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Application of molecular methods in optimizing clinical management of patients with pulmonary multidrug resistant tuberculosis in Tanzania

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APPLICATION OF MOLECULAR METHODS IN OPTIMIZING CLINICAL MANAGEMENT OF PATIENTS WITH PULMONARY MULTIDRUG RESISTANT TUBERCULOSIS IN TANZANIA

Peter M. Mbelele

A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology

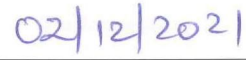**Arusha, Tanzania**

December, 2021

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ABSTRACT

Rifampicin or multidrug-resistant tuberculosis (RR/MDR-TB) accounts for considerable morbidity and mortality. Differences among *Mycobacterium tuberculosis* complex (MTBC) species may predict drug-resistance or treatment success. This thesis aimed at identifying MTBC at species level, their drug-resistance patterns, and mycobactericidal effect of different regimens in patients treated for RR/MDR-TB and drug-susceptible (DS-TB). This was a cross-sectional study nested in a longitudinal design that followed patients on RR/MDR-TB and DS-TB treatment for 4 months. While RR/MDR-TB patients received bedaquiline with or without an injectable-aminoglycoside based regimen, DS-TB patients received a fixed-dose-combination comprised of rifampicin-isoniazid-pyrazinamid-ethambutol (RHZE). The genotype MTBC assay was used to identify MTBC species. WGS-based drug resistant mutations predicted the minimum-inhibitory-concentration (MIC) of antibiotics measured in the MycoTB-Sensitire™ assay. An isolate was categorised as resistant and susceptible if it had a MIC above and at or below the epidemiological-cut-off (ECOFF) value, respectively. Non-linear-mixed effects modelled the MTBC killing rates measured by Tuberculosis molecular-bacterial load assay and culture. The median MIC at their 25th and 75th interquartile range (IQR) were compared using Mann-Whitney U test. Among 126 patients, 89 (71%) had positive culture whereas 87 (98%) were identified as *M. tuberculosis*. Overall, mutant (gNWT-R) MTBC isolates correlated with MIC values above the ECOFF. For instance, the median MIC (µg/mL) for rifampicin-gNWT-R strains was > 4.0 (IQR; 4.0 – 4.0) compared to 0.5 (IQR; 0.38 – 0.50) in non-mutant (gWT-S, $p < 0.001$); isoniazid-gNWT-R > 4.0 (IQR; 2.0 – 4.0) compared to 0.25 (IQR; 0.12 – 1.00) among gWT-S ($p = 0.001$); ethionamide-gNWT-R 15.0 (IQR; 10.0 – 20.0) compared to 2.50 (IQR; 2.50 – 5.00) among gWT-S ($p < 0.001$). There were no detectable mutations in genes previously known to confer fluoroquinolones, aminoglycosides, capreomycin, bedaquiline, delamanid, linezolid, clofazimine, cycloserine, and p-aminosalicylic acid resistance. Compared to the adjusted *M. tuberculosis* killing rate of -0.17 (95% CI; -0.23 to -0.12) for the injectable without bedaquiline reference regimen, the killing rates were -0.62 (95% CI; -1.05 to -0.20) log10 eCFU/mL for the injectable with bedaquiline-containing regimen ($p = 0.019$), -0.35 (95% CI; -0.65 to -0.13) log10 eCFU/mL for the all-oral bedaquiline-based regimen ($p = 0.054$), and -0.29 (95% CI; -0.78 to +0.22) log10 eCFU/mL for RHZE ($p = 0.332$). The detected *M. tuberculosis* was susceptible to core drugs including bedaquiline, fluoroquinolones, linezolid, and clofazimine, supporting their central use in the RR/MDRTB treatment regimens. *M. tuberculosis* killing rates were higher among patients who received bedaquiline but were further improved with addition of an injectable aminoglycoside.

DECLARATION

I, Peter Masunga Mbelele do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that: this is my own original work and that it has not been submitted for consideration of a similar degree award in any other University.
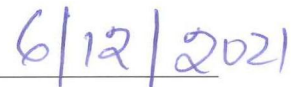
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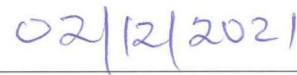Peter M. Mbelele   Date

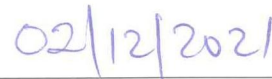This declaration is confirmed by:

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CERTIFICATION

The undersigned, certify that they have read this thesis titled *"Application of Molecular Methods in Optimizing Clinical Management of Patients with Pulmonary Multidrug Resistant Tuberculosis in Tanzania"*, and found it to be in a form acceptable for the award of the Degree of Doctor of Philosophy in Life Sciences of the Nelson Mandela African Institution of Science and Technology.

_____   _____6/12/2021_____

Dr. Elingarami Sauli   Date

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Dr. Emmanuel A. Mpolya   Date

_____   _____

Prof. Sayoki G. Mfinaga   Date

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ACKNOWLEDGEMENTS

DEDICATIONS

I am grateful to dedicate my wife, Ms. Happyness J. Kyekaka and our four children (Brian, Blessing, Ezra and Elisha) for their endless love, prayers, courage, support, and patience while I was undertaking research work and studies. Also, I dedicate to my late mother Ms. Martha Mawishi Seni Matunge whom she passed away during my PhD academic pursuit in 2018, and in memory of my late father, Mr. Mbelele Masunga, who is now in his 9th anniversary since when he passed away in 2012. May our almighty God rest their (mother and father) soul in eternal peace.

Amen.

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LIST OF ABBREVIATIONS AND SYMBOYS

AIDS	Acquired immunodeficient syndrome
AMK	Amikacin
AO-ASPIRE	African Science Partnership for Intervention Research Excellence
BDQ	Bedaquiline
CAP	Capreomycin
CB	Clinical breakpoint
CC	Critical concentration of a drug
CFZ	Clofazimine
CI	Confidence interval
CRF	Clinical report form
CS	Cycloserine
DLM	Delamanid
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
DS-TB	Drug susceptible tuberculosis
ECOFF	Epidemiological cut-off value
EDCTP	European & Developing Countries Clinical Trials Partnership
EMB	Ethambutol
ERC	Ethical review committee
ETH	Ethionamide
FQs	Fluoroquinolones
gCM	Genotype Mycobacterium CM version 2.0
gDST	Genotypic drug susceptibility testing
gMTBC	Genotype MTBC v1.x assay
HIV	Human immunodeficiency virus
ICF	Informed Consent form
INH or H	Isoniazid
KAN	Kanamycin
KIDH	Kibong'oto Infectious Diseases Hospital
LZD	Linezolid
LFX	Levofloxacin
LPA	Line probe assay

MAC	Mycobacterium -aviam-intracellulare complex
MDR-TB	Multidrug resistance tuberculosis
MFX	Moxifloxacin
MIC	Minimum inhibition concentration
MRCC	Medical Research coordinating committee
MTBDRplus	Genotype MTBDRplus assay
MTBDRsl	Genotype MTBDRplus assay
MTC	*Mycobacterium tuberculosis* complex
NIMR	National Institute for Medical research
NM-AIST	Nelson Mandela-African Institution of Science and Technology
NTLP	National Tuberculosis and Leprosy Program
NTM	Non-tuberculous mycobacteria
PAS	*para*-aminosalicylic acid
PCR	Polymerase Chain Reaction
pDST	Phenotypic drug-susceptibility testing
PLWHA	People living with HIV/AIDS
PZA or Z	Pyrazinamide
RIF or R	Rifampicin
RHZE	Fixed dose of rifampicin (R), isoniazid (H), pyrazinamide (Z) and ethambutol (E)
RIB	Rifabutin
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RR-TB	Rifampicin resistant tuberculosis
RR/MDR-TB	Rifampicin resistance or multidrug resistance tuberculosis
RT-qPCR	Reverse transcriptase- quantitative PCR
SLIDs	Second line injectable drugs
STR or S	Streptomycin
TB	Tuberculosis
TB-MBLA	TB-Molecular bacterial load assay
WGS	Whole genome sequencing
WHO	World Health Organization
XDR-TB	Extensive or extremely drug resistant TB

CHAPTER ONE

INTRODUCTION

1.1   Background of the Problem

Tuberculosis (TB), a chronic infectious disease, is an old disease previously described as phthisis in Greece. Tuberculosis in human and animal is caused by a group of genetically-related mycobacterial species called *Mycobacterium tuberculosis* complex (MTBC). The member species in this complex includes *M. tuberculosis*, *M. africanum*, *M. bovis* subsp. *bovis, M. bovis* BCG*, M. caprae*, *M. microti, M. pinnipedii*, *M. canettii,* and *M. mungi* (Riojas *et al.,* 2018). *M. tuberculosis* is a prototype species and was discovered by Robert Koch in 1882 (Sakula, 1982). Using single nucleotide polymorphisms (SNPs) and region of difference (RD) based technologies, the members of MTBC have been genotyped into main phylogenetic lineages and sub-lineages (Coscolla & Gagneux, 2014). Widely known lineages include: (a) the Indo-Oceanic lineage I, (b) East-Asian (Beijing family) lineage 2, (c) East-African-Indian (EAI) lineage 3 [(*M. tuberculosis sensu* stricto and Central-Asian (CAS)], (d) Euro-American lineage 4 which has sub-lineages like Latin-American-Mediterranean (LAM), Haarlem, Uganda and S-type, (e) West African lineage 5 (*M. africanum* I), (f) lineage 6 (*M. africanum* II), (g) lineage 7 (Ethiopian strain); and (i) the recently recovered Rwandan lineage 8 (Comas *et al*., 2009; Ngabonziza *et al.,* 2020; Wiens *et al.,* 2018).

Tuberculosis is curable. During pre-antibiotic era, TB incidence and mortalities was high worldwide. The anti-TB drugs such as streptomycin (S), isoniazid (H), para-aminosalicylic acid (PAS), rifampicin (R), ethambutol (E) and pyrazinamide (Z) became available from 1940s, and in 1970s a fixed dose combination of RHZE became the standard regimen for treating drug susceptible TB (DS-TB) worldwide (Riccardi & Pasca, 2014). Implementing this regimen reduced incidences and mortality tremendously, and TB was considered to be controlled in most part of the world. However, in 1980s through 1990s, TB incidence and mortality resurged. This resurgence was attributed to emergence of human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) and drug resistant *M. tuberculosis* complex strains (Sharma & Mohan, 2013).

Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome weakens the host immune system especially the T lymphocyte cell mediate response. Consequently, it increases

the risk of acquiring active TB disease (Kiazyk & Ball, 2017; Scriba *et al.,* 2017) from 10% in immunocompetent patients to 20% in people living with HIV/AIDS P (MacNeil *et al.,* 2019; World Health Organization [WHO], 2020). In addition, HIV increases the rate of relapse TB and chance for re-infection, posing an increased risk of transmission in the general population including drug resistant TB (Sharma & Mohan, 2013).

Drug resistant TB is a common phenomenon. It was reported for the first time in early 1950s when streptomycin monotherapy was the only active antibiotic for treating drug-susceptible TB (Iseman, 1994), and it continued to escalate despite global implementation of direct observed therapy strategy of the standard RHZE treatment regimen in 1999 (Calver *et al.,* 2010). Principally, drug resistance in *M. tuberculosis* complex strains is fundamentally conferred through spontaneous point mutations in specific gene targets for an anti-tuberculosis drug (Dookie *et al.,* 2018; Hameed *et al.,* 2018). Figure 1 shows that combination of those point mutations can result in multidrug-resistant tuberculosis (MDR-TB), defined as a TB caused by strains resistant to at least rifampicin (due to mutation on *rpoB*) and isoniazid (due to mutation on *katG* genes with or without *inhA* promotor region) (WHO, 2019b). Also, accumulation of point mutations may results to an extensively drug resistant TB (XDR-TB), recently described as MDR-TB with additional mutation(s) on *gyrA* and *gyrB* genes that predicts resistance to at least one fluoroquinolone (levofloxacin, moxifloxacin, ofloxacin, gatifloxacin) and mutation(s) on either *Rv0678*, *atpE*, and *pepQ* genes that was previously linked to bedaquiline resistance or *rrl* and *rplC* genes which confer linezolid resistance (WHO, 2021).

Indeed, emergence of HIV/AIDS and drug resistant TB epidemics challenged routine diagnostic approaches. Smear microscopy is a routine and widely used diagnostic tool but it is neither sensitive nor specific for detecting *M. tuberculosis* complex, and it does not detects drug resistance TB (Steingart *et al*., 2006). Culturing *M. tuberculosis* complex and testing drug resistance on agar-based Lowenstein-Jensen (LJ) solid and Mycobacterium Growth Indicator Tube (MGIT) liquid culture media is sensitive and specific (European Centre for Disease Prevention and Control, [ECDPC] 2018). However, culture is a laborious work and it delays results up to 8 weeks, yet it is prone to contamination in up to 15% of Tanzanian patients (Hoza *et al.,* 2015; Reddy *et al.,* 2014). Subsequently, it further delays clinical decision. Rapid molecular methods such as Xpert® MTB/RIF (Cepheid, USA) and line probe assays (LPA; Hain Life Sciences, Germany), such as the genotype MTBDRplus or MTBDRsl are accurate in detecting *M. tuberculosis* complex and drug resistant tuberculosis (Creswell *et al.,* 2015;

Mbelele *et al.,* 2017). The Xpert® MTB/RIF dually detects *M. tuberculosis* complex and rifampicin resistant (RR) TB. The genotype MTBDRplus detects MTBC and resistance to rifampicin and isoniazid whereas the genotype MTBDRsl detects *M. tuberculosis* complex, and resistance to fluoroquinolones and injectable aminoglycosides/peptides (Mbelele *et al.,* 2018).

Moreover, RR/MDR-TB epidemic revolutionized TB treatment options and clinical decisions. Firstly, RR/MDR-TB treatment regimen requires combination of at least 5 effective second-line anti-TB drugs (Ahmad et al., 2018). The regimen comprises: (a) at least one injectable aminoglycosides like kanamycin, amikacin or a cyclic peptide like capreomycin, (b) at least one of fluoroquinolone such as levofloxacin, moxifloxacin, (c) pyrazinamide, (d) ethionamide, and (e) cycloserine (WHO, 2016b, 2019b). Secondly, RR/MDR-TB increased treatment duration from 6 months in patients with drug-susceptible TB to minimum of 20 months. Here, patients receive a daily intramuscular injection of kanamycin or capreomycin along with other drugs for 8 months of intensive phase. Thereafter, injectable aminoglycoside or capreomycin is removed and patient continue with other 4 drugs for 12 months. In 2019, the World Health Organization shortened the treatment duration from 20 months to 9 to11 months (WHO, 2019b). In this shorter regimen, also known as modified Bangladesh regimen, patients received a total of 7 anti-TB drugs including kanamycin or capreomycin injection, moxifloxacin, clofazimine, prothionamide, pyrazinamide, high dose isoniazid and ethambutol for 4 to 6 months intensive phase followed by 5 months continuation phase of injectable free containing regimen (Sotgiu *et al.,* 2016).

Unfavourably to the patients, injectable aminoglycosides and capreomycin causes nephrotoxicity and permanent ototoxicity (hearing loss) leading to cryptically treatment interruption and overall poor treatment outcomes (Javadi *et al.,* 2011; Sagwa *et al.,* 2015). For these reasons, the WHO has transitioned the treatment regimen from injectable aminoglycosides/capreomycin to all-oral bedaquiline based longer regimen or shorter under operational research (WHO, 2019b). The new proposed injectable free regimen is comprised of at least 5 anti-drugs mainly belonging to WHO group A, such as fluoroquinolones, bedaquiline and linezolid, and antibiotic in WHO group B such as clofazimine and cycloserine or terizidone (WHO, 2018a). The drugs in WHO group C such as delamanid, pyrazinamide, ethionamide, p-aminosalicylic acid and ethambutol can be used in accordance with clinical and local evidences. Success of this new regimen requires optimal diagnostic tools for monitoring

the regimen's efficacy and patient's health outcomes (Gaikwad & Gaikwad, 2018; Rockwood *et al.,* 2016).
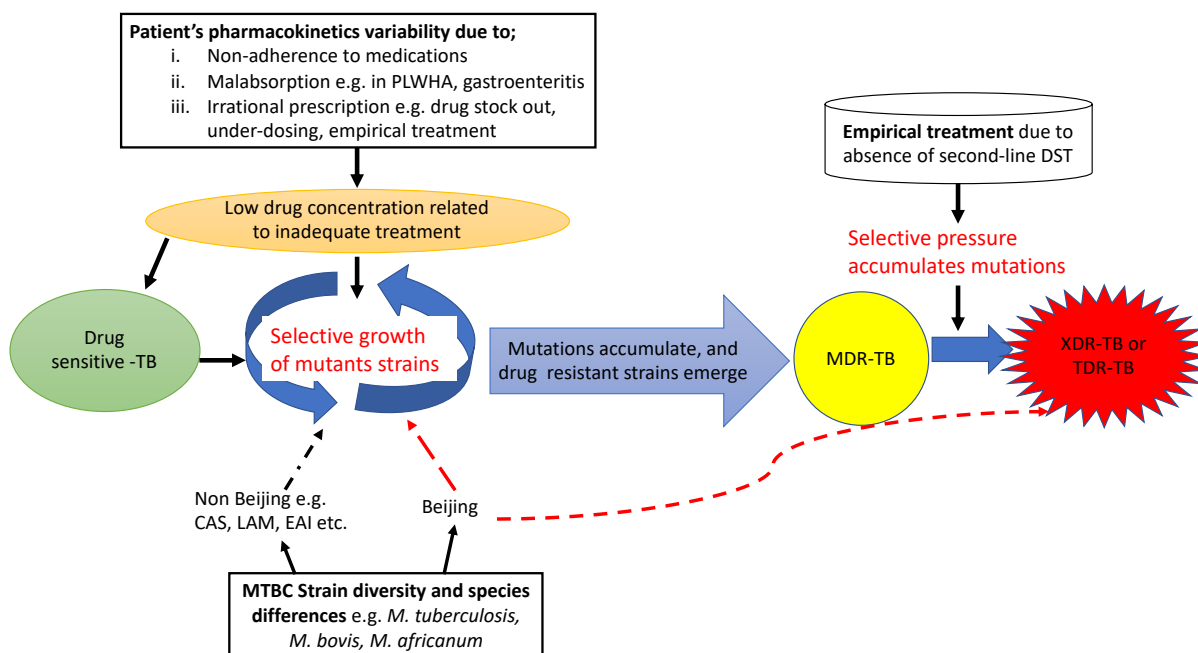
Figure 1: Evolution of drug resistance in *M. tuberculosis* complex

Beijing strains are associated with high risk of transmission and rapid acquisition of drug resistance than non-Beijing one such as Central-Asian (CAS), Latin-American-Mediterranean (LAM), East-African-Indian (EAI) lineages (Dookie *et al*., 2018; Wiens *et al*., 2018). Drug susceptibility testing (DST) is important to confirm multidrug resistant (MDR), extensive drug resistant (XDR) and some patients may be infected with isolates with total drug resistant (TDR) tuberculosis. Malabsorption is common in people living with HIV/AIDS (PLWHA).

