^{-6}$ to $10^{-8}$ per replication, and when combined with selection pressure from inadequate anti-tuberculosis treatment (due to incorrect treatment regimens, interrupted drug supply, or poor patient adherence) drug resistant strains of *Mtb* emerge.(8, 9) Drug resistance that emerges during treatment (acquired resistance) can be further amplified (amplified resistance) as mutations associated with additional drug resistance accumulate.(10) Recent increases in drug resistance, however, are thought to be caused primarily by direct transmission of drug resistant strains (primary or transmitted resistance).(4, 11) Bacterial mutations can impact the reproductive fitness of a microbe, either inhibiting or facilitating its survival in the pathogen population. Mathematical models and laboratory studies have shown that *Mtb* can and does develop compensatory mutations that restore reproductive

fitness, and in some instances, mutation-containing strains grow better than their wild type counterparts, resulting in continued propagation of mutations in the pathogen population.(12-16)

Resistance to the two most effect first-line anti-tuberculosis drugs, INH and RIF, is defined as multidrug-resistant, or MDR-TB.(17) Moreover, an increased prevalence of MDR-TB is also associated with an increased risk of extensively drug resistant TB or XDR-TB which is defined as MDR-TB with additional resistance to a flouroquinolone (FQ) and a second-line injectable drug (SLID), such as amikacin (AMK), kanamycin (KAN), or capreomycin (CAP).(18)

## Mechanisms of Drug-resistance

Although certain drug resistance mechanisms have been extensively studied and the genes associated with this resistance are well characterized, other resistance mechanisms are not completely understood despite ongoing studies. Novel or previously undetected mutations associated with drug resistance continue to emerge and have yet to be exhaustively characterized. Evidence also suggests the frequency and geographic distribution of specific mutations vary significantly and are influenced by a multitude of factors, including host characteristics and selection pressure based on local treatment regimens.(19) The following paragraphs briefly describe the known resistance mechanism for drugs typically used in first-line treatment and the most commonly used drugs for second-line treatment (FQs and SLIDs). Although other second-line drugs exist, many are bacteriostatic, more toxic and less efficacious and are excluded from further discussion as they are not currently recommended for routine clinical use.(20, 21)

Resistance to INH, the most frequently encountered form of drug resistance, is notoriously complex; however, it is the cornerstone of first-line anti-TB treatment

and provides the most potent early bactericidal activity.(22) Mutations on the *katG*, *inhA*, *ahpC, oxyR*, *kasA*, *ndh*, *efpA*, and *iniBAC* genes have all been associated with INH resistance.(23, 24) INH is a pro-drug and must be activated by the catalase-peroxide enzyme that is encoded by the *katG* gene.(9) Mutations on the *ahpC* and *oxyR* genes can also inhibit INH activation as mutations occurring on those genes reduce the peroxide availability and impact the regulation of oxidative stress.(17, 25) Once activated, INH primarily targets the NADH-specific enoyl-acyl carrier protein, which is encoded by *inhA*. Mutations on *inhA* inhibit binding to NADH.(3) Although numerous genes have been shown to confer resistance, INH resistance has been primarily associated with mutations which occur in just two gene regions, *katG* and *inhA* with the dominate mutation (*katG*315) associated with 40 to 90% of all isoniazid resistance.(26-29) High level INH resistance is generally associated with mutations on *katG*, while low level isoniazid resistance is associated with mutations on *inhA*.(30, 31)

Utilization of RIF is also a key component of first line anti-tuberculosis treatment since it has a sustained sterilizing effect among bacteria that only sporadically reproduce.(9) Rifampicin binds to the B-subunit of RNA polymerase (encoded by *rpoB* gene) of the *Mtb* and inhibits RNA transcription and protein synthesis.(3, 10, 32) RIF resistance appears to be almost exclusively associated with mutations on *rpoB*. Greater than 95% of RIF resistant isolates harbor mutations on the 81 base pair region of the *rpoB* and less than 10% of RIF resistant isolates harbor mutations on the N-terminal.(10, 33-35) An estimated 30 to 75% of RIF resistant strains harbor a mutation at *rpoB* 531. Mutations occurring at codons 516, 526, and 531 are typically associated with higher level resistance, and codons occurring at 511, 518, and 522 are associated with lower level resistance. RIF resistance typically

occurs in conjunction with INH resistance and is often used a proxy indicator of MDR-TB in diagnostic testing.(36)

Resistance to other first line anti-TB agents, either previously or currently used, pyrazinamide (PZA), ethambutol (EMB), and streptomycin (STR), are described below. PZA has a unique sterilizing effect on semi-dormant bacilli and resistance is primarily associated with mutations on *pncA* and *rspA*; however, no single mutation is predominant.(26) PZA is a pro-drug and must be activated by pyrazinamidase, which is encoded by the *pncA* gene and binds to the ribosomal protein S1 encoded by *rpsA.* (3, 17, 37) Seventy to 95% of PZA resistance is associated with mutations on the *pncA* and *rpsA* genes.(20, 38) Mechanisms of resistance for EMB are not fully understood, but target enzymes used for synthesis and polymerization of cell walls are encoded by the following three genes: *embC*, *embA*, and *embB*. Mutations on *embB* 306 have been reported in 30 to 68% of EMB resistant strains.(20, 39) STR binds to a ribosomal protein and 16S rRNA, which interrupts protein synthesis and is encoded by *rpsL* and *rrs*. Thirty to 70% of STR resistant strains have *rrs* or *rpsL* mutations. Low level STR resistance has also been associated with mutations on the *gidB* gene.(12)

Resistance to FQs is associated with particularly poor treatment outcomes.(40) FQ resistance includes resistance to ofloxacin (OFX), levofloxacin (LVX), moxifloxacin (MOX), or gatifloxacin (GAT).(9) FQs inactive DNA gyrase and inhibit DNA replication. Approximately 70 to 90% of FQ resistance is associated with mutations which occur on the *gyrA,* with the most common occurring at codons 90, 91, and 94.(41, 42) Mutations occurring on the *gyrB* gene also contribute to drug resistance, however, this frequency is reported at less than 5% of phenotypically FQ resistant strains.(42)

Resistance mechanisms to second line injectable drugs (or SLIDs) inhibit protein synthesis and are primarily associated with mutations on the *rrs* gene.(9) An estimated 75 to 100% of AMK resistance is associated with mutations on the *rrs* gene, specifically at codon 1401.(26, 43) CAP resistance is associated with mutations on both *rrs* and *tlyA* genes.(43, 44) And KAN resistance is associated with both mutations on *rrs* gene and the *eis* promoter.(26)

## Epidemiology of Drug-resistant TB

Accurately quantifying MDR-TB incidence in countries without systematic surveillance systems or mortality records is challenging.(18, 45, 46) Global estimates for 2013 suggest that 3.5% of new cases and 20.5% of previously treated cases of TB were multi-drug resistant.(47) The proportion of incident TB cases which are MDR slowly increased from 1990 until 2008 and now accounts for approximately 5% of all global cases.(48, 49) The burden and distribution of drug resistant TB is uneven with India, China, and the Russian Federation accounting for over half of all new MDR-TB cases.(50) The highest incidence rates of MDR-TB occur in Eastern Europe and central Asia, with subnational rates as high as 35% among new cases and 75% among previously treated cases.(50) Globally, the proportion of MDR-TB cases that harbor XDR mutations is estimated to be 9%. However, in several eastern European countries more than 20% of MDR-TB cases are also classified as XDR-TB.(50) Reports indicate mixed trajectories for rates of MDR-TB; some regions have recorded increasing rates and others have begun to see decreasing rates.(46) This variability underscores the need for a regionalized approach in drug resistance detection.

## Treatment Outcomes for Drug-resistant TB

Long-term prognosis for TB patients without anti-tuberculosis treatment is very poor. A study of the natural history of TB prior to the introduction anti-tuberculosis

therapy discovered that approximately 70% of individuals with active TB died within 10 years.(51) Drug resistance in general is associated with higher morbidity and mortality than drug susceptible TB, particularly within the context of an HIV immunocompromised population.(4) In the seminal report on XRD-TB outcomes from KwaZulu-Natal, South Africa, 52 of 53 patients died of XDR-TB in a single hospital based outbreak, with a mean time to death of 16 days post sputum collection.(18, 52) According to most recent TB statistics, in 2013 86% of global drug-susceptible TB patients were treated successfully; in comparison, only 48% of MDR-TB patients had successful treatment outcomes.(49) Not only are both MDR and XDR-TB more difficult to treat because they do not respond to standard six-month treatment regimens, often requiring up to two years of specialized treatment; but also because they require treatment with drugs that are significantly more costly than the standard treatment.(53, 54) Individualized treatment, or treatment that accounts for previous treatment history and individual resistance profiles, demonstrated success rates as high as 70% among patients with MDR-TB.(55-57) The importance of appropriate individualized treatment cannot be underestimated; but it can only occur when drug resistance has been accurately detected.(58)

## DRUG SUSCEPTIBILITY TESTING

Timely diagnosis of TB is particularly challenging as no single test is ideally sensitive, specific, and rapid. Currently, a combination of tests and an evaluation of clinical symptoms are used to definitively diagnose active TB. The acid-fact bacilli (AFB) smear is the most commonly used method to detect the presence of *Mtb* in sputum for diagnosis of active TB in low resource settings.(59, 60) However, the sensitivity of AFB smear microscopy is less than ideal, ranging from 20 to 80%

depending on the study.(5, 61) AFB smear tests perform particularly poorly among children, individuals who are immunocompromised, and those with drug-resistant TB.(11) More fundamentally, though, AFB smear only detects the presence of *Mtb* and cannot detect the difference between susceptible and drug resistant organisms. Culture based drug susceptibility testing (DST) remains the gold standard for detection of drug resistance. During the past decade, increased funding in rapid diagnostics development and the advent of new technologies has fueled an increase in new diagnostics being used for the detection of drug resistance.(62)

**Phenotypic Drug Susceptibility Testing**

Culture based phenotypic DST methods identify drug resistance based on the growth of *Mtb* in media containing varying amounts of anti-tuberculosis drugs. Conventional culture in solid media, either Lowenstein-Jenson or Middlebrook agar, is commonly used to detect drug susceptibility using absolute concentration, agar proportion, or resistance ratio method; but it can take from 8 to 12 weeks to produce a definitive result.(33) These methods rely on the differential growth of *Mtb* in untreated and drug containing media to detect resistance.

Refined phenotypic techniques using liquid culture, such as the BACTEC 460 TB (Becton Dickinson Diagnostics, Sparks, MD, USA), the BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system (Becton Dickinson Diagnostics, Sparks, MD, USA), the MB/BacT (Organon Teknika, Turnhout, Belgium), and the VersaTREK culture system (TREK Diagnostic Systems, Cleveland, Ohio, USA) reduce time to results to 3 to 7 weeks.(33, 63) These liquid culture methods employ a variety of technologies to detect growth; the MGIT 960 system uses an oxygen quenched fluorochrome which fluoresces when multiplying bacteria consume oxygen, and the VersaTREK monitors pressure as bacteria consume oxygen and the reduction in

pressure is detected.(64) Although liquid culture methods are commercially available and typically automated, inoculation can be cumbersome and biosafety protocols must be strictly adhered to during testing.

Rapid phenotypic methods, such as Microscopic Observations of Drug Susceptibility (MODS), the colorimetric redox indicator methods, phage amplification assays, and nitrate reduction assays further reduce turnaround time to 1 to 4 weeks.(33) MODS does not require complex laboratory equipment and can be performed directly on sputum. Wells with anti-microbial agents are inoculated and growth in the wells indicate drug resistance. This method does require technical skill and specific biosafety protocols.(59)

**Genotypic Drug Susceptibility Testing**

Molecular based genotypic DST methods detect drug resistance by identifying resistance conferring mutations on *Mtb* genes. Methods include sequencing, Polymerase chain reaction (PCR) based hybridization assays, real time PCR based methods, and other nucleic acid amplification techniques.(65-67) In contrast to phenotypic growth based methods, molecular based methods are not dependent on the notoriously slow growth of *Mtb*, and the time to detection of drug resistance can be greatly reduced.(68-70)

Sanger sequencing is considered the gold standard for molecular based methods as it directly detects precise mutations, however, sequencing requires extensive technical skill and time and sequencing remains prohibitively costly to be practical for routine testing.(26, 71) Pyrosequencing, a rapid real-time method for sequencing provides an alternative method by sequencing small segments of *Mtb* for the detection of resistance conferring mutations.(72, 73) Next generation sequencing platforms capable of sequencing thousands of sequences concurrently require

sophisticated software to analyze data output. Relatively high concentrations of DNA are also necessary, rendering more useful for research and not for rapid detection of drug resistance.(26)

PCR based hybridization assays use DNA extraction, amplification, and reverse hybridization to identify known gene mutations associated with resistance. Line probe assays immobilize specific oligonucleotides for either wild type or mutation sequences on a membrane strip. And as hybridization occurs, a colored band appears indicating the presence of either a wild type or mutation sequence. Turnaround time for these assays can be as little as six hours, but the real world results average is closer to two days.(19, 33) In 2008 line probe assays became the first molecular method endorsed by the WHO for detection of drug resistance (MDR-TB). Commercially available line probe assays for MDR-TB detection include the INNO-LiPARif.TB (Innogenetics, Zwijndrecht, Belgium) designed for use in culture isolates, and the Genotype MTBDRplus (Hain LifeScience GmbH, Nehren, Germany), which can be used for both sputum sediments and culture isolates. The risk of cross contamination and need for high level laboratory skill make line probe assays best suited for use in reference and district level laboratories.

Real time PCR based methods include molecular beacon, taqman probes, fluorescence resonance energy transfer probes, single stranded conformation polymorphism, and high performance liquid chromatography analysis. The most widely used commercially available molecular beacon assay, the GeneXpert MTB/RIF TB assay (Cepheid, Sunnyvale, CA), has a turnaround time of two hours.(74) Extraction, amplification, and detection process occur in an automated closed cartridge system. The GeneXpert MTB/RIF is ideally suited as a point of care test

since relatively unskilled laboratory workers can perform the test. However, necessary equipment for utilizing the test can be costly.(19)

A manual TB detection method based on the loop-mediated isothermal amplification platform (LAMP) LoopAMP MTBC detection kit (Eiken Chemical Company, Japan) is currently waiting WHO endorsement.(19) LAMP technology is based on autocycling strand displacement and DNA synthesis.(19) It is ideal for use in lower resource settings or in rural areas where access to a district or regional testing laboratory is unavailable. Minimal equipment is necessary and the test is easy to interpret since results can be read with the naked eye.

A notable difference between all culture based and molecular based testing is the amount of bacteria needed to perform the test. Culture based methods can detect resistance in ten or fewer colony forming units per milliliter (CFU/mL) of sputum the most sensitive molecular tests require ten times the number of CFU/mL to yield a result. It is anticipated that next generation sequencing tests also will only require CFU of similar magnitude to culture for *Mtb* detection.(19, 75) In contrast, culture based methods can also detect the presence of drug resistance even when the proportion of resistant organisms comprise less than 1% of the bacterial population.(28) Other documented disadvantages of phenotypic methods include the need for specialized laboratory equipment and training, advanced biosafety protocols, and the significant delay between testing and results. For molecular based methods, a significant reduction in time to result is gained, and proper biosafety conditions are only required during specimen processing and DNA extraction. These tests can also detect non-viable DNA. The primary drawback to molecular based tests is that the sensitivity of the test depends directly on the exhaustiveness of characterized mutations. Phenotypic resistance is not always associated with a mutation on a target

gene, and sometimes mutations occurring on structural genes do not confer resistance.(28) Until all mutations are fully characterized, genotypic susceptibility testing methods will likely need to be coupled with confirmatory phenotypic testing, particularly when treatment appears to be failing.

