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Molecular diagnostics for drug-resistant tuberculosis: Can genetic tests replace conventional diagnostics as predictors of drug resistance and clinical outcome?

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

in

Public Health (Global Health)

by

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The Dissertation of Sophia B. Georghiou is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter 3, "Frequency and Distribution of Tuberculosis Resistance-associated Mutations in a Multisite Study," has been accepted for publication in *Antimicrobial Agents and Chemotherapy*, a journal of the American Society for Microbiology. Marva Seifert, Donald Catanzaro, Richard Garfein, Faramarz Valafar, Valeriu Crudu, Camilla Rodrigues, Thomas Victor, Antonino Catanzaro and Timothy Rodwell are co-authors.

Chapter 4, “Increase in Mortality for Tuberculosis Patients with Mutations Associated with Second-line Drug Resistance in a Multisite Study,” is currently being prepared as a submission for publication. Marva Seifert, Donald Catanzaro, Richard Garfein, Valeriu Crudu, Camilla Rodrigues, Thomas Victor, Antonino Catanzaro and Timothy Rodwell are co-authors. All co-authors have given me approval to submit these manuscripts as evidence of my independent work for purposes of a dissertation.

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2. **Georghiou, S.B.;** Seifert, M.; Catanzaro, D.; Garfein, R.S.; Valafar, F.; Crudu, V.; Rodrigues, C.; Victor, T.C.; Catanzaro, A.; Rodwell, T.C. (in press) Frequency and Distribution of Tuberculosis Resistance-Associated Mutations in a Multisite Study. *Antimicrobial Agents and Chemotherapy*.

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ABSTRACT OF THE DISSERTATION

Molecular diagnostics for drug-resistant tuberculosis:
Can genetic tests replace conventional diagnostics as predictors of drug resistance and clinical outcome?

by

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Doctor of Philosophy in Public Health (Global Health)

University of California, San Diego, 2016

San Diego State University, 2016

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Background: Rapid molecular diagnostics, with their ability to quickly identify genetic mutations associated with drug resistance, have great potential to curb the spread of drug-resistant tuberculosis (DR-TB). Unfortunately, the technical and diagnostic performance of these technologies has yet to be thoroughly evaluated in

diverse clinical environments, and the reliability and clinical relevance of the tuberculosis (TB) resistance mutations identified by these assays has not been well characterized.

Methods: These analyses use data from a multisite, observational cohort study conducted by the Global Consortium for Drug Resistant TB Diagnostics (GCDD). Chapter 2 assesses the diagnostic performance of a pyrosequencing assay in this study and applies logistic regression to identify the variables associated with poor assay technical performance. Chapter 3 presents the frequency and distribution of all TB resistance mutations identified between clinical sites. Chapter 4 applies multivariate logistic regression to determine the associations between TB resistance mutations and patient mortality for the GCDD study cohort.

Results: Chapter 2 found pyrosequencing assay performance to be high, overall, though sensitivity fluctuated between sites. Smear negativity, culture negativity, site (Moldova), and sequencing of the *rpoB, gyrA*, and *rrs* genes were associated with poor assay technical performance. Chapter 3 found inclusion of the *ahpC* and *eis* promoter gene regions to be critical for optimal test sensitivity for isoniazid resistance detection in South Africa and kanamycin resistance detection in Moldova. Chapter 4 found that the detection of a mutation associated with high-level fluoroquinolone or kanamycin resistance was significantly associated with higher odds of patient mortality by 52 weeks.

Conclusions: A DR-TB diagnostic demonstrated excellent specificity in a large, multisite study, though performance was influenced by regional genetics and technical errors. Findings of the high prevalence of low-global frequency TB resistance mutations in specific sites emphasizes that these mutations may be important resistance markers in certain regions. The observation that patients with

high-level *gyrA* and *rrs* resistance mutations had higher mortality odds provides strong evidence that certain high-level resistance mutations are clinically relevant. Together, these findings underscore the potential for molecular diagnostics to replace conventional growth-based diagnostics as predictors of phenotypic drug resistance and clinical outcome.

CHAPTER 1: INTRODUCTION

OVERVIEW

Although the incidence of new tuberculosis (TB) cases has been declining over the past decade, the incidence of multi- and extensively drug-resistant TB (M/XDR-TB) has remained stable, undermining TB control efforts. One of the major roadblocks in combating this growing problem has been the lack of diagnostic technology for drug-resistant TB (DR-TB) infections. Growth-based culture methods and phenotypic drug susceptibility testing (DST) remain the gold standard for M/XDR-TB diagnosis, but these methods can take several months to yield results. Rapid molecular diagnostics for M/XDR-TB hold great promise as tools to replace growth-based culture methods and shorten the time to DR-TB diagnosis and appropriate treatment. However, questions remain regarding the performance of these technologies, including the reliability and clinical relevance of the genetic markers of resistance upon which their performance is dependent. The overall goal of this dissertation is to evaluate the potential for molecular diagnostics to replace conventional growth-based diagnostics for M/XDR-TB. The specific aims of this dissertation are (1) to assess the diagnostic and technical performance of a molecular diagnostic assay for M/XDR-TB between three diverse clinical sites, (2) to determine the frequency and distribution of TB resistance mutations between these sites, and (3) to determine the clinical relevancy of TB resistance mutations in a multisite, observational cohort study.

To meet these aims, I determine the sensitivity and specificity of a pyrosequencing assay for the detection of M/XDR-TB between three clinical sites in a global study (Aim 1), I use multivariate logistic regression to identify variables

associated with poor pyrosequencing technical performance across these sites (Aim 1), I identify differences in the frequencies of particular TB resistance mutations between three international sites (Aim 2), and I employ multivariate logistic regression to determine the associations between particular TB resistance mutations and patient mortality up to a 52-week follow-up period (Aim 3). This will contribute to a better understanding of the nuances of molecular diagnostic performance as well as the reliability and relevancy of molecular markers of TB drug resistance worldwide. Results will assist diagnostic developers in designing and optimizing their assays, help clinicians to better interpret molecular diagnostic test results, and ultimately indicate the potential for rapid molecular diagnostics to replace conventional growth-based diagnostics as predictors of TB drug resistance and clinical outcome.

BACKGROUND

Drug-resistant Tuberculosis

TB is an infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). According to the most recent Global TB Report published by the World Health Organization (WHO), 9.6 million new cases of TB and 1.5 million TB-associated deaths were reported globally in 2014 [1]. Although global TB incidence rates have fallen at an average of 1.5% per year since 2000, TB control efforts have been significantly complicated by the rise and spread of DR-TB. In 2014, the WHO estimated that one in every 20 new, active TB infections was drug-resistant, and reported 480,000 new cases of MDR-TB and 190,000 deaths attributed to MDR-TB, defined as TB that has developed resistance to the first-line anti-tuberculosis drugs isoniazid (INH) and rifampicin (RIF) [1]. An estimated 9.7% of those with MDR-TB had XDR-TB, or MDR-TB that has developed additional resistance to the fluoroquinolones

(FQs) and at least one of the injectable compounds [amikacin (AMK), kanamycin (KAN) and/or capreomycin (CAP)].

TB drug resistance typically develops when patients do not take their medications properly, and so *Mtb* bacteria harboring genetic mutations that confer resistance are selected for within the patient. These mutated *Mtb* strains then profligate, surviving low-level drug exposure, while susceptible bacteria without these mutations are killed by the antibiotic. In this way, DR-TB sub-populations slowly become dominant in the patient. However, DR-TB strains can also be transmitted directly from one person to another if they breathe in the drug-resistant bacteria. According to the WHO, 3.3% of new and 20.0% of previously treated TB infections were drug-resistant in 2014 [1]. The prompt and accurate diagnosis of these infections is critical to direct appropriate treatment of DR-TB patients and to prevent further amplification or transmission of drug resistance.

Conventional Diagnostics for Drug-resistant Tuberculosis

Conventional methodologies for the diagnosis of M/XDR-TB rely upon mycobacterial culture and DST in liquid to solid media- methods that have not changed in decades. Unfortunately, the biosafety conditions required for these methods are complex, and many clinics worldwide do not have the stringent, sterile environment necessary to run these tests. Furthermore, the infrastructure required to implement these methods can be cost prohibitive, limiting the use and reliance of these methods in low- and middle-income countries. Even when available, growth-based DST methods can take several weeks to months to yield results [2]. While waiting on culture results to determine the phenotypic drug resistance profiles of TB infections, physicians are forced to treat their patients empirically, only adjusting initial

treatment regimens once culture results become available. Prior to appropriate treatment, M/XDR-TB patients may be given medications that are ineffective and so remain contagious. In this way, inappropriate treatment amplifies resistance, increases the risk of patient mortality, and increases the risk of these patients transmitting DR-TB to their communities.

Molecular Diagnostics for Drug-resistant Tuberculosis

Rapid molecular diagnostics for M/XDR-TB that do not rely upon the culture of the slow-growing *Mtb* pathogen are essential to the future management of M/XDR-TB. Gene-based M/XDR-TB diagnostics, with their ability to quickly identify genetic mutations associated with drug-resistance in *Mtb* clinical specimens [3-5], have shown the most promise to shorten the time to DR-TB diagnosis and effective treatment [6-8]. These technologies focus upon the detection of mutations in *Mtb* genes that have been associated with INH, RIF, FQ, and injectable resistance, and yield results in just one day following specimen collection [9].

Three of the more promising, commercially available M/XDR-TB gene-based molecular diagnostics include hybridization-based tests, molecular beacon tests, and pyrosequencing assays. Hybridization tests, including the MTBDR*plus* and MTBDR*sl* line probe assays (Hain Life Sciences, Tübingen, Germany), rely upon the hybridization of specific mutations in clinical *Mtb* strains to complementary probes. These assay have shown great technical and diagnostic performance for M/XDR-TB detection in field studies [9-12], and they are the only commercially available molecular diagnostic assays currently in broad use for M/XDR-TB detection. Molecular beacon tests have also show excellent utility in the field [13, 14]. Beacon-type assays rely upon the detection of short stretches of DNA that are different from

wildtype, rather than detecting specific mutations. This technology is the basis of the GeneXpert assay, endorsed by the WHO for detection of RIF resistance in *Mtb* [15], but the current assay is limited in its ability to detect TB resistance to only to RIF. Pyrosequencing, in comparison, is a rapid, real-time sequencing method capable of detecting genetic mutations associated with *Mtb* drug resistance. The technology has been recently shown to compare favorably against culture DST methods and line probe assay results in a large, multinational field study of M/XDR-TB patients [9]. Although all three gene-based molecular diagnostic assays rely upon the detection of known resistance-conferring mutations in the *Mtb* genome to diagnose drug-resistance in clinical specimens, pyrosequencing will be the molecular diagnostic method of focus in this dissertation, as it is the only assay that can provide detailed sequencing data for relevant gene regions of interest and is open-format, allowing for the addition of new gene targets [16-18].

Global Consortium for Drug-resistant TB Diagnostics

The Global Consortium for Drug-resistant TB Diagnostics (GCDD) was formed in 2008 to characterize the genetic basis of drug resistance and develop and evaluate rapid DST methods for DR-TB detection. From April 2012 to June 2013, the GCDD enrolled 1128 patients in three countries in an observational cohort study. The primary focus of this study was to perform a head-to-head assessment of three different rapid diagnostic tools for DR-TB, including a modified pyrosequencing assay for DR-TB detection [9]. Subjects at risk for DR-TB were enrolled in Mumbai, India; Chisinau, Moldova; and Port Elizabeth, South Africa. Clinical data were gathered from a combination of patient interviews, chart reviews, and laboratory testing at each site's reference laboratory [19]. This clinical trial generated a large body of clinical

and laboratory data for one of the largest populations of M/XDR-TB patients studied to date [9]. Although the primary outcome of interest in this study was reduction in time from specimen arrival at the laboratory to rapid DST result availability [19], as compared to phenotypic DST, the procurement of a large and informative body of clinical and laboratory data for a diverse M/XDR-TB patient population allowed for many additional, informative diagnostic and genetic analyses.

AIMS AND HYPOTHESES

Using the dataset described above, and following a review of the relevant literature on genetic markers of TB drug resistance and associations with diagnostic performance and patient outcomes, this dissertation has the following specific aims and corresponding hypotheses:

Aim 1: To assess the diagnostic and technical performance of a pyrosequencing platform, analyzing nine gene targets to diagnose M/XDR-TB, in a multisite study of 1128 patients in India, Moldova and South Africa. *Hypothesis 1.1*: The diagnostic performance of the pyrosequencing platform is expected to be high between all clinical sites. *Hypothesis 1.2:* There will be similar sensitivity improvements across all sites for the detection of KAN resistance when the *eis* promoter is added to the pyrosequencing platform. *Hypothesis 1.3:* Technical performance of the diagnostic assay is expected to be low for acid-fast bacilli smear-negative samples and even lower for culture-negative samples, and technical performance will vary according to the different gene targets included in the pyrosequencing assay.

Aim 2: To determine the frequency and approximate the global distribution of TB resistance mutations by analyzing diagnostic pyrosequencing results from

patients suspected of having M/XDR-TB in Mumbai, India; Moldova; and the Eastern Cape; South Africa. *Hypothesis 2*: The frequencies of mutations in specific gene regions will be higher in certain geographical locations than in others.

Aim 3: To investigate the associations between the genetic mutations characterizing M/XDR-TB and patient mortality by a 52-week follow-up period in a multisite, observational cohort study. *Hypothesis 3*: There will be significant associations between high-level resistance-conferring mutations and patient mortality outcomes, compared to low-level resistance-conferring mutations, for a diverse cohort of M/XDR-TB patients.

GLOBAL HEALTH IMPLICATIONS

Findings from this research on TB resistance mutations and M/XDR-TB molecular diagnostics have significant global health implications. First, these analyses use data collected from one of the largest, most diverse diagnostic studies of M/XDR-TB patients to date. The findings of these analyses will allow us to comment upon the global relevance of TB resistance markers in diverse clinical environments, and how molecular diagnostic platforms that rely upon these resistance markers might be expected to perform globally. These results will be relevant to diagnostic developers, who will be able to predict how their technologies will perform in similar sites, and thereby make informed decisions on what mutations to include in their assays to maximize diagnostic performance in different environments. By investigating the diagnostic reliability and clinical relevancy of specific TB resistance mutations, these results will also help clinicians to interpret molecular diagnostic results and tailor drug-treatment regimens according to M/XDR-TB genotypes to ultimately improve patient outcomes. Together, these findings

provide evidence for the potential for rapid M/XDR-TB molecular diagnostics to replace conventional growth-based methods in global clinics and significantly impact TB control efforts worldwide.

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CHAPTER 2: SHEDDING LIGHT ON THE PERFORMANCE OF A PYROSEQUENCING ASSAY FOR DRUG-RESISTANT TUBERCULOSIS DIAGNOSIS

ABSTRACT

Background: Rapid molecular diagnostics, with their ability to quickly identify genetic mutations associated with drug resistance in *Mycobacterium tuberculosis* clinical specimens, have great potential as tools to control multi- and extensively drug-resistant tuberculosis (M/XDR-TB). The Qiagen PyroMark Q96 ID system is a commercially available pyrosequencing (PSQ) platform that has been validated for rapid M/XDR-TB diagnosis. However, the details of the assay's diagnostic and technical performance have yet to be thoroughly investigated in diverse clinical environments.

Methods: This study evaluates the diagnostic performance of the PSQ assay for 1128 clinical specimens from patients from three areas of high TB burden. We report on the diagnostic performance of the PSQ assay between the three sites and identify variables associated with poor PSQ technical performance.

Results: In India, the sensitivity of the PSQ assay ranged from 89-98% for the detection of phenotypic resistance to isoniazid, rifampicin, fluoroquinolones, and the injectables. In Moldova, assay sensitivity ranged from 7-94%, and in South Africa, assay sensitivity ranged from 71-92%. Specificity was high (94-100%) across all sites. The addition of *eis* promoter sequencing information greatly improved the sensitivity of kanamycin resistance detection in Moldova (7% to 79%). Nearly all (89.4%) sequencing reactions conducted on smear-positive, culture-positive specimens and

most (70.8%) reactions conducted on smear-negative, culture-positive specimens yielded valid PSQ reads. An investigation into the variables influencing sequencing failures indicated smear negativity, culture negativity, site (Moldova), and sequencing of the *rpoB, gyrA*, and *rrs* genes were highly associated with poor PSQ technical performance (adj. OR > 2.0).

Conclusions: This study has important implications for the global implementation of PSQ as a molecular TB diagnostic, as it demonstrates how regional factors may impact PSQ diagnostic performance, while underscoring potential gene targets for optimization to improve overall PSQ assay technical performance.

INTRODUCTION

In 2014, 9.6 million new cases of tuberculosis (TB) and 1.5 million TB-associated deaths were reported worldwide [1]. Although the incidence of new TB cases has continued to fall over the past decade, the incidence of multi- and extensively drug-resistant TB (M/XDR-TB) has been stable, undermining TB eradication efforts. MDR-TB is defined as TB that has developed resistance to the first-line anti-tuberculosis drugs isoniazid (INH) and rifampicin (RIF). XDR-TB is MDR-TB that has developed additional resistance to the fluoroquinolones (FQs) and at least one of the injectable compounds [amikacin (AMK), kanamycin (KAN) and/or capreomycin (CAP)]. An estimated 480,000 people developed MDR-TB while 190,000 deaths were attributed to MDR-TB in 2014 [1]. Alarmingly, only 26% of the estimated MDR-TB infections globally were detected in 2014 [1]. This means that over one third of a million people suffered from undiagnosed and untreated drug-resistant TB, which is a significant risk for high mortality and continued transmission of M/XDR-TB.

The conventional methodology for diagnosis of drug-resistant TB (DR-TB) has not changed for decades, and relies upon mycobacterial culture and drug susceptibility testing (DST) in solid or liquid media. These methods yield results only after weeks to months of cell culture, and require biosafety conditions that are complex and expensive to implement in low- and middle-income countries. MDR- and XDR-TB patients waiting for growth-based diagnostic test results before appropriate treatment is started remain contagious and at increased risk of death.

Rapid molecular diagnostics for M/XDR-TB have great potential to shorten the time to DR-TB diagnosis and appropriate treatment. Pyrosequencing (PSQ) is a real time, rapid method for sequencing fragments of genomic DNA. PSQ assays have

been previously established as valid technologies to rapidly and accurately identify mutations associated with drug resistance in *Mycobacterium tuberculosis* (*Mtb*) isolates and in clinical specimens [2-8]. The commercially available Qiagen PyroMark Q96 PSQ platform has been validated as an M/XDR-TB diagnostic assay and is currently in use in by the Microbial Diseases Laboratory in the California Department of Public Health for rapid detection of M/XDR-TB in the United States, having been validated for clinical use [9, 10]. One study validating this PSQ assay against conventional methods in high burden settings found strong correlations with phenotypic DST, with sensitivity values ranging from 86-100% and specificity values ranging from 99-100% for all drugs tested [11]. The Global Consortium for Drug-resistant TB Diagnostics (GCDD) also conducted a large-scale, multisite study to evaluate the ability of this assay to accurately predict TB phenotypic drug resistance profiles, and found test performance to vary across diverse clinical environments. Overall assay sensitivity ranged from 50-95%, and the number of interpretable results ranged from 73-88% among *Mtb* culture-positive specimens [12]. These variations highlight potential limitations of the assay. This study examines the detailed diagnostic and technical performance of a PSQ assay for M/XDR-TB diagnosis in three diverse clinical sites and describes modifications that could improve overall diagnostic and technical performance of the PSQ assay.

MATERIALS AND METHODS

Study Population

Briefly, three diverse clinical sites (Chisinau, Moldova, Port Elizabeth, South Africa, and Mumbai, India) were selected for this study [13]. Newly-presenting TB patients over five years of age were eligible for the study if they were known to be

acid-fast bacilli (AFB) smear-positive (defined as 1+ or greater within prior 14 days) or suspected of having active pulmonary TB and having one or more reason to be considered to have DR-TB, and provided informed consent for the study. Patients unable to provide 7.5mL of sputum were excluded, along with subjects who had second-line DST in the prior three months. A total of 1128 patients meeting the above criteria were enrolled in the GCDD study from April 24, 2012 to June 27, 2013.