1.2   Statement of the Problem

The RR/MDR-TB remains a disease of public health crisis (WHO, 2017). Its incidence has doubled from 250 000 in 2010 to 463 000 cases in 2019 worldwide. Southern-East Asia and sub-Sahara Africa countries bear the highest burden accounting for 171 000 and 77 000 RR/MDR-TB cases, respectively (WHO, 2020). In Tanzania, case notification has increased from 34 in 2010 to 449 in 2019 (National Tuberculosis and leprosy Programme, 2018). Globally, mortality is 39% (WHO, 2020) and it is about 20% in Tanzania (Ismail *et al.,* 2018; Leveri *et al.,* 2019). Without optimal medical interventions, incident cases will increase by 17% and deaths by 22% in 2050, whereas the MDR-TB will become the dominant form of TB (Kendall *et al.,* 2017; Mehra *et al.,* 2013).

Delayed diagnosis is among the potential RR/MDR-TB challenges. Drug resistance testing using phenotypic solid Lowenstein Jensen and liquid MGIT culture media is a gold standard (Chihota *et al.,* 2010; Lu *et al.,* 2017). However, it neither quantifies the level of resistance nor allows clinicians to adjust drug dosage. Minimum inhibitory concentration testing of a drug by microdilution methods such as MycoTB Sensitire® (MycoTB) addresses this limitation (Heysell *et al*., 2015; Mpagama *et al*., 2013). Despite that it is a gold standard, culture is usually contaminated in 15% of patients (Hoza *et al.,* 2015), and it delays results for clinical action for up to 3 months (van Zyl-Smit *et al.,* 2011). Molecular methods endorsed by the WHO such as Xpert® MTB/RIF and genotype MTBDRplus or MTBDRsl are rapid and accurate, but they only detect drug resistance in specific region of a gene. While Xpert® MTB/RIF detects mutations on *rpoB* gene which confers rifampicin resistance, the genotype MTBDRplus detects additional mutations on *katG* gene and *inhA* promoter region previously linked with isoniazid resistance, and the genotype MTBDR*sl* detects mutations on *gyrA* and *gyrB* associated with fluoroquinolones resistance as well as mutations on *rrs, eis,* and *tylA* associated with injectable aminoglycosides or cyclic peptides (e.g. capreomycin) resistance (Seifert *et al.,* 2016; Solari *et al.,* 2020). Consequently, it limits the design of optimal RR/MDR-TB treatment regimens. Whole genome sequencing (WGS) is now an alternative for predicting resistance to all anti-TB drugs including the new and repurposed drugs such as bedaquiline, delamanid, clofazimine and linezolid (Heyckendorf *et al.,* 2018; Katale *et al.,* 2020; Ruesen *et al.,* 2018).

Despite advancement in molecular TB diagnostics in the past decade since 2010, there has been no optimal treatment regimen and adequate approach for monitoring patient's response to medication. Anti-TB drugs composition of RR/MDR-TB treatment regimens are less efficacious requiring prolonged treatment duration ranging from 9 to 12 months to 20 to 24 months (Sotgiu *et al.,* 2016; WHO, 2019b). Also, injectable aminoglycosides or capreomycin causes irreversible hearing loss (ototoxicity) and nephrotoxicity (Harris *et al.,* 2016). Consequently, the WHO transitioned from injectable to all-oral bedaquline based regimens (WHO, 2019b). However, evidences about mycobactericidal efficacy of the proposed regimens to support this transition are either scarce or not available. In addition, routine monitoring of regimen's efficacy and patient's health outcome by smear microscopy & culture are not reliable. Yet, rapid DNA based molecular tests like Xpert® MTB/RIF and genotype MTBDRplus or MTBDRsl detect both viable and non-viable DNA of *M. tuberculosis* complex. In recurrent TB, DNA of dead cells may be detected beyond 5 years after successful treatment,

limiting use of DNA based assays in monitoring treatment response (Nicol, 2013; Theron *et al.,* 2016). Currently, there is a growing evidence from patents with drug susceptible TB that molecular bacterial load assay (TB-MBLA) is accurate and it detects viable *M. tuberculosis* 16S rRNA in a similar way as culture (Honeyborne *et al.,* 2011a; Sabiiti *et al.,* 2020). To uphold this evidence, the current study utilized different molecular methods in order to characterize *M. tuberculosis* complex and monitor its mycobactericidal rates of regimens among adult patients with RR/MDR-TB.

1.3   Rationale of the Study

The primary purpose of this study was to generated evidences that will not only support the design of simple and shorter treatment regimens but also optimize RR/MDR-TB treatment outcomes. These evidences include updating the local knowledge about the clinical application of molecular methods for identification of *M. tuberculosis* complex to the species level, and their lineages among patient tread for MDR-TB, the list of curated mutations for predicting the level of drug resistance in *M. tuberculosis* complex, and mycobactericidal activities of different regimens for treating RR/MDR-TB patients. Ultimately, these evidences would guide policy for adopting new diagnostic algorithms, treatment regimens as well as infection, prevention & control in Tanzania and elsewhere

In addition, this study aimed to build research capacity and clinical skills to the PhD trainee as well as participating clinical team and institution at large. This capacity is important in fostering innovations and clinical application of molecular technologies for infectious diseases such as RR/MDR-TB. This contribute toward achieving the WHO END-TB strategy 2015 to 2035 and the 2030 Agenda for Sustainable Development Goals on new innovations and technologies for ensuring human wellbeing (United Nations, 2016; WHO, 2015b),

1.4   Study Objectives

1.4.1   General Objective

To deploy molecular methods for characterizing the members of *Mycobacterium tuberculosis* complex and determining mycobactericidal effect of different treatment regimens among adult patients with pulmonary RR/MDR-TB.

1.4.2 Specific Objectives

(i)   To determine the performance of the genotype MTBC assay in diagnosing tuberculosis from direct sputa collected from patients with presumed MDR-TB, using culture and genotype MTBDRplus/sl reference methods.

(ii)   To describe the *M. tuberculosis* complex species in patients with presumed MDR-TB.

(iii)   To compare WGS resistance associated variants to minimum inhibitory concentrations of anti-TB drugs as measured by MycoTB Sensitire® Microdilution plate in *M. tuberculosis* complex among people treated for MDR-TB.

(iv)   To compare the killing rate of *M. tuberculosis* in patients receiving second line regimen for treating RR/MDR and standard regimen for drug-susceptible TB.

(v)   To review molecular methods for diagnosis and monitoring treatment response in adults with RR/MDR-TB.

1.5   Research Questions

(i)   What is the rate of *M. tuberculosis* killing from the sputum, as measured by TB molecular bacterial load assay among adult patients treated using different RR/MDR-TB regimens?

(ii)   What is the performance of genotype MTBC v1.x in detecting *M. tuberculosis* complex from direct sputum samples of adult patients with pulmonary RR/MDR-TB?

(iii)   What are the common members of *M. tuberculosis* complex species and lineages in adult patients with pulmonary RR/MDR-TB?

(iv)   What are the whole genome sequencing gene mutations for predicting level of drug resistance in adult patients with pulmonary RR/MDR-TB?

1.6   Significance of the Study

This research has added knowledge to physicians and laboratory personnel working in TB and scientific community on clinical application of molecular diagnostics for simplifying RR/MDR-TB treatment. For the first time in Africa, this study has optimized the genotype

MTBC and Mycobacterium CM 2.0 assays, and utilized them in detecting *M. tuberculosis* complex and non-tuberculous mycobacteria to the species level from direct sputum, respectively. Clinical application of genotype Mycobacterium CM 2.0 assays in detecting *M. intracellulare* infection led to treatment alteration from MDR-TB and XDR-TB to NTM-based regimen. Secondly, WGS updated the curated list of mutations for predicting level of drug resistance as measured by MycoTB sensitire® plate. This information not only guide clinician in designing optimal and simple MDR-TB treatment regimen but also allows bioinformatician to update bioinformatic tools. Thirdly, deploying TB-MBLA deciphered that combination of bedaquiline and kanamycin had superior rate of *M. tuberculosis* complex killing in patients with MDR-TB. With the current transition from injectable to bedaquiline without injectable containing regimen, this study added knowledge to clinician and scientific community about the needs for searching an alternative drug with high early mycobactericidal activity, such as high dose levofloxacin and moxifloxacin.

Importantly, the research project has enabled technology transfer to Tanzania through collaborative initiatives. As a PhD trainee, I have acquired knowledge and skills in clinical application of WGS, TB-MBLA and other TB diagnostics. Moreover, I have developed and acquired high level of competence and expertise in the analysis and application of scientific research methods to undertake high quality research in infectious diseases diagnostics, which will subsequently guide patient's clinical decisions. As it serves as a benchmark for my career development, the research work has transitioned me to a senior researcher level. Identified potential research avenue will be developed into research programs for addressing patients and public challenges in health.

1.7   Delineation of the Study

This study aimed to build research capacity and clinical skills to the PhD trainee as well as participating clinical team and institution at large. The primary purpose of this study was to generated evidences that will not only support the design of simple and shorter treatment regimens but also optimize RR/MDR-TB treatment outcomes. This thesis aimed at identifying MTBC at species level, their drug-resistance patterns, and mycobactericidal effect of different regimens in patients treated for RR/MDR-TB and drug-susceptible (DS-TB). This was a cross-sectional study nested in a longitudinal design that followed patients on RR/MDR-TB and DS-TB treatment for 4 months.

CHAPTER TWO

LITERATURE REVIEW

2.1 Conceptual Framework of the Research Project

Figure 2 illustrates key infectious diseases diagnostics and factors to be considered when designing an optimal regimen that will lead to favourable outcomes in patients treated for RR/MDR-TB. Achieving a favourable health outcome requires early detection of drug resistance in *M. tuberculosis* complex. However, detection is affected by host factors such that HIV-infected patients and those without chest cavity are usually paucibacillary and they test negative with most diagnostics. Also, the species of *M. tuberculosis* complex bears variable susceptibility to anti-TB drugs and each responds differently to anti-TB regimens. Hence, optimal diagnostics to confirm diagnosis and provide guidance for designing an optimal regimen are described here.

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┌────────────────────────────────┐   ┌────────────────────────────────┐
│ Host: Patient's characteristics,│   │ Diagnostics: Optimal diagnostic│
│   such as HIV status, initial    │   │   technologies for diagnosis and│
│   bacterial load, chest cavity and│  │   monitoring treatment response │
└────────────────────────────────┘   └────────────────────────────────┘
                    ↘                       ↙
              ┌─────────────────────────────────┐
              │   Optimal RR/MDR-TB treatment    │
              │      regimens and outcomes       │
              └─────────────────────────────────┘
                    ↗                       ↖
┌────────────────────────────────┐   ┌────────────────────────────────┐
│ Regimens: Optimal treatment     │   │  Pathogen: M. tuberculosis     │
│   regimens, either an injectable or│ │  complex to species level and  │
│   injectable free beddaquiline based│ │ lineage, and their susceptibility to│
└────────────────────────────────┘   └────────────────────────────────┘
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Figure 2: **Conceptual framework for optimizing RR/MDR-TB treatment regimens and outcomes**

2.2 Distribution of M. tuberculosis Complex Species

Epidemiological studies showed that species within the *M. tuberculosis* complex, the causative agent for drug susceptible and resistant TB, have geographical distributions (Correa-Macedo

et al., 2019; Yeboah-Manu *et al*., 2017). For instance, *M. tuberculosis* is the commonest causative agent for TB in human, accounting for 95% of TB cases worldwide (Addo *et al*., 2017). The *M. africanum* is an important cause of TB in West Africa, and rarely detected in East African countries, including Tanzania (Yeboah-Manu *et al*., 2017; Zumla *et al*., 2017). Moreover, previous comparative genomic studies for *M. tuberculosis* complex have described important variations among species in the complex with differences in host association, drug-resistance, virulence, and epitope diversity (Jia *et al.,* 2017). Unfortunately, these studies were predominantly done in countries where TB burden is low but have high capacity for genomic testing and bioinformatics analyses (Colman *et al.,* 2019). In the past 20 years, one cross-section study reported *M. bovis* as the causative pathogen in 10% of patients with drug-susceptible TB in Tanzania (Mfinanga *et al*., 2004), but there is limited or no data on the later species, particularly in patients with rifampicin and or multidrug-resistant TB (RR/MDR-TB). Certainly, the scarcity of these data in resource limited countries, such as East Africa countries like Tanzania could partially be attributed to inadequate infrastructure and laboratory capacity for detecting *M. tuberculosis* complex to the species level (Colman *et al*., 2019). Consequently, it may partly limit not only the understanding of the global distribution, and susceptibility patterns of *M. tuberculosis* complex species to anti-TB drugs but also on how they respond to therapy.

2.3   Methods for Genotyping M. tuberculosis Complex to Species Level

Detection of *M. tuberculosis* complex to the species level can be traditionally performed phenotypically using biochemical characteristics of cultured isolates (European Centre for Disease Prevention and Control., 2018; Niemann *et al.,* 2000). However, culturing *M. tuberculosis* complex is a time-consuming process, and data generated are rarely inferred to their phenotypes such as biochemical properties (Ngabonziza *et al*., 2020).

In recent years, molecular genotyping has advanced the understanding of *M. tuberculosis* complex species. For instance, an insertion sites (IS6110)-based restriction fragment- length polymorphism (RFLP) analysis is often used for genotyping of *M. tuberculosis* complex. However, RFLP requires a large quantity of genomic DNA, and it's not high throughput, yet has poor discriminatory power for species with low numbers of insertion sites such as *M. bovis* (Bauer *et al*., 1999). Spacer oligonucleotide typing, often referred as spoligotyping, is a PCR-based method which genotype *M. tuberculosis* complex by detecting presence or absence of 43

unique spacers within the genomic region of difference (Zeng *et al.*, 2016). Advantageously, spoligotyping requires smaller amounts of genomic DNA, which favours the discrimination of strains with low IS6110 copy numbers. Nonetheless, its overall discriminatory power is lower than that of RFLP. Mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR), another PCR- based assay, is an alternative to RFLP and spoligotyping (De Beer *et al.*, 2014). Principally, MIRU-VNTR amplifies multiple loci (12, 15 or 24 loci) using primers specific for each repeat. The Locus's flanking regions correspond to specific *M. tuberculosis* complex species. In recent years, targeted gene or whole genome sequencing (WGS) uncovered that MIRU-VNTR has a poor predictive value of the close genetic relatedness, and yet it varies with the lineages of *M. tuberculosis* complex strains (Wyllie *et al.*, 2018). The targeted sequencing of 23 rRNA, *gyrB* and *hsp65* genes for example, utilizes single nucleotide polymorphism (SNPs), also known as single nucleotide variants, to identify *M. tuberculosis* complex to the species level (Colman *et al.*, 2019; Shea *et al.*, 2017). In addition to high resolution and discriminatory power over IS6110-RFLP, Spoligotyping and MIRU-VNTR, WGS can predict drug resistant phenotypes (Witney *et al*., 2016). However, all genotyping methods are labour-intensive, yet expensive in resource limited countries like Tanzania, and they require cultured isolates, specialized mycobacteriology laboratory infrastructures such as laboratory biosafety level 3 containment to manipulate cultured isolates, and technical experience for bioinformatics to interpret sequence data (Colman *et al.*, 2019; Shea *et al.*, 2017). Collectively, these pre-requisites, limit their wide application in routine clinical settings.

The genotype MTBC v.1x assay (gMTBC) is a commercial line probe assay platform (Hain LifeSciences, Germany) that can discriminates *M. tuberculosis* complex to the species level from cultured isolates (Safianowska *et al.,* 2009; Somoskovi *et al.,* 2008). This gMTBC amplifies multiple species specific primers and probes for the contiguous regions of 23 rRNA, *gyrB* and RD1 genes (Safianowska *et al.*, 2009). Based on whether mutation(s) are present or absent on 23 rRNA, *gyrB* and RD1 genes, the gMTBC allows differential identification of the *M. tuberculosis* complex to *M. tuberculosis/M. canettii, M. africanum, M. microti*, *M. bovis* subsp. *bovis, M. bovis* subsp. *caprae*, and *M. bovis* BCG only (LifeSciences, 2012). Favourably, gMTBC do not requires new investment, rather it utilizes the widely available laboratory infrastructures for genotype MTBDRplus and MTBDRsl. Ten years ago, few scholars validated the gMTBC for testing cultured isolates and reported the test performance

to be 93% (Safianowska *et al*., 2009; Somoskovi *et al*., 2008). Considering the challenges of culturing *M. tuberculosis* complex (Barac *et al*., 2019), and the growing evidence from clinical trials that the genotype MTBDRplus and MTBDRsl can be used to test direct sputum samples of patients with MDR-TB (Alipanah *et al*., 2019), optimizing gMTBC protocol for testing direct sputum samples is now desirable. Contrary to culture which requires special safety containment, manipulating sputum on these molecular tests can be performed in laboratory biosafety level 2 containment within a biosafety cabinet class II, and still contamination rates are rare, mainly due to cross contamination between samples (Mbelele *et al*., 2018).

2.4 Methods for Detecting Drug Resistance in M. tuberculosis Complex

2.4.1 Phenotypic Methods

Phenotypic drug susceptibility testing (DST) to confirm drug resistance in *M. tuberculosis* complex can be routinely performed at a single concentration recommended by the WHO, historically known as critical concentration or breakpoint by proportion method (WHO, 2018b). This proportion method can either be done on Lowenstein-Jensen solid and liquid MGIT 960 culture (BD, Franklin Lakes, NJ, USA) (Chihota *et al*., 2010; Mpagama *et al*., 2012). Previous studies have underscored that testing at a single critical concentration of a drug by proportion method may either over or under estimate resistance in *M. tuberculosis* complex, and yet it does not allow dose adjustment (Ängeby *et al*., 2012). The WHO has proposed measuring the minimum inhibitory concentration (MIC) of anti-TB drugs as an alternative to proportional method. The MIC is determined when MTBC strains are tested at multiple serial concentrations of anti-TB drugs that have been previously reported and employed clinically (Schön *et al.,* 2019; WHO, 2018c). This allows the understanding of the level of drug resistance in *M. tuberculosis* complex and further drug dosage adjustment (Schön *et al*., 2019; WHO, 2018c). Previous studies have highlighted a high yield in detecting drug resistance in *M. tuberculosis* by MIC compared to proportion method (Banu *et al*., 2014; Heysell *et al*., 2015). In most cases, proportion methods is associated with high discordance between resistant and susceptible *M. tuberculosis* complex isolates in up to 15 - 30% for ethambutol on solid Loewenstein-Jensen or liquid MGIT culture media compared to 5% of ethambutol and 10% of streptomycin on MycoTB (Banu *et al*., 2014). Ultimately, this can lead to over or under estimation of the drug specific resistance in up to 49% of ethambutol, for example (Banu *et al*., 2014). Nonetheless, interpretation criteria for the quantitative range of MIC values requires

further workout to facilitate accurate interpretation of drug resistance-associated mutations detected by genotypic methods (Ismail *et al.*, 2020; Schön *et al.*, 2019).