## Data Sources

Data used for this dissertation was drawn from two sources: a systematic review and a prospective cohort study conducted by the Global Consortium for Drug-resistant TB Diagnostics (GCDD), a University of California San Diego based international research collaborative. Data for the systematic review was extracted from publications obtained via electronic search using PubMed. All English-language peer-reviewed publications that assessed mutations associated with INH resistance published between January 1, 2000 and August 31, 2013 were evaluated. PubMed key search terms included: (INH OR isoniazid) AND (resistance or resistant) AND (tuberculosis) AND (mutation OR sequence). Publications were excluded if they did not present original data, did not use clinical strains of *Mtb*, did not describe the phenotypic DST method used as reference standard, did not use DNA sequencing as a means of characterizing mutations, or did not include individual level amino acid mutation data. Study details, exact sequences characterized, and individual isolate or specimen mutation information were extracted from each publication and compiled in a database for analysis.

Data used for the assessment of molecular diagnostic performance was collected through a multicenter, prospective, non-randomized, observational study focused on the evaluation of rapid tests for the detection of drug resistant TB conducted by the GCDD.(76) Between 2012 and 2013, patients with risk factors for drug-resistant TB were screened at participating study sites in South Africa, Moldova,

and India. Un-institutionalized men and women, regardless of HIV status, greater than age five, and willing and able to provide age appropriate informed consent were invited to participate. Participants had to have previously received more than one month of TB treatment for a prior TB episode, be failing TB treatment with a positive smear after three months of standard treatment, have had close contact with a known drug-resistant TB case, be newly diagnosed with MDR-TB within the previous 30 days, or be previously diagnosed with MDR-TB and be failing current treatment with a positive smear after three months of treatment to be included in the study. Both biological specimens and patient data used for the current studies were collected from participants at baseline. Sputum samples were used to perform multiple diagnostic tests including: AFB smear microscopy; MODS, a non-commercial rapid growth-based assay redesigned to detect XDR-TB; two line probe assays GenoType MTBDRplus v2 and GenoType MTBDR*sl* (Hain Lifesciences, Nehren, Germany); a commercially available pyrosequencing platform, the PyroMark Q96 platform (Qiagen, Valencia, CA, USA), repurposed for *Mtb* resistance detection; and a phenotypic reference standard, Mycobacterial Growth Indicator Tube 960 (BD Biosciences, Sparks, MD, USA). *Mtb* culture positive specimens were tested for drug susceptibility to INH, RIF, MOX, OFX, KAN, CAP, and AMK.

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# CHAPTER 2

## Genetic Mutations Associated with Isoniazid Resistance in *Mycobacterium tuberculosis*: A Systematic Review

PLOS | ONE

RESEARCH ARTICLE

# Genetic Mutations Associated with Isoniazid Resistance in *Mycobacterium tuberculosis*: A Systematic Review

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## Abstract

### Background

Tuberculosis (TB) incidence and mortality are declining worldwide; however, poor detection of drug-resistant disease threatens to reverse current progress toward global TB control. Multiple, rapid molecular diagnostic tests have recently been developed to detect genetic mutations in *Mycobacterium tuberculosis (Mtb)* genes known to confer first-line drug resistance. Their utility, though, depends on the frequency and distribution of the resistance associated mutations in the pathogen population. Mutations associated with rifampicin resistance, one of the two first-line drugs, are well understood and appear to occur in a single gene region in >95% of phenotypically resistant isolates. Mutations associated with isoniazid, the other first-line drug, are more complex and occur in multiple *Mtb* genes.

### Objectives/Methodology

A systematic review of all published studies from January 2000 through August 2013 was conducted to quantify the frequency of the most common mutations associated with isoniazid resistance, to describe the frequency at which these mutations co-occur, and to identify the regional differences in the distribution of these mutations. Mutation data from 118 publications were extracted and analyzed for 11,411 *Mtb* isolates from 49 countries.

### Principal Findings/Conclusions

Globally, 64% of all observed phenotypic isoniazid resistance was associated with the *katG*315 mutation. The second most frequently observed mutation, *inhA*-15, was reported among 19% of phenotypically resistant isolates. These two mutations, *katG*315 and *inhA*-15, combined with ten of the most commonly occurring mutations in the *inhA* promoter and the *ahpC-oxyR* intergenic region explain 84% of global phenotypic isoniazid resistance. Regional variation in the frequency of individual mutations may limit the sensitivity of molecular diagnostic tests. Well-designed systematic surveys and whole genome sequencing are needed to identify mutation frequencies in geographic regions where rapid molecular tests

**Competing Interests:** The authors have declared that no competing interests exist.

are currently being deployed, providing a context for interpretation of test results and the opportunity for improving the next generation of diagnostics.

## Introduction

While tuberculosis (TB) incidence and mortality declined over the past decade, there were still an estimated 8.6 million new cases of TB and 1.3 million deaths attributed to TB worldwide in 2012 [1]. Additionally, progress toward the reduction of TB morbidity and mortality has been largely overshadowed by the increasing prevalence of TB that fails to respond to standard antibiotic treatment. Multidrug resistant TB (MDR-TB), or TB that does not respond to either isoniazid (INH) or rifampicin (RIF), the "backbone" of the current recommended treatment, accounted for approximately 3.6% of all new TB cases and 20.2% of recurring, or previously treated TB cases reported in 2012 [1]. On a local level, incidence of MDR-TB is even more alarming, with individual countries detecting MDR-TB among as many as 28% of new cases and 62% of previously treated cases [2].

Drug resistance arises when mutations, or chromosomal replication errors, occur in genes that encode drug targets or drug metabolism mechanisms and impact the efficacy of anti-tuberculosis therapies [1–7]. Suboptimal treatment and poor adherence to drug therapies contribute to resistance amplification; while lack of detection and subsequent inappropriate treatment increase the risk of direct transmission of drug-resistant *Mtb* [5,8,9]. In 2012 it was estimated that less than 25% of MDR-TB cases were detected worldwide [1].

Low MDR-TB detection rates are due in part to the lack of rapid, accurate, and inexpensive diagnostic tests. The most commonly used method for detection of TB disease—sputum smear microscopy—does not detect drug resistance [1]. Standard drug susceptibility testing (DST), based on mycobacterial culture on solid or liquid media, can take weeks to months to produce results and requires significant biosafety resources, making it unfeasible in many low resource settings [10–12]. Multiple genotypic DST methods based on the detection of mutations that confer resistance have been developed during the past decade, including DNA sequencing, line probe assays, hybridization on DNA chips, single-strand-conformation-polymorphisms, pyrosequencing, and real-time PCR [12–14]. In order for these genotypic methods to accurately identify all resistant *Mtb* phenotypes, the genes and specific mutation locations associated with resistance must be known and included in the diagnostic test. However, global frequency and geographic distribution of INH resistance-conferring *Mtb* mutations have not yet been systematically quantified in the pathogen population [15].

Multiple reviews have identified genes that encode drug targets and have summarized the various mechanisms of resistance to both INH and RIF.[6,16–20] While greater than 95% of RIF resistance is associated with mutations in an 81 base pair section of the *rpoB* gene, INH resistance appears more complex and has been associated with multiple genes, most frequently *katG* and *inhA* [17,20–25]. Current rapid molecular diagnostic tests for INH resistance have focused on the detection of the "canonical" mutations in codon 315 of *katG* and position-15 in the *inhA* promoter region. Previous studies have identified highly variable frequencies of these mutations; with *katG*315 mutations accounting for 42 to 95% and *inhA*-15 mutations accounting for 6 to 43% of phenotypic INH resistance [6,19,20,26,27].

To date no systematic review has assessed the geographic variability of the most common mutations associated with INH resistance. Nor have the frequencies for multiple or co-occurring mutations been aggregated in order to understand the overall proportion of phenotypic INH

resistance explained by the existing canonical mutations. It is critically important to understand the frequency and geographic distribution of mutations associated with INH resistance. A failure to account for these variations limits the local effectiveness of molecular diagnostic tools currently available and constrains the development of improved genotypic diagnostic tests [28]. The aims of our systematic review were to quantify the frequency and co-occurrence of the most common mutations associated with phenotypic INH drug resistance and to describe the regional differences in the distribution of these mutations as reported in the published literature.

## Methods

A PubMed search was conducted to identify all English-language peer-reviewed publications that assessed mutations associated with INH resistance. In order to capture publications not aggregated in previous reviews, the search was restricted to studies published between January 1, 2000 and August 31, 2013. PubMed key search terms included: (INH OR isoniazid) AND (resistance or resistant) AND (tuberculosis) AND (mutation OR sequence).

### Publication Selection Criteria

Publications were selected for inclusion if they met the following criteria: 1) presented original data; 2) used clinical strains of *Mtb* (not laboratory strains); 3) described the phenotypic DST method used as reference standard; 4) used DNA sequencing (including pyrosequencing) as a means of characterizing mutations; and 5) included individual level amino acid mutation data. Data from studies evaluating commercial or in-house molecular drug susceptibility tests were included if the results were verified by DNA sequencing.

Our primary goal was to assemble mutation data that was representative of the global distribution of drug resistance phenotypes. Studies were excluded if DNA sequencing was only performed on isolates with discrepancies between phenotypic and genotypic tests, as data from these studies would not be generalizable to the pathogen population. Studies were also excluded if DNA sequencing was performed on non-random isolate subsets or if studies reported sequential sequencing, i.e. sequencing *katG* on all isolates then sequencing *inhA* among isolates without *katG* mutations then sequencing *ahpC-oxyR* among isolates without mutations in either *inhA* or *katG*. An exception was made for studies where identifying sequencing results for the initial gene sequenced was easily discernible and in these instances, the sequencing results for only the first gene sequenced were included.

### Data Extraction

The following information was extracted from each publication: primary author, publication year, geographic origin of specimens, year(s) of specimen collection, reference strains, phenotypic drug susceptibility testing method and cutoff, genotypic testing method, gene loci sequenced, primers used for DNA sequencing, and total number of resistant and susceptible isolates tested. Individual isolate mutation information included: location of gene mutation, amino acid and/or nucleotide changes, number of resistant and susceptible isolates with identified mutations, and number of resistant and susceptible isolates tested. Mutations in codon 463 of *katG* were not extracted as previous studies have identified *katG*463 as a genetic marker and not a marker of resistance [29,30]. All data were extracted and compiled using MS Excel software (Microsoft, Redmond, WA).

### Mapping of Primer Sequences and Mutation Locations

In order to accurately assess which gene fragments had been sequenced for each individual isolate, the exact start and end points of the sequenced sections were determined using one of two

methods. If primer sequences were included or referenced in the selected studies, sequence endpoint coordinates were identified by entering primer sequences into the NCBI Basic Local Alignment Search Tool (BLAST) and mapping the primers onto the complete *Mtb* H37Rv genome (Accession number NC_000962.3) [31]. If primer sequences were not published, sequenced endpoints coordinates were inferred by using the outermost identified mutations as sequence endpoints. If several primers were included and sequenced fragments overlapped, the final dataset included only the outermost/inclusive primer coordinates. Mutations identified in studies by traditional gene and nucleotide or codon nomenclature were converted to NCBI *Mtb* H37Rv complete genome (Accession number NC_000962.3) coordinates using the conversions described in S1 Table. In short, mutations occurring in the promoter or regulatory regions of the identified genes were mapped by exact nucleotide position and mutations occurring in the coding region of the gene were mapped by the center nucleotide position of the codon. Due to inconsistencies in the nomenclature and reporting of insertions, deletions, and frameshifts, they were excluded from analysis.

### Calculation of Single Mutation Frequencies

As described in 2012 by Georghiou et al., mutation frequencies among phenotypically resistant isolates were calculated by aggregating the number of occurrences of a specific mutation among phenotypically resistant isolates and dividing it by the total number of phenotypically resistant isolates that could have identified that mutation, i.e. all phenotypically resistant isolates that had been sequenced at the location of the specific mutation [32]. Mutation frequencies among phenotypically susceptible isolates were calculated using the same method; the number of occurrences of a specific mutation among phenotypically susceptible isolates was divided by the total number of phenotypically susceptible isolates sequenced at the location of the mutation being assessed. Additionally, mutation frequency data were stratified by WHO region based on specimen origin and regional frequencies were calculated as described above [33].

### Calculation of Cumulative Mutation Frequencies

Studies that reported co-occurring or multiple mutations in phenotypically resistant isolates were further analyzed to assess cumulative frequencies of the most commonly occurring mutations. Cumulative mutation frequencies were calculated first for individual genes, then across multiple genes simultaneously. Isolates were only included in data subsets if they had been sequenced at each of the mutation locations being evaluated. Individual mutation frequencies were reassessed in each data subset for global comparison, then the cumulative frequency for all mutations being evaluated was calculated by aggregating the number of isolates containing any of the mutations being assessed and dividing it by the total number of isolates in the subset.

## Results

Of the 466 publications identified, 348 were excluded as they did not meet inclusion criteria (S1 Fig.). Mutation data, including gene, mutation location, original amino acid and/or nucleotide, and mutated amino acid and/or nucleotide, were extracted for 11,411 *Mtb* isolates described in the selected publications. Study size ranged from 4 to 502 isolates per publication. Seventy-seven percent (8,786) of the isolates were phenotypically resistant to INH. Geographic origin of the isolates was reported for 94% (10,745) of the isolates and included 49 countries (S4 Table). Primers were extracted (>96% of publications included primer information) for the three most commonly inspected genes and their surrounding regions: *furA-katG*, *fabG1-inhA*, and *aphC-oxyR*.

Mutations associated with INH resistance were reported in 15 unique gene regions, the most frequently being *furA-katG* (115 publications), *fabG1-inhA* (72 publications), and *ahpC-oxyR* (24 publications). Mutations were also reported in *efpA*, *fadE24*, *iniA*, *iniB*, *iniC*, *kasA*, *nat*, *ndh*, Rv1772, Rv1592c, Rv0340, and *srmR* genes, however data extracted for these genes were very limited and were excluded from further analysis.

