Defining the Role of High-dose Isoniazid in the Treatment of Multi-Drug Resistance Tuberculosis: Isoniazid Resistant Profiling

By

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PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Centre for the AIDS Programme for Research in South Africa (CAPRISA) BSL3 Tuberculosis Laboratory, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Dr. Navisha Dookie and Prof Kogieleum Naidoo.

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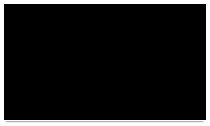

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DECLARATION

I, **Senamile Lale Ngema** declare that:

(i) The research reported in this dissertation, except where otherwise indicated is my original work.

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Signed:  Date: 8 December 2021

DEDICATION

To my family and friends, I could not have done this without you. Thank you for the support through my Masters journey.

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AMK	Amikacin
ART	Antiretroviral therapy
BDQ	Bedaquiline
BMI	Body mass index
CAPRISA	Centre for the AIDS Programme for Research in South Africa
CAP	Capreomycin
CB	Clinical breakpoint
CC	Critical Concentration
CFZ	Clofazimine
CLSI	Clinical and Laboratory Standards Institute
CS	Cycloserine
DLM	Delamanid
DR-TB	Drug-Resistant Tuberculosis
DST	Drug susceptibility Testing
DS-TB	Drug Susceptible Tuberculosis
EMB	Ethambutol
ETH	Ethionamide
FQ	Fluoroquinolone
GU	Growth Unit
HIV	Human Immune Deficiency Virus
INH	Isoniazid
INHhd	High-dose Isoniazid
INH-NAD	Isoniazid- Nicotinamide adenine dinucleotide
INH S	Isoniazid susceptible
KAN	Kanamycin
LFX	Levofloxacin
LPA	Line Probe Assay

LZD	Linezolid
MIC	Minimum inhibitory concentration
MFX	Moxifloxacin
MDR-TB	Multidrug Resistant Tuberculosis
MGIT	Mycobacterial Growth Indicator Tube
M.tb	Mycobacterium Tuberculosis
NAATs	Nucleic Acid Amplification Tests
NADH	Nicotinamide adenine dinucleotide
NAT2	*N*-acetyl transferase type-2
OADC	Oleic Albumin Dextrose Catalase
PANTA	Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin
PAS	para-aminosalicylic
PTO	Prothionamide
PZA	Pyrazinamide
RIF	Rifampicin
RR-TB	Rifampicin Resistant Tuberculosis
SLID	Second Line Injectable Drugs
SL	Second line
STREAM	Standardized Treatment Regimen of Anti-tuberculosis Drugs for Patients with MDR-TB
STR	Streptomycin
TB	Tuberculosis
TRZ	Terizidone
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug resistant

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ABSTRACT

Background: High-dose isoniazid is recommended in short-course regimens for multidrug-resistant tuberculosis (MDR-TB). However, there is no substantial evidence supporting its use in the presence of INH resistant mutations. Therefore, this study aimed to establish the efficacy of INH in the presence INH resistance associated mutations.

Methods: We selected 94 clinical isolates obtained from 65 patients from the IndEX (CAP020) study specimen biorepository. Isolates were selected based on whole genome sequencing results showing evidence of INH resistant conferring mutations. Twenty-one isolates had *inhA* promoter gene and/ *inhA* coding region mutations, 35 had *katG* mutations, and 20 had both *inhA* promoter and/ *inhA* coding region plus *katG* mutations. Additionally, 18 INH susceptible clinical isolates were included in this analysis. Minimum inhibitory concentrations (MICs) were done in different concentration ranges depending on the mutation present. INH susceptible and H37Rv (0.016-0.256) µg/ml, *inhA* (0.256-4.0) µg/ml, *katG* (1.0-16.0) µg/ml and *inhA* plus *katG* (4.0-16) µg/ml.

Results: Among 94 isolates, 36 were excluded: 11 MPT64 antigen negative, 23 non-growers and two were contaminated. Fifty-eight isolates from 55 patients were left for analysis. Eleven isolates had *inhA* mutations, 23 *katG* mutations, 12 had double mutations in *inhA* and *katG*, and 12 were INH susceptible. MICs obtained varied within isolates ranging from 0.016 to >64.0 µg/ml. *InhA*, *katG*, *inhA* plus *katG* mutations and INH susceptible isolates had median INH MIC of 8.0 (4.0-64.0), 4.0 (95% CI, 4.0-8.0), 64.0 (95% CI, 64.0-64.0), and 0.48 (95% CI, 0.32-1.0) µg/ml, respectively, confirming the association between INH MICs and genotypic profile. The MDR-TB and pre/XDR-TB had median INH MIC of 8.0 (95% CI, 8.0-32.0) and 48.0 (4.0-64.0) µg/ml, respectively. We found association between cavitary disease and increase in INH MICs for *inhA* mutants, median of 64.0 (64.0-64.0) µg/ml, and previous TB history and increased INH MICs (8.0[95% CI, 8.0-64].

Conclusion: This study demonstrated highly variable MIC range with significant overlap in MIC range among the mutant groups. Furthermore, *inhA* mutants demonstrated unexpectedly high MICs raising a concern for the ongoing use of the high-dose INH in our setting. Our findings suggest that the current one-size-fits all approach to MDR-TB short-course regimen requires urgent review.

CHAPTER 1

LITERATURE REVIEW

1.1 Tuberculosis

Tuberculosis (TB) is an infectious disease caused by infection with *Mycobacterium tuberculosis* (*M.tb*), though it can affect any organ in the body, it predominantly affects the lungs (1). TB is an airborne infection and is easily transmitted via aerosols. *M.tb* is an effective intracellular pathogen belonging to the *Mycobacteriaceae* family. It is an aerobic, non-motile organism, called a tubercle bacillus. When an individual inhales the organism they get infected, which may result in either latent infection or active TB disease (2). People who are latently infected with TB develop immune responses to *M.tb*, however, they do not present with clinical symptoms of TB and can remain in this state for years (3). On the contrary, active TB infection results in several symptoms as weight loss, fever and persistent coughing and is clinically known as active TB (1).

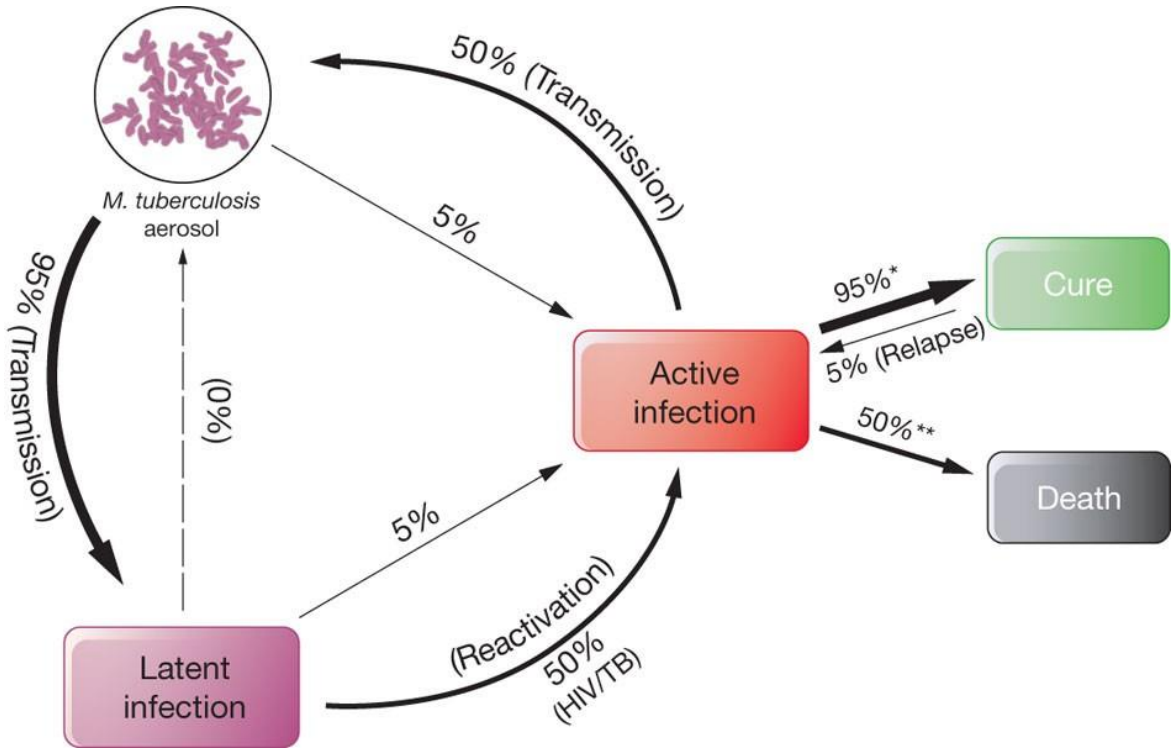


Figure 1.1 *Mycobacterium tuberculosis* infection manifested at different stages. The transmission of *M. tuberculosis* aerosols can result in active TB disease or non-infectious (latent) TB disease. Majority of people (95%) are latently infected, however, years later, 50% of these people may have reactivation resulting in the active disease. Latent TB reactivation commonly arises in patients with immune suppression and is common with HIV. People infected with drug-susceptible (DS)-TB have a high chance of cure (95%) after treatment, only a minority (5%) relapse. The mortality rate among those patients who remain untreated is as high as 50% (4).

1.2 Epidemiology

TB remains a significant global public health concern, leading to high morbidity and mortality rates (5, 6). According to the World Health Organization (WHO), 9.9 million incident cases of TB cases were reported in 2020 and more than 1.3 million people died from the disease. Of the 1.3 million deaths, 214 000 deaths were among Human Immunodeficiency Virus (HIV) TB co-infected patients (7), this was a 10% increase from 2019 (1). Despite great progress in curbing the TB epidemic over the last two decades, efforts to control TB are threatened by the ongoing transmission of drug-resistant TB (DR-TB) (8). In 2020, WHO reported 132 222 cases of rifampicin-resistant/ multidrug-resistant TB (RR-TB/MDR-TB) i.e resistance to at least isoniazid (INH) and rifampicin (RIF). Among these patients 25 681 cases had additional resistance to fluoroquinolones (FQ) or second-line injectable drugs (SLIDs) presenting with pre- extensively drug-resistant (XDR)-TB or XDR-TB (7, 9). INH resistant incident cases reported in 2019 were 1.4 million, of which 79% were INH mono-resistant (10). This represented the most significant type of resistance overall. South Africa remains among a few countries globally with a TB incidence exceeding 500 cases per 100 000 population with approximately 328 000 cases of TB reported during 2020 (7). South Africa features prominently among the 30 high-burden TB countries with significantly overlapping epidemics of TB, HIV and MDR-TB. During 2020, 6 784 and 733 cases of laboratory-confirmed RR/MDR-TB and XDR-TB were reported in South Africa, respectively. The overall treatment success rate was 79% for drug-susceptible TB, 65% for RR-TB/ MDR-TB and 57% for XDR-TB (based on 2019 cohort data) (11).

1.3 Diagnosis of *Mycobacterium tuberculosis* and drug-resistant Tuberculosis

There are four predominant methods for the diagnosis of TB; these include chest x-ray, sputum smear microscopy, molecular and culture-based tests (12). Sputum smear is one of the oldest front-line diagnostic techniques that is still used to date to detect acid-fast bacilli (AFB) directly from the sputum. This is a simple and cheap technique, but its sensitivity is limited to the number of bacilli available in the sample and results are subject to interpretation by the reader which can result in variability of results (12, 13). The presence of *M.tb* can also be determined from clinical specimens using various solid and liquid culture media such as (i) Lowenstein – Jensen and (ii) Middlebrook 7H10/11 and (iii) Middlebrook 7H9 (14). The disadvantage of using solid culture media is the turn-around time; it takes 3 -6 weeks for *M.tb* growth and isolation (15). Remarkable progress in improving TB diagnostics has been made in the past decades. The cultivation and susceptibility testing of *M.tb* has evolved from solid culture to automated BACTEC - Mycobacteria Growth Indicator Tube [MGIT] and other liquid media (14). Availability of liquid media for

culture have substantially reduced the time of growth detection, with growth reported within 10-20 days (16).

Since 2010, molecular assays based on the GeneXpert technology known as Xpert MTB/RIF and Ultra have been used to diagnose TB and detect resistance to rifampicin (17). This is a polymerase chain reaction based technique that detects the presence of *Mt.b* and resistance to RIF from a clinical specimen (18). The transition to a new next-generation Xpert MTB/RIF (Ultra) cartridge has shown better sensitivity in *M.tb* detection (19, 20). Molecular assays are highly sensitive and specific, require minimal labour and have a rapid turnaround time (17, 21, 22). The limitation for Xpert is the false positivity among recently treated TB patients and that it can only detect resistance to RIF, not other first-line drugs (12, 23). Subsequently, patients that are presenting RIF resistance are subjected to second-line drug resistance testing using line probe assay (LPA). GenoType MTBDR*plus* assay (LPA1) can detect RIF and INH resistance allowing the diagnosis of MDR-TB (24). Additionally, GenoType MTBDR*sl* assay (LPA2) detects FQ resistance and SLIDs, allowing diagnosis of pre-XDR and XDR-TB (18, 24). However, WHO has recently updated the definition of XDR-TB, whereby, MDR-TB with additional resistance to a FQ, bedaquiline (BDQ) and/ linezolid (LZD) now constitutes XDR-TB. This limits the relevance of LPA2 to the detection of FQ resistance. LPA has a few limitations such as limited probes/limited to specific mutations; only high confidence mutations can be detected, secondly, it cannot detect resistance to new drugs and repurposed drugs such as BDQ and clofazimine (CFZ) which are now widely used (25). There is a recent significant update on molecular assays, (i) Moderate complexity automated Nucleic Acid Amplification Tests (NAATs), for detection of *M.tb* and RIF and INH resistance; (ii) Low complexity automated NAATs for INH resistance detection and second-line injectable; (iii) High complexity hybridization-based NAATs for the detection of resistance to pyrazinamide (PZA) (26). Another promising technique is whole-genome sequencing (WGS) and targeted sequencing technology has shown great promise, is highly sensitive and specific in detecting resistance compared to conventional methods (27). This technique allows the detection of a complete drug resistance profile for known resistance-conferring mutations (27, 28), however, it is limited by our understanding of the mechanisms that mediate resistance to drugs and thus, is currently limited for novel drugs.

