Undetected isoniazid mono resistance in rural Eastern Cape Province

A risk for the emergence of multidrug-resistant TB

by

Charnay Janine van der Merwe

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology in the Faculty of Medicine and Health Sciences at Stellenbosch University

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

Supervisor: Dr Marisa Klopper

Co-supervisor: Dr Elizabeth Streicher

March 2021

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

Date: March 2021

Abstract

The emergence of drug-resistant tuberculosis (TB) remains a major challenge in South Africa, particularly the Eastern Cape, being one of the most severely affected provinces in the country. Due to resource limitations, many isoniazid (INH) mono-resistant TB cases remain undiagnosed, as TB control programmes generally focus on rifampicin-resistant strains. Rifampicin resistance, which is a marker of multidrug-resistant (MDR) TB, is more difficult to treat than INH mono-resistant (IMR) TB, often resulting in high morbidity and mortality.

The effectiveness of INH, an important first-line anti-TB drug, has been compromised by resistance, which arises through spontaneous mutations in the genome of *Mycobacterium tuberculosis* (*M. tuberculosis*). Occurrence of these mutations is often missed by the current diagnostic algorithm, which fails to detect IMR-TB cases at diagnosis, hence threatening the efficacy of TB treatment. Consequently, these patients are likely to be treated with a weakened regimen, which may increase the risk of treatment failure or relapse. Previous studies have reported IMR as the source for the emergence of MDR-TB.

This study aimed to provide the first in-depth analysis of the molecular epidemiology of IMR-TB in the Eastern Cape. Clinical isolates from patients with rifampicin-susceptible TB were obtained via the National Health Laboratory Services (NHLS) in Port Elizabeth and analysed by using a series of microbiological and molecular techniques. These tests were done to identify IMR-TB cases, describe the molecular mechanisms of INH resistance, identify cases of acquisition of IMR and MDR, as well as to describe risk factors associated with IMR at diagnosis (baseline). We also used spoligotyping to classify isolates into their respective lineages and strain families.

Phenotypic INH drug susceptibility testing on solid media identified 107 (13.9%) cases of IMR among the cohort of 993 TB cases enrolled, which was nearly double the estimated national average. No association between patient demographic or clinical parameters was identified. This may be due to the inaccuracies of the electronic TB database. Genetic drug susceptibility testing only identified causal mutations in 25 baseline isolates, while 4 baseline isolates showed evidence of heteroresistance, possibly masking the detection of underlying INH-resistant populations. This was confirmed in a small sub-analysis using a highly sensitive targeted deep sequencing approach. Subsequent analysis of serial isolates showed acquisition of IMR in 9 cases, as well as loss of IMR in 12 cases. Repeat analysis identified

heteroresistance as the possible cause of the observed flip flopping of the IMR phenotype. Spoligotyping failed to identify reinfection as a major mechanism causing the flip flopping IMR phenotype. IMR was associated with the Atypical Beijing genotype ($p < 0.0001$).

This study highlights the need to change TB policy through: (1) understanding the local epidemiology of IMR to identify potential risk factors for targeted interventions and to strengthen current first-line regimens for the continuation phase of TB treatment, (2) improving surveillance studies in neglected rural areas by monitoring IMR to inform policy, (3) developing new rapid molecular technologies to ensure early identification of IMR-TB cases and close monitoring of patients following appropriate treatment and care. These strategies will be essential to contain the spread of IMR-TB, improve outcomes and prevent progression of disease to more severe forms of drug resistance often culminating in death.

Opsomming

Die opkoms van middelweerstandige tuberkulose (TB) bly 'n groot uitdaging in Suid-Afrika, veral die Oos-Kaap, as een van die provinsies in die land wat die ergste geraak word. As gevolg van hulpbronbeperkte kapasiteit, bly baie isoniasid (INH) mono-weerstandige TB gevalle nie gediagnoseer nie, aangesien TB-beheerprogramme oor die algemeen fokus op rifampisin-weerstandige stamme. Rifampisin weerstand is 'n merker van multimiddel-weerstandige (MDR) TB, wat moeiliker is om te behandel as INH mono-weerstandige (IMR) TB, wat dikwels lei tot hoë morbiditeit en mortaliteit.

Die effektiwiteit van INH, 'n belangrike eersterangse middel teen TB, word verminder deur weerstand wat ontstaan deur spontane mutasies in die genoom *Mycobacterium tuberculosis* (*M. tuberculosis*). Die voorkoms van hierdie mutasies word dikwels gemis deur die huidige diagnostiese algoritme, wat nie IMR-TB-gevalle kan opspoor tydens diagnose nie. Gevolglik word die doeltreffendheid van TB-behandeling bedreig, aangesien hierdie pasiënte meer geneig is om met 'n verswakte behandeling behandel te word, wat die risiko van versuim of terugval kan verhoog. Vorige studies het IMR as die bron vir die opkoms van MDR-TB gerapporteer.

Hierdie studie het ten doel gehad om die eerste diepgaande analise van die molekulêre epidemiologie van IMR-TB in die Oos-Kaap te bied. Kliniese isolate van pasiënte met rifampisin-vatbare TB is verkry deur die National Health Laboratory Services (NHLS) in Port Elizabeth en geanaliseer deur gebruik te maak van 'n reeks mikrobiologiese en molekulêre tegnieke. Hierdie toetse is gedoen om IMR-TB-gevalle te identifiseer, die molekulêre meganismes van INH-weerstand te beskryf, gevalle van verkryging van IMR en MDR te identifiseer, asook om risikofaktore wat verband hou met IMR tydens diagnose (basislyn) te beskryf. Ons het ook spoligotipering gebruik om isolate in hul onderskeie geslagte en stamfamilies te klassifiseer.

Fenotipiese toetsing vir vatbaarheid vir INH-geneesmiddels op vaste media het 107 (13,9%) gevalle van IMR geïdentifiseer onder die groep 993 TB-gevalle wat ingeskryf is, wat byna dubbel die geskatte nasionale gemiddelde was. Geen verband tussen pasiënte se demografiese of kliniese parameters is geïdentifiseer nie. Dit kan te wyte wees aan die onakkuraathede van die elektroniese databasis. Die toetsing van vatbaarheid vir genetiese geneesmiddels het slegs oorsaaklike mutasies in 25 basislyn-isolate geïdentifiseer, terwyl vier basislyn-isolate bewyse

van heteroresistensie getoon het, wat moontlik die opsporing van onderliggende INH-weerstandige populasies kon wegsteek. Dit is bevestig in 'n klein sub-analise deur gebruik te maak van 'n baie sensitiewe, gerigte diepvolgorde-benadering. Daaropvolgende ontleding van reeksisolate het die verkryging van IMR in 9 gevalle getoon, asook verlies aan IMR in 12 gevalle. Heranalise het heteroresistensie geïdentifiseer as die moontlike oorsaak van die waargenome “flip-flopping” van die IMR-fenotipe. Spoligotipering kon nie herinfeksie identifiseer as 'n belangrike meganisme wat die IMR-fenotipe omkeer. IMR word geassosieer met die Atipiese Beijing-genotipe ($p < 0.0001$).

Hierdie studie beklemtoon die behoefte om TB-beleid te verander deur: (1) die plaaslike epidemiologie van IMR te verstaan om potensiële risikofaktore vir geteikende intervensies te identifiseer en die huidige eerste-lyn-regimes vir die voortsettingsfase van TB-behandeling te versterk, (2) verbetering van toesigstudies by verwaarloosde landelike gebiede deur IMR te monitor om beleid in te lig, (3) die ontwikkeling van nuwe vinnige molekulêre tegnologieë om vroeë identifikasie van IMR-TB-gevalle te verseker en noukeurige monitering van pasiënte na toepaslike behandeling en sorg. Hierdie strategieë is noodsaaklik om die verspreiding van IMR te beperk, die uitkomste te verbeter en die progressie van siektes na meer ernstige vorme van middelweerstandigheid te voorkom, wat dikwels op die dood uitloop.

Acknowledgements

First and foremost I would like to thank the Almighty who has provided me with compelling strength to make this thesis a success. I express my sincere gratitude to my supervisor Dr Marisa Klopper, co-supervisor Dr Elizabeth Streicher and Professor Rob Warren for their continued valuable support, proficiency and guidance throughout my research. The immense input and patience served of inspiration and helped facilitate submission of this thesis. I am sincerely humbled and appreciative of the granted opportunity. In addition, I would like to thank the TB Genomics group for the consistent encouragement and fair share of vital insight to all proposed questions.

I am exceedingly grateful for the financial assistance awarded by the National Research Foundation (NRF), in partnership with the DST-NRF Centre of Excellence for Biomedical TB Research (CBTBR), the DAAD-NRF German Academic Exchange Service and South African Medical Research Council (SAMRC) Centre for Tuberculosis Research. Such support was essential for this study.

To my dearly beloved parents, Edith and Cyril van der Merwe, thank you for your endless love, motivation and support. I am forever indebted for all you have sacrificed towards my education. You have installed much value and inspired me to achieve my dreams through hard work and perseverance and have always encouraged me to do my best. A token of appreciation through my success is one I owe to you, my role models. In addition, I would like to thank all family and friends for their support and prayers, I truly appreciate it.

Dedication

I wish to dedicate this thesis to:

Mom and Dad, who inspired me to “strive for that greatness of spirit that measures life not by its disappointments but by its endless possibilities”

And

In loving memory of my grandfather, Jan Steenkamp, who always showed considerable interest and appreciation towards my field of study and for believing in my capabilities

You are my Angel.

Table of contents

List of figures

List of tables

List of abbreviations

%	Percentage
<	Less than
>	More than
≥	Greater than or equal to
=	Equals
±	Plus-minus
˚C	Degrees Celsius
µg	Microgram
µl	Microlitre
µM	Micromolar
β	Beta
A	Adenine
T	Thymine
G	Guanine
C	Cytosine
C+	Positive control
ACP	Acyl carrier protein
AFB	Acid-fast bacilli
AM	Amplification mix
AO	Auramine O
Approx	Approximately
ART	Antiretroviral therapy

ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guérin
BD	Becton Dickinson
BDQ	Bedaquiline
BMRC	British Medical Research Council
bp	Base pair
BSL3	Biosafety level 3
CAF	Central Analytical Facility
CB	Clinical breakpoint
CC	Critical concentration
CXR	Chest X-ray
dH_2O	Distilled water
DLM	Delamanid
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment, short-course
DR	Direct repeat
DST	Drug susceptibility testing
DTH	Delayed-type hypersensitivity
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EMB	Ethambutol
et al.	And others

ETR	Electronic TB Register
FAS II	Fatty Acid Synthase II
FDA	Food and Drug Administration
g	Gram
GC	Growth control
GXP	GeneXpert
HIV	Human immunodeficiency virus
hr	Hour
HREC	Health Research Ethics Committee
I	Intermediate
IBM	International Business Machines Corporation
IC	Internal control
i.e.	In other words
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma release assay
INH	Isoniazid
IMR	Isoniazid mono-resistant
IPT	Isoniazid preventative therapy
IUATLD	International Union Against TB and Lung Disease
L	Litre
LATE	Linear-after-the-exponential
LED	Light-emitting diode
LJ	Löwenstein–Jensen

LPA	Line probe assay
LTBI	Latent tuberculosis infection
m^2	Square metre
MDR	Multidrug-resistant
mg	Milligram
$MgCl_2$	Magnesium Chloride
MGIT	Mycobacterial growth indicator tube
min	Minutes
ml	Millilitre
mM	Millimolar
M. bovis	*Mycobacterium bovis*
M. tuberculosis	*Mycobacterium tuberculosis*
MTBDR	*Mycobacterium tuberculosis* drug resistance
NAA	Nucleic acid amplification
NAD	Nicotinamide adenine dinucleotide
NADH	1,4-Dihydronicotinamide adenine dinucleotide
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NHLS	National Health Laboratory Services
NTC	No-template control
NTF	Noise transfer function
NTM	Non-tuberculous mycobacteria
NY	New York

OADC	Oleic Albumin Dextrose Catalase
PA-824	Pretomanid
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim and Azlocillin
PAS	Para-aminosalicylic acid
PCR	Polymerase chain reaction
pH	Potential of hydrogen
pmol	Picomole (picomolar)
PPD	Purified protein derivative
PZA	Pyrazinamide
R	Resistant
RIF	Rifampicin
RNA	Ribonucleic acid
RR	Rifampicin-resistant
RRDR	Rifampicin resistance determining region
S	Susceptible
SDS	Sodium dodecyl sulphate
sec	Seconds
SM	Streptomycin
SpolDB4	Spoligotype database
SSPE	Saline sodium phosphate-EDTA
TAC	Thioacetazone
TB	Tuberculosis

TBE	Tris-borate-EDTA buffer
TDM	Therapeutic drug monitoring
TDS	Targeted deep sequencing
TGEN	Translational Genomic Research Institute
Tm	Melting temperature
Tris	Hydroxymethyl
TST	Tuberculin skin test
U	Units
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
v/v	Volume per volume
WGS	Whole genome sequencing
WHO	World Health Organization
x	Times
xg	Times gravity
XDR	Extensively drug-resistant
ZN	Ziehl-Neelsen

Chapter 1: General introduction

1.1 Background

Tuberculosis (TB), a chronic and highly infectious airborne disease caused by the pathogen *Mycobacterium tuberculosis* (*M. tuberculosis*), remains a global public health concern and is the ninth leading cause of death worldwide, according to the World Health Organization (WHO) (1). The nature of this disease is particularly fatal in individuals co-infected with the human immunodeficiency virus (HIV) and not receiving antiretroviral therapy (ART).

To combat TB disease, global TB control effort predominantly focuses on interrupting transmission of the causative agent through chemotherapeutic intervention in active infectious disease. As such, it aims to cure the majority of TB cases, thereby reducing mortality and eventually eradicating the disease. However, this strategy largely relies on self-reporting, which often is delayed due to poor health seeking behaviour. The efficacy of this strategy is further compromised by the emergence of drug resistance, fueled by improper TB case management and ineffective control strategies. Failure to curb the spread of drug-resistant TB therefore continues to threaten TB control programmes on a global scale.

TB treatment includes a combined drug regimen, consisting of multiple medications taken concurrently in order to improve efficiency and limit the emergence of resistance. These include first-line anti-TB drugs rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA), recommended by the WHO as standard short-course therapy (2).

The anti-TB drug INH has been the backbone of the standardized TB treatment regimen for decades, as a fundamental component used to treat drug-susceptible TB and in some instances drug-resistant TB (high-dose INH). This drug demonstrates unique potent bactericidal characteristics, targeting both actively replicating and dormant bacilli (3).

INH is a prodrug activated by the catalase-peroxidase enzyme KatG, encoded by the *katG* gene (4). The activated form of the drug inhibits the Fatty Acid Synthase II (FAS II) pathway, preventing mycolic acid synthesis, which is required for integrity of the *M. tuberculosis* cell wall (5).

The antibiotic effect of INH results in the extreme vulnerability of the pathogen under optimal conditions due to destabilization of the cell wall. However, this bacterium rapidly

evolves resistance during periods of monotherapy, as was seen soon after its introduction in 1952 (6).

INH resistance most commonly arises through spontaneous mutations in *katG* (the activator of INH), limiting conversion of the prodrug to the activated form, thereby preventing inhibition of mycolic acid biosynthesis. The second most common mechanism of resistance is mutations in the *inhA* promoter. This promoter mutation leads to upregulation of InhA (the target of INH-adduct), leading to titration of nicotinamide adenine dinucleotide (NAD) allowing mycolic acid synthesis to continue. Canonical mutations known to confer INH resistance are most frequently detected in codon 315 of *katG* and position -15 in the *inhA* promoter region (7–10). However, several other genes have also been implicated in INH resistance, including *furA-katG*, *inhA*, *fabG1-inhA*, *ahpC-oxyR* and *kasA* among others (11–16).

Despite considerable efforts to improve molecular-based tests, constraints in current diagnostics remain a cause for concern, as this results in delayed and/or incorrect diagnosis, subsequent inappropriate treatment of patients and poor adherence to drug therapies. The inability to detect INH resistance may contribute to the amplification of resistance, hence increasing the likelihood of unfavourable treatment outcomes and progression to multidrug-resistant (MDR; resistant to at least INH and RIF) TB, if not managed accordingly (17–19).

MDR-TB persists as a major threat to global health security with over 400,000 new MDR-TB cases occurring each year (20). South Africa is reportedly one of the worst affected countries in the Afro-region (estimated incidence of 520 active TB cases per 100,000 population, 59% HIV co-infection and MDR-TB burden of 3.4% new TB cases and 7.1% previously treated cases) (20). The largely rural Eastern Cape Province is an area of particular concern, harbouring some of the highest number of confirmed MDR-TB cases in South Africa (21).

Previous drug resistance surveys have reported increased prevalence of mono- and poly-resistant TB strains (22,23), with several studies having identified isoniazid mono-resistant (IMR; resistant to INH only) TB as a precursor to MDR-TB (24–28). The most recently conducted nationwide survey in South Africa (2012-2014) (21) showed a higher prevalence of any INH resistance (9.3%) compared to any RIF resistance (4.6%). In addition, reports described a notable increase in IMR-TB (4.9%) in the current survey compared to a previous survey (2001-2002) (2.7%), regardless of previous treatment history. The provincial prevalence rates of INH resistance irrespective of resistance to other first-line drugs was 5.4%

among new cases and 7.2% among previously treated cases (overall 6.4%) in the Eastern Cape. This is particularly worrying due to the limited availability of additional drug susceptibility testing (DST) beyond those diagnosed with RIF-resistant (RR; resistant to RIF detected using genotypic or phenotypic methods, with or without resistance to other first-line anti-TB drugs) TB, likely resulting in missed diagnostic opportunities. In addition, the burden of resistance may influence the usefulness of isoniazid preventative therapy (IPT).

IPT is a safe and low-cost intervention, used in HIV-infected persons to treat latent tuberculosis infection (LTBI). However, though being increasingly promoted, the use of INH monotherapy poses a risk for developing INH resistance, following the improper diagnosis of active TB cases that should receive a combined drug regimen for optimal efficacy. This suggests the need for ongoing surveillance among populations for INH resistance that may be attributed to the wider use of IPT.

In South Africa, the current TB diagnostic algorithm utilizes the widely implemented GeneXpert (Xpert) MTB/RIF test as a screening tool for the diagnosis of both TB and RIF resistance. Following a RIF-resistant result on Xpert, a line probe assay (LPA) (GenoType MTBDR*plus* v2) is used to confirm RIF resistance and determine INH resistance. Confirmed RR-TB cases are progressed to second-line treatment, on the premise that RIF resistance is a surrogate marker for MDR-TB. Follow-up by culture-based DST (requiring several weeks) for INH, if not detected by the MTBDR*plus* assay and second-line DST for fluoroquinolones and aminoglycosides are then done using the MTBDR*sl* v2 assay. However, this algorithm disregards the possibility of IMR-TB (or poly- resistance to INH and other first-line drugs, except RIF), leaving RIF-susceptible TB cases on standard first-line therapy. Thus, during the 4-month continuation phase of treatment (when treatment includes INH and RIF only), IMR-TB patients are treated with only one effective drug. Further drug resistance investigation is only conducted when the sputum specimen remains smear-positive after 7 or 23 weeks of treatment, with treatment adapted only by extending the intensive phase in cases where IMR-TB is identified. This strategy increases the risk of acquiring additional resistance, as well as the transmission of drug-resistant TB to the surrounding community.

Efforts to improve and develop rapid diagnostic tools are thus crucial in future TB control to ensure early case detection, followed by prompt initiation with an appropriate treatment regimen to potentially halt the progression of IMR-TB. Identifying alternative mechanisms of resistance and associated risk factors that fuel the IMR-TB epidemic may help influence

diagnostic algorithms to triage patients at risk, thereby ensuring initiation of effective therapy with concomitant improvement in treatment outcomes, reduced rates of relapse and lower numbers of acquired resistance.

1.2 Problem statement

Current diagnostic algorithms ignore the possibility of new and retreatment TB cases presenting with IMR-TB. Treatment monitoring is poorly implemented; consequently, patients are treated with a substandard regimen, thereby increasing the risk of treatment failure, raising the chance of relapse and acquisition of additional resistance, and exacerbating transmission.

1.3 Hypothesis

We hypothesize that a significant proportion of IMR-TB in the Kouga sub-district, Eastern Cape will not be identified by the current diagnostic algorithm, therefore these patients are at an increased risk of acquiring RIF resistance and poorer outcomes compared to patients infected with pan drug-susceptible TB.

1.4 Aims

This study aimed to describe the epidemiology and risk factors associated with IMR-TB in the Eastern Cape Province of South Africa.

1.5 Objectives

1.5.1 To describe the epidemiology of IMR-TB at a community level in a rural region of the Eastern Cape.

a) Determine the *M. tuberculosis* population structure of drug-susceptible and INH-resistant TB in the Kouga sub-district of the Eastern Cape Province with the use of spoligotyping.
b) Determine the prevalence of IMR in the region by phenotypic DST.
c) Characterize mutations responsible for INH resistance in the cohort using Linear-after-the-exponential (LATE)-PCR or FluoroType® MTBDR and Sanger sequencing.

1.5.2 To determine the risks associated with IMR-TB in this setting.

a) Quantify the risk that IMR poses for the acquisition of additional resistance, in particular RIF resistance, during standardized first-line treatment.
b) Determine whether IMR-TB cases display different (worse) outcomes compared to drug-susceptible cases.

1.6 Chapter overview

Chapter 2 is the literature review, which focuses on the evolution of TB diagnostic and treatment strategies and how these developments impact current TB policies. Chapter 3 describes the chosen methodology for this study and includes detailed protocols for each procedure. Chapter 4 sets out the results obtained following each method and Chapter 5 aims to comprehensively discuss the results in detail and draws conclusions. Chapter 6 addresses the limitations of the study and highlights future recommendations. Chapter 7 presents an overall conclusion of the study.

Chapter 2: Literature review

A step back in time: Evolutionary advances in tuberculosis control

Abstract

Tuberculosis (TB) remains a global health threat, with the emergence of drug resistance as a formidable obstacle to TB elimination. Notably, South Africa encounters one of the world's worst TB epidemics driven by the human immunodeficiency virus (HIV) and high multidrug-resistant (MDR) TB burden, raising concerns to potential significant threats relevant to TB treatment and control programmes. Despite major advances over the years, a significant breakthrough that would revolutionize TB diagnosis and treatment is yet to be accomplished. Case finding and treatment of TB disease are essential for controlling transmission and reducing incidence. However, shortcomings of current management protocols impose a major impact on the economy and quality of life due to costly and time-consuming interventions, which impede the progress of TB elimination. Reinforcing the importance of strengthening TB policies by improving current diagnostic and therapeutic practices is thus necessary to ensure accurate and early diagnosis, followed by optimum treatment of TB cases. This review presents a historical perspective on the evolution of TB diagnostic and treatment control strategies over the past century. Understanding the strengths and weaknesses thereof may guide possible future approaches to help inform and shape TB control policy in the twenty-first century.

2.1 Introduction

Tuberculosis (TB) has plagued mankind since the Neolithic era, reaching epidemic proportions in Europe and North America during the 18th and 19th centuries, earning the sobriquet, “Captain of all these men of Death” (29). TB is a poverty-related disease, endemic in most developing countries with high rates of human immunodeficiency virus (HIV) infection, displaying higher disease prevalence often accompanied by worse clinical outcomes, including mortality (30–35), as seen in South Africa. These effects can be attributed to various risk factors (e.g. behaviours and other co-morbidities) that limit access to diagnosis and care, which are essential in the fight against TB (36).

Despite decades of significant transformation in diagnostic and treatment options, the TB epidemic continues to rage in many developing countries. In 2018, an estimated 10.0 million people developed TB and 1.5 million died from the disease, among which 251,000 were HIV-positive (20). An added complication is the emergence of resistance against currently available short-course first-line anti-TB drugs, with major concern directed towards the rise and spread of multidrug-resistant TB (MDR-TB; defined as resistance to multiple first-line anti-TB drugs, including at least isoniazid and rifampicin).

Standard first-line therapy (6 months rifampicin (RIF) and isoniazid (INH); 2 months pyrazinamide (PZA) and ethambutol (EMB)) is recommended for treatment of newly diagnosed active pulmonary TB cases and is effective in most cases if taken according to prescription (2,37). However, drug efficacy can be compromised by various factors, including poor adherence or inadequate drug levels due to poor quality medicines, drug-drug interactions or patient-specific pharmacodynamics, which could contribute to the development of resistance to one or more drugs (38,39). Thus, optimal combination therapy is the key approach for successful treatment of cases.

MDR-TB is an enormous global health crisis, with an estimated 484,000 new RIF-resistant TB (RR-TB; defined as resistance to RIF detected using genotypic or phenotypic methods, with or without resistance to other first-line anti-TB drugs) cases reported in 2018 (20), of which 78% were MDR. Among these, approx. 6.2% had extensively drug-resistant TB (XDR-TB; defined as MDR-TB that is additionally resistant to a second-line injectable anti-TB drug as well as any fluoroquinolone). South Africa has one of the highest recorded TB incidences worldwide and reports one of the largest numbers of diagnosed MDR-TB cases in

Africa (20). This is concerning, as second-line anti-TB treatment is toxic, poor treatment outcomes are common and inappropriate or interrupted treatment may further promote amplification of resistance.

Resistance can either be primary by infection with an already drug-resistant strain or acquired as the result of intra-patient evolution of the bacillus, followed by the selection and growth of resistant populations (3). Drug resistance is thus not only restricted to ineffectively treated patients exhibiting unknown resistance but also includes undetected or incorrectly diagnosed cases, which may ultimately expose the surrounding communities to the risk of outbreaks (40,41).

Underdiagnosed TB is often ascribed to economic barriers due to limited primary care access and poor resource capacity found particularly in high burden low-income settings. Culture-based (phenotypic) drug susceptibility testing (DST) is the current gold standard for detection of drug-resistant TB. Phenotypic detection is a highly sensitive method, allowing for a definite diagnosis; however, it is costly, time-consuming and technically challenging, often causing delayed initiation of the appropriate therapy. Furthermore, it is not standardized for all anti-TB drugs. The demand for new advances in molecular detection tools thus emerged with the GeneXpert (Xpert) MTB/RIF and line probe assay (LPA), allowing for rapid and accurate diagnosis, and the potential to improve treatment outcome. However, though current technologies appear promising, much remains to be explored in terms of novel tools with suitable accuracy for use at point of care.