## Single Mutation Frequencies

Global frequencies for all mutations occurring in the *katG* coding region, *inhA* coding region, *inhA* promoter region, and *ahpC-oxyR* intergenic region were evaluated, however only mutations occurring at a cumulative frequency of >0.1% and identified in at least two publications are summarized in Table 1 (for a complete listing of all mutations see S2 Table). Of the 8,416 phenotypically resistant isolates sequenced at *katG*315, 5,400 (64.2%) harbored a mutation in

**Table 1. Global frequencies for selected mutations among phenotypically isoniazid resistant and phenotypically isoniazid sensitive isolates.**

| Gene | Loci | H37Rv Coordinate | Frequency among Resistant specimens (%) | Resistant specimens (n) | Frequency among Susceptible specimens (%) | Susceptible specimens (n) |
|---|---|---|---|---|---|---|
| *katG* | 315 | 2155168 | 64.2 | 8416 | 0.1 | 2462 |
| | 309 | 2155186 | 0.5 | 7967 | 0.0 | 2277 |
| | 316 | 2155165 | 0.4 | 8199 | 0.0 | 2393 |
| | 311 | 2155180 | 0.3 | 8181 | 0.0 | 2382 |
| | 299 | 2155216 | 0.3 | 7456 | 0.0 | 2052 |
| | 321 | 2155150 | 0.2 | 7875 | 0.0 | 2288 |
| | 275 | 2155288 | 0.2 | 6713 | 0.0 | 2015 |
| | 328 | 2155129 | 0.2 | 7825 | 0.0 | 2288 |
| | 155 | 2155648 | 0.2 | 2105 | 0.0 | 538 |
| | 110 | 2155783 | 0.2 | 2031 | 0.0 | 511 |
| *inhA* Promotor | -15 | 1673425 | 19.2 | 6192 | 0.3 | 2063 |
| | -8 | 1673432 | 1.3 | 5955 | 0.0 | 2048 |
| | -47 | 1673393 | 0.4 | 5914 | 0.0 | 1994 |
| | -17 | 1673423 | 0.3 | 6143 | 0.0 | 2063 |
| *inhA* | 94 | 1674482 | 1.2 | 2225 | 0.0 | 444 |
| | 21 | 1674263 | 1.1 | 1685 | 0.0 | 434 |
| | 194 | 1674782 | 1.1 | 996 | 0.0 | 286 |
| | 3 | 1674209 | 0.5 | 1713 | 0.0 | 434 |
| | 258 | 1674974 | 0.3 | 926 | 0.0 | 286 |
| | 190 | 1674770 | 0.3 | 996 | 0.0 | 286 |
| ahpC-oxyR Intergenic Region | -10 | 2726141 | 1.3 | 2321 | 0.0 | 536 |
| | -46 | 2726105 | 1.2 | 2321 | 0.9 | 536 |
| | -6 | 2726145 | 1.1 | 2321 | 0.0 | 536 |
| | -39 | 2726112 | 0.8 | 2321 | 0.0 | 536 |
| | -48 | 2726103 | 0.7 | 2424 | 0.0 | 576 |
| | -15 | 2726136 | 0.6 | 2321 | 0.0 | 536 |
| | -12 | 2726139 | 0.5 | 2321 | 0.0 | 536 |
| | -9 | 2726142 | 0.5 | 2321 | 0.0 | 536 |
| | -100 | 2726051 | 0.4 | 2321 | 0.2 | 536 |
| | -30 | 2726121 | 0.3 | 2321 | 0.0 | 536 |
| | -32 | 2726119 | 0.3 | 2321 | 0.2 | 536 |
| | -81 | 2726070 | 0.2 | 2321 | 0.0 | 536 |

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Table 2. Amino acid and nucleotide mutation frequency of 4,505 *katG*315 (wild type AGC) isolates.

| Amino Acid | Nucleotide | Total Number | % |
|---|---|---|---|
| **Threonine** | | | **95.3** |
| | ACC | 4208 | 93.4 |
| | ACA | 73 | 1.6 |
| | ACG | 7 | 0.2 |
| | ACT | 7 | 0.2 |
| **Asparagine** | | | **3.6** |
| | AAC | 161 | 3.6 |
| **Isoleucine** | | | **0.5** |
| | ATC | 23 | 0.5 |
| **Arginine** | | | **0.4** |
| | AGA | 8 | 0.2 |
| | CGC | 5 | 0.1 |
| | AGG | 4 | 0.1 |
| **Glycine** | | | **0.2** |
| | GGC | 9 | 0.2 |
| **Total** | | **4505** | **100.0** |

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*katG*315 whereas only 2 of 2,462 (<0.1%) phenotypically susceptible isolates harbored a mutation at that location. The remaining mutations identified in *katG* had frequencies of less than 1% among phenotypically resistant isolates. The second most commonly occurring mutation in INH resistant isolates, at position-15 of the *inhA* promoter, was identified in 1,189 of 6,192 (19.2%) phenotypically resistant and 7 of 2,063 (0.3%) phenotypically susceptible isolates. A second mutation in *inhA* promoter region, -8, was identified among 1.3% of phenotypically resistant isolates. Additionally, mutations occurring in the coding region of the *inhA* gene at codon positions 21, 94, and 194 were identified in approximately 1% of the isolates. Mutations in the *ahpC-oxyR* intergenic region reported at positions-10, -46, and -6 occurred in just over 1% of phenotypically resistant isolates. However, the mutation at position-46 of *ahpC-oxyR* intergenic region was also reported in 0.9% of susceptible isolates as well making it a non-specific marker of INH resistance.

Four-thousand, five-hundred and five isolates had nucleotide information reported in addition to amino acid information for the *katG*315 mutation (Table 2). The most common nucleotide change for the *katG*315 codon, AGC to ACC (serine to threonine), occurred among 93.4% of isolates. AGC to AAC (serine to asparagine), the second most common nucleotide change, occurred among 3.6% of isolates.

Mutation data at the country level was sparse, allowing for meaningful aggregation only at the regional level. Aggregating mutation frequencies by WHO regions revealed geographic differences in the individual frequencies for the two most commonly occurring mutations, *katG*315 and *inhA*-15 (Table 3). Among phenotypically INH resistant isolates, South East Asia had the highest frequency (78.4%) and the Western Pacific Region had the lowest frequency (55.5%) of the *katG*315 mutation. Frequencies also varied widely for *inhA*-15 mutation with the Americas region reporting the highest frequency (24.6%) and the Eastern Mediterranean region reporting the lowest frequency (13.0%) of this mutation in phenotypically INH resistant isolates.

Table 3. Regional distribution of the individual frequencies for *katG*315 and *inhA*-15.

| WHO Region | *katG*315 | | | *inhA*-15 | | |
|---|---|---|---|---|---|---|
| | Frequency (%) | Resistant w/ mutation | Total Resistant | Frequency (%) | Resistant w/ mutation | Total Resistant |
| Africa | 73.5 | 317 | 431 | 17.1 | 71 | 415 |
| Americas | 61.6 | 939 | 1525 | 24.6 | 262 | 1067 |
| E Mediterranean | 64.1 | 334 | 521 | 13.0 | 25 | 192 |
| Europe | 69.4 | 1652 | 2379 | 18.7 | 332 | 1771 |
| South East Asia | 78.4 | 438 | 559 | 13.5 | 56 | 416 |
| Western Pacific | 55.5 | 1378 | 2483 | 19.8 | 371 | 1873 |
| Unknown[a] | 66.0 | 342 | 518 | 15.7 | 72 | 458 |
| Total | 64.3 | 5400 | 8416 | 19.2 | 1189 | 6192 |

[a] Origin of specimens not identified in publication.

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## Cumulative Mutation Frequencies

The cumulative frequencies of multiple or co-occurring mutations associated with INH resistance, were assessed first by individual genes and then in combination across multiple gene regions (Table 4). Eighty-nine publications assessed 6,134 phenotypically resistant isolates for multiple mutations in *katG* inclusive of codon positions 306 to 316. The mutation frequency for *katG*315 in this subset of INH resistant isolates was 66.2%. After the inclusion of mutations at codons 311, 309, and 316 as markers of INH resistance, an additional 9 phenotypically resistant isolates were identified, for a total cumulative frequency of 66.3%. For the *inhA* promoter region, a total of 4,484 isolates from 51 publications were sequenced inclusive of positions -8 to -47. The cumulative frequency of *inhA* -15 in this subset was 19.0%, however unlike *katG*, other *inhA* promoter mutations did not typically co-occur with *inhA* -15. By including mutations at *inhA* -8, *inhA* -47, and *inhA* -17 as markers of resistance, an additional 72 (1.6%) phenotypically resistant isolates were identified. Seventeen publications, consisting of 1,696 phenotypically resistant isolates assessed mutations in the *ahpC-oxyR* intergenic region. In this subset no co-occurring mutations in the *ahpC-oxyR* intergenic region were observed. Mutations at positions -48, -39, -15, -12, -10, -9, and -6, of the *ahpC-oxyR* intergentic region occurred independently, resulting in a global cumulative frequency of 5.4% for *ahpC-oxyR* mutations among phenotypically resistant isolates in this subset.

Forty-six studies, consisting of 4,179 phenotypically resistant isolates, assessed mutations in both the *katG* gene and the *inhA* promoter region, specifically encompassing *katG*315, *inhA* -15, *inhA* -8, and *inhA* -17. Frequencies for *katG*315 and *inhA* -15 in this subset were 65.2% and 19.0% respectively. *KatG*315 and *inhA* -15 mutations co-occurred in 181 isolates, resulting in a combined cumulative frequency for *katG*315 and *inhA* -15 of 79.9%. Only fifteen publications, consisting of 1,582 individual isolates, assessed mutations in *katG* coding region, *inhA* promoter region, and *ahpC-oxyR* intergenic region. In this subset, *katG*315 occurred among 66.8% of phenotypically resistant isolates and *inhA* -15 occurred among 16.2% of phenotypically resistant isolates. With the addition of *inhA* -8, *inhA* -47, and *inhA* -17, cumulative frequency reached 80.3%; and with the addition of the *ahpC* -10, *ahpC* -6, *ahpC* -39, *ahpC* -48, *ahpC* -15, *ahpC* -12, and *ahpC* -9 cumulative frequency reached 83.9%.

Table 4. Single and cumulative mutation frequencies on ***katG***, ***inhA***, and ***ahpC-oxyR*** among data subsets which assessed co-occurring mutations.

| Gene Name (number of isolates) | Mutation type | Codon or Nucleotide Location | Resistant w/ Mutation | Cumulative Frequency (%) |
|---|---|---|---|---|
| *katG* (n = 6,134) | Single | 315 | 4059 | 66.2 |
| | | 309 | 36 | 0.6 |
| | | 316 | 27 | 0.4 |
| | | 311 | 27 | 0.4 |
| | Cumulative | 315, 309, 316, and/or 311 | 4068 | 66.3 |
| *inhA* (n = 4,484) | Single | -15 | 854 | 19.0 |
| | | -8 | 46 | 1.0 |
| | | -47 | 15 | 0.3 |
| | | -17 | 11 | 0.2 |
| | Cumulative | -15, -8, -47, and/or -17 | 926 | 20.6 |
| *ahpC-oxyR* (n = 1,696) | Single | -10 | 20 | 1.2 |
| | | -6 | 25 | 1.5 |
| | | -39 | 13 | 0.8 |
| | | -48 | 3 | 0.2 |
| | | -15 | 13 | 0.8 |
| | | -12 | 9 | 0.5 |
| | | -9 | 8 | 0.5 |
| | Cumulative | -10, -6, -39, -48, -15, -12, and/or -9 | 91 | 5.4 |
| *katG* & *inhA* (n = 4,179) | Single | katG315 | 2723 | 65.2 |
| | | inhA -15 | 795 | 19.0 |
| | Cumulative | katG315 and/or inhA -15 | 3337 | 79.9 |
| *katG* & *inhA* & *ahpC-oxyR* (n = 1,582) | Single | katG315 | 1056 | 66.8 |
| | | inhA -15 | 257 | 16.2 |
| | Cumulative | katG315 and/or inhA -15 | 1257 | 79.5 |
| | | katG315 and/or inhA -15, -8, -47, -17 | 1271 | 80.3 |
| | | katG315 and/or inhA -15, -8, -47, & -17 and/or ahpC -10, -6, -39, -48, -15, -12, & -9 | 1328 | 83.9 |

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## Discussion

Few studies to date have attempted to assess the prevalence of the most commonly occurring "canonical" mutations associated with phenotypic INH resistance among a globally representative set of isolates, making it difficult to compare the global frequencies calculated in this systematic review to previous global estimates. However, a study conducted by Lin et al. using 127 INH resistant isolates from California, a population which is thought to mirror global MDR-TB diversity due to immigration, identified a global frequency of 61% for *katG*315, 23% for *inhA* -15 mutations, and 83% for the cumulative frequency of either mutation, approximating the frequencies of these mutations as quantified in this systematic review [34]. In contrast, Campbell et al., using 212 INH resistant isolates from both WHO and CDC laboratory archives estimated the global frequency of the *katG*315 mutation to be 85%, *inhA* -15 to be 17%, and their cumulative frequency 91%; however, isolates used for that study were selected to provide a diverse set of mutation patterns, and therefore may not accurately represent true global frequencies [35]. Finally, a more recent study conducted by Rodwell et al. using 348 INH resistant isolates from four geographically diverse countries estimated the global frequencies of *katG*315 and *inhA* -15 to be significantly higher at 86% and 34% respectively. The cumulative frequency

of both mutations with the addition of *inhA -17* was 96% [36]. However, as with the previously mentioned study, isolates were selected to maximize diversity, potentially affecting the generalizability of the frequency estimates.

Prevalence of drug resistant TB is commonly understood to vary significantly by region, making it reasonable to expect regional variation in the frequencies of specific mutations that drive INH resistance [37,38]. Based on this systematic review, frequencies of specific mutations do in fact appear to vary quite significantly by geographic region. Although the total number of isolates analyzed varied by country and for some countries data was very sparse, we suspect that regional differences in the frequencies of the most common mutations could play a significant role in the performance of molecular-based diagnostic tests in some regions. For example, of the 808 INH resistant isolates sequenced from Japan and Korea, only 43% harbored the *katG*315 mutation, in contrast *katG*315 mutations in the post-soviet states of Belarus, Lithuania, Kazakhstan, Latvia, Moldova, and Russia were identified in 94% of the 751 INH resistant isolates analyzed from those countries. This regional variability of the *katG*315 mutation frequency may contribute to the poor performance of molecular diagnostics for INH resistance detection in Japan [39]. However, due to the lack of large, geographically diverse cross-sectional studies, regional patterns in single mutations should be interpreted with caution.

The frequency patterns of the most common mutations associated with INH resistance appear to differ between individual genes. It is clear that the overwhelming majority (64%) of phenotypic INH resistance among *Mtb* isolates is associated with a single mutation, *katG*315. The dominance of this mutation is hypothesized to be the result of a low or zero fitness cost for this mutation, allowing it to propagate without negative selection pressure [40,41]. Mutations other than *katG*315 in the *katG* gene appear to occur at low (<1%) frequencies and occur overwhelmingly in conjunction with the *katG*315 mutation. Patterns of co-occurring mutations in the *inhA* promoter region appear to differ markedly from co-occurring mutations in the *katG* gene. Although the *inhA* -15 mutation is the dominant (19%) mutation in the *inhA* promoter region, other resistance associated mutations (~1%) in the *inhA* promoter region appear to occur independently of the *inhA* -15 mutation and frequently contribute to the detection of INH resistance. In contrast, no single mutation in the *ahpC-oxyR* intergenic region showed an individual frequency above 1.3%; rather multiple mutations (at positions -48, -39, -15, -12, -10, -9, and -6,) in the *ahpC-oxyR* intergenic region occurred at a total cumulative frequency of 5.4% among phenotypically resistant isolates.

Equally important to our understanding of the distribution of INH resistance associated mutations is their cumulative frequency across genes. Analysis of the cumulative frequencies of mutations in *katG*, *inhA*, and *ahpC-oxyR* was performed only in a small subset of the data (n = 1,582), as few publications sequenced portions of all three genes of interest. In this subset, 80% of the isolates had at least one mutation reported at the following locations: *katG*315, *inhA* -15, *inhA* -8, *inhA* -47, or *inhA* -17. With the addition of mutations occurring at *ahpC* -48, *ahpC* -39, *ahpC* -15, *ahpC* -12, *ahpC* -10, *ahpC* -9, or *ahpC* -6, the percentage of isolates with at least one reported mutation increased to 84%. This approaches the cumulative frequency previously documented by Lin et al. of 88% [42]. The increased detection capacity achieved by including mutations in the *ahpC-oxyR* intergenic region appears to contradict previous studies which have found no significant contribution of INH resistance detection from mutations occurring in the *ahpC-oxyR* intergenic region [18,27,43].