Acid-Fast Bacilli Smear and Drug-Susceptibility Testing

AFB smear testing was performed on all isolates, and smear grading was determined in the first two weeks following enrollment. All phenotypic drug susceptibility profiles were established using the Mycobacterial Growth Indicator Tube (MGIT) 960 platform. These MGIT DST results served as reference standard in our study. All specimens were tested for resistance to INH, RIF, two FQs [moxifloxacin (MOX) and ofloxacin (OFX)], and three injectable drugs (AMK, KAN and CAP) using standard manufacturer protocols [14] and previously-published and World Health Organization-recommended critical concentrations for MGIT-based DST [13, 15].

DNA Extraction, PCR and Molecular Targets

Crude DNA was extracted from each decontaminated, concentrated sputum (sediment) by heating the cell suspensions in a water bath at 100°C [11, 13]. PCR master mixes were prepared and amplification reactions were carried out as previously reported [11]. Table 2.1 lists all primers used for PCR and sequencing reactions. Our PSQ assay included one reaction to identify *Mtb* and seven reactions to detect specific mutations in drug resistance-associated gene regions. The molecular target IS*6110* was considered confirmatory for identification of *Mtb*.

However, since the marker is not 100% reliable, especially for Indian strains of *Mtb* [16-20], we included findings for specimens deemed indeterminate for presence of the IS*6110* marker via PSQ as long as at least one other *Mtb* gene yielded a sequencing result. This practice is in accordance with similar PSQ studies as, apart from the *rrs*, the primers utilized in sequencing reactions are highly specific for *Mtb* [11]. A negative H_2O control was used for every target in each run.

Pyrosequencing

We used the PyroMark Q96 ID system (Qiagen, Valencia, CA) to perform PSQ on specific regions of the *ahpC* and *inhA* promoters and the *katG*, *rpoB*, *gyrA*, and *rrs* genes, sequencing two different parts of *rpoB* in two separate reactions, as described previously [9]. Sequencing of these targets was completed at the respective clinical sites. Sequenced gene regions are outlined in Table 2.1. Variants relative to the *Mtb* H37Rv reference strain (ATCC 27294) were identified automatically from generated PSQ pyrograms using IdentiFire software (Qiagen, Valencia, CA). All samples that did not provide PSQ queries with a 100% match to library wildtype or mutant sequences were repeated in duplicate. Samples that still did not provide confirmatory sequence and samples for which contradictory hits were obtained for any given target were deemed genotypically indeterminate.

Upon completion of the study, *eis* promoter sequencing capabilities were added to the platform by designing primers specific for sequencing the *eis* promoter of *Mtb* (Table 2.1), and updating the system's library for query read identification via the IdentiFire software. *eis* sequencing reactions used PCR and PSQ parameters identical to the other assay targets. In sequencing the *eis* promoter, DNA extracted from specimens from India were sequenced on-site, while DNA extracted from

specimens from Moldova and South Africa were sequenced using a PyroMark Q96 ID system at the University of California, San Diego. As for the other targets, all *eis* queries that did not 100% match reference library sequences were repeated in duplicate.

Pyrosequencing Diagnostic Performance

In order to comment on the validity of the PyroMark PSQ platform in establishing *Mtb* drug resistance profiles, we calculated sensitivity and specificity for each drug by comparing PyroMark findings to conventional MGIT phenotypic DST results in each clinical site. Sensitivity was calculated as the number of phenotypically resistant specimens in which a resistance-associated mutation was found via PSQ, divided by the number of phenotypically resistant specimens. Specificity was calculated as the number of phenotypically susceptible specimens in which no resistance-associated mutation was found via PSQ, divided by the number of phenotypically susceptible specimens. INH resistance was determined with PSQ via the presence of known resistance-conferring mutations in at least one of three genes (*inhA*, *katG* and *ahpC*), RIF resistance through presence of at least one resistance-associated mutation in one of two *rpoB* gene regions, injectable resistance through the presence of the 1401G or 1402T mutation in the *rrs* gene, and FQ resistance via the presence of resistance-conferring mutations in the *gyrA* gene. KAN resistance was determined via the presence of the 1401G or 1402T mutation in the *rrs* gene or the presence of a resistance-associated mutation in the *eis* promoter. Confidence intervals for sensitivity and specificity of individual mutations were determined using the score/efficient score method with continuity correction [21, 22]. Diagnostic performance differences were noted between the sites based upon the presence of

non-overlapping confidence intervals for sensitivity or specificity calculations for any particular drug.

Analysis of Pyrosequencing Technical Performance

Sequencing success was first determined for smear- and culture-negative and positive samples by calculating the proportion of the total PSQ reactions conducted for those samples that yielded interpretable sequencing results. The variables associated with poor PSQ technical performance, defined as the inability to obtain interpretable sequencing results, were then investigated by logistic regression, using STATA 13.1 Software (StataCorp, College Station, TX, USA). The outcome variable was an “indeterminate” result, or the inability to obtain a PSQ read that generated a 100% match with a target library sequence. Covariates evaluated included: smear negativity, culture negativity, clinical site and gene target. A bivariate analysis was first conducted to generate unadjusted odds ratios for each variable. Variables with p-value <0.20 were considered for inclusion in the final model. Covariates included in the final multivariate model with p-value <0.05 were considered significant.

In order to further comment on the underlying reasons for PSQ failures, all indeterminate results were categorized according to type of observed error. Categories included: no read error, homopolymer error, instrument error, mixed population, new mutation, or other error. No read errors were defined as unresolved errors where few or no peaks were seen in the resulting PSQ pyrograms in all sequencing reactions for a given gene target. Homopolymer errors were unresolved errors that occurred due to IdentiFire software mischaracterization of pyrogram peak height at one or more bases in any PSQ reaction for a given gene target. Instrument errors were unresolved errors resulting from incorrect instrument reagent

dispensation or camera detection errors, where one or more peaks in the resulting pyrogram were seen as a split peak (two small peaks) below IdentiFire peak detection threshold. Mixed populations occurred when all three pyrograms obtained for any gene target were identical but did not match a confirmatory sequence in the sequencing library due to the presence of two peaks in a given mutation region-representing both wildtype and mutant sequences. New or novel mutations were confirmed when three unambiguous, identical pyrograms were obtained for any gene target but did not match a sequence in the reference sequence library of known wildtype and common mutations in that region. Finally, the other error category included all other errors, including unknown errors or a combination of error types that could not be attributed to a single source. All PSQ indeterminates were characterized according to one of these reasons for error, and the numbers of errors falling into the different categories were summarized for each gene target.

Human Research Conduct

Our study was approved by the Institutional Review Board of the University of California, San Diego and by the Institutional Review Boards of the respective clinical sites.

RESULTS

Culture and Drug Susceptibility Testing Results

Of 1128 patients enrolled in the study, 914 (81%) provided *Mtb* culture-positive pulmonary sputum samples. One of the remaining 214 samples was contaminated, and the rest were *Mtb* culture-negative. MGIT DST could not be performed, or did not yield results, for seven of the 914 culture-positive clinical

specimens. One additional specimen did not yield a valid DST result for the evaluation of phenotypic MOX resistance. Four hundred fifty-four (40%) of the 1128 patients enrolled in the study had MDR-TB and 80 (7%) had XDR-TB, as determined by MGIT DST results. Thus, 906/907 results were available for this analysis.

Sensitivity and Specificity of Pyrosequencing as Compared to Phenotypic Testing

PSQ diagnostic performance for each TB treatment drug in each clinical site is detailed in Table 2.2. No major differences in the specificity of the PSQ assay for the detection of resistance to any antibiotic were observed between the three sites, with assay specificity ranging from 94-100% for all drugs in all sites prior to the addition of the *eis* promoter. The PSQ assay did, however, show differences in diagnostic sensitivity for various drugs between the three sites. For the detection of INH resistance, as seen in the presence of distinct 95% confidence intervals, the assay demonstrated lower sensitivity in South Africa (71%) than in either India (98%) or Moldova (94%). For the detection of RIF resistance, the assay demonstrated lower sensitivity in South Africa (77%) than in India (98%). For the detection of FQ resistance, the assay demonstrated lower sensitivity in Moldova (64-67%) than in India (96%). The sensitivities of the PSQ assay for the detection of resistance to the injectable drugs varied greatly between the three sites. The sensitivity of the assay for the detection of AMK resistance was 94% in India, 33% in Moldova, and 92% in South Africa. The sensitivity for the detection of CAP resistance was 94% in India, 40% in Moldova, and 85% in South Africa. The sensitivity of the assay for the detection of KAN resistance showed the greatest variation of all the injectables between the three sites: 89% in India, 7% in Moldova, and 92% in South Africa. For

the detection of injectable resistance, our PSQ assay demonstrated lower sensitivity in Moldova than in India for all drugs, though 95% confidence intervals overlapped with South African estimates for all but KAN resistance detection. By far, the most notable difference in assay sensitivity between the three sites was for the detection of KAN resistance in Moldova, where only 7% (95% CI 0.02-0.18) of the 57 phenotypically KAN-resistant specimens were found to have the *rrs* 1401G mutation, compared to 89% in India and 92% in South Africa.

Kanamycin Resistance Detection upon *eis* Promoter Addition

Prior to the addition of *eis* promoter sequencing capabilities to the PyroMark platform, overall sensitivity of the PSQ assay was lowest for the overall detection of KAN resistance (50.4%) [12]. The addition of *eis* promoter mutations as predictors of KAN resistance increased the sensitivity estimate to 85.8%, but decreased overall specificity for KAN from 99.3% to 93.3%. In India, the addition of the *eis* promoter region to the assay increased test sensitivity for KAN resistance from 89% to 93%, but decreased test specificity from 100% to 91%. The addition of the *eis* promoter greatly increased test sensitivity for KAN resistance in Moldova, from 7% to 79%, but decreased test specificity from 99% to 95%. In South Africa, test sensitivity remained unchanged upon the addition of *eis* promoter sequencing capabilities, as no *eis* promoter mutations were identified in South African specimens.

Pyrosequencing Success by Smear and Culture Result

PSQ of the IS*6110*, *katG*, *inhA*, *ahpC*, *gyrA*, *rrs*, and two *rpoB* gene targets (regions outlined in Table 2.1) was performed on all samples, regardless of culture- and smear-status, at the respective clinical sites. Altogether, 9016 gene target

regions were pyrosequenced between the three sites. Overall, 86.7% of all smear-positive specimens and 86.4% of all culture-positive specimens yielded valid PSQ reads, while 54.9% of all smear-negative specimens and 43.1% of all culture-negative specimens gave valid sequence reads for the given gene targets. Figure 2.1 summarizes PSQ reaction success for each target gene region, stratified by smear- and culture-result. The IS*6110* gene marker had the highest frequency of successful PSQ reactions for all reactions, at 88%, followed by *inhA*, *katG*, *ahpC*, *rrs*, *gyrA*, *rpoB2*, and finally *rpoB1* at 85%, 83%, 81%, 80%, 71%, 69%, and 67%, respectively. In this study, 5493/6144 (89.4%) of PSQ reactions performed on culture- and smear-positive specimens and 500/1240 (40.3%) of PSQ reactions performed on culture- and smear-negative specimens gave valid sequencing results. For smear-negative, culture-positive samples, 821/1160 reactions (70.3%) provided useable sequence information, and for culture-negative, smear-positive samples, 234/464 (50.4%) reactions provided valid sequencing results.

Pyrosequencing Indeterminate Analysis

Results of the logistic regression analysis evaluating the variables associated with poor PSQ technical performance (PSQ indeterminate results) are displayed in Table 2.3. Multivariate logistic regression analysis showed that independent factors highly associated (adj OR > 2.0) with poor PSQ technical performance (indeterminate results) were: culture negativity (adj OR=7.74), sequencing of either of two *rpoB* gene targets (adj OR=5.29 and 4.65), sequencing of *gyrA* (adj OR=4.07), sequencing in a Moldovan (adj OR=2.86) site, sequencing of the *rrs* gene target (adj OR= 2.24), and AFB smear negativity (adj OR=2.19). Sequencing in a South African site and sequencing of any gene target other than the IS*6110* marker were also significantly

associated with increased odds of sequencing failures, adjusting for other covariates, though at lower levels than for the other variables (adj OR < 2.0).

The number and type of errors observed for each PSQ gene target are shown in Table 2.4. No read errors were the most commonly occurring error for any gene target (49-91% of all indeterminate PSQ reactions). The *gyrA* gene target had the highest percentage of no read errors (91%) of any gene target. Homopolymer errors were a common cause of PSQ indeterminate results for the *katG* target (40% of all indeterminate reactions). Instrument errors, mixed populations, and new mutations made up a minority of PSQ indeterminate calls for any gene target.

DISCUSSION

Our investigation into the diagnostic and technical performance of PSQ in the GCDD study demonstrated the following: 1) The PSQ assay showed differences in diagnostic performance between clinical sites, especially with regards to the sensitivity of the assay in detecting KAN resistance in Moldova, 2) As an open sequencing platform, new gene targets may be added to the PSQ assay to improve diagnostic performance and accommodate our evolving knowledge of the molecular basis of TB drug resistance, and 3) The current PSQ assay protocols may be further improved by optimizing primers and PCR and sequencing parameters for each gene target included in the assay in order to decrease the number of indeterminate PSQ results. As this PSQ assay has great potential to curb the spread of M/XDR-TB, and its performance has recently been validated in a large multisite study, these results have important implications for future assay use and performance in diverse clinical environments while highlighting key areas for assay optimization.

Pyrosequencing Diagnostic Performance between Sites

Differences in diagnostic sensitivity were noted for various drug compounds between the three clinical sites. South Africa showed lower sensitivity for the detection of INH resistance (71%, 95% CI 0.53-0.85) than India or Moldova, as the PSQ assay did not detect resistance-associated mutations in 25 of 35 phenotypically INH-resistant specimens evaluated in South Africa. This result suggests that these strains do not to have the expected *katG*, *inhA*, and/or *ahpC* mutations found in approximately 94% of INH-resistant strains, globally [23]. One reason for this discordance might be the failure of our assay to include additional gene regions associated with INH resistance, such mutations in the *fab*G1 gene or outside regions of *katG* [24, 25], including *katG* mutations at codons 139, 142, 269, 385, 387 and 541, recently associated with high INH minimum inhibitory concentrations (>10μg/mL) [25]. If any of these mutations are common in the South African population enrolled in our study, then it may be worthwhile to incorporate one or more of these gene regions into the next version of our PSQ assay. However, as only 35 phenotypically INH-resistant South African specimens were available for analysis, and the confidence intervals for the calculation included values as high as 85%, the low sensitivity estimate we observed may also have been an artifact of small sample size.

The PSQ assay also demonstrated lower sensitivity for the detection of RIF resistance in South Africa (77%, 95% CI 0.54-0.91) than in India. The assay did not detect mutations in five of the 22 phenotypically RIF-resistant samples evaluated in South Africa. As it is unlikely that these specimens lacked the resistance-associated *rpoB* mutations found in approximately 96% of all RIF-resistant strains [23], this result was also likely due to the small sample size of phenotypically RIF-resistant South African samples available for analysis, as the confidence intervals for this calculation

included values as high as 91%. However, it might also be worth investigating *rpoB* gene regions outside of those evaluated in this study, to ensure that no rare mutations are present in these samples in future studies.

For the detection of FQ resistance, PSQ demonstrated lower sensitivity in Moldova (64-67%, 95% CI 0.36-0.87) than in India. As 93% of all FQ-resistant strains have mutations in the *gyrA* gene region included in our assay [23], this result was lower than expected. However, our diagnostic sensitivity measures were in the range of those reported by Lacoma et al., who reported 40% sensitivity for detection of OFX resistance and 70.8% for the detection of MOX resistance for a PSQ assay including the same *gyrA* gene regions as our study, tested against strains from Spain and Lithuania [26]. Furthermore, only 14-15 phenotypically MOX- and OFX-resistant specimens were analyzed in Moldova, which may have led to a chance oversampling of specimens missing these common mutations. This possibility is reflected in the upper limits of the confidence intervals for this estimate, which include values as high as 87%. As with the detection of phenotypic INH and RIF resistance, although the point estimates for the sensitivity of the detection for phenotypic FQ resistance were lower in one clinical site, no significant differences could be confirmed based upon the spread of the confidence intervals surrounding those sensitivity estimates.

For the detection of injectable resistance, our PSQ assay showed lower sensitivity in Moldova than in India for all drugs. The sensitivity of the assay for the detection of CAP resistance in Moldova (40%, 95% CI 0.14-0.73) was lower than expected, as only four of the 10 phenotypically CAP-resistant specimens evaluated in this site were found to have the expected *rrs* 1401G mutation, previously documented to occur in 88% of CAP-resistant specimens, globally [23]. For the detection of AMK resistance in Moldova, the assay also demonstrated lower sensitivity (33%, 95% CI

0.11-0.65) than expected, as only four of 12 phenotypically AMK-resistant specimens were determined to have the *rrs* 1401G mutation found in approximately 84% of all AMK-resistant specimens, globally [23]. Although these discordances are likely related to the small sample size of AMK- and CAP-resistant specimens evaluated in Moldova, these observed discordances might also result from the failure of our assay to include additional gene regions associated with injectable resistance, such as the *rrs* 1484T mutation or *tlyA* mutations [27]. Indeed, other studies of tests relying upon the *rrs* 1401G mutation for AMK and CAP resistance detection have reported sensitivities as low as 57% [28], and so this result may be worth further investigation. There is also the possibility that *rrs* 1401 or 1402 mutations were present in the specimens, but were missed by our PSQ assay for some reason, which would call for a closer look into the ability of PSQ to accurately sequence this gene region. These specimens are currently being further evaluated by whole genome sequencing to identify the molecular basis of phenotypic injectable resistance. By far, however, the most notable difference in observed sensitivity for any injectable between the sites was for the detection of KAN resistance in Moldova, where only 7% (95% CI 0.02-0.18) of the 57 phenotypically KAN-resistant specimens were found to have the *rrs* 1401G mutation, versus 89% in India and 92% in South Africa.

Diagnostic Performance Following *eis* Promoter Addition

In Moldova, a high number of specimens showed resistance to KAN but not to the other injectable compounds (AMK and CAP). This fact is unsurprising, as kanamycin was widely used for TB treatment in the former Soviet Union, selecting for resistance to this compound [29, 30]. The high number of KAN-resistant *Mtb* clinical specimens without *rrs* mutations in Moldova (n=53) suggested that other genes or

gene regions were involved in conferring KAN resistance in this site. Upon the addition of *eis* promoter sequencing capability to the PSQ assay, a dramatic change in sensitivity for KAN resistance detection was observed in Moldova (7% to 79%), confirming the role of *eis* promoter mutations in conferring KAN resistance in this population. Notably, the addition of the gene region to the assay in India also resulted in a sensitivity improvement for the detection of KAN resistance (89% to 93%). However, the improved sensitivity came at a loss to assay specificity in both sites for KAN resistance detection, due to the presence of *eis* promoter mutations in KAN-susceptible specimens. In order to comment upon this discrepancy, 15 KAN-susceptible Indian specimens confirmed to have *eis* promoter mutations were subjected to repeat phenotypic KAN DST at the critical concentration (2.5μg/mL). All DST reactions were run in duplicate. Eleven of the 15 specimens (73%) showed a resistant phenotype in at least one of the two duplicate DST runs, but five of these results were mixed (one run being susceptible, the other resistant). Four discrepant specimens were KAN susceptible in both DST runs. These results underscore the fact that mutations in this gene region should to be studied further to quantitate their association with phenotypic KAN resistance, especially as these mutations confer only low-level KAN resistance, which may or may not be picked up by phenotypic DST at just one critical concentration (2.5μg/mL, in our study) [31]. Although these results suggest a reexamination of the critical concentration to establish KAN phenotypic resistance, the addition of this gene region into our assay confirms the adaptability of our molecular diagnostic platform for diverse clinical environments.

Pyrosequencing Technical Performance across Sites

A current limitation of PSQ as an M/XDR-TB diagnostic is its high rate of sequencing failure. In our study, 25% of all sequencing reactions failed to generate an interpretable sequencing read [12], with results varying by specimen smear- and culture-status. The variable most highly associated with poor PSQ technical performance was culture negativity, over AFB smear negativity. This difference is unsurprising as culture is a more sensitive test for *Mtb* compared to AFB smear. However, over 40% of sequencing reactions conducted on smear- and culture-negative samples still yielded sequencing results. Although culture-negative samples are generally considered to be samples in which the *Mtb* bacteria is not present, it is likely that our assay was indeed detecting *Mtb* DNA, as the primers designed for our assay are highly specific for *Mtb*. This DNA may have come from dead *Mtb* bacteria present in the samples, which is likely seen when processing samples from patients previously treated for TB infections.