Moreover, phenotypic methods have remained a gold standard for TB drug resistance detection for decades, with the ability to differentiate resistance from susceptible clinical isolates for different anti-TB agents (29). However, a major limitation with this method is that the result turn-around is 4-6 weeks, impacting rapid initiation of treatment (30). In addition, phenotypic DST requires highly-skilled personnel, strictly biosafety containment laboratory infrastructure and results are not always reproducible (29). However,

despite apparent limitations, a combination of phenotypic and genotypic assays are still currently in use to provide a comprehensive DST.

1.4 Treatment of Drug-Resistant Tuberculosis: The current guidelines

The treatment and management of DR-TB has evolved significantly in the past decade with the introduction of new and repurposed drugs. While DS-TB can be successfully treated with first-line drugs (INH, RIF, PZA and ethambutol (EMB)) achieving success rates of up to 85% (1), their use in DR-TB is limited. The treatment of DR-TB requires the use of a complex second-line drug regimen for a longer duration to eradicate *M.tb*. Over the last five years, DR-TB treatment has evolved drastically with the introduction of a short-course regimen of 9-12 months for RR/MDR-TB and a recent transition to the injection-free regimens (31, 32). The introduction of a short-course treatment arose from the results of the short-course "Bangladesh" regimen (31). These patients were closely monitored during the intensive phase of the treatment as they were hospitalized. Among patients that received a Bangladesh shorter treatment regimen, the relapse-free cure reported was 84.4% (33). Reported treatment failure and relapse among 11 patients was associated with the high-level FQ resistance, compounded by background PZA resistance. Nevertheless, the overall results showed the potential of this regimen as the alternative treatment option. Following the success in Bangladesh, other African countries i.e. Niger and Cameroon evaluated this regimen and reported treatment success >85% (34, 35). This regimen was further evaluated in a randomized clinical trial; STREAM (Standardized Treatment Regimen of Anti-tuberculosis Drugs for Patients with MDR-TB) study, conducted in seven countries (Ethiopia, Georgia, India, Republic of Moldova, Mongolia, South Africa and Uganda). The STREAM study compared this regimen with the 18-24 months conventional regimen. Main findings from the STREAM study, showed that the effect of the shorter regimen was non-inferior to the longer regimen (80% vs 79%) (36). Recently, the role of second-line injectable drugs in DR-TB treatment have been downgraded, due to the increased risk of treatment failure and relapse associated with their use in longer MDR-TB regimens (37). The injectable has been replaced with BDQ (38), however, some patients still receive the injectable based regimen (39). The current standard treatment regimens recommended by WHO are presented in Table 1.1. Patients who are not eligible for the standard short-course regimen are treated using longer BDQ-based regimens. These patients include those that have been previously exposed to RR/MDR-TB regimen for more than a month, present with extensive pulmonary disease or cavities and present with both *inhA* and *katG* mutations (39). In addition, successful treatment is impacted by factors such as patient adherence, adverse drug reactions, comorbidities, potential drug-drug interactions and patient tolerability (40).

Table 1.1 Current standardised regimens for the treatment of drug-susceptible and Rifampicin Resistant/ Multidrug-resistant Tuberculosis.

Treatment regimen	Composition	Duration
DS-TB standard regimen	(2) INH-RIF-EMB-PZA/ (4) INH-RIF	4-6 months
Standard short-course regimen - Injection containing	(4) AMK-MFX-CFZ- INHhd -PTO-EMB-PZA/ (5) MFX-CFZ-EMB-PZA	9-12 months
Standard short-course regimen - All oral	(4-6) BDQ-LFX (or MXF)- LZD, INHhd-CFZ-EMB-PZA/ (5) LFX-CFZ-EMB-PZA	9-12 months
Standard longer regimen – all oral	(6) BDQ-LFX-CFZ-LZD/(12) LFX-CFZ-LZD	18-20 months

Abbreviations: AMK, amikacin; BDQ, bedaquiline; CFZ, clofazimine; DS-TB, drug-susceptible TB; EMB, ethambutol; INH, isoniazid; INHhd, high-dose isoniazid; LFX, Levofloxacin; LZD, linezolid; MFX, moxifloxacin; PTO, prothionamide; PZA, pyrazinamide; RIF, rifampicin

1.5 Isoniazid – an overview and mechanism of action

INH is a synthetic derivative of nicotinic acid displaying anti-mycobacterial activity and is one of the most important drugs used in the treatment of TB (41, 42). INH was first synthesized in 1912, but its anti-TB properties were discovered and reported in 1952 (42). The bactericidal activity of INH is selective and specific to the species of mycobacteria and the *M.tb* complex (43). Despite the seemingly simple structure of INH, its mode of action is one of the most complex of all antibiotics because it interferes with nearly every metabolic pathway in *M.tb*. INH displays potent early bactericidal activity against actively growing *M.tb* (44). It is a pro-drug, which enters through passive diffusion into *M.tb* cytoplasm. It requires activation by catalase-peroxidase, an enzyme encoded by the *katG* gene found in *M.tb* (45). This is followed by binding the enoyl-acyl carrier protein reductase encoded by the *inhA* gene, inhibiting mycolic acid synthesis (44, 46). Due to its high bactericidal activity, remarkable intracellular penetration, high bioavailability and low cost, INH has been one of the excellent antimicrobial agents (47). The drug currently forms a key component in DS-TB regimens, displaying high bactericidal activity during the intensive phase of treatment (44).

1.6 Mechanisms of Isoniazid resistance: Genotypic resistance

Resistance to INH is complex and results from mutations found in various genomic loci (48). These mutations include *katG*, *inhA*, *inhA* promoter gene (*mabA*), *ahpC* (49–51). However, resistance to INH is mediated mainly through mutations in two regions, *katG* and *inhA* promoter genes (52, 53). The most predominant mutation, S315T, in the *katG* gene (44, 45), accounts for approximately 90% of INH resistant isolates (50, 54). This mutation results in INH pro-drug failing to form the INH-nicotinamide adenine dinucleotide (INH-NAD) adduct required for its mechanism of action (41, 55). Various reports have associated S315T mutation with a high level of resistance to INH and are frequently reported in MDR-TB strains (55). On the contrary, *InhA* promoter region mutations result in overexpression of *inhA* and reduce the affinity of INH-NAD adduct (46). The most prevalent *inhA* mutation is found in position -15C/T and is associated with low-level resistance. Moreover, *inhA* mutations confer cross-resistance to ethionamide (ETH) as they share the same target (49, 56). Mutations in *ahpC* gene encoding for alkyl hydroperoxidase reductase was initially thought to be associated with INH resistance and mutations in its promoter region was assumed a marker of INH resistance (57). Subsequently, it was confirmed that these mutations are compensatory mutations for the loss of catalase-peroxidase resulting from *katG* mutations rather than resistance mutations (58). Compensatory evolution is a phenomenon defined as acquiring the second mutation that compensates for the fitness cost resulting from the original mutation (59). This mechanism result in *M.tb* increased fitness without interfering with the resistant phenotype (60). In context with INH, compensatory evolution results when a second mutation occurs in the *ahpC* regulatory region, resulting in the overexpression of alkyl hydroperoxidase reductase, compensating for the fitness cost resulted from *katG* mutation (58).

1.7 Mechanisms of Isoniazid resistance: Phenotypic resistance

Phenotypic resistance is confirmed using culture-based DST, which tests for critical concentration (CC). CC is defined as "the lowest concentration of an anti-TB agent *in vitro* that will inhibit the growth of 99% of phenotypically wild type strains of *M.tb* complex" (61). In its latest technical manual for DST, WHO recommends INH critical concentration values using various media. The recommended critical concentration values are 0.2 µg/ml in Lowenstein-Jensen, 0.2 µg/ml in Middlebrook 7H10, 0.2 µg/ml in 7H11, and 0.1 µg/ml in BACTEC MGIT liquid culture (29). For the past decade, Clinical and Laboratory Standards Institute (CLSI) has been using two concentrations to stratify drugs such as INH into low and high-level resistance (62). According to CLSI the concentrations used to stratify the low-level and high-level INH resistance is 0.1 and 0.4 µg/ml, respectively (62). To date, WHO has not adopted this

recommendation from CLSI, as they still recommend DST at CC of 0.1 µg/ml in MGIT and 0.2 µg/ml in solid media for low-level INH resistance (29). Isolates with mutations in *the inhA* gene are associated with an approximately ten-fold increase in minimum inhibitory concentration (MIC) corresponding to 0.25-2 µg/ml (63). Isolates bearing *katG* mutations are associated with an 80-fold increase in MIC corresponding to 1–16 µg/ml. Isolates with a combination of both mutations have a 320-fold increase in MIC, corresponding to 8–64 µg/ml. This is indicative that *katG* mutations are associated with a wide range of overlapping MICs, presenting a moderate to high level of phenotypic resistance.

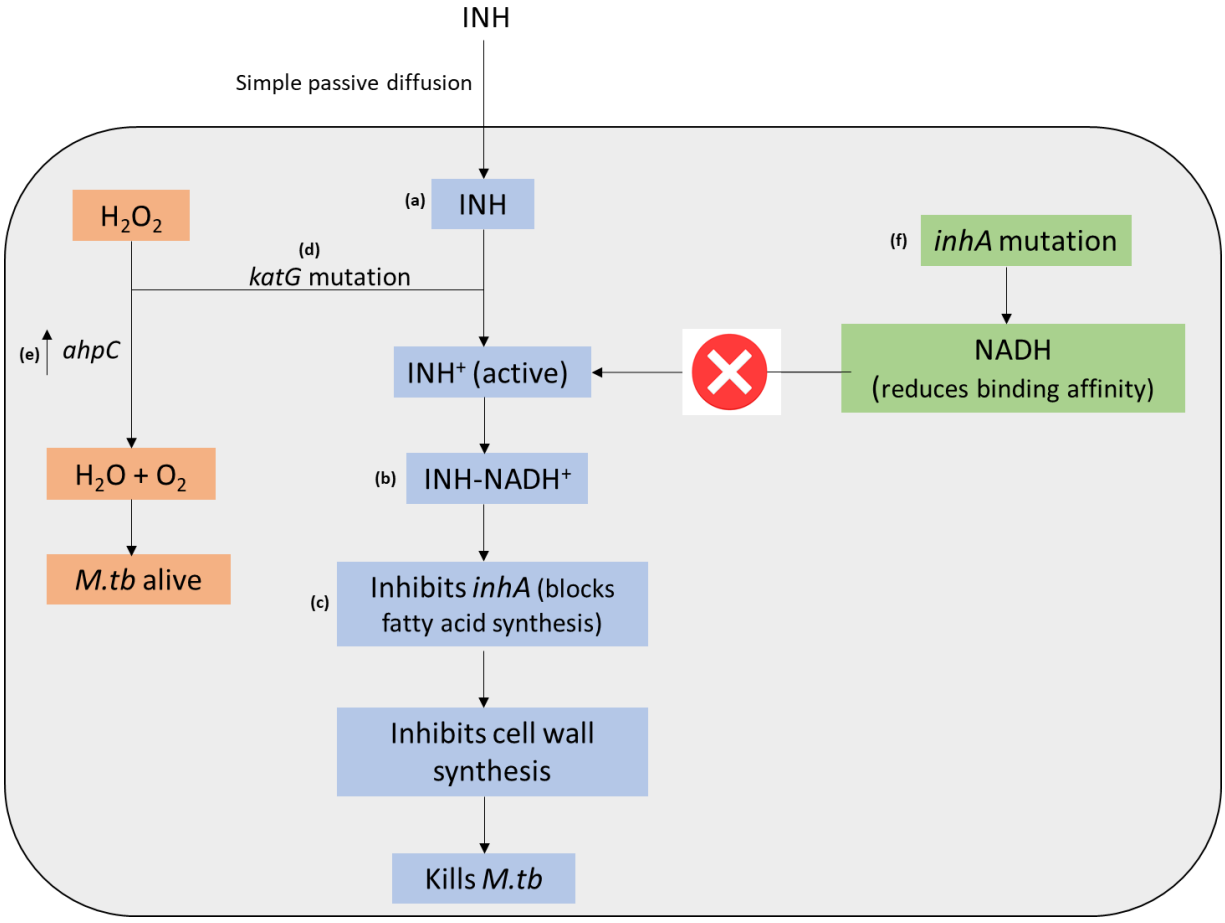


Figure 1.2 An overview of INH mechanism of action and resistance in *Mycobacterium tuberculosis*. (**a**) INH enters through simple passive diffusion and gets activated by catalase-peroxidase, encoded by *katG*. **(b)** Activated INH/isonicotinyl radical interacts with nicotinamide adenine dinucleotide (NADH) in the presence of *inhA*, which is involved in the synthesis of mycolic acid, to form INH-NADH, (**c)** thereby inhibiting the synthesis of mycolic acid. (**d)** In contrast when resistance rise in the bacterium due to mutations in the *katG*315, the drug remains inactivated and INH-NADH adduct cannot be formed, therefore *inhA* is able to continue with mycolic acid synthesis leading to the survival of the bacterium. (**e)** Further, the occurrence of the compensatory mutation in the *ahpC* gene, results in the overexpression of *ahpC* which

takes up the function of *katG* of decomposing hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2), hence the bacterium survives. (**f)** Additionally, the mutations in the *inhA* results in the reduced binding affinity of NADH to form INH-NADH, hence mycolic acid synthesis takes place and the bacterium survives. (Figure adapted from Unissa *et al*., 2016) (64).