## Limitations

Given the nature of a systematic review, we were unable to obtain data from all geographic regions, thereby introducing a theoretical potential for bias in the global cumulative frequencies

calculated. However, given that the total number of isolates reviewed exceeded 11,000, the cumulative frequencies most likely approximate true global frequencies of the most common mutations associated with INH resistance. Additionally, there is a potential for publications to selectively sequence isolates that did not show the most common mutations as detected on rapid molecular tests which may have led to artificially lower cumulative frequencies of the most common mutations. To reduce the potential for this reporting bias, we specifically excluded publications that indicated any non-random selection of isolates for sequencing analysis.

## Conclusions

The performance of molecular-based diagnostic tests for drug resistant TB is intrinsically linked with not only the regional frequencies of mutations being detected, but also the diversity of resistance-conferring mutations being detected by diagnostic tests. Both of these factors influence the maximum sensitivity which rapid molecular tests can be expected to achieve.

Based on the data we evaluated, approximately 80% of global *Mtb* isolates with phenotypic resistance to INH appeared to contain mutations in codon 315 of the *katG* gene or position -15 in the *inhA* promoter. Taken together with other independently occurring mutations in the *inhA* promoter and the *ahpC-oxyR* intergenic region that have been previously documented to confer INH resistance, a minimum of 84% of all global *Mtb* isolates with INH resistance phenotypes should be detectable with molecular diagnostics based on analysis of these mutations. Additionally, as these mutations almost never (<0.1%) occurred in INH susceptible isolates, they should have specificities in excess of 99% as markers of phenotypic INH resistance. However, the cumulative frequencies of these mutations do appear to differ by region which could lead to variation in the sensitivity of molecular diagnostics if they are based only on these mutations. Further research, especially in regions with low prevalence of *katG* and *inhA* canonical mutations, is needed to determine if unknown genes or specific mutations account for the unexplained phenotypic INH resistance, or if a more inclusive combination of mutations in previously identified genes such as the *inhA* promoter, *inhA* coding, *ahpC-oxyR* intergenic region, or *katG* gene account for the remaining phenotypic resistance. Regional surveys of the most commonly occurring mutations, including mutations reported in the *ahpC-oxyR* intergentic region should continue to be conducted in geographic regions where rapid molecular tests are being deployed. This would allow for tailoring of molecular tests to specific regions, better interpretation of the molecular tests being used, and improved therapy recommendations.

## Supporting Information

**S1 Fig. PRISMA Flow Diagram.**
(DOCX)

**S1 PRISMA Checklist. PRISMA Checklist.**
(DOC)

**S1 Table. Calculations for H37Rv gene coordinate locations.**
(DOCX)

**S2 Table. Frequency of reported mutations on *furA-katG*, *fabG1-inhA*, and *ahpC-oxyR* gene regions.**
(DOCX)

**S3 Table. Drug Susceptibility Testing (DST) Methods Employed in Publications.**
(DOCX)

**S4 Table. Details of Studies Included in Review.**
(DOCX)

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## Author Contributions

Conceived and designed the experiments: TCR AC. Performed the experiments: MS. Analyzed the data: MS. Contributed reagents/materials/analysis tools: DC. Wrote the paper: MS TCR DC.

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Chapter 2, in full, is a reprint of the material as it appears in PLoS ONE: Seifert M, Catanzaro D, Catanzaro A, Rodwell TC. (2015) Genetic Mutations Associated with Isoniazid Resistance in *Mycobacterium tuberculosis*: A Systematic Review. *PLoS ONE* 10(3): e0119628. Seifert M, Catanzaro D, Catanzaro A, Rodwell TC. The dissertation author was the primary author of this material.

# CHAPTER 3

## A Performance Evaluation of MTBDR*plus* (version 2) for the Diagnosis of Multidrug-Resistant Tuberculosis

**ABSTRACT**

BACKGROUND: Delays in the detection of multidrug-resistant tuberculosis (MDR-TB) threaten global tuberculosis control efforts. A recently updated rapid molecular diagnostic test, MTBDR*plus* version 2 (v2), was designed to detect drug resistance in both acid-fast bacilli (AFB) smear negative and positive specimens. However, the performance of this assay has not been extensively evaluated in a large diverse cohort.

METHODS: Sputum samples from patients at risk for MDR-TB were tested using MTBDR*plus* v2 and results were compared to MGIT960 drug susceptibility testing results. Logistic regression was used to assess the relationship of participant HIV status, diabetic status, history of previous treatment, and AFB smear gradation to the likelihood of obtaining an interpretable (resistant or susceptible) MTBDR*plus* v2 result.

RESULTS: Among the 914 culture positive samples evaluated, sensitivity and specificity of the MTBDR*plus* v2 compared to MGIT960 for MDR-TB detection was 95.1% (95%CI 92.6, 96.9) and 99.1% (95%CI 97.1, 100.0) among both AFB smear positive and negative specimens, respectively. Sensitivity for MDR-TB detection among exclusively smear negative specimens was considerably lower, 82.8% (95%CI 63.5, 93.5). This difference was driven primarily by significantly lower sensitivity for isoniazid resistance detection, 81.6% (95%CI 65.1, 91.7) among AFB smear negatives compared to 94.9% (95%CI 92.4, 96.6) among AFB smear positives. A dose-response relationship was found between the proportion of interpretable test results and AFB smear bacterial load after adjusting for BMI, HIV status, previous treatment, and diabetic status.

CONCLUSION: While MTBDR*plus* v2 performs well among both AFB smear positive and negative specimens, smear gradation appears to influence both the probability of obtaining an interpretable result and test sensitivity and specificity, indicating a significant association between bacillary load and assay performance.

**BACKGROUND**

Although rates of tuberculosis (TB) have been decreasing globally in recent years, rates of multidrug-resistant TB (MDR-TB), or TB that is resistant to both isoniazid (INH) and rifampicin (RIF), the two most commonly used drugs for first-line treatment, have remained constant.(1) According to World Health Organization (WHO) global estimates, in 2013, approximately 3.5% of new and 20.5% of previously-treated cases of TB were multidrug-resistant.(2) These global estimates, however, mask variation of the MDR-TB epidemic, as subnational rates of MDR-TB have risen to as high as 35% among new cases and 75% among previously treated cases in several eastern European and central Asian countries.(2)

Historically, phenotypic drug susceptibility testing (DST) of Mycobacterium tuberculosis (Mtb) on solid media took months to produce results.(3) The advent of broth-based media reduced the time to results to weeks, and the recent introduction of rapid molecular-based diagnostics has further reduced this time to less than a day.(4-6) Despite these gains, including WHO approval of the GenoType MTBDR*plus* assay (Hain, Nerhen, Germany) and Xpert MTB/RIF Assay (Cephid, Sunnyvale, USA) in 2008 and 2010, gaps in MDR-TB diagnosis remain. In 2013, less than half (45%) of MDR-TB cases were detected globally.(2) Differences in diagnostic test characteristics partially explain this gap in MDR-TB diagnosis. For example, Xpert MTB/RIF was developed to detect RIF resistance, but does not assess INH

resistance. The MTBDR*plus* version 1 (v1) assay detects resistance to both RIF and INH, however given its performance, WHO recommended its usage among Acid-Fast Bacilli (AFB) smear positive samples only.(7)

In 2012, version 2 (v2) of the MTBDR*plus* assay addressed performance issues associated with AFB smear status. Several small-scale studies have assessed the performance of this new version among homogenous populations, but a large-scale study in a diverse population has yet to be performed.(8-10) Additionally, the impact of AFB smear gradation, HIV status, history of previous treatment, and other clinical factors associated with disease progression, on the performance of MTBDR*plus* v2 has yet to be assessed.(11-14)

Expanding the rapid testing and detection of MDR-TB cases is one of the five priority actions outlined by WHO in their goals to address the global MDR-TB crisis.(2) Field trials of rapid point-of-care diagnostics provide valuable information for clinical decision making in resource limited settings.(15) In this report we describe on the performance of the MTBDR*plus* v2 compared with mycobacterial growth indicator tube (MGIT) 960 (Becton Dickinson, Sparks, MD) in a large diverse field trial among both AFB smear positive and negative specimens. (16) We also assessed whether various clinical and demographic factors were associated with test performance.

## METHODS

### Study design

This prospective cohort (diagnostic) study was conducted through the Global Consortium for Drug-resistant TB Diagnostics (GCDD), a collaboration between UCSD and study sites in India, Moldova, and South Africa. Details of patient recruitment and selection have been reported previously.(16) In brief, between 2012

and 2013, TB patients with risk factors for drug-resistant TB were screened at participating study sites and those meeting eligibility criteria were invited to enroll. Biological specimens were collected and participants were interviewed at baseline and at 52 weeks post-enrollment. Medical record reviews were conducted at baseline, 30 days post-enrollment, and 52 weeks post-enrollment. However, only data and specimens collected at baseline were used for the current study. Informed consent was obtained from all patients prior to study enrollment. This study was approved by institutional review boards at the University of California, San Diego as well as the participating institutions at the three study sites.

**Specimen collection and processing**

Three sputum samples were collected at baseline from each study participant: at the clinic site during initial enrollment (S1), by the participant upon waking the following morning (S2), and again when the participant returned to the clinic (S3). Sputum samples S1 and S2 were pooled and used to perform multiple diagnostic tests including AFB smear, MTBDR*plus* v2 line probe assay, and MGIT 960 culture which was used as the inoculum for MGIT 960 DST. All tests were performed according to manufacturer instructions or per standard procedures.(17, 18) Mtb culture positive specimens were tested for drug susceptibility to RIF and INH resistance at concentrations of 1.0 and 0.1 (μg/ml) respectively, according to WHO recommendations.(19)

**MTBDR*plus* v2**

The updated MTBDR*plus* v2 assay strip contains 27 hybridization probes. Two probes, the conjugate and amplification probes, ensure test function. Four control probes confirm the presence of Mtb (TUB probe) and identify wild type loci sequences for the *rpoB*, *katG* and *inhA* promoter gene regions. Eight *rpoB* wild type

probes overlap gene regions between codons 505 and 533. Four *rpoB* mutant probes identify the specific mutations D516V, H526Y, H526D, and S531L. One wild type probe is specific for the *katG*315 codon, and two mutant probes identify S315T mutations at this codon. Two *inhA* wild type probes cover the *inhA* promoter from position -15 to -8. And, four mutant probes identify specific nucleic acid changes in the *inhA* promotor region (C-15T, A-16G, T-8C, and T-8A). The absence of one or more wild type probes for a given resistance-associated gene region, or the absence of one or more wild type probes in addition to the presence of one or more mutant probes for a given gene region, was considered indicative of resistance to either INH or RIF. The assay result was considered indeterminate if either of the test function probes were absent; if the TUB probe or gene loci probe were absent; or if all wild type probes, in addition to mutation probes, were present.(20)

**Data Analysis**

Performance of MTBDR*plus* v2 was described by computing sensitivity, specificity, likelihood ratio positive (LR+), and likelihood ratio negative (LR-); confidence intervals for all proportions were calculated using the Wilson Score method.(21) Impact of AFB smear status was determined by stratifying by AFB smear status for RIF resistance, INH resistance, and MDR-TB. For further analysis of test performance, results for RIF and INH resistance were categorized into valid (either resistant or susceptible) and invalid (indeterminate) results. Multivariable logistic regression was used to determine if any demographic or clinical data were associated with obtaining valid MTBDR*plus* v2 results. Analysis was performed using Stata 13 (College Station, TX). Statistical significance was established at an alpha level of 0.05.

## RESULTS

### Test Performance

A total of 1128 specimens were evaluated for resistance to RIF and INH. Nine-hundred fourteen specimens were culture positive for Mtb among which 768 (84%) were AFB smear positive. Resistance to RIF was correctly identified by MTBDR*plus* v2 in 462 (86%) out of 540 RIF resistant isolates; and resistance to INH was correctly identified in 478 (82%) out of 592 INH resistant isolates. (Table 1) Thirty-two out of 58 INH mono-resistant and five out of 6 RIF mono-resistant specimens were correctly identified by MTBDR*plus* v2. Among the 214 culture negative specimens, MTBDR*plus* v2 identified 42 (20%) specimens that demonstrated either INH or RIF resistance or susceptibility.

Overall, sensitivity and specificity of MTBDR*plus* for MDR-TB were 95.1% (95%CI 92.6, 96.9) and 99.1% (95%CI 97.1, 99.8), respectively. For RIF, sensitivity and specificity were 96.7% (95%CI 94.5, 98.0) and 97.9% (95%CI 95.5, 99.1); and for INH, sensitivity and specificity were 93.9% (95%CI 91.4, 95.8), and 99.6% (95%CI 97.7, 100.0). Sensitivity and specificity were also calculated and stratified by AFB smear status for MDR-TB, RIF resistance, and INH resistance (Table 2). Point estimates for sensitivity and specificity were consistently lower and confidence intervals were larger for AFB negative specimens than for AFB positive specimens.

### Indeterminates

One-hundred two (11%) of the 914 Mtb culture positive specimens were indeterminate for RIF resistance by MTBDR*plus* v2 assay. Of these 102 indeterminate results, 5 (5%) were classified indeterminate due to test failure: either there was no conjugate probe (2) or there was no amplification probe (3) present. Seventy (69%) were indeterminate because there was no TUB probe present,

indicating no detection of Mtb. Of the 70 results lacking the Mtb probe, 36 had no other probes present, 17 had either wild type probe 4 or wild type probe 7 (9 had both) present and no other probes, and 17 had the *rpoB* loci probe present in addition to multiple wild type and mutant probes. One (1%) was classified as indeterminate because, although the TUB probe was present, there was no *rpoB* loci probe present. The remaining 26 (25%) were classified as indeterminate because they had all wild type probes present in addition to at least one mutant probe for the relevant gene region.

One-hundred twenty-one (13%) of the 914 Mtb culture positive specimens were indeterminate for INH resistance by MTBDR*plus* v2 assay. Of the 121 indeterminate results, 5 (4%) were classified indeterminate because of test failure: either there was no conjugate probe (2) or there was no amplification probe (3) present. Seventy (58%) were classified as indeterminate because there was no Mtb probe present. Of those 70 results without an Mtb probe, 51 had no other probes present and the remaining 19 had either the *katG* loci or *inhA* loci present (6 had both). Thirteen (11%) were classified as indeterminate because they were missing either the *katG* or *inhA* loci probe and thirty-three (27%) were classified as indeterminate because they had all wild type probes present for *katG* and *inhA* in addition to at least one mutant probe for either gene.

**Correlates of Test Performance**

Demographic and clinical factors associated with disease severity and their distribution among valid and invalid results for RIF and INH resistance are presented in Table 3. In univariate analysis, AFB smear grade of +1 or greater was significantly associated with valid results for both RIF and INH. Body mass index (BMI) appeared borderline significantly inversely associated with obtaining a valid result for INH, and

significantly inversely associated with obtaining a valid result for RIF, indicating that those with a higher BMI were more likely to have an invalid test result. The likelihood of obtaining a valid test was not significantly associated with age, sex, HIV status, previous treatment, diabetic status, or study site.