2.4.2 Molecular Methods

In addressing challenges of phenotypic methods, such as the long turn-around time, and dependence of cultured isolates which may also be contaminated (Barac *et al.,* 2019; Reddy *et al.,* 2014), the WHO approved the Xpert® MTB/RIF and two line probe assays (LPA) including the genotype MTBDR*plus* and MTBDRsl (Hain LifeSciences, Germany) for dual detection of *M. tuberculosis* complex and drug resistance (Mbelele *et al.*, 2018).The Xpert® MTB/RIF amplifies the target 560 region of *M. tuberculosis* complex and 81-bp rifampicin resistant determining region in the *rpoB* gene, a proxy biomarker for rifampicin resistant TB (Steingart *et a*l., 2014). The sensitivity and specificity of Xpert® MTB/RIF is about 95% and 99% in detecting rifampicin resistant TB compared to phenotypic methods, respectively (Chikaonda *et al.*, 2017; Guenaoui *et al.*, 2016; Huang *et al.*, 2018; Rice *et al.*, 2017). Nonetheless, Xpert® MTB/RIF does not differentiate viable from non-viable *M. tuberculosis* complex, accounting for over 10% false positivity compared to culture (Aricha *et al.*, 2019). This is more prominent in patients with a chest radiograph that is inconsistent with TB and those with paucibacillary (Theron *et al.*, 2016, 2018). Xpert® MTB/RIF Ultra (Ultra) assay (Cepheid, Sunnyvale, California, USA) is more sensitive and has few false positivity/negativity than conventional Xpert® MTB/RIF (Chakravorty *et al.*, 2017; Dorman *et al.*, 2018). However, Ultra is not widely implemented in resource-constrained countries including Tanzania.

The LPA yields additional susceptibility information than Xpert® MTB/RIF, but they require more steps, infrastructure and repeats due to invalid results. The genotype MTBDR*plu*s dually detects *M. tuberculosis* complex and mutations on *rpoB* and both *katG & inhA* genes previously known to confer resistant to rifampicin and isoniazid, respectively (World Health Organization, 2008). Compared to phenotypic methods, the genotype MTBDR*plus* has sensitivity, specificity and accuracy of 84%, 98%, and 83% respectively (Abanda *et al.*, 2017; Karimi *et al.*, 2018). On the other hand, the genotype MTBDRsl detects mutations on the *rrs* gen*e & eis* promotor regions that predict phenotypic resistance to injectable aminoglycosides/capreomycin, and on *gyrA & gyrB* genes which confer fluoroquinolones resistance (Gardee *et al.*, 2017; Tagliani *et al.*, 2015; Yadav *et al.*, 2018). However, the genotype MTBDRplus and MTBDRsl are less sensitive in samples with paucibacillary state, such as those collected from people living with

HIV/AIDS, who usually do not form a high bacterial burden containing chest cavity (Park *et al*., 2019). Generally, LPA and Xpert® MTB/RIF interrogate specific resistance associated variants in high fidelity regions of genes (Seifert *et al*., 2016). Consequently, discordances between phenotypic and molecular methods in detecting drug resistance in *M. tuberculosis* complex are frequently reported (Mwanza *et al*., 2018; Variava *et al*., 2020).

Importantly, Whole genome sequencing (WGS) interrogate and can detects most putative resistance associated mutations across the entire *M. tuberculosis* genome (Colman *et al*., 2019; Mokaddas *et al*., 2015). Unlike Xpert® MTB/RIF and LPA, WGS interrogates all resistance associated mutations on genes linked with potential anti-TB drugs used to treat RR/MDR-TB. Examples of these drug and specific target genes include bedaquiline (*Rv0678*, *atpE*, and *pepQ*), delamanid (*ddn, fgd1, fbiA*, and *fbiC),* linezolid (*rrl and rplC*), or clofazimine (*Rv0678, Rv1979c, pepQ*) and cycloserine (alr, ddl, cyA) (Coll *et al*., 2018; Dookie *et al*., 2018; Ramirez *et al*., 2020). Depending on anti-TB drugs, the concordance, sensitivity, and specificity of WGS in detecting phenotypic resistance range from 83-99%, 83-100% and 78-99%, respectively, compared to proportion method in solid Lowenstein Jensen and liquid MGIT 960 culture systems (Chatterjee *et al*., 2017; Quan *et al*., 2017; Shea *et al*., 2017; Walker *et al*., 2015). Despite WGS advantages over other molecular methods, much work remains in determining putative resistance associated with mutations in drugs like ethambutol, and whether certain mutations confer lower or higher levels of resistant phenotypes as determined against the MIC of the isolates (Heyckendorf *et al*., 2018; Ismail *et al*., 2020; Ruesen *et al*., 2018).

2.5   Methods for Monitoring RR/MDR-TB Treatment Response

2.5.1   Phenotypic Methods

The TB treatment response measurements in endemic settings largely depend on detecting acid-fast bacilli from sputum samples by smear microscopy (Mitnick *et al*., 2016). However, the smear microscopy detection threshold is at least 10^3 *M. tuberculosis* colony-forming-units in 1 mL (CFU/mL) of sputum sample (Magalhães *et al.,* 2018). Many patients with TB/HIV co-infection have paucibacillary disease and may not produce quality sputa to detect acid-fast-bacilli by smear microscopy (Das *et al.,* 2019; Park *et al*., 2019). Besides, smear microscopy doesn't distinguish viable from non-viable *M. tuberculosis,* and therefore does not inform the response to treatment for patients with RR/MDR-TB (Das *et al.,* 2019). Culturing *M. tuberculosis* in either the Lowenstein-Jensen solid medium or the MGIT liquid culture system

is the gold standard for monitoring patients treated for RR/MDR-TB. It is sensitive with a detection limit of $10 - 100$ CFU/mL of sputum in Lowenstein-Jensen solid and ≤ 10 CFU/mL in MGIT liquid culture media (van Zyl-Smit *et al.,* 2011). Nonetheless, culture can miss the non-culturable strains, thereby limiting the ability to take appropriate and timely clinical actions (Barac *et al.*, 2019; Reddy *et al.*, 2014).

2.5.2 Molecular Methods

The Xpert® MTB/RIF and LPA like genotype MTBDRplus and MTBDRsl are rapid and accurate, but they detect the DNA of *M. tuberculosis* complex (Boehme *et al.*, 2011; World Health Organization, 2016a). Nevertheless, the DNA is a stable molecule that persists after cell death and may be detected by Xpert® MTB/RIF and LPA for up to 5 years after successful TB treatment (Nicol, 2013; Theron *et al.*, 2016). Previous attempts to distinguish dead and live DNA of *M. tuberculosis* complex by Xpert® MTB/RIF assay included pre-treating the sputum samples with propidium monoazide (Biotium Inc., Hayward, California, USA), a chemical compound which selectively intercalates the dead DNA and therefore inhibits its amplification and detection (Kayigire *et al.*, 2016; Nikolayevskyy *et al.*, 2015). This approach achieved low specificity at 53 to 80% in detecting viable *M. tuberculosis* complex compared to mycobacterial culture, and therefore limiting its clinical application in monitoring TB treatment response (Kayigire *et al.*, 2016; Nikolayevskyy *et al.*, 2015).

Because of its shorter half-life compared to DNA, ribonucleic acid (RNA) families was previously reported as a surrogate biomarker for microbial viability (Cangelosi & Meschke, 2014; Desjardin *et al.*, 1999; Li *et al.*, 2017). The RNA families, such as 16S rRNA of *M. tuberculosis,* have previously been quantified in sputum using tuberculosis molecular bacterial load assay (TB-MBLA), a RT-qPCR (Honeyborne *et al.*, 2011a; Sabiiti *et al.*, 2020). Using TB-MBLA, bacterial decline rate over time was found to concur with that in phenotypic culture methods (Honeyborne *et al.*, 2011a; Mtafya *et al.*, 2019; Sabiiti *et al.*, 2020). Moreover, TB-MBLA has been used to guide clinical decision in a patient who was failing a treatment regimen for drug-susceptible TB (Evangelopoulos *et al.*, 2017). However, evidence to support its use in monitoring patients treated for RR/MDR-TB is missing or scarce. Patients with RR/MDR-TB represents a diverse population compared to those of drug-susceptible TB, and therefore it limits the generalizability of TB-MBLA findings from the former population.

Recently, the WHO has proposed a transition from injectable to the all-oral-based bedaquiline RR/MDR-TB treatment regimen (World Health Organization, 2018a). To align with this transition, countries where TB is endemic, including Tanzania, have adopted new and repurposed TB medicines, such as bedaquiline, delamanid and linezolid, and constructed regimens with limited microbiological evidence of effectiveness in patients with RR/MDR-TB. For this limitation, testing how the killing of *M. tuberculosis* complex measured from the sputa by TB-MBLA correlates with time-to-culture conversion among patients with RR/MDR-TB receiving variable antibiotic regimens was desirable to inform clinical decision

CHAPTER THREE

MATERIALS AND METHODS

3.1   Study Settings

This study was conducted from September 2018 through March 2019. Patients were recruited at Kibong'oto Infectious Diseases Hospital (KIDH), a national centre of excellence for clinical management of drug resistant TB, located in the Siha district of the Kilimanjaro region in Tanzania. The KIDH is a public hospital with 320 bed capacity. Each year, the hospital provides services to over 200 and 600 patients with drug-resistant and susceptible TB, respectively (Mbelele *et al.,* 2017). Patients are usually referred to KIDH if they have: (a) multidrug-resistant TB with or without comorbidities like diabetes, (b) confirmed pre/XDR-TB, and (c) if are living far from ambulatory treatment centres. The hospital has laboratory capacity for testing all forms of TB using smear microscopy, Lowenstein-Jensen (LJ) solid culture media, Xpert® MTB/RIF and line probe assays including genotype MTBDRplus, MTBDRsl and genotype MTBC (Mbelele *et al*., 2018). At the time of writing this thesis, KIDH had functional infrastructure for MGIT liquid culture, and a 90% completed construction of public health laboratory with biosafety level 3 containment. Minimum inhibitory concentration testing and DNA extraction for use in whole genome sequencing (WGS) were performed at the Kilimanjaro Clinical research Institute (KCRI) biotechnology located in Moshi, Kilimanjaro Region in Tanzania. The WGS and bioinformatics analysis was performed at the Research Centre Borstel, in Germany. Tuberculosis molecular bacterial load assay was performed at the National Institute for Medical Research, Mbeya Medical Research Centre branch, Tanzania, given the laboratory's prior experience with the assay (Mbelele *et al.,* 2021; Mtafya *et al.,* 2019).

3.2   Study designs

This thesis utilized three observational study designs to address the study objectives. These study designs included:

3.2.1   Cross-sectional study design

A cross-sectional design was used to identify Mycobacteria species from direct sputa of patients with drug-susceptible TB and presumptive MDR-TB (objective #1 &2). A case of

unrecognized *M. intracellulare* in a patient living with HIV/AIDS, who had repeatedly been treated for drug-susceptible- and -MDR-TB was observed and reported in order to highlight the best clinical practice and guide integration of non-tuberculous mycobacteria screening services into programmatic TB testing algorithms (objective #1). The design was also used to predict and compare whole genome-sequencing mutations associated with resistance (SNPS and INDELs) in *M. tuberculosis* with MIC of anti-TB drugs (objective #3)

3.2.2 Longitudinal cohort study with treatment regimens

A longitudinal cohort study was conducted in which patients were followed for 16 weeks of receiving different anti-TB regimens in order to determine the *M. tuberculosis* killing kinetics [objective #4]. The treatment regimens for patients with RR/MDR-TB were as follows: (a) an injectable without bedaquiline-containing regimen composed of a daily dosed kanamycin injection (15 mg/kg), levofloxacin (at a dose of 750 mg for patients weighing < 50 kg and 1000 mg for those weighing ≥ 50 kg), pyrazinamide (25 mg/kg), ethionamide (750 mg), and cycloserine (750 mg); (b) an all-oral-based regimen composed of bedaquiline (400 mg daily for 2 weeks, and then 200 mg thrice per week), linezolid (600 mg/day), levofloxacin, pyrazinamide, and ethionamide; (c) an injectable with bedaquiline-containing regimen composed of kanamycin injection, bedaquiline, levofloxacin, pyrazinamide, and ethionamide. On the other hands, patients with drug susceptible TB received a standard fixed-dose combination containing rifampicin (150 mg), isoniazid (75 mg), pyrazinamide (400 mg), and ethambutol (275 mg), termed RHZE. Patients weighing < 50 kg received three tablets of RHZE, and those weighing ≥ 50 kg received four tablets of RHZE per day

3.2.3 Systematic meta-narrative review

A meta-narrative systematic review was conducted in order to update and appraise the literature on the currently available molecular methods for diagnosis and monitoring of MDR-TB treatment in TB-endemic settings (Objective #5). The review was conducted in accordance to the RAMESES recommendations (Mbelele *et al*., 2018; Wong *et al*., 2013). First, a protocol containing a set of eligibility criteria was developed and approved by the thesis supervisors and mentors. Articles were included in the review if they met the following criteria: (a) being an original article published in the English language from January 2013 to June 2018, (b) a cross-sectional or cohort studies reporting performance characteristics (sensitivity, specificity, and accuracy or concordance, as well as laboratory infrastructure required) of the molecular method

for either diagnosis or monitoring of RR/MDR-TB treatment in adult participants aged ≥18 years with presumptive pulmonary TB. On the other hands, case reports, reviews, commentary, short communication and original articles reporting molecular epidemiology, drug resistance profile in M. tuberculosis were excluded from the review. Moreover, an article describing an outmoded version of the molecular method was also excluded. Also, articles reporting immunological or host biomarkers either for diagnosis of or monitoring MDR-TB therapy were excluded.

Second, relevant articles published in English language from January 2013 to June 2018 were searched on electronic databases including the Medline/PubMed and Google Scholar, and through scanning of references. Key search terms for relevant articles included: (molecular OR genotype* OR "polymerase chain reaction" OR "PCR") AND ("drug resistant* tuberculosis") AND diagnosis AND ("multidrug resistant* tuberculosis") AND monitor* AND ("tuberculosis treatment response" OR "anti-tuberculosis therapy").

Third, the title and abstracts of the articles retrieved form databases were screened for by two independent reviewers (Peter Mbelele and Sagal Mohamed) in accordance to eligibility criteria. An article was read in full if the abstract mentioned, in some capacity, the performance of the molecular method for either diagnosis or monitoring of MDR-TB treatment. Duplicates were removed. A final consensus was discussed between the two reviewers. An opinion from a third reviewer (Stellah Mpagama) was sought in case of any disagreement between the two. Ultimately, eligible articles were archived using Mendeley-reference Management Software (www.mendeley.com).

Data were extracted, analysed and synthesized to features different themes including the principle of the test, technical performance (accuracy), advantages and limitations based on simplicity, turnaround time, laboratory infrastructure, and logistics required. The details of the protocol and findings are presented in a published article in a peer reviewed, International Journal of Mycobacteriology (Mbelele *et al*., 2018) which is also attached in this thesis

3.3   Sample size estimation

The sample size required to test whether or not the *M. tuberculosis* complex can be detected by the genotype MTBC from direct patients' sputa was estimated based on the 10th rule for binary logistic regression model as previously decribed (van Smeden *et al*., 2019). For this rule, an

estimated sample size (N) is a function of a disease prevalence (P) and the number of factors predicting the detection of *M. tuberculosis* complex. In summary, five predictors of detecting *M. tuberculosis* complex such as: (a) bacterial load, as measured by Xpert® MTB/RIF, (b) chest cavity, (c) HIV status, (d) repeated TB treatments, and (e) smoking habit, were considered (Park *et al*., 2019; Theron *et al*., 2016). Using a TB prevalence of 43% as reported in patients suspected of having pulmonary TB reported from the same setting (Mbelele *et al*., 2017), a minimum sample size of 116 patients were estimated from a formula

$$N = 10 \text{ x } \frac{\text{Number of predictors}}{\text{Prevalence (P) of the disease}}$$

$$N = 10 \text{ x } \frac{5}{0.43} = 116$$

Ultimately, 126 patients were recruited. Of these patients, 86 had pretreatment sputum culture positive. Because of budget limit, Whole genome sequence was performed in only 50 *M. tuberculosis* isolates, which were randomly selected from 86 positive cultures. The random numbers for all 86 isolates were generated in a computer using R programming language (http://www.R-project.org).

On the other hand, the number of patients needed for the longitudinal cohort study design to determine the differences in mycobactericidal activity of different treatment regimens over time, were calculated as previously (Guo *et al*., 2013). A Spearman's rank correlation of 0.51, and a baseline *M. tuberculosis* burden of 5.5 log10 eCFU/mL, as well as daily *M. tuberculosis* decline and a decay rate of 0.42 and 0.05 log10 eCFU/mL, respectively were used to determine the number of patients required (Honeyborne *et al*., 2011a; Sabiiti *et al*., 2020). Hence, at least 7 patients were needed per regimen to reach a statistical power of 80% with a two-sided type I error of 5%. For the past 10 years in Tanzania, at least 75% of patients with MDR-TB complete the treatment regimens successfully (Leveri *et al*., 2019). Therefore, adjusting for at least 25% of patients who were likely to have unsuscceful treatment due to lost to follow-up, not evaluated due to negative microbiological results at baseline, and/or died, a minimum of 37 patients were needed. Overall, 59 patients were enrolled in a longitudinal study design, of whom only 37 had complete TB-MBLA and a positive culture results for analysis.

3.4   Study population and eligibility criteria

3.4.1   Study population

Patients with pulmonary TB, including those with presumed MDR-TB, participated in this study. Presumptive patients were those who presented with symptoms or signs and risk factors suggestive of MDR-TB. These patients included those with prior history of treatment for drug-sensitive TB, and people living with HIV/AIDS (World Health Organization, 2014). Patients with rifampicin resistance in *M. tuberculosis* complex confirmed by Xpert® MTB/RIF assay were also considered to be presumptive MDR-TB. All patients were conveniently recruited, and were included in the study if they met the following eligibility criteria:

3.4.2   Eligibility Criteria

(i)   Inclusion criteria

(a)   Adult patients aged ≥18years

(b)   Patients had prior history of TB treatment with confirmed rifampicin susceptibility or resistance in *M. tuberculosis* complex by the Xpert® MTB/RIF assay

(c)   Patients who consented to stay at KIDH for at least 4 months of treatment. This was an eligible for patients participating in longitudinal cohort design

(d)   Patients who consented to participate in the study and signed a witnessed oral or written informed consent form.