1.8 Current Evidence for high-dose Isoniazid in Multi-drug Resistant Tuberculosis treatment

The use of high-dose INH has been debated ever since its introduction to the short-course regimen. To date, evidence on its use against drug-resistant strains of *M.tb* is lacking, but the recommendation remains the same. In response to the endorsement of standard course regimen containing high-dose INH, a study in the Republic of Moldova, evaluating first-line resistance found 2638/4570 (58%) strains were resistant to INH and RIF indicating MDR-TB (65). Additionally, 2323/2638 (88%) of MDR-TB strains had *katG* S315T mutation. According to the authors, this was the indication of high-level resistance as per TBNET/RESIST-TB consensus statement (66) and strongly advocated against the use of high-dose INH as part of the standardised regimen in their setting as it will offer no benefit. In contrast, other reports suggested that the presence of *katG* mutation alone should not lead to the exclusion of INH within regimens (67, 68). Rieder *et al*. reported that *katG* mutations have variable resistance levels and those moderate levels of resistance can still be treated with high-dose INH. The authors concluded that depriving patients of the effective drug in the absence of evidence may be unnecessary (67). Otto-Knapp and co-workers defined moderate level resistance by MICs of >1 and <5 µg/ml on solid media (68), however, there is no official standard to define moderate level INH resistance (69). Furthermore, a study on 52 *M.tb* strains with *katG* mutations reported an MIC range of 4-32 µg/ml on solid media, with most isolates demonstrating a 30-fold increase compared to the clinical breakpoint (CB) of 0.2 µg/ml (70). Similarly, Lempens and co-workers reported that *katG* mutation is associated with the low-moderate resistance level with MIC range of 3.2-19.2 µg/ml (71). In addition, *katG* mutation in combination with *inhA* mutation was associated with a high resistance level with MIC ≥ 19.2 mg/l. Most MICs from *katG* mutation were below serum peak (15 mg/l) upon the recommended dose of 15 mg/kg (5). It is believed that INH can still be applicable or have activity when used in a treatment regimen. When both mutations were present MIC was above serum peak (≥19.2 mg/L), making it difficult for INH to work even at the higher dose (71).

Several studies conducted a retrospective analysis to establish the efficacy of high-dose INH among patients treated with the regimen and its impact on treatment outcomes. A study conducted in Haiti examined the effect of INH high-dose in MDR-TB treatment among HIV-negative patients. Of a total 187 patients, 99

patients received high-dose INH. Among those that received high-dose INH, 22/99 (25%) were given a dose of 18-21mg/kg, and 20/99 (20%) were given 12.1-15.9 mg/kg (72). Patients who received high-dose INH had rapid culture conversion compared to those who did not (7.0 weeks (95% CI, 6.3-8.3 vs 9.1 weeks (95% CI: 7.9-10.4) ($P < .001$) (72). These results suggested that adding high-dose INH in the regimen (FQ, kanamycin (KAN), cycloserine (CS), ETH, and PZA) was effective in MDR-TB treatment. However, the limitation of this study was that they were unable to determine specific mutations linked to INH resistance.

Previous studies reported treatment outcomes greater than 85% in MDR-TB patients given standardised treatment with high-dose INH (73). These results were later confirmed by other researchers in Bangladesh and other African countries (74), where the use of short-course MDR-TB regimen with high-dose INH was associated with high cure rates (33–35), previously discussed under current treatment guidelines. In a double-blind, randomised controlled trial, Katiyer and co-workers reported faster culture conversion in patients receiving high-dose INH compared to those on the standard dose (75). Patients had 2.38 times faster sputum negative (95% CI 1.45-3.91, $p = 0.001$) and showed 2.37 times (95% CI 1.46-3.84, $p < 0.001$) potential of obtaining sputum-negative results at six months, thus meeting the primary outcome of the study. These results showed that including high-dose INH could improve the treatment outcomes for MDR-TB (72). Furthermore, in a study of 65 patients who received a high-dose INH based regimen, findings showed cure in 58/64 (90%) patients, death in 6/65 (8%) patients and 1/65 (2%) default, in a six-month follow-up (34). Additionally, 49/65 (75%) patients remained culture-negative.

1.9 Rationale, aims and objectives

1.9.1 Rationale

Increasing prevalence rates in TB drug resistance coupled with the paucity of novel antibiotics warrants exploration of new approaches for treating TB. One such approach is exploring the use of higher drug dosing to overcome low-level resistance. This has been formally endorsed in the case of MFX, whereby a daily dose of 800mg compared to the standard dose of 400mg can be used to treat low-level resistance to FQ. Similarly, the possibility of leveraging INH for high-dose treatment is appealing, given its potent early bactericidal activity, limited pharmacokinetic interactions and well-established safety profile. Mutations described in the key targets for INH resistance, the *inhA* and *katG* genes, lead to low to moderate increases in MIC, reported in the range of 0.2-2 µg/ml and 1-16 µg/ml, respectively. However, INH displays dose-dependent early bactericidal activity, thus, high doses may result in exposures that overcome resistance mediated through these mutations. Several clinical trials have demonstrated improved time to culture conversion, improved treatment outcomes and successful implementation of short-course all oral treatment with the inclusion of high-dose INH. However, the independent bactericidal effect of high-dose INH on *M.tb* isolates with and without INH resistance mutations remains uncertain. Thus, the current study will assess the efficacy of high-dose INH among *M.tb* isolates with various commonly occurring INH resistance-conferring gene mutation profiles. The study will provide valuable insights on the role of INH in DR-TB, one of our most valuable therapeutic options.

1.9.2 Aim

- To characterise the efficacy of INH in *M.tb* clinical isolates with *inhA*, *katG* and both *inhA* and *katG* mutation profiles

1.9.3 Objectives

- To establish the INH MICs of clinical isolates with *katG* with or without *inhA* mutations from the InDEX study (CAP020)
- To observe whether the MICs of *M.tb* clinical isolates fall within the selected concentration ranges for INH resistant categories
- To correlate the MICs with genotypic profile, clinical characteristics and previous TB history

1.9.4 Hypothesis

INH resistance in clinical isolates of *M.tb* will have an overlapping MIC distribution amongst isolates with all three INH-mutation profiles; *viz*. *inhA* mediated resistance, *katG* mediated resistance or a combination of both.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study design

This study was performed on stored clinical isolates obtained from the CAPRISA 020 InDEX study. The InDEX study is an ongoing randomised controlled trial assessing the effectiveness of an individualised treatment regimen for DR-TB (NCT03237182). Patients were recruited and treated at the CAPRISA Springfield Research Site located at the King Dinuzulu Hospital in KwaZulu-Natal, Durban. The study included participants who were 18 years and older, presenting with drug-resistant pulmonary TB. Patient demographic and clinical data was collected at enrollment. As part of the study procedures the following tests were performed at the Centre for Tuberculosis at the National Institute of Communicable Diseases: Xpert MTB/RIF, LPA, DST at critical concentration (CC), and whole genome sequencing (WGS). Additionally, culture DST for treatment response monitoring was done as per national guidelines at the National Institute of Communicable Diseases. All other tests and procedures were administered according to the national standard of care guidelines, including, body mass index (BMI), chest radiography, HIV testing and monitoring. Patients received individualised treatment regimens based on the WGS result. Written informed consent for study participation and provision of samples for storage and analysis was obtained from patients prior to enrolment. Ethical approval was obtained from the University of KwaZulu-Natal Biomedical Research Ethics Committee (BREC REF NO: BFC584/16) and (BREC/00001449/2020) (Appendix 1 and 2).

2.2 *Mycobacterium tuberculosis* clinical isolates

A total of 94 *M.tb* clinical isolates were requested from the IndEX (CAP020) study specimen biorepository. Isolates were selected based on the presence of INH resistant conferring mutations based on the WGS results, which was the part of CAP020 study. The 94 clinical isolates were from 65 patients (some patients had multiple isolates from follow up visits). Twenty-one isolates had *inhA* promoter gene and/ *inhA* coding region mutations, 35 had *katG* mutations, and 20 had both *inhA* promoter and/ *inhA* coding region and *katG* mutations. In addition, 18 clinical isolates were INH susceptible. H37Rv was added as a reference strain.

2.3 Reviving of *Mycobacterium tuberculosis* isolates

2.3.1 Reconstitution of PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin)

All laboratory assays were performed in a biosafety cabinet in the Biosafety level 3 TB laboratory. The lyophilized MGIT PANTA was reconstituted with 15.0 ml MGIT growth supplement containing OADC (Oleic acid, Albumin, Dextrose and Catalase); PANTA-OADC solution was mixed until completely dissolved. This mixture was stored at 2-8 °C and used within five days.

2.3.2 Inoculation of the Isolates in Mycobacterium Growth Indicator Tube Medium

Each MGIT tube was labelled with corresponding specimen numbers. The selected *M.tb* isolates for this study were thawed at room temperature. In each MGIT tube, 800 µl of reconstituted PANTA solution was added. Thereafter, 100 µl of the thawed isolate was added to the respective tubes. For quality control, 100 µl of the H37Rv strain was added to the control MGIT tube.

2.3.3. Incubation in the Mycobacterium Growth Indicator Tube instrument

Following inoculation, MGIT tubes were incubated in the BACTEC MGIT 960 (Becton Dickinson (BD), New Jersey, USA) instrument. The instrument maintains a temperature of 37 °C. These MGIT tubes were incubated until the instrument flagged them positive (following the protocol length of 42 days). Positive MGIT tubes were scanned out and observed visually (*M.tb* growth is granular and settled at the bottom of the tube while contaminating bacteria appeared turbid) (Figure 2.1).

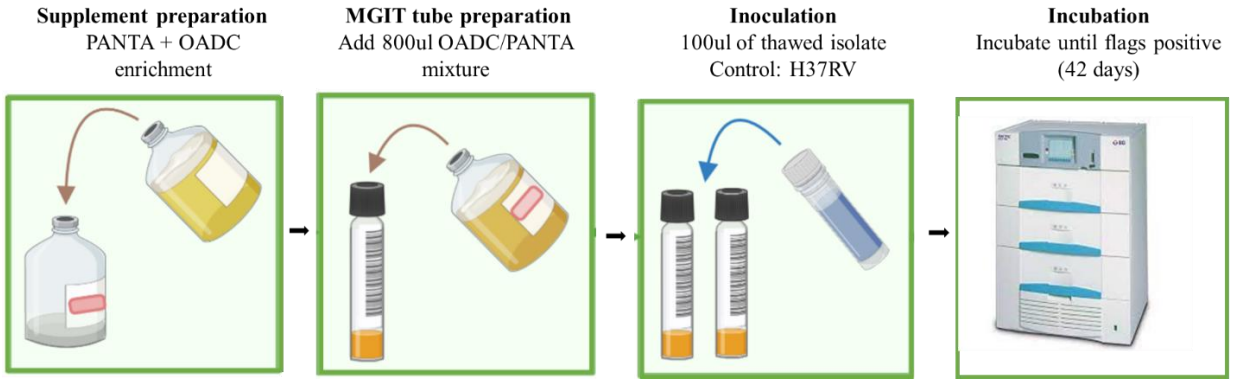


Figure 2.1 Flow diagram for preparation of reviving *Mycobacterium tuberculosis* isolates

2.4 *Mycobacterium tuberculosis* speciation testing

2.4.1 Becton Dickinson Mycobacterium Growth Indicator Tube Tuberculosis complex identification test

The BD MGIT TB complex identification test [(TBc ID) Becton, Dickinson and Company, Sparks, MD, USA] was used to detect *M.tb* complex antigen as per manufacturers guidelines (76). TBc ID is a rapid immunochromatographic assay for the qualitative detection of MPT64 antigen (MPT64 Ag). Briefly, each testing device was brought to the safety cabinet and placed flatly on the surface. The inoculated MGIT tube was mixed thoroughly by inverting, thereafter 100 µl of sample was added into the sample well and left to stand at room temperature for 15 minutes. Every batch had a positive (H37Rv) and negative control (uninoculated MGIT).

2.4.2 Kinyoun Staining (cold method)

Upon a positive result detected by the TBc ID test, Kinyoun staining was performed to observe roping on AFB. Smear: the positive MGIT was mixed by inverting; the loopful was taken from the tube and spread on the glass slide with the AFB smear fixative. The slides were placed on a hot plate set at 70 °C for one hour under UV light. Staining: (i) The smear was flooded with carbol fuchsin for five minutes and rinsed with water until the water became clear, (ii) The slide was flooded with acid alcohol for one minute thereafter the slide was rinsed with water until the water became clear, (iii) Lastly the slide was flooded with methylene blue for three minutes, and it was rinsed until the water became clear (Figure 2.2). The slide was air-dried before viewing using microscopy at 1000× oil immersion.