Although South Africa has made remarkable strides to ensure prevention and cure, with reduced prevalence and deaths, and improved treatment outcomes; TB, unfortunately, continues to cause extensive suffering. Nevertheless, South Africa has the means to overcome this challenge by implementing proper medical diagnosis in conjunction with the appropriate treatment, which is essential in the management and control of TB.

This review aims to explore the historical landmarks in the development of TB diagnostic and treatment control strategies over time, discussing current implications thereof as a clinical concern regarding emerging drug resistance, and addressing gaps and opportunities to improve public health policies.

2.2 History

2.2.1 TB diagnostics

Over the millennia, many trials and triumphs were faced in efforts to successfully diagnose and treat “phthisis” or “consumption”, having had one of the greatest impacts on society. The discovery of the tubercle bacillus in 1882 was a major milestone in the history of medicine, a turning point in the understanding and conquest of the deadly disease (42).

Throughout much of the 1800s, consumptive patients sought “the cure” in sanatoriums, with a strict regimen of bed rest, fresh air at all times, a healthy diet and gradual increase in activity levels (43). Patients were only examined for TB using a stethoscope (invented in 1816) when the disease was fairly well-advanced, following a prolonged cough (with blood at times), often associated with additional symptoms (e.g. weight loss, night sweats, fever, fatigue and chest pain). Early cases were discovered following routine examinations in the case of nurses, entrants to the teaching profession and public service, miners etc. Today, modern methods used for laboratory diagnosis of TB are continually evolving in order to achieve less expensive, more rapid and accurate results.

Immunological testing

The first TB diagnostic test was the Mantoux tuberculin skin test (TST) (**Table 2.1**), developed in 1907-1908, which later became the international standard (44–46). The TST is an intradermal technique performed by injecting an antigen (purified protein derivative (PPD)) into the inner surface of the forearm. The principle of the test is based on the demonstration of a delayed-type hypersensitivity (DTH) immune response once the person is exposed to the bacterial proteins within 48-72 hrs, leading to the formation of induration at the inoculation site, indicating active or past TB infection (47). Despite its long-term use, the TST is limited by suboptimal specificity and sensitivity. False-positive results occur due to induced responses upon infection with non-tuberculous mycobacteria (NTM). Additionally, individuals with a prior *Mycobacterium bovis* (*M. bovis*) Bacille Calmette-Guérin (BCG) vaccination (first administered in 1921; universal BCG vaccination of infants at birth implemented in South Africa in 1973) may also have a positive TST reaction (47–49). In contrast, though infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), some individuals may not react to the TST. False-negative results are common among those who are immunosuppressed (e.g. due to immunosuppressive treatment, uncontrolled HIV infection

or malnutrition) (50). Other causes may include inappropriate storage of the tuberculin antigen, incorrect inoculation of the antigen or inaccurate interpretation of the induration at the test site. Most concerning is that false-negative TST results may occur in patients with active TB disease due to a diminished immune response, as seen in HIV-infected or immunosuppressed patients (51).

Table 2.1: Implementation of the tuberculin skin test. yr. 1907-1908

Advantages	Disadvantages
• Basic test (*M. tuberculosis* infection) • Low-cost • Contact tracing • Risk assessment: Active TB	• Scarce (shortage of supplies) • Return visit required • Cross-reactivity • Human error/bias • Variable sensitivity and specificity • Does not assess drug resistance

The more sophisticated interferon-gamma release assays (IGRAs) (QuantiFeron-TB Gold-In-Tube and the T-SPOT.TB) (**Table 2.2**) (first approved in 2001; upgraded in 2005) (52,53) were designed using whole blood to detect immune response to specific *M. tuberculosis* antigens, which are not present in BCG or certain NTM. Both tests are based on the T cell response of an individual with acquired TB infection by secretion of cytokine interferon-gamma (IFN-γ) when re-stimulated with *M. tuberculosis* antigens (52,54,55). Several publications have shown improved accuracy, predictive value and cost-effectiveness of the TB IGRA since being commercially available (56–59). Compared to the TST, IGRAs are more advantageous as they do not require patient follow-up visits and avoid cross-reactivity with BCG, thus eliminating the complication of a false-positive result. The World Health Organization (WHO) published recommendation against the use of IGRAs in low-and middle-income countries, since most IGRA studies were done in high-income countries and mere extrapolation to low-and middle-income settings with a background of high TB infection rates would not be appropriate (60). In addition, systematic reviews on IGRA performance differs in high versus low TB/HIV incidence settings, with relatively lower sensitivity in high burden settings (61).

Table 2.2: Implementation of interferon-gamma release assays. yr. 2001

Advantages	Disadvantages
• Ex vivo tests • Single patient visit • Rapid turn-around time (24 hrs) • Independent of BCG vaccination • No booster effect	• Blood samples required • Time-sensitive • High-cost • Technical errors (↓ accuracy) • High laboratory resource demand • Lack of prospective studies • Does not assess drug resistance • Inability to differentiate between active and latent disease state

Screening test

The chest X-ray (CXR) (**Table 2.3**) (discovered in 1895) was the first imaging technique, valuable in detecting and confirming the diagnosis of active TB disease for management and infection control, often in sanatoria (62). This approach was suitable to track TB progression with the purpose to evaluate the effect of the bacterium on the individual suspected of having TB (63). However, it was slow, inflexible and a relatively costly procedure with limited availability, hence reserved for high-risk groups only. This technique was later employed for hospital diagnosis in the 1930s, utilizing chest photofluorography (1936) for mass civilian screening of TB in the 1940s, when antibiotics became available. Thus, radiography played an essential role in identifying those eligible for treatment (64). However, though once a mainstay for TB control, chest radiography was discouraged when bacteriology became the standard for TB diagnosis (65).

Table 2.3: Implementation of radiography. yr. 1895

Advantages	Disadvantages
• Inexpensive • Fast • High sensitivity • Widely available (urban areas)	• Additional testing required • Low specificity • Limited availability in low- and middle income countries (rural areas) • Not a definite diagnostic test • Active TB cases missed (approx. 30% HIV) • Does not assess drug resistance • Special equipment required • Trained personnel required • Exposure to low radiation dose

Microbiological testing

Up to the 1950s, bacteriological diagnosis was mainly performed by light microscopy examination of Ziehl-Neelsen (ZN) (**Table 2.4**) (developed in the early-mid 1890s) stained smears, directly from sputum specimens, and has remained the most conventional test available for many years. However, smear microscopy has since been replaced by the Xpert MTB/RIF assay and is no longer used as a primary tool for diagnosing TB in South Africa (66,67). ZN staining is a "hot staining" method, which requires the use of heat (steam) to help drive the primary stain (carbol fuchsin) into the waxy cell wall of mycobacterial cells. It is a simple, rapid, inexpensive and highly specific method; however, is limited by variable sensitivity (20-80%) compared to auramine O (AO) fluorescent staining, which is more sensitive. As such, it was gradually replaced by fluorescence microscopy (introduced in the early 1900s), which is more sensitive, more specific and five times more rapid than ZN but was limited by high-cost light sources, the need for regular maintenance and a required dark room (68,69). This prompted the development of light-emitting diode (LED) microscopy (first commercially introduced in 1968; WHO approved for TB diagnosis in 2009), which was less costly than mercury vapour lamps and had fewer maintenance requirements. Following standard appropriate evaluation to assess efficacy and accuracy, including its effect on patients and public health, LED microscopy was shown to be more sensitive

compared to the ZN method. It became the most favoured, with qualitative, operational and cost advantages over both conventional ZN and fluorescence microscopy, and was later recommended to replace conventional fluorescence microscopy and/or be phased in as an alternative for ZN light microscopy. Though sputum smear examination was vital, it did not confirm a diagnosis of TB (not all acid-fast bacilli (AFB) are *M. tuberculosis*) and cannot identify drug-resistant strains. Therefore, clinicians were advised to perform culture confirmation on all initial samples.

Table 2.4: Implementation of sputum smear microscopy. yr. mid-late 1800's

Advantages	Disadvantages
• Simple, inexpensive and rapid • Suitable for low- and high-level laboratories (including labs with low biosafety levels) • Treatment monitoring • Widely available	• Relies on sputum • Pulmonary TB (only) • Low sensitivity (50%) among all cases; suboptimal in children, persons with HIV, extrapulmonary TB • Cannot distinguish *M. tuberculosis* from other related bacteria (NTM) • Does not assess drug resistance • Difficult to implement good quality assurance

Cultures (**Table 2.5**) were rarely done until the 1950s (solid culture: Löwenstein–Jensen (LJ) medium; developed in 1932) but since became the gold standard with an exceptional sensitivity and specificity compared to smear microscopy (70–72). The technique allows for crucial DST of mycobacterial strains and aids in monitoring treatment progress (73,74). Traditionally, primary isolation and culturing of mycobacteria from sputum (or other body sample thought to contain the TB pathogen) is performed on LJ media or 7H10/7H11 agar, allowing ample time for growth to occur followed by visual inspection to determine the presence of bacteria. Though culturing on solid media is a cost-effective approach, the major drawback is the long confirmatory time due to the extreme slow growth of the bacterium, resulting in diagnostic and treatment initiation delays. This may permit further spread of drug-resistant strains and potentially promote selection of strains with even greater resistance

(75). Liquid culture systems (e.g. BACTEC 460 system, BACTEC MGIT 960 system, MB/BacT, and Versa Trek system) are more sensitive and rapid compared to traditional solid cultures and in turn enable faster execution of the scheduled treatment. However, liquid systems are more prone to bacterial contamination, with increased frequency of NTM species. Conversely, solid cultures provide all this information by simple observation of colonies. Therefore, current guidelines recommend that all specimens cultured on liquid media also be inoculated on solid media to ensure sufficient culture purity for diagnosis of TB (76). Although highly recommended for process efficiency, lack of resources and technical expertise serve as major limitations in high prevalence settings.

Table 2.5: Implementation of culture. yr.1932

	Advantages	**Disadvantages**
Solid media:	• Definite diagnosis of TB • High sensitivity • Early detection of cases • Provides strains for DST • Monitoring TB treatment • Diagnosis of drug resistance	• Intricate and expensive • Results delayed (slow growth) • Complex handling of specimens • Skilled technicians • High biosafety level requirements
Liquid media:	• Process larger sample size • Faster	• Prone to contamination; requires rapid transport of specimens • More expensive if using MGIT • Higher biosafety level requirements (large volumes of infectious samples)

Nucleic acid amplification (NAA) technology (guidelines: first published in 1996; updated in 2009) has become essential in clinical microbiology as a valuable, rapid, yet underexploited molecular tool that enables both detection and identification of *M. tuberculosis* through enzymatic amplification of DNA. NAA tests are often used for verification of smear-positive results (within hours) or for primary case diagnosis (in combination with other methods). Though a reliable way to increase specificity of diagnosis, testing is often hindered by poor sensitivity to rule out disease, especially in smear-negative cases where clinical diagnosis is unclear and the clinical need greatest (77,78). Compared with AFB smear microscopy, the added value of NAA testing demonstrates high sensitivity (73-100%) and specificity (96-100%), and greater positive predictive value (PPV) (>95%) with AFB smear-positive

specimens in settings where NTM are common. In addition, NAA tests are able to rapidly confirm the presence of *M. tuberculosis* in 50-80% of smear-negative, culture-positive specimens. Similarly, when compared with culture, these tests are able to produce results weeks earlier for 80-90% of patients suspected to have pulmonary TB, which are then ultimately confirmed by culture (77–79). Although NAA testing is recommended for initial diagnosis of suspected cases, it should not be routinely advised when clinical suspicion of TB is low, as these cases often render a low PPV (<50%). Due to high cost and complexity with possibly lower specificity in clinical conditions, the use of these methods are restricted to developed countries; however, their introduction to developing countries is gradually progressing.

In 2008, the WHO endorsed the use of the first line probe assay (LPA) (**Table 2.6**), the GenoType MTBDR*plus* for rapid detection of MDR-TB (80). The LPA is an alternative approach recommended over conventional testing in sputum smear-positive or culture proven cases at risk of MDR-TB, such as previously treated patients (80,81). LPA has generally been available in rapid DST as a molecular assay that allows for the detection of specific gene markers associated with RIF resistance (*rpoB*) alone or in combination with INH (*katG* and *inhA* promoter). This technology utilizes DNA strip-based tests (embedded with genus-and species-specific probes) to determine the drug resistance profile of a *M. tuberculosis* complex strain. This is achieved through the pattern of binding of amplicons to probes targeting the most common resistance associated mutations to first-and second-line drugs (82,83). Probes targeting the corresponding wild-type DNA sequence are also included, allowing identification of resistance caused by less common markers (inferred by the lack of hybridization). The post-hybridization reaction would then lead to the development of coloured bands on the test strip, detecting probe binding. However, similar to other rapid tests, LPAs have some limitations such as: (1) the inability to detect mutations conferring resistance outside the regions covered by the test, (2) the possibility of systematic errors due to synonymous and non-synonymous mutations (e.g. phylogenetic markers) (84) and (3) reduced test efficiency in finding heteroresistance (a phenomenon described as the co-existence of susceptible and resistant *M. tuberculosis* variants associated with discrete resistance-conferring mutations within a single specimen), which could be missed if the resistant population is less than 95% of the total bacterial population (85,86). As such, additional phenotypic DST may be necessary to provide a full assessment. Overall sensitivities and specificities are generally high for different drugs targeted by LPAs; RIF

resistance (≥97% and ≥99%, respectively), INH resistance (≥90% and ≥99%, respectively), fluoroquinolone resistance (≥86% and 99%, respectively) and second-line injectable drug resistance (≥97% and 99%, respectively) (87,88). The use of LPA in clinical routine care aims to improve time to diagnosis for drug-resistant TB (results available within 4-6 hours), particularly when used for direct testing of smear-positive sputum specimens. Early detection of drug resistance using LPAs therefore provides earlier initiation of appropriate patient therapy with the potential to better patient treatment outcomes.

Table 2.6: Implementation of the line probe assay. yr. 2008

Advantages	Disadvantages
• Detects *M. tuberculosis* and both RIF and INH resistance • Rapid turn-around time (48 hrs) • High throughput • Specific for *M. tuberculosis* complex • Low biosafety requirements	• Not for treatment monitoring • Relies on smear results • Culture and DST still required • Contamination- and human error prone • Lower sensitivity to detect INH resistance (approx. 85%) • Discordant results: LPA vs. DST • Well-equipped labs required Intensive training of personnel

In 2010, the WHO approved the current recommended Xpert MTB/RIF assay (**Table 2.7**) for initial diagnosis of individuals suspected of having MDR-TB or HIV-associated TB (89). Xpert MTB/RIF is a rapid and automated molecular-based test that has shown remarkable sensitivity over smear microscopy in the diagnosis of pulmonary (90), extrapulmonary (91) and paediatric TB (92,93), hence promoting large-scale global rollout. This cartridge-based (Cepheid) NAA test is capable of detecting *M. tuberculosis* and its resistance to RIF directly from sputum within 2 hrs of collection (94). It uses hemi-nested real-time PCR to amplify a defined sequence of the *rpoB* gene, which is subsequently probed with molecular beacons for mutations within the rifampicin resistance determining region (RRDR), providing rapid results (95). A major advantage of the Xpert MTB/RIF test is that it allows for accurate administration with minimal hands-on technical time. The ability to detect TB and RIF resistance without referral to a specialist laboratory has been hailed as a game changer in TB

diagnostics. Reports on the sensitivity (89%) and specificity (99%) of this test have shown to be acceptable for the detection of TB (95,96). Xpert MTB/RIF has higher sensitivity for the detection of TB in smear-positive patients than smear-negative patients; nevertheless, this test may be valuable as an add-on following smear microscopy in patients previously found to be smear-negative (90). While it should be noted that RIF mono-resistance is found in nearly 5% of RIF-resistant strains, a high proportion RIF resistance is associated with concurrent resistance to INH (approx. 95%). Thus, detection of RIF resistance can be used as a marker for MDR-TB with high-profile accuracy.

Table 2.7: Implementation of the Xpert MTB/RIF assay. yr. 2010

Advantages	Disadvantages
• Detects *M. tuberculosis* and RIF resistance • Rapid turn-around time (2 hrs) • High sensitivity (89%) and high specificity (99%) • Low biosafety level requirements • Minimal training • Pulmonary TB and extrapulmonary TB	• Not for treatment monitoring • Does not detect INH resistance • Discordance: GeneXpert vs. DST results • Stable power required • Operating temp. <30°C; cartridge storage temp. <28°C • Cartridge shelf life 22 months • Instrument accuracy checked (calibrated)

2.2.2 First-line anti-TB treatment

History recounts major discoveries of effective first-line TB medications **(Figure 2.1)**.

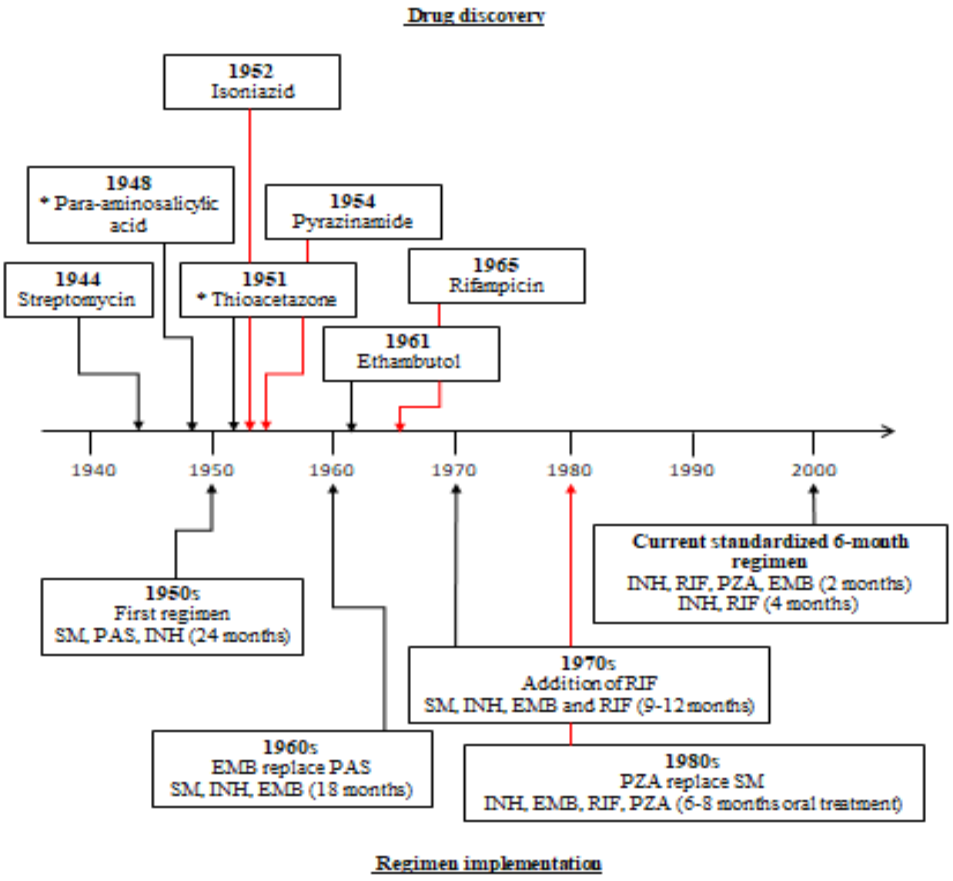

Figure 2.1: Discovery timeline of currently available first-line TB drugs. Note: * indicate drugs currently not first in class.

Streptomycin (SM) and para-aminosalicylic acid (PAS) were the first clinically introduced antibiotics in 1944/45, showing activity against *M. tuberculosis* (97,98). SM was the first effective and most widely used anti-TB treatment drug, proving that TB infected patients could be cured. However, despite improved conditions during initial treatment, SM soon revealed severe limitations after long term therapy, including the evolution of drug-resistant strains and adverse events such as hearing loss in some patients (99,100). Hearing loss is a serious adverse event, which makes the use of SM undesirable but not ineffective. For the next 20 years, studies were hence directed at preventing the emergence of drug resistance, which remains a continual primary focus of TB research today.

Accordingly, in 1950, a clinical trial by the Medical Research Council of the United Kingdom, documented the remarkable value of combined antimicrobial therapy for the treatment of pulmonary TB to suppress the emergence of resistance compared to either SM or PAS alone (101,102). Results demonstrated the combination to be more effective at achieving cure and preventing acquired resistance, thus providing insight that would shape future treatment trials. Combination therapy hence became standard as subsequent years of research proved effective treatment to rely on an array of anti-TB drugs. These advances had an important impact on TB treatment and would transform TB from being a fatal disease (in severely infected cases) to one that is curable with medications. In addition, patients would no longer need to remain at bed rest in sanatoria for prolonged periods nor be surgically treated.

Isoniazid (INH), introduced in 1952, was the next major discovery in the modern era of TB chemotherapy, with profound benefit in both experimental and human TB, displaying the optimal properties of potent early bactericidal activity, greater than any drug previously tested, and appeared to be non-toxic, inexpensive and well-tolerated (103–106). Due to the promising nature of the drug, it was thought that INH monotherapy could be quite effective; however, patients subsequently relapsed, with an approximate three-fourths having developed resistance to INH, once more demonstrating that multidrug therapy was necessary.

This paved the way for a series of trials (1952 to mid-1960s) to define the optimal combination of INH with SM and/or with PAS (107). In 1955, The British Medical Research Council (BMRC) conducted the first national drug resistance survey, which showed nearly all strains with primary resistance to be resistant to one drug only (108,109). This led to exploration of a treatment regimen with an initial three-drug phase (SM, PAS and INH) lasting 2-3 months, followed by a continuation phase of two oral drugs (PAS and INH) for 9 months (110,111). Inclusion of INH with PAS and SM (“triple therapy”) resulted in cures for 90-95% of patients. Unfortunately this strategy required up to 24 months of continuous treatment. Nevertheless, the proposed therapy was adopted and remained the standard treatment in the Western world for nearly 15 years (104). Despite the successes, TB chemotherapy remained far from optimal, with large numbers of affected people, continual side effects and increased resistance patterns, which drove further drug development exploration. In addition, drugs were too expensive to be widely used in developing countries, particularly for PAS. As such, further studies in East Africa were established on thioacetazone (TAC) as a cheaper substitute for PAS (112,113).

In 1960, a study at the Tuberculosis Chemotherapy Centre, Madras, India, revealed that domiciliary chemotherapy could be as effective as treatment in expensive hospitals or sanatoria (114,115). However, this immediately raised the question of how to ensure regular drug taking during a year of treatment (116). To address this matter, further investigation was conducted in two ways: (1) by the development of fully supervised intermittent regimens, and (2) by shortening the treatment period.

Pyrazinamide (PZA) was developed in the late 1940s, based on observational research, demonstrating nicotinamide activity against *M. tuberculosis* in animal models. Motivated by this finding, PZA was later identified (1952) as the most active pyrazine analogue of nicotinamide (117,118). This following a series of long-term experiments (1950s and 1960s) on TB treatment in mice (explored at Cornell University, Ithaca, NY, USA), which established the exceptional ability of PZA to destroy bacilli that persisted in organs after treatment with INH and SM (119). Though considered a good sterilizing drug, as a result of the presence of non-culturable bacilli, relapses eventually occurred. In addition, initial development of PZA had suggested associated hepatotoxicity at the doses used for first-line therapy (120). However, subsequent clinical trials on the possibility of using lower doses of PZA played an important role in shortening the treatment course (113,121).

In 1961, ethambutol (EMB) was uncovered by random screening of compounds active against *M. tuberculosis* in vitro and was shown to be effective on INH- and SM-resistant organisms upon treating TB in mice (122). EMB was initially used in retreatment regimens and found to be well-tolerated. This prompted subsequent clinical trials, which showed EMB to be as effective as PAS when combined with INH, ultimately leading to the replacement of PAS and for reduced treatment duration to 18 months (123,124). In subsequent years later when HIV infection led to increased toxicity of TAC as to render it unusable, EMB was instead given. Although EMB was effective in preventing failure in intermittent regimens, high relapse rates unfortunately followed (125).

Rifamycin was discovered in 1957, based on an investigation into the antibiotic properties of *Nocardia mediterranei*, followed by first clinical use of the modified compound rifampicin (RIF) (designed for oral delivery) in the late 1960s (126,127). The duration of therapy varied from 1 to 2 years. As with PZA, similar experiments later performed at the Pasteur Institute, Paris, showed high sterilizing activity of RIF (128,129). In vitro experiments substantiated this finding with probable reason ascribed to the speed at which the drug killed bacilli as they

recovered from dormancy and not to a specific rapid kill of slow growing bacilli (130). Studies showed RIF to be relatively non-toxic and comparably efficacious when used to replace other drugs in combination regimens; most importantly, as seen in patients with resistance to 7 or more antimicrobial drugs at the time (131). Early trials (132,133) on regimens including RIF showed good results, demonstrating that practical combinations of RIF, INH, SM and EMB could improve cure rates in >95% of cases, and enabled therapy to be shortened to 9 months (134). The inclusion of PZA (at low doses) further demonstrated the efficacy of a basic 6-month regimen when combined with RIF, INH and SM; shown to radically reduce the relapse rate (113,135). This proved to be a game changer over the course of the next decade, with RIF at the forefront as the keystone in the development of modern short-course TB chemotherapy regimens. Other drugs developed during this time, including cycloserine (136), kanamycin (137), ethionamide (138) and capreomycin (139) have each played a valuable role in treating drug-resistant TB.

2.3 Treatment guidelines throughout the years

Though early combination therapies were geared towards reducing drug resistance, ease of administration was prioritised to allow ambulatory treatment, which did not require administration by a medical professional. Simplified, less-frequent dosing schedules were soon explored, and SM, which required injections every 3 days was now replaced with oral medication (113).