(e)   Ability to provide two quality early morning sputa for laboratory testing. Quality sputum was defined by an adequate volume of > 5 mL and absence of food particles in the sputum.

(ii)   Exclusion criteria

(a)   Critically ill or moribund patients. The critically ill or moribund patients were previously defined as patients who had a severe form of illness requiring either respiratory or cardiovascular support, including those in comatose condition (Robertson

& Al-Haddad, 2013). These were excluded because they would have not been able to provide quality sputa for laboratory testing.

(b)   Pregnant and breastfeeding women. These were excluded because most anti-TB treatment regimen have not been well studied in this population

3.5   Mycobacteriology Laboratory procedures

3.5.1   Sputum sample collection

Patients provided 5 mL of quality early morning sputum for testing at day 0 (baseline) and at days 3, 7, 14, 28, 56, 84, and 112 of anti-TB treatment. They were instructed to rinse their mouth with water, take a deep breath and expectorated sputum into calibrated wide-mouthed-screw capped container. Each sputum container was uniquely labelled and capped before submitting it for laboratory testing. Saliva, nasal secretions and food particle containing sputa were excluded. No sputum induction was performed to patients who were unable to expectorate quality sputum.

3.5.2   Sputum processing, storage and transport

Patients' sputa were transported in a cool box at $2 - 8\,°C$ to the laboratory within 30 minutes. on arrival, the sputum was homogenized at room temperature for 30 minutes using a sterile magnetic stirrer. Then, 1mL of homogenized sputum was treated using 4 mLs of 4 M guanidine thiocyanate (GTC) containing 1 M Tris–HCl (pH 7.5) and 1% (Vol/Vol) of β-mercapto-ethanol, and was frozen at $-80°C$ in order to preserve the *M. tuberculosis* RNA from degrading. The Sputum in GTC was shipped at $-80°C$ to NIMR Mbeya for RNA extraction and testing by TB-MBLA. The remaining 4 mLs of sputum sample was decontaminated and sedimented using N-Acetyl-L-Cysteine - Sodium Hydroxide (NALC-NaOH). Briefly, 4 mls of 1% NALC-NaOH were added to 4 mLs of homogenized sputa. The mixture was vortexed and left to stand at room temperature for 15 minutes. The tubes containing 4 mLs of sputa and 4mLs of 1% NALC-NaOH were inverted so that all contents are exposed to NaOH-NALC solution. Modified Petroff's method, which utilizes 4% of NaOH only, was used to decontaminate blood containing sputum (Mbelele *et al*., 2017; Tripathi *et al*., 2014). Tubes were left at room temperature for 20 minutes before Phosphate buffer solution (PBS, pH 6.8) was added up to the 50 mL mark of a falcon tuber. The *M. tuberculosis* complex in sputum was concentrated

by centrifugation at 3000 g for 15 minutes at 4°C. Supernatants were discarded into a container with 25% phenol. Sediments were suspended in 1.5 mLs of PBS and tested for *M. tuberculosis* complex using Light-emitting diode fluorescence microscopy, Loewenstein-Jensen culture and line probe assays.

3.5.3 Detecting acid-fast bacilli on smear microscopy

Sputum sediments were examined for acid-fast bacilli on Light-emitting diode fluorescence microscopy in accordance with the standard operating procedure at KIDH and elsewhere (Thapa *et al.,* 2015). In brief, a slide was smeared with sediments, dried and heat-fixed and flooded with 0.1 % auramine phenol for 7 to10 minutes. The stained slide was left at room temperature for 20 minutes, and was gently rinsed under running tap water. The stained slide was decolorized using 3 % acid alcohol for 2 minutes and rinsed with running tap water. The decolorized slide was counterstained with 0.1 % potassium permanganate solution for 45 minutes. Thereafter, the counterstained slide was rinsed under running tap water and air-dried in the dark. The dried slides were examined for acid fast bacilli under Light-emitting diode fluorescence microscopy. According to the IUTLD/WHO and KIDH grading system, smear results were either reported as negative, if no acid-fast-bacilli, or positive, if at least scanty (1 – 19 per 100 field) bacilli were seen (Thapa *et al*., 2015).

3.5.4 Culturing M. tuberculosis complex on Lowenstein-Jensen media

Mycobacteria species were cultured from the remaining sputum samples, collected at six time-points on days 0, 14, 28, 56, 84, and 112 of treatment, on Lowenstein-Jensen (LJ) slants in accordance with the Clinical and Laboratory Standard Institute and others (CLSI, 2008; Tripathi *et al*., 2014). In brief, 200 μL of sputum sediments were inoculated into two LJ slants containing medium of either glycerol to support the growth of *M. tuberculosis* or pyruvate, which favours the growth of *M. bovis* and other *Mycobacterium* species (Faburay *et al*., 2016). While a standard laboratory strain, *M. tuberculosis* H37Rv, was used as positive control, an uninoculated LJ medium was used as negative control. Inoculated LJ slants were incubated at 37 °C, and were read on a weekly basis to detect mycobacterial growth for up to 8 weeks. The incubated LJ slants were deemed negative if there was no growth at week 8 of incubation. The sputum sediments remaining after tests were stored in cryo-vials at 2 to 8°C until a valid test was available. Sediments were re-cultured if both LJ slants were contaminated. The *M. tuberculosis* complex colonies were identified and reported according to local existing and

CLSI standard operating procedures. Any growth on LJ slants was confirmed as TB if smear microscopy and *M. tuberculosis* complex MPT64 antigen of cultured isolate was positive (Arora *et al.*, 2015; Thapa *et al.*, 2015).

3.5.5 Minimum Inhibitory Concentration testing by Sensititre® MycoTB plate

The Minimum Inhibitory Concentration (MIC) of anti-TB drugs for cultured MTBC isolates was measured by the MycoTB assay as described previously (Heysell *et al.,* 2015; Lee *et al.,* 2014; Mpagama *et al.,* 2013). The MycoTB was customized by Trek to be able to test 13 different first and second-line anti-TB drugs per plate and sample: rifampicin, isoniazid, ethambutol, levofloxacin, moxifloxacin, kanamycin, amikacin, streptomycin, capreomycin, clofazimine, cycloserine, ethionamide, and p-aminosalicylic acid. Individual drug concentrations tested per drug are shown in Table 1. In brief, suspensions of cultured isolates and the laboratory reference strain *M. tuberculosis* H37Rv (ATCC 27294) were prepared and adjusted to 0.5 McFarland standard turbidity. A total of 100µL of suspension was inoculated into each well of the MycoTB plate, and was incubated aerobically at 37°C for up to 21 days. Unless it was contaminated in the first run, an isolate was tested only once. The MIC value was visually recorded by two independent readers at day 10 and/or day 21. A third opinion was sought if the MIC values reported by the two independent readers were different. The tentative epidemiological cut-off (ECOFF) values published by Ismail *et al.* (2020) on MycoTB plate were used to categorize an isolate as susceptible to a drug, if its MIC value was at, or lower than the ECOFF, and resistant if it was above this ECOFF (Ismail *et al.,* 2020). For cycloserine, the published breakpoint derived from datasets, including a similar Tanzanian study population from the same study location, was used (Deshpande *et al.,* 2018; Yu *et al.,* 2018).

Table 1: Drug concentration tested and epidemiological cut-off values in MycoTB

Anti-TB Drugs	Concentrations (µg/mL)	Tentative ECOFF (µg/mL)
Isoniazid	0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0	0.25
Rifampicin	0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0	0.5 & 0.125
Rifabutin	0.125, 0.25, 0.5 and 1.0	0.125
Ethambutol	1.0, 2.0, 4.0, 8.0, 16.0 and 32.0	2.0 & 4.0
Streptomycin	0.25, 0.5, 1.0, 2.0, 4.0 and 8.0	2.0
Amikacin	0.0625, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0	2.0
Kanamycin	0.3, 0.6, 1.2, 2.5, 5.0, 10.0 and 20.0	2.5 & 5.0
Capreomycin	0.3, 0.6, 1.2, 2.5, 5.0, 10.0 and 20.0	2.5 & 5.0
Levofloxacin	0.125, 0.25, 1.0, 2.0, 4.0 and 8.0	1.0
Moxifloxacin	0.125, 0.25, 1.0, 2.0, 4.0 and 8.0	0.5
Clofazimine	0.06, 0.12, 0.25, 0.5, 1.0, 2.0 and 4.0	0.25 &1.0
Ethionamide	0.6, 1.2, 2.5, 5.0, 10.0 and 20.0	5.0
Cycloserine	4.0, 8.0, 16.0, 32.0, 64.0 and 128.0	64.0
p-aminosalicylic acid	0.25, 0.5, 1.0, 2.0, 4.0 and 8.0	4.0

Each well of Sensitire® MycoTB plate was coated with a defined concentration per drug, at which an isolate was tested to determine the minimum inhibitory concentration (MIC). The tentative epidemiological cut-off (ECOFF) values published by Ismail et al (2020) on MycoTB plate were used to categorize an isolate as susceptible to a drug, if its MIC value was at, or lower than the ECOFF, and resistant if it was above this ECOFF (Ismail *et al.,* 2020)

3.5.6 Xpert MTB/RIF Assay

The Xpert® MTB/RIF was used to screen eligibility of study participants in which patients' sputum samples were tested as previously (Mbelele *et al.,* 2017). In summary, the sputum was homogenized using sample reagent (SR) at a ratio of 1:2, and was incubated at room temperature for 15 minutes to reduce *M. tuberculosis* complex viability. In total, 2 mL of homogenized sputum were transferred into the cartridge and loaded into the Xpert® MTB/RIF module to continue with automatic DNA extraction, amplification and detection of *M. tuberculosis* complex and rifampicin susceptibility according to the manufacturer instructions. Depending on the Xpert® MTB/RIF quantification cycle (Cq) value attained at detection, the

M. tuberculosis complex density was semi-quantified into very low, low, medium and high at Cq values of >28, 23–28, 16–22 and <16 cycles, respectively (Ssengooba *et al*., 2016). The test was negative if none or only one probe was amplified and detected.

3.5.7   Line probe assays

The Line Probe Assays (LPA) procedures included DNA extraction, master mix preparation, polymerase chain reaction (PCR) and reverse hybridization. All assays (genotype MTBC; genotype Mycobacterium CM; genoyype MTBDRplus, and MTBDRsl) were performed according to the manufacturer's instructions. In each run, the *M. tuberculosis* H37Rv and non-tuberculous mycobacteria (*M. intracelulare, M. kansasii*) reference strains were used as positive quality control markers for *M. tuberculosis* complex and non-tuberculous mycobacteria, respectively. Nuclease free water was used as a negative control.

(i)   DNA extraction

The DNA was extracted from 500μL of sputum sediments using the GenoLyse® kit as instructed by the manufacturer. The DNA extracted was stored at -20℃ until amplification, hybridization and detection by an appropriate LPA. In each run, the *M. tuberculosis* H37Rv and non-tuberculous mycobacteria reference strains were spiked into sputum from a healthy individual, and were extracted in the same way as the patient's samples.

(ii)   Master mix preparation

In each of 5 μL of extracted DNA, a master mix contained 10μL of amplification, Mix A (AM-A), composed of 5 μL buffer, 2 μL MgCl2, 3 μL of molecular grade water, and 0.2 μL Taq DNA polymerase, and 35 μL of amplification, and a Mix B (AM-B) made up of nucleotides, biotinylated primers and dye master mix (MMX) specific for genotype MTBC (gMTBC), genotype Mycobacterium CM v2.0 (gCM), genotype MTBDRplus and MTBDRsl kits.

(iii)   Polymerase chain reaction

Except for gMTBC and gCM assays, a 5 μL of extracted DNA and 45 μL MMX were multiplex amplified with biotinylated primers on TC 4000 thermal cycler as per prior studies and the manufacturer's instructions of the genotype MTBDRplus version 2.0 and MDBDRsl version 2.0 kits (Addo *et al*., 2017; Liu *et al*., 2017). The gMTBC and gCM were previously validated

by the manufacturer and others for testing clinical isolates, and therefore some procedures were modified to support testing direct sputa (Somoskovi *et al.*, 2008). For gMTBC, the cycling conditions were modified. Briefly, one cycle at 95 °C for 15 minutes, followed by 20 cycles at 95 °C for 30 seconds and at 58 °C for 2 minutes in the first stage. In the second stage, this was followed by 30 cycles at 95 °C for 25 seconds, at 53 °C for 40 seconds and at 70 °C for 40 seconds before a single extensions cycle at 70 °C for 8 min. The M. *tuberculosis* H37RV DNA reference strain and sterile molecular grade water were run together with DNA extractants, as a positive and negative control for *M. tuberculosis* complex, respectively. As instructed by the manufacturer and recently reported by Ahmed *et al.*, 2020 , a gCM was used to test direct sputa samples and cultured isolates which tested negative for *M. tuberculosis* complex by the MTBDRplus/sl and gMTBC (Ahmed, 2020). In brief, 1 cycle at 95 °C for 15 minutes, followed by 20 cycles at 95 °C for 30 seconds and at 65 °C for 2 minutes in the first stage. In the second stage, this was followed by 30 cycles at 95 °C for 25 seconds, at 50 °C for 40 seconds and at 70 °C for 40 seconds before a single extensions cycle at 70 °C for 8 min.

(iv)   Reverse hybridization and result interpretations

Amplicons were finally held at 4°C until the DNA strip-based hybridization, and downstream detection steps on twin-Incubator. The results were interpreted according to the manufacturer instructions and a previous publication (Addo *et al.*, 2017). Visualization and reading of bands on the DNA strips were done by naked eye, and interpretation of results was performed by aligning corresponding strips of an appropriate LPA kit (gMTBC, gCM, MTBDRplus and MTBDRsl) results interpretation chart (Hain LifeScience, Germany). A valid LPA result was defined by the presence of a test specific *M. tuberculosis* complex, conjugate controls (CC) and amplification control (AC) bands in conjunction with the target gene locus control for *M. tuberculosis* complex. A test was positive for *M. tuberculosis* complex and non-tuberculous mycobacteria or its member species if a corresponding band on the interpretation chart was formed and was negative for, if no band was formed. If LPA was negative but positive on Xpert® MTB/RIF for *M. tuberculosis* complex, the Xpert® MTB/RIF was re-tested using sputum sediments as previously described (Mbelele *et al.*, 2017). The LPA was declared negative if a repeat Xpert® MTB/RIF was also negative.

3.5.8   Whole genome sequencing and bioinformatic analysis

(i)   DNA extraction

The MTBC isolates stored in trypticase soy broth supplemented with 10% glycerol were sub-cultured on LJ medium. The DNA was extracted from positive LJ slants using the cetyltrimethylammonium bromide protocol described previously (Somerville *et al.,* 2005; Van Soolingen *et al.,* 1991), and all procedures were performed at KCRI biotechnology laboratory. Briefly, 2 loops of bacteria cells were heat-killed and lysed using 50 µL of 10 mg/mL lysozyme and 75 µL of 10% sodium dodecyl sulfate/proteinase K mixture (Promega Inc.). Then, 750 µL chloroform/isoamyl alcohol mix (24:1) were added to separate the aqueous DNA-containing layer. The genomic DNA (gDNA) was precipitated and washed using 5 M sodium chloride and 70% ethanol. The gDNA was dried and resuspended in 80 µL of 10X TE (100 mL Tris/HCl, pH 8.0 and 10 mL EDTA mixture) buffer and was frozen at -20^0C before shipment to the Research Center Borstel in Germany for WGS and genomic analysis.

(ii)   Whole genome sequencing

Libraries for next generation sequencing (NGS) were prepared from gDNA using a modified Nextera protocol (Baym *et al.,* 2015) and were sequenced with 2 x 150 bp paired-end reads on an Illumina NextSeq 500 platform as instructed by the manufacturer (Illumina, San Diego, CA).

(iii)   Bioinformatic analysis for predicting drug resistance phenotypes

The FASTQ files (raw sequencing data) were analysed with MTBseq v1.0.3, a semi-automated bioinformatics pipeline for the analysis of MTBC isolates (Kohl *et al.,* 2018). Briefly, reads were mapped to the *M. tuberculosis* H37Rv reference genome (GenBank ID: NC_000962.3), and alignments were refined with regard to base quality re-calibration and alignment corrections for possible PCR artefacts. WGS datasets with an average read coverage of ≤ 50 fold and coverage breadth of $\leq 95\%$ as well as samples contaminated with other bacteria as detected by Kraken 2 were excluded (Wood *et al.,* 2019). Variants were called by changing the default variant detection parameters to read coverage of a minimum of two for each forward and reverse orientation, two reads of a phred score of at least 20 and 5% allele frequency. The MTBseq was run with the additional parameter –lowfreq_vars to allow the detection of low-frequency variants.

Mutations such as short insertions/deletions (INDELS) and single nucleotide polymorphisms (SNPs) from a curated mutation catalogue employed at the Research Center Borstel (2020-05-10) were considered as resistance determinant (Grobbel *et al.,* 2021). Furthermore, uncharacterized amino acid changes in pncA (pyrazynamide) as well as unknown INDELS and stop codons in the following genes were also considered as resistance determinants: (a) ethA & ethR (ethionamide/prothionamide), (b) pncA (pyrazinamide), (c) rpoB (rifampicin, rifabutin), (d) Rv0678c, mmpL5 (bedaquiline, clofazimine), (e) ald , cycA, pykA & PPE 22 (cycloserine), (f) katG (isoniazid), (g) gid & rpsL (streptomycin), and (h) fbiC & ddn (delamanid) (Grobbel *et al.,* 2021). Genotypic resistance was inferred on the basis of mutations listed in the WHO guide (World Health Organization, n.d.), the CRyPTIC mutation catalogue (Allix-Béguec *et al.,* 2018), and an interpretation catalogue for pncA gene mutations (Yadon *et al.,* 2017). Strains harbouring mutations clearly linked to phenotypic drug-resistance as well as strains following the above-mentioned exception rules were reported as resistant. Other uncharacterized mutations were considered as unknown. The interpretation of SNPS and INDELS were performed without prior knowledge of the MIC results.

3.5.9 Tuberculosis Molecular bacterial load assay

The *M. tuberculosis* quantification by tuberculosis molecular load assay (TB-MBLA) was performed as described by Gillespie *et al.* (2017). The TB-MBLA steps included preservation of *M. tuberculosis* RNA in GTC at -80^{0}C, RNA extraction and DNA removal, RT-qPCR and translating RNA to estimated colony forming unit in 1 mL (eCFU/ml), corresponding to bacterial load.