Three models were generated to determine the relationship between clinical and demographic factors and the likelihood of a successful test. The first model included only AFB smear gradation, the only significant factor associated with valid results for both RIF and INH in the univariate analysis. The second model included BMI, based on the biologically plausible assumption that individuals with low BMI would likely produce sputum with larger amount of bacilli due to disease progression. The third model included three more variables, HIV status, a known confounder of test performance, previous TB treatment, and diabetic status, both associated with disease progression. The models were compared using the likelihood ratio test; models two and three were not statistically superior to model one. After controlling for smear status, BMI was no longer significantly associated with valid results in any of the models. AFB smear grade was significantly associated with both RIF and INH test performance, after controlling for HIV status, previous TB treatment status, BMI, and diabetic status. Higher amounts of bacteria in the sputum as defined by AFB smear status were associated with significantly more valid test results. (Table 4) A dose response relationship was evident; interpretable test results increased with each progressive AFB smear gradation, culminating in an AFB smear +3 being more than 10 times more likely to result in an interpretable MTBDR*plus* v2 result than smear negative specimens.

**DISCUSSION**

Overall, MTBDR*plus* v2 performed well among both AFB smear positive and negative specimens. However, performance of the test, particularly sensitivity, was affected by AFB smear status. Sensitivity for the detection of MDR-TB among AFB smear positive specimens was 96.0% (95%CI 93.5, 97.6) compared to 82.8% (95%CI 63.5, 93.5) among AFB smear negative specimens. This difference in sensitivity between AFB smear negative and positive specimens was significant for INH resistance, 81.6% (95%CI 65.1, 91.7) versus 94.9% (95%CI 92.4, 96.6). This difference between AFB smear negative and positive specimens was not significant for RIF 91.4% (95%CI 75.8, 97.8) compared to 97.1% (95%CI 94.9, 98.4). Additionally, the proportion of results that were valid was strongly correlated with AFB smear gradation. After adjusting for BMI, HIV status, diabetic status, and history of previous TB treatment, AFB smear +3 specimens were at least 10 times more likely to result in a valid result when compared to AFB smear negative specimens.

Among the 914 culture positive specimens, less than one percent (5) of MTBDR*plus* v2 test results were indeterminate due to test failure (data not shown). Mtb was detected (as evidenced by the presence of the TUB probe) in 92% of all culture positive specimens. This result is slightly higher than two smaller studies evaluating MTBDR*plus* v2 among a mix of AFB smear positive and negative specimens, which reported sensitivities to Mtb of 73% and 88%.(8, 9) When comparing performance among only AFB smear negative specimens, the Mtb sensitivity for the present study, 74%, was lower than the 79-80% reported by Crudu et al. and higher than the 57% reported by Bernard et al.(8, 9) As a comparison, MTBDR*plus* v1, although not recommended for use on AFB smear negative samples, had previously reported Mtb detection rates of exclusively AFB smear negative

specimens to be 46%, 48% and 65%.(22-24) When compared to the Cochrane review of the performance of Xpert, the Mtb sensitivity of the current study among AFB smear positive specimens, 92%, was lower than that of the pooled Mtb sensitivity reported by the review of 98%; however the Mtb sensitivity among AFB smear negative specimens of the current study, 74%, to was higher than the pooled sensitivity of 67% among AFB smear negative specimens reported for Xpert.(25) The MTBDR*plus* v2 also identified Mtb among 24% of culture negative specimens. Probe hybridization in these cases may have been caused by the presence of non-viable bacteria in the specimen or may indicate that bacteria were present in amounts too small to sustain culture growth.

Direct comparison of test results by smear status to previously published MTBDR*plus* v2 results was difficult as few previous studies have reported these results. The sensitivity and specificity of the test for RIF resistance in this study, as stratified by smear status, was similar (within 95% confidence intervals) to those reported by Crudu et al.(8) The only difference in INH resistance performance measures between the current study and the Crudu et al. study was the sensitivity and specificity of MTBDR*plus* v2 for AFB smear negative specimens. INH sensitivity and specificity among AFB smear negative specimens in this study was 81.6% and 98.1% respectively, compared to 93.5% and 82.3% reported by Crudu et al. N'guessan et al. reported RIF sensitivity and specificity only among AFB smear positive specimens, and the only evident difference in assay performance was the sensitivity of RIF resistance (73.2%), which was significantly lower than reported in the current study (97.1%).(10) The sensitivity and specificity of MTBDR*plus* v2, as reported by Barnard et al., were 100% for both RIF and INH resistance, higher than both estimates from the current study.(9)

Given an absence of large diverse studies assessing the overall performance of MTBDR*plus* v2, sensitivity and specificity for RIF and INH could only be compared to the performance of MTBDR*plus* v1, as reported by the WHO Expert Group Report and a meta-analysis of the MTBDR*plus* v1 by Ling et al.(7, 26) Overall, the sensitivity for RIF resistance found in this study (96.7%) was slightly lower than reported by the WHO (98.4%) and by Ling et al. (98.4%).(7, 26) In contrast, overall sensitivity for INH resistance in this study (93.9%) was slightly higher than reported by the WHO (91.4%) and by Ling et al. (88.7%). Specificities of both RIF and INH resistance from the current study were similar to those reported by the WHO and Ling et al.
Smear gradation was the strongest predictor of obtaining valid test results (either susceptible or resistant) for either RIF and INH, even after adjusting for BMI, previous history of TB treatment, HIV status (which is a known confounder), and diabetic status. As smear positivity increased, so did the odds of obtaining a valid test result. This correlates with the WHO findings for version 1 of the MTBDR*plus* assay, where a significant association was found between smear gradation and the proportion of interpretable or valid results.(7)

**Limitations**

This study did not repeat the MTBDR*plus* v2 assay if results were indeterminate, potentially reducing sensitivity estimates and making direct comparisons to previous studies where the assay was repeated if a result was indeterminate difficult. Additionally for the three variables, HIV status, diabetic status, and history of previous treatment, data was either self-reported, or was extracted from medical records which were not always compete.

**Conclusions**

While MTBDR*plus* v2 performs well on both AFB smear positive and negative specimens. The sensitivity and specificity for the detection of RIF resistance, INH resistance, and MDR-TB by MTBDR*plus* v2 were 96.7% and 97.9%, 93.9% and 99.6%, and 95.1% and 99.1%, respectively. Smear gradation appears to influence both the probability of obtaining an interpretable result and test sensitivity and specificity, indicating a significant association between bacillary load and test performance. MTBDR*plus* v2 does however appear to consistently provide accurate results for both RIF and INH resistance.

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**Table 3.1** Detection of Rifampicin and Isoniazid Resistance by MTBDR*plus* version 2 versus MGIT 960

| **Rifampicin** | | | | | |
|---|---|---|---|---|---|
| **MTBDRplus** | **MGIT Results** | | | | |
| **Results** | **Resistant** | **Susceptible** | **Mtb Negative** | **No Result** | **TOTAL** |
| Resistant | 462 | 7 | 9 | 0 | 478 |
| Susceptible | 16 | 324 | 32 | 3 | 375 |
| Indeterminate | 62 | 36 | 173 | 4 | 275 |
| Total | 540 | 367 | 214 | 7 | 1128 |
| **Isoniazid** | | | | | |
| **MTBDRplus** | **MGIT Results** | | | | |
| **Results** | **Resistant** | **Susceptible** | **Mtb Negative** | **No Result** | **TOTAL** |
| Resistant | 478 | 1 | 5 | 0 | 484 |
| Susceptible | 31 | 280 | 33 | 3 | 347 |
| Indeterminate | 83 | 34 | 176 | 4 | 298 |
| Total | 592 | 315 | 214 | 7 | 1128 |

**Table 3.2** Performance Parameters of MTBDR*plus* v2 in Detecting Rifampicin (RIF), Isoniazid (INH), and Multidrug-resistant TB (MDR-TB) Compared to MGIT960 Drug Susceptibility Testing, Stratified by Smear Status.[1]

| Drug Resistance<br>**Smear Status** | Isolates[2]<br>n | Sensitivity % | Specificity % | LR+ | LR- |
|---|---|---|---|---|---|
| **RIF** | 809 | 96.7 (94.5, 98.0) | 97.9 (95.5, 99.1) | 46 (22, 95) | 0.03 (0.02, 0.06) |
| **AFB +** | 712 | 97.1 (94.9, 98.4) | 98.5 (96.0, 99.5) | 65 (25, 173) | 0.03 (0.02, 0.05) |
| **AFB -** | 97 | 91.4 (75.8, 97.8) | 95.2 (85.6, 98.7) | 19 (6, 57) | 0.09 (0.03, 0.27) |
| **INH** | 790 | 93.9 (91.4, 95.8) | 99.6 (97.7, 100.0) | 264 (37, 1867) | 0.06 (0.04, 0.09) |
| **AFB +** | 699 | 94.9 (92.4, 96.6) | 100.0 (97.9, 100.0) | ∞ | 0.05 (0.03, 0.08) |
| **AFB -** | 91 | 81.6 (65.1, 91.7) | 98.1 (88.6, 99.9) | 43 (6, 303) | 0.19 (0.10, 0.37) |
| **MDR-TB** | 775 | 95.1 (92.6, 96.9) | 99.1 (97.1, 99.8) | 102 (33, 316) | 0.05 (0.03, 0.07) |
| **AFB +** | 686 | 96.0 (93.5, 97.6) | 99.2 (97.0, 99.9) | 126 (32, 502) | 0.04 (0.03, 0.06) |
| **AFB -** | 89 | 82.8 (63.5, 93.5) | 98.3 (89.9, 99.9) | 50 (7,349) | 0.18 (0.08, 0.39) |

[1]Values are percentages with 95% confidence interval in parentheses.

[2]Samples that did not have both interpretable phenotypic and genotypic results were excluded from analysis.

**Table 3.3** Univariate Analysis of Patient Demographic and Clinical Characteristics by Validity of MTBDR*plus* version 2 Results for Rifampicin (RIF) and Isoniazid (INH).[1]

| | RIF results | | | INH results | | |
|---|---|---|---|---|---|---|
| | Valid (n=812) | Invalid (n=102) | p-value | Valid (n=793) | Invalid (n=121) | p-value |
| Smear gradation | | | | | | |
| Negative (ref) | 98 (12.1) | 48 (47.1) | | 92 (11.6) | 54 (44.6) | |
| Rare or Scanty | 58 (7.1) | 17 (16.7) | 0.12 | 55 (6.9) | 20 (16.5) | 0.13 |
| Few (+1) | 171 (21.1) | 14 (13.7) | <0.001 | 166 (20.9) | 19 (15.7) | <0.001 |
| Many (+2) | 168 (20.7) | 7 (6.9) | <0.001 | 165 (20.8) | 10 (8.3) | <0.001 |
| TNTC (+3) | 317 (39.0) | 16 (15.7) | <0.001 | 315 (39.7) | 18 (14.9) | <0.001 |
| Age (continuous) | 33 (24,45) | 33 (24, 47) | 0.39 | 33 (24,45) | 33 (24, 46) | 0.63 |
| Male (vs. Female) | 525 (64.7) | 61 (59.8) | 0.34 | 514 (64.8) | 72 (59.5) | 0.26 |
| Positive HIV results (vs. Negative/Unknown) | 99 (12.2) | 14 (13.7) | 0.66 | 97 (12.2) | 16 (13.2) | 0.75 |
| Retreatment/Previously treated (vs. New treatment/other) | 629 (77.5) | 78 (76.5) | 0.82 | 619 (78.1) | 88 (72.7) | 0.20 |
| BMI (continuous) | 18.0 (16.0, 20.4) | 19.6 (17.3, 21.8) | 0.005 | 18.0 (16.0, 20.4) | 18.8 (16.9, 21.3) | 0.06 |
| Diabetes (vs. No Diabetes/Unknown) | 42 (5.2) | 7 (6.9) | 0.49 | 43 (5.4) | 6 (5.0) | 0.83 |
| Site | | | | | | |
| India (ref) | 439 (53.8) | 53 (52.0) | | 433 (54.6) | 59 (48.8) | |
| Moldova | 204 (25.1) | 22 (21.6) | 0.67 | 193 (24.3) | 33 (27.3) | 0.33 |
| South Africa | 169 (21.4) | 27 (26.5) | 0.27 | 167 (21.1) | 29 (24.0) | 0.32 |

1 Categorical data are reported as percentages; continuous data as medians with interquartile ranges (Q1, Q3)

**Table 3.4** Adjusted odds of obtaining a valid RIF or INH result.[1]

**RIF Resistance**

| | Model 1 | | Model 2 | | Model 3 | |
|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | **OR** | **95% CI** | **OR** | **95% CI** |
| **Smear gradation** | | | | | | |
| **Negative (ref)** | | | | | | |
| **Rare** | 1.7 | 0.9, 3.2 | 1.6 | 0.9, 3.1 | 1.8 | 0.9, 3.4 |
| **Few** | **6.0** | **3.1, 11.4** | **6.6** | **3.0, 11.1** | **6.6** | **3.4, 12.7** |
| **Many** | **11.8** | **5.1, 27.0** | **11.6** | **5.1, 26.7** | **12.4** | **5.4, 28.8** |
| **TNTC** | **9.7** | **5.3, 17.8** | **8.9** | **4.8, 16.6** | **10.6** | **5.6, 20.1** |
| **BMI** | | | 0.9 | 0.9, 1.0 | 0.9 | 0.9, 1.0 |
| **Previously Treated** | | | | | 0.6 | 0.4, 1.11 |
| **HIV status** | | | | | 1.3 | 0.7, 2.7 |
| **Diabetic status** | | | | | 0.7 | 0.3, 1.7 |

**INH Resistance**

| | Model 1 | | Model 2 | | Model 3 | |
|---|---|---|---|---|---|---|
| | **OR** | **95% CI** | **OR** | **95% CI** | **OR** | **95% CI** |
| **Smear gradation** | | | | | | |
| **Negative (ref)** | | | | | | |
| **Rare** | 1.6 | 0.9, 3.0 | 1.6 | 0.9, 3.0 | 1.7 | 0.9, 3.2 |
| **Few** | **5.1** | **2.9, 9.2** | **5.1** | **2.8, 9.1** | **5.3** | **2.9, 9.6** |
| **Many** | **9.7** | **4.7, 20.0** | **9.6** | **4.7, 19.8** | **10.0** | **4.8,20.6** |
| **TNTC** | **10.3** | **5.7, 18.4** | **10.0** | **5.5, 17.9** | **10.6** | **5.8, 19.5** |
| **BMI** | | | 1.0 | 0.9, 1.0 | 0.9 | 0.9, 1.0 |
| **Previously Treated** | | | | | 0.9 | 0.5, 1.4 |
| **HIV status** | | | | | 1.3 | 0.7, 2.5 |
| **Diabetic status** | | | | | 1.0 | 0.4, 2.5 |

[1] Significant results in bold.

# CHAPTER 4

## MTBDRplus and MTBDRsl Assays: The Absence of Wild Type Probe Hybridization and Implications for the Detection of Drug-resistant Tuberculosis

**ABSTRACT**

Background: Rapid accurate detection of drug-resistant tuberculosis is imperative for effective treatment and subsequent reduction in transmission of drug-resistant Mycobacterium tuberculosis. The sensitivity and specificity of line probe assays, although generally very good, has been less than optimal for several individual drugs.