The ability of our assay to sequence a large portion of AFB smear-negative *Mtb* clinical specimens underscores the utility of this molecular diagnostic for a diverse range of clinical samples. Indeed, our 70.8% sequencing success for smear-negative, culture-positive specimens is better than reported for the GeneXpert assay (55%) [32]. As many laboratories lack the sterile conditions or equipment necessary to perform AFB smear, culture and DST of *Mtb*, and a large portion of TB infections remain smear-negative despite clinical and radiological signs of disease, PSQ presents a valid alternative to conventional growth-based diagnostic methods [33]. Although the presence of *Mtb* DNA does not necessarily confirm the presence of viable bacteria in a sample (as with culture-negative samples), PSQ can potentially provide the clinician with information about a portion of smear-negative infections when a diagnosis is otherwise elusive, as long as the results are considered in the

context of the patient's clinical presentation and past and current TB treatment regimens [34].

In addition to culture and smear result, the proportion of interpretable sequencing results in our study appeared to vary significantly by gene. Interestingly, after culture negativity, sequencing of the *rpoB* gene target in either one of two sequencing reactions was the variable with the highest adjusted odds of sequencing failure. Poor *rpoB* sequencing success is likely a result of the higher order DNA structures present in the *Mtb* genomic DNA at this gene region, preventing DNA access and therefore resulting in PCR and/or sequencing failure [35]. Although these higher order DNA structures are inherent to the *rpoB* gene, their presence might be addressed by altering PCR and/or sequencing reaction conditions, such as increasing the melting and extension temperatures during PCR or introducing reaction additives to prevent the formation of such structures. Additionally, the two *rpoB* sequencing products were the longest in this study. This factor appeared to contribute to the occurrence of indeterminate results by increasing the number of unresolvable homopolymer errors seen in these reactions [24]. Increasing the number of sequencing reactions for a given target, thereby shortening the length of the sequencing products, may rectify any issues related to gene target sequencing length such as seen with the *rpoB* gene targets. Sequencing of the *gyrA* target had the next highest odds of indeterminate results. The high number of indeterminate results seen for *gyrA* target sequencing reactions appeared to result from amplification errors, or primer hybridization during PCR, as the majority of *gyrA* sequencing errors were characterized as no read errors. This factor may be addressed in future versions of the PSQ assay by redesigning the *gyrA* PCR primers for this reaction. The *gyrA* gene is also a G/C rich gene in the *Mtb* genome, meaning that the PCR primers designed

for this gene region may show performance improvements following PCR parameter adjustments. The variable with the next highest odds of sequencing failure was sequencing in Moldova. This association is likely tied to observed clinical laboratory factors affecting PCR and PSQ in Moldova versus other sites, such as long delays between DNA extraction, PCR and sequencing. In our study, the Moldovan site was known to batch *Mtb* samples more than the other two sites, performing PSQ only once a week- a factor that may explain technical performance differences between the sites. A final gene target highly associated with indeterminate results was the *rrs* gene. The majority of *rrs* indeterminate results were classified as no read errors, indicating potential for PCR and sequencing optimization, similar to the *gyrA* target. This analysis highlights important areas for assay technical performance improvement, and many of these problematic gene targets may be easily optimized in future versions of the assay.

CONCLUSIONS

Although our PSQ assay was generally a high performing M/XDR-TB diagnostic across three diverse clinical environments, notable reductions in sensitivity were identified between the three sites, especially for KAN resistance detection in Moldova. The flexibility of the PSQ assay allowed us to quickly update the platform when this performance lapse was identified, improving assay sensitivity for KAN resistance detection. Additionally, we found the PSQ assay to generate data for a large proportion of smear-negative samples, comparable to GeneXpert, and our analysis of the additional variables associated with poor PSQ technical performance highlighted gene targets for optimization to further improve the assay's technical performance. These results have important implications for the use and interpretation

of PSQ assays as M/XDR-TB diagnostics, and may serve to inform other molecular M/XDR-TB diagnostics that interrogate similar gene targets in clinics across the globe.

DECLARATIONS

Ethical Approval and Consent to Participate

Clinical sites for the study included P.D. Hinduja National Hospital in Mumbai, India, the Phthisiopneumology Institute in Chisinau, Moldova, and multiple primary care clinics in Port Elizabeth, South Africa in collaboration with Stellenbosch University. Patients were treated at both public and private hospitals and clinics depending on study site. Our study, registered with ClinicalTrials.gov (#NCT02170441), was reviewed and approved by institutional review boards at University of California, San Diego and each of the study sites. All study participants provided written informed consent.

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Chapter 2, “Shedding Light on the Performance of a Pyrosequencing Assay for Drug-resistant Tuberculosis Diagnosis,” has been prepared and submitted for publication. Marva Seifert, Shou-Yean Lin, Donald Catanzaro, Richard Garfein, Lynn Jackson, Valeriu Crudu, Camilla Rodrigues, Tommie Victor, Antonino Catanzaro, and Timothy Rodwell are co-authors. Sophia Georghiou, the dissertation author, is the primary author of this material.

TABLES AND FIGURES

Table 2.1: Primers utilized in PCR and pyrosequencing reactions.

Genes	Target	Forward primer	Reverse primer	Sequencing primer	Detection range	Reference
ID of Mtb	IS6110	Biotin-CCGCCAACT	CAGGCCGAGTTTGC	GGCCACCTCGATGC	Multiple	[11]
Isoniazid-R	katG	Biotin-CGGAACCGC	CCATTTCGTCGGGG	TCCATACGACCTCG	Codons 312 to 316	[11]
	inhA	Biotin-ACGCTCGTG	CAGTGGCTGTGGCA	TGTGGCAGTCACCC	Position -4 to -20	[11]
	ahpC	TCCTCATCATCAAA	Biotin-CGATGCCGA	CATTTGGTTGCGAC	Position -4 to -23	[6, 11]
Rifampin-R	rpoB1	GGAGGCGATCACA	Biotin-CCTCCAGCC	GCGATCAAGGAGT	Codons 507 to 521	[6, 11]
	rpoB2	TTTCGATCACACCG	Biotin-AAAGGCAC	CAGAACAACCCGCT	Codons 522 to 533	[6, 11]
Fluoroquinolone-R	gyrA	AATGTTCGATTCCG	Biotin-CGGGCTTCG	CAACTACCACCCGC	Codons 88 to 95	[2]
Injectable-R	rrs	TAAAGCCGGTCTCA	Biotin-CAGCTCCCT	CTTGTACACACCGC	Position 1397 to 14	[11]
	eis-pt	Biotin-GGCTACACA	GCCAGACACTGTCG	CAGACACTGTCGTC	Position -5 to -47	This study

Mtb, *Mycobacterium tuberculosis*
-R, -resistant

Table 2.2: Pyrosequencing diagnostic performance by clinical site.

	INDIA (n=492)			MOLDOVA (n= 226)			SOUTH AFRICA (n= 196)		
	Sensitivity	Specificity	Agreement	Sensitivity	Specificity	Agreement	Sensitivity	Specificity	Agreement
INH	98% (0.96-0.99)	97% (0.90-1)	98% (0.96-0.99)	94% (0.88-0.97)	96% (0.87-0.99)	95% (0.90-0.97)	71% (0.53-0.85)	94% (0.87-0.98)	88% (0.81-0.93)
RIF	98% (0.95-0.99)	100% (0.94-1)	98% (0.96-0.99)	94% (0.86-0.98)	100% (0.92-1)	97% (0.92-0.99)	77% (0.54-0.91)	98% (0.91-1)	94% (0.87-0.97)
MOX	96% (0.92-0.98)	96% (0.92-0.98)	96% (0.94-0.98)	67% (0.39-0.87)	100% (0.97-1)	97% (0.92-0.99)	82% (0.48-0.97)	99% (0.96-1)	98% (0.94-1)
OFX	96% (0.93-0.98)	99% (0.96-1)	97% (0.95-0.99)	64% (0.36-0.86)	99% (0.96-1)	96% (0.91-0.98)	90% (0.54-0.99)	99% (0.96-1)	99% (0.95-1)
AMK	94% (0.82-0.98)	100% (0.98-1)	99% (0.98-1)	33% (0.11-0.65)	99% (0.96-1)	95% (0.91-0.98)	92% (0.60-1)	98% (0.94-0.99)	98% (0.93-0.99)
KAN	89% (0.76-0.95)	100% (0.98-1)	99% (0.97-0.99)	7% (0.02-0.18)	99% (0.95-1)	71% (0.64-0.77)	92% (0.60-0.1)	98% (0.94-0.99)	97% (0.94-0.99)
KAN (+*eis*)	93% (0.81-0.98)	91% (0.88-0.94)	91% (0.88-0.94)	79% (0.66-0.88)	95% (0.90-0.98)	90% (0.85-0.94)	92% (0.60-1)	98% (0.93-0.99)	97% (0.93-0.99)
CAP	94% (0.81-0.98)	99% (0.98-1)	99% (0.97-0.99)	40% (0.14-0.73)	99% (0.96-1)	96% (0.92-0.98)	85% (0.54-0.97)	98% (0.94-0.99)	97% (0.92-0.99)

INH, isoniazid
RIF, rifampicin
MOX, moxifloxacin
OFX, ofloxacin
AMK, amikacin
KAN, kanamycin
CAP, capreomycin

Table 2.3: Multivariate logistic regression model of variables associated with poor pyrosequencing technical performance.

Variable	Crude OR	95% CI	Adjusted OR	95% CI
AFB Smear				
Negative	**5.30**	4.76-5.90	**2.19**	1.91-2.51
Positive	1.00		1.00	
Culture				
Negative	**8.44**	7.51-9.49	**7.74**	6.67-8.99
Positive	1.00		1.00	
Site				
Moldova	**2.07**	1.84-2.34	**2.86**	2.47-3.31
South Africa	**1.86**	1.64-2.10	**1.50**	1.29-1.74
India	1.00		1.00	
Target				
katG	**1.52**	1.19-1.92	**1.69**	1.29-2.21
inhA	**1.30**	1.02-1.66	**1.38**	1.05-1.81
ahpC	**1.71**	1.35-2.16	**1.96**	1.50-2.55
rpoB1	**3.65**	2.93-4.55	**5.29**	4.12-6.79
rpoB2	**3.31**	2.65-4.12	**4.65**	3.62-5.97
gyrA	**2.99**	2.39-3.73	**4.07**	3.16-5.23
rrs	**1.90**	1.50-2.39	**2.24**	1.72-2.91
IS6110	1.00		1.00	

Table 2.4: Indeterminate pyrosequencing results: number and type of errors by gene target.

TARGET	No Sequence Error	Homopolymer Error	Instrument Error	Other Error	Mixed Population	New Mutation	TOTAL	Error Rate (/1128 reactions)
IS6110	112 (85%)	12 (9%)	4 (3%)	4 (3%)	-	-	132	11.7%
katG	91 (49%)	74 (40%)	1 (1%)	19 (10%)	1 (1%)	-	186	16.5%
inhA	121 (73%)	9 (5%)	3 (2%)	29 (17%)	2 (1%)	2 (1%)	166	14.7%
ahpC	182 (87%)	13 (6%)	6 (3%)	9 (4%)	-	-	210	18.6%
rpoB1	221 (61%)	76 (21%)	15 (4%)	45 (12%)	1 (0%)	3 (1%)	361	32.0%
rpoB2	269 (78%)	40 (12%)	7 (2%)	28 (8%)	-	1 (0%)	345	30.6%
gyrA	294 (91%)	2 (1%)	6 (2%)	18 (6%)	1 (0%)	1 (0%)	322	28.5%
rrs	149 (66%)	30 (13%)	-	48 (21%)	-	-	227	20.1%

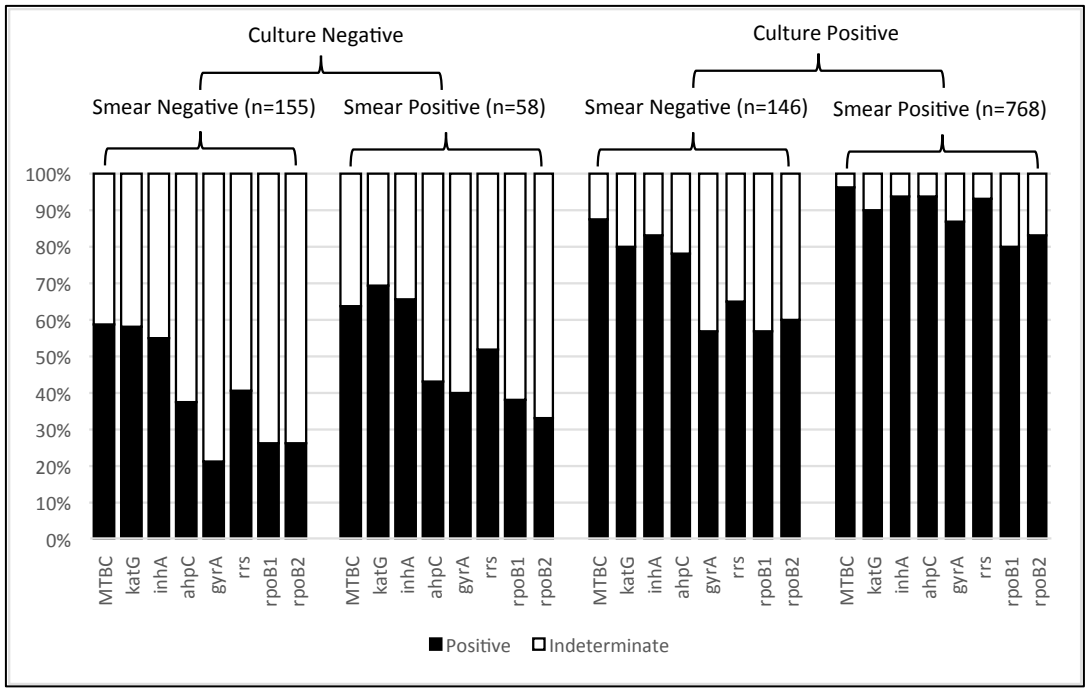

Figure 2.1: Pyrosequencing technical performance (sequencing success) by acid-fast bacilli smear and culture result.

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CHAPTER 3: FREQUENCY AND DISTRIBUTION OF TUBERCULOSIS RESISTANCE-ASSOCIATED MUTATIONS BETWEEN MUMBAI, MOLDOVA, AND EASTERN CAPE

ABSTRACT

Background: Molecular diagnostics, with their ability to rapidly detect resistance-associated mutations in bacterial genes, are promising technologies to control the spread of drug-resistant tuberculosis (DR-TB). Sequencing assays provide detailed information for specific gene regions and can help diagnostic developers prioritize mutations for inclusion in their assays.

Methods: We performed pyrosequencing of seven Mycobacterium tuberculosis gene regions (*katG*, *inhA*, *ahpC*, *rpoB*, *gyrA*, *rrs*, and *eis*) for 1128 clinical specimens from India, Moldova, and South Africa. We determined the frequencies of each mutation among drug-resistant and -susceptible specimens based on phenotypic drug susceptibility testing results and examined mutation distributions by country.

Results: The most common mutation among isoniazid-resistant (INHR) specimens was *katG* 315ACC (87%). However, in the Eastern Cape, INHR specimens had a lower frequency of *katG* mutations (44%) and a higher frequency of *inhA* (47%) and *ahpC* (10%) promoter mutations. The most common mutation among rifampicin-resistant (RIFR) specimens was *rpoB* 531TTG (80%). The mutation was common in RIFR specimens in Mumbai (83%) and Moldova (84%), but not the Eastern Cape (17%), where the 516GTC mutation appeared more frequently (57%). The most common mutation among fluoroquinolone-resistant specimens was *gyrA* 94GGC (44%). The *rrs* 1401G mutation was found in 84%, 84%, and 50% of amikacin,

capreomycin, and kanamycin-resistant (KANR) specimens, respectively. The *eis* promoter mutation -12T was found in 26% of KANR and 4% of KANS specimens. **Conclusions:** Inclusion of the *ahpC* and *eis* promoter gene regions was critical for optimal test sensitivity for detection of INH resistance in the Eastern Cape and KAN resistance in Moldova.

INTRODUCTION

In 2014, an estimated 9.6 million people developed tuberculosis (TB) and 1.5 million people died of their infection [1]. Although global TB incidence rates have fallen an average of 1.5% per year since 2000, the rise of drug-resistant TB (DR-TB) globally has complicated TB control efforts [1]. The World Health Organization (WHO) estimates that as many as one in every 20 new, active TB infections is now drug-resistant [1]. One of the major roadblocks in combating this growing problem has been the lack of diagnostic technology for DR-TB. Current growth-based culture and drug susceptibility testing (DST) methods can take several weeks to months to yield results [2]. While waiting on culture results, physicians are forced to treat their patients empirically, only adjusting treatment regimens once DST results become available. As a result, many undiagnosed DR-TB patients are being given medications that are ineffective, which amplifies resistance, increases the risk of mortality and increases the risk of transmitting DR-TB infections in the community.

Rapid molecular diagnostics for DR-TB have the potential to curb this spread of resistance by shortening the time to TB diagnosis and effective treatment. These technologies identify and characterize DR-TB infections based upon the presence or absence of known resistance-conferring mutations in the *Mycobacterium tuberculosis* (*Mtb*) genome [3-5]. Unfortunately, the vast majority of rapid molecular DR-TB diagnostic tests, including line probe and microarray assays, rely on a closed set of mutations for resistance detection [6-8]. The decision of which mutations to include in these assays is generally based upon the global frequencies of known resistance-associated mutations and the strength of association between these mutations and phenotypic drug resistance to corresponding anti-tuberculosis drugs of interest. The relationship between phenotypic drug resistance and mutation status is not always

100%, however, and although recent systematic reviews have given us a better idea of the relationship between particular mutations and phenotypic drug resistance [9-12], data is still lacking for rare and novel mutations. Furthermore, little is known about regional distributions of TB resistance-associated mutations, which could affect the performance of molecular diagnostics implemented globally.

Global studies providing phenotypic as well as complete genotypic sequence information for DR-TB clinical specimens are necessary in order to further inform the development of molecular diagnostic assays. Unlike most rapid DR-TB molecular diagnostics, such as the line probe assays and real time amplification-based assays, sequencing assays yield long sequencing reads, allowing for the detailed genetic analysis of diverse clinical specimens. Sequencing technologies have the additional advantage of being open assays, meaning that they can be easily modified to accommodate our evolving knowledge of the genetic basis of TB phenotypic drug resistance. The Global Consortium for Drug-resistant Tuberculosis Diagnostics (GCDD) conducted a large, multisite study evaluating the diagnostic performance of a modified pyrosequencing diagnostic for DR-TB in three diverse clinical environments [13], and in doing so generated sequencing data for epidemiologically different populations of DR-TB patients. This study presents the frequency and distribution of all identified resistance-associated mutations and considers the implications of these findings on the expected performance of rapid molecular diagnostics in diverse clinical environments.

MATERIALS AND METHODS

Study Population

Three epidemiologically diverse clinical sites (Chisinau, Moldova; Port Elizabeth, South Africa; and Mumbai, India) were selected for this study. In India, patients were enrolled at P.D. Hinduja Hospital and Medical Research Centre, the main DR-TB referral center for the city of Mumbai and state of Maharashtra. In Moldova, TB patients were enrolled in four Regional TB Hospitals: two in Chisinau, one in Vorniceni and one in Balti. All patient samples were processed at the Phthisiopneumology Institute in Chisinau, a scientific research, medical consultation, and training center that is the central unit of the Moldovan National TB Control Program. In Port Elizabeth, patients were enrolled at one regional hospital and six Primary Health Care facilities spread throughout the region. Newly-presenting TB patients over five years of age were eligible for the study if they were known to be acid-fast bacilli smear-positive or suspected of having active pulmonary TB and having one or more reason to be considered to have DR-TB, and provided informed consent for the study. Of the eligible patient population, 52 patients were excluded for inability to provide 7.5mL of sputum (n=35) or for other or unknown reasons (n=17). A total of 1128 patients with risk factors for DR-TB were enrolled from April 24, 2012 to June 27, 2013 [14].