(i) RNA extraction

The *M. tuberculosis* RNA in 1ml of homogenized sputum, preserved in 4 mL of guanidine thiocyanate (GTC) at -80°C, was extracted using the RNA pro kit (FastRNA Pro BlueKit; MP Biomedical, CA, USA) as instructed by the manufacturer and others (Gillespie, 2017; Honeyborne *et al.*, 2011b). In summary, 100 μL of internal (extraction) control were added into 4 mLs of sputa containing 4M GTC and centrifuged at 3000 g at room temperature for 30 minutes. Then, 950 μL RNApro solution (lysis buffer) were dispensed into each sample tube containing the sputum sediments and were homogenized using the FastPrep machine (MP biomedical) programmed for 40 seconds. This step was followed by a series of centrifugation before 300 μL of chloroform was added. The upper layer containing RNA/DNA was transferred

to another tube. This was followed by a series of washing steps using 70% and 100% ice-cold ethanol and dried at 50^0C in the hot block for 25 minutes. Dried RNA/DNA was suspended in 100 μL RNase-free water, before was incubated at -80^0C for 15 minutes. The extract was treated to remove DNA from the dead cells using a master mix containing 1 μL DNase I enzyme (TURBO DNA-Free Kit Ambion) and 10 μL of Turbo DNase I 10x buffer, vortexed and incubated at 37^0C for 30 minutes. Thereafter, 1 μL DNase I enzyme was added and incubated at the same temperature for 30 minutes. After this incubation, 10 μl of DNase inactivation reagent was added, vortexed, and incubated at room temperature for 5 minutes before it was centrifuged at 13 000 g for 2 minutes. Supernatant containing pure RNA of *M. tuberculosis* was stored at -80^0C until RT-qPCR

(ii) RT-qPCR

The *M. tuberculosis* 16S rRNA, a biomarker for viable cells, was amplified and quantified by RT-qPCR using specific primers and probes on a Rotor gene Q 5plex platform (Qiagen) according to the manufacturer instructions and others (Gillespie H Stephen, 2017; Mtafya *et al*., 2019). Principally, each sample was run in triplicate reaction, in which 2 reactions utilized the reverse transcriptase enzyme (RT+) to synthesize and amplify complementary DNA (cDNA) from RNA extracts. The remaining 1 reaction was run without the enzyme in the mix (RT-) to synthesize cDNA. A TB-MBLA result was valid if there was no amplification in the RT- samples, suggesting a complete removal of DNA from the sample. The RT-qPCR quantification cycle (Cq) was translated into the bacterial load as eCFU/mL using standard curves prepared from a known concentration for *M. tuberculosis* and internal control. The cut-off for TB-MBLA positivity was a 30 Cq value that corresponds to 1.0 $\log_{10}$eCFU/mL, beyond which the test was considered negative (Gillespie, 2017; Sabiiti *et al*., 2020)

3.6   Data management and statistical analysis

3.6.1   Data quality control and assurance

Data were recorded in a clinical case report form, entered in a Microsoft Excel 2018 Mac OS and cleaned before statistical analysis and visualization on R programming language, version 4.0.2 (http://www.R-project.org). The PhD 3 developed protocol and trained it to the research assistants and the documents for quality data collection. A standard operating procedure was made available for the research team for them to ensure reproducibility of sputum sample

collection for a test assay. The clinician investigator or sub-investigator collected data in the health facilities and laboratory. Except for WGS, all laboratory procedures as well as statistical data analyses were performed by the candidate. Before entry, the PhD candidate verified the correctness of the filed clinical report form to ensure that data are attributable, legible, completeness, original and accurate.

Patients who completed 8 treatment visits and who had positive pre-treatment TB-MBLA and culture test results were analysed. For better fitting in the model, only the first visit with negative TB-MBLA result after the positive results at baseline was retained. The rests were removed from the final analysis.

Additionally, patients whose isolate had poor quality of sequencing by WGS and those without MIC data were excluded from the final analysis. Resistance-associated variants were classified as previously defined by Heyckendorf et al (Heyckendorf et al., 2018). For example, an isolate without mutations in resistance-associated genes or with only phylogenetic polymorphisms relative to the *M. tuberculosis* H37Rv reference sequence, with a MIC (μg/mL) value at or below the epidemiological cut-off value (ECOFF) was defined as genotypically wildtype and phenotypically susceptible (gWT-S). The isolate with a mutation known to result in MIC (μg/mL) increases above the highest breakpoint was considered as genotypically non-wildtype and phenotypically resistant (gNWT-R). The isolate with a yet uncharacterized mutation for which too little was known or no MIC values were available to make a judgement was considered to be genotypically non-wildtype unclear (gNWT-U).

3.6.2 Statistical analyses

(i) Descriptive statistics

Statistical analyses were objective-specific. Demographic and clinical data, including age, HIV status, prior history of exposure to anti-TB medications, weight (Kg), height in meters, and body mass index (Kg/m^2) were retrieved from the patient's clinical charts and reported totals and proportions. Accordingly, Chi-Square or Fischer's exact test compared proportions. Normality of continuous data was assessed using Sharpiro's test. Data were considered to be not normally distributed at a p-value of < 0.05. Normally distributed continuous data were summarized as mean and its 95% confidence interval and were compared using independent student T-test. Non-normally distributed continuous data were reported as median and 25th and

75th interquartile range (IQR). Two and three medians were compared using Mann-Whitney U (Wilcox rank) and Kruskal-Wallis tests, respectively.

(ii)   Performance of diagnostic assays

Using genotype MTBDRplus/sl and MycoTB as reference methods for gMTBC and WGS, respectively, assays performance was presented as sensitivity, specificity, positive and negative predictive values. An accuracy was defined as the measure of proportion of all tests where gMTBC agrees with genotype MTBDRplus or culture as well as where WGS agrees with the MycoTB sensitire® plate. Drug resistance predicted by WGS, as well as concordance and discordance between WGS and MycoTB were summarized as proportions. The weighted Cohen's kappa (κ) statistic measured the level of agreement between genotype MTBC assays with other tests. A κ of < 0.60, $0.60 - 0.79$, $0.80 - 0.90$ and > 0.90 was interpreted as an inadequate, moderate, strong and almost perfect agreement between the two tests (McHugh, 2012). The median MIC at their 25th and 75th interquartile range (IQR) were compared using Mann-Whitney U (Wilcox rank) test. The significance level was set at $p < 0.05$ and 95% confidence interval. Relationships of resistance-associated variants and MIC for an individual drug were visualized using bar plots.

(iii)   Regression models for binary outcome and repeated measures

Modified Poisson regression model was used to estimate the incident rate of detecting *M. tuberculosis* complex by gMTBC, and was adjusted against gender, age, presence of cavitary disease, HIV status, prior history of anti-TB exposure, cigarette smoking, smear microscopy results, and bacterial load measured by Xpert® MTB/RIF. In principle, Modified Poisson regression model measures the incident rate or relative risk. The model utilizes a robust error variance estimator to rectify overestimation of the risk (Zou, 2004). This bacterial load was categorized as low (sum of low and very low) *M. tuberculosis* complex quantity if it was detected at the Cq of 23 to >28, and high (sum of high and medium) if it was detected at the Cq value of < 23 Xpert® MTB/RIF.

To determine the rate of *M. tuberculosis* killing ($\log_{10}$eCFU/mL) of different treatment regimens measured by TB-MBLA, a quartic polynomial nonlinear-mixed-effects model for repeated measures was fitted as previous (Movshovitz-Hadar & Shmukler, 1991; Rustomjee *et al*., 2008). In this model, initial bacterial load bacterial load measured by TB-MBLA, chest

cavity, HIV, silicosis and gender were used as fixed effects. Individual patients were accounted for a random effect. A model was reliably selected if it had low Akaike-information-criterion but high intraclass-correlation-coefficient in Table 2. The mean difference in *M. tuberculosis* load, due to two different regimens received by patients at the end of 4 months of treatment was compared using one-way analysis-of-variance (ANOVA) and Tukey's test for repeated measures (Hazra & Gogtay, 2016). An injectable without bedaquiline regimen was used as a reference regimen.

(iv)   Survival analysis

The median time to TB-MBLA and culture conversion to negative was estimated using the Kaplan-Meier method and was compared across different regimens using a log-rank test (Gillespie *et al.*, 2014). Cox Proportional-Hazards regression models were used to estimate the hazard ratios (HR) for *M. tuberculosis* killing, and was adjusted for the effects of HIV, baseline bacillary load measured by TB-MBLA, chest cavity, silicosis, gender, prior history of treatment for drug susceptible TB and initial kiling rate. Computed overall mean *M. tuberculosis* load of 4.0 log10 eCFU/mL was used to categorize a patient's bacterial load as "high bacterial load" and "low bacterial load" if patients had detectable *M. tuberculosis* above and below this mean respectively. A p value < 0.05 was considered significant. A 95% confidence interval (CI) of the mean killing rate and HR was included

Table 2: Fitting and model selection in polynomial nonlinear mixed effect models

Polynomial models (degree)	Intercepts (log10 eCFU/mL)	ICC	Standard deviation	AIC	Likelihood ratio test	p value
Non-poly (model 1)	3.00	0.54	0.81	722.89		
Quadratic (model 2)	2.99	0.63	0.67	634.63	1 vs. 2	< 0.001
Cubic (model 3)	3.00	0.65	0.63	611.59	2 vs.3	< 0.001
Quartic (model 4)	3.20	0.67	0.61	592.7	3 vs. 4	< 0.001
Quintic (model 5)	2.89	0.68	0.60	588.58	4 vs. 5	0.020

Vallejo *et al.* (2014)

Model 4 had the lowest Akaike information criterion (AIC) and within variability (SD) but high Intraclass correlation coefficient (ICC) values, the key selection criteria for a reliable model, and hence it was used to model *M. tuberculosis* killing rates

3.7   Ethical considerations

All research work and procedures in this thesis were carried out in accordance with International Council of Harmonization- Good Clinical Practice (ICH-GCP), the ethical principles that have their origin in the Declaration of Helsinki and the applicable regulatory requirements. First, the Department of Global Health and Biomedical Science, School of Life Science and Bio-engineering (LiSBe) of the Nelson Mandela African Institution of Science and Technology (NM-AIST) granted permission to conduct this research. Secondly, the research protocol was granted ethical approval by the National Health Research Ethics Sub-Committee (NatHREC) with the secretariat at the National Institute for Medical Research (NIMR) in Tanzania (NIMR/HQ/R.8a/Vol. IX/2662. Permission to conduct the study was granted by authorities of the Kibong'oto Infectious Diseases Hospital (KIDH) and NIMR, Mbeya Medical Centre.

Prior to any study procedure, all patients provided an informed consent to participate and provide sputum samples for laboratory testing. During the informed consent process, patients were informed about the study problem, procedures to be undertaken, potential risk and benefit for participating in the study. Participation in the study was voluntary and free from due influence and coercion. They were allowed to withdraw from the study any time they wished to do so without penalties on their medical care. Importantly, all patients received anti-TB and anti-retroviral medications as per treatment guidelines in Tanzania. Informed consent forms detailing the study information and contacts for investigators and the ethical committee was given to patients to read at their own time for at least 1 day before enrolment and procedures. Both investigators and patients signed a witnessed written and dated informed consent form (ICF), written in the Kiswahili language. Illiterate patients were given the explanation in the presence of a literate witness or authorized representative chosen by a patient and who was not part of the investigators' team. In this case, an impartial witness signed the ICF, and the participant had a thumbprint.

Patient's information was kept confidential, and their security was ensured. For instance, the original signed ICF was kept in a master file, locked in a cabinet, and was made available to the study team only. A copy of signed ICF was provided to the patient. A unique identifier (ID) number was used to de-identify the patient's information. Electronic database and computer used to capture patient's information were password protected.

3.8   Dissemination of findings

Findings from this thesis were disseminated in different settings, including presentation during the: (a) graduate seminars at NM-AIST, (b) clinical team at Kibong'oto Infectious Diseases Hospital for them to adjust or guide patient's management, (c) summer schools organized by the Afrique One ASPIRE, (d) scientific conferences, including the Union TB and lung health, (e) at the Virtual symposium for the 2021 world TB day Webinar in Ghana and social media like Twitter. Also, findings were published in open access peer-reviewed journals with the candidate being a corresponding author. Other candidate's roles were published in accordance to the International Committee of Medical Journal Editors (ICMJE) authorship criteria (McNutt et al., 2018).A policy brief will also be written and submitted to potential TB stakeholders for future clinical decisions.

3.9   Data availability

The RNA extracts used for TB-MBLA were achieved at the Kibong'oto Infectious Diseases Hospital mycobacteriology laboratory. The datasets containing the minimum inhibitory concentration values of *M. tuberculosis* to anti-TB drugs, WGS data, and other datasets used to generate this thesis are available on request to the research team including Peter Mbelele, the PhD candidate, supervisors/mentors and the sponsors. The raw sequence data (FASTQ files) were deposited in European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJE9680 (https://www.ebi.ac.uk/ena/browser/view/PRJEB9680), and per run accession number of 50 isolates ranging from ERR459685 to ERR4596944.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1   Results

4.1.1   Socio-demographic and clinical characteristics of patients

A total of 126 patients participated in the study, of whom 83 (66%) were male. Their mean age (95% CI) was 42 (40 – 45) years. Baseline demographic and clinical characteristics of patients are in Table 3. Patients with positive gMTBC test results had lower mean (95% CI) age at 41 (38 – 44) compared to mean age of 45 (41 – 49) years for those with negative gMTBC (p = 0.073, Table 3). Patients with cavity on chest radiograph, low median body mass index (BMI) and HIV-infection at a high median CD4+ T cell counts were significantly associated with a positive gMTBC test result compared to those without chest cavity, low CD4counts and high BMI (p ≤ 0.027), respectively (Table 3).

Table 3 Baseline characteristics of studied patients (N = 126)

Variable	Overall (N = 126)	gMTBC positive (n = 82)	gMTBC negative (n = 44)	*p* value
Mean age (95% CI) in years	42 (40 – 45)	41 (38 – 44)	45 (41 – 49)	0.073
Male gender	83 (66%)	51 (62%)	32 (73%)	0.321
Cigarette smoking	60 (48%)	39 (48)	21 (48)	0.982
Feature of Silicosis	43 (34%)	28 (34%)	15 (34%)	0.941
HIV positive	45 (37%)	29 (35%)	16 (36%)	0.654
Median CD4+ (IQR) in cells/μL (n = 45)	186 (90 – 308)	253 (147 – 354)	104 (75 – 152)	0.021
Chest cavity	85 (67%)	76 (93%)	9 (20%)	< 0.001
Prior TB history	70 (56%)	45 (55%)	25 (57%)	0.914
Median BMI (IQR) in Kg/m^2	17.8 (16.5 – 20.0)	17.4 (16.3 – 19.9)	18.3 (17.4 – 20.7)	0.027
High *M. tuberculosis* complex quantity	75 (60%)	72 (88%)	3 (7%)	< 0.001

BMI, body mass index; gMTBC is the genotype MTBC. The Chi Square test or fisher's exact test if 20% of the cell had a value ≤ 4 computed the p values and compared proportions

4.1.2 Detection of M. tuberculosis complex

Among 126 patients with positive Xpert® MTB/RIF results, 51 (40%) had low *M. tuberculosis* complex quantity. Figure 3A – 3D shows that patients with chest cavity had high *M. tuberculosis* complex quantity, and were likely to test positive by any of culture and line probe assays [genotype MTBDRplus/sl (MTBDR) and genotype MTBC (gMTBC)]. Overall, 41 (80%) of patients with low *M. tuberculosis* complex quantity had negative culture and any of the MTBDR and gMTBC compared to 3 (4%) of 75 patients with high *M. tuberculosis* complex quantity (Fig. 3D, p < 0.001).
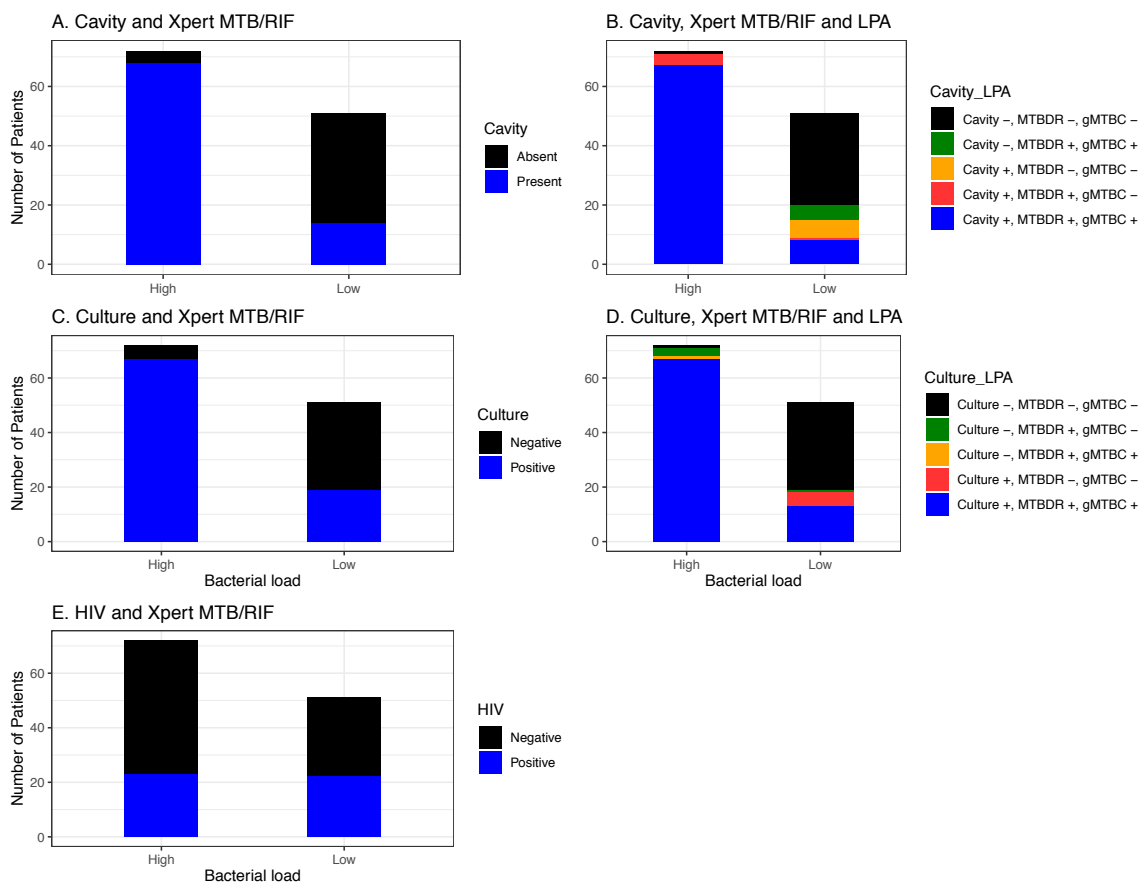
Figure 3: Detection of *M. tuberculosis* complex by additional tests against patient's bacterial load measured by Xpert® MTB/RIF

Bacterial load was quantified as low and high at the Xpert® MTB/RIF quantification cycle of 23 to >28, and <23, respectively. Patients were tested using culture and line probe assay (LPA) including the genotype MTBC (gMTBC) and genotype MTBDRplus/sl (MTBDR).