Despite progressive reduction in the treatment duration required for cure from 24 to 6 months, non-compliance or prescription abandonment remained one of the major impediments to effective therapy. In an effort to combat these factors, direct observation was incorporated to minimize the risk of developing resistance by ensuring compliance with the appropriate drug regimen for the entire treatment period (113).

During the 1990s, the WHO introduced the current standard 6-month regimen, known as the directly observed treatment, short course (DOTS) strategy (140). This regimen consists of an initial intensive phase of 2 months of INH, RIF, EMB and PZA, followed by a continuation phase of 4 months of INH and RIF (141), and is considered highly effective for treatment of drug-susceptible TB (142,143). The DOTS programme was the first to address the issue of developing fully intermittent regimens (less than daily), with the idea of shortening the treatment period. This strategy solely relied on the use of smear microscopy for diagnosis

(despite shortcomings) and subsequent treatment with first-line anti-TB drugs only (144). This approach was increasingly implemented as part of global TB policy, particularly in poor countries, saving many lives and averting many new cases. However, although the DOTS approach served as an important development, this strategy provided limited options for prompt diagnosis and cure among children with TB, people with advanced disease from HIV and the increasing proportion of patients already infected with drug-resistant *M. tuberculosis* strains. This prompted breakthrough of the GeneXpert MTB/RIF assay, which revolutionized TB control by contributing to rapid diagnosis of TB disease and drug resistance. **Figure 2.2** describes the revised algorithm for TB diagnosis and management of patients based on test results.

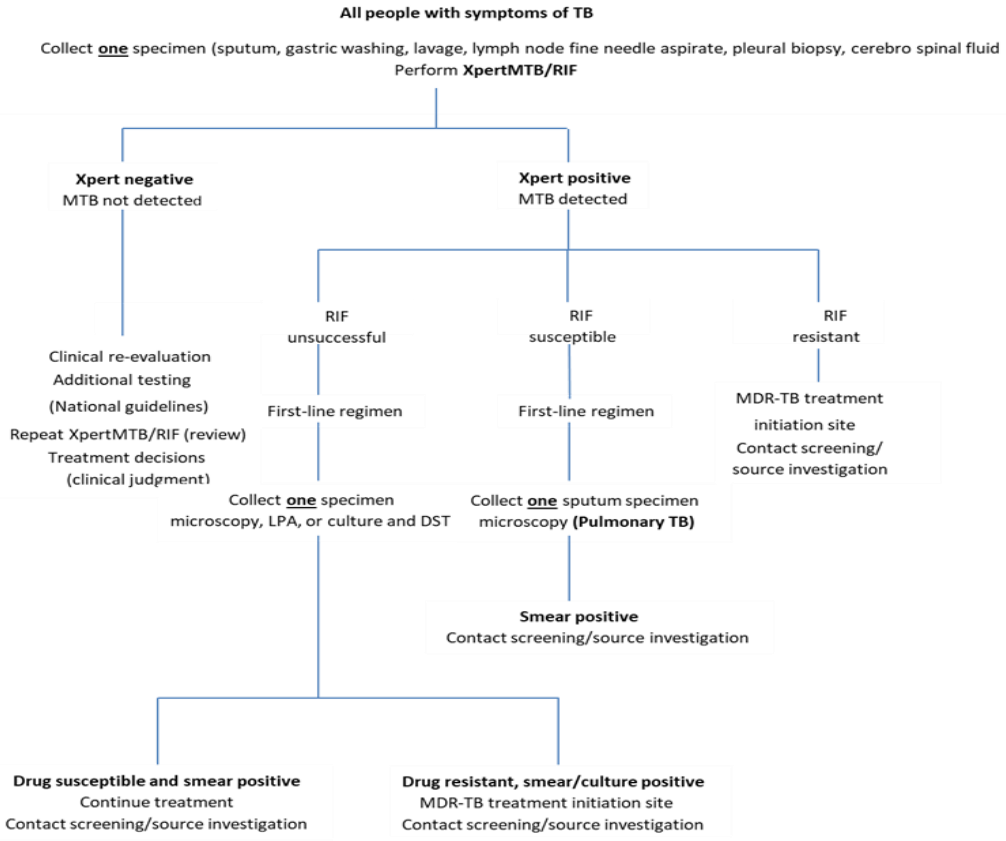

Figure 2.2: National TB diagnostic algorithm.

Shortened treatment duration and reduction of adverse drug effects are critical for improving patient treatment adherence, which is known to influence the evolution of drug resistance (145). However, despite the establishment of combination therapies displaying high cure and low relapse rates (146), drug-resistant strains continued to emerge, with frequent evolution of MDR-TB variants along with increasing HIV prevalence in different parts of the world

(27,147). Furthermore, differences in the standard of public health care systems contributed to the spread of drug-resistant TB disease, leading to varying distribution of drug-resistant TB incidence rates, as we observe today (20).

At first, the rising numbers of MDR-TB was primarily attributed to acquired resistance emerging during poor first-line treatment through poor patient adherence, inadequate treatment regimens or lower drug quality (148–150). Several other studies suggested transmission to be the main thrust to the rising epidemic (151–153), with the possibility of XDR-TB outbreaks (154), thus threatening TB control. On account of this experience, a blueprint was offered, one that was quite different from the DOTS strategy. It consisted of diagnosis with the use of mycobacterial culture and fast-track DST, access to second-line anti-TB medications, proper infection control and delivery of medications under direct observation (142). However, the principle standard-setting for many resource poor countries continued to advocate the use of smear microscopy and first-line treatment for combating epidemics. Policymakers thought MDR-TB treatment to be too expensive and complex, and considered it more important to cure new TB cases at first diagnosis, through effective application of the DOTS programme (140,155). Such reasoning may have been based on the notion that effective first-line treatment would reduce drug-resistant TB prevalence (156). In 2000, MDR-TB guidelines were officially endorsed and the DOTS-Plus implementation actively supported from 2001 (157). Yet, despite being credited with preventing emergence of resistance, the approach had little effect on current MDR-TB cases, with consistently poor treatment outcomes. Hence, emphasizing the importance of clinical and public health management to identify drug resistance as early as possible to ensure effective treatment. With the aid of current advanced molecular-based techniques, time to diagnosis has been shortened dramatically to facilitate prompt initiation with the appropriate treatment.

2.4 Challenges in the detection and treatment of drug-resistant TB

Clearly, efforts in diagnostic-drug discovery and development have the potential to add new, innovative approaches. These are targeted towards more advanced diagnostic modalities and more efficacious agents compared to the current regimen, focusing on shortening treatment duration to improve compliance; and administering safe, tolerable drugs (fewer) in correct combination and correct dosage, with the goal of increasing cure rates and reducing resistance.

Given the complex genetics of drug-resistant TB disease, it is evident that a single molecular diagnostic test would less likely cover the broad spectrum of mutations associated with a large number of drugs/drug classes used to treat drug-resistant TB (158). However, the logical extension of genotypic DST, is whole genome sequencing (WGS) (159–162) as a technologically advanced approach for routine diagnosis, drug resistance detection and strain typing (163,164). The extent to which a genetic variant confers resistance and the clinical relevance thereof should be considered when using WGS as a diagnostic tool (158,165). Hence, rapidly expanding databases (165–168) that link genetic polymorphisms associated with drug resistance along with clinical metadata would be instrumental in this regard.

Furthermore, WGS identifies heteroresistance, which can arise as a result of infection with different *M. tuberculosis* strains or through mutation within a clonal population and is found in 5.38% of drug-resistant TB cases, depending on the setting, specific drug(s) and the method used to detect resistance (169). Complications hereof, include masked heteroresistance within samples upon culturing isolates prior to DST, which may have important implications for phenotypic testing of drug resistance (170), and hence, adverse consequences for patient management, especially if underlying drug resistance is not detected by conventional DST (169). Thus, the ability to detect minority variants earlier may avoid inappropriate first-line treatment and improve treatment outcomes for drug-resistant TB patients (169).

Preventing the emergence of drug-resistant TB remains a focus, as adequate treatment may avert the emergence of drug-resistant forms, which have a worse prognosis and less therapeutic options. Treatment with so-called second- and third-line drugs is less effective, more toxic and more expensive than first-line drugs. Nonetheless, to obtain a clinical and microbiological cure, protocol is to treat patients for longer periods, due to decreased effectiveness of second- and third-line drugs. Such prolonged exposure to medication is unfortunately characterized by a poor safety and tolerability profile, which reduces patient adherence (38).

One of the key factors in the management of drug-resistant strains is the prescription of an efficacious drug regimen, which could be established based on DST results. Currently available rapid molecular tests can assess resistance of *M. tuberculosis* strains to INH and RIF, allowing administration of an early tailored anti-TB drug regimen.

Until recently, the WHO widely proposed the prescription of a standardized regimen of at least four active drugs during the intensive phase of MDR-TB treatment. This consisted of pyrazinamide (the backbone of the regimen), one of the injectable second-line drugs (amikacin, capreomycin or kanamycin), a new generation fluoroquinolone, ethionamide (or prothionamide) and cycloserine (or PAS). Other drugs would be administered in the case of resistance to one or more of the backbone drugs. According to the South African drug regimen policy framework, patients presenting for the first time with no prior second-line drug exposure for more than one month are prescribed a five drug regimen: kanamycin, moxifloxacin, ethionamide, terizidone and pyrazinamide. Treatment duration would depend on the culture conversion but should last between 4 and 6-8 months, whereas the continuation phase should be 18 months (moxifloxacin, ethionamide, terizidone and pyrazinamide) post culture conversion.

Current recommendations for longer MDR-TB treatment regimens (18-20 months) include three groups: Group A medicines (levofloxacin/moxifloxacin, bedaquiline and linezolid), Group B medicines (clofazimine and cycloserine/terizidone); and in the case when agents from Group A and Group B cannot be used, drugs from Group C (ethambutol, delamanid, pyrazinamide, imipenem-cilastatin, meropenem, amikacin (streptomycin), ethionamide/ prothionamide and *p*-aminosalicylic acid) can be included to complete the regimen. Moreover, the WHO no longer recommends the use of kanamycin and capreomycin in longer MDR-TB regimens due to the increased risk of treatment failure and relapse.

Alternatively, in the case of MDR/RR-TB patients who have not previously been treated with second-line medicines for longer than 1 month or in whom resistance to fluoroquinolones and second-line injectable agents has been excluded, a standardized, shorter regimen (9-12 months) is suggested instead of longer regimens. The shorter MDR-TB regimen consists of moxifloxacin (or gatifloxacin), clofazimine, pyrazinamide and ethambutol throughout the treatment period, supplemented by kanamycin, prothionamide and high-dose isoniazid during the intensive phase of treatment.

In the view of XDR-TB management, no formal standard regimen has been recommended thus far. Patients generally receive up to 24 months of treatment with multiple agents, often with substantial adverse effects and cure rates of approx. 14%. However, the US Food and Drug Administration (FDA) recently announced the approval of pretomanid in combination with bedaquiline and linezolid. This was following an open-label study in South Africa of a

26-week course of these agents, which has shown activity in patients with XDR-TB or treatment-intolerant/non-responsive MDR-TB (171).

2.5 Novel drugs

In recent years, new therapeutic options have been proposed for the management of drug-resistant TB, with the approval of bedaquiline (BDQ), delamanid (DLM) and pretomanid (PA-824) among several other compounds. Bedaquiline, a novel compound belonging to the diarylquinoline group offers a new mechanism of anti-TB drug action by specifically inhibiting mycobacterial adenosine triphosphate (ATP) synthase, thereby leading to intracellular ATP depletion (172,173). Bedaquiline-containing regimens increase the probability by 12 times of sputum culture conversion in MDR-TB patients during the first 2-6 months of treatment and prevents further emergence of resistance to drugs included in the backbone regimens, thus offering the possibility of a shorter treatment duration in the future. Delamanid, a first-in-class bicyclic nitroimidazole acts by inhibiting the synthesis of methoxy mycolic acid and ketomycolic acid. Delamanid-containing regimens have shown both short- and long-term efficacy in culture conversion. Improved mortality rates were observed in individuals completing ≥6 months of treatment (1%) compared to individuals with short-term or no exposure to the drug (8%). Pretomanid, a nitroimidazooxazine that inhibits mycolic acid biosynthesis and subsequently prevents mycobacterial cell wall production, also acts as a respiratory toxin against nonreplicating bacteria after nitric oxide release under anaerobic conditions (174,175). Pretomanid exhibits in vitro activity against both drug-susceptible and drug-resistant (including XDR) *M. tuberculosis* strains and has in vivo activity in animal TB models (176,177). Another new promising drug in clinical trial phase includes sutezolid, which belongs to the same chemical family of linezolid, and has shown its ability in the reduction of colony forming units. Linezolid, although efficacious is characterized by several hematological side effects, peripheral nervous system complications and gastrointestinal toxicity. However, it has been proven that therapeutic monitoring of its blood levels (TDM) could allow for dosage adjustment, followed by a reduced probability in the occurrence of adverse events. Furthermore, observational studies have shown the efficacy of meropenem-clavulanate and cotrimoxazole, with favored sputum smear (88%) and culture (84%) conversion in >80% of MDR-TB cases, and was associated with an optimal safety profile. Although evaluated in vitro in a few cases, its efficacy remains to be proven in experimental

controlled studies. The development of new therapeutic options along with new experimental clinical trials is thus needed to assess clinical drug profiles.

2.6 Conclusion: Future perspectives

The high proportion of therapeutic failure, recurrence and acquisition of additional resistance development underscores the critical importance of early identification and close monitoring of drug-resistant TB cases. Improved rapid and cost-effective diagnostic tools with high sensitivity are needed to enable rapid diagnosis of resistance to old, new and repurposed drugs (especially where phenotypic methods have not been standardized). Therefore, the use of new technology to detect full-spectrum resistance profiles is required, not only for surveillance purposes but also to inform treatment choices necessary to improve outcomes and prevent progression of disease. Ideally, shorter, simpler anti-TB drug treatments administered in the right combination and dosage should be implemented. Such changes could help avoid recurrence of past programmatic error and would have a significant impact on disease control by improving patient adherence and inhibiting the development and spread of drug resistance, thereby improving treatment completion rates. Though this may aim to "cure", it also serves to be easier to administer and keep record of. Also, the ability to identify potential risk factors for targeted interventions helps strengthen current regimens and maintains an enhanced routine surveillance system for monitoring existing and new drug resistance. Unfortunately, one caveat is the absence of reliable and standardized testing for new drugs. In this regard, the combined effort of stakeholders, including funding agencies and national/international bodies of research experts, clinicians and regulators will be instrumental in addressing described challenges, and in consolidating and expanding the design of novel drugs and favourable DST for challenging resistance patterns. This in turn would have a meaningful impact on TB policy, towards reaching sustainable goals proving to be practical, safe and effective in the treatment of both drug-sensitive and drug-resistant TB.

Chapter 3: Materials and methods

3.1 Overview

The purpose of this study was to investigate the prevalence of IMR-TB in the Kouga sub-district of the Eastern Cape Province, South Africa and to describe the *M. tuberculosis* population structure in terms of strain type and molecular mechanisms conferring resistance to INH. This was achieved using various standardized techniques. These included: 1) culture and drug susceptibility testing (DST) to identify phenotypic INH resistance, 2) Linear-after-the-exponential (LATE)-PCR or FluoroType® MTBDR and Sanger sequencing to identify the genetic basis of INH resistance, 3) targeted deep sequencing (TDS) to identify heteroresistance and 4) spoligotyping to determine strain type. The experimental workflow is outlined below (**Figure 3.1**).

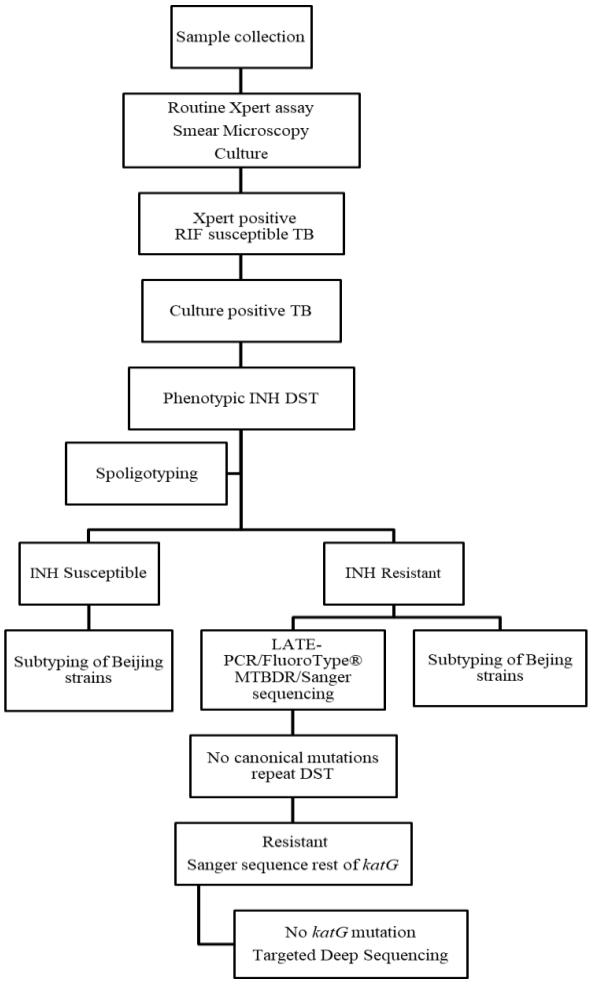

Figure 3.1: Experimental workflow of molecular techniques applied in the study in relation to phenotypic INH resistance.

3.2 Research setting

The study focused on a rural area of the Eastern Cape Province, namely the Kouga sub-district. The area is characterized by a low population density of 37/m^2 and surrounds the Nelson Mandela Bay Metropole.

3.3 Study population

Patients (excluding children <18 years) from 10 high TB burden clinics in the Kouga sub-district of the Eastern Cape Province with Xpert TB positive, RIF-susceptible or TB culture positive results were enrolled in the study between April 2016 and October 2017, following the signing of informed consent. All clinical information including HIV status if available, treatment given, DST results and outcome was extracted from the national electronic TB register (ETR.net). This information remained password protected and only accessible to clinical researchers. Ethical approval was obtained from Stellenbosch University Health Research Ethics Committee (HREC) (reference number N15/07/058) and the Eastern Cape Department of Health (reference number EC_2015RP9_641). This study did not disrupt the standard of care for RIF-susceptible TB.

As part of standard routine procedures, patients with an Xpert TB positive result are requested to return to the clinic and a second sputum specimen is collected for smear microscopy. At this point, one additional sample was collected from each study participant before treatment was initiated (baseline), as well as at routine follow-up visits at two time points: 7 weeks (end of the intensive phase) and 23 weeks (end of the continuation phase). Sputum specimens were sent to Stellenbosch University to be decontaminated, cultured and an aliquot of crude DNA prepared by boiling for further analysis.

3.4 Electronic TB register

3.4.1 Design

A prospective record review of routinely collected data was conducted during the study period for RIF-susceptible TB cases registered in the Eastern Cape provincial electronic TB database, with the purpose of evaluating patient treatment outcomes.

3.4.2 Data source

ETR.net is a Microsoft.net-based computer software program built on the recording formats of the WHO and International Union Against TB and Lung Disease (IUATLD), for more efficient and useful collection, assortment and analysis of TB data. TB data in the Eastern Cape is recorded at the primary health care facility in a paper-based TB register, which is then captured on the ETR.net and stored by the Provincial Department of Health.

3.4.3 Extraction of data

Each patient was routinely assigned a TB registration number ("blue folder number"). These numbers are assigned chronologically by each clinic and starts at one each year. We created unique patient identification numbers by combining the TB registration number, the year of registration and the clinic name to link ETR.net information to study participants, and to check for internal consistency for key fields of interest. Next, duplicate case entries were deleted from the database and patient names removed before the data was extracted for analysis.

Information was extracted based on socio-demographic variables such as age and gender, as well as clinical variables including HIV status, ARV status, previous treatment, IPT, pre-treatment sputum smear result, disease classification and treatment outcomes.

3.4.3.1 Treatment outcomes: definitions

"Unsuccessful" TB outcomes included cases that failed treatment, died, defaulted or progressed to MDR. Treatment "failure" indicates an initial baseline smear/culture positive result and remaining positive at the end of treatment or reverted to smear positive status. This includes "resistance acquired" cases of patients with RR-TB or progression to MDR-TB during treatment. "Died" includes death for any reason during the course of treatment. "Default" indicates that treatment was interrupted for at least two months during the treatment period. "Successful" treatment outcomes include cases that were cured or completed treatment. "Cured" was classified based on a positive baseline smear/culture at the start of treatment and a negative smear/culture in the last month of treatment, or on at least one previous occasion. In addition, patients had to have a negative smear/culture at least 30 days prior to completing treatment. Treatment "completed" was defined in patients with a positive baseline smear/culture at the start of treatment and who completed treatment without

evidence of failure but had no negative smear/culture during the last month of treatment, or on at least one previous occasion, because tests were not done or results were unavailable.

3.4.4 Statistical analysis

We assessed differences between IMR and drug-susceptible TB episodes using the chi-squared test and descriptive statistics for categorical variables and the Wilcoxon rank-sum test for continuous variables. In addition, we fitted logistic regression models to compare treatment outcomes of TB episodes with IMR to episodes with drug-susceptible TB after controlling for other variables. All data analyses were done by Professor van der Heijden, collaborator from Vanderbilt University, USA. Statistical software used for analysis included IBM SPSS Statistics for Windows, version 26.0 (IBM Corp., Armonk, N.Y., USA).

3.5 Culture and Drug susceptibility testing

Sputum specimens were decontaminated and subjected to culture and DST using the agar proportion method on 7H10 agar plates (178) containing INH. Repeat INH DST were done on selected isolates (see **Figure 3.1**) using the BACTEC^{TM} Mycobacterial growth indicator tube (MGIT)^{TM} 960 culture system for mycobacteria (179). As part of health and safety regulations, inoculation of *M. tuberculosis* was done in the Biosafety Level (BSL) 3 laboratory.

3.5.1 Procedure

3.5.1.1 NALC-NaOH decontamination method

Sputum specimens were decontaminated using the N-acetyl-L-cysteine (NALC)-sodium hydroxide (NaOH) (Sigma-Aldrich) method. Briefly, each sputum specimen (at least 2 ml) was transferred into a sterile 50 ml centrifuge tube in a class 2 biosafety cabinet and an equal volume of NALC-NaOH-sodium citrate solution was added (mix NaOH (4%) and Trisodium citrate•3H_2O (2.94%); add 0.5 g NALC to 4 ml aliquots). The tube was capped and mixed on a vortex until the specimen liquefied (15 min). Thereafter, phosphate buffer (0.067 mol/L, pH 6.8) was added to the solution (at least double the amount or up to 50 ml), mixed well and tubes centrifuged for 15 min at 3000 x g. The supernatant fluid was carefully decanted and the sediment resuspended by adding a small quantity of phosphate buffer (between 0.5 and 2 ml). Smears were then prepared for microscopy using Ziehl-Neelsen (ZN)-fixative before inoculation of MGIT tubes.

3.5.1.2 Smear preparation

Following digestion/decontamination, concentration and resuspension of the pellet; the specimen was then well-mixed with a pipette and one drop (or two-three loopfulls) were placed on a clean microscope slide, spread and allowed to air dry. The smear was then heat-fixed on a slide warmer (heating block) at 85˚C for 2 hrs.

3.5.1.3 ZN staining

The 'hot' ZN method is a differential staining technique used to identify acid-fast bacilli (AFB), mainly mycobacteria.

The prepared bacterial smear was covered with carbol fuchsin (Sigma-Aldrich) stain and gently heated (without boiling) for 5 min by passing a flame beneath the slide until vapour began to rise (i.e. approx. 60˚C). This allows the stain to penetrate and bind to the mycolic acid in the bacterial cell wall. The heated stain was then allowed to cool and gently rinsed with clean H_2O until no colour appears in the effluent. Hereafter, the smear was covered with 3% v/v acid alcohol (or 20% sulfuric acid) (Thermo Scientific, USA) for 2 min until sufficiently decolourised (i.e. pale pink), then gently rinsed with clean H_2O. The acid decolourising solution removes the stain from nonacid-fast cells, while mycobacteria retain the stain. Next, the smear was covered with methylene blue (counter stain) for 1-2 min and gently rinsed with clean H_2O. Methylene blue stains the background material, providing a contrast colour against which the red AFB can be seen. The smear was allowed to air dry and examined under the microscope using 100x oil immersion objective.

3.5.1.4 Sputum inoculation in MGIT™ 960 tubes

MGIT tubes each contain an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the base of the tube. The medium contains free oxygen that is utilized during bacterial growth and is subsequently replaced with carbon dioxide. The instrument exploits the fluorescence of the oxygen sensor in the tube when exposed to ultraviolet (UV) light. The intensity of fluorescence is equivalent to the level of oxygen present in the media and is thus a measure of bacterial growth.

At least 500 µl of decontaminated sputum was inoculated into a MGIT tube for initial growth and incubated at 37˚C in the BACTEC™ MGIT™ 960 instrument until test tube flagged positive (for a maximum period of 42 days), with the remaining specimen stored at -70˚C in a

proteose peptone sterile tube with beads. Further inoculation was then performed on the day of positivity. However, if the reading was below 100 (between 70 and 100), incubation was allowed to proceed for another day before inoculating on solid medium.

3.5.1.5 Drug susceptibility testing using solid media - 7H10 Middlebrook agar

7H10 Middlebrook agar is a solid growth medium used for culturing of *M. tuberculosis* and has been proposed for both primary isolation and DST of tubercle bacilli (180). The solid media method for DST is designed to provide a suggested interpretation of susceptibility. The same principle applies to the BACTEC™ MGIT method for DST as described in 3.5.2.