Drug Susceptibility Testing

Mycobacterial Growth Indicator Tube (MGIT) 960 cultures were performed. MGIT DST results served as the phenotypic reference standard in our study. All specimens were tested for resistance to isoniazid (INH), rifampicin (RIF), moxifloxacin (MFX), ofloxacin (OFX), amikacin (AMK), kanamycin (KAN), and capreomycin (CAP) using standard manufacturer protocols and critical concentrations recommended by the WHO and as published previously for MGIT-based drug susceptibility testing at

the time of our study: 0.1 µg/ml for INH, 1.0 µg/ml for RIF, 2.0 µg/ml for OFX, 0.25 µg/ml for MFX, 1.0 µg/ml for AMK, 2.0 µg/ml for CAP [15, 16], and 2.5 µg/ml for KAN [17, 18]. For the purposes of this analysis, specimens resistant to either MFX or OFX via MGIT DST were considered fluoroquinolone (FQ)-resistant. Specimens that were not phenotypically resistant to MFX and OFX were considered FQ-susceptible.

DNA Extraction, Molecular Targets and PCR

DNA was extracted from each decontaminated, concentrated sputum (sediment) by heating the cell suspensions in a water bath at 100°C [14, 19]. Our pyrosequencing assay included eight reactions: one to identify *Mtb* and seven to detect mutations in drug resistance-associated gene regions. All primers used in this study, other than the *rrs* primers, are specific for *Mtb* and do not show cross-reactivity with other TB species [19]. We associated INH resistance with mutations in the *ahpC* promoter (position -4 to -23), the *inhA* promoter (position -4 to -20), and *katG* (codons 312 to 316). RIF resistance was associated with mutations within *rpoB* (codons 507 to 533); FQ resistance with mutations other than the natural polymorphism 95ACC in *gyrA* (codons 88 to 95) [20]; and resistance to the injectable drugs (KAN, AMK, and CAP) with mutations in the *rrs* (position 1397 to 1406). PCR primers for these gene regions have been previously described [19]. Upon completion of this study, *eis* promoter (position -5 to -47) sequencing capability was added to our platform, ensuring specificity for *Mtb* via hybridization analysis for cross-reactivity to other TB species. Mutations in the *eis* promoter, in addition to *rrs* mutations, were associated with KAN resistance [21]. PCR master mixes were prepared and amplification reactions were carried out for all targets as previously reported [19].

Pyrosequencing

Pyrosequencing was performed according to standard manufacturer procedures and modified for sequencing of mutations associated with DR-TB, as described previously [4, 19]. We utilized the PyroMark Q96 ID system (Qiagen, Valencia, CA) to perform pyrosequencing on the nine targets detailed above, sequencing two parts of *rpoB* in two separate reactions. Variants were identified automatically following pyrosequencing using IdentiFire software (Qiagen, Valencia, CA) [12, 22]. Samples that did not provide sequencing queries that 100% matched library sequences were repeated in duplicate. Samples that still did not provide confirmatory sequence, and samples for which contradictory hits were obtained, were deemed genotypically indeterminate.

Cumulative Mutation Frequencies

Cumulative mutation frequencies were established for every mutation identified in our study across all clinical sites. Mutation frequencies were determined for all relevant drug-resistant and -susceptible specimens for which sequence data was obtained. For each gene region of interest (i.e. “only *katG*”), only those specimens with both a relevant phenotypic DST result and a sequencing result for the given region(s) of interest were included in mutation sensitivity and specificity calculations.

Mutation Distributions between Clinical Sites

Site-specific mutation frequencies were also established for every mutation identified in our study. The number of times a mutation appeared in relevant drug-resistant and -susceptible specimens was summarized for each gene region of

interest for each clinical site. As with cumulative mutation frequencies, only those mutations with both sequencing data and a phenotypic DST result for each relevant drug were considered when establishing site-specific mutation frequencies. Mutation frequencies among drug-resistant specimens between the different clinical sites are presented as bar graphs.

Human Research Conduct

Our study, registered with ClinicalTrials.gov (#NCT02170441), was reviewed and approved by the Institutional Review Board of the University of California, San Diego and by the Institutional Review Boards of the participating institutions at the three study sites. All participants provided written informed consent. Participation did not alter the standard of care.

RESULTS

Drug Susceptibility Testing Results

Nine hundred fourteen (81%) of the 1128 patients enrolled in the study provided *Mtb* culture-positive pulmonary sputum samples. Of these 914 samples, 768 (84%) were smear positive. Of the remaining 214 samples, one was culture-contaminated and 213 were *Mtb* culture-negative. Seven of the 914 culture-positive samples either did not have MGIT DST performed or did not yield results for any of the anti-tuberculosis drugs evaluated. Of the original 1128 patients, 454 (40%) had multidrug-resistant TB (MDR-TB) and 80 (7%) had extensively drug-resistant TB (XDR-TB) (data not shown).

Cumulative Mutation Frequencies

Cumulative mutation frequencies are presented in Tables 3.1-3.4.

Isoniazid (INH) Resistance-Associated Mutations. The *katG* 315ACC mutation was the most common INH resistance-associated mutation identified in this study. This mutation was found in 480 specimens across all sites, including 139 (29%) co-occurrences with *inhA* or *ahpC* promoter mutations. Overall, the 315ACC mutation was found in 473 (87%) INH-resistant (INH^R) and seven (3%) INH-susceptible (INH^S) specimens. Within the *inhA* promoter, the -15T mutation was most commonly identified, appearing in 135 specimens: 133 (24%) INH^R specimens and two (1%) INH^S specimens. The -15T mutation co-occurred with *katG* or *ahpC* promoter mutations in 110 (81%) of these 135 specimens. Within the *ahpC* promoter, the -10A mutation was most commonly identified, appearing in seven (1%) INH^R specimens, including four co-occurrences with *katG* or *inhA* promoter mutations. Twenty-six (5%) of the total 516 INH^R specimens with sequencing reads for all three gene regions were found to be wildtype. The mutations we identified in the *katG*, *inhA* promoter, and *ahpC* promoter gene regions sequenced in this study explained 95% of phenotypic INH resistance across the three clinical sites.

Rifampicin (RIF) Resistance-Associated Mutations. The *rpoB* 531TTG mutation was the most common RIF resistance-associated mutation identified in this study, appearing in 360 (80%) RIF^R specimens across all sites. Fourteen (4%) of the total 389 RIF^R specimens with sequencing reads for both *rpoB* gene regions were found to be wildtype. Interestingly, *rpoB* mutations were also identified in 10 RIF^S specimens (Table 3.2). Together, all of the mutations identified in the *rpoB* gene region

encompassing codons 507 to 533 helped to explain 97% of phenotypic RIF resistance in our study.

Fluoroquinolone (FQ) Resistance-Associated Mutations. Within the *gyrA* gene, the 94GGC mutation was the most common resistance-associated mutation, identified in 121 (44%) FQ^R specimens and one (0%) FQ^S specimen. Eighteen (7%) of the 278 FQ^R specimens with *gyrA* sequencing reads were wildtype or only contained the 95ACC mutation. Altogether, the resistance-associated *gyrA* mutations identified in this study explained 94% of phenotypic FQ resistance.

Injectable Resistance-Associated Mutations. The *rrs* 1401G mutation was identified in 84%, 50%, and 84% of AMK^R, KAN^R, and CAP^R specimens, respectively, and 1% of all injectable-susceptible specimens. The C-12T mutation was the most common mutation in the *eis* promoter, occurring in 37 (26%) KAN^R specimens and 31 (4%) KAN^S specimens. Eleven to twelve (16%) of the total 70-73 AMK^R and CAP^R specimens with *rrs* sequencing reads were wildtype, while 18 (15%) of the total 119 KAN^R specimens with *rrs* and *eis* sequencing reads were wildtype. After inclusion of the *eis* promoter gene target, the combined sensitivity of all identified resistance-associated mutations in the seven *Mtb* gene regions sequenced in this study was 84-97% for DR-TB drugs.

Mutation Distributions

Isoniazid (INH) Resistance-Associated Mutations. Differences were noted in the frequencies of mutations associated with INH resistance between the three clinical sites (Figure 3.1). The *katG* 315ACC mutation was present in 89% of INH^R samples

in India and 92% of INH^R samples in Moldova, but only 44% of the INH^R specimens in South Africa. A greater percentage of South African INH^R specimens had mutations in the *inhA* (47%) or *ahpC* promoter (10%) gene regions than seen in India or Moldova. Although Moldova had a large number of INH^R specimens with the *inhA* -15T mutation (46%), almost all co-occurred with a *katG* 315 mutation.

Rifampicin (RIF) Resistance-Associated Mutations. Differences were also observed in the frequencies of mutations associated with RIF resistance between the three sites (Figure 3.2). The *rpoB* 531TTG mutation was present in 83-84% of RIF^R specimens in India and Moldova, but only 17% of RIF^R specimens in South Africa. Instead, the *rpoB* 516GTC mutation was found more frequently (57%) among RIF^R specimens in South Africa.

Fluoroquinolone (FQ) Resistance-Associated Mutations. Unlike the mutations associated with INH or RIF resistance, none of the *gyrA* resistance-associated mutations identified in our study were found in more than 50% of the FQ^R specimens in any clinical site (Figure 3.3). The 94GGC *gyrA* mutation was the most frequent mutation identified among FQ^R specimens in India (46%) and South Africa (46%), but the mutation was only identified in 6% of FQ^R specimens in Moldova. Instead, the 90GTG (19%), 94GCC (19%) and 91CCG (13%) mutations were more commonly identified among the FQ^R specimens evaluated in Moldova. The 90GTG mutation was also identified in 66 (26%) FQ^R specimens in India.

Injectable Resistance-Associated Mutations. The *rrs* 1401G mutation showed notable differences in its frequency among injectable-resistant specimens between the clinical

sites (Figure 3.4). The mutation appeared in 85-94% of the injectable-resistant specimens evaluated in India and South Africa, although it was less common in Moldova, appearing in 33%, 7%, and 40% of Moldovan AMK^R, KAN^R and CAP^R specimens, respectively. Mutations in the *eis* promoter were more common than *rrs* mutations among the KAN^R specimens evaluated in Moldova. The *eis* promoter -12C/T mutation was found in 37 (53%) KAN^R specimens in Moldova. The -14C/T, -10G/A and -37G/T mutations were also identified in eight (11%) KAN^R specimens in Moldova. These *eis* promoter mutations were also found in India (nine specimens), although they appeared in a mix of KAN^R and KAN^S specimens. The -15C/G mutation was identified in one (2%) KAN^R specimen in India. Notably, no *eis* promoter mutations were found in any South African specimens in this study and no *eis* promoter mutations co-occurred with *rrs* mutations.

DISCUSSION

Large-scale sequencing studies remain critical for DR-TB molecular diagnostic development as they enable assay developers to prioritize resistance-associated mutations for optimal diagnostic performance and to predict diagnostic assay performance globally. We conducted a large, multisite DR-TB sequencing study to determine the frequencies of all significant resistance-conferring mutations across three diverse clinical sites, and characterized differences in the distribution of the mutations between the sites. Notably, inclusion of the *ahpC* and *eis* promoter gene regions was found to be critical for optimal assay sensitivity for INH resistance detection in the Eastern Cape and KAN resistance detection in Moldova.

Isoniazid (INH) Resistance-Associated Mutations

The most common *katG*, *inhA*, and *ahpC* promoter mutations in our study were identified at frequencies similar to previously reported findings [11, 23, 24]. However, our finding of 315ACC mutations among INH^S specimens (2.8%) was unexpected. The *katG* 315ACC mutation has been associated with an INH MIC of 3 to >16µg/mL by liquid media DST [25-28], and was shown to confer INH resistance at 5µg/mL in an allelic exchange study [29]. Therefore, it is highly unlikely that any *Mtb* specimens with the *katG* 315ACC mutation would have an INH MIC below the critical concentration used in this study (0.1µg/mL). The seven discordant results observed were likely false positives, resulting from PSQ failure rather than DST error. Of the *inhA* promoter mutations identified in our study, only the -15T and -17T mutations were identified in INH^S specimens, both at low frequencies of 0.7%. This finding is slightly higher than reported global frequencies of 0.0-0.3% [11], but *inhA* promoter mutations generally convey lower levels of resistance to INH than *katG* mutations (MICs range from 0.1 to >16.0µg/mL) [30-32], and so it is possible that the four study specimens with these mutations had MIC values close to the INH critical concentration and were interpreted as INH^S by liquid culture. Although *inhA* and *ahpC* mutations occurred independently in a small proportion (1-6%) of INH^R specimens evaluated, they nonetheless contributed to the overall prediction of phenotypic INH resistance, supporting their inclusion as molecular markers of INH resistance in our diagnostic assay.

Important differences were seen in the distribution of INH resistance-associated mutations between the clinical sites. Although *ahpC* promoter mutations were only identified in a low proportion (3.1%) of the INH^R specimens in our study, these mutations appeared in a substantial proportion (10.5%) of the INH^R specimens in the Eastern Cape. A previous study conducted in KwaZulu-Natal found a similar

frequency of *ahpC* promoter mutations among INH^R isolates (12.6%), though all co-occurred with *katG* mutations [33]. In contrast, our study identified many *ahpC* mutations without co-occurring *katG* or *inhA* promoter mutations. Previous studies have suggested that the selection of *ahpC* mutations occurs only after the accumulation of *katG* mutations [34], yet our study finds *ahpC* mutations to be independent markers of resistance in different patient populations, similar to the findings of Silva et al. [24] Despite *ahpC* promoter mutations being rare globally (5.4% of all INH^R specimens) and often co-occurring with *katG* mutations [11, 34], they may play a significant role in explaining regional phenotypic INH resistance patterns. If these mutations are excluded from molecular diagnostic tests, then these tests may experience significant decreases in sensitivity in certain geographical regions. The inclusion of the *ahpC* promoter in our assay in the Eastern Cape, for example, was critical to our detection of INH resistance in that region. This finding also has important implications for the performance of other DR-TB molecular diagnostics, such as the Hain MTBDR*plus* line probe assay, which does not include the *ahpC* promoter mutations [35]. If this assay were used to diagnose INH resistance in our South African study population, approximately 8.6% of INH^R strains would have been missed without the addition of *ahpC* promoter mutations. Adding this gene target to DR-TB molecular diagnostic assays could improve assay sensitivity for INH resistance detection both regionally and globally.

Rifampicin (RIF) Resistance-Associated Mutations

The most common *rpoB* mutations identified, 531TTG (80.0%) and 516GTC (5.2%), appeared across study sites at frequencies comparable to those previously reported for a set of RIF^R *Mtb* isolates in a multisite study (68.8% and 6.8%) [4]. All

other mutations in the 81bp *rpoB* RIF resistance-determining region appeared to explain a smaller (14.8%) but still significant proportion of RIF resistance in this study, confirming that the inclusion of this entire gene region in rapid molecular diagnostics is important to best predict phenotypic RIF resistance. Interestingly, resistance-associated *rpoB* mutations were also identified in 10 RIF^S specimens, suggesting that they were poor predictors of phenotypic RIF resistance, as opposed to previous findings [36]. This discrepancy is likely related to the complexities of the liquid culture-based DST we used to determine RIF resistance for these low MIC mutants. Liquid and solid media DST methods both use WHO-endorsed critical concentrations of RIF that are ideally equivalent, however, for these particular *rpoB* mutations resulting in low MIC RIF resistance, the result is consistently RIF^S by liquid (MGIT960) and RIF^R by solid media. Therefore, these mutants likely have MICs near the WHO-recommended RIF critical concentration established for liquid DST [37, 38]. It is critical to understand the relationship between these mutations and the levels of phenotypic resistance they confer in order to accurately interpret of the results of molecular diagnostic assays that rely upon these genetic markers to predict phenotypic RIF resistance.

The frequency of the *rpoB* 531TTG mutation was much higher in India (83%) and Moldova (84%) than in the Eastern Cape (17.4%). The inclusion of the 516GTC mutation (56.5% of RIF^R specimens in the Eastern Cape) appeared to be more important for molecular test performance in this region. Most molecular diagnostic assays, including the Hain MTBDR*plus* and Cepheid GeneXpert MTB/RIF assays, include the 81bp RIF resistance-determining region of the *rpoB* gene region sequenced in this study and therefore would be expected to detect all major mutations we identified between the three sites.

Fluoroquinolone (FQ) Resistance-Associated Mutations

Mutations conferring resistance to the FQ compounds were most often identified at the *gyrA* 90 (25.5%) and 94 (60.4%) codons, in line with previous findings [9, 39]. As with *rpoB*, mutations spread throughout the entire *gyrA* quinolone resistance-determining region contributed to the prediction of phenotypic FQ resistance, confirming the need to include this entire gene region in molecular diagnostic tests. Our study also provided information regarding rare *gyrA* mutations 88GCC and 88TGC, which were found exclusively in FQ^R specimens. Although these mutations only contributed 1.8% to the prediction of phenotypic FQ resistance in this study, their reliability as FQ resistance markers may support their inclusion in molecular diagnostic assays such as the Hain MTBDR*sl* assay [40].

No large differences were identified between clinical sites in regards to the frequencies of the various mutations in the *gyrA* gene region, and no single mutation appeared in more than 46% of FQ^R specimens in any site. The inclusion of the 88 to 95 codon quinolone resistance-determining region of the *gyrA* gene was adequate to detect the majority (64-96%) of FQ resistance in each of the three sites, and observed variations in the sensitivity of the assay for FQ resistance detection between the sites were similar to previous reports [41-43].

Injectable Resistance-Associated Mutations

The *rrs* 1401G mutation was identified in injectable-resistant and –susceptible specimens in the range of reported global frequencies (56-78% and 0-7%) [12]. *eis* promoter mutations were also identified among injectable-resistant specimens within the range of global estimates (0-22%) [12], but many *eis* promoter mutations also

appeared in KAN^S specimens. In order to investigate this discrepancy, 15 KAN^S specimens with *eis* mutations were subjected to repeat phenotypic KAN DST at the critical concentration (2.5µg/mL), with all repeated MGIT DST reactions run in duplicate. Eleven specimens (73%) were KAN^R in at least one of the two duplicate DST runs, but five of these results were discordant between the two runs, suggesting a possible mixture of KAN^S and KAN^R isolates in the sample that then grew out either resistant or susceptible when cultured. The other four specimens were KAN^S in both DST runs, indicating that these mutants had MICs below the tested KAN critical concentration. While *eis* promoter mutations have been well documented to confer only low-level KAN resistance [21, 44], recent studies have found *Mtb eis* mutants to have broad KAN MICs ranges (0.625-32µg/mL) via liquid DST methods [45, 46]. As such, the *eis* promoter mutants identified in our study may have had MICs around the critical concentration. Although *eis* promoter mutations do not appear to be reliable predictors of KAN resistance above 2.5µg/mL, this is probably as much a reflection of the uncertainties around our understanding of KAN^R phenotypes and the critical concentrations we use for measuring resistance as the uneven expression of resistance in these mutants. Despite these limitations, the inclusion of these mutations in our diagnostic assay helped to explain 86% of phenotypic KAN resistance in our study, compared to 50% based solely upon the *rrs* 1401G mutation.

Our initial pyrosequencing assay, based solely upon the detection of mutations at the 1401 *rrs* gene region, predicted 84.6-93.9% of injectable resistance in India and the Eastern Cape. However, the mutation only appeared in a few injectable-resistant specimens in Moldova. The inclusion of the *eis* promoter resulted in a large gain in platform sensitivity for KAN resistance detection in Moldova (7% to 79%), in line with previously published studies, as *eis* promoter mutations have been

documented to occur at a high frequency in countries once part of the former Soviet Union, due to heavy reliance upon KAN in TB treatment regimens [47, 48]. Interestingly, no *eis* mutations co-occurred with *rrs* mutations. Previous studies have documented *eis* promoter mutation selection prior to *rrs* mutation selection [47], as *rrs* mutants have already evolved high-level resistance to KAN and would not benefit from the addition of *eis* promoter mutations. Additionally, no *eis* promoter mutations were found in the Eastern Cape. This finding is important, as technologies lacking the *eis* promoter gene target would show high sensitivity for KAN resistance detection in our South African population, but low sensitivity for KAN resistance detection in our Indian and Moldovan population.