4.1.3   Incident rate of detecting M. tuberculosis complex by genotype MTBC

In a multivariate modified Poisson regression model, patients with cavity on chest radiograph and high *M. tuberculosis* complex quantity were 2.94 (95% CI: 1.12 – 8.77, p = 0.038) and 2.66 (95% CI: 1.25 – 6.41, p = 0.019) times more likely to have detectable *M. tuberculosis* complex from direct sputa by the gMTBC compared to patients without chest cavity and low *M. tuberculosis* complex (Table 4).

Table 4: Predictors of detecting *M. tuberculosis* complex by genotype MTBC (N = 126)

Variable	Univariate Crude incident rate ratio (95% CI)	p value	Multivariate Adjusted incident rate ratio (95% CI)	*p* value
Gender				
Female	Ref:		Ref:	
Male	0.85 (0.55 - 1.35)	0.992	0.98 (0.63– 1.56)	0.938
HIV status				
Negative	Ref:		Ref:	
Positive	0.98 (0.62 – 1.54)	0.706	1.12 (0.69 – 1.92)	0.407
Cigarette smoking				
Non-smokers	Ref:		Ref:	
Smokers	1.00 (0.64 – 1.53)	0.974	0.88 (0.56 – 1.37)	0.250
Prior TB treatment				
New	Ref:		Ref:	
Retreatment	0.97 (0.63 – 1.51)	0.862	0.96 (0.62 – 1.50)	0.572
Chest cavity				
Absent	Ref:		Ref:	
Present	6.11 (2.90 – 15.73)	<0.001	2.94 (1.12 – 8.77)	0.038
MTBC quantity				
Low	Ref:		Ref:	
High	4.90 (2.65 – 10.10)	<0.001	2.66 (1.25 – 6.41)	0.019
Smear microscopy				
Negative	Ref:		Ref:	
Positive	2.08 (1.34 – 3.23)	0.001	1.10 (0.69 – 1.79)	0.692

Modified Poisson regression model determined the incident rate of detecting *M. tuberculosis* complex (MTBC) by the genotype MTBC. The MTBC was quantified as either low at a cycle threshold (CT) value of > 23 or high at a CT value of ≤ 22 using the Xpert® MTB/RIF.

4.1.4   Performance of genotype MTBC in direct sputum samples

Overall, there was a strong concordance between the gMTBC assay and genotype MTBDR (Cohen's kappa, κ= 0.89), and between LJ culture (κ= 0.84) in detecting *M. tuberculosis* complex. The number of patients with positive and negative results by all tests as well as sensitivity, specificity and accuracy of the genotype MTBC assay compared to the genotype

MTBDRplus or MTBDRsl and LJ culture in detecting *M. tuberculosis* complex from direct sputa were summarised in Table 5.

Table 5: Performance of the genotype MTBC v1.x assay in sputum samples (N = 126)

Reference Methods		genotype MTBC assay, n % (95% Confidence interval)					
		POS	NEG	Sensitivity	Specificity	Accuracy	Cohen's kappa
Genotype MTBDR	POS	82	4	100 (94 - 100)	97 (92 - 99)	100 (89 - 100)	0.89 (0.76 - 99)
	NEG	0	40				
LJ culture	POS	80	9	98 (91 - 100)	91 (85 - 95)	95 (80 - 99)	0.84 (0.69 - 97)
	NEG	2	35				

LJ, Lowenstein-Jensen solid media; MTBDR is genotype MTBDRplus or MTBDRsl assays for detecting *M. tuberculosis* complex and drug resistant; NEG and POS, negative and positive test results respectively

4.1.5 Detection of M tuberculosis complex to the species and lineage level

Among 126 patients, 89 (71%) had positive culture, including 86 (98%) and 82 (65%) *M. tuberculosis* complex that were detected by the genotype MTBC and MTBDR, respectively. All *M. tuberculosis* complex tested positive by MPT64 antigen (Fig. 4). Unexpectedly, 37 (29%) of 126 patients with positive Xpert® MTB/RIF test results had neither TB nor non-tuberculous mycobacteria by any of additional tests (Fig. 4). Figure 4 shows the proportion of patients with positive test results by different diagnostic method in the testing algorithm.

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                  ┌─────────────────────────────────────────┐
                  │ 126 patients studied and had positive    │
                  │ Xpert® MTB/RIF results                   │
                  └─────────────────────────────────────────┘
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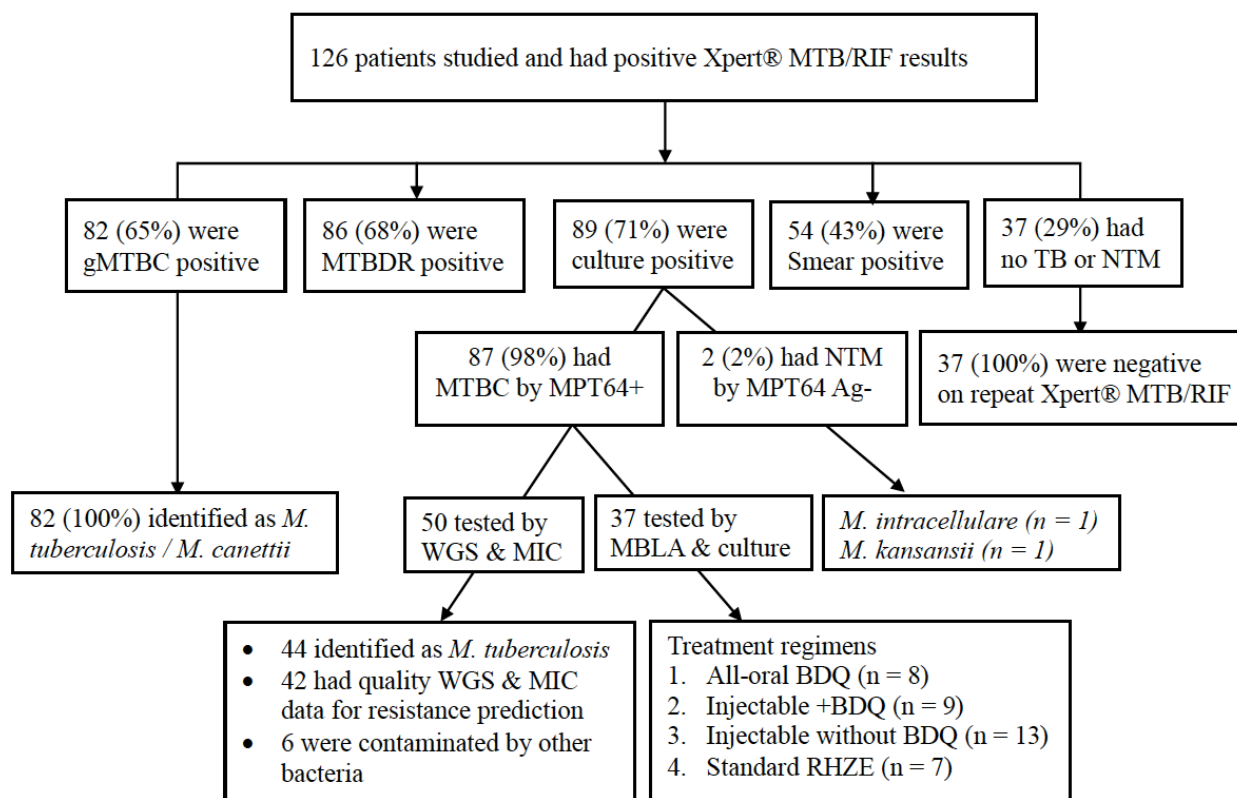

Figure 4: Testing algorithm of study participants

The gMTBC, genotype MTBC v1.x; NTM, non-tuberculous mycobacteria detected using the genotype Mycobacterium CM v2.0 kit; MPT64 Ag is an antigen for *M. tuberculosis* complex (MTBC); WGS is whole genome sequencing; MIC, minimum inhibitory concentration; TB-MBLA, Tuberculosis Molecular Bacterial Load Assay; RHZE, fixed dose combination of rifampicin, isoniazid (H), pyrazinamide (Z) and ethambutol (E); and BDQ, bedaquiline, a key drug in multidrug-resistant tuberculosis treatment regimens.

4.1.6   *M. tuberculosis* lineages in patients with RR/MDR-TB

Among 50 *M. tuberculosis* complex isolates sequenced from 50 patients with RR/MDR-TB, *6* (12%) had poor sequence coverage and were identified as mixtures of different bacteria with Kraken 2 including *Ralstonia pickettii* (n = 2), *Streptomyces spp* (n = 2), *Tsukamurella tyrosinosolvens* (n = 1) and *Gordonia bronchialis* (n = 1). The remaining 44 were identified as *M. tuberculosis* and were further identified to lineage and sub lineages. In total, 41 (93%) of *M. tuberculosis* belonged to East-African-Indian lineage 3 (n = 19) and Euro-American lineage 4 (n = 22). Figure 6 shows different lineage and sub-lineages of *M. tuberculosis* isolated from patients with RR/MDR-TB.
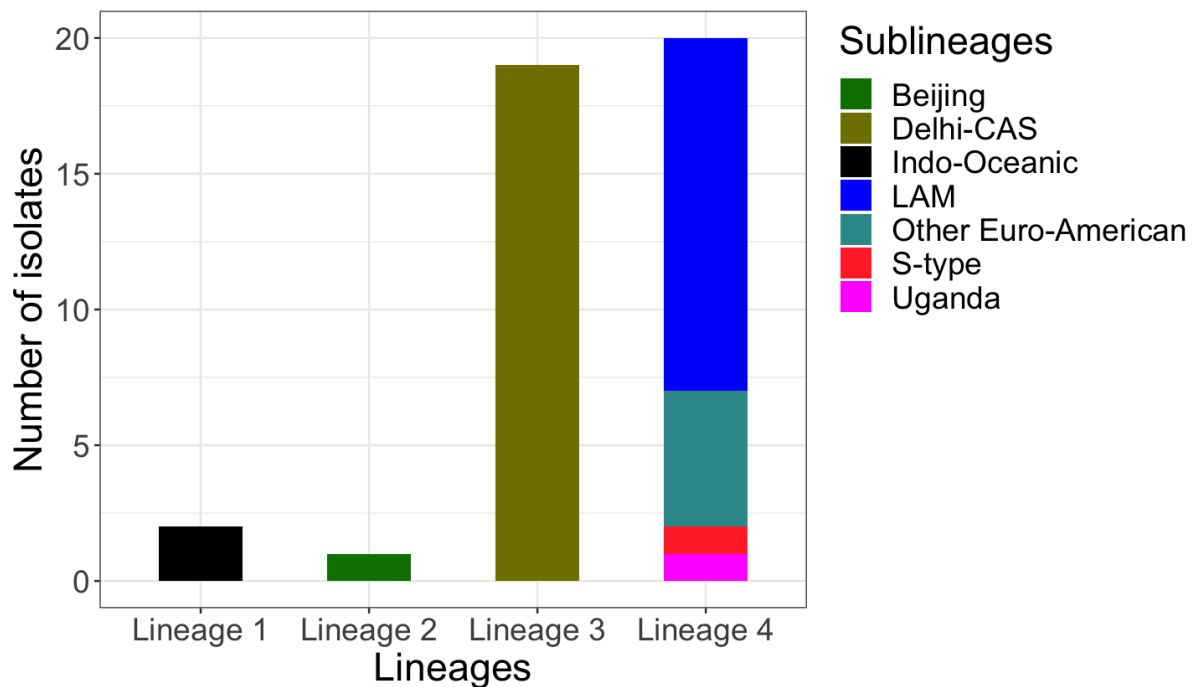
Figure 5: Different lineages and sub-lineages of *M. tuberculosis* among RR/MDR-TB patients

4.1.7 Detection and clinical outcomes of non-tuberculous mycobacteria species

Among 89 (71%) patients with positive culture, 2 (2%) tested positive for non-tuberculous mycobacteria by genotype Mycobacterium CM v2.0 assay, with 1 being *M. intraceullare* and the other 1 being *M. kansasii* (Fig. 4*)*. The *M. intracellulare* was detected from a 51-years-old Tanzanian woman who presented at the Kibong'oto Infectious Diseases Hospital with productive cough without haemoptysis of 2 years, and 6-months duration of chest pain, breathless, progressive weight loss, fever and excessive night sweats. She had multiple prior histories of TB treatment up to 7 episodes including 2 episodes of MDR-TB (Table 6). Also, the patient was living with HIV/AIDS, and had been on antiretroviral-therapy since 2004. Her current HIV viral load was undetectable. She had extensive lung destruction characterized by cavity, bronchiectasis, cystic and fibrosis on chest radiograph (Fig. 5). In the current episode, she was treated for multidrug and extensively-drug resistant TB, but could not achieve microbiological cure measured by smear and culture. Ultimately, the *M. intracellulare* was detected from both cultured isolate and direct sputa. Upon detection, a macrolide was added to bedaquiline, linezolid, moxifloxacin, pyrazimanide (PZA) and amoxycylin-clavulinic acid in order to treat both M/XDR-TB and the later pathogen. While on revised regimen, the patient converted to culture and smear negative at month 3, but reverted to positive at month 6. She

had now completed 24 months of revised regimen and was discharged to TB clinic to continue attending pulmonary clinic for symptomatic care.
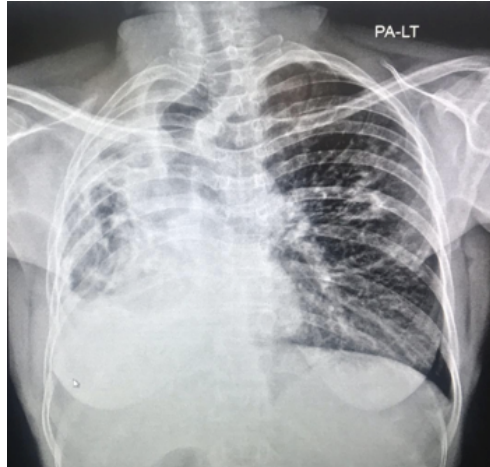
Figure 6: **Posterior-anterior chest x-ray showing extensive right pulmonary fibrosis, focal bronchiectasis and reduced volume**

Table 6: Prior histories of TB treatment and clinical outcomes of a patient with *M. intracellulare*

Years	Form of TB	Diagnostic tool	Treatment Category	Treatment Regimens	Outcomes	Diagnostic improvement
2008	DS-TB	Positive Smear Microscopy (SM)	Category I	Rifampicin, isoniazid, pyrazinamide & ethambutol (RHZE)	Completed	No culture & DST offered
2009	DS-TB	Positive SM	Category II	Streptomycin plus RHZE	Cured	
2010*	DS-TB	Positive SM and culture	Category II	Streptomycin plus RHZE	Failure	no DST offered on cultured isolate
November 2010	MDR-TB	Phenotypic culture-based DST	MDR-TB regimens	Levofloxacin, Kanamycin Pyrazinamide Cycloserine and Ethionamide	Reverted to culture positivity	Neither second-line DST offered nor testing by Xpert® MTB/RIF
July 2011	Presumed NTM	Positive culture but negative MPT64 Ag RAPID® test	MDR-TB regimen was stopped and discharged	Not provided	Unknown	Neither testing nor treatment for NTM offered
2012 to 2016†	DS-TB	Positive SM	Category II	Streptomycin plus RHZE	Failure	No DST offered
February 2017	MDR-TB	Phenotypic DST and MTBDRplus	MDR-TB regimens	Levofloxacin, Kanamycin, Pyrazinamide, Cycloserine and Ethionamide	Failure	No second-line DST offered

- *Treatment failure at month 7 defined by positive smear microscopy and culture. Phenotypic drug-susceptibility-testing (DST) in November 2010 revealed resistance to rifampin (R), isoniazid (H), ethambutol (E), and streptomycin (S); MDR-TB, is multidrug resistant tuberculosis
- † Lapse in patient's records from 2012-2016. Patient was treated twice for drug-susceptible tuberculosis (DS-TB) using category II regimen.
- Phenotypic DST revealed was MDR-TB with additional resistant to ethambutol and streptomycin in late 2016.
- NTM, non-tuberculous mycobacterium

4.1.8 Prediction of drug susceptibility in M. tuberculosis complex

Drug resistance in 86 *M. tuberculosis* was predicted by using genotype MTBDRplus and MTBDRsl. Of these 86%, 50 cultured isolates form patients with RR/MDR-TB underwent WGS. In total, 42 (84%) of 50 sequenced isolates passed the sequencing quality thresholds and had complete MIC results, and hence were analysed. The WGS derived genotypic drug-resistance prediction for *M. tuberculosis* isolates at the drug's ECOFF in MycoTB plate are summarized in Table 7. The prediction of drug resistance was correctly made by WGS for streptomycin in 67% (8/12), for ethionamide in 80% (4/5), and for ethambutol in 81% (13/16). For the drugs primarily used to treat RR/MDR-TB like bedaquiline, fluoroquinolones, and linezolid, no resistance-associated mutations were detected via WGS (Table 7). Nonetheless, MycoTB detected phenotypic resistance to levofloxacin (n = 2), moxifloxacin (n = 4), clofazimine (n = 1), cycloserine (n = 1), p-aminosalicylic acid (n = 2), kanamycin (n = 8), capreomycin (n = 4) and amikacin (n = 1). Discordances between WGS and MycoTB are shown in Table 7, with Ethambutol (at ECOFF 2.0 & 4.0 µg/mL) and streptomycin bore the highest discordance. Distribution of MIC values of the first, second-line and injectable aminoglycosides/peptides anti-TB drugs among genotypically wildtype and non-wildtype isolates are shown in figure 7, 8 and 9, respectively. Overall, strains harbouring mutations had MIC values above the ECOFF values in MycoTB Sensitire® plate.

(i) Resistance prediction for first line anti-TB drugs

Rifamycin (rifampicin and rifabutin)

Among 86 isolate*s*, 64 (74%) had rifampicin resistance detected by either by the genotype MTBDRplus or WGS. At ECOFF value of 0.5 and 0.125 µg/mL, WGS predicted rifampicin resistance in 36 (95%) and all 38 (100%) of 38 resistant *M. tuberculosis* complex isolates, respectively. The isolates with *rpoB* gene mutations had MIC values above the ECOFF value (Fig. 7A & 7B). The median MIC value (µg/mL) for gNWT-R for rifampicin was ≥ 4.0 (IQR; 4.0 – 4.0) compared to 0.5 (IQR; 0.38 – 0.50) among gWT-S isolates (p < 0.001). The *rpoB* S450L was the most frequent mutation associated with rifampicin/rifabutin accounting for 23 (61%) of 38 gNWT-R isolates. Based on gNWT-R alone, WGS predicted rifampicin and isoniazid resistance in 29 (76%) and it increased to 32 (84%) when isolates with gNWT-U for isoniazid were added to those with gNWT-R (Table 7).