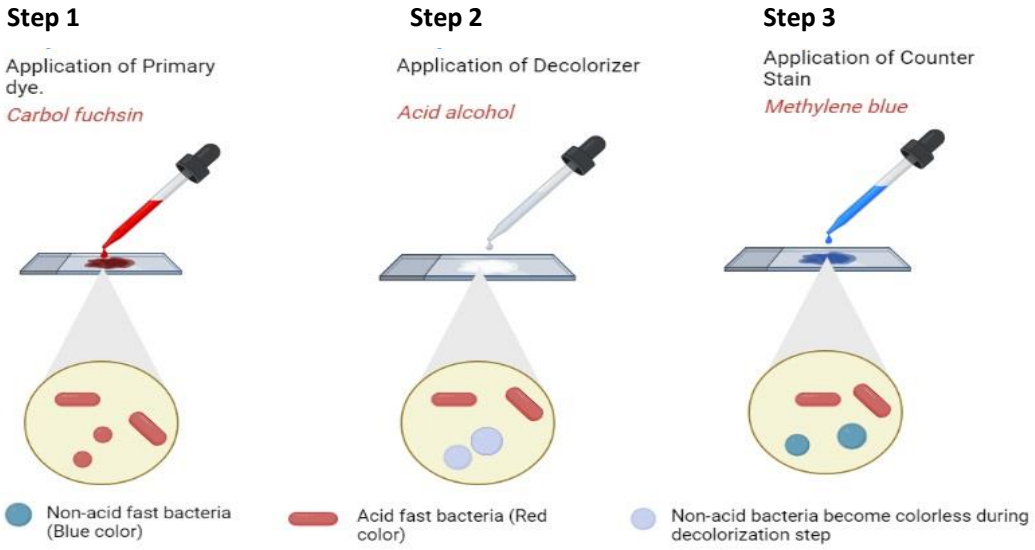


Figure 2.2 Kinyoun Staining (cold method).

2.4.3 Detection of contamination from the Positive Mycobacterium Growth Indicator Tubes

To ensure that the positive MGIT is due to *M.tb* and not other contaminants, a loopful from the tube was sub-cultured in the trypticase soy agar supplied with 5% sheep blood plate. The plate was divided into two equal parts, and each part was labelled with a correct specimen number. Two specimens were carefully inoculated on a plate in their corresponding specimen number (small streak for each specimen). These subcultures were incubated at 37 °C and observed after 24-48 hours. Those that show no contamination proceeded for MIC testing.

2.5 Minimum Inhibitory Concentration Testing

2.5.1 Isoniazid Stock Solutions

Table 2.1 Displays the various stock solutions (ready to use), volume added to the MGIT tube and their desired concentration in the MGIT tube. The stock concentrations were purchased commercially from Media Mage Company Pty Ltd.

Anti-TB drug	Stock concentration received	Volume added to MGIT tubes for test	The concentration of the drug in the MGIT tube
INH	1.344 µg/ml	100 µl	0.016 µg/ml
INH	2.688 µg/ml	100 µl	0.032 µg/ml
INH	5.376 µg/ml	100 µl	0.064 µg/ml
INH	10.752 µg/ml	100 µl	0.128 µg/ml
INH	21.504 µg/ml	100 µl	0.256 µg/ml
INH	43.008 µg/ml	100 µl	0.512 µg/ml
INH	84 µg/ml	100 µl	1.0 µg/ml
INH	168 µg/ml	100 µl	2.0 µg/ml
INH	336 µg/ml	100 µl	4.0 µg/ml
INH	672 µg/ml	100 µl	8.0 µg/ml
INH	1344 µg/ml	100 µl	16.0 µg/ml
INH	2688 µg/ml	100 µl	32.0 µg/ml
INH	5376 µg/ml	100 µl	64.0 µg/ml
INH	10752 µg/ml	100 µl	128.0 µg/ml

MICs were done in serial 2-fold dilutions from INH stock solution to provide a final test range of; 0.016-0.256 µg/ml for INH susceptible strains, 0.256-4.0 µg/ml for *inhA* promoter mutant isolates with/without a concurrent *inhA* coding mutation, 1.0-16.0 µg/ml for *katG* (S315T/N) mutant isolates; 4.0-64.0 µg/ml for isolates with double mutations (*inhA* promoter + *katG*). Those isolates that showed resistance at the highest concentration tested within their range were repeated at a higher range.

2.5.2 Registering the Specimen on the Epicenter/TB EXist

The Epicentre software (version V7.45A) with the TB Exist module was installed and connected to the MGIT instrument to read MICs results. Each specimen was registered with its unique identity number, and

the specific test for the specific drug and concentrations was selected. i.e INH low (growth control, 0.016, 0.032, 0.064, 0.128, 0.256 µg/ml). Each concentration had its MGIT tube scanned; when all tubes were scanned for concentrations, the data was saved, and the labels for TB exist printed from the Epicentre printer. To ensure that the tubes were labelled correctly, the MGIT tube barcode was checked against the correct concentration of the drug (concentration must correspond with the correct sequence of the MGIT tube).

2.5.3 Inoculum preparation from Pure Positive Mycobacterium Growth Indicator Tubes

The first day of the instrument positive MGIT tube was considered as day zero, and no MICs were done on this day; only quality control tests were done (refer to section 2.4). For the preparation of the test inoculum, a positive MGIT tube was used the day after it first became positive on the MGIT instrument, which was recorded as day one, up to including day five after the instrument had flagged positive. If the tube was day one or day two positive, MGIT broth suspension was used for the inoculation procedures. It was mixed well and proceeded to "inoculation procedure for MIC". If the tube was day three, day four, or day five positive, it was mixed well then diluted. A positive tube longer than five days was re-subcultured to a fresh MGIT tube containing the reconstituted PANTA solution and incubated in the MGIT instrument (refer to section 2.3.2 - 2.3.3) until positive.

2.5.4 Procedure for dilutions

The 1:5 dilution of the inoculum was prepared by taking 1.0 ml of suspension from the positive MGIT tube and adding it into 4.0 ml of normal saline (**Tube 1**). The second dilution of 1:100 was prepared by adding 100µl of previously made dilution into 9.9ml of normal saline (**Tube 2**). The 1:100 dilution has a low yield and was used for the growth control.

2.5.5 Inoculation procedure for Minimum Inhibitory Concentration testing

Each isolate had six MGIT tubes (a growth control tube and five tubes for two-fold serial dilution concentrations). 800 µl of OADC was added to each tube. Aseptically, the appropriate drug concentration was added to the appropriately labelled tube. No drug was added to the growth control. 500 µl of the 1:100 dilution was added to the tube labelled "growth control". For drug-containing tubes, 500 µl of the organism suspension or 1:5 dilution was added to all the drug-containing MGIT tubes. The tubes were closed tightly

and mixed thoroughly by gentle inversion 3-4 times. The tubes were entered into the BACTEC MGIT instrument.

2.5.6. Minimum Inhibitory Concentration determination

The determination of MICs was performed within the epicentre/TB Exist and MGIT 960 instrument. This was done by filtering the reports on epicentre/TB Exist. The minimum and the maximum days of the protocol was four and 24 days, respectively, and the minimum for the growth units (GU) for the control was 400. A test isolate whose growth control reached 400 GU before four days or 400 GU after 24 days was recorded as a growth control fail, and the MIC testing was repeated. The growth unit of 0-99 was considered susceptible, and ≥100 was deemed to be resistant. In a series of 2-fold concentrations tested, the first concentration with a growth unit less than 100 was recorded as the MIC. Those isolates that showed resistance in all tested concentrations within their range were repeated at higher concentrations.

2.6 Statistical analysis

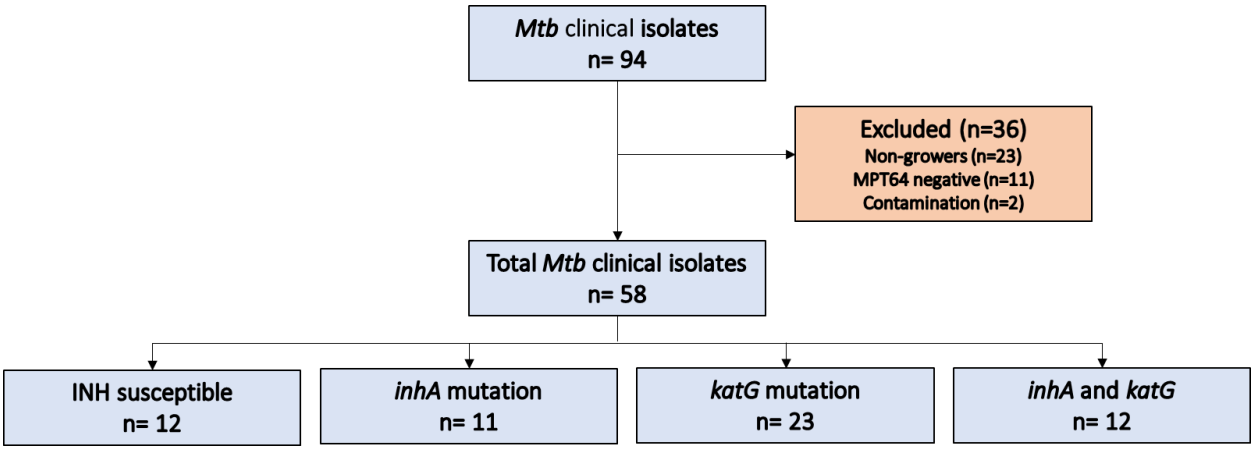
Data was captured on Excel for Microsoft Office® version 16.11.1 (Redmond, WA, USA). Descriptive statistics was used to analyze demographic data, such as age, sex and BMI in IBM SPSS Statistics for Windows, Version 27. Clinical data with MICs were analyzed using custom tables and chart builder with a summary statistic of median, 95% lower and upper confidence interval for the median.

CHAPTER 3

RESULTS

3.1 *Mycobacterium tuberculosis* clinical isolates

Of 94 *M.tb* clinical isolates from the 65 patients that were selected in this study, 36 clinical isolates were excluded: 11 negative MPT64 antigen and non-roping AFB (the roping AFB vs non-roping AFB is presented in Appendix 3), 23 non-growers and two were contaminated. The final sample size included in this analysis was 58 isolates from 55 patients and was further stratified based on INH mutation type (Figure 3.1).



Abbreviations: *M.tb:* Mycobacterium tuberculosis; INH: isoniazid

Figure 3.1. Flow diagram of *Mycobacterium Tuberculosis* clinical isolates analysed in this study.

3.2 Patient demographic and clinical characteristics

All patient information (demographic, medical history other than TB, HIV status, viral load, CD4 count, X-ray results) was extracted from electronic medical records (DFdiscover Data Management) from the CAP020 study database. Fifty-five patients with confirmed active TB were included in this study. The mean age was 34.8 ± 9.9 years (range, 19-59 years). Twenty-six (47.3%) patients were male and 29 (52.7%) were female. Based on HIV status, 37 (67.3%) patients were positive, of which 27 (73%) were on antiretroviral therapy (ART). Among the 27 patients on ART, one had DS-TB (3.7%), two RIF mono-resistant (7.4%), one INH mono-resistance (3.7%), 18 MDR-TB (66.7%) and five (18.5%) pre-XDR/XDR-TB. Seventeen (30.9%) patients had previous history of TB; 24 (43.6%) and 30 (54.5%) patients showed bilateral and unilateral disease on chest X-ray, respectively. Demographic and clinical information is presented in Table 3.1.