LIMITATIONS

All results presented in this study should be considered specific to our study populations in the large cities of Mumbai, Chisinau and Port Elizabeth, and not necessarily the countries of India, Moldova or South Africa. It is therefore possible that observed variations in mutation frequencies between the sites may be representative of localized DR-TB outbreaks or the persistence of endemic drug-resistant clones in these locales. However, our results highlighted a diversity of DR-TB strains with unique genetic combinations, suggesting that the vast majority of studied infections were not clonal. Our results are noteworthy if they reflect true regional differences, rather than local outbreaks, as these genetic variances will affect the performance of rapid molecular diagnostic technologies in larger regions. Additionally, although the mutations we identified provide a larger picture of the genetic basis of phenotypic anti-tuberculosis drug resistance, they do not represent a complete genetic profile of the DR-TB specimens evaluated in this study. The

inclusion of other gene regions, such as novel *katG* and *fabG1* mutations recently associated with INH resistance [32], may further increase the sensitivity of rapid molecular diagnostics for DR-TB detection. Additional sequencing studies investigating other genes and gene regions, such as *tlyA* and *gidB* mutations and their association with injectable resistance [12], will be necessary to identify the genetic basis of drug resistance for the 4-16% genetically wildtype, drug-resistant specimens in this study.

CONCLUSIONS

We conducted a large, multisite DR-TB sequencing study and found a wide diversity of mutations that varied in frequency between three diverse clinical sites. Altogether, the 46 resistance-associated mutations identified in seven gene targets were sufficient to detect 84-97% of XDR-TB phenotypes in this study. Inclusion of the *ahpC* and *eis* promoter gene regions was critical for optimal test sensitivity for INH resistance detection in the Eastern Cape and KAN resistance detection in Moldova. The identification of *rpoB* and *eis* promoter mutations in a large number of RIF^S and KAN^S specimens in this study emphasizes the need for future studies to address discordant phenotypic results for these low-MIC mutations and verify the clinical relevance of these mutations. These findings may help diagnostic assay developers to prioritize gene regions and mutations for inclusion in their assays, though DR-TB diagnostics that include all known resistance-associated mutations will likely remain the best option for optimal sensitivity of molecular diagnostics for DR-TB detection.

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Chapter 3, “Frequency and Distribution of Tuberculosis Resistance-Associated Mutations between Mumbai, Moldova and Eastern Cape,” has been accepted for publication in the American Society for Microbiology journal, *Antimicrobial Agents and Chemotherapy*. Marva Seifert, Donald Catanzaro, Richard Garfein, Faramarz Valafar, Valeriu Crudu, Camilla Rodrigues, Tommie Victor,

Antonino Catanzaro and Timothy Rodwell are co-authors. Sophia Georghiou, the dissertation author, is the primary author of this material.

TABLES AND FIGURES

Table 3.1: Cumulative frequencies of all mutations among *Mycobacterium tuberculosis* specimens resistant or susceptible to isoniazid (INH).

Gene	Observed mutation(s) by gene location	No. of INH-R specimens sequenced	No. of INH-S specimens sequenced	No. of INH-R specimens with mutation	No. of INH-S specimens with mutation	Frequency of mutation among INH-R specimens*	Frequency of mutation among INH-S specimens**
***katG* only**							
	315ACC	546	254	334	6	61.2%	2.4%
	315ACA	546	254	7	0	1.3%	0.0%
	315AAC	546	254	1	0	0.2%	0.0%
***inhA* only**							
	-15T	553	277	24	1	4.3%	0.4%
	-17T	553	277	5	2	0.9%	0.7%
	-8C	553	277	2	0	0.4%	0.0%
***ahpC* only**							
	-10A	542	287	3	0	0.6%	0.0%
	-6T	542	287	1	0	0.2%	0.0%
	-12A	542	287	1	0	0.2%	0.0%
	-9T	542	287	1	0	0.2%	0.0%
katG* and *inhA							
	315ACC/-15T	530	237	106	1	20.0%	0.4%
	315ACC/-17T	530	237	14	0	2.6%	0.0%
	315ACC/-8C	530	237	6	0	1.1%	0.0%
	315ACC/-8G	530	237	3	0	0.6%	0.0%
	315ACC/-8A	530	237	1	0	0.2%	0.0%
	315GGC/-15T	530	237	1	0	0.2%	0.0%
	315ACA/-8C	530	237	1	0	0.2%	0.0%
katG* and *ahpC							
	315ACC/-6T	526	235	4	0	0.8%	0.0%
	315ACC/-10A	526	235	3	0	0.6%	0.0%
	315ACC/-12A	526	235	1	0	0.2%	0.0%
	315ACC/-4A insertion	526	235	1	0	0.2%	0.0%
inhA* and *ahpC							
	-15T/-6T	528	263	1	0	0.2%	0.0%
	-15T/-10A	528	263	1	0	0.2%	0.0%
No mutations in *katG*, *inhA* or *ahpC*							
	Wildtype	516	225	26	222	5.0%	98.7%

INH, isoniazid
-R, -resistant
-S, -susceptible
*Frequency of mutation among INH-R specimens= No. of INH-R specimens with mutation/No. of INH-R specimens sequenced
**Frequency of mutation among INH-S specimens=No. of INH-S specimens with mutation/No. of INH-S specimens sequenced

Table 3.2: Cumulative frequencies of all mutations among *Mycobacterium tuberculosis* specimens resistant or susceptible to rifampicin (RIF).

Gene	Observed mutation(s) by gene location	No. of RIF-R specimens sequenced	No. of RIF-S specimens sequenced	No. of RIF-R specimens with mutation	No. of RIF-S specimens with mutation	Frequency of mutation among RIF-R specimens*	Frequency of mutation among RIF-S specimens**
rpoB							
	531TTG	450	266	360	0	80.0%	0.0%
	516GTC	427	262	22	1	5.2%	0.4%
	526GAC	450	266	9	0	2.0%	0.0%
	531TGG	450	266	9	0	2.0%	0.0%
	533CCG	450	266	6	2	1.3%	0.8%
	526TAC	450	266	5	1	1.1%	0.4%
	516TAC	427	262	3	3	0.7%	1.1%
	526AAC	450	266	3	1	0.7%	0.4%
	526TGC	450	266	3	0	0.7%	0.0%
	515ATA & 526AAC	389	222	1	0	0.3%	0.0%
	511CCG	427	262	1	2	0.2%	0.8%
	511CGG & 516TAC	427	262	1	0	0.2%	0.0%
	513AAA	427	262	1	0	0.2%	0.0%
	526CGC	450	266	1	0	0.2%	0.0%
	526CTC	450	266	1	0	0.2%	0.0%
	526GGC	450	266	1	0	0.2%	0.0%
No mutations in *rpoB*							
	Wildtype	389	222	14	212	3.6%	95.5%

RIF, rifampicin
-R, -resistant
-S, -susceptible
*Frequency of mutation among RIF-R specimens= No. of RIF-R specimens with mutation/No. of RIF-R specimens sequenced
**Frequency of mutation among INH-S specimens=No. of RIF-S specimens with mutation/No. of RIF-S specimens sequenced

Table 3.3: Cumulative frequencies of all mutations among *Mycobacterium tuberculosis* specimens resistant or susceptible to fluoroquinolones (FQs).

Gene	Observed mutation(s) by gene location	No. of FQ-R specimens sequenced	No. of FQ-S specimens sequenced	No. of FQ-R specimens with mutation	No. of FQ-S specimens with mutation	Frequency of mutation among FQ-R specimens*	Frequency of mutation among FQ-S specimens**
gyrA							
	94GGC & 95ACC	278	467	117	1	42.1%	0.2%
	90GTG & 95ACC	278	467	62	2	22.3%	0.4%
	94GCC & 95ACC	278	467	23	0	8.3%	0.0%
	91CCG & 95ACC	278	467	15	0	5.4%	0.0%
	94AAC & 95ACC	278	467	11	0	4.0%	0.0%
	94TAC & 95ACC	278	467	10	0	3.6%	0.0%
	90GTG	278	467	8	0	2.9%	0.0%
	94GGC	278	467	4	0	1.4%	0.0%
	88TGC & 95ACC	278	467	3	0	1.1%	0.0%
	94CAC & 95ACC	278	467	2	0	0.7%	0.0%
	88GCC	278	467	1	0	0.4%	0.0%
	90GTG, 91CCG & 95ACC	278	467	1	0	0.4%	0.0%
	94AAC	278	467	1	0	0.4%	0.0%
	88GCC & 95ACC	278	467	1	0	0.4%	0.0%
	91CCG	278	467	1	0	0.4%	0.0%
	95ACC	278	467	17	427	6.1%	91.4%
No mutations in *gyrA*							
	Wildtype	278	467	1	37	0.4%	7.9%

FQ, fluoroquinolone
-R, -resistant
-S, -susceptible
*Frequency of mutation among FQ-R specimens= No. of FQ-R specimens with mutation/No. of FQ-R specimens sequenced

**Frequency of mutation among FQ-S specimens=No. of FQ-S specimens with mutation/No. of FQ-S specimens sequenced

Table 3.4: Cumulative frequencies of All mutations among *Mycobacterium tuberculosis* specimens resistant or susceptible to amikacin (AMK), kanamycin (KAN) and/or capreomycin (CAP).

Gene	Mutation	INJ	No. of INJ-R specimens sequenced	No. of INJ-S specimens sequenced	No. of INJ-R specimens with mutation	No. of INJ-S specimens with mutation	Frequency of mutation among INJ-R specimens*	Frequency of mutation among INJ-S specimens**
***rrs* only**								
	1401G	AMK	73	728	61	5	83.6%	0.7%
		KAN	121	680	61	5	50.4%	0.7%
		CAP	70	731	59	7	84.3%	1.0%
***eis* only**								
	-12c/t	AMK	78	793	1	67	1.3%	8.4%
		KAN	141	730	37	31	26.2%	4.2%
		CAP	75	796	1	67	1.3%	8.4%
	-10g/a	AMK	78	793	0	6	0.0%	0.8%
		KAN	141	730	5	1	3.5%	0.1%
		CAP	75	796	0	6	0.0%	0.8%
	-14c/t	AMK	78	793	1	6	1.3%	0.8%
		KAN	141	730	3	4	2.1%	0.5%
		CAP	75	796	0	7	0.0%	0.9%
	-37g/t	AMK	78	793	0	4	0.0%	0.5%
		KAN	141	730	3	1	2.1%	0.1%
		CAP	75	796	0	4	0.0%	0.5%
	-10g/c	AMK	78	793	0	3	0.0%	0.4%
		KAN	141	730	0	3	0.0%	0.4%
		CAP	75	796	0	3	0.0%	0.4%
	-15c/g	AMK	78	793	0	1	0.0%	0.1%
		KAN	141	730	1	0	0.7%	0.0%
		CAP	75	796	0	1	0.0%	0.1%
No mutations in *rrs*								
	Wildtype	AMK	73	728	12	723	16.4%	99.3%
		KAN	121	680	60	675	49.6%	99.3%
		CAP	70	731	11	724	15.7%	99.0%
No mutations in *rrs* or *eis*								
	Wildtype	AMK	71	714	9	631	12.7%	88.4%
		KAN	119	666	18	622	15.1%	93.4%
		CAP	68	717	9	631	13.2%	88.0%

INJ, injectable
AMK, amikacin
KAN, kanamycin
CAP, capreomycin
-R, -resistant
-S, -susceptible
*Frequency of mutation among INJ-R specimens= No. of INJ-R specimens with mutation/No. of INJ-R specimens sequenced
**Frequency of mutation among INJ-S specimens=No. of INJ-S specimens with mutation/No. of INJ-S specimens sequenced

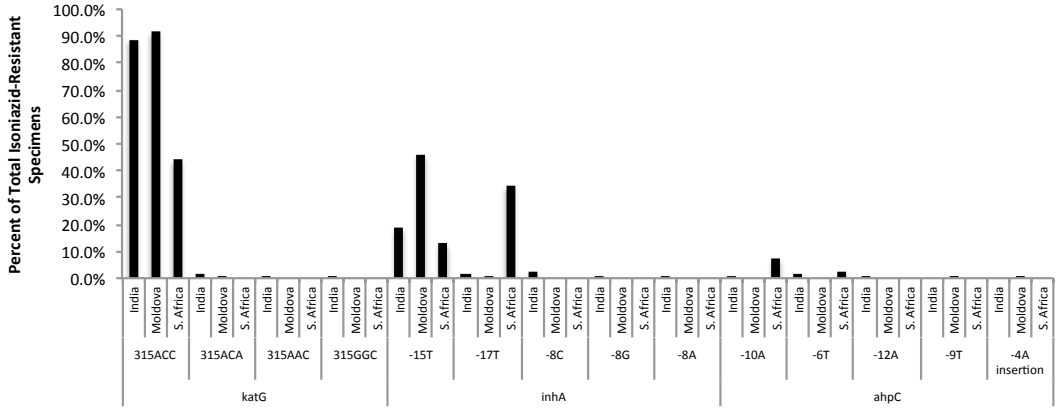

Figure 3.1: Frequency of mutations associated with isoniazid (INH) resistance identified among INH-resistant (INH^R) specimens by clinical site.

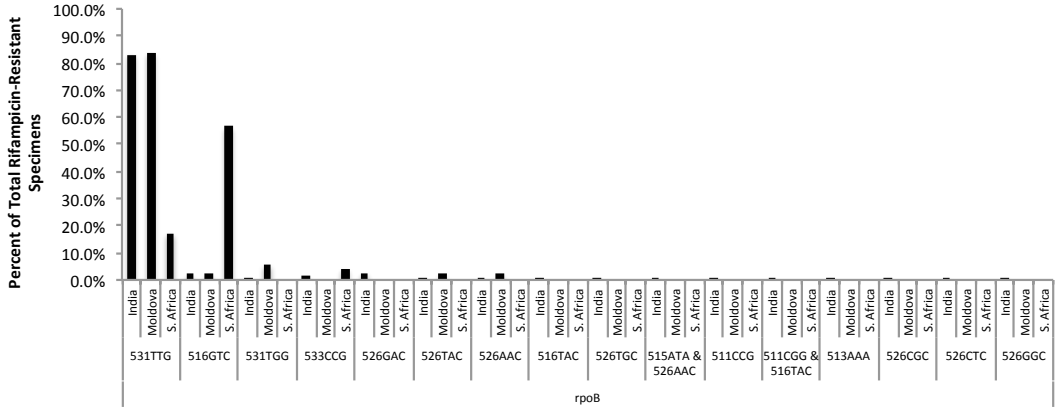

Figure 3.2: Frequency of mutations associated with rifampicin (RIF) resistance identified among RIF-resistant (RIF^R) specimens by clinical site.

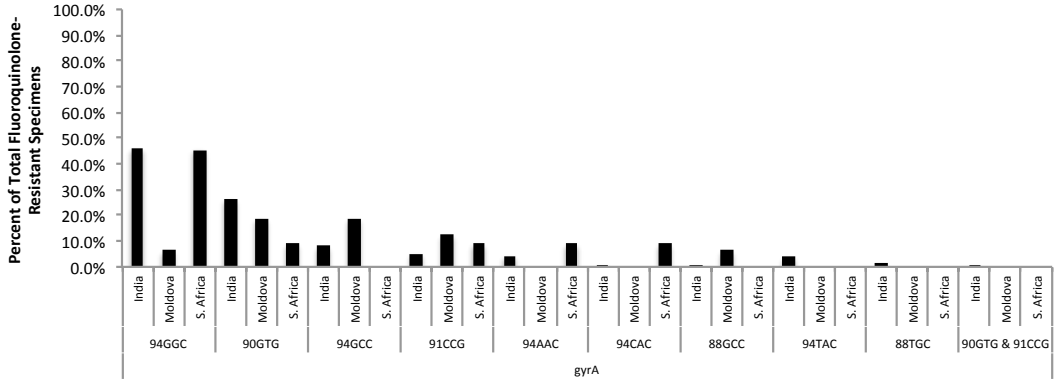

Figure 3.3: Frequency of mutations associated with fluoroquinolone (FQ) resistance identified among FQ-resistant (FQ^R) specimens by clinical site.

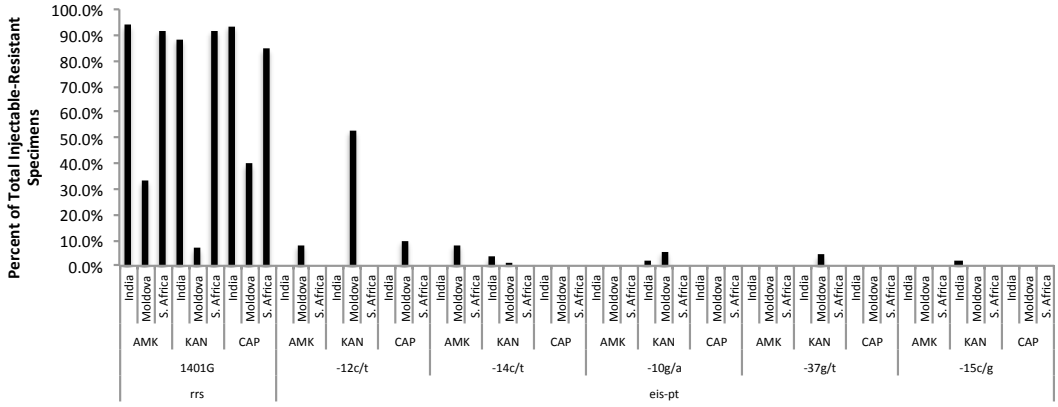

Figure 3.4: Frequency of mutations associated with injectable resistance identified among amikacin (AMK), kanamycin (KAN) and/or capreomycin (CAP)-resistant (AMK^R, KAN^R and/or CAP^R) specimens by clinical site.

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CHAPTER 4: INCREASE IN MORTALITY FOR TUBERCULOSIS PATIENTS WITH MUTATIONS ASSOCIATED WITH SECOND-LINE DRUG RESISTANCE IN A MULTISITE STUDY

ABSTRACT

Background: Rapid molecular diagnostics, which rely upon the identification of resistance mutations in specific gene regions, have great potential to curb the spread of drug-resistant tuberculosis. However, there are many factors complicating the interpretation of gene-based diagnostic results, such as variations in the levels of phenotypic drug resistance conferred by different *Mycobacterium tuberculosis* mutations. The clinical relevancy of these mutations is still unclear.

Methods: We analyzed clinical data for 453 patients enrolled in a multisite observational cohort study in relation to generated pyrosequencing data to determine the associations between *M. tuberculosis* resistance mutations and patient mortality.

Results: Notably, detection of a single *rrs* 1401G mutation was associated with significantly higher odds of patient mortality [adj OR=5.43 (95% CI 1.52 to 19.33)], even after adjusting for patient clinical characteristics and other mutations. Further analysis of these mutations, categorized as either low- or high-level resistant to various drug compounds, found the detection of mutations associated with high-level fluoroquinolone resistance [OR 3.66 (95% CI 1.06-12.62)] or kanamycin resistance [OR 6.59 (95% CI 1.99-21.84)] to be significantly associated with higher odds of patient mortality compared to patients with genetically wildtype infections, even after adjusting for clinical site, patient age, BMI, diabetes, HIV status, and other mutations.

Conclusions: These results imply that high-level *gyrA* and *rrs* resistance mutations may be reliable markers of patient outcomes for diverse, drug-resistant tuberculosis populations. This discovery has important implications for the interpretation of molecular diagnostic results as diagnostic standards move towards genotypic drug susceptibility testing.

INTRODUCTION

Although global tuberculosis (TB) incidence rates have declined over the past decade, the rise and spread of multi- and extensively drug-resistant TB (M/XDR-TB) strains continue to undermine TB eradication efforts. In 2014, the World Health Organization (WHO) reported 480,000 new cases of MDR-TB and 190,000 deaths attributed to MDR-TB, defined as TB that has developed resistance to the first-line anti-tuberculosis drugs isoniazid (INH) and rifampicin (RIF) [1]. Of those with MDR-TB, an estimated 9.7% had XDR-TB, or MDR-TB that has developed additional resistance to the fluoroquinolones (FQs) and at least one of the injectable compounds [amikacin (AMK), kanamycin (KAN) and/or capreomycin (CAP)] [1].