Isoniazid

Among 86 *M. tuberculosis,* 37 (43%) had isoniazid resistance detected by either MTBDRplus or WGS. In total, 5 (14%) of these 37 *M. tuberculosis* isolates had isoniazid monoresistance. Among 42 sequenced isolates*,* 29 (69%) had mutations previously known to confer isoniazid resistance (gNWT-R), but WGS was discordant with MycoTB in 8 (19%) isolates (Table 7). At ECOFF 0.25 µg/mL, WGS predicted isoniazid resistance in 27 (93%) of gNWT-R isolates. Four isolates had gNWT-U mutations on *katG* gene and all had MIC above ECOFF value (Fig. 7C). The gNWT-R for isoniazid had median MIC of > 4.0 (IQR; $2.0 – 4.0$) µg/mL compared to 0.25 (IQR; $0.12 – 1.00$) µg/mL in gWT-S isolates ($p = 0.001$); In total, 21 (72%) of isoniazid resistant isolates had the *katG* S315T and 2 (7%) the *fabG1* -15c>t mutations previously known to confer isoniazid resistance, showing MIC values ≥ 2.0 µg/mL (Fig. 7C).

Ethambutol

In total, 16 (38%) of 42 sequenced isolates were gNWT-R (Fig. 7E). At ECOFF value of 2.0 and 4.0 µg/mL, WGS predicted ethambutol resistance in 14 (88%) and 13 (81%) of gNWT-R isolates, respectively (Table 7). All 16 isolates had known resistance-mediating mutations in the *embB* gene with MIC values ≥ 8.0 µg/mL (Fig. 7E). Of the remaining 26 isolates, 25 had unclear mutations representing 13 that had MIC values of ≤ 4.0 µg/mL and 12 isolates that had MIC values ≥ 8.0 µg/mL suggesting ethambutol susceptible and resistant, respectively (Fig. 7E). The *embR* F376L was the commonest unclear mutation often found with additional mutations (Fig. 7E & Table 8). Nonetheless, distribution of ethambutol's MIC values varied significantly between gNWT and gWT isolates (Fig. 7E). For example, 3 isolates were genotypically resistant including one with an *embB* M306I mutation but had a phenotypically susceptible MIC ≤ 4.0 µg/mL.

Pyrazinamide

A total of 11 (26%) sequenced isolates had mainly mutations in the *pncA* gene known to confer pyrazinamide resistance. Six isolates harbored stop codons and five isolates had non-synonymous mutations in the *pncA* gene, whereas in one strain the known mutation *rpsA* R212R was identified. Additional unclear mutations for pyrazinamide resistance were found in 39 (48%) isolates, with *Rv3169* A190G being the most common (Fig. 7D, Table 8). However, there were no MICs to evaluate the mutations (Fig. 7D).

Figure 7: Distribution of minimum inhibitory concentration for first line anti-TB drugs in genotypically wildtype and non-wildtype isolates

The isolates with MIC (μg/mL value below the tentative epidemiological cut-off value (ECOFF) was defined as genotypically wildtype and phenotypically sensitive (gWT-S). The isolate with a mutation known to result in MIC (μg/mL) increases above the highest breakpoint was considered as genotypically non-wildtype and phenotypically resistant (gNWT-R). The isolate with unclear mutation or a mutation for which too little was known or no MIC values were available to make a judgement was considered to be genotypically non-wildtype unclear (gNWT-U). The MIC testing for pyrazinamide was not done. A plus sign denotes that mutation presented co-existed with *embR* F376L.

(ii) **Resistance prediction for second-line anti-TB drugs**

Fluoroquinolones (Levofloxacin and Moxifloxacin)

All 86 *M. tuberculosis* had no mutations on *gyrA* and *gyrB* genes previously known to confer levofloxacin and moxifloxacin as measured by either genotype MTBDRsl or WGS. At ECOFF values of 1.0 µg/mL for levofloxacin and 0.5 µg/mL for moxifloxacin, WGS and MycoTB was discordant in 2 (5%) and 4 (10%) of 42 isolates, respectively (Table 7). However, eight (19%) isolates had unclear mutations including six with mutations in both *gyrA* and *gyrB* genes and two in *eccB5* and *eccC5* genes associated with levofloxacin and moxifloxacin resistance (Fig. 8A & 8B). Of these eight isolates seven had MIC below the ECOFF for levofloxacin and moxifloxacin with only 1 isolate with *eccC5* K835R mutation having MIC of 1.0 µg/mL suggesting potential resistance against moxifloxacin (Fig. 8B). Table 8 shows the number of unclear novel mutations on genes that could possibly confer fluoroquinolone resistance in *M. tuberculosis*.

Clofazimine and Cycloserine

In all 42 isolates, no mutations in genes previously linked to clofazimine (Rv0484 gene) were found (Fig. 8C). However, at ECOFF of 0.25 and 1.0 µg/mL, WGS and MycoTB was discordant in 6 (14%) and 1 (2%) of isolates (Table 7). For cycloserine, one isolate showed a MIC of 128 µg/mL indicating resistance, but it was genotypically wildtype. In total, 8 (19%) out of 42 isolates had unclear mutations in the *pykA (n= 3)* and *PPE22* (n = 5) genes, but all with MICs below the cycloserine breakpoint (Table 7, Fig. 8D

Para-aminosalicylic acid

No mutations were detected by WGS in genes conferring resistance to p-aminosalicylic acid. At ECOFF 4.0 µg/mL, two (5%) gWT isolates had a MIC of 8 µg/mL suggesting p-aminosalicylic acid phenotypic resistance. Another three (7%) isolates had unclear mutations in *thyA* (n = 2) and *folC* (n = 1), putative resistance conferring genes for this drug, but both had MIC values below the breakpoint (Fig. 8E).

Ethionamide

Figure 8F shows that gNWT-R isolates for ethionamide showed median MIC of 15.0 (IQR; 10.0 – 20.0) compared to 2.50 (IQR; 2.50 – 5.00) among gWT-S isolates (p < 0.001). In total,

5 (12%) of *M. tuberculosis* isolates were gNWT-R, including 3 and 2 in *ethA* and *fabG1* genes respectively. The WGS predicted resistance in 4 (80%) of gNWT-R isolates. At ECOFF 5.0 µg/mL for ethionamide, all mutations in the *ethA* gene or *fabG1* promotor region known to confer resistance had MICs ≥ 10.0 µg/mL. Four additional isolates had unclear mutations on ethR (n = 2) and ethA (n = 2) genes, which all showed MICs below or at the ECOFF (Fig. 8F)

Bedaquiline, delamanid and linezolid

There were no mutations that have previously linked with bedaquiline, delamanid and linezolid resistance in all 42 *M. tuberculosis* isolates. The MIC testing for these drugs were not done to compare with the unclear mutations (Fig. 8G, 8H & 8I). The numbers of isolates with unclear mutations per drug are shown in Table 8 and in Fig. 8. All participants were bedaquline and delamanid naïve.

Injectable aminoglycosides and cyclic peptides

All 86 *M. tuberculosis* had no mutations on genes previously known to confer aminoglycosides class of antibiotics (*rrs* and *eis*) like amikacin and kanamycin and cyclic peptide like capreomycin (*rrs* and *tlyA*) resistance detectable by the genotype MTBDRsl and WGS. All 42 sequenced isolates had no known mutations in genes linked to resistance of the aminoglycosides class of antibiotics (*rrs* and *eis*) such as amikacin and kanamycin and cyclic peptide such as capreomycin (*rrs* and *tlyA*). Five (12%) isolates had unclear mutations (gNWT-U) in the *rrs* gene for kanamycin, amikacin, capreomycin and streptomycin are shown in Fig. 9 A – 9D. The number of gNWT-U isolates and distribution of MIC are shown in Table 8 and Fig. 9, respectively. Discordances between gNWT-U measured by WGS and MycoTB plate in predicting kanamycin, amikacin, capreomycin and streptomycin are shown Table 7. Overall, there were high discordance between WGS and MycoTB plate in predicting streptomycin resistance in *M. tuberculosis* (Table 7). Using gNWT-R, WGS was 19% (8/42) discordant with MycoTB in predicting streptomycin resistance and it increased to 29% (12/42) when gNWT-U mutations were added (Table 7). In total, 17 (40%) isolates were genotypically non-wildtype (gNWT) and had mutations in *rpsL* (n = 13), *gidB* (n = 2) and *rrs* (n = 2) genes linked to streptomycin resistance (Fig. 9D). Of these 17 isolates, 4 (24%) had mutations on *rpsL* (n = 2) and *gidB* (n = 2) genes previously associated with streptomycin resistance (gNWT-R). While the MIC values for the *gidB* mutants were at or below 1.0 µg/mL, that of *rpsL* ranged from 2 – 8 µg/mL, suggesting streptomycin resistance. Also, 8 (47%) had *rpsL* K88M mutations

previously reported in the literature to confer resistance to streptomycin and had a median MIC of 8 (IQR; 4 – 8) µg/mL, and 5 (29%) had unclear mutations on either *rpsL* or *rrs* genes (Fig. 9D).
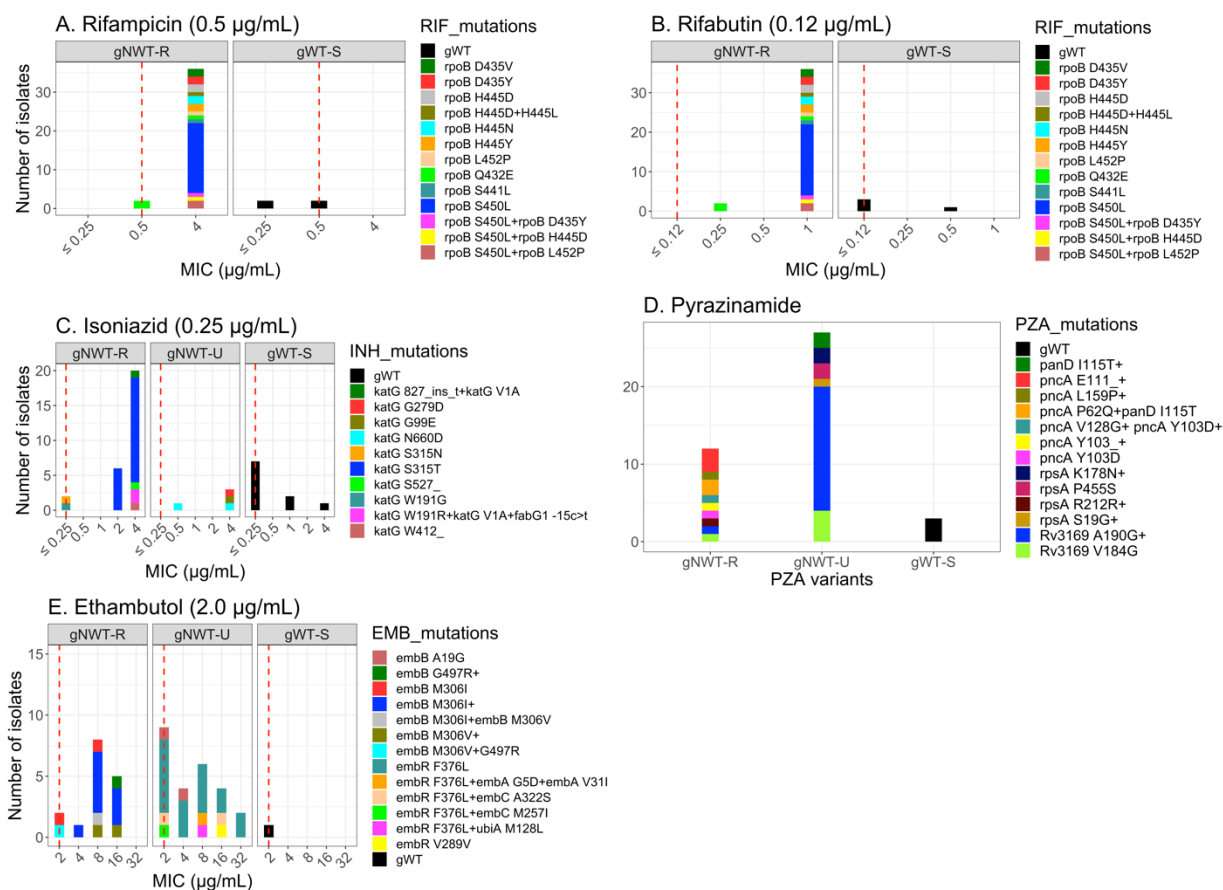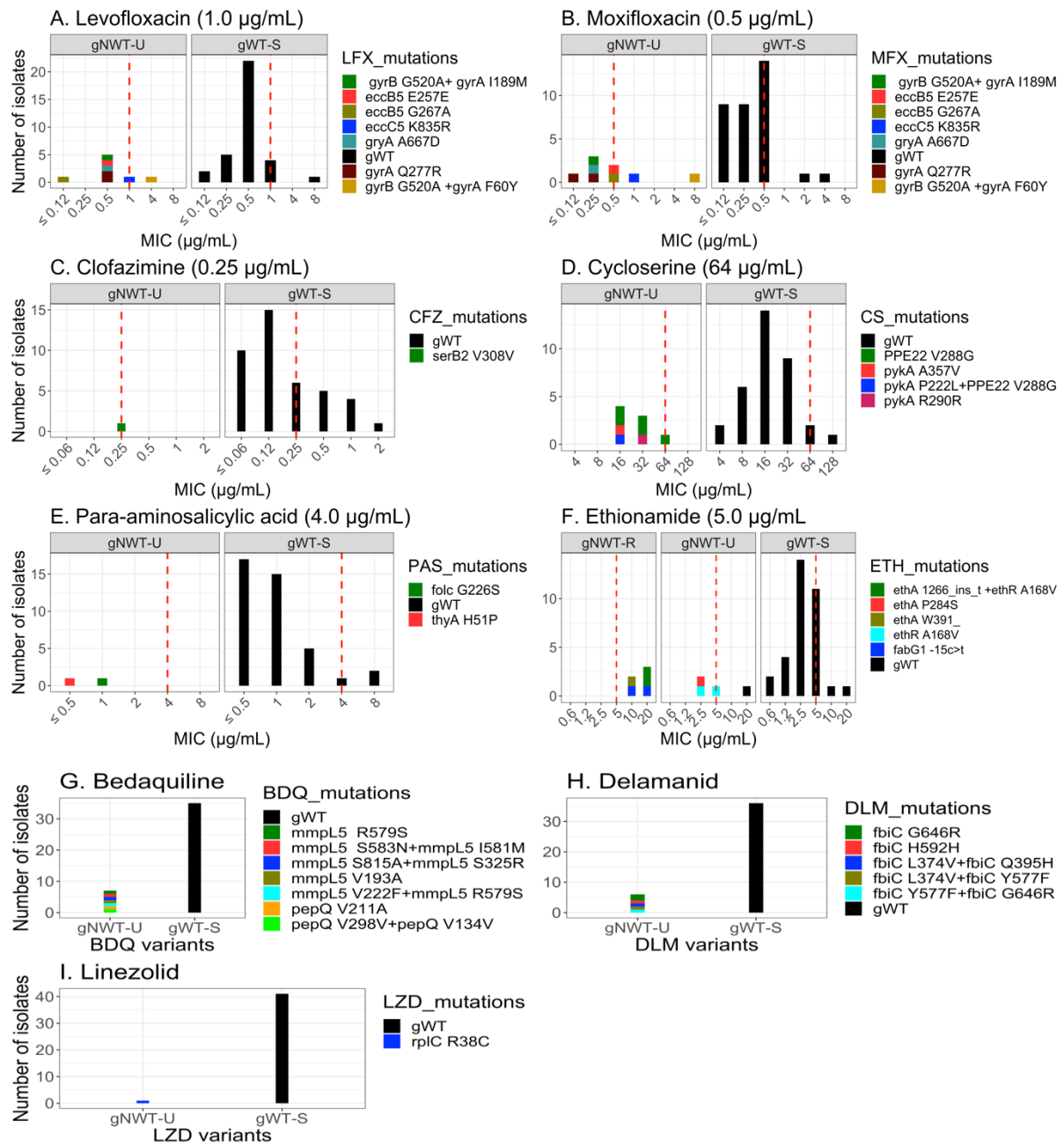

Figure 8: Distribution of Minimum inhibitory concertation for second line anti-TB drugs in genotypically wildtype and non-wildtype isolates

The isolates with MIC (µg/mL value below the tentative epidemiological cut-off value (ECOFF) was defined as genotypically wildtype and phenotypically sensitive (gWT-S). The isolate with a mutation known to result in MIC (µg/mL) increases above the highest breakpoint was considered as genotypically non-wildtype and phenotypically resistant (gNWT-R). The

isolate with unclear mutation or a mutation for which too little was known or no MIC values were available to make a judgement was considered to be genotypically non-wildtype unclear (gNWT-U). The MIC testing for pyrazinamide was not done. MIC testing for bedaquiline, delamanid and linezolid was not done.
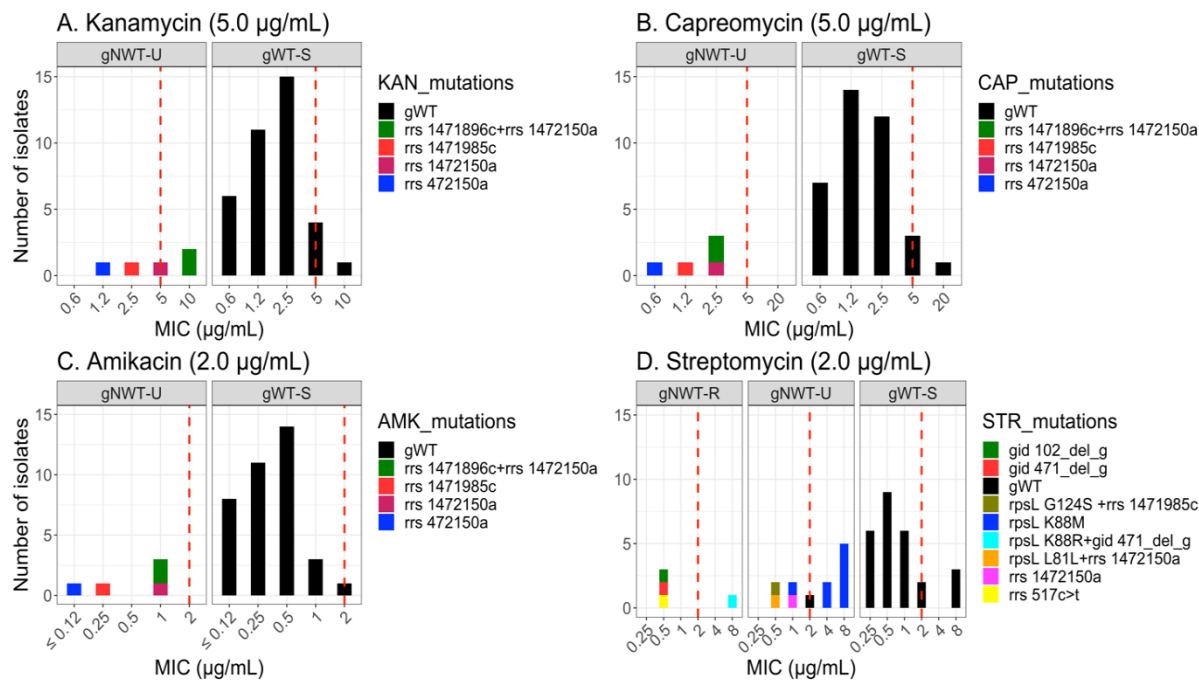
Figure 9: **Distribution of Minimum inhibitory concertation values injectable aminoglycosides/ capreomycin in genotypically wildtype and non-wildtype**

The isolates with MIC (μg/mL value below the tentative epidemiological cut-off value (ECOFF) was defined as genotypically wildtype and phenotypically sensitive (gWT-S). The isolate with a mutation known to result in MIC (μg/mL) increases above the highest breakpoint was considered as genotypically non-wildtype and phenotypically resistant (gNWT-R). The isolate with unclear mutation or a mutation for which too little was known or no MIC values were available to make a judgement was considered to be genotypically non-wildtype unclear (gNWT-U).