Solid medium was prepared by suspending 7.6 g of 7H10 agar base (Sigma-Aldrich) in 360 ml of dH_2O, followed by the addition of 2 ml of glycerol, and autoclaved at 121˚C for 10 min. A stock solution of the drug (INH) was prepared to a concentration of 10 mg/ml in dH_2O (potency 1000 mg/g = 100%) and sterilized by filtration through a 2 µm Millipore filter. Drug stocks were stored at -70˚C for a maximum of 6 months. Following sterilization of the medium, the 7H10 base broth was then allowed to cool in a water bath to 52-56˚C, then aseptically supplemented with 40 ml Oleic Albumin Dextrose Catalase (OADC) (Sigma-Aldrich) and INH at concentrations of 0.2 and 1 µg/ml, and mixed well before dispensing 5 ml into pre-labelled quadrant petri dishes. Hereafter, 100 µl of the well-mixed MGIT culture was spread on each quadrant containing 0.2 and 1 µg/ml INH. Each test included a growth control (GC) (i.e. undiluted culture) and a 1:100 control (i.e. diluted culture) spread onto quadrants with supplemented 7H10 media in the absence of INH. Following incubation at 37˚C for 2-3 weeks, the plates were read and growth in the presence of INH was compared to the 1:100 control (in the absence of INH).

3.5.2 Quantitative drug susceptibility testing - BACTEC™ MGIT™ 960 culture system

MGIT960 DST was performed on all clinical isolates found to be resistant on agar proportion method but for which no resistance conferring markers could be identified by genotypic testing with LATE-PCR or FluoroType® MTBDR, as described in **3.6**. This was done to confirm phenotypic resistance in these culture isolates.

The BACTEC™ MGIT™ 960 culture system, in conjunction with the Becton Dickinson (BD) EpiCenter™ software is a highly sensitive method for the detection of mycobacterial growth (181–184). This method allows for qualitative testing of *M. tuberculosis* resistance to one or more drugs, under specific growth conditions (refer to MGIT manual) (185).

Qualitative DST is based on a single critical concentration (CC) (breakpoint) as a standard to discriminate whether an isolate is susceptible or resistant to a drug, in this case INH (186). A 1% critical proportion is typically used to differentiate between drug-resistant and drug-susceptible strains, whereby growth of the test isolate at a standard inoculum size in the presence of a drug is compared to the growth of a 1/100 dilution of the same culture in the absence of the drug. This implies that ≥1% bacterial growth in the presence of the critical concentration of a drug compared with the growth in the absence of the drug is considered resistant. Conversely, <1% growth indicates that the culture is susceptible. This is documented by the BACTEC™ MGIT™ 960 instrument, which continuously monitors tubes for increased fluorescence and recorded by the epicenter. A resistant strain shows better growth in the presence of an antimicrobial as opposed to the 1% GC. In contrast, a susceptible strain shows better growth in the GC lacking drug compared to the drug-containing tube, indicating more than 99% inhibition.

In the MGIT system, low-level resistance to INH is when there is growth between 0.1 and 1 µg/ml INH. High-level resistant status is designated to a sample if there is growth at concentrations higher than 1 µg/ml INH.

3.5.2.1 Procedure

To culture from frozen stock samples, freshly prepared MGIT tubes were each labelled with the corresponding sample number and 800 µl of reconstituted PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin)/OADC supplement was aseptically added to each tube. Two glass beads coated with *M. tuberculosis*, stored at -70˚C were removed from the Nunc vials under sterile conditions and added to the respective MGIT tubes. The inoculated tubes were then incubated at 37˚C in the BACTEC™ MGIT™ 960 instrument, until tubes flagged positive. Subsequently, 250 µl of the positive inoculum and 800 µl of Middlebrook OADC were transferred into fresh MGIT tubes and incubated at 37˚C.

As per the manual, the first day of positivity is considered Day 0. Thereafter, sub-cultures are to be directly used as inoculum for susceptibility testing, following an additional 2 days of incubation (Day 2).

Day 2 cultures were thoroughly mixed by vortexing and the undiluted cultures were subsequently used for inoculation of drug tests. However, a positive tube may be used for DST up to and including the 5th day (Day 5) after the instrument flagged it as positive. Thus,

cultures that became positive on Day 3, 4 or 5 were firstly diluted (1:5) by adding 1 ml of the positive suspension into 4 ml sterile saline, then vortexed and used for inoculation. An additional purity check was done of the inoculum by streaking the test culture specimen onto blood agar plates and preparing a slide for ZN staining and microscopy.

Sub-cultures of clinical isolates were grown at 2 concentrations of INH (0.1 and 1 µg/ml) to distinguish between susceptible and resistant strains. Furthermore, growth between 0.1 and 1 µg/ml is indicative of low-level resistance and if growth occurs at concentrations equal to or higher than 1 µg/ml, it is considered high-level resistance. A no-drug control tube was also included.

Fresh MGIT tubes were prepared for each positive sub-cultured specimen, including one GC tube and 2 INH drug tubes to be tested at concentrations of 0.1 and 1 µg/ml respectively. The above-mentioned concentrations were calculated as follows: An 840 µg/ml drug stock solution was prepared by measuring 8.4 mg of INH to be dissolved in 10 ml sterile dH_2O. This was freshly diluted to appropriate concentrations for further use. An 800 µl aliquot of OADC was added to each MGIT tube, followed by 100 µl of reconstituted drug (in the appropriate concentrations) to the drug labelled tubes to obtain final concentrations of 0.1 and 1 µg/ml INH. In preparation of the GC MGIT inoculation, positive sub-cultured specimens were vortexed and 100 µl thereof transferred to a 10 ml sterile saline containing tube (1:100 dilution) and inverted. A 500 µl aliquot of the newly inoculated saline tube was added to the respective GC MGIT tubes with no drug, together with 500 µl of the undiluted MGIT culture to the drug-containing tubes. MGIT tubes were mixed well, subsequently scanned and entered into the BACTEC™ MGIT™ 960 instrument for incubation at 37˚C.

Results were monitored on the BD EpiCenter™ system and incubation terminated 7 days after the control reached 400 growth units. Results were interpreted as follows: an isolate is considered resistant (R) when the GC reached a growth unit ≥400, while the growth unit of the drug tube ≥100; an isolate is susceptible (S) when the growth unit of the drug tube <100 until 7 days after the GC reaches a growth unit ≥400 and an isolate is intermediate (I) when the growth unit of the drug tube >100 in a 7 day period after the GC reaches a growth unit ≥ 400 (185).

3.6 Strain typing

3.6.1 Spoligotyping

All clinical isolates from positive cultured sputum specimens that formed part of the study were subjected to spoligotyping, a molecular strain typing method, described by Kamerbeek et al. in 1997 (187). This was done to categorize isolates according to strain families, to gain insight to the most prevalent strain type observed among patients and to identify possible cases of mixed infections, which could potentially indicate underlying resistance.

Spoligotyping is a rapid, reliable and informative deoxyribonucleic acid (DNA) amplification-based technique that has been used extensively in TB research, enabling the identification and characterization of a diverse spectrum of *M. tuberculosis* strains (187–189). This technique is an internationally standardized reverse line probe assay, based on polymorphisms of the chromosomal direct repeat (DR) locus of *M. tuberculosis*, which contains a variable number of numerous short DR sequences with unique spacer sequences that are well-conserved (**Figure 3.2**). These loci are interspersed with 34-41 base pair (bp) non-repetitive spacer sequences, which can be amplified through PCR using primers designed from the DR sequence. It also includes strain-dependent hybridization patterns of in vitro-amplified DNA with multiple spacer oligonucleotides (187,190).

The initial step in this methodology entails PCR amplification of the DR region, followed by linkage of synthetic oligonucleotides, specific to each spacer, to an activated membrane in parallel lines. The PCR products are then hybridized onto the membrane, perpendicular to the oligo lines, which can be visualized using chemiluminescence and autoradiography. Differentiation between *M. tuberculosis* isolates depends on the presence or absence of spacer sequences, generating a characteristic pattern for each strain analysed.

Spoligotyping can be used to assess strain population structure in different settings and enables rapid identification of strain families that are overrepresented in a given setting (191). In addition, spoligotyping can aid in determining whether a particular TB episode arises from relapse or reinfection in cases where reinfection is caused by a different strain type (192).

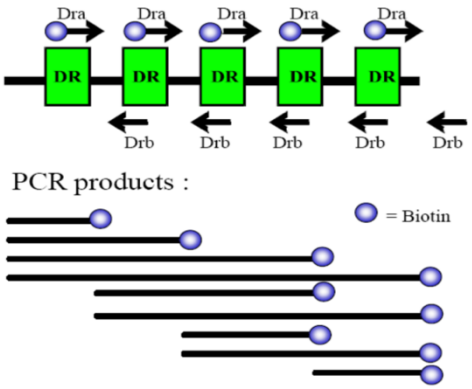

Figure 3.2: The principle of in vitro PCR amplification of DNA within the DR region of the *M. tuberculosis* complex using primers "a" (Dra) and "b" (Drb) (187).

For the purpose of this study, the method cited in Kamerbeek (187) was followed, with minor adaptations. Isolates were organized into strain families in accordance to the international spoligotype database (SpolDB4) (193).

3.6.1.1 Procedure

3.6.1.1.1 PCR amplification

M. tuberculosis isolates were spoligotyped with the internationally standardized PCR protocol constituting a PCR mix with primers DRa (biotinylated) and DRb (0.2 µmol/µl each) as summarized in **Table 3.1** and cycling conditions detailed in **Table 3.2**. This allows for amplification of spacers by means of amplifying the entire DR region. Aliquots of 23 µl each of this mixture was added into PCR strip tubes, followed by 2 µl of template DNA. To prevent contamination, PCR amplification was conducted in a physically separated workstation. In each experiment, a negative control was included to identify any possible amplicon contamination. In addition, *M. tuberculosis* strain H37Rv and BCG was used for estimation of analytical accuracy.

Table 3.1: PCR amplification mix for genotyping of DNA.

PCR mix	**25 µl**
dH_2O	6.5 µl
DRa (5′biotin) (GGT TTT GGG TCT GAC GAC)	2 µl
DRb (CCG AGA GGG GAC GGA AAC)	2 µl
Kapa Taq readymix	12.5 µl
DNA template	2 µl

Table 3.2: Standard PCR cycling for spoligotyping.

Cycle step	Temperature	Time	Number of cycles
Activation	95˚C	3 min	
Denature	94˚C	1 min	
Annealing	55˚C	1 min	x30
Extension	72˚C	30 sec	
Final Extension	72˚C	10 min	

3.6.1.1.2 Hybridization

Following amplification, biotin labelled PCR products were hybridized to the immobilized spacer oligos representing spacers of known sequence. Amplified PCR products (25 µl) was added to 150 µl 2x SSPE/0.1% SDS (2x saline sodium phosphate-EDTA (ethylenediamine tetra-acetic acid)/0.1% sodium dodecyl sulphate) (Thermo Scientific, USA). Next, the diluted PCR product was heat-denatured at 99˚C for 10 min and immediately cooled on ice for the annealing of single strand DNA. The membrane (Mapmygenome^{TM}, India) was prepared by washing at 60˚C in 2x SSPE/0.1% SDS for 5 min and placed on support cushion on the miniblotter (Mapmygenome^{TM}, India), perpendicular to the line pattern of the applied oligonucleotides. Residual fluid was removed by aspiration and the slots were each filled with 150 µl diluted denatured PCR product, taking care to avoid air bubbles and avoiding contamination of neighbouring slots. Hybridization occurred for 60 min at 60˚C on a horizontal surface without shaking. Next, the samples were removed from the miniblotter by aspiration and the membrane transferred and washed twice in 2x SSPE/0.5% SDS at 60˚C for 5-10 min. After this, 50 ml 2x SSPE/0.5% SDS (42˚C) with 12.5 µl Streptavidine-peroxidase conjugate (500 U/ml) (Roche, Germany) was added and incubated at 42˚C for 45-60 min. The membrane was then washed twice with 2x SSPE/0.5% SDS at 42˚C for 5-10 min. Next, the membrane was rinsed with 2x SSPE for 5 min at room temperature and incubated in 20 ml (10 ml solution 1 + 10 ml solution 2) enhanced chemiluminescence (ECL) mix (Amersham ECL Direct^{TM} nucleic acid labelling and detection system, GE Healthcare Limited, Little Chalfont, UK) for 1 min 30 sec. The membrane was hereafter exposed to an X-ray film for 5-20 min and developed with a hyperprocessor. Lastly, the membrane was regenerated by washing in 1% SDS at 80˚C for 30 min-1 hr and stored at 4˚C in 20 mM EDTA (Sigma-Aldrich) pH8 overnight.

3.6.2 Differentiation between Typical and Atypical Beijing

All baseline (drug-resistant/susceptible) isolates with a characteristic Beijing spoligotype were further characterized as modern/typical or ancestral/atypical Beijing sublineages, based on the presence or absence of an IS*6110* insertion sequence in the noise transfer function (NTF) region (Rv0001-Rv0002) (194,195).

3.6.2.1 Procedure

3.6.2.1.1 PCR amplification

Two separate PCR amplification reactions were performed on each baseline isolate from patients infected with a Beijing strain using primers (Whitehead Scientific) in **Table 3.3** (196). A universal forward primer, binding in the IS*6110*, and two primers (IS21-4 and IS21-19) binding outside of the IS*6110* at unique locations, were used in combination with internal control primers to determine the presence (Typical Beijing) or absence (Atypical Beijing) of IS*6110* elements to specific chromosomal loci (197,198). The PCR mix used for amplification is outlined in **Table 3.4** and PCR cycles in **Table 3.5** (196).

Table 3.3: Primer sequences for PCR amplification.

Primer set	Primer name	Primer sequence (5' to 3')	Tm[b]
	Internal control	TCCCAGTGACGTTGCCTTC	62˚C
	Internal control	GAGCAGCAGTGGAATTTCGC	62˚C
	Universal Forward	TTCAACCATCGCCGCCTCTAC	62˚C
1	IS21-4 (1592)[a]	CAGCGACACTCACAGCCAATTGAACC	62˚C
2	IS21-19 (3493910)[a]	GAACCCCGGCTCCGCCTCGATGAACC	62˚C

[a] chromosomal position of IS*6110* insertion sites
[b] Tm, melting temperature

Table 3.4: PCR DNA amplification mix for genotyping of Beijing strains.

PCR mix	25 µl
dH_2O	8.375 µl
Q solution	5 µl
10x buffer	2.5 µl
MgCl2 (25 mM)	2 µl
dNTPs (10 mM)	4 µl
Primers (50 pmol/µl)	1 µl
Qiagen Hotstar Taq polymerase	0.125 µl
DNA template	1 µl

Table 3.5: Standard Hotstart PCR amplification cycles to distinguish between Typical and Atypical Beijing strains.

Cycle step	Temperature	Time	Number of cycles
Activation	95˚C	15 min	
Denature	94˚C	1 min	x35-40
Annealing	62˚C	1 min	
Extension	72˚C	1 min	
Final Extension	72˚C	10 min	

3.6.2.1.2 Electrophoretic evaluation

PCR amplification products were electrophoretically fractionated in a 2% agarose gel in 1x tris-borate-EDTA (TBE) buffer (pH 8.3) at 100-150 V for ± 1 hr, and visualized by staining with SYBR Green (Thermo Scientific, USA) under UV light using the Bio-Rad ChemiDoc imaging system (Bio-Rad, UK). The sizes of amplified DNA samples were determined by a 100 bp Plus DNA ladder (GeneRuler, Thermo Scientific, USA). PCR products were analysed according to intensity and expected band size (200 and 300 bp respectively), as well as the number of bands observed (typical Beijing: 4 band pattern; atypical Beijing: 3 band pattern).

3.7 Genotypic testing

3.7.1 Linear-after-the-exponential PCR or FluoroType® MTBDR

A new and highly innovative PCR technique, known as LATE-PCR was used for genotypic testing of INH and RIF resistance in baseline isolates that were phenotypically resistant, as well as follow-up isolates to determine emergence of resistance. This method was later substituted with FluoroType® MTBDR, which is the validated commercial form of the test.

The molecular genetic assay is a qualitative in vitro test that allows for automated detection of the *M. tuberculosis* complex and its resistance to RIF and/or INH, using DNA extracted from decontaminated sputum specimens or cultivated isolates. RIF resistance was detected by mutations in the rifampicin resistance determining region (RRDR) of the *rpoB* gene (coding for the β-subunit of RNA polymerase) (199,200). High-level INH resistance is detected by certain mutations of the *katG* gene (coding for catalase-peroxidase) (201,202) and low-level resistance by mutations in the promoter region of the *inhA* gene (coding for NADH-enoyl-ACP reductase) (203,204).

LATE-PCR/FluoroType® MTBDR is an advanced approach to non-symmetric PCR. The assay incorporates both limiting and excess primer pairs to generate double-stranded DNA. This is followed by the production of single-stranded amplicons with predictable kinetics for many cycles beyond the exponential phase. Sets of Lights-On/Lights-Off probe pairs are used, which are considered reliable, enhances allele discrimination and increases signal strength relative to symmetric PCR, making it useful for real-time qualitative analysis.

Conventional symmetric PCR permits rapid and quantitative identification of unique DNA targets. However, it is inefficient and difficult to optimize as it results in a decreased melting temperature below the reaction annealing temperature when the concentration of a single primer is limited. As a result, reactions tend to slow down and plateau stochastically because reannealing of the template strands gradually outcompete primer binding to the template strands. Hence, the more binding of additional PCR products to the target, the less efficient the reaction because no de novo synthesis occurs.

LATE-PCR/FluoroType® MTBDR is based on a functional approach, which combines asymmetric excess PCR with Lights-On/Lights-Off probe pairs. It uses pairs of limiting and excess primers at unequal concentrations to avoid the problem of amplicon strand reannealing. It further modifies the exponential enrichment of PCR products into linear, single-stranded DNA available for probe hybridization to the target sequence at a wide temperature range. Primers are designed with adjusted melting temperatures to increase amplification efficiencies and to provide a rational framework for the reliable amplification of single-stranded DNA with improved sensitivity (no plateau) and flexible use of primer ratios.

Specific variants are identified by nucleotide sequence-specific probes that are complementary to specific target sequences. Adjacent sets of Lights-On/Lights Off probes, labelled either with fluorophores and quenchers (Lights-On) or with quenchers only (Lights-Off) are used to scan hundreds of nucleotides for the detection of drug resistance conferring mutations in *rpoB, katG* and the promoter area of *inhA*. These low temperature Lights-On/Lights-Off consensus probes are used at end-point to coat the accumulated single-stranded amplicon by decreasing the temperature. Because each probe is a mismatch tolerant, the temperature at which it hybridizes to its complementary region within the target is sequence dependent. Once bound to the amplicons, a unique fluorescent signature is released depending on which fluorophores were used to label Lights-On probes. In the unbound

Lights-On probe, the fluorescence emission is intrinsically quenched. Lights-On and Lights-Off probes then detach from the amplicons at their specific melting temperatures while stringency (temperature) is gradually increased. The fluorescence emitted is then measured on the FluoroCycler® 96 apparatus. Normalization of fluorescent signatures allows for easy comparison of different samples for the presence of variants. In addition, fluorescent signatures using the same targets and probes are considered to be highly reproducible.

3.7.1.1 Procedure

3.7.1.1.1 LATE-PCR: *rpoB, inhA* and *katG* gene amplification

Both probes and primers were pre-prepared by dissolving it in 10 mM Tris (hydroxymethyl) aminomethane buffer to a final concentration of 100 µM. For all excess primers and OFF probes, a working stock was prepared by transferring an aliquot of the 100 µM stock solution to a new test tube. For all limiting primers and ON probes, a working stock of 10 µM was prepared in Tris buffer.

A PCR mix (**Table 3.6**) was prepared for gene-specific amplifications from the above mentioned working stocks to a final concentration of: 0.15 µM OFF probes (*rpoB* Probe 1, *rpoB* Probe 3, *rpoB* Probe 6, *inhA*, *katG*); ON probes: 0.05 µM (*rpoB* Probe 4, *inhA*, *katG*), 0.025 µM (*rpoB* Probe 5) and 0.075 µM (*rpoB* Probe 5_G); 1 µM excess primers (*rpoB*, *katG*, *inhA*) and 0.05 µM limiting primers (*rpoB*, *katG*, *inhA*).

In addition, the following working stocks (10x PCR, 10 mM dNTPs, 50 mM Mg^{2+}, 5 U/µl Taq) were prepared to a final concentration of: 1x PCR, 0.3 mM dNTPs, 3 mM Mg^{2+}, 2 U/µl Taq and dH_2O added to the mix. A 23 µl aliquot PCR mix was added to new test tubes, followed by 2 µl of crude DNA sample and analysis performed using the Stratagene MXP5001 real-time instrument. Two no-template controls (NTC) and H37Rv were included as PCR controls. PCR cycling conditions used are presented in **Table 3.7**. Following completion of the PCR reaction, samples were then heated for 10 min at 75˚C, followed by a cooling step at 25˚C for 10 min, which would allow for all probes to anneal to their target. This was then followed by a melting protocol with 1˚C increments at 1 min intervals to 99˚C. A fluorescent reading was taken by the instrument at each interval. The fluorescence data analysis of the probe-target hybridizations following amplification were analysed by anneal curve analysis using the first derivative.

Table 3.6: PCR mix for gene amplification.

PCR mix		**25 µl**
Probes		
OFF	*rpoB* Probe 1, *rpoB* Probe 3, *rpoB* Probe 6, *inhA*, *katG*	0.0375 µl
ON	*rpoB* Probe 4, *inhA*, *katG*	0.125 µl
	rpoB Probe 5	0.0625 µl
	rpoB Probe 5 G	0.1875 µl
Primers		
Excess	*rpoB*, *katG*, *inhA*	0.25 µl
Limiting	*rpoB*, *katG*, *inhA*	0.125 µl
PCR		2.5 µl
dNTPs		0.75 µl
Mg^{2+}		1.5 µl
Platinum Taq		0.40 µl
DNA template		2 µl

Table 3.7: Thermocycling conditions for LATE-PCR.

Cycle step	**Temperature**	**Time**	**Number of cycles**
Activation	95˚C	3 min	
Denature	98˚C	10 sec	x60
Annealing	75˚C	40 sec	
Extension	75˚C	40 sec	

3.7.1.1.2 FluoroType® MTBDR: *rpoB*, *inhA* and *katG* gene amplification

FluoroType® MTBDR is a simple and rapid commercial assay based on the principles of LATE-PCR. In preparation of the master mix (pre-PCR), each amplified mix: AM-A (contains buffer, Taq polymerase and bovine serum albumin) and AM-B (contains buffer, specific oligonucleotides, nucleotides, bovine serum albumin and salts) was thawed and gently mixed. Fresh PCR master mix was then prepared, containing 6 µl of AM-A and 14 µl of AM-B per sample and mixed thoroughly. A 20 µl aliquot of the master mix was then transferred into the respective sample wells (including that for downstream PCR controls) of the prepared PCR plate.

To avoid contamination, the following steps post-PCR (FluoroLyse kit, Hain Lifescience) was done in a separate designated area. A 1 µl aliquot of internal control (IC) was added to

fresh tubes representing each sample. Next, 20 µl of negative and positive (C+) control samples were added to the respective control wells. A 100 µl aliquot of supernatant DNA from each sample was then transferred into the corresponding tubes containing 1 µl of IC. Tubes were then vortexed and 20 µl of sample transferred into the respective sample wells. The PCR plate was then sealed and briefly centrifuged to ensure uniformity of the sample mix in all wells before running it on the FluoroCycler® 96 apparatus (Hain Lifescience, Germany). The Fluoro-Software® IVD automatically compiles a report on the outcome for each sample, along with its respective fluorescence signature. Interpretation of probable resistance causing mutations compiled from the report was analysed using the available manual.

3.7.2 Sanger sequencing of *rpoB, katG* and *inhA* promoter

DNA samples with inconclusive results and/or discrepancies (phenotypically resistant on solid media but wild-type by LATE-PCR or FluoroType® MTBDR) were subjected to PCR amplification and Sanger sequencing of the *rpoB*, *katG* 315 and *inhA* promoter regions. This was done to detect mutations conferring resistance in drug-resistant isolates and/or determine acquired resistance in follow-up susceptible isolates.

3.7.2.1 Procedure

3.7.2.1.1 Primer design

PCR primers (**Table 3.8**) were designed for amplification of the *rpoB*, *katG* 315 and *inhA* promoter regions from genomic DNA using Primer-BLAST software (205) and *M. tuberculosis* H37Rv laboratory strain (206).

Table 3.8: Primers for PCR amplification of target genes (*rpoB*, *katG* and *inhA* promoter).

Gene	Primer	Primer sequence (5' to 3')	Tm	Size (bp)
katG	RTB59_F	TGGCCGCGGCGGTCGACATT		
	RTB38_R	GGTCAGTGGCCAGCATCGTC	62˚C	419
***inhA* promoter**	inhAP5	CGCAGCCAGGGCCTCGCTG		
	InhAP3	CTCCGGTAACCAGGACTGA	55˚C	246
rpoB	rpoB_F	TGGTCCGCTTGCACGAGGGTCAGA	78˚C	
	rpoB_R	CTCAGGGGTTTCGATCGGGCACAT	76˚C	437

3.7.2.1.2 PCR amplification

DNA samples were subjected to PCR amplification using a standard Hotstart PCR protocol for the *inhA* promoter and *katG* 315-region, and Touchdown PCR for the *rpoB* region using annealing temperatures 72-69˚C, using an Applied Biosystems thermal cycler 2720 (Thermo Scientific, USA). In the case of an inconclusive genotypic test result, PCR amplification was performed using primer sets for all three genes (*rpoB, katG* and *inhA* promoter). The PCR amplification mix (**Table 3.9**) and respective cycling conditions (**Table 3.10 and 3.11**) for each primer set is outlined below.

Table 3.9: PCR mix for amplification of target genes (*rpoB, katG* and *inhA* promoter).

PCR mix	**25 µl**
dH_2O	8.375 µl
Q solution	5 µl
10x buffer	2.5 µl
MgCl2 (25 mM)	2 µl
dNTPs (10 mM)	4 µl
Primer_F (50 pmol/µl)	1 µl
Primer_R (50 pmol/µl)	1 µl
Qiagen Hotstar Taq polymerase	0.125 µl
DNA template	1 µl

Table 3.10: Standard Hotstart PCR cycling for amplification of target genes (*katG* and *inhA* promoter).