Methods: Using data collected during a large, multisite diagnostic study, hybridization probe results from both line probe assays, MTBDRplus and MTBDRsl, were compared to sequencing and diagnostic performance (sensitivity and specificity) for each individual mutation and wild type probe results was assessed. Additionally, resistant line probe assay results due to the failure of wild type probe hybridization were compared to sequencing to determine which mutations were inhibiting wild type probe hybridization. Finally, the overall contribution of wild type probe hybridization failure to the detection of drug-resistance was assessed via comparison to a phenotypic reference standard.

Results/Conclusions: Mutation probes demonstrated significantly higher specificity when compared to wild type probes. Conversely, wild type probes demonstrated marginally higher sensitivities when compared to mutation probes. This combination is ideal for detecting the presence of a mutation, while yielding the fewest number of false positive results. The failure of wild type probe hybridization was not overwhelmingly the result of novel or rare mutations, but the result the failure of mutation probe hybridization. When compared to phenotypic culture-based drug susceptibility testing, the absence of wild type probe hybridization significantly contributes to the detection of rifampicin and flouroquinolone resistance with negligible increases in false positive results.

**BACKGROUND**

While number of incident cases of tuberculosis (TB) has been steadily declining over the past decade, the proportion of multidrug-resistant (MDR) TB, or TB that is resistant to both isoniazid (INH) and rifampicin (RIF), has remained constant.(1, 2) The World Health Organization (WHO) estimated that 5% of all TB cases in 2013 (3.5% of new and 20.5% of previously treated cases) were MDR. Furthermore, 9% of these MDR cases were extensively drug-resistant (XDR), or resistant to at least one fluoroquinolone (FQ) and a second-line injectable drug (SLID) ((amikacin (AMK), kanamycin (KAN), or capreomycin (CAP)). Although detection of M/XDR-TB is imperative for effective disease management, less than half of all MDR-TB cases were detected in 2013.(2, 3) This detection gap is due, in part, to the lack of low-cost, rapid, and accurate diagnostic technologies for M/XDR-TB.(3-5)

Conventional culture-based phenotypic drug susceptibility testing (DST) methods can take months to yield results.(6) Molecular-based tests, however, can produce results in less than a day.(4, 7-11) The WHO has endorsed both line probe hybridization and molecular beacon assays for the detection of drug resistance in high incidence settings.(12) Although these molecular-based tests have significantly increased M/XDR-TB detection, no test has yet replaced phenotypic drug susceptibility testing as the gold standard for M/XDR-TB diagnosis. The approved molecular beacon assay, GeneXpert (Cepheid, Sunnyvale, CA), detects only RIF resistance, and fails to provide information about INH, FQ, or SLID resistance. The line probe assay, MTBDR*plus* version 2 (v2) (Hain Lifesciences, Nehren, Germany), detects both INH and RIF resistance, and the MTBDR*sl* (Hain Lifesciences, Nehren,

Germany) is designed to detect FQ, SLID, and ethambutol resistance. However the sensitivity of these assays remains less than ideal for INH and SLID.(13-15)

Line probe assays utilize a combination of two methods for classifying specimens as resistant. 1) If a probe containing a mutation sequence hybridizes on the test strip, the presence of the mutation can be assumed and the specimen is considered resistant. 2) If no mutation probes hybridize on the test strip, but a probe containing a wild type sequence also fails to hybridize, the presence of a mutation can also be assumed as no hybridization occurred with the wild type sequence and the specimen is considered resistant. Two recent studies assessing the performance of the MTBDR*sl* assay detected an unusually high proportion of FQ-resistant specimens that were eventually determined to be falsely positive after comparison to sequencing and/or conventional culture results.(16, 17) False positive FQ resistance results from the two studies were determined to be the result of absent wild type probe hybridization (denoting the presence of a non-wild type sequence), rather than direct detection of a pre-specified mutation.(16, 17) Given the significant impact of FQ resistance on MDR-TB treatment and the potential for the absence of wild type probe hybridization to disproportionately increase the number of false positive test results for all drugs being assessed, an evaluation of individual probe performance was undertaken to understand the underlying subtleties of MTBDR*plus* v2 and MTBDR*sl* assays results.(18)

Using data collected during a large, multisite diagnostic study, hybridization probe results from both MTBDR*plus* and MTBDR*sl* assays were compared to sequencing (as determined by pyrosequencing) and diagnostic performance (sensitivity and specificity) for each individual mutation and wild type probe results was assessed. Additionally, resistant MTBDR*plus* v2 and MTBDR*sl* results due to the

failure of wild type probe hybridization were compared to sequencing to determine which mutations were inhibiting wild type probe hybridization. Finally, the overall contribution of wild type probe hybridization failure to the detection of drug-resistance was assessed via comparison to a phenotypic reference standard.

## METHODS

### Study Design

Specimens were collected as part of a prospective cohort study (Clinical trial number: NCT02170441) conducted by the Global Consortium for Drug-resistant TB Diagnostics (GCDD); a research collaboration with study sites in India, Africa, and Moldova. This study was approved by the institutional review board at the University of California San Diego and by participating institutions at their respective study sites. Detailed descriptions of the study protocol and major findings have been published elsewhere.(19) In brief, patients presenting with suspected M/XDR-TB were invited to participate at the respective clinic sites. Sputum specimens were tested using two line probe assays, GenoType MTBDR*plus* v2 and GenoType MTBDR*sl*; a commercially available pyrosequencing platform, the PyroMark Q96 platform (Qiagen, Valencia, CA, USA), repurposed for *Mycobacterium tuberculosis* (*Mtb*) resistance detection; and a phenotypic reference standard, Mycobacterial Growth Indicator Tube (MGIT) 960 (BD Biosciences, Sparks, MD, USA). All culture positive specimens were tested for drug susceptibility to INH, RIF, MOX, OFX, KAN, CAP, and AMK, using WHO recommended critical concentrations of 0.1, 1.0, 0.25, 2.0, 2.5, 2.0, and 1.0 ug/ml, respectively. All phenotypic DST were performed according to manufacturer instructions or as per standard procedures.

### Line Probe Assays

Both MTBDR*plus* v2 and MTBDR*sl* utilize polymerase chain reaction (PCR) amplification followed by reverse hybridization to specific, immobilized oligonucleotide probes to detect either *Mtb* wild type sequences or mutations associated with RIF, INH, FQ, and/or SLID resistance.(20, 21) MTBDR*plus* relies upon a series of probes that hybridize DNA in the codons 505 to 534 of the *rpoB* gene, codon 315 of the *katG* gene, and positions -1 to -22 on the *inhA* regulatory region. Specific mutations associated with RIF resistance are detected by MUT probes *rpoB*MUT1, which detects the D516V amino acid change, as well as *rpoB*MUT2A(H526Y), *rpoB*MUT2B(H526D) and *rpoB*MUT3(S531L). Mutations associated with INH resistance are detected by MUT probes *katG*MUT1(AGC315ACC), *katG*MUT2(AGC315ACA), *inhA*MUT1(C15T), *inhA*MUT2(A16G), *inhA*MUT3A(T8C), and *inhA*MUT3B(T8A).(20, 22) Although MTBDR*sl* detects resistance to FQ, SLID, and ethambutol, only resistance to FQ and SLID were assessed in this study. MTBDR*sl* probes detect mutations in codons 85 to 97 of the *gyrA* gene and position 1401 to 1402 and position 1484 of the *rrs* gene.(16, 21) Mutations associated with FQ resistance are detected by MUT probes *gyrA*MUT1 (A90V), *gyrA*MUT2(S91P), *gyrA*MUT3A(D94A), *gyrA*MUT3B(D94N/Y), *gyrA*MUT3C(D94G), and *gyrA*MUT3D(D94H); and mutations associated with resistance to SLID are detected by MUT probes *rrs*MUT1(A1401G) and *rrs*MUT2(G1484T).

**Line Probe Assay Interpretation**

Both MTBDR*plus* v2 and MTBDR*sl* assays contain a series of control probes. The conjugate and amplification probes document successful substrate reaction, and successful DNA extraction and amplification. The *Mtb* identification probe and specific gene loci probes confirm the presence of *Mtb* and each gene region being assessed. If any control probes are absent, or if the gene loci probe for the wild type (WT) and

mutation (MUT) probes being assessed is absent, (denoting test failure or absence of *Mtb*) the result is considered indeterminate. If all WT probes are present and all MUT probes are absent for a specific gene region, the result is considered susceptible to the drug for which resistance is being assessed. Resistance can be detected by one of two methods. 1) If a MUT probe hybridizes on the test strip, the presence of the mutation can be assumed and the specimen is considered resistant. 2) If no MUT probes hybridize on the test strip, but a WT probe also fails to hybridize, the presence of a mutation can also be assumed as no hybridization occurred with the wild type sequence and the specimen is considered resistant. These two different methods of resistance detection are unique to the line probe assays, as they provide an indirect method through which to identify novel or rare mutations located in those regions covered by the WT probes. If all WT probes AND at least one MUT probe is present, then interpretation is difficult, and a repeat test or confirmation by sequencing is recommended. For this study, however, the assay was not repeated and such results were classified as indeterminate.(23)

**Sequencing**

Pyrosequencing (PSQ), used as the reference sequencing standard for MTBDR*plus* v2 and MTBDR*sl* results, consists of DNA extraction, amplification by PCR and real-time sequencing. Primers are used to synthesize nucleotides into growing DNA chains of no larger than 30 base pairs.(4) The gene regions sequenced include: codons 507 to 521 and 522 to 533 of the *rpoB* gene, codons 312 to 316 of *katG*, the *inhA* promotor region -4 to -20, gyrA codons 88 to 95, and the rrs gene region from nucleotides 1397 to 1406.(19, 24)

**Statistical Analysis**

Standard sensitivity and specificity calculations were used to compare each individual MUT and WT probe of the MTBDR*plus* v2 and MTBDR*sl* assays with sequencing in every instance where PSQ generated a valid sequencing read at the target location. Ninety-five percent confidence intervals were calculated using the Wilson score method.(25) Next, MTBDR*plus* v2 and MTBDR*sl* results for RIF, INH, FQ, and SLID were dichotomized as either resistant by MUT probe hybridization, or resistant by failure of the WT probe to hybridize. Specimens identified as resistant by failure of the WT probe to hybridize were compared with PSQ results to confirm if a novel, rare, or expected mutation was present. The final analysis compared MTBDR*plus* v2 and MTBDR*sl* results for RIF, INH, FQ, and SLID to phenotypic DST results for all culture positive specimens. Resistance based only on the presence of MUT probe hybridization was compared to resistance based on both the presence of MUT probe hybridization and the failure of WT probe hybridization by comparing the area under the receiver operating characteristic curve for equality (AUC). Analysis was performed using Stata 13 (College Station, TX). Statistical significance was established at an alpha level of 0.05.

## RESULTS

### Resistance Detection by Method

One thousand, one hundred and twenty-eight specimens were assessed by LPA, PSQ, and MGIT960. The number of specimens detected as resistant, susceptible or indeterminate/no result for each drug or drug classification tested is presented in Table 1. Indeterminate results varied for both line probe assays (ranging from 24% for RIF to 38% for SLIDs) and PSQ (ranging from 20% for SLIDS to 37% for RIF). Two hundred and fourteen specimens were culture negative for *Mtb* and

therefore did not undergo phenotypic DST, accounting for almost all indeterminate/no result for MGIT DST results. Comparison to phenotypic testing was assessed using only *Mtb* culture positive specimens with either resistant or susceptible results.

**Individual Probe Performance**

Sixteen specific MTBDR*plus* v2 and MTBDR*sl* MUT probes and 11 corresponding WT probes, were designed to interrogate 10 gene regions with potential resistance conferring mutations. The sequence of each of these regions was examined independently in each isolate using targeted PSQ as the reference sequencing method (Table 2). Sensitivity of MUT probes compared to reference sequence ranged from 0.48 to 1.00, and specificity from 0.96 to 1.00. MUT probes for *gyrA* codon 94 (*gyrA*MUT3A and *gyrA*MUT3B), were the least sensitive. WT probe sensitivity ranged from 0.76 to 0.95 and specificity ranged from 0.39 to 0.97. WT probes *rpoB*WT7 (codon 526) and *inhA*WT2 (nucleotide position -8) demonstrated the lowest specificity. Figure 1 shows the point estimates for sensitivity by 1-specificity for both WT and MUT probes.

**MTBDRplus v2 and MTBDRsl Resistance Determination**

The majority of resistance results from the MTBDR*plus* v2 and MTBDR*sl* assays was due to the direct detection of a resistance conferring mutation by hybridization of genomic *Mtb* DNA to a MUT probe. However, 13% (60) of RIF resistance, 12% (19) of *inhA* associated INH resistance, 6% (15) of FQ resistance, and 2% (1) of SLID resistant results were due to failure of the genomic DNA to hybridize to the WT probe, indicating the absence of a wild type sequence and indirectly indicating the presence of a mutation. (Table 3) Of these 95 resistant isolates classified as resistant based on the failure of a WT probe to hybridize, 24 could not be compared to sequencing as PSQ results were indeterminate for the

corresponding sequence region. Of the 71 with comparable sequence data, 11 were discordant with sequencing (no hybridization occurred on the MTBDR*plus* v2 or MTBDR*sl* probe while PSQ reported a wild type sequence in the corresponding region), 56 were concordant with sequencing results (no hybridization occurred on the probe and PSQ detected a mutation in the corresponding region), and 4 were mixed (no hybridization occurred on multiple MTBDR*plus* v2 or MTBDR*sl* probes and PSQ reported wild type sequence in one region and a mutation sequence in another region). Among the 56 specimens concordant with sequencing (no hybridization occurred on the MTBDRplus v2 or MTBDRsl probe and PSQ detected a mutation in the corresponding region), the mutation identified by sequencing was identical to a MUT probe in 28 (50%) cases, indicating that the corresponding MUT probe failed to hybridize. The most frequent MUT probe to fail to hybridize was *rpoB*MUT3(531TTG) which occurred in 17 instances.

**Contribution to Resistance by the Failure of WT Probe Hybridization**

In order to determine if the inclusion of resistant results due to failure of the genomic DNA to hybridize to the WT probe contributed significantly to the molecular detection of drug resistance, resistance classification based on the presence of MUT probe hybridization was compared to resistance classification based on both the presence of MUT probe hybridization AND the failure of WT probe hybridization. For RIF, MOX, and OFX, the inclusion of resistance classification based on the failure of WT probe hybridization significantly improved detection of phenotypic resistance when the area under the curve was compared. (Table 4) This was also true for *inhA*-associated INH resistance, the inclusion of resistance classification based on the failure of WT probe hybridization significantly improved the detection of phenotypic INH resistance. However, when assessed in conjunction with *katG*-associated INH

resistance, the added value was not significant, as the majority of *inhA*–associated INH resistance co-occurred with a *katG*315 mutation, and would have been detected based upon *katG*-associated INH resistance.