Molecular diagnostics, with their ability to rapidly detect resistance-associated mutations in bacterial genes, have great potential to shorten the time to M/XDR-TB diagnosis and appropriate treatment. The accuracy of these technologies is dependent upon the strength of association between specific *Mycobacterium tuberculosis* (*Mtb*) mutations and the phenotypic resistance of the isolate with that mutation. However, this relationship is not always binary. Recent studies have demonstrated that different mutations, even those occurring within the same gene region, can confer different levels of phenotypic resistance, and have different drug minimum inhibitory concentrations (MICs), to anti-tuberculosis drugs. For the detection of INH resistance, resistance-associated mutations at the *katG* 315 codon have been associated with higher INH MICs than *inhA* promoter mutations [2-4]; select mutations in the *rpoB* gene have been noted to have higher RIF MICs than other *rpoB* mutations [2, 5, 6]; mutations at the *gyrA* 94 codon have been associated with higher FQ MICs than the 90 and 91 codon mutations [7-9]; and the *rrs* 1401G mutation has been shown to have higher KAN MICs than *eis* promoter mutations [10,

11]. Studies defining the relationship between these mutations and the levels of phenotypic resistance they confer have been critical in order to accurately interpret of the results of molecular diagnostic assays, but the relationship between individual mutations and patient clinical outcomes is still unclear.

As *Mtb* diagnostic standards move towards genotypic drug susceptibility testing (DST), it is becoming increasingly important to independently verify the clinical relevance of these resistance mutations, rather than rely upon their prediction of phenotypic resistance as defined by a single critical concentration. Few studies to date have investigated the clinical relevancy of different *Mtb* mutations [12-15], and none have evaluated the relevancy of individual mutations in the broader context of M/XDR-TB genotypes and patient clinical environment. The Global Consortium for Drug-resistant TB Diagnostics (GCDD) conducted a large, multisite observational cohort study evaluating the diagnostic performance of a modified pyrosequencing diagnostic for M/XDR-TB [16], and followed up with the patients 52 weeks after enrollment to determine clinical outcomes. This analysis investigates the associations between different *Mtb* mutations and patient mortality outcomes in the context of M/XDR-TB genotypes and clinical environment.

MATERIALS AND METHODS

Study Population

Three epidemiologically diverse clinical sites (Chisinau, Moldova; Port Elizabeth, South Africa; and Mumbai, India) were selected for this study. In India, patients were enrolled at P.D. Hinduja Hospital and Medical Research Centre, the main drug-resistant TB (DR-TB) referral center for the city of Mumbai and state of Maharashtra. In Moldova, TB patients were enrolled between four Regional TB

Hospitals: two in Chisinau, one in Vorniceni and one in Balti. All patient samples were processed at the Phthisiopneumology Institute in Chisinau, a scientific research, medical consultation and training center that is the central unit of the Moldovan National TB Control Program. In Port Elizabeth, patients were enrolled at one regional hospital and six Primary Health Care facilities spread throughout the region. Newly-presenting TB patients over five years of age were eligible for the study if they provided informed consent and were known to be acid-fast bacilli smear-positive or suspected of having active pulmonary TB and having one or more reason to have DR-TB [17]. Risk factors for DR-TB included:

- Previously receiving >1 month of treatment for a prior TB episode
- Failing TB treatment with positive sputum smear or culture after ≥3 months of a standard TB treatment
- Close contact with a known DR-TB case
- Newly diagnosed with MDR-TB within the last 30 days
- Previously diagnosed with MDR-TB and failed TB treatment with positive sputum smear or culture after ≥3 months of a standard MDR-TB treatment regimen

Patients were excluded if they were unable to provide a sufficient quantity of sputum (7.5mL) or if they requested to be withdrawn from the study.

Drug-Susceptibility Testing and Patient Treatment

Mycobacterial Growth Indicator Tube (MGIT) 960 cultures were performed, with all specimens tested for resistance to INH, RIF, the FQs [moxifloxacin (MFX) and ofloxacin (OFX)], AMK, KAN, and CAP using standard manufacturer protocols and critical concentrations recommended by the WHO and as published previously for

MGIT-based drug susceptibility testing at the time of our study [18-21]. These phenotypic results provided the basis for TB treatment decisions at each site, which were determined and administered by local TB clinicians without GCDD input or recommendation. Phenotypic results are not included in the following analyses, as only sequencing results were considered in establishing genotypic drug resistance profiles in this study.

Clinical Data Collection

Clinical data were gathered from a combination of patient interviews and chart reviews [17]. Interviews were conducted at baseline and at the 52-week follow-up period. Briefly, upon screening and after obtaining informed consent, patients were asked to provide a spot sputum specimen and complete a baseline interview. 52 weeks following enrollment, patients were asked to return for a final sputum specimen and complete a clinical examination. All interviews collected information about patient age, race, ethnicity, gender, TB risk factors, and clinical history. Chart reviews were also conducted at baseline, 30 days into the study and at the 52-week follow-up with the subject's consent. Chart reviews noted TB treatment history and HIV status. The primary outcome of interest for this study was patient mortality, which was determined based upon data from any or all of these sources. Any patients lost to follow-up or transferred out of the study by the 52-week follow-up period were excluded from these analyses.

DNA Extraction, Molecular Targets and PCR

DNA was extracted from each decontaminated, concentrated sputum (sediment) by heating the cell suspensions in a water bath at 100°C [22]. PCR master

mixes were prepared and amplification reactions were carried out for all targets as previously reported [22]. Our sequencing assay included seven reactions to detect specific mutations in *Mtb* drug resistance-associated gene regions: the *inhA* promoter (position -4 to -20), *katG* (codons 312 to 316), *rpoB* (codons 507 to 533), *gyrA* (codons 88 to 95), *rrs* (1401 gene region) and the *eis* promoter (position to -5 to -47) [23]. All primers used in this study, other than the *rrs* primers, are specific for *Mtb* and do not show cross-reactivity with other TB species [22].

Pyrosequencing

Pyrosequencing was performed according to standard manufacturer procedures and modified for sequencing of mutations associated with M/XDR-TB, as described previously [22]. *eis* promoter sequencing capability was added to the assay following initial study completion by designing an *eis* promoter sequencing primer suitable to run at given pyrosequencing parameters. We utilized the PyroMark Q96 ID system (Qiagen, Valencia, CA) to perform pyrosequencing on the targets detailed above, sequencing two parts of *rpoB* in two separate reactions. Variants were identified automatically following pyrosequencing using IdentiFire software (Qiagen, Valencia, CA) according to manufacturer specifications and procedures and as described by Lin et al., generating a pyrosequencing report [22]. Samples that did not provide sequencing queries that 100% matched library sequences were repeated in duplicate. Samples that still did not provide confirmatory sequence, and samples for which contradictory hits were obtained for any given target, were deemed genotypically indeterminate and were not included in these analyses.

Mutations and Resistance Levels

Following our initial analyses investigating the association between individual mutations and patient mortality, mutations were categorized as susceptible, low-level, high-level, or very high-level resistant to different anti-tuberculosis drugs based upon literature findings. For mutations associated with INH resistance, *katG* mutations were considered to confer high levels of phenotypic INH resistance while *inhA* promoter mutations were considered to confer low levels of phenotypic INH resistance [2-4], and *katG* and *inhA* double mutants were included as a very high-level resistance category [24, 25]. *rpoB* mutations 531TTG, 531TGG, 526TAC, and 526GAC were considered high-level resistant to RIF, while mutations 511CCG, 516TAC, 526TGC, 526AAC, and 533CCG were considered low-level resistant to RIF [2, 5]. Other *rpoB* mutations identified in this study (513AAA, 515ATA & 526AAC, 516GTC and 522TTG) were considered to belong to an "unknown" RIF resistance category, having variable or undefined phenotypic RIF resistance profiles [5]. FQ resistance was associated with mutations other than the natural polymorphism 95ACC in *gyrA* (codons 88 to 95): *gyrA* mutations 88GCC, 94AAC, and 94GGC were associated with high-level FQ resistance, while mutations 90GTG, 91CCG and 94GCC were associated with low-level FQ resistance [9, 26]. High-level KAN resistance was associated with the *rrs* 1401G mutation while low-level KAN resistance was associated with *eis* promoter mutations [10, 11].

Data Analysis

All data analyses were performed in Stata (version 13.1; Stata Corp., College Station, TX). For comparison of categorical variables chi-squared tests were used. Associations of patient mortality with explanatory clinical variables were expressed as odds ratios (ORs). Confounding effects for these relationships were investigated by

multivariable logistic regression. In the analysis of patient outcomes, first individual mutations and then mutations categorized by resistance levels (above) were used as explanatory variables in multivariable logistic regression modeling along with any covariates that showed confounding effects. Potential confounders included site, age, gender, BMI, HIV status, diabetes, and other *Mtb* resistance mutations. HIV, diabetes and age are well-established confounders for TB and DR-TB negative treatment outcomes and were retained in the final model [27-29], along with any additional variables that showed confounding effects for the association between resistance mutations and TB patient mortality by follow-up. Outcome analyses were conducted both prior to and following restriction of our data to patients with complete genetic information for the gene regions of interest to ensure the final patient population included in this analysis was representative of the larger study population. All tests were performed using a significance level of 5%.

Human Research Conduct

Our study, registered with ClinicalTrials.gov (#NCT02170441), was reviewed and approved by the Institutional Review Board of the University of California, San Diego and by the Institutional Review Boards of the participating institutions at the three study sites. All participants provided written informed consent. Participation did not alter the standard of care.

RESULTS

Study Population

During enrollment, 52 patients were excluded for inability to provide 7.5mL of sputum (n=35), by request (n=3), or for other or unknown reasons (n=14). A total of

1128 patients with risk factors for DR-TB were enrolled in our initial study from April 24, 2012 to June 27, 2013 [17]. Clinical characteristics of this patient population have been described previously [16]. Of the 1128 total patients enrolled in the study, 518 (45.9%) transferred out before study completion or were lost to follow-up before the 52-week follow-up period. Of the remaining 610 patients, 157 were lacking relevant clinical data and were excluded from analyses. Clinical and outcome data were available for a total of 453 (40.2%) of the original 1128 enrolled patients, as shown in Figure 4.1. Clinical characteristics for these 453 patients were as follows: 297 (66%) patients were male; 90 (20%) were under the age of 25 while 91 (20%) were 50 years of age or older; 212 (47%) patients were underweight with a BMI < 18.50; 317 (70%) had been previously treated for TB; 166 (37%) were from India, 165 (36%) from Moldova and 122 (27%) from South Africa; 21 (5%) had diabetes and 68 (15%) were HIV-positive.

Associations between Baseline Clinical Characteristics and Patient Mortality

Eighty-nine (20%) of the total 453 patients with full clinical and outcome data died by the 52-week follow-up period (Table 4.1). Prior to adjustment for other clinical characteristics, patients with a previous history of TB treatment had significantly higher odds of mortality by 52 weeks [OR=2.70 (95% CI 1.47 to 4.97)], and patients in South Africa had significantly higher odds of mortality by 52 weeks [OR=2.13 (95% CI 1.09 to 4.17)] compared to patients in Moldova. These associations were no longer significant following adjustment for other relevant clinical variables. BMI, clinical site and HIV were all significantly associated with patient mortality in this population, both prior to and following adjustment for all other clinical variables: patients with a normal BMI (18.50 to <25) had significantly lower odds of mortality by 52 weeks [adj

OR=0.39 (95% CI 0.23 to 0.68)] compared to those with a low BMI (<18.50); patients from India had significantly higher odds of mortality by 52 weeks [adj OR=2.83 (95% CI 1.13 to 7.13)] compared to patients in Moldova; and HIV-positive patients had significantly higher odds of mortality by 52 weeks [adj OR=2.74 (95% CI 1.22 to 6.18)] compared to HIV-negative patients. Patient gender, age, and diabetes were not significantly associated with patient mortality by the 52-week follow-up period in either the adjusted or unadjusted models. When later evaluating the associations between baseline clinical characteristics and patient mortality for the final 204 specimens with complete sequencing data (data not shown), no additional, relevant clinical variables were identified.

Sequencing Results

Sequence data was generated for 277-400 specimens for each gene region evaluated (Table 4.2). The most commonly identified resistance mutations, each appearing in 10 or more specimens, were: *katG* 315ACC (n=200), *inhA* -15T (n=69), *rpoB* 531TTG (n=144), *gyrA* 94GGC (n=41), *gyrA* 90GTG (n=25), *gyrA* 94GCC (n=10), *rrs* 1401G (n=32), and *eis* -12T (n=35).

Associations between Individual Resistance Mutations and Patient Mortality

Few of the individual TB resistance mutations identified in this study were significantly associated with patient mortality (Table 4.3). Notably, only the detection of a *katG* 315ACA [OR=12.22 (95% CI 1.25 to 119.26)], *gyrA* 94GGC [OR=2.20 (95% CI 1.09 to 4.44)], or *rrs* 1401G [OR=4.23 (95% CI 2.00 to 8.93)] mutation was associated with significantly higher odds of mortality prior to adjustment for patient clinical characteristics or other resistance mutations. Following adjustment for

relevant clinical characteristics, only the katG 315ACA [adj OR=11.94 (95% CI 1.18 to 120.81)] and *rrs* 1401G [adj OR=3.36 (95% CI 1.46 to 7.73)] mutations remained associated with significantly higher odds of mortality by the 52-week follow-up period for the 453 patients with full clinical and outcome data. The *rpoB* 511CCG and *gyrA* 94AAC mutations appeared exclusively in patients who died by the follow-up period, though at low frequency (two to three patients in each instance).

Prediction of Patient Mortality

Individual Resistance Mutations. Two hundred four specimens had full clinical, outcome, and sequencing data. The *katG* 315ACA mutation, found to be associated with higher odds of patient mortality following adjustment for relevant clinical characteristics in the expanded population of 453 patients (Table 4.3), was only identified in patients who died by study completion in this restricted population (Table 4.4), along with the *rpoB* 511CCG and *gyrA* 94AAC mutations. After adjusting for patient age, BMI, HIV status, diabetes, clinical site, and all other TB resistance mutations, only the *rrs* 1401G mutation was found to be associated with significantly higher odds of patient mortality [adj OR=5.43 (95% CI 1.52 to 19.33)].

High- versus Low-Level Resistance Mutations. After categorizing mutations into groups high-level resistant, low-level resistant, or susceptible to relevant anti-tuberculosis drug compounds, we found that only the *gyrA* and *rrs* resistance mutations were significantly associated with higher odds of patient mortality by 52 weeks following adjustment for patient age, BMI, HIV status, diabetes, and clinical site (Table 4.5). Detection of either a *gyrA* high-level resistant [adj OR=4.04 (95% CI 1.37 to 11.89)] or low-level resistant [adj OR=4.18 (95% CI 1.47 to 11.87)] mutation

was significantly associated with increased odds of patient mortality in this analysis prior to adjustment for other high- or low-level TB resistance mutations. Following adjustment for additional mutations, however, only the detection of a high-level *gyrA* [adj OR=3.66 (95% CI 1.06 to 12.62)] or *rrs* [adj OR=6.59 (95% CI 1.99 to 21.84)] resistance mutation was significantly associated with higher odds of patient mortality by 52 weeks, in comparison to genetically wildtype strains. Detection a low- or high-level INH or RIF resistance mutation was not significantly associated with patient mortality by 52 weeks before or after adjustment for other resistance mutations.

DISCUSSION

It is becoming increasingly important to confirm the clinical relevance of TB resistance mutations as genotypic DST methods for M/XDR-TB diagnosis are standardized. We conducted a multisite, observational cohort study and determined associations between *Mtb* resistance mutations and patient mortality outcomes by a 52-week follow-up period. We found that the detection of mutations associated with high-level FQ or KAN resistance was associated with significantly higher odds of patient mortality compared to wildtype specimens, even after adjusting for the presence of other resistance mutations and relevant clinical factors. This same association was not observed for the mutations associated with first-line drug resistance. Our results demonstrate that, in the broader context of M/XDR-TB genotypes and patient clinical environment, the detection of *gyrA* and *rrs* high-level resistance mutations might be more important than the detection of low-level resistance mutations, or any of the mutations associated with INH and RIF resistance, in predicting patient clinical outcomes.

Associations between Clinical Characteristics and Patient Mortality

Clinical site played a significant role in the prediction of patient mortality outcomes in this study. Notably, patients in India had significantly higher odds of mortality than patients in Moldova, even after accounting for the many clinical factors that may help to explain this relationship. Although differences in patient quality of care or treatment between sites could have contributed to the higher odds of patient mortality in India, it is more likely that the observed association between clinical site and patient mortality was related to differences in the proportions of patients being enrolled under different enrollment criteria in each site. In India, patients were enrolled at a major DR-TB referral hospital, and so these patients were more likely to have been previously treated for TB or DR-TB and have failed treatment prior to enrollment in our study. Indeed, 138/166 (83%) patients enrolled in India were failing TB treatment for presumed or confirmed TB or MDR-TB, versus 17/165 (10%) of the patients enrolled in Moldova. We would expect these patients to have more progressed TB infections and worse outcomes compared to patients who were enrolled in our study based upon other criteria, such as contact with DR-TB patients. In Moldova, 111/165 (67%) patients had contact with a known DR-TB patient, compared to 52/166 (31%) patients enrolled in India. These differences in enrollment criteria, and their believed contribution to observed patient clinical outcomes, led us to retain site as an important covariate in our final models. Patient HIV status and BMI, which have been well established as risk factors for TB patient mortality [28, 30], were also significantly associated with patient mortality in this population and were retained in all models. Patient age and diabetes were not found to be significantly associated with patient mortality in this population, but are known risk factors for patient mortality in TB patients and so were also retained in all models [27, 29].

Association between First-Line Resistance Mutations and Patient Mortality

None of the individual first-line drug (INH or RIF) resistance mutations were significantly associated with patient mortality in the patients with full clinical, outcome and sequence data in this study after accounting for relevant clinical characteristics and additional resistance mutations. Although the *katG* 315ACA and *rpoB* 511CCG mutations were identified exclusively in patients that died by study completion, few patients were available for analysis in each instance, and so the association between these specific mutations and patient mortality could not be confirmed. However, there is some evidence that the *rpoB* 511CCG mutation may be directly associated with adverse TB patient clinical outcomes [31], and so the finding of this mutation exclusively among patients who died by study completion might be worth further investigation. The *katG* 315ACA mutation, in contrast, encodes the same amino acid (threonine) as the common 315ACC mutation, and so would be expected to confer the same phenotypic resistance level as the ACC mutations, meaning that these two mutation groups could possibly be combined for analyses.

However, when *katG* and *inhA* mutations were categorized according to resistance level, none of these categories predicted significantly higher odds of patient mortality compared to the wildtype (susceptible) specimens. These findings are different from those of Huyen et al., who found that *katG* mutations were associated with patient mortality while *inhA* mutations were associated with patient relapse [13], and Tolani et al., who determined that *katG* mutations were associated with poor clinical outcomes [32]. However, these previous studies did not account for important clinical factors, such as HIV, or the presence of other DR-TB resistance mutations in their analyses. Studies that did account for additional clinical factors,

including HIV and patient weight [33], and studies that restricted their analyses to INH mono-resistant TB cases [34], failed to find significant associations between specific INH resistance mutations and patient outcomes. Jacobson et al. even demonstrated that the treatment of TB infections harboring *katG* 315T mutations with high-dose INH was associated with increased odds of favorable treatment outcomes [33], suggesting that even high-level INH resistance mutations might be overcome with targeted treatment regimens. Our results agree with these latter studies, implying that the *katG* and *inhA* promoter mutations commonly associated with INH resistance are not reliable predictors of patient outcomes.

As with the *katG* and *inhA* mutations, few studies to date have evaluated the direct associations between *rpoB* mutations and patient outcomes. However, there has been some evidence to suggest that, along with *rpoB* high-level resistance mutations, even disputed, low-level *rpoB* resistance mutations are associated with poor clinical outcomes. Van Deun et al., for example, determined that 61-80% of patients with *rpoB* high-level resistance mutations (531Leu, 526Tyr or 516Val) experienced treatment failure or relapse, compared to 40-71% of patients with *rpoB* low-level resistance mutations (511Pro, 516Tyr, 526Asn or 533Pro) [31]. Other studies have reported the recurrence, persistence or progression of TB disease for patients with low-level RIF resistance mutations [35], even on standard first-line treatment regimens [15, 36]. The finding that *rpoB* mutations are associated with treatment failures is especially concerning if true, as many of these mutations have been shown to test RIF-susceptible via phenotypic DST methods in liquid media [35, 36], and so these potentially clinically relevant infections may be misdiagnosed. However, in the broader context of M/XDR-TB genotypes and patient clinical environment, these low- and high-level RIF resistance mutations were not found to be

predictive of patient mortality. Still, as few low-level RIF resistance mutations were available for analysis in this study (n=7), these particular *rpoB* resistance mutations should be further evaluated by future clinical relevance studies, taking into account relevant patient clinical factors and other resistance mutations in order to determine their association with patient outcomes.