Cycle step	**Temperature**	**Time**	**Number of cycles**
Activation	95˚C	15 min	
Denature	94˚C	1 min	x40
Annealing	62˚C (*katG*) 55˚C (*inhA*)	1 min	
Extension	72˚C	1 min	
Final Extension	72˚C	10 min	

Table 3.11: Touchdown PCR cycling (*rpoB*).

Cycle step	Temperature	Time	Number of cycles
Activation	95˚C	15 min	
Denature Annealing Extension	94˚C 72˚C 72˚C	1 min	x2
Denature Annealing Extension	94˚C 71˚C 72˚C	1 min	x2
Denature Annealing Extension	94˚C 70˚C 72˚C	1 min	x2
Denature Annealing Extension	94˚C 69˚C 72˚C	1 min	x40
Final Extension	72˚C	10 min	

3.7.2.1.3 DNA sequencing

PCR amplified products (confirmed by gel electrophoresis) were subjected to post PCR manual bead clean-up and sequenced with only the forward primer from each primer set using Sanger sequencing at the Central Analytical Facility (CAF), Stellenbosch University. Gene sequencing files received from CAF were aligned to the *M. tuberculosis* H37Rv laboratory reference sequence using DNAMAN (Lynnon, Quebec, Canada) and BioEdit version 7.2, while Chromas version 2.4 (Technelysium, Australia) was used to visually inspect chromatograms of sequences for underlying variants (represented as double peaks).

3.7.3 Sequencing of the entire coding region of *katG*

Phenotypically confirmed resistant isolates (by agar proportion and MGIT DST methods) with no detectable mutations following standard genotypic resistance testing in the region of *inhA* promoter or *katG* were further investigated by sequencing the entire coding region of the *katG* gene (namely fragment 4, fragment 3 and fragment 1) (**Figure 3.3**). These exclude fragment 2, which surrounds the *katG* 315 region (described above). This was done with the purpose of identifying mutations outside the well-known drug resistance hotspot position. Isolates with no identifiable mutations in these regions were subsequently selected and DNA

extracted for TDS to identify underlying INH-resistant *M. tuberculosis* populations with canonical *katG* gene and *inhA* promoter mutations. Analyses were done in collaboration with the Translational Genomic Research Institute (TGEN) based in Arizona, USA.

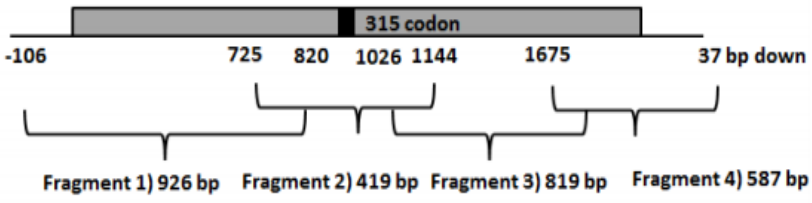

Figure 3.3: Amplification of the entire coding region of *katG* (fragments 1-4) via Sanger sequencing; fragment size indicated as "bp" and numbers above square brackets as relative genetic position.

3.7.3.1 Procedure

Fragment-specific primers (**Table 3.12**) were designed for PCR amplification using Primer software 3 version 0.2 (207) and *M. tuberculosis* H37Rv reference strain. PCR amplification was done using the standard Hotstart PCR protocol (**Table 3.9** and **3.10)** and amplified PCR products sent for Sanger sequencing at CAF.

Table 3.12: Primers for PCR amplification of *katG* fragments.

Gene (katG)	Primer	Primer sequence (5'-3')	Tm	Size (bp)
Fragment 1	katG1_F	GCCCGATAACACCAACTCCT		
	katG1_R	GGCGCCATGGGTCTTACC	62˚C	926
Fragment 3	katG3_F	GCTGACGAAGAGCCCTGC		
	katG3_R	TTCGCCTTGTCGAGCAGCAT	62˚C	819
Fragment 4	katG4_F	GCTGGCCACAACATCACGG		
	katG4_R	GTTGTGGTTGATCGGCGGG	62˚C	587

3.8 Sample preparation for targeted deep sequencing

3.8.1 Procedure

3.8.1.1 DNA extraction from cultured *M. tuberculosis* INH drug-resistant isolates (without mutations in the entire *katG* gene or *inhA* promoter region)

Middlebrook 7H9 liquid media was prepared by suspending 2.35 g of 7H9 powder (Sigma-Aldrich) in 450 ml of dH_2O, with the addition of 1 ml of glycerol and 0.25 g of polysorbate 80 to the stock solution, and sterilized by autoclaving at 121˚C for 10 min. After cooling to 56˚C, 50 ml of OADC enrichment was added. Culturing of *M. tuberculosis* for DNA extraction was done in the BSL3 by inoculating 5 ml liquid culture with 1 ml of positive MGIT sample (section **3.5.2**) and incubating at 37˚C for 10-21 days. Following sufficient growth, 5 ml of liquid culture was poured into 15 ml tubes and heat killed at 80˚C for 1 hr before being removed from the BSL3.

Tubes were centrifuged at 4000 x g for 30 min at room temperature and supernatant discarded (ensure that all supernatant is removed). The remaining pellet was resuspended in 400 µl TE (Tris EDTA) (Sigma-Aldrich) buffer pH 8.0 and 50 µl lysozyme (10 mg/ml) (Thermo Scientific, USA) was added, mixed well and incubated at 37˚C overnight. Thereafter, 70 µl 10% SDS and 5 µl Proteinase K (10 mg/ml) (Sigma-Aldrich) was added to each tube, mixed and incubated at 65˚C for 10 min with intermittent mixing. Next, 100 µl 5 M NaCl was added and mixed well, followed by 100 µl CTAB/NaCl solution (pre-warmed at 65˚C) to be vortexed until the solution appeared milky, then incubated at 65˚C for 10 min. Hereafter, 750 µl chloroform/isoamyl alcohol (24:1) (Sigma-Aldrich) was added to each corresponding tube, vortexed for at least 10 sec and tubes centrifuged at 12000 x g for 5 min. The top (aqueous) phase was aspirated into a clean 1.5 ml tube (without disturbing the interphase) and 0.6 x volume of ice cold isopropanol added, inverted and incubated at 20˚C for 1 hr. Tubes were then centrifuged at room temperature at 12000 x g for 30 min (note: if small volume of DNA is expected, centrifuge at 4˚C). The supernatant was removed without disturbing the pellet, with the addition of 1 ml ice cold 70% ethanol and centrifuged (in the same orientation) at 12000 x g for 15 min. The remaining ethanol was aspirated without disturbing the pellet and tubes allowed to air dry at room temperature for at least 30 min. Subsequently, the DNA was rehydrated by adding 50 µl of TE pH 8.0 to each tube, gently mixed and the pellet allowed to resuspend overnight. Samples were then stored at 4˚C for short-term use or -80˚C for long-term storage. The DNA concentration was determined using the NanoDrop™ 2000/2000c spectrophotometer (Thermo Scientific, USA).

Chapter 4: Results

4.1 Participant characteristics

During the study period, 3,953 patient entries were captured in the ETR database with a diagnosis of RIF-susceptible TB. A total of 1,120 patients were enrolled into the study, of which 88 patients were excluded (22 not matched to the ETR; 41 under the age of 18 years; 4 RIF-resistant; 21 no baseline sample). In addition, for patients with multiple entries (n=39), duplicates were removed. In total, 993 patients met the inclusion criteria (**Figure 4.1**).

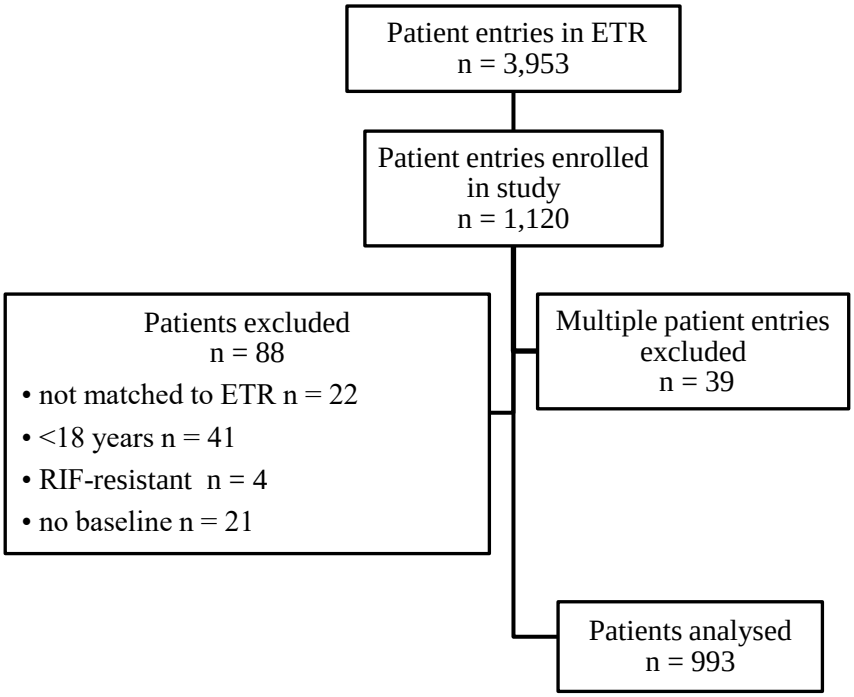

Figure 4.1: Flow diagram to describe patients included in the study.

Of the 993 patients analysed, 618 (62.2%) patients were male, while most patients (n=345; 34.7%) were in the age group 28 to 37 years. At enrolment, 740 (74.5%) were new TB cases and 253 (25.5%) were retreatment TB cases (including 168 (16.9%) documented relapse cases). Four hundred and seventy six (47.9%) patients were HIV-positive and of those with a known positive status, 425 (89.3%) were already on ART at the time of enrolment and treatment initiation (**Table 4.1)**.

Table 4.1: Demographic and clinical characteristics of study participants.

	Category	Number of participants (%)
Age group (n = 993)	18–27 years	228 (23)
	28–37 years	345 (34.7)
	38–47 years	226 (22.8)
	≥48	194 (19.5)
Gender (n = 993)	Male	618 (62.2)
	Female	375 (37.8)
History of previous TB (n = 993)	New	740 (74.5)
	Retreatment	253 (25.5)
HIV status (n = 993)	Positive	476 (47.9)
	Negative	496 (49.9)
	Unknown	21 (2.1)
Use of ARTs in HIV positive (n = 476)	Yes	425 (89.3)
	No	42 (8.8)
	Missing	9 (1.9)

4.2 Phenotypic drug susceptibility testing

In order to identify INH resistance in patients with RIF-susceptible TB, sputum specimens were collected from 993 patients at enrolment (baseline) (**Figure 4.2**) and from 643 patients during treatment (follow-up) (**Figure 4.3**). Of the baseline specimens, 766 (77.1%) were culture positive and 227 (22.9%) were culture negative. Of the follow-up specimens, only 137 (21.3%) were culture positive. **Table 4.2** describes the patient characteristics at baseline: culture positive vs culture negative specimens.

Phenotypic INH testing on 7H10 growth media identified 107 (13.9%) baseline isolates and 19 (13.9%) follow-up isolates to be resistant to INH. Of the INH-resistant baseline and follow-up isolates, 48 (44.9%) and 10 (52.6%) showed low-level resistance (INH concentration ≥0.2 and <1 µg/ml on 7H10), respectively. High-level resistance (INH concentration of ≥1 µg/ml on 7H10) was observed in 59 (55.1%) and 9 (47.4%) baseline and follow-up isolates, respectively. The remaining 659 (85.8%) baseline isolates and 118 (86.1%) follow-up isolates were susceptible to INH at a concentration of <0.2 µg/ml on 7H10. **Table 4.3** and **Table 4.4** describe the patient demographic and clinical parameters at baseline: INH-resistant vs INH-susceptible.

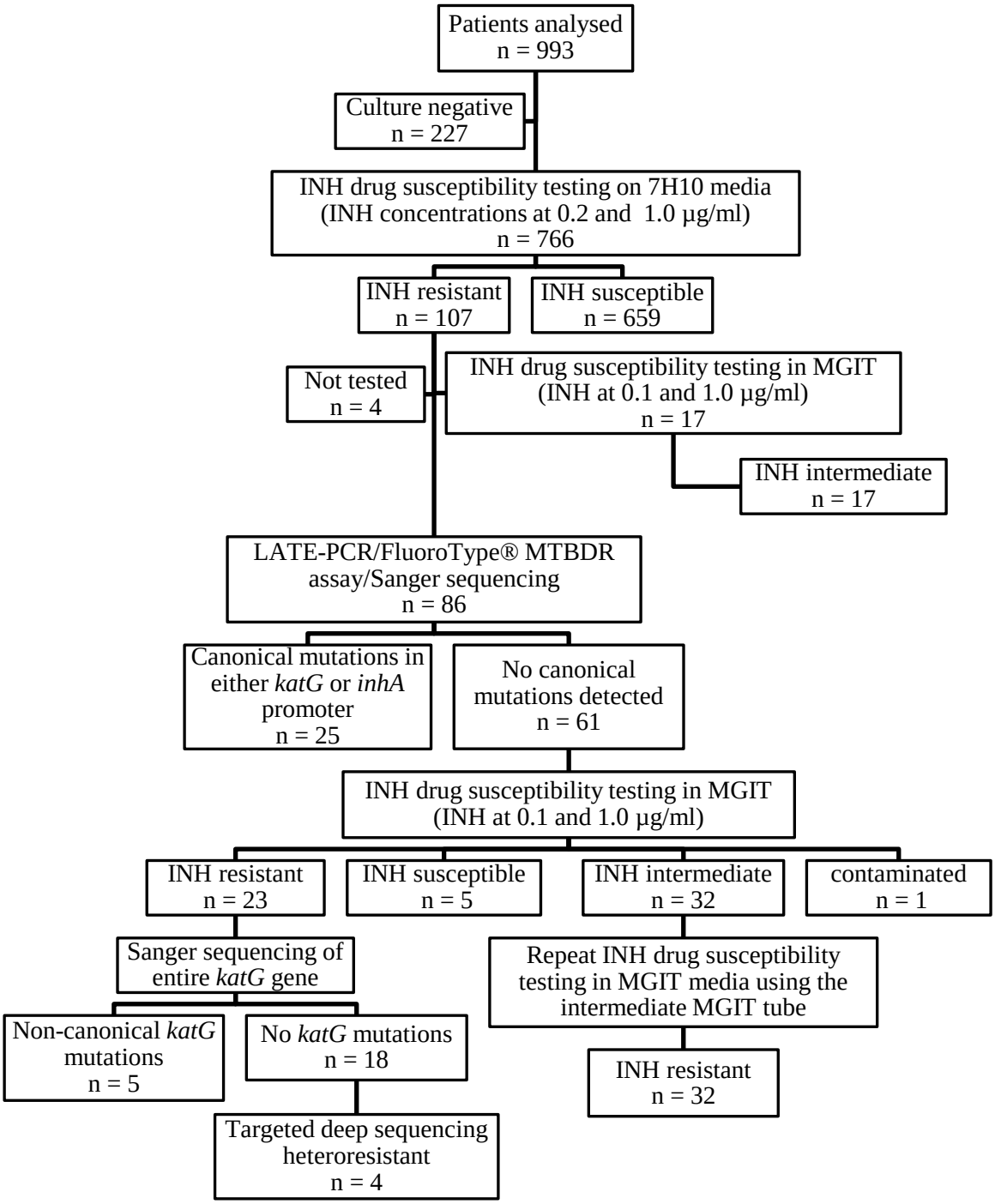

Figure 4.2: Flow diagram illustrating analyses done on baseline isolates and the corresponding phenotypic and genotypic results.

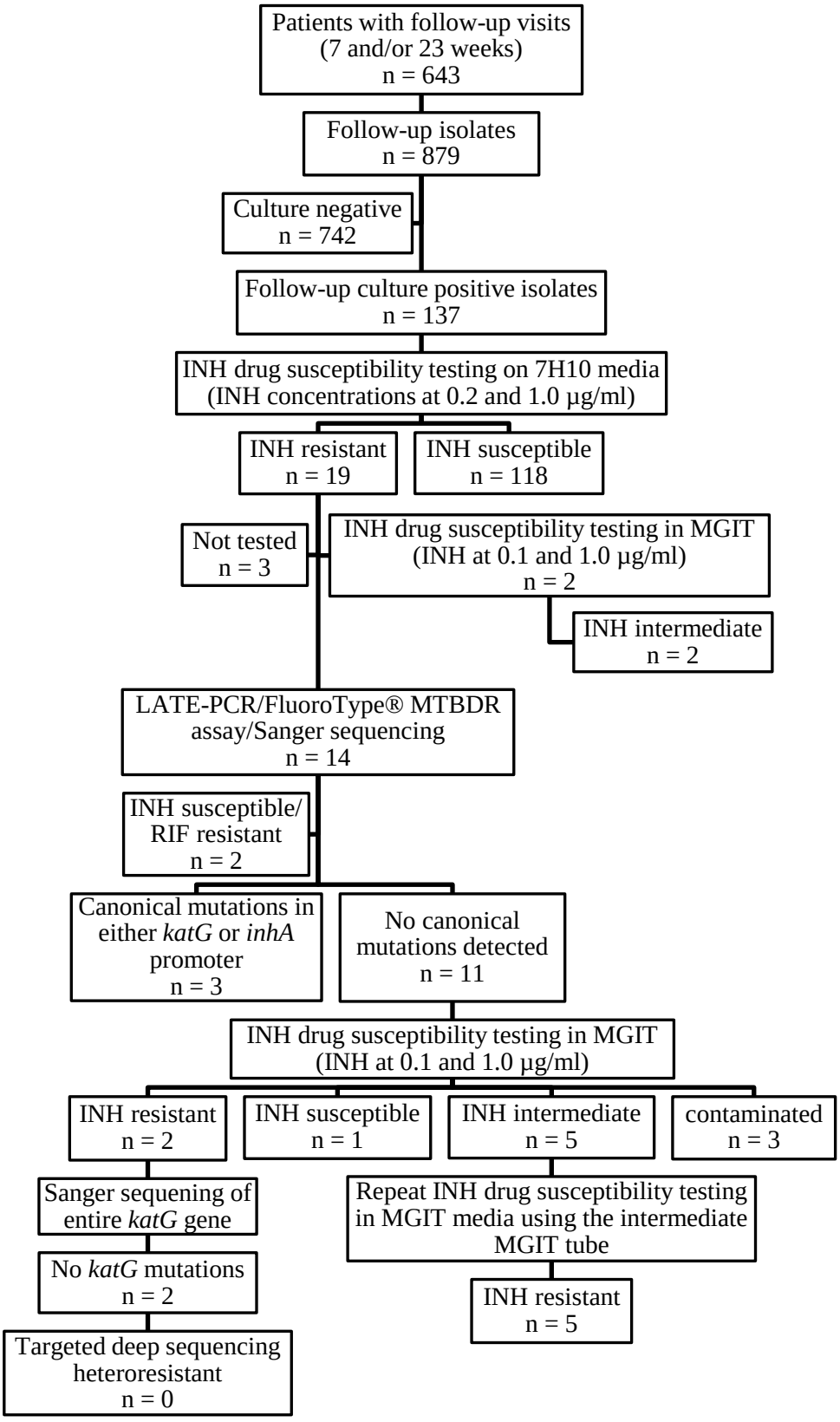

Figure 4.3: Flow diagram illustrating analyses done on follow-up isolates and the corresponding phenotypic and genotypic results.

Comparison of the patient characteristics at baseline showed that the patients with culture negative specimens were older (p <0.001), more likely to be HIV-positive (p = 0.008) and smear negative (p <0.001) (**Table 4.2**).

Table 4.2: Demographic and clinical characteristics of study participants (baseline culture positive vs. culture negative).

	Total Patients n = 993 (%)	**Patients culture positive n = 766 (%)**	**Patients culture negative n = 227 (%)**	**P value**
Age: median years (IQR)	35.1 (28.2- 44.3)	34.3 (27.7- 43.5)	38.3 (30.4- 46.8)	<0.001
Sex				
Male	618 (62)	479 (63)	139 (61)	0.72
Female	375 (38)	287 (37)	88 (39)	
HIV status				0.008
HIV positive	476 (48)	347 (45)	129 (57)	
HIV negative	496 (50)	403 (53)	93 (41)	
Unknown	21 (2)	16 (2)	5 (2)	
ARV status among HIV positive				0.08
On ARVs before TB treatment	70/476 (15)	45/347 (13)	25/129 (19)	
ARVs started during TB treatment	60/476 (13)	42/347 (12)	18/129 (14)	
On ARVs but unknown timing	295/476 (62)	228/347 (66)	67/129 (52)	
Not on ARVs	42/476 (9)	26/347 (7)	16/129 (12)	
Unknown	9/476 (2)	6/347 (2)	3/129 (2)	
Retreatment TB				0.29
New	740 (75)	577 (75)	163 (72)	
Retreatment after default, relapse, or failure	253 (25)	189 (25)	64 (28)	
Isoniazid Preventive Therapy				0.08
Received	40 (4)	26 (3)	14 (6)	
Did not receive	168 (17)	124 (16)	44 (19)	
Unknown	785 (79)	616 (80)	169 (74)	
Pre-treatment AFB smear status				<0.001
Positive	463 (47)	415 (54)	48 (21)	
Negative	263 (26)	148 (19)	115 (51)	
Unknown	267 (27)	203 (27)	64 (28)	
Isoniazid susceptibility at baseline				-
Resistant	107 (11)	107 (11)	0	
Susceptible	659 (66)	659 (66)	0	
Indeterminate	2 (0)		2 (1)	
Culture negative	225 (23)		225 (99)	
Acquired rifampicin resistance	4/128 (3)	3/116 (3)	1/12 (8)	0.28
Acquired isoniazid resistance	9/126 (7)	9/116 (8)	0/10	0.36

Treatment outcome				<0.001
Cured	124 (12)	110 (14)	14 (6)	
Treatment completed	163 (16)	105 (14)	58 (26)	
Treatment failed	5 (1)	5 (1)	0	
Lost to follow up	108 (11)	86 (11)	22 (10)	
Died	30 (3)	21 (3)	9 (4)	
Transferred out	24 (2)	18 (2)	6 (3)	
Not evaluated	539 (54)	421 (55)	118 (52)	

The demographic and clinical parameters at baseline (including treatment outcomes) were not shown to be associated with IMR (**Table 4.3**).

Table 4.3: Demographic and clinical characteristics of 766 study participants with known baseline INH DST results (resistant vs. susceptible).

	Isoniazid Resistant n = 107 (%)	**Isoniazid Susceptible n = 659 (%)**	**P value**
Age: median years (IQR)	34.7 (26.8, 43.0)	34.3 (27.9, 43.9)	0.41
Sex			0.29
Male	62 (58)	417 (63)	
Female	45 (42)	242 (37)	
HIV status			0.47
HIV positive	53 (50)	294 (45)	
HIV negative	53 (50)	350 (53)	
Unknown	1 (1)	15 (2)	
ARV status among HIV positive (n= 347)			0.57
On ARVs before TB treatment	10/53 (19)	35/294 (12)	
ARVs started during TB treatment	6/53 (11)	36/294 (12)	
On ARVs but unknown timing	33/53 (62)	195/294 (66)	
Not on ARVs	4/53 (8)	22/294 (8)	
Unknown	0	6/294 (2)	
Retreatment TB			0.74
New	82 (77)	495 (75)	
Retreatment after default, relapse, or failure	25 (23)	164 (25)	
Isoniazid Preventive Therapy			0.28
Received	2 (2)	24 (4)	
Did not receive	13 (12)	111 (17)	
Unknown	92 (86)	524 (80)	
Pre-treatment AFB smear status			0.47
Positive	61 (57)	354 (54)	
Negative	16 (15)	132 (20)	
Unknown	30 (28)	173 (26)	
Acquired rifampicin resistance	1/17 (6)	2/99 (2)	0.35
Acquired isoniazid resistance	0/12	9/104 (9)	0.29
Treatment outcome			0.53
Cured	18 (17)	92 (14)	

Treatment completed	9 (8)	96 (15)
Treatment failed	1 (1)	4 (1)
Lost to follow up	11 (10)	75 (11)
Died	5 (5)	16 (2)
Transferred out	3 (3)	15 (2)
Not evaluated	60 (56)	361 (55)

Surprisingly; retreatment, age, gender or HIV-positive status was not associated with IMR (**Table 4.4**).

Table 4.4: Univariate and multivariate logistic regression (baseline INH-resistant vs. INH-susceptible) using four predetermined variables: retreatment TB status, age, sex, HIV status. HIV status and the multivariate model used 750 participants due to missing data.

	Univariate		**Multivariate**	
	OR (95% CI)	**P value**	**OR (95% CI)**	**P value**
Retreatment TB	0.92 (0.57-1.49)	0.74	0.86 (0.53-1.41)	0.55
Age (per year)	0.99 (0.97-1.01)	0.38	0.99 (0.97-1.01)	0.40
Sex (1=male, 0=female)	0.80 (0.53-1.21)	0.29	0.82 (0.53-1.25)	0.35
HIV positive	1.19 (0.79-1.80)	0.41	1.16 (0.76-1.76)	0.50

4.3 Strain typing

4.3.1 Spoligotyping

Of 766 baseline and 137 follow-up culture positive isolates, 747 and 131 available isolates were genotyped using spoligotyping and classified according to the SITVIT database to determine the distribution of genotypes among baseline and follow-up isolates (**Table 4.5**). A total of 10 spoligotype lineages were identified: Beijing (48.9%), Latin American Mediterranean (LAM) (17.9%), T (14.8%), S (6.9%), X (5.6%), MANU (1.4%), Haarlem (1.4%), Central Asia (CAS) (1.1%), East African Indian (EIA) (0.3%) and U (0.1%). The remaining 1.5% of isolates could not be classified by SITVIT and were assigned an unknown genotype. One isolate failed to amplify. Among the resistant isolates, the Beijing spoligotype was considered the most dominant among both baseline (42.9%) and follow-up (44.4%) isolates.