Table 3.1 Patients baseline demographic and clinical characteristics**.**

Characteristics	Patients at baseline (n=55)
Mean age (range) (years)	34.8 (19-59)
Sex - total no. (%)	
Female (%)	29 (52.7)
Median BMI (kg/m^2) - (range)	20.5 (14.2-29.5)
Co-morbidities no. (%)	
HIV positive no. (%)	37 (67.3)
Diabetes	04 (7.3)
Hypertension	02 (3.6)
Epilepsy	01 (1.8)
Peripheral neuropathy	02 (3.7)
Asthma	02 (3.6)
TB diagnosis by LPA no. (%)	
RIF mono-R	10 (18.8)
MDR-TB	35 (64.2)
Pre-XDR/XDR-TB	09 (17.0)
[#]Previous TB history (%)	17 (30.9)
Sputum culture positive week 8 no (%)	11 (20.0)
Chest X-ray (%)	
Bilateral	24 (43.6)
Unilateral	30 (54.5)
Lung abnormalities no. (%)	
Cavities	29 (52.8)
Infiltrates	45 (81.8)
Pleural disease	01 (1.9)
Pleural effusion	01 (1.9)

[#] All had previous drug-susceptible tuberculosis

Abbreviation: BMI: body mass index; HIV: human immunodeficiency virus; LPA, line probe assay; MDR-TB: multi-drug resistant tuberculosis; RIF mono-R: rifampicin mono-resistant; pre-XDR/XDR-TB, pre-extensively drug-resistant tuberculosis

3.3 Highly variable Isoniazid Minimum Inhibitory Concentrations with overlapping distribution between Isoniazid susceptible and resistance-conferring mutations

Isolates obtained from the patients were grouped based on INH resistant mutations: INH susceptible group (n=12), *inhA* mutation group (n=11) *katG* mutation group (n=23), and both *inhA* and *katG* mutation group (n=12). 9/12 INH susceptible isolates and the H37Rv reference strain had an INH MIC range of 0.016 to 0.064 µg/ml. The remaining three isolates fell out of the susceptible range with INH MICs of 1.0, 8.0 and 16.0 µg/ml. Isolates within the *inhA* group demonstrated a highly variable range of MICs. 3/11 isolates had MICs that fell within the expected ranges at 0.256 (n=2) and 1.0 µg/ml. The remaining isolates demonstrated high-level resistance, with four isolates recording MICs >64.0 µg/ml. The *katG* mutant group (n=23) fell within the expected range with MICs ranging between 1.0 and 16.0 µg/ml. Only one isolate recorded an MIC of 32.0 µg/ml. The group with both *inhA* and *katG* mutations (n=12) demonstrated high-level resistance with all MICs falling within the range of 16.0 to >64.0 µg/ml. INH mutants demonstrated a highly variable MIC range, demonstrated by a series of overlapping MIC distributions. Figure 3.2 shows the MICs of these clinical isolates based on their INH mutations. The median INH MICs for INH susceptible isolates, *inhA*, *katG*, and combination of *inhA* and *katG* mutants was 0.48 (95% CI, 0.32-1.0), 8.0 (95% CI, 4.0-64.0), 4.0 (95% CI, 4.0-8.0) and 64.0 (95% CI, 64.0-64.0) µg/ml, respectively (Table 3.2).

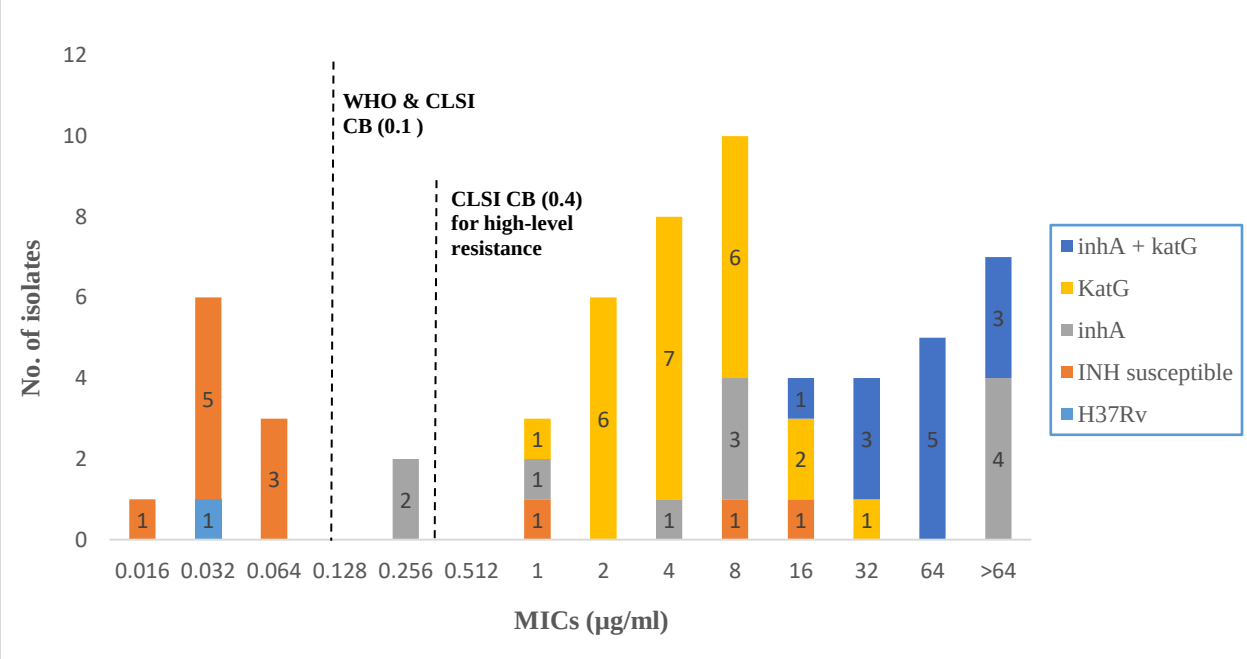


Figure 3.2 INH MIC distribution of 58 clinical isolates obtained from CAP020. MICs are presented separately for INH susceptible (no mutations linked to INH), *inhA* mutation, *katG* mutation and *inhA* +

katG mutations. (CB, clinical breakpoint; CC, critical concentration; CLSI, clinical laboratory standard institute; MIC, minimum inhibitory concentration

Table 3.2 The median INH MICs and 95% confidence interval (CI) for *Mycobacterium tuberculosis isolates*.

Mutation	**Median INH MIC (µg/ml)**	**95% CI (µg/ml)**
INH susceptible	0.48	0.32-1.0
inhA	8.0	4.0-64.0
katG	4.0	4.0-8.0
inhA plus *katG*	64.0	64.0-64.0

Abbreviation:INH, isoniazid; MIC, minimum inhibitory concentration.

Table 3.3 Detailed resistance data, INH MIC data, treatment regimen initiated and week 8 culture conversion data for 55 patients.

Patient	Resistance Profile	INH Mutation Profile	INH MIC (ug/ml)	Additional TB drug Resistance	Past TB history	DR-TB Treatment Regimen Initated	Week 8 Culture Conversion
1	MDR	*inhA;* C-15T*	>64.0	RIF, PZA, EMB, STR	None	INHhd, PZA, MFXld, KAN, ETO	Positive
2	MDR	*katG*; S315T	8.0	RIF, PZA, EMB	DS-TB	INHhd, PZA, MFXld, KAN, ETO	Negative
3	MDR	*katG*; S315T	4.0	RIF, PZA	None	INHhd, PZA, MFXld, KAN, ETO, Rifabutin, CFZ	Negative
4	MDR[#]	*inhA;* C-15T*	8.0, 8.0, 8.0	RIF, PZA, EMB	DS-TB	INHhd, PZA, MFXld, KAN, ETO	Positive
5	DS	-	0.064	-	None	INHhd, PZA, EMB, MFXld, ETO, CFZ, BDQ	Negative
6	DS[ǂ]	-	0.032, 0.064	-	None	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Positive
7	MDR	*katG*; S315T	2.0	RIF, EMB, STR	None	INHhd, PZA, LFX, ETO, CFZ, BDQ	Negative
8	RIF + other	-	8.0	PZA, EMB	None	INHhd, PZA, EMB, MFXld, ETO, PTM, CFZ	Negative
9	MDR	*katG*; S315T	4.0	RIF, PZA, EMB, STR	None	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
10	MDR	*InhA*; T-8A* *katG*; S315T	64.0	RIF, PZA, EMB	None	PZA, EMB, MFXld, ETO, TRZ, CFZ, BDQ	Negative
11	MDR	*katG*; S315T	16.0	RIF, PZA, EMB, STR	None	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Negative

12	Pre-XDR	*InhA*; T-8A* *katG*; S315T	64.0	RIF, PZA, EMB, PAS, SLIDs	None	PZA, EMB, KAN, ETO, CFZ	Negative
13	Pre-XDR	*inhA;* C-15T*	>64.0	RIF, PZA, EMB, STR, FQ	DS-TB	PZA, EMB, MFXld, ETO, CS, CFZ	Positive
14	RIF-mono	-	0.032	-	DS-TB	INHhd, PZA, EMB, MFXld, KAN, ETO, CFZ	Negative
15	MDR	*katG*; S315T	4.0	RIF, STR	None	INHhd, PZA, EMB, MFXld, KAN, ETO, CFZ	Negative
16	MDR	*inhA;* C-15T*	0.256	RIF	DS-TB	INHhd, PZA, EMB, MFXld, KAN	Positive
17	Pre-XDR	*katG*; S315T	2.0	RIF, PZA, EMB, SLIDs	DS-TB	PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
18	MDR	*katG*; S315T	2.0	RIF, PZA, EMB, STR	None	INHhd, PZA, EMB, LFX, KAN, ETO, CFZ	Negative
19	MDR	*katG*; S315T	4.0	RIF, PZA, EMB, STR	None	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
20	MDR	*inhA;* C-15T*	1.0	RIF, PZA, STR	None	INHhd, PZA, MFXld, KAN, ETO, CFZ	Negative
21	MDR	*InhA*; T-8A* *katG*; S315T	64.0	RIF, PZA, EMB	None	INHhd, EMB, MFXld, KAN, ETO, CFZ	Negative
22	MDR	*katG*; S315T	2.0	RIF, STR	DS-TB	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
23	MDR	*katG*; S315T	8.0	RIF	DS-TB	PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
24	RIF mono	-	0.016	-	DS-TB	PZA, EMB, LFX, MFXhd, ETO, KAN, ETO, CFZ, BDQ	Negative

25	MDR	*katG*; S315T	8.0	RIF, EMB, STR	None	INHhd, PZA, EMB, KAN, ETO,CFZ, amoxicillin/clavulanate	Positive
26	RIF mono	-	0.032	-	DS-TB	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
27	RIF mono	-	0.032	-	None	INHhd, PZA, EMB, MFXld, KAN, ETO, CFZ	Positive
28	MDR	*InhA*; T-8A* *katG*; S315T	64.0	RIF, PZA, EMB	DS-TB	INHhd, PZA, LFX, MFXld, ETO, KAN, CFZ	Negative
29	MDR	*InhA*; T-8A* *katG*; S315T	32.0	RIF, PZA, EMB	None	PZA, EMB, MFXld, KAN, ETO, CFZ	Negative
30	MDR	*inhA;* C-15T*	>64.0	RIF, PZA, EMB, STR	None	INHhd, EMB, LFX, ETO, CFZ, BDQ	Negative
31	MDR	*InhA*; T-8A* *katG*; S315T	16.0	RIF, PZA, EMB	DS-TB	INHhd, PZA, LFX, ETO, CFZ, BDQ	Negative
32	MDR	*InhA*; C-15T* *katG*; S315T	32.0	RIF, PZA, EMB, STR	None	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
33	RIF mono	-	0.064	-	None	PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
34	RIF mono	-	16.0	-	None	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ, LZD	Negative
35	MDR	*katG*; S315T	8.0	RIF, PZA, EMB	DS-TB	INHhd, PZA, EMB, LFX, ETO, CFZ, BDQ	Negative
36	MDR	*katG*; S315T	2.0	RIF, PZA, EMB, ETO, STR	None	INHhd, PZA, EMB, LFX, LZD, CFZ, BDQ	Negative

37	RIF + other	-	1.0	EMB, STR	None	INHhd, PZA, EMB, LFX, LZD, CFZ, BDQ	Negative
38	MDR	*katG*; S315T	8.0	RIF, PZA, EMB, STR	None	INHhd, PZA, EMB, LFX, LZD, CFZ, BDQ	Negative
39	MDR	*katG*; S315T	1.0	RIF, PZA, EMB, STR	None	INHhd, PZA, RIF, LFX, LZD, BDQ	Negative
40	MDR	*InhA*; C-15T*	4.0	RIF, PZA, STR,	None	INHhd, PZA, EMB, LFX, CFZ, BDQ	Negative
41	MDR	*InhA*; C-15T* *katG*; S315T	>64	RIF, PZA, EMB	DS-TB	INHhd, PZA, EMB, LFX, LZD, CFZ, BDQ	Negative
42	MDR	*katG*; S315T	32.0	RIF, PZA, EMB, STR	None	EMB, LFX, LZD, CFZ, BDQ, DLM	Negative
43	RIF mono	-	0.032	-	None	LFX, TRZ, LZD, CFZ, BDQ	Positive
44	MDR	*InhA*; C-15T*	0.256	RIF, EMB	None	INHhd, PZA, EMB, LFX, LZD, CFZ, BDQ	Negative
45	MDR	*katG*; S315T*	16.0	RIF, STR, PAS	None	INHhd, PZA, EMB, LFX, LZD, CFZ, BDQ, PAS	Negative
46	Pre-XDR	*InhA*; T-8A* *katG*; S315T	64	RIF, PZA, EMB, STR, FQ	DS-TB	PZA, EMB, MFXld, ETO, KAN, CFZ	Negative
47	Pre-XDR	*InhA*; T-8A* *katG*; S315T	32.0	RIF, PZA, EMB, STR, FQ	None	PZA, EMB, MFXld, KAN, ETO, CFZ, BDQ	Positive

48	MDR	*katG*; S315T	8.0	RIF, PZA, EMB	None	INHhd, PZA, EMB, MFXld, CAP, ETO, CFZ	Negative
49	Pre-XDR	*InhA*; C-15T* *katG*; S315T	>64	RIF, PZA, EMB, FQ	None	PZA, EMB, MFXld, KAN, ETO, CFZ	Positive
50	XDR	*katG*; S315T	4.0	RIF, PZA, EMB, STR, FQ, SLIDs	None	PZA, EMB, MFXld, KAN, ETO, CFZ	Positive
51	INH mono	*katG*; S315T	4.0	-	None	INHhd, PZA, EMB, MFXld, KAN, ETO, CFZ	Negative
52	Pre-XDR	*InhA*; C-15T*	>64	RIF, EMB, STR, FQ	DS-TB	PZA, EMB, MFXld, KAN, ETO, CFZ	Negative
53	Pre-XDR	*katG*; S315T	4.0	RIF, PZA, EMB, STR, SLIDs	DS-TB	LFX, TRZ, LZD, CFZ, BDQ, PAS	Negative
54	Pre-XDR	*katG*; S315T	2.0	RIF, PZA, EMB, ETO, STR, FQ,	None	PZA, EMB, LFX, ETO, LZD, CFZ, BDQ, DLM, PAS	Negative
55	MDR-TB	*InhA*; T-8A* *katG*; S315T	>64	RIF, PZA	None	PZA, EMB, LZD, LFX, BDQ, TRZ	Negative

Abbreviation: DR-TB, drug-resistant tuberculosis; BDQ, bedaquiline; CAP, capreomycin; DLM, delamanid; DS, drug-susceptible; DS-TB, drug-susceptible tuberculosis; EMB, ethambutol; ETO, ethionamide; FQ; fluoroquinolone; INH, isoniazid; INHhd, high-dose INH; KAN, kanamycin; LFX, levofloxacin; LZD, linezolid; MDR, multidrug-resistant; MFX, moxifloxacin; MFXhd/ld, moxifloxacin high-dose/low-dose; PAS, *p*-aminosalicylic acid; PZA; pyrazinamide; RIF, rifampicin; SL, second-line; SLIDs, second-line injectable drugs; STR, streptomycin;

#Patient had three isolates, baseline, month three and six.