**DISCUSSION**

The MUT probes included on the MTBDR*plus* v2 and MTBDR*sl* tests demonstrated significantly higher specificity when compared to WT probes. Conversely, WT probes demonstrated marginally higher sensitivity when compared to MUT probes. Together, these diagnostic differences are ideal for determining drug resistance profiles, yielding the fewest number of false positive results as determined by sequencing. When a WT and corresponding MUT probe fail to hybridize it can mean that either hybridization has failed (technical problem) or that there is a different mutation present at that site than the one for which the MUT probe was designed to detect. By default, the interpretation of MTBDR*plus* v2 and MTBDR*sl* assumes that the failure of both WT probe hybridization and its corresponding MUT probe is due to the presence of a rare or novel mutation. In our study, however, for any gene region, the absence of the WT probe often indicated a failure of MUT probe hybridization, rather than the presence of novel or rare mutations. PSQ confirmed the presence of expected mutations (for which there were MUT probes) in approximately 50% of instances where the WT probe was absent.

Significant variability however, was evident when assessing the sensitivity of the various MUT probes, including probes covering the same codons. For example, 2 different *gyrA*94 mutations (GCC and TAC/AAC) had low sensitivities of 0.60 and 0.48, while two others (GGC and CAC) had high to very high sensitivities of 0.89 and 1.00. Initially this poor *gyrA* sensitivity was thought to be caused by differential co-

occurrence of non-resistance conferring mutation at *gyrA*95. However upon closer inspection, of the 180 specimens with *gyrA*94 mutations, more than 97% harbored a co-occurring mutation at *gyrA*95, indicating that the presence of the gyrA95 mutation did not inhibit binding to the gyrA94 MUT probe.

The poor sensitivity seen for the *gyrA* 94 GCC and TAC/AAC MUT probes may be due instead to poor mutant template binding. The low sensitivity of the TAC/AAC probe, in particular, might result from the MUT probe having to recognize two different mutations, ultimately resulting in poor sensitivity. Specificity was also found to be low for *rpoB* WT probes at codons 516, 526, and 531, all less than 0.67. These low specificities may also be attributable to differences in strand accessibility between wild type and mutant sequences, making the WT probes less likely to bind the template DNA, versus their corresponding MUT probes.(26)

Among those specimens classified as resistant by MTBDR*plus*, only 13% of RIF resistance was due to the failure of WT probe hybridization for *rpoB*. This is lower than the 21-33% contribution of resistance due to the failure of WT probe hybridization reported in India, France, and Vietnam.(27-29) Interestingly, in the current study, no *katG*-associated INH resistance was due the failure of WT probe hybridization at *katG*, as opposed to 6% of results previously reported in India and 9% in Vietnam.(27, 30) Approximately 12% of *inhA*-associated INH resistance in the current study was attributable to the failure of WT probe hybridization at the *inhA* promoter, higher than the 9% reported in India and 0% reported in Vietnam. Six percent of FQ resistance, as classified by MTBDR*sl*, was due to the failure of WT probe hybridization. Our results contrast significantly with the results of two studies conducted in the Congo and the Democratic Republic of Congo, where over 60% of FQ resistance was attributable to the failure of WT probe hybridization.(16, 17) Our

results more closely resemble those reported by Huang et al. and Brossier et al., where less than 20% of FQ resistance was attributable to the failure of WT probe hybridization.(31, 32) Given the regional proximity of the two studies, this may indicate the local presence of a mutation that inhibits MTBDR*plus* v2 and MTBDR*sl* performance. This underscores the need for continued evaluation of regional mutation frequencies and a greater understanding of oligonucleotide binding mechanisms to continue to ensure optimal MTBDR*plus* v2 and MTBDR*sl* performance.

Rare or novel mutations, as detected by sequencing, did not account for the majority of resistance determination based only on the failure of WT probe hybridization in this study. Rather, half of the specimens contained mutations that should have hybridized to the corresponding MUT probes, but failed to do so. The MUT hybridization probes that most commonly failed to hybridize were *rpoB*MUT3 531TTG (17) and *gyrA*MUT3B 94TAC (8). This may indicate the need for continued assay optimization and not for the inclusion of novel MUT probes.

When compared to phenotypic DST, resistance determination based on the failure of WT probe hybridization detected an additional 10% of phenotypically RIF resistant isolates and 6% of phenotypically FQ resistant isolates, with less than 1% false positive results. These differences in performance were deemed significant by AUC analysis. The failure of WT probe hybridization resistance classification provides a unique fail-safe method for indirect detection of novel or rare mutations in this study.

**Limitations**

If PSQ did not yield an interpretable result, MTBDRplus/sl results could not be compared to sequencing, and the presence of a novel mutation may have been missed.

In conclusion, resistance determination based on the failure of WT probe hybridization is critical for detecting drug resistance by MTBDR*plus* v2 and MTBDR*sl* and other fixed probe hybridization systems. The inclusion of these results significantly improved resistance detection for RIF and FQs in our study, with negligible increases in false positive results.

Chapter 4, in full, is currently being prepared for submission for publication. Seifert M, Georghiou SB, Catanzaro D, Rodrigues C, Crudu V, Victor TC, Garfein RS, Catanzaro A, Rodwell TC. The dissertation author was the primary author of this material.

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**Table 4.1** Line Probe Assay, Pyrosequencing, and MGIT DST Results.

| | **Resistant** | **Susceptible** | **Indeterminate/No Result**[1] |
|---|---|---|---|
| | n (%) | n (%) | n (%) |
| **Line Probe Assay** | | | |
| RIF | 478 (42) | 375 (33) | 275 (24) |
| INH | 484 (43) | 347 (31) | 297 (26) |
| FQ | 237 (21) | 541 (48) | 350 (31) |
| SLID | 46 (4) | 656 (58) | 426 (38) |
| **Pyrosequencing** | | | |
| RIF | 448 (40) | 264 (23) | 416 (37) |
| INH | 608 (54) | 282 (21) | 238 (21) |
| FQ | 277 (25) | 528 (47) | 323 (29) |
| SLID | 74 (7) | 825 (73) | 229 (20) |
| **MGIT DST** | | | |
| RIF | 540 (48) | 368 (33) | 220 (20) |
| INH | 592 (52) | 316 (28) | 220 (20) |
| MOX | 310 (27) | 597 (53) | 221 (20) |
| OFX | 314 (28) | 594 (53) | 220 (20) |
| AMK | 82 (7) | 826 (73) | 220 (20) |
| CAP | 79 (7) | 829 (73) | 220 (20) |
| KAN | 145 (13) | 763 (68) | 220 (20) |

[1] Includes culture negative results for MGIT DST.

**Table 4.2** Sensitivity and specificity estimates for individual MTBDR*plus* and MTBDR*sl* mutation and wild type probes, and true positive, false positive, false negative and true negative (TP, FP, FN, and TN) values associated with probe diagnostic performance.

| | | Sensitivity | 95%CI | Specificity | 95%CI | TP | FP | FN | TN | Sensitivity | Specificity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MUT Probes | rpoBMUT1 (516 GAC) | 0.87 | (0.65, 0.97) | 0.99 | (0.99, 0.98) | 20 | 5 | 3 | 729 | | |
| | rpoBMUT2A (526 TAC) | 0.83 | (0.36, 0.99) | 1.00 | (1.00, 0.99) | 5 | 2 | 1 | 773 | | |
| | rpoBMUT2B (526 GAC) | 1.00 | (0.63, 1.00) | 0.99 | (0.99, 0.99) | 9 | 4 | 0 | 768 | | |
| | rpoBMUT3 (531 TTG) | 0.88 | (0.85, 0.91) | 0.96 | (0.96, 0.93) | 335 | 16 | 44 | 386 | | |
| | katGMUT1 (315 ACC) | 0.83 | (0.80, 0.86) | 0.97 | (0.97, 0.95) | 457 | 12 | 92 | 376 | | |
| | katGMUT2 (315 ACA) | 0.88 | (0.47, 0.99) | 1.00 | (1.00, 0.99) | 7 | 0 | 1 | 929 | | |
| | inhAMUT1 (-15 T) | 0.89 | (0.82, 0.93) | 1.00 | (1.00, 0.99) | 129 | 4 | 16 | 809 | | |
| | inhAMUT3A (-8 C) | 0.90 | (0.54, 0.99) | 1.00 | (1.00, 0.99) | 9 | 0 | 1 | 948 | | |
| | inhAMUT3B (-8 A) | 1.00 | (0.05, 1.00) | 1.00 | (1.00, 1.00) | 1 | 0 | 0 | 957 | | |
| | gyrAMUT1 (90 GTG) | 0.86 | (0.75, 0.92) | 0.99 | (0.99, 0.98) | 65 | 5 | 11 | 724 | | |
| | gyrAMUT2 (91 CCG) | 0.88 | (0.62, 0.98) | 1.00 | (1.00, 0.99) | 15 | 3 | 2 | 785 | | |
| | gyrAMUT3A (94 GCC) | 0.60 | (0.41, 0.77) | 1.00 | (1.00, 0.99) | 18 | 3 | 12 | 772 | | |
| | gyrAMUT3B (94 TAC/AAC) | 0.48 | (0.27, 0.69) | 1.00 | (1.00, 0.99) | 11 | 3 | 12 | 779 | | |
| | gyrAMUT3C (94 GGC) | 0.89 | (0.81, 0.93) | 0.97 | (0.97, 0.96) | 110 | 18 | 14 | 663 | | |
| | gyrAMUT3D (94 CAC) | 1.00 | (0.20, 1.00) | 1.00 | (1.00, 0.99) | 2 | 2 | 0 | 801 | | |
| | rrsMUT1 (1401 G) | 0.72 | (0.60, 0.81) | 1.00 | (1.00, 0.99) | 53 | 4 | 21 | 821 | | |
| WT Probes | rpoBWT3 (516WT) | 0.92 | (0.90, 0.94) | 0.63 | (0.63, 0.44) | 671 | 11 | 56 | 19 | | |
| | rpoBWT4 (516WT) | 0.93 | (0.91, 0.95) | 0.67 | (0.67, 0.47) | 679 | 10 | 48 | 20 | | |
| | rpoBWT7 (526WT) | 0.95 | (0.93, 0.96) | 0.39 | (0.39, 0.24) | 705 | 22 | 40 | 14 | | |
| | rpoBWT8 (531WT) | 0.86 | (0.82, 0.89) | 0.97 | (0.97, 0.95) | 330 | 11 | 53 | 387 | | |
| | katGWT (315WT) | 0.84 | (0.80, 0.88) | 0.96 | (0.96, 0.94) | 318 | 23 | 60 | 536 | | |
| | inhAWT1 (-15WT) | 0.84 | (0.82, 0.87) | 0.92 | (0.92, 0.86) | 686 | 12 | 127 | 133 | | |
| | inhAWT2 (-8WT) | 0.87 | (0.84, 0.89) | 0.57 | (0.57, 0.30) | 817 | 6 | 127 | 8 | | |
| | gyrAWT2 (90WT) | 0.88 | (0.85, 0.90) | 0.95 | (0.95, 0.86) | 640 | 4 | 89 | 72 | | |
| | gyrAWT2 (91WT) | 0.81 | (0.78, 0.84) | 0.76 | (0.76, 0.50) | 640 | 4 | 148 | 13 | | |
| | gyrAWT3 (94WT) | 0.90 | (0.88, 0.92) | 0.91 | (0.91, 0.86) | 564 | 16 | 61 | 164 | | |
| | rrsWT1 (1401WT) | 0.76 | (0.73, 0.79) | 0.81 | (0.81, 0.70) | 631 | 14 | 194 | 60 | | |
| | | | | | | | | | | 0.0 0.2 0.4 0.6 0.8 1.0 | 0.0 0.2 0.4 0.6 0.8 1.0 |

**Figure 4.1** Sensitivity vs. 1-specificity scatter plot of MTBDR*plus/sl* mutation and wild type probes.

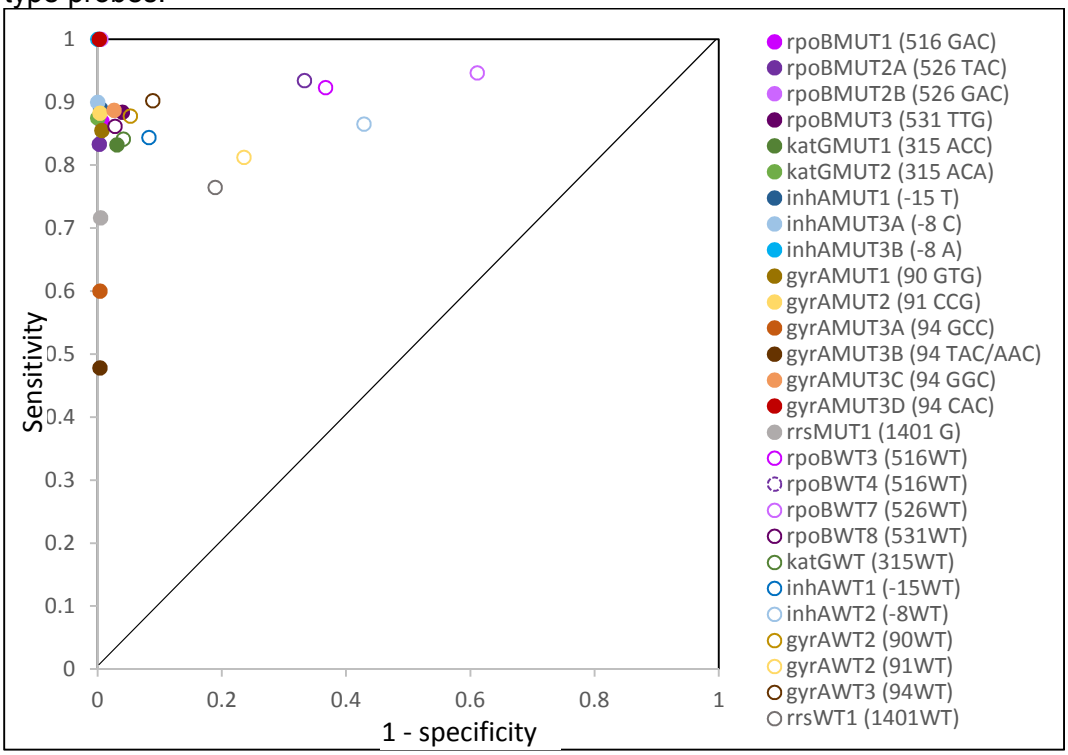


**Table 4.3** Specimens classified as resistant by failure of wild type probe hybridization and their PSQ results.[1]

| # of Resistant Specimens by Line Probe Assay | # of Absent-WT Resistant Specimens | Absent LPA WT probes | Corresponding PSQ result |
|---|---|---|---|
| **RIF n=478** | **60 (13%)** | | |
| | 19 | Combination of WT1, WT2, WT3, WT4, WT6, WT7, and/or WT8 | Indeterminate |
| | 7 | *WT8* | No mutations |
| | 1 | *WT3 & WT4* | No mutations |
| | 1 | **WT2 & WT3** | 511CGG & 516TAC |
| | 1 | *WT1 & WT2 & WT8* | 515ATA |
| | 1 | *WT1* & **WT7** | 515ATA & 526AAC |
| | 1 | **WT3** | 516TAC |
| | 1 | **WT3 & WT4** | 516TAC |
| | 2 | **WT4** | 516TAC |
| | 1 | **WT7** | 526AAC |
| | 1 | **WT7** | 526TGC |
| | 1 | *WT8* | 526CTC |
| | 1 | *WT8* | 526TGC |
| | 4 | **WT8** | 531TCG/TGG |
| | 17 | **WT8**[2] | 531TTG |
| | 1 | **WT8** | 533CCG |
| **INH n=484** *(326 katG, 128 inhA, 30 inhA & katG)* | | | |
| ***katG*** | **0 (0%)** | | |
| ***inhA*** | **19 (12%)** | | |
| | 4 | WT1 | Indeterminate |
| | 13 | **WT1** | -17 T |
| | 2 | **WT1**[3] & *WT2* | -15 T |
| **FQ n=237** | **15 (6%)** | | |
| | 1 | WT2 | Indeterminate |
| | 1 | **WT1** & *WT3* | 88GCC |
| | 1 | **WT1** | 88GCC & 95ACC |
| | 1 | **WT1** | 88TGC & 95ACC |
| | 1 | **WT1 & WT2 & WT3** | 90GTG & 95ACC |
| | 2 | **WT3**[4] | 94GGC & 95ACC |
| | 8 | **WT3**[5] | 94TAC & 95ACC |
| **SLID n=46** | **1 (2%)** | | |
| | 1 | **WT1**[6] | 1401G |