Table 4.5: Spoligotype signatures of *M. tuberculosis* isolates identified in the Eastern Cape.

Lineage		Strain family		SIT	Spoligotype pattern	Baseline		Follow-up	
	no. of isolates		no. of isolates			no. of resistant isolates	no. of susceptible isolates	no. of resistant isolates	no. of susceptible isolates
Beijing	429	Beijing	429	1		45	309	8	67
CAS	10	CAS	2	2391			1		
				2269			1		
		CAS1_DELHI	5	1092			4		1
		CAS1_KILI	3	Orphan			1		1
				21			1		
EAI	3	EAI5	2	Orphan				1	1
		EAI3-IND	1	11			1		
Haarlem	12	H1	1	47			1		
		H3	10	50		4	4		1
				not in SITVIT			1		
		H3/LAM3	1	2402					1
LAM	157	LAM1	3	20		2	1		
		LAM3	111	33		15	59	2	7
				719		1	7	1	1
				2302		1	4		
				not in SITVIT					1
				not in SITVIT			1		
				not in SITVIT			1		
				2402			3		
				2527			1		
				2015			1		
				130			2		
				ORPHAN			1		
				1222			2		
		LAM3 ans S/convergent	1	4			1		
		LAM4	17	60		1	8		
				811		1	4		3
		LAM5	2	136			2		
		LAM9	10	42		1	3		1
				492			1		
				ORPHAN			1		
				2165			1		
				81			1		1
		LAM11	13	1873		1	2		1
				2017			3		
				ORPHAN			3		
				ORPHAN			1		
				2196			1		
				813			1		

MANU	12	MANU2	12	54			1		
				ORPHAN			1		
				583		1			
				2229			3		
				226			2		1
				1096			1		
				2117			1		1
S	61	S	61	34		5	33		4
				1333		2	1		
				2367		1		1	1
				71			10	1	
				2181			2		
T	130	T1	90	53		4	37		8
				2296			4		1
				766		1	12	2	1
				719		1	1		
				334		1	4		1
				244			2		
				535			1		
				1324			2		1
				888			2		
				1202			1		
				136			1		
				278			1		
				1105			1		
		T1_RUS2	2	280			2		
		T2	4	52		1	3		
		T2-T3	18	73			16		2
		T3	3	158			2		
				37			1		
		T5	1	44			1		
		T5_RUS1	11	254		1	9		1
		T-TUSCANY	1	1737			1		
U	1	U	1	1241			1		
X	49	X1	16	119		1	9		
				2226		1	4		
				336			1		
		X2	10	2016		2	5		1
				137			2		
		X3	23	92		7	11	2	2
				2020		1			
unknown	13		13	not in SITVIT		1			
				not in SITVIT		1			1
				2328			1		
				not in SITVIT			2		
				not in SITVIT			1		
				not in SITVIT			1		
				not in SITVIT			1		
				not in SITVIT			1		
				not in SITVIT			1		
				not in SITVIT			1		
				not in SITVIT			1		
no result	1		1			1			
Total	**878**		**878**			**105**	**642**	**18**	**113**

4.3.2 Sub-typing of the Beijing genotype

Differentiation of Beijing genotype isolates into typical or atypical subclades was done in order to determine whether the ability of these Beijing subclades to gain INH resistance may differ. Of the 354 baseline Beijing genotype isolates, 326 (92.1%) were typical and 28 (7.9%) were atypical. Thirty (9.2%) and 15 (53.6%) of the typical and atypical Beijing isolates were resistant to INH, respectively ($p < 0.0001$). These results explain variation in association with drug resistance.

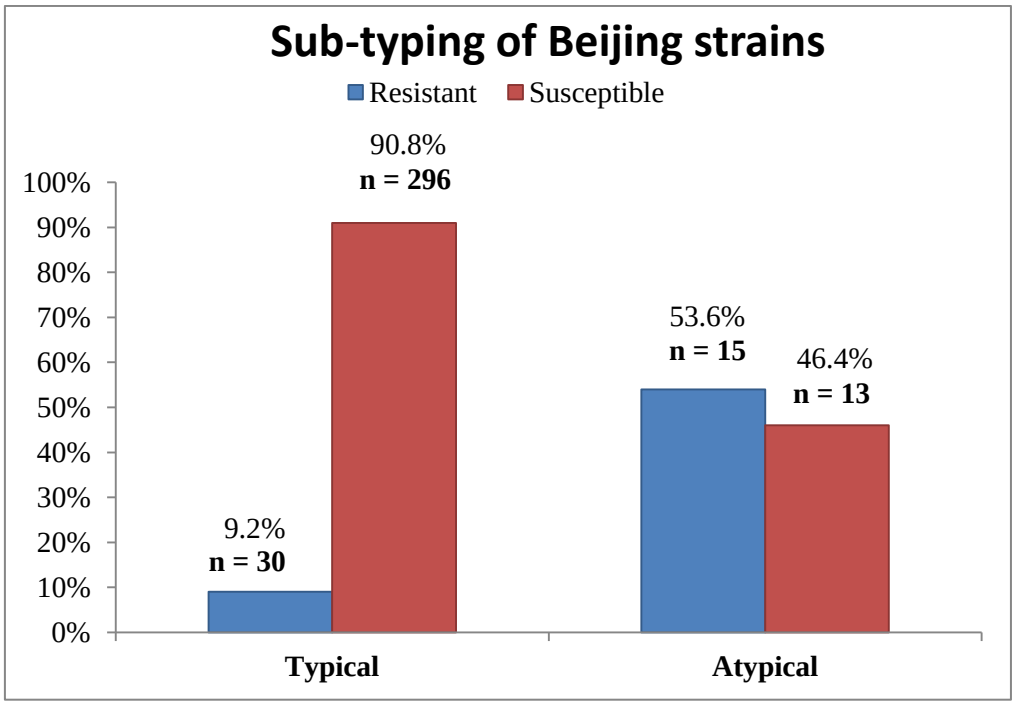

Figure 4.4: Frequency distribution of typical and atypical Beijing strains.

4.4 Genotypic drug resistance testing

To identify mutations conferring INH resistance, INH-resistant baseline (n=86) and follow-up (n=14) isolates were analysed using either LATE-PCR, FluoroType® MTBDR or Sanger sequencing (21 baseline and 5 follow-up isolates not analysed). No differences were observed between clinical and demographic parameters of patients whose isolates were subjected to genotypic analysis compared to those where no genotypic analysis was done (**Table 4.6**).

Table 4.6: Demographic and clinical characteristics of study participants with known baseline INH DST results (resistant vs. intermediate).

	Isoniazid Resistant (R or LLR) n = 86 (%)	**Isoniazid Intermediate R or LLR n = 17 (%)**	**P value**
Age: median years (IQR)	35.1 (27, 41.9)	32.2 (28.5, 51.1)	0.86
Sex			0.94
Male	52 (58)	10 (59)	
Female	38 (42)	7 (41)	
HIV status			0.66
HIV positive	43 (48)	10 (59)	
HIV negative	46 (51)	7 (41)	
Unknown	1 (1)	0	
ARV status among HIV positive (n=53)			0.23
On ARVs before TB treatment	9/43 (21)	1/10 (10)	
ARVs started during TB treatment	6/43 (14)	0	
On ARVs but unknown timing	24/43 (56)	9/10 (90)	
Not on ARVs	4/43 (9)	0	
Unknown	0	0	
Retreatment TB			0.99
New	69 (77)	13 (76)	
Retreatment after default, relapse, or failure	21 (23)	4 (24)	
Isoniazid Preventive Therapy			0.82
Received	2 (2)	0	
Did not receive	11 (12)	2 (12)	
Unknown	77 (86)	15 (88)	
Pre-treatment AFB smear status			0.92
Positive	51 (57)	10 (59)	
Negative	14 (16)	2 (12)	
Unknown	25 (28)	5 (29)	
Acquired rifampicin resistance	0/2	1/15 (7)	0.71
Treatment outcome			0.31
Cured	15 (17)	3 (18)	
Treatment completed	7 (8)	2 (12)	
Treatment failed	0	1 (6)	
Lost to follow up	9 (10)	2 (12)	
Died	5 (6)	0	
Transferred out	3 (3)	0	
Not evaluated	51 (57)	9 (53)	

Analysis of the genotypic data identified canonical mutations in 25 (29.1%) baseline and 3 (21.4%) follow-up isolates in codon 315 (ACC and AAC) of the *katG* gene or in the *inhA* promoter region (-8T/C, -15C/T and -17T/G). Canonical mutations were not identified in 61 (70.9%) phenotypically INH-resistant baseline and 11 (78.6%) follow-up isolates. The results are summarized in **Table 4.7** and **Table 4.8**.

Table 4.7: Frequency of mutations known to confer INH resistance in baseline isolates.

Mutation identified	n = 86	(%)	High-level resistant	Low-level resistant	Strain family (n)	Typical Beijing *	Atypical Beijing *
katG 315ACC	14	16.3	14		MANU (1), LAM 3 (1), S (1), Beijing (11)	3	8
katG 315AAC	1	1.2	1		Beijing (1)	1	
inhA prom -15C/T	10	11.6	3	7	LAM 1 (2), LAM 3 (2), Beijing (6),	2	4
Wild-type	61	70.9	36	25			

*determined by PCR analysis

Table 4.8: Frequency of mutations known to confer INH resistance in follow-up isolates.

Mutation identified	n = 14	(%)	High-level resistant	Low-level resistant	Strain family (n)
inhA prom -15C/T	1	7.1		1	LAM 3 (1)
rpoB 516GTC and *katG* 315ACC and *inhA* prom -17T/G	1	7.1	1		S (1)
rpoB 516GTC and *katG* 315ACC and *inhA* prom -8T/C	1	7.1	1		Beijing (1)
Wild-type	11	78.6	5	6	

Baseline (n=61) and follow-up (n=11) INH-resistant isolates, which were wild-type for the *katG* 315 region and *inhA* promoter region were subjected to repeated phenotypic testing in MGIT media at INH concentrations of 0.1 and 1 µg/ml. Of these, 23/61 (37.7%) baseline isolates and 2/11 (18.2%) follow-up isolates were confirmed as INH-resistant. To identify possible causative mutations in these isolates, the entire coding region of the *katG* gene was Sanger sequenced. Mutations were identified in 5/23 (21.7%) baseline isolates at codons 430

(CAG), 454 (ACA), 454 (ACA) and 503 (TGG), 483 (TAC), and 538 (GAG), while the remaining 18 isolates had no *katG* mutations. None of the sequenced INH-resistant follow-up isolates (n=2) had *katG* mutations.

Isolates (baseline n=18; follow-up n=2) for which no resistance conferring mutations were found, were subjected to TDS to identify potential heteroresistance. These sequencing results identified the presence of causal mutations in four baseline isolates in *katG* at frequencies of 4.5%, 33.5% and 44.8%; and the *inhA* promoter region at a frequency of 11.2%, demonstrating heteroresistance. Isolates exhibiting resistance for which no resistance mutations could be identified are currently undergoing whole genome sequencing (WGS).

Of the remaining 38/61 baseline and 9/11 follow-up isolates, 32 (52.5%) and 5 (45.5%) showed an intermediate INH susceptibility phenotype, respectively at either or both of the drug concentrations tested in MGIT media. Five baseline isolates and one follow-up isolate were classified as susceptible in MGIT media; one baseline and three follow-up isolates were contaminated. All of the isolates with an intermediate INH susceptibility phenotype were subjected to a further round of susceptibility testing in MGIT media but using the culture that showed an intermediate INH susceptibility in the previous test as the inoculum. This was done to enrich for the INH-resistant subpopulations present in the original isolate. Thirty-two baseline and five follow-up isolates were subsequently classified as INH-resistant, suggesting that the original isolates harboured resistant subpopulations (heteroresistance).

In order to identify acquisition of resistance during treatment, baseline and follow-up isolates from 123 patients were analysed. In 95 patients both baseline and follow-up isolates were susceptible to INH, while in 7 patients INH resistance was detected in both baseline and follow-up isolates. INH resistance (low-level n=5; high-level n=4) was acquired in 9 patients during treatment. Subsequent drug susceptibility testing of the follow-up isolates in MGIT (n=6) confirmed INH susceptibility in one, INH resistance in one and an intermediate phenotype in four follow-up isolates. Causal mutations were identified in only one follow-up isolate (**Table 4.9**). However, INH resistance was confirmed after retesting of the isolates with an intermediate phenotype in MGIT media (using the intermediate MGIT culture as the inoculum), suggesting heteroresistance, which in turn could be masking the detection of resistance causing mutations. These isolates will be subjected to TDS, as well as WGS to confirm heteroresistance and identify the genetic basis of resistance.

In one follow-up isolate (KG0347) that acquired INH resistance, an *rpoB* mutation (516GTC) was also acquired, while in a second patient's follow-up isolate (KG0625), an *rpoB* mutation (deletion of codon 517-518) was acquired in the absence of INH resistance (**Table 4.9**).

In 12 patients, INH resistance detected in the baseline isolates reverted to INH susceptibility in the follow-up isolates. Of the 12 patients whose baseline isolates were INH-resistant (low-level n=3; high-level n=9) on 7H10, three were confirmed to be resistant in MGIT media; however, only one isolate showed a causative *katG* mutation (315ACC) (**Table 4.9**). The remaining 6 baseline isolates showed an intermediate INH susceptibility phenotype on MGIT testing. Subsequent repeat testing in MGIT media using the intermediate MGIT tube from the previous test, confirmed that all 6 isolates were INH-resistant, suggesting heteroresistance at baseline. Canonical resistance causing mutations were not identified in these 6 baseline isolates or the follow-up isolates, possibly as a result of either masking by the dominant INH-susceptible population or due to another mechanism not explored in this study.

In order to determine whether acquisition of INH resistance or reversion to INH susceptibility was associated with a changing spoligotype pattern, the baseline and follow-up spoligotypes from 123 patients were analysed. A change in spoligotype pattern was only observed in one of the nine patients who acquired INH resistance during treatment (Beijing to X3) (**Table 4.9**). Similarly, a change in spoligotype was only observed in one patient whose isolates reverted to INH susceptibility. In this instance, the baseline spoligotype was classified as MANU2 (**Table 4.9**), which is thought to represent a mixed infection between Beijing and T family. The T family was absent in the follow-up isolate. No change in the spoligotype patterns was observed in patients who acquired RIF resistance. However, in 26 patients with INH susceptible TB, difference in strain types were observed between baseline and follow-up isolates (different lineage n=24; same lineage n=2).

Table 4.9: Drug resistance profiles of patients' isolates that increased or decreased in INH susceptibility, and increased in RIF susceptibility.

Category	Baseline						Follow-up					
Study numbers	MIC on 7H10 (µg/ml)	MGIT at (0.1 µg/ml)	Retesting in MGIT using the intermediate tube	Strain family	*katG* gene and *inhA* promoter	TDS	MIC on 7H10 (µg/ml)	MGIT at (0.1 µg/ml)	Retesting in MGIT using the intermediate tube	Strain family	*katG* gene and *inhA* promoter	TDS
Increase in INH susceptibility												
KG0077	>1	ND		T1	ND	ND	<0.2	ND		T1	wt	ND
KG0079	>1	ND		LAM4	ND	ND	<0.2	ND		LAM4	wt	ND
KG0114	<1	R		X3	wt	ND	<0.2	ND		X3	wt	ND
KG0222	>1	I	R	Beijing	wt	ND	<0.2	ND		Beijing	wt	ND
KG0228	>1	R		X3	wt	S	<0.2	ND		X3	wt	ND
KG0371	>1	R		MANU2	*katG* 315ACC	ND	<0.2	ND		Beijing	wt	ND
KG0399	>1	I	R	Beijing	wt	ND	<0.2	ND		Beijing	wt	ND
KG0433	>1	I	R	LAM3	wt	ND	<0.2	ND		LAM3	wt	ND
KG0477	>1	I	R	Beijing	wt	ND	<0.2	ND		Beijing	wt	ND
KG0621	>1	I	R	Beijing	wt	ND	<0.2	ND		Beijing	wt	ND
KG0676	<1	I	R	Beijing	wt	ND	<0.2	ND		Beijing	wt	ND
KG1204	<1	ND		LAM9	ND	ND	<0.2	ND		LAM9	wt	ND
Decrease in INH susceptibility												
KG0096	<0.2	ND		Beijing	ND	ND	>1	I	R	Beijing	wt	ND
KG0115	<0.2	ND		LAM3	ND	ND	<1	S		LAM3	wt	ND
KG0191	<0.2	ND		Beijing	ND	ND	<1	I	R	Beijing	wt	ND
KG0193	<0.2	ND		Beijing	ND	ND	>1	I	R	Beijing	wt	ND
KG0214	<0.2	ND		T1	ND	ND	<1	ND		T1	ND	ND
KG0227	<0.2	ND		X3	ND	ND	>1	I	R	X3	wt	ND
KG0347	<0.2	ND		S	ND	ND	>1	ND		S	**katG* 315ACC and -17T/G *inhA* prom	ND
KG0351	<0.2	ND		Beijing	ND	ND	<1	R		X3	wt	S
KG0978	<0.2	ND		Beijing	ND	ND	<1	ND		Beijing	ND	ND
Decrease in RIF susceptibility												
KG0347	<0.2	ND		S	ND	ND	>1	ND		S	**katG* 315ACC and -17T/G *inhA* prom	ND
KG0625	<0.2	ND		Beijing	ND	ND	<0.2	ND		Beijing	**wt	ND

**rpoB* mutation (516GTC) acquired (presence of INH resistance)

***rpoB* mutation (deletion of codon 517-518) acquired (absence of INH resistance)

Chapter 5: Discussion

IMR-TB lacks priority in TB control programmes despite a global prevalence of 8% (5-11%) (208), yet most research and policy efforts are solely focused on RIF resistance as a proxy for MDR-TB. Literature suggests that patients infected with IMR-TB (if not detected) are clinically managed as if pan-susceptible, with a substantially increased risk of treatment failure or relapse and a greater propensity to acquire further resistance (209). The implementation of the Xpert MTB/RIF assay as the primary TB screening tool demoted the diagnostic importance of IMR to the extent that treatment response has become the primary indicator to initiate additional DST (210,211). Consequently, patients are treated with suboptimal therapy, increasing their risk of developing MDR-TB despite new WHO treatment guidelines (212,213). Major flaws in national surveillance systems along with limited laboratory capacity is a worrisome problem, as failure to implement effective TB control programmes and to correctly manage TB cases could seriously jeopardize the progress in the fight against TB and in particular IMR-TB.

This is the first population-based study of IMR in a rural setting where healthcare is a challenge. The current study aimed to describe the IMR-TB epidemic in a high HIV prevalence region to determine the association between INH resistance and treatment outcomes.

Following analysis of a convenience sample of patients diagnosed with RIF-susceptible TB, we identified IMR in 13.9% (high-level 55.1%; low-level 44.9%) using a solid media proportion method. The proportion of patients with IMR at baseline was higher than reported in the national drug resistance survey (6.1%) between 2012 and 2014, thus highlighting the severity of the growing burden of disease in South Africa (21). Current standard of care does not have an algorithm to identify IMR at baseline, resulting in treatment with a standardized short-course regimen. This could potentially impact treatment outcome (reduced chance of anti-TB treatment success) and raise the risk of acquiring RIF resistance during treatment. RR-TB rates are known to be high in this setting (3.3%) (21,214).

In order to determine whether a targeted genetic diagnostic approach could be implemented to rapidly identify IMR at diagnosis, we analysed the hotspot region of the *M. tuberculosis* genome (*katG* gene and *inhA* promoter region) known to confer resistance in approx. 90% of INH-resistant clinical isolates (215,216). Surprisingly, canonical INH resistance causing

mutations were only detected in 29% of baseline isolates with an INH resistance phenotype, possibly suggesting either resistance in these patients was mediated via mechanisms outside of the hotspot regions, or the solid media DST method overscored INH resistance.

When mutations in these gene regions are identified, they are associated with either high- (*katG*) or low-level (*inhA* promoter) INH resistance. However, contrarily, our study showed that certain *inhA* promoter mutations were present in high-level INH-resistant isolates (baseline n = 3), possibly suggesting the presence of an alternative, unidentified mutation. Taken together, these data suggest that identifying only the canonical mutations is not sufficient to inform individualized treatment.

Repeat testing in MGIT confirmed resistance in 37.7% of baseline isolates, while the remaining 52.5% showed an intermediate result, suggesting a reduced INH susceptibility that was not sufficiently high to be scored as resistant. Upon repeat MGIT testing using the intermediate inoculum, we confirmed the presence of INH-resistant bacilli within these isolates, thereby suggesting heteroresistance in the original isolates. This in part explains our genotyping results, as the proportion of resistant clones would have been below the threshold of detection in the methods used. This was confirmed in a limited subset of isolates using TDS, which could detect subpopulations of resistant bacilli at or below 1%. These findings demonstrate that culture-based DST should not be excluded but rather be supplemented by genotypic resistance testing in order to achieve the highest possible sensitivity for the detection of drug-resistant isolates.

Within this programmatic setting, follow-up specimens were only available from 123 (16%) patients, suggesting that the majority of patients were responsive to therapy using the standard short-course regimen. However, in 116 patients culture positivity was noted at 7 weeks and 7 were culture positive at 23 weeks, suggesting slow response to antibiotic therapy. In order to establish whether the delay in culture conversion was associated with INH resistance at baseline or acquisition of resistance during treatment, we compared the INH susceptibility phenotypes at baseline and follow-up in patients with serial isolates. A decrease in susceptibility to INH (acquisition of resistance) was observed in 9 patient follow-up isolates. Spoligotyping data suggested reinfection in one patient, while Sanger sequencing confirmed acquisition of an INH resistance causing mutation in another patient. However, resistance confirming mutations could not be identified in four follow-up isolates despite confirmation of an intermediate INH susceptibility, suggesting that the proportion of resistant

bacilli was below the detection threshold (heteroresistance). Together these results suggest either unmasking of pre-existing INH resistance, early acquisition of INH resistance or reinfection with an already INH-resistant strain. All three mechanisms have previously been described for RR- and MDR-TB; however, these studies have largely focused on treatment failure (212,217–220).

In this study we also observed the counterintuitive loss of INH resistance during treatment (increase in susceptibility) in 12 patient follow-up isolates. Subsequent review of nine MGIT results confirmed resistance in three baseline isolates, while six baseline isolates had an intermediate INH susceptibility, thereby reflecting heteroresistance. Resistance causing mutations were only identified in one baseline isolate, which showed a MANU spoligotype at baseline and a Beijing spoligotype at follow-up. We propose that the MANU spoligotype was a composite of two spoligotypes, which included a Beijing spoligotype and a T-type spoligotype. During treatment the T-type strain was lost leading to an INH-susceptible phenotype. A similar scenario could explain the increase in INH susceptibility (loss of resistance) in the remaining 8 follow-up isolates. This implies that within these patients the combination of 4 anti-TB drugs was sufficient to reduce the proportion of INH-resistant bacilli to below the level of phenotypic testing.

However, the observed decrease or increase in INH susceptibility may also be explained by fact that the two sputum specimens do not necessarily originate from the same lesion within the lung. A similar hypothesis was presented to explain the emergence of different clones of *M. tuberculosis* harbouring different causal mutations in serial isolates.

In two patients, emergence of RIF resistance was observed. In one patient RIF resistance was also associated with INH resistance in the follow-up isolate. In both instances the spoligotype was the same for both baseline and follow-up isolates. Given that the patterns of resistance causing mutations in the follow-up isolates matched known MDR-TB isolates from the region, we hypothesize either reinfection or unmasking of an underlying MDR-TB strain. The absence of a RIF-resistant genotype in the baseline isolates of these patients may be explained by the sensitivity of the Xpert MTB/RIF assay to detect resistant subpopulations. According to Ng et al., 2019 (see also Blakemore et al., 2010), the Xpert MTB/RIF assay was only able to detect RIF resistance if the proportion of resistant bacilli was above 60% (221,222). Acquisition of RIF resistance was observed in one patient, where RIF resistance

was encoded by a 6 bp deletion in the RRDR region of the *rpoB* gene. This is a unique mechanism that has not previously been described.

Our analysis of the clinical and demographic parameters failed to identify risk factors for IMR. This finding is contrary to what we would have expected, given previous studies demonstrating strong association between retreatment and drug resistance (212). It is possible in this data set that the definitions of retreatment vs. new cases are not accurate, thereby masking any association. Furthermore, the absence of an association between HIV status and IMR suggests that IMR is not driven through malabsorption/metabolism of INH. In this study it was not possible to identify an association through prior exposure to IPT. This may be related to the limited amount of data captured. An alternative explanation could be an overestimation of cases with IMR due to the methodology that we used. Previous studies may have missed these cases when using MGIT as the primary DST tool, as this method would have scored an intermediate phenotype as susceptible.

Chapter 6: Limitations and Future studies

6.1 Limitations of study

The data presented in this study aimed to describe the epidemiology and risk factors associated with IMR-TB in a high endemic area, with the purpose of highlighting the need for more rapid and accessible diagnostic tools in resource-limited settings. However, there were a number of limitations that could have influenced the results obtained. Firstly, the collection of sputum specimens was not comprehensive, thus it is not possible to exclude potential bias, which may have inflated the relative proportion of patients with IMR. Secondly, culturability may have been compromised due to shipment of sputum specimens from the study setting to the laboratory. This could explain the large proportion of baseline specimens that were culture negative despite having been diagnosed as TB positive by the Xpert MTB/RIF assay in the routine laboratory. Our comparison of patient characteristics with culture and those without culture showed that culture negativity was strongly associated with age, HIV and smear gradation. This suggests that bacterial burden in the sputum specimens influenced culturability during shipment. Thirdly, a folder review was not possible for each patient and therefore we had to rely on routine data that was captured into the electronic database. Unfortunately, the capture of this data was incomplete with a significant proportion of patients lacking a documented outcome. Furthermore, we identified numerous duplicate entries within the database, which may highlight inadequacies in the TB control programme. This requires further analysis in conjunction with a folder review to establish treatment outcomes. Fourthly, the high number of patients with reduced susceptibility to INH has not been confirmed as true heteroresistance, which may have overinflated our estimate of IMR in the region. This can only be done using TDS, which was not available at the time for analysing all of our samples. Fifthly, we were not able to identify causative mutations in all of the isolates that were confirmed to be resistant. This may reflect the presence of mutations outside of the hotspot region and therefore can only be clarified by WGS, provided that the proportion of resistant bacilli is above the threshold for next generation sequencing. Lastly, the use of spoligotyping compromised our ability to identify chains of transmission accurately. Therefore, we recommend that future studies should use highly discriminating tools such as WGS to determine whether IMR is being transmitted in this setting.