Association between Second-Line Resistance Mutations and Patient Mortality

No individual *gyrA* mutation was significantly associated with patient mortality in this study following adjustment for patient clinical factors and other resistance mutations. Although the *gyrA* 94AAC mutation was identified exclusively in patients that died by study completion, only two patients were available for analysis after adjustment, and so the association between this mutation and patient mortality could not be definitively confirmed. However, when *gyrA* mutations were categorized as either high- or low-level resistant to the FQs according to literature findings, the combined, high-level resistance mutations were found to be significantly associated with higher odds of patient mortality by the 52-week follow-up period compared to *gyrA* wildtype infections. This finding did not hold for *gyrA* low-MIC mutations after accounting for the presence of other resistance mutations. While Leung et al. demonstrated that the presence of any gyrA mutation is predictive of poor treatment outcomes [14], our findings are in line those of Rigouts et al., who found *gyrA* mutations at codon 94 (other than 94GCC) to predict high-level FQ resistance and poor treatment outcome in MDR-TB patients [12]. These results suggest that the high-level *gyrA* mutations are the major contributors to the poor outcomes observed by Leung et al., and that *gyrA* high-MIC mutations may be some of the most reliable markers of clinical outcomes for diverse DR-TB patient populations.

Along with the *gyrA* high-level resistance mutations, the *rrs* 1401G mutation was a reliable predictive marker of TB patient mortality in our study. This result is in line with the findings of Leung et al., who found a strong correlation between *rrs* 1401G mutants and KAN treatment failure, though they did not account for relevant clinical factors that could influence this relationship [14]. To date, we are unaware of any additional studies evaluating the clinical relevancy of *rrs* mutations to DR-TB patient outcomes. Unlike the *rrs* 1401G mutation, *eis* promoter mutations were not significantly associated with patient mortality outcomes in our study, despite their association with low-level KAN resistance [10]. This is the first study, to our knowledge, investigating the direct effect of *eis* mutations on TB patient outcomes [37]. Our findings suggest that these low-MIC mutations are not as relevant as *rrs* mutations in predicting patient outcomes. However, this observation may reflect the fact that other injectable compounds (AMK and/or CAP) were available for treatment in this study, and so the lower mortality rates seen for patients with *eis* mutations may have resulted from these patients being treated with these alternative compounds to which their infections would be susceptible. Future studies should investigate the role of different treatment regimens in predicting treatment outcomes for patients with these low-level resistance mutations.

No studies to date have found high-level *gyrA* or *rrs* resistance mutations to influence *Mtb* virulence [38], and so it is likely that these mutations instead play a role in patient mortality by rendering FQ- and injectable-based treatment regimens ineffective. FQs, for example, are known have concentration-dependent antibacterial activity, and pharmacodynamic parameters such as peak serum concentration (Cmax)/MIC and 24-hour area-under-the-curve (AUC_{24})/MIC ratios have been identified as potential predictors of treatment success [39]. The reported Cmax values

for OFX and MFX are 4.0 and 4.34μg/mL per 70kg at a 400mg dosing, with AUC_{24} values of 48.0 and 39.3μg*h/mL per 70kg [40]. These data yield a Cmax/MIC ratio of 2.0 for OFX and 9.0 for MFX, as MFX has a longer half-life than OFX, and AUC_{24}/MIC ratios of 24 for OFX and 96 for MFX [40]. Studies have demonstrated that the greatest bactericidal activity occurs when the Cmax/MIC ratio is 8-10 or more, and the AUC_{24}/MIC ratio is 100-125 or more, and so MFX has superior pharmacodynamic parameters to OFX and may represent a more effective treatment option for TB patients [40]. Indeed, MFX MICs have been reported to be as low as 0.25μg/mL for *Mtb* isolates with low-level *gyrA* mutations [41], correlating with a Cmax/MIC of 17.36 and AUC_{24}/MIC of 157.2, and suggesting that a proportion of infections with these genotypes might be overcome with high MFX dosing, as opposed to other FQs. For high-level *gyrA* resistance mutations at the 94 codon, in contrast, MFX MICs were reported to be 1-8μg/mL [41], corresponding with a Cmax/MIC of 0.54-4.34 and AUC_{24}/MIC of 4.91-39.3. These infections would be unlikely to be affected by high-level FQ dosing regimens that include even the most potent FQs, and so alternative treatment options must be made available upon the detection of *gyrA* high-MIC mutations.

As with the FQs, KAN has concentration-dependent antibacterial activity [42], with a Cmax of 22μg/mL [43]. KAN MICs of 0.625-1.25μg/mL have been demonstrated for *Mtb* clinical isolates without *rrs* or *eis* promoter mutations via liquid media DST methods [44]. These results yield a Cmax/MIC ratio of about 18-35, representing a potentially effective treatment option for infections with *rrs* and *eis* wildtype sequences. Isolates with the 1401G mutation, however, have been shown to have KAN MICs >20μg/mL [44], yielding a Cmax/MIC ratio of ≤1.1, and perhaps reflecting the significantly higher odds of poor clinical outcomes observed in this study

for patients with the 1401G mutation. Although AMK represents a second aminoglycoside for DR-TB treatment, high levels of cross-resistance have been observed between the two compounds for isolates with *rrs* mutations [45], making the compound an ineffective alternative treatment option in many cases. A high-level of cross-resistance to CAP has also been seen for isolates with the *rrs* 1401G mutation, also making it an unlikely treatment option [45]. In one clinical outcome study where 80% of the population had the *rrs* 1401G mutation, no therapeutic benefit was found for treatment of XDR-TB patients with CAP [46]. These findings underscore the importance in detecting these high-level resistance markers and highlight the potential need for new, appropriate DR-TB treatment options for patients with these high-level TB resistance mutations.

LIMITATIONS

Although these analyses were conservative, accounting for many TB resistance genotypes and patient clinical characteristics, differences in drug treatment regimens were not accounted for. For the purposes of this study we assumed that different mutation groups were treated similarly, so that this factor did not increase mortality risk for any group, but by not accounting for this important factor we were limited in our ability to confirm the independent association of resistance mutations with TB patient mortality. Additionally, there was substantial loss to follow-up in this study, as less than half of the original population had outcome data for the 52-week follow-up period. It is possible that these patients may have been sicker than the final population available for analysis, and had unconfirmed deaths prior to the follow-up period, which might bias our results towards the null if these lost patients had associated M/XDR-TB resistance mutations. Despite the high number of patients

without clinical outcome data, however, our study had adequate power to confirm significant associations between *gyrA* and *rrs* high-level resistance mutations and patient mortality. Another limitation of our study was that our sequencing assay was restricted in its ability to provide a complete genetic resistance profile of the DR-TB specimens evaluated in this study. We did not associate M/XDR-TB drug resistance with mutations occurring outside of the *katG, inhA*, *rpoB*, *gyrA*, *rrs* and *eis* gene regions sequenced, meaning that we may have missed relevant resistance mutations, such as *ahpC* promoter mutations [47], other *katG* mutations lying outside of the gene region included in the assay [48], or *tlyA* and *gidB* mutations [49]. Our failure to sequence these additional gene regions might have biased our results towards the null, if the patients with genetically wildtype infections that died in this study in fact harbored these other resistance mutations.

CONCLUSIONS

We analyzed mutation and clinical data from a multisite, observational cohort study and found high-level *gyrA* and *rrs* resistance mutations to be significantly associated with patient mortality. The rapid detection of these mutations by M/XDR-TB molecular diagnostics can help clinicians to quickly tailor appropriate treatment of these resistant infections and improve patient clinical outcomes.

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Chapter 4, “Increase in Mortality for Tuberculosis Patients with Mutations Associated with Second-line Drug Resistance in a Multisite Study,” is currently being prepared for publication. Marva Seifert, Donald Catanzaro, Richard Garfein, Faramarz Valafar, Valeriu Crudu, Camilla Rodrigues, Tommie Victor, Antonino Catanzaro and Timothy Rodwell are co-authors. Sophia Georghiou, the dissertation author, is the primary author of this material.

TABLES AND FIGURES

Table 4.1: Associations between baseline clinical characteristics and patient mortality by 52-week follow-up period for patients with complete sequencing data for at least one gene of interest (n=453).

Characteristic or Parameter	No. Patients	No. Deceased	%	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Total No. of Patients	453	89	19.6%		
Sex					
Male	297	58	19.5%	0.98(0.60-1.59)	1.42(0.81-2.47)
Female	156	31	19.9%	1.00	1.00
Age (yrs)					
<25	90	20	22.2%	1.00	1.00
25 to 49	272	54	19.9%	0.87(0.49-1.55)	1.13(0.59-2.17)
≥50	91	15	16.5%	0.69(0.33-1.45)	1.28(0.54-3.04)
BMI					
<18.50	212	58	27.4%	1.00	1.00
18.50 to <25	212	25	11.8%	**0.35(0.21-0.59)****	**0.39(0.23-0.68)****
≥25	29	6	20.7%	0.69(0.27-1.79)	0.87(0.30-2.50)
TB Treatment					
New	136	14	10.3%	1.00	1.00
Previously Treated	317	75	23.7%	**2.70(1.47-4.97)****	1.24(0.49-3.14)
Site					
Moldova	165	17	10.3%	1.00	1.00
India	166	48	28.9%	**3.54(1.94-6.48)****	**2.83(1.13-7.13)***
South Africa	122	24	19.7%	**2.13(1.09-4.17)***	0.93(0.32-2.71)
Diabetes					
Yes	21	5	23.8%	1.29(0.46-3.63)	1.37(0.44-4.29)
No	432	84	19.4%	1.00	1.00
HIV Positive					
Yes	68	20	29.4%	**1.91(1.07-3.42)***	**2.74(1.22-6.18)***
No	385	69	17.9%	1.00	1.00

*p≤0.05
**p≤0.01
Adjusted model is adjusted for all other variables

Table 4.2: Tuberculosis resistance mutations detected by pyrosequencing for patients with full clinical and outcome data (n=453).

Gene	Mutation	No. Patients	No. Deceased	%
katG	315ACC	200	47	24%
	315ACA	4	3	75%
	No mutation	150	22	15%
inhA	-15T	69	15	22%
	-17T	5	2	40%
	-8C	3	2	67%
	No mutation	294	63	21%
rpoB	531TTG	144	38	26%
	516GTC	7	1	14%
	526TAC	5	0	0%
	533CCG	3	0	0%
	526GAC	3	0	0%
	526TGC	2	1	50%
	526AAC	2	1	50%
	531TGG	4	0	0%
	516TAC	1	0	0%
	515ATA & 526AAC	1	0	0%
	513AAA	1	0	0%
	511CCG	3	3	100%
	522TTG	1	0	0%
	No mutation	100	18	18%
gyrA	94GGC	41	15	37%
	90GTG	25	9	36%
	94GCC	10	3	30%
	91CCG	6	3	50%
	94AAC	2	2	100%
	88GCC	2	0	0%
	No mutation	220	38	17%
rrs	1401G	32	16	50%
	No mutation	319	61	19%
eis	-12T	35	6	17%
	-10A	4	0	0%
	-14T	2	1	50%
	-37T	2	0	0%
	No mutation	357	77	22%

Table 4.3: Associations between tuberculosis resistance mutations and patient death for patients with full clinical and outcome data (n=453).

Mutation	No. Patients	No. Deceased	%	Unadjusted OR (95% CI)	Adjusted OR_1 (95% CI)
Total No. of Patients	453	89	19.6%		
katG 315ACC Mutation					
Yes	200	47	23.5%	1.59(0.92-2.72)	1.18(0.60-2.30)
No	154	25	16.2%	1.00	1.00
katG 315ACA Mutation					
Yes	4	3	75.0%	**12.22(1.25-119.26)***	**11.94(1.18-120.81)***
No	350	69	19.7%	1.00	1.00
inhA -15T Mutation					
Yes	69	15	21.7%	0.97(0.52-1.84)	1.10(0.55-2.18)
No	302	67	22.2%	1.00	1.00
inhA -17T Mutation					
Yes	5	2	40.0%	2.38(0.39-14.51)	2.07(0.30-14.41)
No	366	80	21.9%	1.00	1.00
inhA -8C Mutation					
Yes	3	2	66.7%	7.20(0.64-80.42)	5.99(0.48-75.29)
No	368	80	21.7%	1.00	1.00
rpoB 531TTG Mutation					
Yes	144	38	26.4%	1.63(0.91-2.90)	1.83(0.87-3.83)
No	133	24	18.0%	1.00	1.00
rpoB 511CCG Mutation					
Yes	3	3	100.0%		
No	274	59	21.5%		
rpoB 526AAC Mutation					
Yes	2	1	50.0%	3.51(0.22-56.91)	6.00(0.25-146.13)
No	275	61	22.2%	1.00	1.00
rpoB 526TGC Mutation					
Yes	2	1	50.0%	3.51(0.22-56.91)	3.42(0.19-62.68)
No	275	61	22.2%	1.00	1.00
rpoB 516GTC Mutation					
Yes	7	1	14.3%	0.57(0.07-4.83)	0.49(0.05-4.43)
No	270	61	22.6%	1.00	1.00
gyrA 94GGC Mutation					
Yes	41	15	36.6%	**2.20(1.09-4.44)***	1.61(0.70-3.66)
No	265	55	20.8%	1.00	1.00
gyrA 94AAC Mutation					
Yes	2	2	100.0%		
No	304	68	22.4%		
gyrA 94GCC Mutation					
Yes	10	3	30.0%	1.46(0.37-5.82)	1.11(0.26-4.69)
No	296	67	22.6%	1.00	1.00
gyrA 91CCG Mutation					
Yes	6	3	50.0%	3.48(0.69-17.63)	2.70(0.47-15.55)
No	300	67	22.3%	1.00	1.00
gyrA 90GTG Mutation					
Yes	25	9	36.0%	2.03(0.85-4.82)	1.54(0.61-3.90)
No	281	61	21.7%	1.00	1.00
rrs 1401G Mutation					
Yes	32	16	50.0%	**4.23(2.00-8.93)****	**3.36(1.46-7.73)****
No	319	61	19.1%	1.00	1.00
eis -12T Mutation					
Yes	35	6	17.1%	0.76(0.31-1.90)	1.25(0.46-3.39)
No	365	78	21.4%	1.00	1.00
eis -14T Mutation					
Yes	2	1	50.0%	3.80(0.23-61.32)	2.57(0.14-47.91)
No	398	83	20.9%	1.00	1.00

*$p \leq 0.05$
**$p \leq 0.01$
Adjusted OR_1: Models are adjusted for site, HIV, diabetes, age, BMI
This analysis excluded mutations that were not identified in any patients that died by the 52-week follow-up period

Table 4.4: Associations between tuberculosis resistance mutations and patient death for patients with full clinical and outcome data and complete genetic sequence data (n=204).

Mutation	No. Patients	No. Deceased	%	Unadjusted OR (95% CI)	Adjusted OR_1 (95% CI)	Adjusted OR_2 (95% CI)
Total No. of Patients	204	46	22.5%			
katG 315ACC Mutation						
Yes	133	32	24.1%	1.29(0.64-2.62)	1.00(0.41-2.43)	0.51(0.13-1.98)
No	71	14	19.7%	1.00	1.00	1.00
katG 315ACA Mutation						
Yes	2	2	100.0%			
No	202	44	21.8%			
inhA -15T Mutation						
Yes	41	9	22.0%	0.96(0.42-2.19)	1.10(0.45-2.66)	0.51(0.17-1.52)
No	163	37	22.7%	1.00	1.00	1.00
inhA -17T Mutation						
Yes	1	1	100.0%			
No	203	45	22.2%			
inhA -8C Mutation						
Yes	1	1	100.0%			
No	203	45	22.2%			
rpoB 531TTG Mutation						
Yes	115	32	27.8%	**2.07(1.02-4.16)***	2.26(0.96-5.30)	2.34(0.57-9.51)
No	89	14	15.7%	1.00	1.00	1.00
rpoB 511CCG Mutation						
Yes	1	1	100.0%			
No	203	45	22.2%			
rpoB 526TGC Mutation						
Yes	2	1	50.0%	3.49(0.21-56.89)	3.09(0.17-55.68)	9.96(0.46-217.02)
No	202	45	22.3%	1.00	1.00	1.00
rpoB 516GTC Mutation						
Yes	4	1	25.0%	1.15(0.12-11.31)	0.91(0.09-9.50)	1.28(0.06-29.95)
No	200	45	22.5%	1.00	1.00	1.00
gyrA 94GGC Mutation						
Yes	32	12	37.5%	**2.44(1.09-5.46)***	1.76(0.69-4.50)	2.40(0.68-8.52)
No	172	34	19.8%	1.00	1.00	1.00
gyrA 94AAC Mutation						
Yes	2	2	100.0%			
No	202	44	21.8%			
gyrA 94GCC Mutation						
Yes	8	3	37.5%	2.13(0.49-9.29)	1.42(0.31-6.56)	1.62(0.25-10.47)
No	196	43	21.9%	1.00	1.00	1.00
gyrA 91CCG Mutation						
Yes	4	3	75.0%	**10.95(1.11-107.97)***	7.46(0.72-77.73)	10.52(0.77-144.46)
No	200	43	21.5%	1.00	1.00	1.00
gyrA 90GTG Mutation						
Yes	21	7	33.3%	1.85(0.70-4.89)	1.52(0.54-4.29)	1.49(0.36-6.11)
No	183	39	21.3%	1.00	1.00	1.00
rrs 1401G Mutation						
Yes	23	14	60.9%	**7.24(2.89-18.18)****	**6.55(2.39-17.97)****	**6.25(1.85-21.07)****
No	181	32	17.7%	1.00	1.00	1.00
eis -12T Mutation						
Yes	20	4	20.0%	0.85(0.27-2.67)	1.66(0.46-5.99)	2.38(0.52-10.87)
No	184	42	22.8%	1.00	1.00	1.00

*p≤0.05
**p≤0.01
Adjusted OR_1: Models are adjusted for site, HIV, diabetes, age, BMI
This analysis excluded mutations that were not identified in any patients that died by the 52-week follow-up period

Table 4.5: Multivariate associations for categories of resistance mutations and death by 52 weeks (n=204)

Resistance	Total	No.	%	Adjusted OR_1 (95% CI)	Adjusted OR_2 (95% CI)
INH resistance					
Susceptible, wildtype	64	11	17.2%	1.00	1.00
Highest MIC mutation	38	10	26.3%	1.85(0.57-6.02)	0.54(0.07-3.99)
High MIC mutation	97	24	24.7%	1.21(0.43-3.42)	0.60(0.11-3.21)
Low MIC mutation	5	1	20.0%	0.90(0.08-10.66)	0.13(0.01-3.12)
RIF resistance					
Susceptible, wildtype	67	11	16.4%	1.00	1.00
High MIC mutation	124	32	25.8%	1.60(0.61-4.19)	1.18(0.24-5.87)
Low MIC mutation	7	2	28.6%	1.40(0.22-9.08)	1.87(0.25-13.71)
Unknown MIC mutation	6	1	16.7%	0.73(0.07-7.74)	0.40(0.02-6.91)
FQ resistance					
Susceptible, wildtype	135	19	14.1%	1.00	1.00
High MIC mutation	36	14	38.9%	**4.04(1.37-11.89)***	**3.66(1.06-12.62)***
Low MIC mutation	33	13	39.4%	**4.18(1.47-11.87)***	2.66(0.76-9.36)
KAN resistance					
Susceptible, wildtype	157	28	17.8%	1.00	1.00
High MIC mutation	23	14	60.9%	**6.87(2.47-19.08)****	**6.59(1.99-21.84)****
Low MIC mutation	24	4	16.7%	1.70(0.47-6.20)	1.44(0.34-6.22)

*p≤0.05
**p≤0.01
$_1$ Models are adjusted for HIV status, age, diabetes, BMI, and site. Site is significant (p<0.01) in all models except the model evaluating FQ resistance.
$_2$ Model is adjusted for HIV status, age, diabetes, BMI, site, and other resistance mutations. None of these other covariates were found to be significant in the final model.