ǂPatient had two isolates, baseline and month two.

*Cross-resistance with ETO

3.4 Patients narrative based on resistance pattern, Isoniazid Minimum inhibitory Concentrations, previous TB history and week 8 culture conversion

Overall, among the 55 patients in the cohort, two were DS-TB (subsequently screened out of the main study), one was INH mono-resistant, nine were RIF resistant TB, 33 were MDR-TB and 10 were pre-XDR/XDR –TB (Table 3.3). Previous TB history did not impact MIC, however, the presence of additional resistance increased the MIC.

The two DS-TB patients displayed discordant results on the screening GeneXpert MTB/RIF test and LPA results at baseline compared to the WGS result obtained at study enrolment. This was likely due to a mixed-strain infection. MICs for these patients fell within the susceptible range. However, the subsequent isolate for patient six showed a 1-fold increase in MIC and the patient remained culture positive at week 8.

While the RIF resistant strains did not display any genotypic resistance to INH, we assessed the phenotypic resistance levels in relation to resistant isolates. Among the nine patients with RIF resistant TB, seven were RIF mono-resistant and one patient had additional resistance to STR and EMB and the remaining patient had additional resistance to EMB and PZA. 5/7 RIF mono-resistant isolates had an MIC of 0.032 µg/ml and 3/5 patients had a previous history of DS-TB. The remaining two RIF mono-resistant isolates had MICs of 0.064 µg/ml and 16.0 µg/ml, respectively with no previous TB history. The latter displaying approximately 7-fold higher than the WHO CC.

The remaining patients displaying RIF resistance with PZA and EMB resistance had an MIC of 8.0 µg/ml with no previous TB history and the patient with RIF resistance with STR and EMB had an MIC of 1.0 µg/ml with a previous TB history, both in the INH resistance range.

Among the MDR-TB patients, 7/33 (21.2 %) had mutations in the *inhA* gene, 18/33 (54.5 %) had *katG* mutations and 8/33 (24.3%) had a combination of both *inhA* and *katG* mutations.

The resistance patterns varied among the MDR-TB patients with 2 (6.1%) patients demonstrating no additional resistance, 1 (3.0%) MDR patient with EMB resistance, 2 (6.1%) MDR patients with STR resistance, 2 (6.1%) MDR patients with PZA resistance, 10 (30.3%) MDR patients with PZA, EMB and STR resistance, 10 (30.3%) MDR patients with PZA and EMB, 2 (6.1%) MDR patients with EMB and STR, 1 (3.0%) MDR patient with PZA, EMB, ETO and STR, and 1 (3.0%) MDR patient with STR and PAS. 3/7 patients in the *inhA* mutation group recorded MICs in the expected range of 0.25-2 µg/ml, with MICs of 0.256 (n=2), and 1.0 (n=1) µg/ml.

Patient 16 had an MIC 0.256 with a previous history of DS-TB and remained culture positive at week 8. Three patients within this group displayed high MICs of 4.0, 8.0 and >64.0 (n=2) µg/ml. Patient 1 had 320-

fold higher MIC than the expected MIC for *inhA* mutations and remained culture positive at week 8. Patient 4 had 40-fold higher MIC (4.0 µg/ml) with previous history of DS-TB and remained culture positive at week 8.

Patients within the *katG* group (n=18) displayed a highly variable range of MICs, one patient had an MIC of 1.0 µg/ml, four patients had an MIC of 2.0 µg/ml, four patients had an MIC of 4.0 µg/ml, six patients had an MIC of 8.0 µg/ml, two patients had an MIC of 16.0 µg/ml, one patient had an MIC of 32.0 µg/ml. Four patients had a previous history of TB but did not demonstrate any significant change in INH MIC. Patient 25 remained culture positive at week 8.

Among patients with both *inhA* and *katG* mutations, one patient had an MIC of 16.0 µg/ml, two patients had an MIC of 32.0 µg/ml, three patients had an MIC of 64.0 µg/ml, and two patients had an MIC of >64.0 µg/ml, as expected for double mutants. Patients 28, 31 and 41 had previous TB history.

Among pre-XDR patients, 2/9 (22.2%) had a mutation in the *inhA* gene mutation, 3/9 (33.3%) had *katG* mutation, and 4/9 (44.5%) had a combination of *inhA* and *katG* mutations. In 3 (33.3%) patients, pre-XDR resulted from SLID resistance, while in 6 (66.7%) patients it resulted from FQ resistance. 2/2 patients in the *inhA* group recorded an MIC of >64.0 µg/ml. Both patients (13 and 52) had previous history of DS-TB, but only patient 13 remained culture positive at week 8.

Among patients within *katG* group (n=3), two patients recorded an MIC of 2.0 µg/ml, and one patient had an MIC of 4.0 µg/ml. Patient 17 and 53 had previous history of TB

Among patients with both *inhA* and *katG* mutations (n=4), one patient had an MIC of 32.0 µg/ml, two patients had an MIC of 64.0 µg/ml, and one patient had an MIC >64.0 µg/ml. Patient 46 had previous TB history, further patient 49 remained culture positive at week 8.

One patient (patient 50) had XDR-TB, and this patient displayed *katG* mutation and recorded the MIC of 4.0 µg/ml, and remained culture positive at week 8

3.5 Resistance pattern, Isoniazid Minimum inhibitory Concentrations and regimens initiated among patients that remained culture positive at week 8

Patient 6 with DS-TB remained culture positive at week 8, the baseline isolate had an MIC of 0.032 µg/ml, a subsequent isolate obtained at month three showed 1-fold increase in INH MIC (0.064 µg/ml). Additionally, this patient was initiated on the standard short-course regimen with high-dose INH for 4 months.

Patient 27 and 43 had RIF-monoresistant TB, and were susceptible to INH, both recording an MIC of 0.032 µg/ml. These patients were initiated on different regimens. Patient 27 received the standard short-course regimen with high-dose INH, while patient 43 received LFX, TRZ, LZD, CFZ and BDQ

Among MDR-TB patients, four patients had positive culture at week 8; with patient 1, 4, and 16 displaying *inhA* mutation and patient 25 displaying *katG* mutation. Patient 1 had additional resistance to PZA, EMB and STR, and recorded an MIC >64.0 µg/ml. Patient 4 had additional resistance to PZA and EMB, and recorded an MIC of 8.0 µg/ml, there was no change in MICs from subsequent isolates obtained at month 3 and 6. Patient 16 had no additional resistance and had an MIC of 0.256 µg/ml. Patient 25 had additional resistance to EMB and STR and had an MIC of 8.0 µg/ml. All four patients were initiated on a short-course regimen with high-dose INH.

Among pre-XDR-TB, three patients remained culture positive at week 8; with patient 13 displaying *inhA* mutation, patient 47 and 49 had both *inhA* and *katG* mutations. Patient 13 and 47 had additional resistance to PZA, EMB, STR and FQ, and recorded an MIC > 64.0 and 32.0 µg/ml, respectively. Patient 49 had additional resistance to PZA, EMB and FQ, and had an MIC >64.0 µg/ml. All three patients were excluded on a standard short-course regimen.

Patient 50 with XDR-TB had *katG* mutation, and additional resistance to PZA, EMB, STR, FQ, SLIDs, and had an MIC of 4.0. µg/ml, this patient was excluded from the standard short-course regimen.

3.6 Correlation of Isoniazid Minimum Inhibitory Concentrations with drug resistance profiles, clinical characteristics, previous TB history, and week 8 sputum culture conversion

3.6.1 INH MICs in relation to the drug-resistance profile of the isolate.

Median INH MICs varied in relation to the drug resistance profile of the isolate demonstrating an increase in MIC with an expanding drug resistance profile. In the INH susceptible group, DS-TB and RIF mono-resistant isolates had median INH MIC of 0.064 and 0.032, and 4.50 µg/ml respectively. Interestingly, RIF resistant isolates with additional resistance to EMB, STR and PZA demonstrated higher MICs compared to RIF mono-resistant isolates. Clinical isolates that were MDR-TB and pre-XDR/XDR-TB demonstrated higher median INH MICs of 8.0 (CI, 8.0-32.0) and 48.0 (CI, 4.0-64.0) µg/ml, respectively. Table 3.5 shows the increase in INH MIC associated with various drug-resistance profiles, as well as increasing MICs with resistance amplification.

Table 3.4 Gene Mutation Profiles of *Mycobacterium tuberculosis* clinical isolates and their median INH MIC and 95% Confidence Interval (n=58).

Drug-resistant profile	**No. of isolates (%)**	**Median MICs – (95% CI) µg/ml**
INH susceptible		
DS-TB	03 (5.2)	0.064 – (0.064-0.064)
RIF mono-resistant	07 (12.1)	0.032 – (0.032-16.0)
RIF + EMB and STR/PZA	02 (3.4)	4.50 – (1.0-8.0)
INH mono-resistant	01 (1.7)	4.0
MDR-TB (at least INH + RIF resistance)	35 (60.3)	8.0 – (8.0-32.0)
Pre-XDR/XDR-TB	10 (17.2)	48.0 – (4.0-64.0)

Abbreviation: DS-TB, *drug-susceptible tuberculosis;* EMB; ethambutol; FQ, INH, *isoniazid*; MDR-TB, *multidrug-resistant tuberculosis;* RIF, *rifampicin;* STR, *streptomycin*

3.6.2 Correlation of Clinical Characteristics with Isoniazid Minimum Inhibitory Concentrations

3.6.2.1 Cavitary Disease and Infiltrates

Overall, median MICs in patients with extensive disease displaying *inhA* mutations were higher at 64.0 µg/ml, compared to MIC in patients with no cavitation., which was 8.0 µg/ml. Compared to patients with cavities that were INH susceptible, those with unilateral and bilateral cavities displaying *inhA* mutation had MICs that were approximately 200-fold higher, while those displaying *katG* mutations had MICs that were 12 and 25-fold higher, respectively. This association of mutations and MICs with extensive cavitary disease was not observed with extensive infiltrative disease, Table 3.5.

Table 3.5 The median INH MICs and 95% CI for INH MICs in association with cavitary disease, infiltrates and INH linked mutations.

			MICs (µg/ml)	
		Mutation	**Median**	**95% CI**
Cavities	***Bilateral***	INH S	0.32	0.12-0.64
		inhA	64.0	-
		katG	4.0	-
		inhA + katG	64.0	64.0-64.0
	Unilateral	INH S	0.48	0.32-8.0
		inhA	64.0	64.0-64.0
		katG	8.0	8.0-32.0
		inhA + katG	64.0	64.0-64.0
	None	INH S	0.64	0.32-1.0
		inhA	8.0	8.0-64.0
		katG	4.0	4.0-16.0
		inhA + katG	48.0	16.0-64.0
Infiltrates	***Bilateral***	INH S	0.32	0.32-16.0
		inhA	8.0	8.0-64.0
		katG	4.0	2.0-16.0
		inhA + katG	64.0	64.0-64.0
	Unilateral	INH S	0.48	0.32-1.0
		inhA	32.13	0.256-64.0
		katG	4.0	4.0-32.0
		inhA + katG	48.0	32.0-64.0
	None	INH S	0.64	0.32-8.0
		inhA	1.0	-
		katG	8.0	4.8-8.0
		inhA + katG	64.0	-

Abbreviation: INH S, *isoniazid susceptible*; CI, *confidence interval;* MIC, *minimum inhibitory concentration*.

3.6.3 Correlation of previous TB history and INH MICs

Overall, previous TB history was associated with a two-four-fold increase in INH MICs, however, it was not significant (p = 0.687). The median MIC obtained was 8.0 (95% CI, 8.0-64.0) µg/ml, while no TB history had median MIC of 4.0 (95% CI, 4.0-16.0) µg/ml.