6.2 Future studies

The findings of this study emphasize the need for expanding IMR-TB research in South Africa to establish its impact on treatment outcome and to determine whether IMR increases the risk of treatment failure, recurrence and emergence of MDR-TB. To achieve this aim, it will be necessary to conduct a study with sufficient funding in order not to be reliant of the already overburdened healthcare services for the capture of data. Ideally, such a study should include two sites representing both an urban and rural community. The future study should have a similar design, although the number of participants should be increased to accommodate the two study sites and take into account loss to follow-up. Patients should be followed up for the full duration of treatment to ensure that accurate outcomes are recorded, which did not occur in the current study. The follow-up period should be for at least two years to also identify recurrence.

The discordance between INH DST on solid and liquid media requires further analysis. Understanding the parameters of solid and liquid media DST will influence the study design, as sub-culturing on these media may lead to the loss of less fit INH-resistant strains, thereby potentially influencing the resistance classification on the respective media. To avoid this problem, a future study should do DST and MIC determinations using the primary culture on both solid and liquid media.

In order to understand the genetic mechanisms driving INH resistance, all baseline resistant cultures should be subjected to WGS to determine whether IMR is acquired or transmitted. Similarly, TDS should be done to identify minority INH-resistant subpopulations at baseline and follow-up to establish whether these subpopulations are selected during treatment. However, in order to capture the full spectrum of INH conferring mutations, it will be essential that the TDS method surveys at least the entire *katG* gene, *inhA* promoter and *inhA* gene. To differentiate acquisition from reinfection/superinfection, it will be necessary to purify INH-resistant and -susceptible clones from patients diagnosed with heteroresistance and thereafter, analyse these clones by WGS to determine whether they matched (acquisition) or did not match (reinfection/superinfection).

Our analysis of the electronic database clearly highlighted numerous inadequacies. This data set needs to be further analysed to highlight limitations within the TB control programme to improve and monitor TB treatment.

Chapter 7: Conclusion

This study confirmed the severity of the IMR-TB epidemic in the rural Eastern Cape Province, with nearly 14% of confirmed cases harbouring INH resistance in the absence of RIF resistance. These cases often remain undiagnosed until recurrence or treatment failure is observed, hence increasing the risk of developing MDR-TB. Unfortunately, despite a high prevalence, the impact on treatment outcome could not be assessed due to poor quality of information recorded in the electronic TB database. Furthermore, in this setting IMR was not associated with prior TB treatment or HIV co-infection, which conflicts with previous reports that have highlighted retreatment as a significant risk factor for acquisition of resistance.

In this study canonical mutations could be identified by genetic analysis in only 29% of INH-resistant isolates. This implies that over 70% of IMR-TB in this region would be missed using targeted genetic DST methods such as the MTBDR*plus* line probe assay. Additionally, some of the missed resistant isolates could potentially be explained by heteroresistance at a frequency too low to be detected by the above-mentioned genetic DST methods.

This study highlights the need to change TB policy through: (1) understanding the local epidemiology of IMR to identify potential risk factors for targeted interventions and to strengthen current first-line regimens for continuation phase, (2) conducting frequent drug resistance surveillance studies in neglected areas that would ultimately improve TB prevention and control programmes and (3) developing new rapid modern technologies to ensure early and accurate identification, and close monitoring of IMR-TB patients to allow appropriate treatment and care.

References

1. WHO. Global tuberculosis report 2020 [Internet]. WHO; Available from: https://www.who.int/publications/i/item/9789240013131

2. WHO. Treatment of tuberculosis: Guidelines [Internet]. 4th ed. 2009. Available from: https://www.who.int/tb/features_archive/new_treatment_guidelines_may2010/en/

3. Zhang Y, Yew W. Mechanisms of drug resistance in *Mycobacterium tuberculosis*. Int J Tuberc Lung Dis. 2009;13(11):1320–30.

4. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature. 1992;358:591–3.

5. Slayden RA, Lee RE, Barry CE. Isoniazid affects multiple components of the type II fatty acid synthase system of *Mycobacterium tuberculosis*. Mol Microbiol. 2000;38(3):514–25.

6. Medical Research Council. The treatment of pulmonary tuberculosis with isoniazid. Br Med J. 1952;2:735–46.

7. Blanchard JS. Molecular mechanisms of drug resistance in *Mycobacterium tuberculosis*. Annu Rev Biochem. 1996;65:215–39.

8. Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D, van Embden JDA. Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and-susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: Restricted array of mutations associated with drug resistance. J Infect Dis. 1996;173(1):196–202.

9. Ramaswamy S V, Reich R, Dou S, Jasperse L, Pan X, Wanger A, et al. Single nucleotide polymorphisms in genes associated with isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2003;47(4):1241–50.

10. Zhang M, Yue J, Yang Y, Zhang H, Lei J, Jin R, et al. Detection of mutations associated with isoniazid resistance in *Mycobacterium tuberculosis* isolates from China. J Clin Microbiol. 2005;43(11):5477–82.

11. Kiepiela P, Bishop KS, Smith AN, Roux L, York DF. Genomic mutations in the *katG*, *inhA* and ahpC genes are useful for the prediction of isoniazid resistance in *Mycobacterium tuberculosis* isolates from Kwazulu Natal, South Africa. Tuber Lung Dis. 2000;80(1):47–56.

12. Pym AS, Domenech P, Honoré N, Song J, Deretic V, Cole ST. Regulation of catalase-peroxidase (*KatG*) expression, isoniazid sensitivity and virulence by furA of *Mycobacterium tuberculosis*. Mol Microbiol. 2001;40(4):879–89.

13. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Sun Um K, Wilson T, et al. *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science (80-). 1994;263:227–30.

14. Ando H, Miyoshi-Akiyama T, Watanabe S, Kirikae T. A silent mutation in mabA confers isoniazid resistance on *Mycobacterium tuberculosis*. Mol Microbiol.

2014;91(3):538–47.

15. Lee AS, Lim IH, Tang LL, Telenti A, Wong SY. Contribution of *kasA* analysis to detection of isoniazid-resistant *Mycobacterium tuberculosis* in Singapore. Antimicrob Agents Chemother. 1999;43(8):2087–9.

16. Kelley CL, Rouse DA, Morris SL. Analysis of *ahpC* gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1997;41(9):2057–8.

17. Davies PDO, Pai M. The diagnosis and misdiagnosis of tuberculosis. Int J Tuberc Lung Dis. 2008;12(11):1226–34.

18. van der Werf MJ, Langendam MW, Huitric E, Manissero D. Multidrug resistance after inappropriate tuberculosis treatment: A meta-analysis. Eur Respir J. 2012;39(6):1511–9.

19. Rifat M, Milton AH, Hall J, Oldmeadow C, Islam A, Husain A, et al. Development of multidrug-resistant tuberculosis in Bangladesh: A case-control study on risk factors. PLoS One. 2014;9(8):1–7.

20. WHO. Global tuberculosis report 2019 [Internet]. WHO; Available from: https://www.who.int/tb/publications/global_report/en/

21. NICD South African tuberculosis drug resistance survey. 2015; Available from: http://www.nicd.ac.za

22. WHO. Anti-tuberculosis drug resistance in the world: Report No.4 [Internet]. 2008. Available from: http://whqlibdoc.who.int/hq/2008/WHO_HTM_TB_2008.394_eng.pdf

23. Mak A, Thomas A, Del Granado M, Zaleskis R, Mouzafarova N, Menzies D. Influence of multidrug resistance on tuberculosis treatment outcomes with standardized regimens. Am J Respir Crit Care Med. 2008;178(3):306–12.

24. Báez-Saldaña R, Delgado-Sánchez G, García-García L, Cruz-Hervert LP, Montesinos-Castillo M, Ferreyra-Reyes L, et al. Isoniazid mono-resistant tuberculosis: Impact on treatment outcome and survival of pulmonary tuberculosis patients in Southern Mexico 1995-2010. PLoS One. 2016;11(e0168955):1–16.

25. van der Heijden YF, Karim F, Mufamadi G, Zako L, Chinappa T, Shepherd BE, et al. Isoniazid mono-resistant tuberculosis is associated with poor treatment outcomes in Durban, South Africa. Int J Tuberc Lung Dis. 2017;21(6):670–6.

26. Manson AL, Cohen KA, Abeel T, Desjardins CA, Armstrong DT, Barry III CE, et al. Genomic analysis of globally diverse *Mycobacterium tuberculosis* strains provides insights into emergence and spread of multidrug resistance. Nat Genet. 2017;49(3):395–402.

27. Eldholm V, Monteserin J, Rieux A, Lopez B, Sobkowiak B, Ritacco V, et al. Four decades of transmission of a multidrug-resistant *Mycobacterium tuberculosis* outbreak strain. Nat Commun. 2015;6(7119):1–9.

28. Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, et

al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet. 2014;46(3):279–86.

29. Rubin S. Tuberculosis: Captain of all these men of death. Radiol Clin North Am. 1995;33(4):619–39.

30. Hoa NB, Tiemersma EW, Sy DN, Nhung N V, Gebhard A, Borgdorff MW, et al. Household expenditure and tuberculosis prevalence in VietNam: prediction by a set of household indicators. Int J Tuberc Lung Dis. 2011;15(1):32–7.

31. Muniyandi M, Ramachandran R. Socioeconomic inequalities of tuberculosis in India. Expert Opin Pharmacother. 2008;9(10):1623–8.

32. Siddiqi K, Barnes H, Williams R. Tuberculosis and poverty in the ethnic minority population of West Yorkshire: an ecological study. Commun Dis Public Heal. 2001;4(4):242–6.

33. Spence D, Hotchkiss J, Williams C, Davies P. Tuberculosis and poverty. Br Med J. 1993;307(6907):759–61.

34. Holtgrave DR, Crosby RA. Social determinants of tuberculosis case rates in the United States. Am J Prev Med. 2004;26(2):159–62.

35. World Health Organization. Addressing poverty in TB control: Options for national TB control programmes. Geneva: World Health Organization; 2005.

36. Lonnroth K, Jaramillo E, Williams BG, Dye C, Raviglione M. Drivers of tuberculosis epidemics: The role of risk factors and social determinants. Soc Sci Med. 2009;68:2240–6.

37. Centers for Disease Control and Prevention. Treatment for TB disease [Internet]. 2009. Available from: https://www.cdc.gov/tb/topic/treatment/tbdisease.html

38. Sotgiu G, Centis R, D'ambrosio L, Migliori GB. Tuberculosis treatment and drug regimens. Cold Spring Harb Perspect Med. 2015;5(5):a017822.

39. WHO (2003). Adherence to long-term therapies: Evidence for action. Available from: http://www.who.int/chp/knowledge/publications/adherence_full_report.pdf. Accessed: 6 February 2006.

40. Kompala T, Shenoi S V, Friedland G. Transmission of tuberculosis in resource-limited settings. Curr HIV/AIDS Rep. 2013;10(3):264–72.

41. Marais BJ, Mlambo CK, Rastogi N, Zozio T, Duse AG, Victor TC, et al. Epidemic spread of multidrug-resistant tuberculosis in Johannesburg, South Africa. J Clin Microbiol. 2013;51(6):1818–25.

42. Sakula A. Robert Koch: Centenary of the discovery of the tubercle bacillus, 1882. Can Vet J. 1983;24(4):127–31.

43. Warren P. The evolution of the sanatorium: The first half-century, 1854-1904. Can Bull Med Hist. 2006;23(2):457–76.

44. Turk JL. Von Pirquet, allergy and infectious diseases: A review. J R Soc Med.

1987;80(1):31–3.

45. Mazana JS. Tuberculosis and its eponyms: Charles Mantoux (1877-1947). Rev Española Sanid Penit. 2009;11(1):27–33.

46. Edwards PQ, Edwards LB. Story of the tuberculin test from an epidemiological viewpoint. Am Rev Respir Dis. 1960;81(1):1–47.

47. Joncas JH, Robitaille R, Gauthier T. Interpretation of the PPD skin test in BCG-vaccinated children. Can Med Assoc J. 1975;113(1):127–8.

48. Farhat M, Greenaway C, Pai M, Menzies D. False-positive tuberculin skin tests: What is the absolute effect of BCG and non-tuberculous mycobacteria? Int J Tuberc Lung Dis. 2006;10(11):1192–204.

49. Watkins RE, Brennan R, Plant AJ. Tuberculin reactivity and the risk of tuberculosis: A review. Int J Tuberc Lung Dis. 2000;4(10):895–903.

50. Deck F, Guld J. The WHO tuberculin test. Bull Int Union Tuberc. 1964;34(1):53–70.

51. American Thoracic Society. Targeted tuberculin testing and treatment of latent tuberculosis infection. MMWR Recomm Rep. 2000;49(RR-6):1–51.

52. Andersen P, Munk ME, Pollock JM, Doherty TM. Specific immune-based diagnosis of tuberculosis. Lancet. 2000;356(9235):1099–104.

53. Mazurek GH, Jereb J, LoBue P, Iademarco MF, Metchock B, Vernon A. Guidelines for using the QuantiFERON-TB Gold test for detecting *Mycobacterium tuberculosis* infection, United States. MMWR Recomm Rep. 2005;54(RR-15):49–55.

54. Fenton MJ, Vermeulen MW, Kim S, Burdick M, Strieter RM, Kornfeld H. Induction of gamma interferon production in human alveolar macrophages by *Mycobacterium tuberculosis*. J Infect Immun. 1997;65(12):5149–56.

55. Gallegos AM, van Heijst JW, Samstein M, Su X, Pamer EG, Glickman MS. A gamma interferon independent mechanism of CD4 T cell mediated control of *M. tuberculosis* infection in vivo. PLoS Pathog. 2011;7(5):e1002052.

56. Bocchino M, Bellofiore B, Matarese A, Galati D, Sanduzzi A. IFN-gamma release assays in tuberculosis management in selected high-risk populations. Expert Rev Mol Diagn. 2009;9(2):165–77.

57. Ferrara G, Losi M, D'Amico R, Roversi P, Piro R, Meacci M, et al. Use in routine clinical practice of two commercial blood tests for diagnosis of infection with *Mycobacterium tuberculosis*: A prospective study. Lancet. 2006;367(9519):1328–34.

58. Madariaga MG, Jalali Z, Swindells S. Clinical utility of interferon gamma assay in the diagnosis of tuberculosis. J Am Board Fam Med. 2007;20(6):540–7.

59. Mazurek GH, Jereb J, Vernon A, LoBue P, Goldberg S, Castro K. Updated guidelines for using interferon gamma release assays to detect *Mycobacterium tuberculosis* infection-United States, 2010. MMWR Recomm Rep. 2010;59(RR-5):1–25.

60. Denkinger CM, Dheda K, Pai M. Guidelines on interferon-γ release assays for

tuberculosis infection: Concordance, discordance or confusion? Clin Microbiol Infect. 2011;17(6):806–14.

61. World Health Organization. Use of tuberculosis interferon-gamma release assays (IGRAs) in low-and middle-income countries: Policy statement [Internet]. Geneva: World Health Organization; 2011. Available from: https://apps.who.int/iris/handle/10665/44759

62. Posner E. The early years of chest radiology in Britain. Thorax. 1971;26(3):233–9.

63. Wei C, Tiu C, Chen J, Chou Y, Chang C, Yu C. Computed tomography features of acute pulmonary tuberculosis. Am J Emerg Med. 2004;22(3):171–4.

64. Golub JE, Mohan CI, Comstock GW, Chaisson RE. Active case finding of tuberculosis: Historical perspective and future prospects. Int J Tuberc Lung Dis. 2005;9(11):1183–203.

65. WHO. Framework for effective tuberculosis control [Internet]. Geneva: World Health Organization; 1994. Available from: https://apps.who.int/iris/handle/10665/58717

66. Bishop PJ, Neumann G. The history of the Ziehl-Neelsen stain. Tubercle. 1970;51(2):196–206.

67. Alfred N, Lovette L, Aliyu G, Olusegun O, Meshak P, Jilang T, et al. Optimising *Mycobacterium tuberculosis* detection in resource limited settings. Br Med J. 2014;4(3):1–8.

68. Bennedsen J, Larsen SO. Examination for tubercle bacilli by fluorescence microscopy. Scand J Respir Dis. 1966;47(2):114–20.

69. Steingart KR, Henry M, Ng V, Hopewell PC, Ramsay A, Cunningham J, et al. Fluorescence versus conventional sputum smear microscopy for tuberculosis: A systematic review. Lancet Infect Dis. 2006;6(9):570–81.

70. Sula L, Sundaresan TK. WHO co-operative studies on a simple culture technique for the isolation of mycobacteria: Comparison of the efficacy of lyophilized liquid medium with that of Löwenstein–Jensen (LJ) medium. Bull World Health Organ. 1963;29(5):607–25.

71. Muddaiah RK, James PM, Lingegowda RK. Comparative study of smear microscopy, rapid slide culture, and Löwenstein–Jensen culture in cases of pulmonary tuberculosis in a tertiary care hospital. J Res Med Sci. 2013;18(9):767–71.

72. Olaru ID, Heyckendorf J, Grossmann S, Lange C. Time to culture positivity and sputum smear microscopy during tuberculosis therapy. PLoS One. 2014;9(8):1–6.

73. World Health Organization. Companion handbook to the WHO guidelines for the programmatic management of drug-resistant tuberculosis. World Health Organization. Geneva; 2014.

74. World Health Organization. Early detection of tuberculosis: An overview of approaches, guidelines and tools [Internet]. Geneva: World Health Organization; 2011. Available from: http://www.who.int/iris/handle/10665/70824#sthash.TN5snAtB.dpuf.

75. Kumar K, Abubakar I. Clinical implications of the global multidrug-resistant tuberculosis epidemic. J Clin Med. 2015;15(6):37–42.

76. World Health Organization. Use of liquid TB culture and drug susceptibility testing (DST) in low- and medium-income settings. [Internet]. WHO; 2007. Available from: https://www.who.int/tb/areas-of-work/laboratory/policy_liquid_medium_for_culture_dst/en/

77. Greco S, Girardi E, Navarra A, Saltini C. Current evidence on diagnostic accuracy of commercially based nucleic acid amplification tests for the diagnosis of pulmonary tuberculosis. Thorax. 2006;61(9):783–90.

78. Ling DI, Flores LL, Riley LW, Pai M. Commercial nucleic-acid amplification tests for diagnosis of pulmonary tuberculosis in respiratory specimens: Meta-analysis and meta-regression. PLoS One. 2008;3(2):e1536.

79. Centers for Disease Control and Prevention (CDC). Updated guidelines for the use of nucleic acid amplification tests in the diagnosis of tuberculosis. MMWR Morb Mortal Wkly Rep. 2009;58(1):7–10.

80. World Health Organization. Molecular line probe assay for rapid screening of patients at risk of multidrug-resistant tuberculosis (MDR-TB). Policy statement [Internet]. 2008. Available from: https://www.who.int/tb/laboratory/line_probe_assays/en/

81. Joint Committee for the Revision of Korean Guidelines for Tuberculosis; Korean Centers for Disease Control and Prevention. Korean guidelines for tuberculosis. 2nd ed. Seoul and Cheongwon: Joint Committee for the Revision of Korean Guidelines for Tuberculosis, Korea Centers for Disease Control and Prevention; 2014.

82. World Health Organization. The use of molecular line probe assays for the detection of resistance to isoniazid and rifampicin: Policy update [Internet]. 2016. Available from: https://www.who.int/tb/publications/molecular-test-resistance/en/

83. World Health Organization. The use of molecular line probe assays for the detection of resistance to second-line anti-tuberculosis drugs: Policy guidance [Internet]. 2016. Available from: https://www.who.int/tb/publications/policy-guidance-molecular-line/en/

84. Ajileye A, Alvarez N, Merker M, Walker TM, Akter S, Brown K, et al. Some synonymous and nonsynonymous gyrA mutations in *Mycobacterium tuberculosis* lead to systematic false-positive fluoroquinolone resistance results with the Hain GenoType MTBDRsl assays. Antimicrob Agents Chemother. 2017;61(4):1–8.

85. Folkvardsen DB, Svensson E, Thomsen VØ, Rasmussen EM, Bang D, Werngren J, et al. Can molecular methods detect 1% isonaizid resistance in *Mycobacterium tuberculosis*. J Clin Microbiol. 2013;51(5):1596–9.

86. Folkvardsen DB, Thomsen VØ, Rigouts L, Rasmussen EM, Bang D, Bernaerts G, et al. Rifampin heteroresistance in *Mycobacterium tuberculosis* cultures as detected by phenotypic and genotypic drug susceptibility test methods. J Clin Microbiol. 2013;51(12):4220–2.

87. Nathavitharana RR, Cudahy PG, Schumacher SG, Steingart KR, Pai M, Denkinger

CM. Accuracy of line probe assays for the diagnosis of pulmonary and multidrug-resistant tuberculosis: A systematic review and meta-analysis. Eur Respir J. 2017;49(1):1–23.

88. Theron G, Peter J, Richardson M, Warren R, Dheda K, Kr S. GenoType® MTBDRsl assay for resistance to second-line anti-tuberculosis drugs. Cochrane Database Syst Rev. 2016;9:CD010705.

89. WHO. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF system: policy statement [Internet]. 2011. Available from: https://apps.who.int/iris/handle/10665/44586

90. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev. 2014;(1):CD009593.

91. Tortoli E, Russo C, Piersimoni C, Mazzola E, Dal Monte P, Pascarella M, et al. Clinical validation of Xpert MTB/RIF for the diagnosis of extrapulmonary tuberculosis. Eur Respir J. 2012;40(2):442–7.

92. Nicol MP, Workman L, Isaacs W, Munro J, Black F, Eley B, et al. Accuracy of the Xpert MTB/RIF test for the diagnosis of pulmonary tuberculosis in children admitted to hospital in Cape Town, South Africa: A descriptive study. Lancet Infect Dis. 2011;11(11):819–24.

93. Zar HJ, Workman L, Isaacs W, Dheda K, Zemanay W, Nicol MP. Rapid diagnosis of pulmonary tuberculosis in African children in a primary care setting by use of Xpert MTB/RIF on respiratory specimens: A prospective study. Lancet Glob Heal. 2013;1(2):e97–104.

94. WHO. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children: policy update [Internet]. 2013. Available from: https://apps.who.int/iris/handle/10665/112472

95. Boehme CC, Nabeta P, Hillemann D, Nicol MP, Shenai S, Krapp F, et al. Rapid molecular detection of tuberculosis and rifampin resistance. N Engl J Med. 2010;363(11):1005–15.

96. Weyer K, Mirzayev F, Migliori GB, Van Gemert W, D'Ambrosio L, Zignol M, et al. Rapid molecular TB diagnosis: Evidence, policy making and global implementation of Xpert MTB/RIF. Eur Respir J. 2013;42(1):252–71.

97. Lehmann J. Twenty years afterward: Historical notes on the discovery of the antituberculosis effect of para-aminosalicylic acid (PAS) and the first clinical trials. Am Rev Respir Dis. 1964;90(6):953–6.

98. Schatz A, Bugie E, Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc Soc Exp Biol Med. 1944;55(1):66–9.

99. Hinshaw HC, Feldman WH, Pfuetze KH. Treatment of tuberculosis with streptomycin; a summary of observations on one hundred cases. J Am Med Assoc. 1946;132(13):778–82.

100. Crofton J, Mitchison DA. Streptomycin resistance in pulmonary tuberculosis. Br Med J. 1948;2(4588):1009–15.

101. Medical Research Council. Treatment of pulmonary tuberculosis with streptomycin and para-aminosalicylic acid. Br Med J. 1950;2:1073–85.

102. Medical Research Council. The prevention of streptomycin resistance by combined chemotherapy. Br Med J. 1952;1(4769):1157–62.

103. Bernstein J, Lott WA, Steinberg BA, Yale HL. Chemotherapy of experimental tuberculosis V. Isonicotinic acid hydrazide (Nydrazid) and related compounds. Am Rev Tuberc. 1952;65(4):357–64.

104. Murray JF. A century of tuberculosis. Am J Respir Crit Care Med. 2004;169(11):1181–6.

105. Robitzek EH, Selikoff IJ, Ornstein GG. Chemotherapy of human tuberculosis with hydrazine derivatives of isonicotinic acid; preliminary report of representative cases. Q Bull Sea View Hosp. 1952;13(1):27–51.

106. Robitzek EH, Selikoff IJ. Hydrazine derivatives of isonicotinic acid (Rimifon, Marsilid) in the treatment of active progressive caseous-pneumonic tuberculosis; a preliminary report. Am Rev Tuberc. 1952;65(4):402–28.

107. Medical Research Council. Various combinations of isoniazid with streptomycin or with P.A.S. in the treatment of pulmonary tuberculosis; seventh report to the Medical Research Council by their Tuberculosis Chemotherapy Trials Committee. Br Med J. 1955;1(4911):435–45.

108. Fox W, Wiener A, Mitchison DA, Selkon JB, Sutherland I. The prevalence of drug-resistant tubercle bacilli in untreated patients with pulmonary tuberculosis: A national survey, 1955-56. Tubercle. 1957;38(2):71–84.

109. Mitchison DA, Selkon JB. Bacteriological aspects of a survey of the incidence of drug-resistant tubercle bacilli among untreated patients. Tubercle. 1957;38(2):85–98.

110. Crofton J. Chemotherapy of pulmonary tuberculosis. Br Med J. 1959;1(5138):1610–4.

111. Crofton J. Sputum conversion and the metabolism of isoniazid. Am Rev Tuberc. 1958;77(5):869–71.

112. East African/British Medical Research Council. Isoniazid with thiacetazone (thioacetazone) in the treatment of pulmonary tuberculosis in East Africa-fifth investigation. Tubercle. 1970;51(2):123–51.