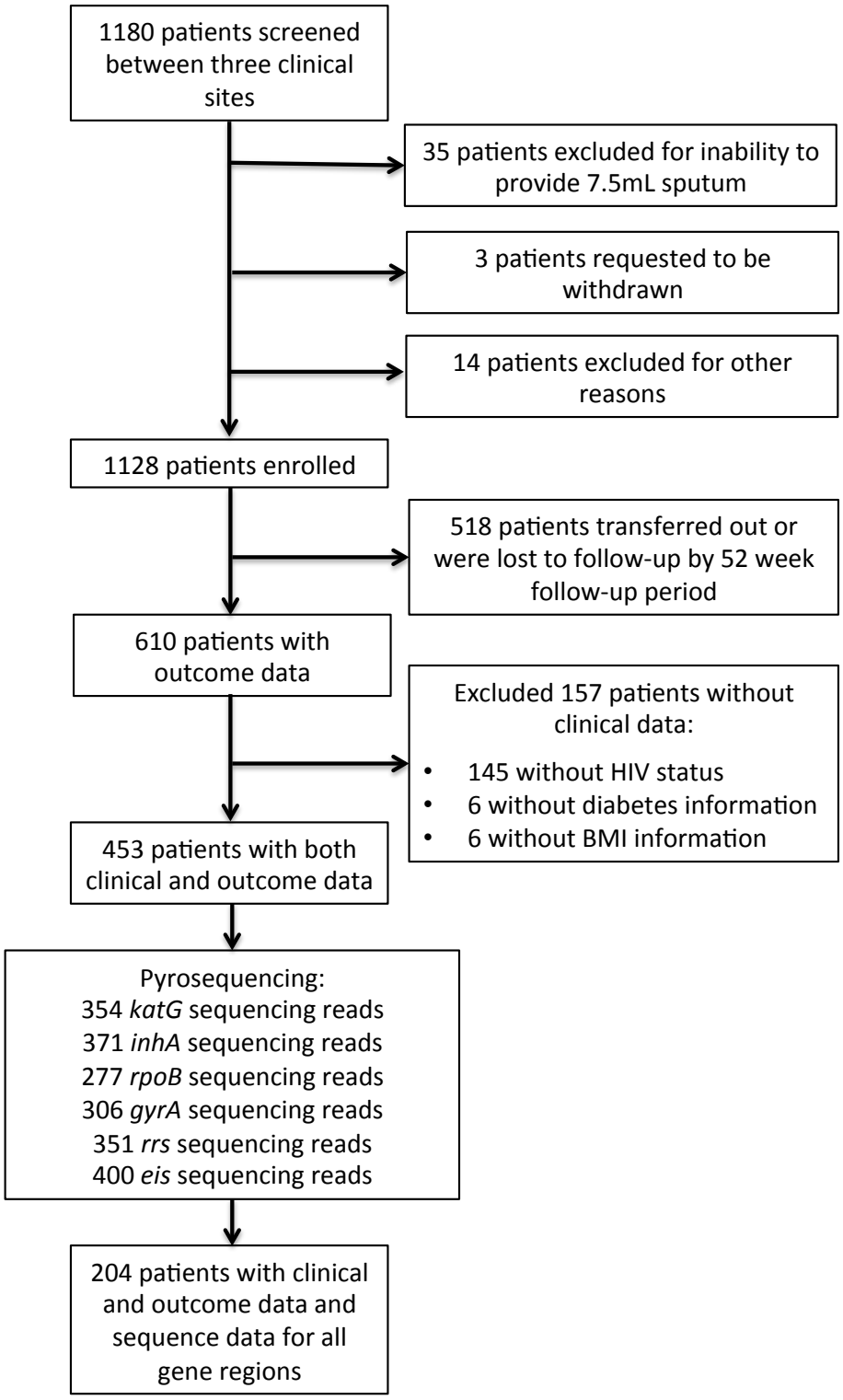

Figure 4.1: Schematic presentation of data availability for outcome analysis.

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CHAPTER 5: DISCUSSION

OVERVIEW

The overall goal of this dissertation research was to determine the potential for molecular diagnostics to replace conventional, growth-based diagnostics for DR-TB. The following aims were addressed by this research: (1) assess the diagnostic and technical performance of a molecular diagnostic assay for M/XDR-TB between three diverse clinical sites, (2) determine the frequency and distribution of TB resistance mutations between these sites, and (3) determine the clinical relevancy of TB resistance mutations in a multisite, observational cohort study. These dissertation results include two important main findings. First, although assay diagnostic and technical performance was high overall, findings identified specific areas for improvement. Second, different gene mutations were found to be reliable, predictive markers of patient clinical outcome.

Specifically, in Chapter 2 (Aim 1), findings showed that among a multinational sample of M/XDR-TB patients, the site-specific sensitivity of the pyrosequencing assay ranged from 89-98% for the detection of INH, RIF, FQ, and injectable-resistance (in India) to 7-94% (in Moldova), though specificity was high (94-100%) across all sites. The addition of *eis* promoter sequencing capability to the platform greatly improved the sensitivity of KAN resistance detection in Moldova (7 to 79%), demonstrating how regional factors can impact molecular diagnostic performance, and how open-format platforms can easily be updated to address these variations and accommodate our evolving knowledge of TB resistance mechanisms. Additionally, although the platform was able to provide sequencing information for a large portion (70.8%) of smear-negative, culture-positive specimens, 25% of all

sequencing reactions failed to generate an interpretable sequencing read [1]. In addition to smear- and culture-negativity, site (Moldova) and sequencing of the *rpoB*, *gyrA* and *rrs* gene targets were found to be highly associated with poor technical performance in this study, presenting diagnostic developers with specific targets and variables for optimization to further improve platform technical performance.

In Chapter 3 (Aim 2), the distribution of TB resistance mutations was not found to be equal between clinical sites. Notably, INH-resistant specimens in South Africa had a lower frequency of *katG* mutations (44%) and a higher frequency of *inhA* (47%) and *ahpC* (10%) promoter mutations. Additionally, the *rrs* 1401G mutation, which confers AMK, KAN and CAP resistance, was only identified in 7% of the KAN-resistant specimens in Moldova. Instead, *eis* promoter mutations appeared at a higher frequency (64%) in the KAN-resistant specimens evaluated in Moldova. The inclusion of the *ahpC* and *eis* promoter gene regions was determined to be critical for optimal diagnostic test sensitivity for the detection of INH resistance in the Eastern Cape and KAN resistance in Moldova. This finding has critical implications for the diagnostic performance of diagnostics that rely upon these low-frequency molecular markers in these regions, and provides evidence of the need for open-format, easily adaptable diagnostic technologies to further accommodate evolving knowledge of TB resistance mechanisms.

In Chapter 4 (Aim 3), detection of a single *rrs* 1401G mutation was found to be associated with significantly higher odds of patient mortality, even after adjusting for relevant patient characteristics and other resistance mutations, compared to those with *rrs* wildtype sequences. When genetic mutations were then characterized according to established phenotypic resistance levels, detection of mutations associated with high-level FQ resistance or KAN resistance were found to be

significantly associated with higher odds of patient mortality, compared to patients with genotypically susceptible infections. These associations held for high-level *gyrA* and *rrs* resistance mutations even following adjustment for clinical site, patient age, BMI, diabetes, HIV status, and the presence of other resistance mutations. Results imply that these high-level, second-line resistance mutations may be the most reliable predictors of patient outcomes for diverse, DR-TB patient populations.

Overall, this research finds an M/XDR-TB molecular diagnostic to demonstrate high diagnostic and technical performance in comparison to conventional diagnostic methods, while identifying specific gene targets and variables for further assay optimization. The analysis of the frequency and distribution of TB resistance mutations provides additional information to help diagnostic developers to prioritize TB resistance mutations for inclusion in their assays to further optimize diagnostic performance. Finally, these dissertation results provide strong evidence of the clinical relevancy of specific molecular markers of resistance that will help clinicians interpret molecular assay results and quickly direct appropriate treatment of DR-TB patients. In the context of a diverse DR-TB patient population, these findings promote the use of M/XDR-TB molecular diagnostics in lieu of, or in conjunction with, phenotypic culture DST results.

IMPLICATIONS

Rapid Molecular Diagnostic Development and Performance

The specific areas for DR-TB molecular diagnostic assay performance improvement identified by this research will have significant implications for the optimization of current diagnostics and development of new rapid M/XDR-TB molecular diagnostics. Although the diagnostic assay evaluated in this dissertation

demonstrated high performance, overall, in comparison to phenotypic testing in one of the largest, multisite diagnostic studies to date [1], important reductions in assay sensitivity were identified between three diverse clinical sites. These performance lapses could be significant, as demonstrated by the poor sensitivity of the assay (7%) for the detection of KAN resistance in Moldova. However, even large platform sensitivity lapses could potentially be overcome with the addition of new gene targets, as demonstrated with the addition of *eis* promoter sequencing capabilities to the pyrosequencing assay. The addition of other resistance-associated gene targets, such as *tlyA, gidB,* and other regions of the *rrs* gene, might be necessary to further improve diagnostic assay sensitivity for the detection of injectable resistance in different clinical environments [2]. The same is true for other mutations associated with FQ resistance, such as *gyrB* mutations [3], which may have helped sensitivity estimates in Moldova, or mutations outside of the *katG* gene region evaluated, which may have improved sensitivity for INH resistance detection in South Africa in this study [4]. These diagnostic performance lapses are notable, as they suggest that diagnostics that include all known TB resistance markers, or open-format molecular diagnostics, which allow for the introduction of new gene targets, would likely be the best options for rapid DR-TB detection in diverse clinical environments.

Another current limitation of many molecular diagnostics is their technical performance, or their ability to obtain a result, due to such factors as bacterial load in the initial patient sample, site-specific factors such as technician training, or molecular factors such as DNA inaccessibility due to formation of higher-order structures. The detailed analysis of the technical performance of the sequencing diagnostic in this dissertation identified variables significantly associated with poor technical performance of a molecular diagnostic. Although the relationship between smear- and

culture-negativity and poor molecular assay technical performance has been well established [5-9], the ability of the tested assay to sequence a large portion of smear-negative clinical samples in this study underscored the utility of molecular diagnostics for a diverse range of clinical samples. This is important, as many laboratories lack the sterile conditions or equipment necessary to perform smear, culture and phenotypic DST of *Mtb*, and a large portion of TB infections remain smear-negative despite clinical and radiological signs of disease [10]. For these reasons, molecular diagnostics present valid alternatives to conventional growth-based diagnostic methods. These technologies can potentially provide the clinician with information about a portion of smear-negative infections when a diagnosis is otherwise elusive, as long as the results are considered in the context of the patient's clinical presentation and past and current TB treatment regimens [11].

The identification of specific problematic gene targets for DR-TB molecular diagnostic assay technical performance improvement will have significant implications for the design and optimization of all diagnostics that assay these sequences. Notably, sequencing of the 81bp RIF resistance-determining region (RRDR) of the *rpoB* gene was determined to be the most problematic gene target included in the sequencing diagnostic assay evaluated in this study. This gene target is necessary for RIF detection worldwide, as 95% of RIF-resistant *Mtb* strains have a mutation in this gene region [12-14]. Unfortunately, there are known higher-order DNA structures present in the *Mtb* genomic DNA at the RRDR, preventing DNA access and therefore resulting in a high rate of PCR and/or sequencing failure [15]. As this gene region is a critical target for all M/XDR-TB diagnostics, findings have important implications for other diagnostic assays, including GeneXpert and the line probe assays, which also query this gene region. Findings recommend further optimization of the PCR and

sequencing conditions of the *rpoB* and other gene-targets, including the resistance-determining *gyrA* and *rrs* gene regions included in the pyrosequencing assay, for the benefit of all M/XDR-TB molecular diagnostics.

Dissertation findings that TB resistance mutations of low global frequency can contribute substantially to molecular diagnostic performance in different geographical regions will also help diagnostic assay developers in designing high-performing assays. Although *Mtb ahpC* and *eis* promoter resistance mutations appear at low frequencies globally [2, 4], and so make a minor contribution to molecular diagnostic sensitivity for INH and KAN resistance detection, they made substantial contributions to assay performance in geographical areas where they appeared at a higher frequency. The *eis* promoter mutation -12T, for example, was found in 53% of KAN-resistant strains in Moldova without co-occurring *rrs* mutations, while the *ahpC* promoter mutations were identified in 9% of INH-resistant specimens without co-occurring *katG* or *inhA* mutations, making them substantial contributors to diagnostic assay sensitivity estimates in those regions. If these mutations are excluded from molecular diagnostic tests, such as the first-generation line probe assays, then those assays would be expected to experience significant decreases in sensitivity in these geographical regions. If DR-TB diagnostic assays are not open-form, allowing for flexibility in resistance detection with the easy addition of new targets, then DR-TB diagnostics that include all known resistance-associated mutations will likely remain the best options for optimal sensitivity for DR-TB detection.

Clinical Relevance of Tuberculosis Resistance Mutations

Genetic mutations associated with high-level second-line (FQ and injectable) drug resistance were found to be reliable predictive markers of patient mortality in the

GCDD study. This finding has important implications for the interpretation of molecular test results, though future clinical studies should work to confirm the independent associations between these mutations and clinical outcomes by establishing MICs for each specimen and accounting for difference in drug treatment regimens.

This finding also has broader implications for the potential for molecular diagnostics to replace conventional phenotypic diagnostics for DR-TB. Notably, the finding of significant associations between specific TB resistance mutations and patient mortality outcomes shows that molecular diagnostic results can potentially provide clinicians with additional information about DR-TB infections that would not be provided by binary culture testing results (which are either resistant or susceptible). In other words, it is important to know whether a patient has a high- or low-level resistance mutation, for that knowledge may help clinicians to direct appropriate treatment of DR-TB patients and save patient lives. In this way, DR-TB molecular diagnostics might have an immeasurable advantage over conventional, growth-based TB diagnostics.

LIMITATIONS

Generalizability of Results

All results presented in this study should be considered specific to GCDD study populations in the large cities of Mumbai, Chisinau and Port Elizabeth, and not necessarily the countries of India, Moldova or South Africa. In Chapters 2 and 3 (Aims 1 and 2), it is possible that observed variations in mutation frequencies, and therefore molecular diagnostic performance, between the sites may be representative of localized DR-TB outbreaks or the persistence of endemic drug-resistant clones in

these locales. However, results highlighted a diversity of DR-TB strains with unique genetic combinations, suggesting that the vast majority of studied infections were not clonal. These results are noteworthy if they reflect true regional differences, rather than local outbreaks, as these genetic variances will affect the performance of rapid molecular diagnostic technologies in larger regions.

In Chapter 4 (Aim 3), patients in Mumbai had significantly higher odds of mortality than patients in Chisinau or Port Elizabeth, even after the adjustment of relevant clinical variables that may explain this relationship. Although it is likely that the observed association between clinical site and patient mortality was related to differences in the proportions of patients being enrolled under different enrollment criteria, this could not be confirmed, and differences in patient quality of care or treatment between sites could have played a significant role in patient mortality. These additional variables were not measured in the GCDD study, and so could not be accounted for in the presented analyses.

Gold Standard: Phenotypic Drug Susceptibility Testing

In the analysis of the diagnostic performance of pyrosequencing for M/XDR-TB detection (Aim 1), and in determining the frequency of TB resistance mutations among resistant specimens between clinical sites (Aim 2), conventional, growth-based DST methods were used as the gold standard. However, DST standards are somewhat controversial and variable, and critical concentrations for M/XDR-TB drug compounds are still under debate [16, 17]. In order to minimize any bias introduced in the GCDD study by an imperfect reference standard, all study sites were asked to perform the same DST methods and re-testing procedures, using the established critical concentrations for M/XDR-TB drugs of interest. However, if errors were made

by phenotypic DST methods in this study, then the reporting of sensitivity and specificity for the diagnostic pyrosequencing platform (Aim 1) may have been biased. Gene-based tests for DR-TB are typically more sensitive for TB than culture methods, and so there is the possibility that pyrosequencing performed better than the gold standard in many instances. For example, when pyrosequencing generated sequencing data for culture-negative specimens, this data was not included in sensitivity and specificity calculations, and so the diagnostic performance of the technology may have been underestimated in the presented analysis.

Sequencing Coverage

The pyrosequencing diagnostic platform was limited in its ability to provide a complete genetic profile of the DR-TB specimens evaluated in the GCDD study. Although the mutations identified via pyrosequencing provide a larger picture of the genetic basis of TB phenotypic drug resistance, the inclusion of other gene regions, such as novel *katG* and *fabG1* mutations recently associated with INH resistance [18], may further increase the sensitivity of rapid molecular diagnostics for DR-TB detection. Additional sequencing studies investigating other genes and gene regions, such as *tlyA* and *gidB* mutations and their association with injectable resistance [2], will be necessary to further improve the diagnostic performance of DR-TB molecular diagnostics (Aim 1, Chapter 2) and identify the genetic basis of drug resistance for the 4-16% genetically wildtype, drug-resistant specimens in the GCDD study (Aim 2, Chapter 3). This limitation could also have a significant impact on analyses in Chapter 4 (Aim 3), as M/XDR-TB drug resistance was not associated with mutations occurring outside of the *katG, inhA, rpoB, gyrA, rrs* and *eis* gene regions sequenced, meaning that the assay could have failed to identify important molecular markers of TB drug

resistance in many patients. If these markers were common in the genetically wildtype patients that died in the GCDD study, then assay failure to sequence these additional gene regions may have biased results towards the null by adding these higher-risk individuals to the reference category.

Prospective Analyses

There was substantial loss to follow-up in the GCDD study prior to the 52-week follow-up period, which limited analyses in Chapter 4 (Aim 3). Altogether, less than half of the original patient population enrolled in the study had clinical outcome data. It is possible that these patients may have been sicker than the final population available for analysis, and had unconfirmed deaths prior to the follow-up period, which might bias results towards the null if these lost patients had associated M/XDR-TB resistance mutations. Despite the high number of patients without clinical outcome data, however, the study had adequate power to confirm significant associations between *gyrA* and *rrs* high-level resistance mutations and patient mortality. There is the additional limitation in that many patients did not have, or did not share, relevant clinical information such as HIV or diabetic status. As these variables were deemed critical to the final analysis, 157 patients without this information were dropped from all analyses. The inclusion of these additional patients may have increased the power of analyses to confirm the associations between particular mutations with wide confidence intervals, such as *gyrA* low-level resistance mutations, and patient mortality outcomes. Furthermore, there was the additional limitation that these prospective analyses did not account for drug treatment data. If drug treatment regimens varied between the investigated mutation groups in this study, then our failure to include this important variable would have biased our results. The direction

in which results would be affected would depend upon which groups (the susceptible/reference category or a resistance level category) were treated differently, and whether or not the different treatment was appropriate.

FUTURE RESEARCH IN GLOBAL HEALTH

In addition to the need for further studies on the performance of M/XDR-TB molecular diagnostics in diverse clinical environments and clinical outcome studies comparing outcomes of patients whose treatment decisions were made based on phenotypic DST to those made based on genotypic DST, future research aims regarding M/XDR-TB molecular diagnostics might focus upon new diagnostic technology development and optimization, assay deployment and implementation in diverse clinical environments, and identify any factors that prevent widespread access to M/XDR-TB molecular diagnostics.

CONCLUSIONS

This dissertation research finds a rapid M/XDR-TB molecular diagnostic to demonstrate good overall technical and diagnostic performance in a large, multisite study while identifying specific gene targets necessary for high diagnostic sensitivity in diverse clinical environments. Results suggest that rapid molecular diagnostics can serve as valuable predictors of TB drug resistance in lieu of, or in absence of, phenotypic culture testing results, while pinpointing areas for further assay improvement. Results also suggest that molecular diagnostics can provide valuable information regarding patient mortality risk in diverse DR-TB patient populations, as certain high-MIC mutations associated with second-line drug resistance appear to be clinically relevant. Findings provide a larger picture of the relevancy of TB molecular

markers as predictors of phenotypic drug resistance in different environments, highlight the clinical relevancy of molecular markers of second-line drug resistance, and suggest that open-format, rapid DR-TB molecular diagnostics have strong potential to replace phenotypic tests in the near future as predictors of drug resistance and clinical outcome.

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