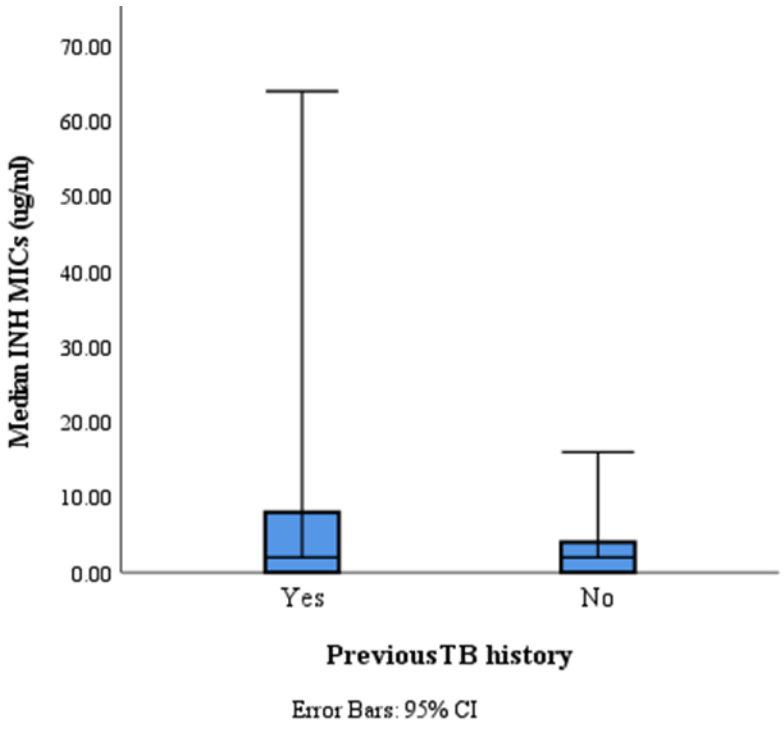


Figure 3.3. The correlation of TB history and INH MICs.

3.6.4 Correlation of Week 8 Sputum Culture conversion and Isoniazid Minimum Inhibitory concentrations

Week 8 culture results showed that 20% of the participants remained culture positive. The median INH MIC for those that were culture positive and negative was 8.0 (95% CI, 8.0-64.0) and 6.0 (95% CI, 4.0-16.0) µg/ml) (p = 0.979).

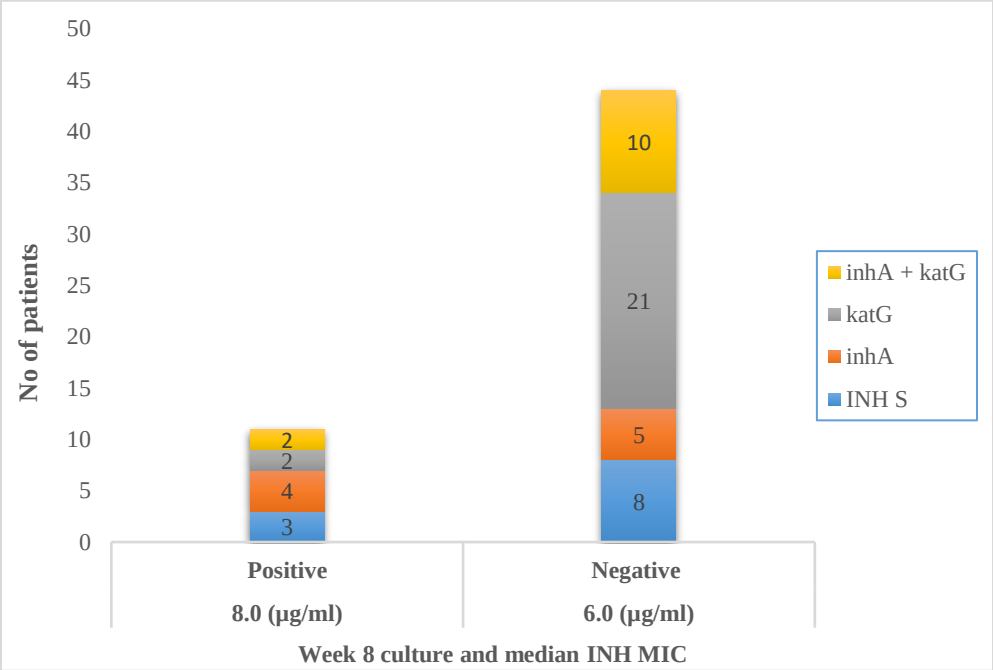


Figure 3.4 Correlation of week 8 culture status and MICs.

CHAPTER 4

DISCUSSION AND CONCLUSION

Discussion

There is an urgent need to optimize existing agents until new ones are widely available to overcome the threat of expanding drug resistance. There is accumulating evidence that higher doses of INH are effective against low- to intermediate-level INH-resistant strains. At the standard dose of 5 mg/kg against DS-TB, INH monotherapy has demonstrated a significant reduction in bacterial burden by 90-95% in the first two days of treatment (77). Further, this activity can be observed at doses as low as 10-20 times the standard dose (77). This suggests that doses higher than 5 mg/kg may overcome low-level resistance caused by mutations in the *inhA* gene, known to confer low-level resistance, in the range of four- to eight-fold increase in INH MICs, whereas *katG* mutations are associated with higher-level resistance, in the range of 10-160 fold increase in INH MICs (71, 78). Several observational studies suggest that high-dose INH is efficacious against MDR-TB, however, there is lack of evidence on the use of high-dose INH in the presence of *katG* mutation (72, 79, 80). There are no current standards set to define moderate-level INH resistance, thus it is not clear how to separate those patients presenting *katG* mutations who can possibly benefit from the high-dose INH from those who cannot (81). Further, the independent effect of INH on *M.tb* strains bearing various high-confidence INH resistance mutations, i.e. *inhA, katG* and both *inhA* and *katG*, remains unknown, leading to the WHO identifying this as a key area of interest requiring further research (38).

Here we examined the efficacy of INH in 58 *M.tb* clinical isolates with *inhA, katG* and both *inhA* and *katG* mutation profiles in a cohort of 55 DR-TB patients enrolled in the CAP020 InDEX study. We further correlated the MICs with genotypic profile, clinical characteristics and previous TB history. In this study cohort, more than 60% were people living with HIV/AIDS 43% presented bilateral TB disease on chest X-ray, and thirty percent with a previous history of DS-TB. We observed a great variation in the MICs obtained for clinical isolates, with significant overlap in the MICs within the selected groups of INH mutations. Apparent differences were observed between the MICs of INH susceptible isolates and those harbouring INH resistance-associated mutations.

Nine of the 12 INH susceptible patients included in the study had MICs that fell within the susceptible range, while the remaining three with significantly higher MICs, fell within the resistance range. INH susceptible isolates were expected to have lower MICs as they had no mutations linked to INH resistance, even though some displayed resistance to other first-line drugs such as RIF. However, 3/12 isolates fell out of the susceptibility reported range (0.016-0.256 µg/ml), showing phenotypic resistance to INH. This discrepancy between the genotypic resistance profile and phenotypic DST has been shown in previous studies. In a study conducted by Ji *et al*. LPA demonstrated that isolates were genotypically susceptible but were phenotypically resistant to INH with MIC >2.0 µg/ml (82). Several authors postulated that the genotypic–phenotypic discrepancy in INH and/RIF when using LPA may be due to probes being limited to

high confidence mutations only. Furthermore, LPAs are limited with respect to: its ability to detect rare mutations associated with increased MICs, detection of mixed infections and in detecting minority resistant populations (82, 83). However, in the current study, WGS was used to screen all targets linked to INH resistance, with no additional resistance detected (27). Other studies using WGS analysis linked INH phenotypic resistance in the absence of conventional mutations on LPA to *ahpC* mutations and stop codons in *katG*, which has been described in both INH susceptible and resistant strains (84, 85). Findings of the current study suggests that there are additional resistance mechanisms in play or there may be other factors influencing the MIC increase (86). The presence of minority variants or mixed infections could be a possibility, given that WGS was done on MGIT culture samples which is known to be selective (87). Interestingly, two of the patients displayed additional resistance to other companion drugs in the regimen. It appears that with an increase in the number of the drugs a patient was resistant to, the MIC for INH increased. However, the numbers in this group were too small for further analysis.

Similar trends were observed in the *inhA* group. Patients within this group demonstrated surprising results. Only three patients isolates with the *inhA* mutation fell within the expect MIC range of (0.256-2 µg/ml). Repeat testing at higher concentrations showed that the isolates in this group had MICs of 4.0 µg/ml (n=1), 8.0 µg/ml (n=3) and >64.0 µg/ml (n=4). This is the level of resistance similar to that reported in double mutants (*inhA* and *katG*), in which the use of high-dose INH is contra-indicated (78). WHO recommends using the standard short-course regimen, including high-dose INH among patients with *inhA* mutations. Additionally, currently implemented SA National department of health guidelines recommends inclusion of high-dose INH regardless of which mutation is present (*inhA*/*katG*) (39).

To the best of our knowledge, there are no previous studies that have reported the high-level resistance for this mutation. Previous studies have consistently reported an MIC ≤4.0 µg/ml for mutations in the *inhA* gene (78, 81, 88, 89). In this study most of the *inhA* mutants had MIC above CB of 0.4 µg/ml for high-level resistance set by CLSI (62). More recently, a phase 11A dose-ranging trial of INH for MDR-TB patients with *inhA* mutations found that, at doses of 10-15mg/kg, INH had measurable activity in patients with low-level INH phenotypic resistance, similar to standard doses in patients with DS-TB (90). In contrast to the rapid clearance observed between days 0-2 in patients infected with drug susceptible strains, the average daily killing of *inhA* mutant strains were higher in days 2-7 (90). The median MIC reported for patients with the *inhA* mutation in this group was 1.0 µg/ml (range 0.05-4 µg/ml). The median MIC in this study was significantly higher at 8.0 µg/ml (range 4-64 µg/ml), although our sample size for this group was much smaller. Further modelling, PK analysis and *NAT2* genotypic information for patients with *inhA* mutant strains showed that 10 and 15 mg/kg doses of INH achieved the therapeutic effect similar to 5 mg/kg used to treat DS-TB, in slow and intermediate acetylators, respectively (91). However, there was a delay in the

bacterial response following exposure to INH. Furthermore, a therapeutic effect could not be achieved in fast acetylators even at a 15 mg/kg (91). This indicates that the presence of the mutation alone should not be a decisive factor for high-dose INH eligibility. In addition to the mutation type, knowing the acetylation status is important to guide the eligibility criteria. Findings from the current study further suggest that even though *inhA* mutants are reported as low-level resistance and therefore eligible to receive the WHO MDR-TB short-course regimen, not all patients infected with strains displaying this mutation may benefit from INH due to strains showing high-level resistance. In addition, a South African study demonstrated that fast acetylators were less common than intermediate and slow acetylators, presenting at 18, 43, and 34% respectively (92). Further, the study demonstrated that fast acetylators had faster INH clearance (2.3 times) compared to slow acetylators, suggesting that INH dosage should depend on the acetylator status of an individual (92). Thus, the impact of infection with a resistant bearing strain as well as patient's acetylator status could potentially compromise treatment success rates and long-term outcomes in INH receiving patient populations. In addition, further research on the role of acetylator status on incidence of INH mutant strain acquisition is warranted.

The INH MICs obtained for *katG* mutants are in keeping with published reports. Most studies have reported an MIC range of 1-16 µg/ml in MGIT (93–95) and a range of 4-32 µg/ml in Middlebrook 7H10 media (70). Seventeen of the 18 isolates in this group fell within the expected range of 1-16 µg/ml. Nevertheless, this is 160-fold higher than the recommended WHO CC and 40 folder higher than the CLSI recommended CB for high-level resistance. The level of resistance displayed by the *katG* mutation is debated as it is still unclear how patients displaying the *katG* mutation are eligible and can possibly benefit from high-dose INH. There are several lines of evidence supporting the role of high-dose INH in the treatment of DR-TB and to date, none of these studies include any data on the type of INH resistance (72, 96, 97). However, global surveillance data indicate that *katG* mutations are more frequent than *inhA* mutations which, given the significant improvement in treatment success rates with high-dose INH, it can be inferred that there is some measurable effect of high-dose INH on *katG*-mutated strains as well. The regimen also includes other agents with excellent sterilizing activity such as BDQ, LZD and CFZ. In contrast, we cannot ignore the high levels of resistance to companion drugs such as PZA and EMB. In this study, among the patients in the *katG* group 14/18 (78%) displayed resistance to EMB and PZA. While WHO recommends against the use of the regimen in presence of resistance to these drugs, resistance to these agents are not routinely tested. Further, there are studies suggesting initial resistance to companion drugs should not be the basis for selection of the regimen as long as FQ susceptibility is preserved (98). This should be applied with caution as the impact on long-term outcomes are unknown. Importantly, further studies on the individual effect of INH in the regimen is required to provide clarity. There is an ongoing study evaluating high-dose

INH and acetylators status in patients infected with *katG* mutated strains (90). The results from this study are expected to give clarity on whether the use of high-dose INH is effective in these patients.

As expected, *M.tb* isolates with a combination *inhA* and *katG* mutations exhibited high INH MICs, and this was consistent with previous studies (81, 89, 99). MICs as high as > 256 µg/ml have been reported in isolates displaying *inhA* and *katG* double mutations, this emphasizes the high-level resistance associated with the presence of both these mutations (52). Our findings support the exclusion of high-dose INH in the presence of both *inhA* and *katG* mutations as per guidelines (38). Patients who present with double mutations receive the longer treatment regimen.