113. Fox W, Ellard GA, Mitchison DA. Studies on the treatment of tuberculosis undertaken by the British Medical Research Council tuberculosis units, 1946-1986, with relevant subsequent publications. Int J Tuberc Lung Dis. 1999;3(10 Suppl 2):S231-79.

114. Tuberculosis Chemotherapy Centre Madras. A concurrent comparison of home and

sanatorium treatment of pulmonary tuberculosis in South India. Bull World Health Organ. 1959;21(1):51–144.

115. Andrews RH, Devadatta S, Fox W, Radhakrishna S, Ramakrishnan C V, Velu S. Prevalence of tuberculosis among close family contacts of tuberculous patients in South India, and influence of segregation of the patient on the early attack rate. Bull World Health Organ. 1960;23(1):463–510.

116. Fox W. The problem of self-administration of drugs; with particular reference to pulmonary tuberculosis. Tubercle. 1958;39(5):269–74.

117. McKenzie D, Malone L, Kushner S, Oleson JJ, SubbaRow Y. The effect of nicotinic acid amide on experimental tuberculosis of white mice. J Lab Clin Med. 1948;33(10):1249–53.

118. Malone L, Schurr A, Lindh H, McKenzie D, Kiser JS, Williams JH. The effect of pyrazinamide (aldinamide) on experimental tuberculosis in mice. Am Rev Tuberc. 1952;65(5):511–8.

119. McCune RM, McDermott W, Tompsett R. The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique. II. The conversion of tuberculous infection to the latent state by the administration of pyrazinamide and a companion drug. J Exp Med. 1956;104(5):763–802.

120. Matthews JH. Pyrazinamide and isoniazid used in the treatment of pulmonary tuberculosis. Am Rev Respir Dis. 1960;81(3):348–51.

121. Muschenheim C, Organick A, McCune RM, Batten J, Deuschle K, Tompsett R, et al. Pyrazinamide-isoniazid in tuberculosis. III. Observations with reduced dosage of pyrazinamide. Am Rev Tuberc. 1955;72(6):851–5.

122. Thomas JP, Baughn CO, Wilkinson RG, Shepherd RG. A new synthetic compound with antituberculous activity in mice: Ethambutol (dextro-2,2'-(ethylenediimino)-di-1-butanol). Am Rev Respir Dis. 1961;83(6):891–3.

123. Doster B, Murray FJ, Newman R, Woolpert SF. Ethambutol in the initial treatment of pulmonary tuberculosis. U.S. Public Health Service tuberculosis therapy trials. Am Rev Respir Dis. 1973;107(2):177–90.

124. Ferebee SH, Doster BE, Murray FJ. Ethambutol: A substitute for para-aminosalicylic acid in regimens for pulmonary tuberculosis. Ann N Y Acad Sci. 1966;135(2):910–20.

125. Tuberculosis Research Centre. Ethambutol plus isoniazid for the treatment of pulmonary tuberculosis: A controlled trial of four regimens. Tubercle. 1981;62(1):13–29.

126. Maggi N, Pasqualucci CR, Ballotta R, Sensi P. Rifampicin: A new orally active rifamycin. Chemotherapy. 1966;11(5):285–92.

127. Sensi P. History of the development of rifampin. Rev Infect Dis. 1983;5(Suppl 3):S402–6.

128. Grumbach F. Activity of rifampicin on experimental tuberculosis in mice. The development of resistance to rifampicin. Therapeutic effects of combinations of

different drugs with rifampicin. Antibiot Chemother. 1970;16(1):392–405.

129. Grosset J. The sterilizing value of rifampicin and pyrazinamide in experimental short-course chemotherapy. Tubercle. 1978;59(4):287–97.

130. Dickinson JM, Mitchison DA. Experimental models to explain the high sterilizing activity of rifampin in the chemotherapy of tuberculosis. Am Rev Respir Dis. 1981;123(4):367–71.

131. Vall-Spinosa A, Lester W, Moulding T, Davidson PT, McClatchy JK. Rifampin in the treatment of drug-resistant *Mycobacterium tuberculosis* infections. N Engl J Med. 1970;283(12):616–21.

132. East African/British Medical Research Council. Second East African/British Medical Research Council Study. Controlled clinical trial of four 6-month regimens of chemotherapy for pulmonary tuberculosis. Second Report. Am Rev Respir Dis. 1976;114(3):471–5.

133. Hong Kong Chest Service/ British Medical Research Council. Controlled trial of 6-month and 8-month regimens in the treatment of pulmonary tuberculosis: The results up to 24 months. Tubercle. 1979;60(4):201–10.

134. British Thoracic and Tuberculosis Association. Short-course chemotherapy in pulmonary tuberculosis: A controlled trial by the British Thoracic and Tuberculosis Association. Lancet. 1976;2(7995):1102–4.

135. East African/British Medical Research Council. Controlled clinical trial of short-course (6-month) regimens of chemotherapy for treatment of pulmonary tuberculosis. Lancet. 1972;299(7760):1079–85.

136. Barclay WR, Russe H. The in vitro action of cycloserine on *M. tuberculosis*. Am Rev Tuberc. 1955;72(2):236–41.

137. Umezawa H. Kanamycin: Its discovery. Ann N Y Acad Sci. 1958;76(2):20–6.

138. Riddell RW, Stewart SM, Somner AR. Ethionamide. BMJ. 1960;2(5207):1207–8.

139. Popplewell AG, Miller JD, Greene ME, Landwehr A. Capreomycin in original treatment cases of pulmonary tuberculosis. Ann N Y Acad Sci. 1966;135(2):989–1005.

140. World Health Organization. TB: WHO report on the tuberculosis epidemic 1997 [Internet]. Geneva; 1997. Available from: https://apps.who.int/iris/handle/10665/63400

141. WHO. Guidelines for TB treatment in adults and children in national TB programmes [Internet]. 1991. Available from: https://www.who.int/tb/publications/1991/en/

142. Frieden TR, Fujiwara PI, Washko RM, Hamburg MA. Tuberculosis in New York City-turning the tide. N Engl J Med. 1995;333(4):229–33.

143. Feng-Zeng Z, Murray C, Spinaci S, Styblo K, Broekmans J. Results of directly observed short-course chemotherapy in 112 842 Chinese patients with smear-positive tuberculosis. Lancet. 1996;347(8998):358–62.

144. Kochi A. Tuberculosis control: Is DOTS the health breakthrough of the 1990s. World

Health Forum. 1997;18(3–4):225–32.

145. Mahmoudi A, Iseman MD. Pitfalls in the care of patients with tuberculosis. Common errors and their association with the acquisition of drug resistance. JAMA. 1993;270(1):65–8.

146. British Thoracic and Tuberculosis Association. Short-course chemotherapy in pulmonary tuberculosis. Lancet. 1975;1(7899):119–24.

147. Cohen KA, Abeel T, Manson Mcguire A, Desjardins CA, Munsamy V, Shea TP, et al. Evolution of extensively drug-resistant tuberculosis over four decades: Whole genome sequencing and dating analysis of *Mycobacterium tuberculosis* isolates from KwaZulu-Natal. PLoS Med. 2015;12(9):1–22.

148. Weyer K, Groenewald P, Zwarenstein M, Lombard CJ. Tuberculosis drug resistance in the Western Cape. South African Med J. 1995;85(6):499–504.

149. South African National Department of Health. The management of multidrug-resistant tuberculosis. Pretoria: NDoH; 1999.

150. WHO. Guidelines for the management of drug-resistant tuberculosis [Internet]. 1997. Available from: https://www.who.int/tb/publications/1997/en/

151. Davies GR, Pillay M, Sturm AW, Wilkinson D. Emergence of multidrug-resistant tuberculosis in a community-based directly observed treatment programme in rural South Africa. Int J Tuberc Lung Dis. 1999;3(9):799–804.

152. Pillay M, Sturm AW. Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. Clin Infect Dis. 2007;45(11):1409–14.

153. van Rie A, Warren RM, Beyers N, Gie RP, Classen CN, Richardson M, et al. Transmission of a multidrug-resistant *Mycobacterium tuberculosis* strain resembling “strain W” among noninstitutionalized, human immunodeficiency virus-seronegative patients. J Infect Dis. 1999;180(5):1608–15.

154. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet. 2006;368(9547):1575–80.

155. Rawlinson J. Multi-drug resistant tuberculosis in South Africa. HST Update. 2000;56:8–10.

156. Espinal MA, Dye C, Raviglione M, Kochi A. Rational “DOTS plus” for the control of MDR-TB. Int J Tuberc Lung Dis. 1999;3(7):561–3.

157. Weyer K. Multidrug-resistant tuberculosis. Continuing Medical Education (CME). 2005;23(2):74–84.

158. Rubin EJ. TB diagnosis from the dark ages to fluorescence. Nat Microbiol. 2018;3(3):268–9.

159. Trauner A, Liu Q, Via LE, Liu X, Ruan X, Liang L, et al. The within-host population dynamics of *Mycobacterium tuberculosis* vary with treatment efficacy. Genome Biol.

2017;18(1):71.

160. Eldholm V, Norheim G, von der Lippe B, Kinander W, Dahle UR, Caugant DA, et al. Evolution of extensively drug-resistant *Mycobacterium tuberculosis* from a susceptible ancestor in a single patient. Genome Biol. 2014;15(11):490.

161. Coll F, Phelan J, Hill-Cawthorne GA, Nair MB, Mallard K, Ali S, et al. Genome-wide analysis of multi- and extensively drug-resistant *Mycobacterium tuberculosis*. Nat Genet. 2018;50(2):307–16.

162. Desjardins CA, Cohen KA, Munsamy V, Abeel T, Maharaj K, Walker BJ, et al. Genomic and functional analyses of *Mycobacterium tuberculosis* strains implicate ald in D-cycloserine resistance. Nat Genet. 2016;48(5):544–51.

163. Heyckendorf J, Andres S, Köser CU, Olaru ID, Schön T, Sturegård E, et al. What is resistance? Impact of phenotypic versus molecular drug resistance testing on therapy for multi- and extensively drug-resistant tuberculosis. Antimicrob Agents Chemother. 2018;62(2):e01550-17.

164. Walker TM, Kohl TA, Omar S V, Hedge J, Del Ojo Elias C, Bradley P, et al. Whole-genome sequencing for prediction of *Mycobacterium tuberculosis* drug susceptibility and resistance: A retrospective cohort study. Lancet Infect Dis. 2015;15(10):1193–202.

165. Miotto P, Tessema B, Tagliani E, Chindelevitch L, Starks AM, Emerson C, et al. A standardised method for interpreting the association between mutations and phenotypic drug resistance in *Mycobacterium tuberculosis*. Eur Respir J. 2017;50(6):1701354.

166. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Med. 2009;6(2):e1000002.

167. Coll F, McNerney R, Preston MD, Guerra-Assunção JA, Warry A, Hill-Cawthorne G, et al. Rapid determination of anti-tuberculosis drug resistance from whole-genome sequences. Genome Med. 2015;7(1):51.

168. Feuerriegel S, Schleusener V, Beckert P, Kohl TA, Miotto P, Cirillo DM, et al. PhyResSE: A Web tool delineating *Mycobacterium tuberculosis* antibiotic resistance and lineage from whole-genome sequencing data. J Clin Microbiol. 2015;53(6):1908–14.

169. Metcalfe JZ, Streicher E, Theron G, Colman RE, Allender C, Lemmer D, et al. Cryptic microheteroresistance explains *Mycobacterium tuberculosis* phenotypic resistance. Am J Respir Crit Care Med. 2017;196(9):1191–201.

170. Metcalfe JZ, Streicher E, Theron G, Colman RE, Penaloza R, Allender C, et al. *Mycobacterium tuberculosis* subculture results in loss of potentially clinically relevant heteroresistance. Antimicrob Agents Chemother. 2017;61(11):e00888-17.

171. Conradie F, Diacon AH, Ngubane N, Howell P, Everitt D, Crook AM, et al. Treatment of highly drug-resistant pulmonary tuberculosis. N Engl J Med. 2020;382(10):893–902.

172. Andries K, Verhasselt P, Guillemont J, Göhlmann HW, Neefs J-M, Winkler H, et al. A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. Science. 2005;307(5707):223–7.

173. Koul A, Vranckx L, Dendouga N, Balemans W, Van den Wyngaert I, Vergauwen K, et al. Diarylquinolines are bactericidal for dormant mycobacteria as a result of disturbed ATP homeostasis. J Biol Chem. 2008;283(37):25273–80.

174. Singh R, Manjunatha U, Boshoff HI, Ha Y, Niyomrattanakit P, Ledwidge R, et al. PA-824 kills nonreplicating *Mycobacterium tuberculosis* by intracellular NO release. Science. 2008;322(5906):1392–5.

175. Manjunatha U, Boshoff HI, Barry CE. The mechanism of action of PA-824: Novel insights from transcriptional profiling. Commun Integr Biol. 2009;2(3):215–8.

176. Tyagi S, Nuermberger E, Yoshimatsu T, Williams K, Rosenthal I, Lounis N, et al. Bactericidal activity of the nitroimidazopyran PA-824 in a murine model of tuberculosis. Antimicrob Agents Chemother. 2005;49(6):2289–93.

177. Stover CK, Warrener P, VanDevanter DR, Sherman DR, Arain TM, Langhorne MH, et al. A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. Nature. 2000;405(6789):962–6.

178. Vestal AL, Kubica GP. Differential colonial characteristics of mycobacteria on Middlebrook and Cohn 7H10 agar-base medium. Am Rev Respir Dis. 1966;94(2):247–52.

179. Hanna BA, Ebrahimzadeh A, Elliott LB, Morgan MA, Novak SM, Rusch-Gerdes S, et al. Multicenter evaluation of the BACTEC MGIT 960 system for recovery of Mycobacteria. J Clin Microbiol. 1999;37(3):748–52.

180. Middlebrook G, Cohn M. Bacteriology of tuberculosis: Laboratory methods. Am J Public Health. 1958;48(7):844–53.

181. Kanchana M V, Cheke D, Natyshak I, Connor B, Warner A, Martin T. Evaluation of the BACTEC ™ MGIT ™ 960 system for the recovery of Mycobacteria. Diagn Microbiol Infect Dis. 2000;37:31–6.

182. Katila M-L, Katila P, Erkinjuntti-Pekkanen R. Accelerated detection and identification of mycobacteria with MGIT 960 and COBAS AMPLICOR systems. J Clin Microbiol. 2000;38(3):960–4.

183. Yan J-J, Huang A-H, Tsai S-H, Ko W-C, Jin Y-T, Wu J-J. Comparison of the MB/BacT and BACTEC MGIT 960 system for recovery of mycobacteria from clinical specimens. Diagn Microbiol Infect Dis. 2000;37(1):25–30.

184. Tortoli E, Cichero P, Piersimoni C, Simonetti MT, Careggi O, Microbiologia L. Use of BACTEC MGIT 960 for recovery of mycobacteria from clinical specimens: multicenter study. J Clin Microbiol. 1999;37(11):3578–82.

185. Siddiqi SH, Rüsch-Gerdes S. For BACTEC™ MGIT 960™ TB System (Also applicable for Manual MGIT) Mycobacteria Growth Indicator Tube (MGIT) Culture and Drug Susceptibility Demonstration Projects [Internet]. 2006 [cited 2021 Feb 23]. Available from: https://www.finddx.org/wp-content/uploads/2016/02/mgit_manual_nov2006.pdf

186. Sirgel FA, Wiid IJF, Van Helden PD. Measuring minimum inhibitory concentrations in mycobacteria. Methods Mol Biol. 2009;465:173–86.

187. Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 1997;35(4):907–14.

188. Glynn JR, Whiteley J, Bifani PJ, Kremer K, van Soolingen D. Worldwide occurrence of Beijing/W strains of *Mycobacterium tuberculosis*: A systematic review. Emerg Infect Dis. 2002;8(8):843–9.

189. Gori A, Bandera A, Marchetti G, Esposti AD, Catozzi L, Nardi GP, et al. Spoligotyping and *Mycobacterium tuberculosis*. Emerg Infect Dis. 2005;11(8):1242–8.

190. Hermans PWM, van Soolingen D, Bik EM, de Haas PEW, Dale JW, van Embden JDA. Insertion element IS987 from Mycobacterium bovis BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. Infect Immun. 1991;59(8):2695–705.

191. Streicher EM, Victor TC, van der Spuy G, Sola C, Rastogi N, van Helden PD, et al. Spoligotype signatures in the *Mycobacterium tuberculosis* complex. J Clin Microbiol. 2007;45(1):237–40.

192. Warren RM, Streicher EM, Charalambous S, Churchyard G, van der Spuy GD, Grant AD, et al. Use of spoligotyping for accurate classification of recurrent tuberculosis. J Clin Microbiol. 2002;40(10):3851–3.

193. Brudey K, Driscoll JR, Rigouts L, Prodinger WM, Gori A, Al-Hajoj SA, et al. *Mycobacterium tuberculosis* complex genetic diversity: Mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. BMC Microbiol. 2006;6(23):1–17.

194. Plikaytis BB, Marden JL, Crawford JT, Woodley CL, Butler WR, Shinnick TM. Multiplex PCR assay specific for the multidrug-resistant strain W of *Mycobacterium tuberculosis*. J Clin Microbiol. 1994;32(6):1542–6.

195. Mokrousov I, Narvskaya O, Otten T, Vyazovaya A, Limeschenko E, Steklova L, et al. Phylogenetic reconstruction within *Mycobacterium tuberculosis* Beijing genotype in northwestern Russia. Res Microbiol. 2002;153(10):629–37.

196. Hanekom M, van der Spuy GD, Streicher E, Ndabambi SL, McEvoy CR, Kidd M, et al. A recently evolved sublineage of the *Mycobacterium tuberculosis* Beijing strain family is associated with an increased ability to spread and cause disease. J Clin Microbiol. 2007;45(5):1483–90.

197. Beggs ML, Eisenach KD, Cave MD. Mapping of *IS6110* insertion sites in two epidemic strains of *Mycobacterium tuberculosis*. J Clin Microbiol. 2000;38(8):2923–8.

198. Iwamoto T, Yoshida S, Suzuki K, Wada T. Population structure analysis of the *Mycobacterium tuberculosis* Beijing family indicates an association between certain sublineages and multidrug resistance. Antimicrob Agents Chemother. 2008;52(10):3805–9.

199. Zaczek A, Brzostek A, Augustynowicz-Kopec E, Zwolska Z, Dziadek J. Genetic evaluation of relationship between mutations in *rpoB* and resistance of *Mycobacterium tuberculosis* to rifampin. BMC Microbiol. 2009;9(10):1–8.

200. Yuen LKW, Leslie D, Coloe PJ. Bacteriological and molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* strains in Australia. J Clin Microbiol. 1999;37(12):3844–50.

201. Ando H, Kondo Y, Suetake T, Toyota E, Kato S, Mori T, et al. Identification of *katG* mutations associated with high-level isoniazid resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2010;54(5):1793–9.

202. Bostanabad SZ, Bahrmand AR, Poorazar S, Abdolrahimi F, Nur-Nemattollahi A, Massomi M, et al. Mutations in codon 315 of the *katG* gene associated with high-level resistance to isoniazid. Tanaffos. 2007;6(3):11–9.

203. Niehaus AJ, Mlisana K, Gandhi NR, Mathema B, James C, Brust M. High prevalence of *inhA* promoter mutations among patients with drug-resistant tuberculosis in KwaZulu-Natal, South Africa. PLoS One. 2015;10(9):e0135003.

204. Abe C, Kobayashi I, Mitarai S, Wada M, Kawabe Y, Takashima T, et al. Biological and molecular characteristics of *Mycobacterium tuberculosis* clinical isolates with low-level resistance to isoniazid in Japan. J Clin Microbiol. 2008;46(7):2263–8.

205. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 1997;25(17):3389–402.

206. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, et al. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature. 1998;393(6685):537–44.

207. Whitehead Scientific, South Africa [Internet]. [cited 2020 Feb 23]. Available from: http://whitesci.co.za/

208. Dean AS, Zignol M, Cabibbe AM, Falzon D, Glaziou P, Cirillo DM, et al. Prevalence and genetic profiles of isoniazid resistance in tuberculosis patients: A multicountry analysis of cross-sectional data. PLoS Med. 2020;17(1):e1003008.

209. Van Der Heijden YF, Karim F, Mufamadi G, Zako L, Chinappa T, Shepherd BE, et al. Isoniazid-monoresistant tuberculosis is associated with poor treatment outcomes in Durban, South Africa. Int J Tuberc Lung Dis. 2017;21(6):670–6.

210. Jhun BW, Koh WJ. Treatment of isoniazid-resistant pulmonary tuberculosis. Vol. 83, Tuberculosis and Respiratory Diseases. 2020. p. 20–30.

211. Chien JY, Chen YT, Wu SG, Lee JJ, Wang JY, Yu CJ. Treatment outcome of patients with isoniazid mono-resistant tuberculosis. Clin Microbiol Infect. 2015;21(1):59–68.

212. Menzies D, Benedetti A, Paydar A, Royce S, Pai M, Burman W, et al. Standardized treatment of active tuberculosis in patients with previous treatment and/or with mono-resistance to isoniazid: A systematic review and meta-analysis. PLoS Med. 2009;6(9):e1000150.

213. WHO treatment guidelines for isoniazid-resistant tuberculosis: Supplement to the WHO treatment guidelines for drug-resistant tuberculosis. WHO [Internet]. 2018; Available from: http://www.who.int/tb/publications/2018/WHO_guidelines_isoniazid_resistant_TB/en/

214. Chingonzoh R, Manesen MR, Madlavu MJ, Sopiseka N, Nokwe M, Emwerem M, et al. Risk factors for mortality among adults registered on the routine drug resistant tuberculosis reporting database in the Eastern Cape Province, South Africa, 2011 to 2013. PLoS One. 2018;13(8):e0202469.

215. Seifert M, Catanzaro D, Catanzaro A, Rodwell TC. Genetic Mutations Associated with Isoniazid Resistance in *Mycobacterium tuberculosis*: A Systematic Review. PLoS One [Internet]. 2015;10(3):e0119628. Available from: https://dx.plos.org/10.1371/journal.pone.0119628

216. Jeeves RE, Marriott AAN, Pullan ST, Hatch KA, Allnutt JC, Freire-Martin I, et al. *Mycobacterium tuberculosis* Is Resistant to Isoniazid at a Slow Growth Rate by Single Nucleotide Polymorphisms in *katG* Codon Ser315. PLoS One. 2015;10(9):e0138253.

217. Gegia M, Cohen T, Kalandadze I, Vashakidze L, Furin J. Outcomes among tuberculosis patients with isoniazid resistance in Georgia, 2007-2009. Int J Tuberc Lung Dis. 2012;16(6):812–6.

218. Jacobson KR, Theron D, Victor TC, Streicher EM, Warren RM, Murray MB. Treatment outcomes of isoniazid-resistant tuberculosis patients, Western Cape Province, South Africa. Clin Infect Dis. 2011;53(4):369–72.

219. Espinal MA, Kim SJ, Suarez PG, Kam KM, Khomenko AG, Migliori GB, et al. Standard short-course chemotherapy for drug-resistant tuberculosis: Treatment outcomes in 6 countries. J Am Med Assoc. 2000;283(19):2537–45.

220. Gegia M, Winters N, Benedetti A, van Soolingen D, Menzies D. Treatment of isoniazid-resistant tuberculosis with first-line drugs: a systematic review and meta-analysis. Lancet Infect Dis. 2017;17(2):223–34.

221. Ng KCS, Supply P, Cobelens FGJ, Gaudin C, Gonzalez-Martin J, De Jong BC, et al. How well do routine molecular diagnostics detect rifampin heteroresistance in *Mycobacterium tuberculosis*? J Clin Microbiol. 2019;57(11):e00717-19.

222. Blakemore R, Story E, Helb D, Kop JA, Banada P, Owens MR, et al. Evaluation of the analytical performance of the Xpert MTB/RIF assay. J Clin Microbiol. 2010;48(7):2495–501.

Supplementary data

Reagents used for MGIT

MGIT medium

A MGIT 960 tube contains 7.0 ml of modified Middlebrook 7H9 broth base. The approximate formula per 1000 ml purified water is:

- Modified Middlebrook 7H9 broth base -------- 5.9 g
- Casein peptone ---------- 1.2 g
- Add supplements as required.

MGIT Growth supplement (OADC)

MGIT growth supplement is used for the preparation of subcultures and inoculate and contains 15 ml of the following per liter of water.

- Bovine albumin ---------- 50.0 g
- Dextrose---------- 20.0 g
- Catalase ---------- 0.03 g
- Oleic acid ---------- 0.1 g
- Polyoxyethyline state ---------- 1.1 g

Solutions used for spoligotyping

Working stocks

500ml 2xSSPE/0.1%SDS:

- 100 ml 10x SSPE
- 5 ml 10% SDS
- dH2O to 500 ml

1000ml 2xSSPE/0.5%SDS:

- 200 ml 10x SSPE
- 50 ml 10% SDS
- dH2O to 1000 ml

1000 ml 1% SDS:

- 100 ml 10% SDS
- dH20 to 1000 ml

500 ml 20 mM EDTA:

- 25 ml 0.5 M EDTA
- dH2O to 500 ml

Stock solutions

10x SSPE:

- 13.7 g Sodium hydrogen Phosphate – 100 mM
- 105.19 g Sodium Chloride – 1.8 M
- 3.36 g EDTA – 10 mM
- dH2O to 1000 ml
- pH = 7.40

10% SDS:

- 50 g SDS
- dH2O to 500 ml

0.5M EDTA:

- 93 g EDTA
- dH2O to 500 ml

Reagents used for FluoroType® MTBDR

	tests: 24	96
Amplification Mix A		
Buffer, Taq Polymerase, bovine serum albumin	144 µl	4x 144 µl
Amplification Mix B		
Buffer, specific oligonucleotides, nucleotides, bovine serum albumin, salts	336 µl	4x 336 µl
Internal Control DNA		
Polynucleotides	192 µl	192 µl
Control DNA		
Polynucleotides	220 µl	220 µl