[1]Discordant pairs are italicized and concordant pairs are in bold.
[2]*rpoB*WT8 did not adhere for mutation 531TTG (as expected), but the corresponding *rpoB*MUT3 is absent
[3] *inhA*WT1 did not adhere for mutation -17T (as expected), but the corresponding *inhA*MUT1 is absent
[4] *gyrA*WT3 did not adhere for mutation 95ACC (as expected), but the corresponding *gyrA*MUT3C is absent
[5] *gyrA*WT3 did not adhere for mutation 95ACC (as expected), but the corresponding *gyrA*MUT3B is absent
[6] *rrs*WT1 did not adhere for mutation 1401G (as expected), but the corresponding *rrs*MUT1 is absent

**Table 4.4** Comparison of line probe assay resistance detection by mutation probe hybridization alone, and mutation probe hybridization plus failure of wild type probe hybridization to MGIT 960 phenotypic results.[1]

| DRUG | LPA Resistance Determination | TP | FP | FN | TN | AUC | P For AUC |
|---|---|---|---|---|---|---|---|
| RIF (N=809) | MUT-present | 415 | 2 | 63 | 329 | 0.9311 | **<0.001** |
| | MUT-present + absent-WT | 462 | 7 | 16 | 324 | 0.9727 | |
| INH *katG* & *inhA* (N=790) | MUT-present | 472 | 0 | 37 | 281 | 0.9637 | 0.17 |
| | MUT-present + absent-WT | 478 | 1 | 31 | 280 | 0.9678 | |
| INH *katG* | MUT-present | 450 | 0 | 59 | 281 | 0.9420 | n/a |
| | MUT-present + absent-WT | 450 | 0 | 59 | 281 | 0.9420 | |
| INH *inhA* | MUT-present | 139 | 0 | 370 | 281 | 0.6365 | **<0.001** |
| | MUT-present + absent-WT | 156 | 1 | 353 | 280 | 0.6515 | |
| MOX (N=742) | MUT-present | 218 | 4 | 25 | 495 | 0.9446 | **<0.001** |
| | MUT-present + absent-WT | 232 | 5 | 11 | 494 | 0.9724 | |
| OFX (N=742) | MUT-present | 221 | 1 | 23 | 497 | 0.9519 | **<0.001** |
| | MUT-present + absent-WT | 234 | 3 | 10 | 495 | 0.9765 | |
| AMK (N=672) | MUT-present | 45 | 0 | 8 | 619 | 0.9245 | 0.32 |
| | MUT-present + absent-WT | 46 | 0 | 7 | 619 | 0.9340 | |
| CAP (N=672) | MUT-present | 43 | 2 | 8 | 619 | 0.9200 | 0.32 |
| | MUT-present + absent-WT | 44 | 2 | 7 | 619 | 0.9298 | |
| KAN (N=672) | MUT-present | 45 | 0 | 51 | 576 | 0.7344 | 0.32 |
| | MUT-present + absent-WT | 46 | 0 | 50 | 576 | 0.7396 | |

[1]Significant p-values for change in AUC identified in bold

# CHAPTER 5

## Discussion and Conclusions

## Overview

This dissertation was undertaken to enhance the understanding of genetic mutations associated with drug resistant Mycobacterium tuberculosis (Mtb) and their role in the performance of molecular based diagnostic tests. In particular, the following questions were addressed. 1) What is the global frequency and distribution pattern of mutations associated with isoniazid (INH) resistance? 2) Does the newly redesigned line probe assay, MTBDRplus version 2.0 (v2), perform equally well among smear positive and negative specimens for the detection of both INH and rifampicin (RIF) resistance? 3) What demographic or clinical characteristics are associated with valid (resistant or susceptible) line probe assay MTBDRplus v2 results? 4) At the individual probe level, how well do line probe assays detect wild type and mutation sequences? And 5) Does the method of line probe assay detection of resistance by failure of wild type probe hybridization improve sensitivity and specificity when compared to culture-based results?

## Frequency and Distribution Pattern of Isoniazid Associated Mutations

Results from the systematic review confirmed that the overwhelming dominance of a single mutation among phenotypically INH resistant isolates, the serine to threonine mutation at codon 315 of the *katG* gene, was detected in 64.2% of all phenotypically INH resistant isolates. The principal nucleotide change (AGC to ACC) occurred in 93% of isolates reporting at the nucleotide level. While the second most commonly occurring mutation at position -15 of the *inhA* promotor was identified in 19.2% of phenotypically INH resistant isolates. Although the prevalence of individual mutations in the pathogen population is important, the cumulative frequency of these two mutations provides a more useful estimate as to what proportion of phenotypic resistance could be captured by molecular based tests.

Among studies that assessed both *katG* and *inhA* mutations, 65% had the *katG*315 mutation and 19% had the *inhA*-15 mutation. The combined cumulative frequency was 80% for both mutations. In the subset of studies that assessed *katG*, *inhA*, and ahpC genes, the cumulative frequency of *katG*315, *inhA* mutations -15, -8, -47, and -17, and ahpC mutations -10, -6, -39, -48, -15, -12, and -9 reached 84%.

Most notable, previously undescribed patterns of mutation frequency were detected in the systematic review dataset. The overwhelming majority of all *katG* mutations occurred at codon 315; however, any other mutations occurring on the *katG* gene almost always co-occurred with the *katG*315 mutation. This indicates that the additional detection of other *katG* mutations would not add any power to the discriminatory ability of a molecular test. Mutations occurring on the *inhA* gene, however, were quite different. While the large majority of mutations on the *inhA* promoter occurred at position -15, other mutations on the *inhA* promoter occurred independently; and therefore, could potentially contribute to the identification of phenotypic resistance. Mutations detected on the ahpC gene demonstrated a completely different pattern, and unlike *katG* and *inhA*, there was no single dominate mutation that accounted for the majority of phenotypic resistance associated with that gene. Rather, mutations on the ahpC gene appeared to occur infrequently (~1%) in the INH resistant pathogen population. But taken together, the ahpC mutations did account for up to five percent of associated phenotypic resistance as they rarely co-occurred with other mutations on ahpC or on either *katG* or *inhA*. Further examination of patterns of mutation co-occurrence of not only mutations associated with INH resistance but also mutations associated with resistance to other anti-tuberculosis drugs could contribute to more efficient design of rapid molecular diagnostic tests.

Additionally, few studies have assessed the presence of INH related mutations among a globally representative population. One study conducted in California, which is considered globally representative of drug resistant TB because of immigration did detect similar cumulative frequencies of the co-occurrence of *katG*315 and *inhA*-15 at 83%.(1) Two other studies detected higher cumulative frequencies of *katG*315 and *inhA*-15 mutations, at 91% and 96%.(2, 3) However, both studies selected isolates for inclusion to maximize the diversity of mutations without consideration of global representativeness, which may have reduced potential extrapolation to global frequencies.

The systematic review also identified regional variations in mutation frequencies. Although these regional frequencies could not be accurately estimated since the data was collected via a systematic review and not through a representative survey, it did appear that there were differences in frequencies between WHO regions and at the country level. In particular, the mutations occurring at *katG*315 accounted for 94% of INH resistance among post-soviet states. In contrast, only 43% of INH resistant isolates harbored the *katG*315 mutation in Japan and Korea, underscoring the importance of continued analysis of country level prevalence data.

**Performance of MTBDRplus v2**

Of the commercially available line probe assays that assess multi-drug resistance, only the Genotype MTBDRplus v2 is designed to detect the presence of both RIF and INH resistance among both AFB smear positive and negative specimens. Other commercially available rapid genotypic tests detect RIF resistance, but use the detection of RIF associated mutations as a proxy for MDR-TB. Testing for INH resistance is critical, though, as demonstrated by a recent country level study where 18% of all the INH resistant strains were mono-resistant.(4)

The MTBDRplus v2 assay was found to perform well among both smear positive and negative specimens, with sensitivity and specificity for MDR-TB of 95.1% (92.6, 96.9) and 99.1% (97.1, 100.0), respectively. However, sensitivity for MDR-TB detection among exclusively smear negative specimens was considerably lower, at 82.8% (63.5, 93.5). The difference in sensitivity appeared to be driven primarily by INH resistance detection. Sensitivity was 81.6% (65.1, 91.7) among AFB smear negatives compared to 94.9% (92.4, 96.6) among AFB smear positives for INH. While sensitivity among AFB smear negatives for RIF was 91.4% (75.8, 97.8) compared to 97.1% (94.9, 98.4) among AFB smear positives

Overall, approximately 10% of Mtb culture positive specimens yielded an uninterpretable (neither resistant nor susceptible) INH or RIF result. Demographic and clinical characteristics associated with either presentation or progression of disease were assessed in this study to determine if there was any association between these characteristics and obtaining a valid (resistant or susceptible) result. Body Mass Index (BMI) was borderline significantly associated with valid results, and AFB smear grade was significantly associated with valid results. In the multivariable model, only AFB smear grade was associated with valid results after controlling for BMI, HIV, history of previous treatment, and diabetic status. A dose response relationship was evident for both INH and RIF resistance; as smear grade increased, so did the likelihood of obtaining a valid results.

The identified differences in test sensitivity for INH, along with the positive association between smear grade and valid result serve to reinforce the findings that test performance is primarily influenced by the bacilli load or the number of bacteria in the specimen, both in yielding a valid result (resistant or susceptible) and in the ability to detect a mutation.

## Comparison of Line Probe Assays to Sequencing

The third study evaluated both line probe assays, MTBDRplus v2 for detecting resistance to INH and RIF, and MTBDRsl for detecting resistance to second-line injectable drugs (SLIDs) and flouroquinolones (FQs), to actual sequencing data. The results provide a better understanding of underlying test performance mechanisms. Sixteen probes containing mutation sequences and eleven corresponding wild type probes, designed to interrogate ten gene regions with potential resistance conferring mutations, was examined independently in each specimen using targeted pyrosequencing (PSQ) as the reference sequencing method. Probes containing mutation sequences demonstrated significantly higher specificity when compared to probes containing wild type sequences. Conversely, probes containing wild type sequences demonstrated marginally higher sensitivity when compared to probes containing mutation sequences. This combination of mutation sequence specificity and wild type sequence sensitivity is an ideal combination for detecting drug resistance, yielding the fewest number false positive results when compared to sequencing.

Both line probe assays evaluated utilize a combination of two methods for classifying specimens as resistant. If a probe containing a mutation sequence hybridizes on the test strip, the presence of the mutation can be assumed and the specimen is considered resistant. If no mutation probes hybridize on the test strip, but a probe containing a wild type sequence also fails to hybridize, the presence of a mutation can be assumed as no hybridization occurred with the wild type sequence and the specimen is considered resistant. In this analysis, 95 individual drug assay results were classified as resistant based on the absence of wild type probe hybridization. These results were compared to sequencing to identify the mutations

inhibiting wild type probe hybridization. Twenty-five percent of the results that were resistant due to lack of hybridization could not be compared to sequencing because pyrosequencing (PSQ) yielded an unreadable result. Twelve percent were discordant (the sequencing results identified wild type sequences), 60% were concordant (sequencing indicated mutations in the area of the absent probe), and 4% were mixed (multiple probes were absent, some being concordant while others were discordant). Examining only the 56 concordant probes revealed that 50% of the mutations identified by sequencing should have been detected by a mutation probe, with the most commonly missed being the *rpoB*531TTG mutation.

A final analysis evaluated whether the lack of wild type hybridization contributed significantly to the overall sensitivity of the line probe assay. Line probe assay results were compared to phenotypic results in a subset of 907 culture positive specimens with drug susceptibility results. It was discovered that the inclusion of specimens as resistant due to the absence of wild type hybridization significantly improved the detection of both RIF and FQ resistance. Lack of wild type hybridization also improved *inhA* related INH resistance detection; however, many INH resistant specimens also harbored a *katG* mutation and would have been classified as INH resistant regardless.

To summarize, a resistance determination based on the failure of the wild type probe hybridization significantly improved overall resistance detection for RIF and FQs in our study, with negligible increases in false positive results.

## Conclusions

While each study examined a different facet of mutation frequency or line probe assay performance, all contributed to an improved understanding of molecular based drug resistance detection. The analysis of mutations associated with INH

resistance provides a more nuanced understanding of mutation patterns both within and between genes. These results are useful for future molecular test improvement and design, potentially leading to increased specificity of TB diagnostics. The identification of geographic variation in mutation frequency also provides justification for more local or country level surveys to provide clinicians a better context in which to interpret molecular based test results, as the performance of molecular-based diagnostic tests for drug resistant TB is inherently linked to both the regional frequencies of mutations being detected and to the diversity of resistance-conferring mutations being detected.

While MTBDRplus v2 performs well among both smear positive and smear negative specimens, smear gradation appears to negatively influence both the probability of obtaining an interpretable result, and test sensitivity and specificity, indicating a significant association between bacillary load and test performance. Continued monitoring of test performance, particularly in a field setting, is vital to ensure optimal function.(5)

Further analysis of the drug resistance detection mechanisms for molecular based line probe assay testing as compared to genetic sequencing is particularly relevant in the context of recent studies indicating that resistance detected by absent wild type probe hybridization has produced false positive results.(6, 7) The current study demonstrates that the inclusion of absent hybridization of wild type probes in resistance classification significantly increases test sensitivity for detection of both RIF and FQ resistance. This has broader implications for diagnostic testing as other rapid molecular based tests have encountered this issue also.(8) Findings can be used to better understand how these tests work on a molecular level, and the

potential limitations of such testing methods. Continued research is need to elucidate all resistant gene determinates and specific diagnostic assay performance.(9) Although culture based or phenotypic resistance detection methods will continue to remain the gold standard for detecting TB and drug resistant TB for the near future, the utilization and development of molecular based or genotypic methods of resistance detection are critically important for successful treatment outcomes. In some instances, genotypic methods are already becoming the preferred method for resistance detection.(10) Clinical benefits provided by earlier detection include both rapid initiation of appropriate therapy, and the reduction of primary transmission of drug resistant strains. Given this, continued research in rapid diagnostics and field evaluation is warranted.

Overall, this in depth evaluation of line probe assay performance provides some confidence for practitioners currently utilizing line probe assays in a clinical setting. The comparison of line probe assay results and the classification algorithms to both sequencing and genotypic culture tests also provide an enhanced understanding of the mechanisms of line probe assays that can be translated into the design of other molecular based diagnostic tests. Together these studies contribute to an enhanced understanding of mutations associated with resistance to anti-tuberculosis drugs and how diagnostic tests evaluating these mutations discriminate between resistant and susceptible Mtb.

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