Correlating the complete genotypic resistance profile with MICs, showed that highly resistant isolates or having more mutations is associated with increased in INH MICs. DS-TB and RIF mono-resistant isolates had similar range of MICs, which showed susceptibility to INH. However, clinical isolates that were resistant to RIF, EMB, STR or PZA showed increasing phenotypic resistance to INH. This may suggest that mechanism of resistance is beyond what is reported and may not be entirely based on reported mutations. Additionally, strain genetic background, non-modulation mechanisms and drug efflux mechanisms may contribute to the variability in the increase of the MICs (100–102). Therefore, it is essential that genotypic results are complemented with additional DST to avoid the inappropriate administration of drugs that may be ineffective and that may contribute to toxicity and side-effects (103). Overall, the patients demonstrated highly variable MICs with significantly overlapping MICs.

In this study there was no significant association between cavitary disease and INH MICs in INH susceptible group. The INH MICs were more or less the same in the presence and absence of cavitary disease. However, the presence of cavities in *inhA* mutants showed higher MICs. Even though the MICs were lower in the absence of cavities for *inhA* mutants, they were still indicating high-level resistance. There was no notable difference in cavitary status among *katG* mutants, and this was similar with combination of *inhA* and *katG* mutants, however all groups showed high-level resistance. Since this is the first study to compare clinical data (cavitation) with INH MICs, we assume that this may be a reason patients with cavitation are excluded from the standard short-course regimen because they are at a higher risk of treatment failure (104). Furthermore, previous studies have shown that INH has a poor penetration to the cavitary lesions, and the resulting concentration at the site of infection is inadequate (105). Therefore, they require extended duration of treatment to have better response to treatment. Furthermore, the presence of infiltrates did not show major effects in all groups. However, comparing with cavities there was slight difference observed in *inhA* group. *i.e*. INH MICs associated with cavities were two times higher than those associated with infiltrates. Suggesting that infiltrates may not be as lethal as cavities.

We found MICs two times higher among those who had previous TB history, though it was not statistically significant. There is no data on TB history and INH MICs, however, higher INH MICs in this group can be supported by the prevalence of DR-TB in patients with TB history (106). Other countries have reported that more than 50% of MDR-TB strains are from patients with previous history of treatment (107). Furthermore, in this study all the previous history TB cases were DS-TB. This would mean they have been previously exposed to INH. It has been previously shown that history of INH exposure is associated with INH mono-resistance and MDR-TB (108). This may explain increased INH MICs in previous TB history group.

Summary of key findings

In this study, we observed isolates with *inhA* only mutations demonstrated a highly variable range of MICs. 3/11 isolates had MICs that fell within the expected ranges of 0.256-2.0 µg/ml. Furthermore, most of the *inhA* mutants had MIC above CB of 0.4 µg/ml for high-level resistance set by CLSI. The remaining isolates demonstrated high-level resistance, with four isolates recording MICs >64.0 µg/ml. This is a novel finding as previous studies have consistently reported an MIC ≤4.0 µg/ml for mutations in the *inhA* gene. INH MICs for *katG* mutants with an MIC range of 1.0-32.0 µg/ml. Nevertheless, this is 160-fold higher than the recommended WHO CC and 40-fold higher than the CLSI recommended CB for high-level resistance. Isolates with a combination *inhA* and *katG* mutations demonstrated high-level resistance with all MICs falling within the range of 8.0 to >64.0 µg/ml.

We assessed the correlation of MICs with clinical characteristics and previous TB history and found that MIC's in patients with extensive disease displaying *inhA* mutations were higher at 64.0 µg/ml, compared to MIC in patients with no cavitation, which was 8.0 µg/ml. Compared to patients with cavities that were INH susceptible, those with unilateral and bilateral cavities displaying *inhA* mutation had MICs that were approximately 200 fold higher, while those displaying *katG* mutations had MICs that were 12 and 25 fold higher, respectively. Patients with a previous TB history demonstrated a two-four-fold increase in INH MICs. Median MICs obtained in patients with previous TB was 8.0 (95% CI, 8.0-64.0) µg/ml, while those with no previous TB history had median MIC of 4.0 (95% CI, 4.0-16.0) µg/ml.

Limitations of study

One of the study limitations was the small sample size, especially for *inhA* group, which precluded our ability to compare INH resistant profiles across MIC ranges, and by clinical characteristics.

CONCLUSION

Patients respond differently on treatment depending on their weight, age, co-morbidities and genetic factors. Hence, the standardized treatment approach may not be beneficial especially among patients infected with resistant strains. Furthermore, the absence of substantial evidence of effectiveness of included drugs in this case high-dose INH, may further cause harm. Our findings suggest that the MDR-TB short-course regimen should not be a one-size-fits all. This study demonstrated highly variable MIC range with significant overlap in MIC range among the mutant groups. Furthermore, *inhA* mutants demonstrated unexpectedly high MICs raising a concern for the use of the high-dose INH in our setting. These findings further highlight that eligibility criteria should not be based only on which mutation linked to INH is present. As it was shown that even *inhA* mutations can be associated with INH high-level resistance. Furthermore, *katG* mutants were found to be consistent, however, patients with *katG* mutations should not be eligible for the standard short-course regimen due to high-level resistance and no evidence on its effectiveness. Improper use of the regimen and patient non-adherence can contribute to amplification of resistance. In addition, rapid molecular assays should be complemented with DST, to guide INH dosage and properly identify those likely to benefit.

Future directions

Time kill experiments need to be performed to assess the early bactericidal activity of INH against INH resistant associated mutations. Moreover, serial isolate sequencing studies need to be conducted for understanding patterns of amplification of INH resistance. Improved understanding on the prevalence and the role of low confidence mutations detected by WGS, and the relationship of RIF resistance mutations to loss of INH function. Detected INH resistance among patients with no INH resistance conferencing mutations with high INH MICs requires further research and understanding.

CHAPTER 5

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CHAPTER 6

APPENDICES

Appendix 1: Ethical approval for the InDEX study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI
RESEARCH OFFICE
Biomedical Research Ethics Administration
Westville Campus, Govan Mbeki Building
Private Bag X 54001
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Tel: 27 31 2604769 - Fax: 27 31 2604609
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx

02 January 2018

Dr N Padayatchi
CAPRISA
K-RIRTH Building - 2nd Floor
NRMSM
Nesri.padayatchi@caprisa.org

Dear Dr Padayatchi

Title: The Individualised M(X) Drug-Resistant TB treatment strategy study (INDEX Study). A strategy to improve treatment outcomes in patients with drug-resistant TB.
Degree: Non-degree
BREC REF NO: BFC584/16

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	24 February 2018
Expiration of Ethical Approval:	23 February 2019

I wish to advise you that your application for Recertification dated 10 November 2017 for the above protocol has been noted and approved by the Biomedical Research Ethics Committee (BREC) at a meeting that took place on **12 December 2017** for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

Yours sincerely

[redacted]

Mrs A Marimuthu
Senior Administrator: Biomedical Research Ethics

Appendix 2: Ethical approval for the current sub-study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

14 June 2021

Miss Senamile Lale Ngema (215001557)
School of Laboratory Medicine & Medical Science
Medical School

Dear Miss Ngema,

Protocol reference number: BREC/00001449/2020
Project title: Defining the Role of High-dose Isoniazid in Multidrug-resistant Tuberculosis Treatment
Degree Purposes: Masters

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**20 July 2021**
Expiration of Ethical Approval:	**19 July 2022**

I wish to advise you that your application for Recertification received on 03 June 2021 for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 13 July 2021.

Yours sincerely

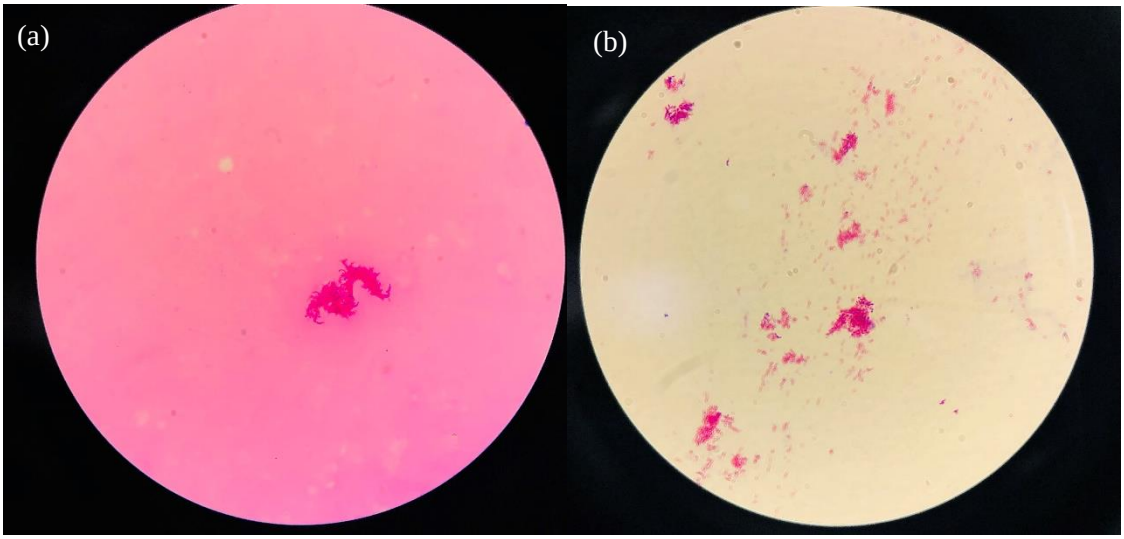
[redacted]

.....

Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee

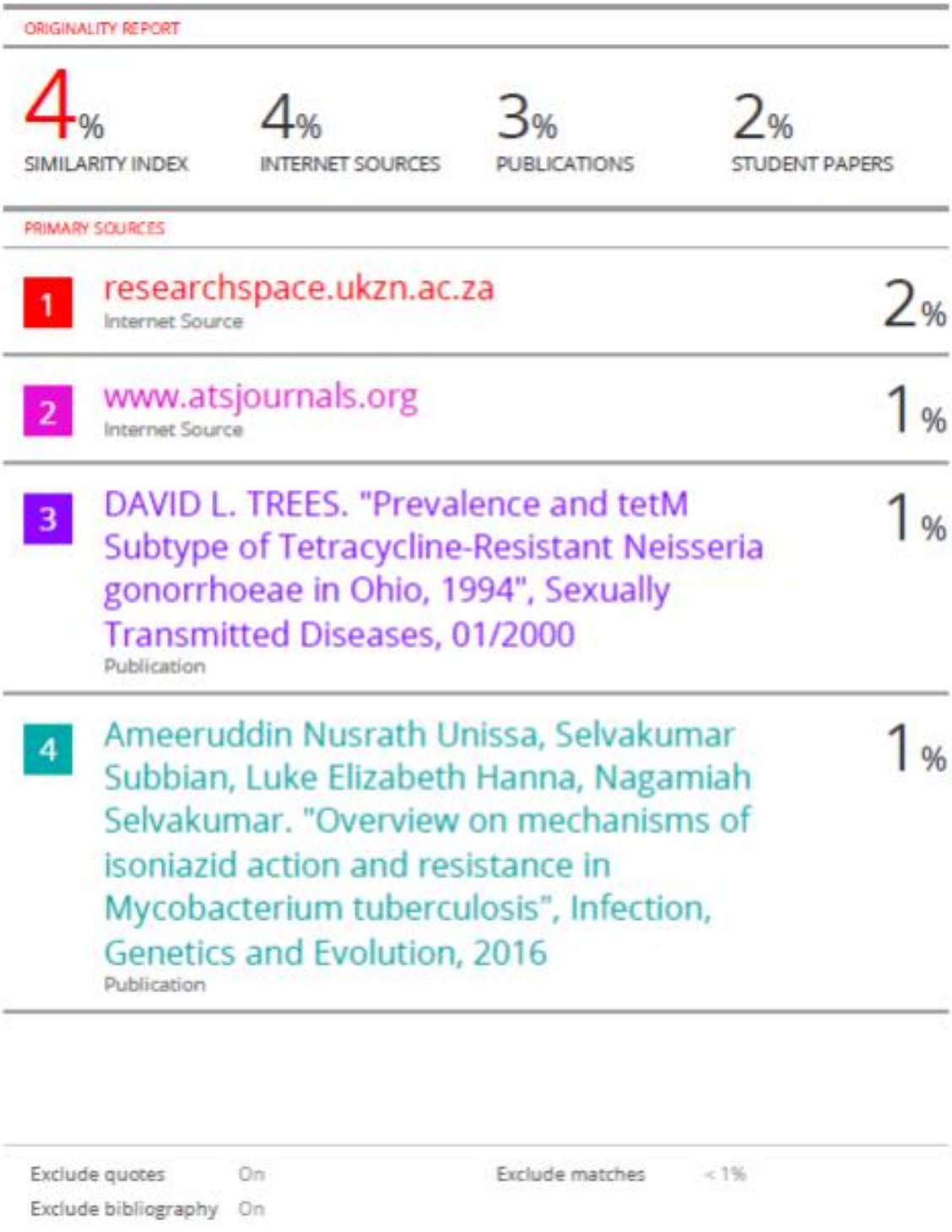
Biomedical Research Ethics Committee
Chair: Professor D R Wassenaar
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Email: BREC@ukzn.ac.za
Website: http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx
Founding Campuses: Edgewood Howard College Medical School Pietermaritzburg Westville

Appendix 3: Images of ZN staining



(a) Roping acid-fast bacilli (AFB) *vs* **(b)** non-roping.

Appendix 4: Turn it in report



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