Table 7: WGS drug resistance predictions of MTBC isolates at an epidemiological cut-off value (ECOFF) in MycoTB assay

Anti-TB drug	ECOFF in μg/mL	Resistance prediction of known mutations (gNWT-R) alone			Resistance prediction of combined gNWT-R and unknown mutations (gNWT-U)		
		Agreement	Resistant predicted	Discordance	Agreement	Resistant predicted	Discordance
Rifampicin	0.5	95% (40/42)	95% (36/38)	5% (2/42)	95 (40/42)	95% (36/38)	5% (2/42)
	0.125	100% (42/42)	100% (38/38)	0% (0/42)	100% (42/42)	100% (38/38)	0% (0/42)
Rifabutin	0.125	100% (42/42)	100% (38/38)	0% (0/42)	100% (42/42)	100% (38/38)	0% (0/42)
Isoniazid	0.25	81% (34/42)	93% (27/29)	19% (8/42)	88% (37/42)	94% (30/32)	12% (5/42)
Ethambutol	2.0	57% (24/42)	88% (14/16)	42% (18/42)	74% (31/42)	73% (30/41)	26% (11/42)
	4.0	64% (27/42)	81% (13/16)	36% (15/42)	62% (26/42)	61% (25/41)	38% (16/42)
Streptomycin	2.0	81% (34/42)	67% (8/12)	19% (8/42)	71% (30/42)	50% (8/16)	29% (12/42)
Ethionamide	5.0	90% (38/42)	80% (4/5)	10% (4/42)	90% (38/42)	64% (7/11)	10% (4/42)
Levofloxacin	1.0	95 (40/42)	None	5% (2/42)	80% (34/42)	13% (1/8)	19% (8/42)
Moxifloxacin	0.5	90% (38/42)	None	10% (4/42)	80% (34/42)	25% (2/8)	19% (8/42)
Clofazimine	0.25	86% (36/42)	None	14% (6/42)	83% (35/42)	0% (0/1)	17% (7/42)
	1.0	97% (41/42)	None	2% (1/42)	98% (40/42)	0% (0/1)	5% (2/42)
Cycloserine	64.0	97% (41/42)	None	2% (1/42)	79% (33/42)	0% (0/8)	21% (9/42)
p-aminosalysilic acid	4.0	95% (40/42)	None	5% (2/42)	90% (38/42)	0% (0/2)	10% (4/42)
Kanamycin	2.5	81% (34/42)	None	19% (8/42)	83% (35/42)	60% (3/5)	17% (7/42)
	5.0	93% (39/42)	None	7% (3/42)	90% (38/42)	60% (3/5)	10% (4/42)
Capreomycin	2.5	90% (38/42)	None	10% (4/42)	79% (33/42)	0% (0/5)	21% (9/42)
	5.0	97% (41/42)	None	2% (1/42)	86% (36/42)	0% (0/5)	14% (6/42)
Amikacin	2.0	100% (42/42)	None	0% (0/42)	88% (37/42)	0% (0/5)	12% (5/42)

Table 8: **Summary of unclear novel mutations on genes that could possibly confer drug resistance in *M. tuberculosis***

Anti-TB drug	Genes	gNWT-U isolates	No. of R isolates	No. of S isolates	Common unclear Mutation(s)
Levofloxacin (n = 8)	*gyrA*	3	0	3	Q277R, A667D
	gyrB	2	1	1	G520A#
	eccB5	2	0	2	G267A, E257E
	eccC5	1	0	1	K835R
Moxifloxacin (n = 8)	*gyrA*	3	0	3	Q277R, A667D
	gyrB	2	1	1	G520A#
	eccB5	2	0	2	G267A, E257E
	eccC5	1	1	0	K835R
Cycloserine (n = 8)	*PPE22*	5	0	5	V288G
	pykA$	3	0	3	R290R, P222L
Clofazimine (n = 1)	*serB2*	1	0	1	V308V
p-aminosalicylic acid (n = 2)	*thyA*	1	0	1	H51P
	folC	1	0	1	G226S
Ethionamide or prothionamide (n = 4)	*ethA*	2	1	1	W391_, P284S1
	ethR	2	2	0	A168V
Streptomycin (n = 12)	*rpsL*	10	7	3	K88M, L81L, G124S
	rrs	2	0	2	517c>t, 1472150a,
Isoniazid (n = 4)	*katG*	4	4	0	G279D, G99E, N660D
Ethambutol (n = 25)	*embR*	17	11	6	F376L, V289V
	*embA**	1	1	0	G5D, V31I
	*embB**	2	1	1	A19G
	*ubiA**	1	1	0	M128L
	*embC**	3	1	2	M257I, A322S

R, resistant and S, Susceptible isolate. Superscripts Asterix (*) denotes combination with embR F376L, a dollar ($) denotes combination with cycA V110V (n = 1), and harsh (#) denotes combination with gyrA F60Y (n= 1) and gyrA I189M (n = 1) mutations

4.1.9 M. tuberculosis killing rates of the treatment regimens

(i) Mycobactericidal activities of different regimens over time

The *M. tuberculosis* load measured by TB-MBLA and culturing in Figure 9 decreased significantly over time (R = - 0.77, p < 0.001). The mean *M. tuberculosis* load in $\log_{10}$ eCFU/mL (95% CI) was reduced from 5.19 (4.40 to 5.78) at baseline to 3.10 (2.70 to 3.50) at day 14, then to 2.52 (2.13 to 2.90) at day 28, 1.88 (1.53 to 2.22) at day 56, and 1.36 (-1.03 to 1.70) at day 84 through 112 of treatment. The overall mean daily *M. tuberculosis* killing was -0.24 (95% CI -0.39 to -0.08) log10 eCFU/mL, and it varied with treatment regimen (Table 3, p < 0.001). An injectable bedaquiline-containing regimen had the highest mean *M. tuberculosis* killing rate, followed by an all-oral bedaquiline-based regimen compared to the injectable but bedaquiline-free reference regimen (Table 7, p = 0.019). Kanamycin-containing regimens in Fig. 10 had rapid bactericidal activity at day 14, but this was not translated into long-term bactericidal effect (p < 0.001). An all-oral bedaquiline-based regimen had a sharp decline after day 28.

(ii) Median time to M. tuberculosis killing

There was moderate positive correlation in time to sputum conversion between TB-MBLA and culture (r = 0.46 [95% CI 0.36 to 0.55]; p < 0.001). The overall median time to sputum TB-MBLA conversion to negative was 56 (IQR: 28 to 84) days. The median times to TB-MBLA conversion to negative were 28, 42, and 84 days among patients on injectable bedaquiline, an all-oral bedaquiline-based regimen, and injectable but bedaquiline-free regimens, respectively. Irrespective of treatment regimen, 92% (34/37) of patients had negative culture results compared to 65% (24/37) of negative TB-MBLA at day 56 (p = 0.037). The number of patients who converted to sputum negative by culture and TB-MBLA per treatment regimen is shown in Fig. 11. Among 13 patients who received the injectable but bedaquiline-free regimen, 2 and 7 of them remained culture and TB-MBLA positive, respectively, whereas all 8 patients who received injectable bedaquiline-containing regimens had negative LJ culture and TB-MBLA at day 56 (Fig. 11A to D). Favorably, all patients on injectable bedaquiline for treating RR/MDR-TB and standard RHZE regimen for treating DS-TB had negative TB-MBLA at days 56 and 84, respectively. Compared to 31% (4/13) of patients who received an injectable but bedaquiline-free regimen, only 11% (1/9) of those who received an all-oral bedaquiline-

containing regimen remained positive TB-MBLA but negative LJ culture at day 112 of treatment (Fig. 11A and B versus Fig.11E and F; p = 0.283).

(iii)   Hazard ratio (HR) of M. tuberculosis killing

The overall mean *M. tuberculosis* load log10 eCFU/ml at baseline was 5.19 (95% CI 4.40 to 5.78), and was similar in all patients treated with any of the 4 regimens (Table 8, *p* = 0.453). The mean *M. tuberculosis* load (log10 eCFU/ml) among female patients was 5.6 (95% CI 5.0 to 6.2) log10 eCFU/mL compared to 4.7 (95% CI 4.3 to 5.2) log10 eCFU/ml among male patients (*p* = 0.017). Patients with chest cavity had mean *M. tuberculosis* load of 5.26 (95% CI 4.45 to 5.87) compared to 4.40 (95% CI 3.91 to 4.75) log10 eCFU/mL in those without cavity (*p* = 0.080). Adjusting for bacterial load, initial killing rate, silicosis, chest cavity, HIV status, and gender, the hazard ratios (HR) for *M. tuberculosis* killing were 12.37 (95% CI 2.87 to 53.30; *p* = 0.001) and 14.31 (95% CI 3.49 to 58.65; *p* < 0.001) for patients who received an all-oral bedaquiline versus injectable bedaquiline-containing regimens, respectively (Table 8). Bacterial load at baseline correlated positively with median time to sputum conversion to negative measured by both TB-MBLA and culture (r = 0.48 [95% CI 0.18 to 0.69]; p = 0.003). High *M. tuberculosis* load and TB with silicosis were independent predictors of slow *M. tuberculosis* killing compared to low *M. tuberculosis* load and TB without silicosis (Table 8, p ≤ 0.033).
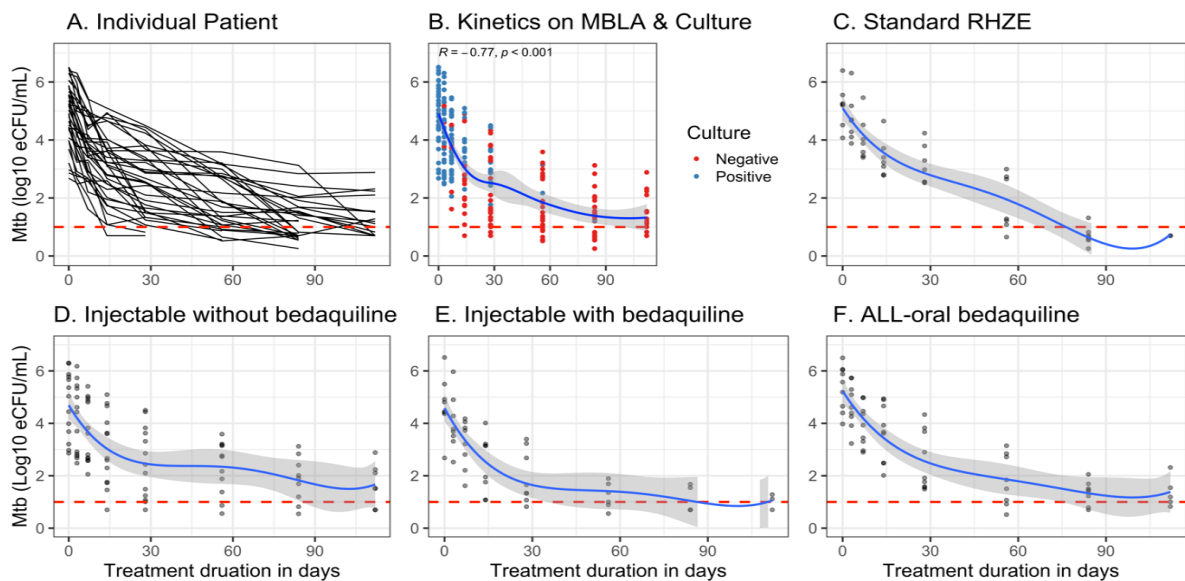
Figure 10:   *M. tuberculosis* **killing during the first 4 months of treatment with different anti-TB regimens**

The red dotted line denotes the cutoff value of a positive tuberculosis molecular bacterial load assay (MBLA). Standard RHZE is a fixed dose combination of rifampicin (R), isoniazid (H), pyrazinamide (Z) and ethambutol (E). *M. tuberculosis* (Mtb) quantity are reported in $\log_{10}$ of estimated colony forming unit per 1 mL ($\log_{10}$ eCFU/mL).
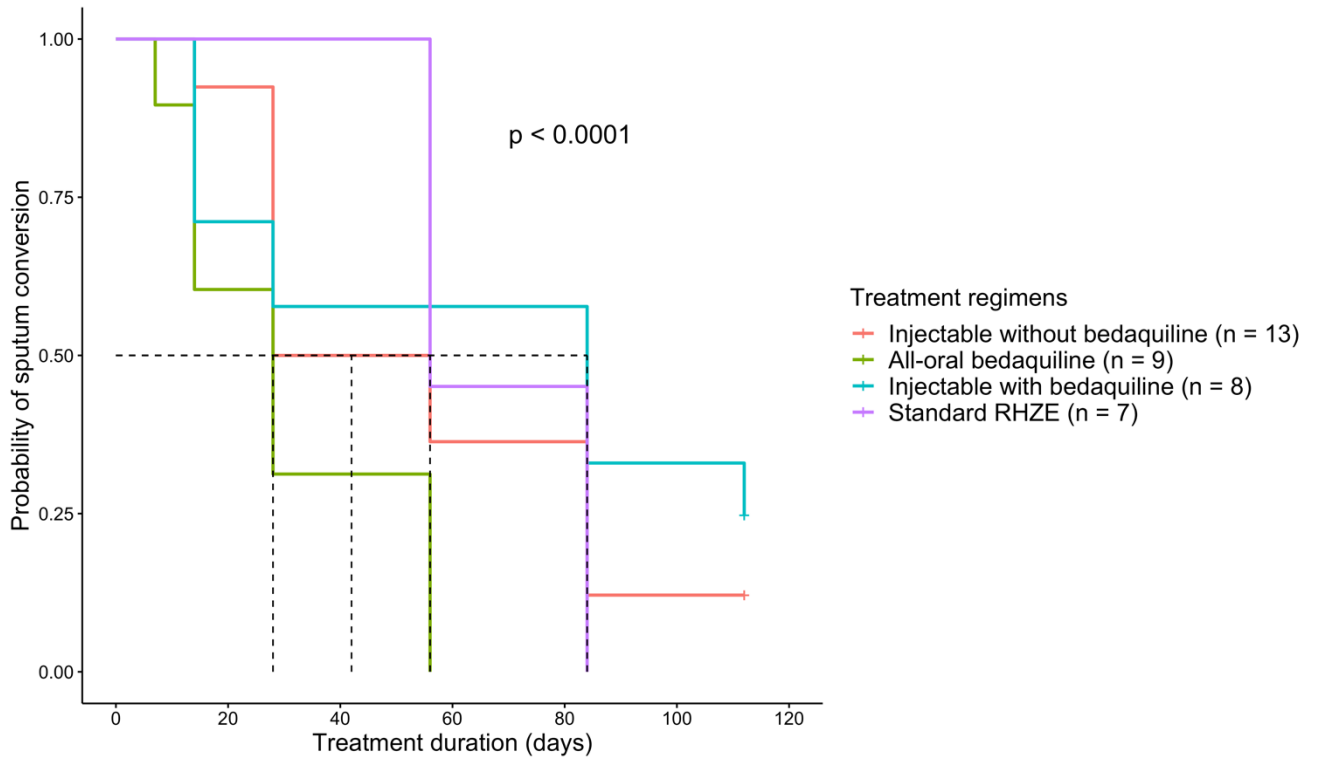
Figure 11: **Kaplain Kaplan-Meier curves showing median time to *M. tuberculosis* killing in patient sputum per treatment regimen**

The dotted lines denote the median time of sputum conversion from positive to negative.

Table 9: Mean daily *M. tuberculosis* killing rates (log$_{10}$ eCFU/mL) and corresponding burden at day 0 and 112 of treatment

Treatment regimens	Mean *M. tuberculosis* killing rates				Mean (95% CI) *M. tuberculosis* load	
	Unadjusted model for covariates		Adjusted model for covariates			
	Rates (95% CI)	p-value	Rates (95% CI)	p-value	Day 0 (baseline) [†]	Day 112 *
1. Reference (injectable-BDQ free)	-0.18 (-0.27 to -0.08)		-0.17 (-0.23 to -0.12)		4.73 (4.13 – 5.32)	2.77 (2.51- 3.04)
2. Injectable-bedaquiline	-0.48 (-1.25 to +0.28)	0.239	-0.62 (-1.05 to -0.20)	0.019	4.63 (3.95 – 5.47)	2.08 (1.81 - 2.36)
3. All-oral bedaquiline	-0.26 (-0.48 to +1.00)	0.507	-0.35 (-0.65 to -0.13)	0.054	5.36 (4.65 – 6.08)	2.47 (2.20 - 2.74)
4. Standard RHZE	-0.23 (-0.57 to +1.02)	0.593	-0.29 (-0.78 to +0.22)	0.332	5.17 (4.36 – 5.99)	2.51 (2.18 - 2.85)

[†]Baseline mean *M. tuberculosis* load in all regimens were comparable (ANOVA, p = 0.453). An asterisk (*) denotes p -values for mean difference in *M. tuberculosis* load for regimen pairwise comparison at day 112: regimen 1 & 2, p < 0.001; regimen 2 & 3, p = 0.031; regimen 1 & 3, p = 0.077; and regimen 2 & 4, p = 0.040. Reference regimen was the injectable-bedaquiline (BDQ) free regimen composed of kanamycin (KAN), levofloxacin (LFX), pyrazinamide (PZA), ethionamide (ETH) and Cycloserine (CS); Injectable-bedaquiline regimen was comprised of KAN, BDQ, LFX, PZA and ETH; All-oral bedaquiline regimen contained BDQ, LFX, linezolid (LZD), PZA and ETH; and the RHZE for rifampicin, isoniazid, PZA and ethambutol (E) Covariates adjusted included baseline bacterial load, cavity, gender, HIV and silicosis, *M. tuberculosis* killing rates varied among regimens
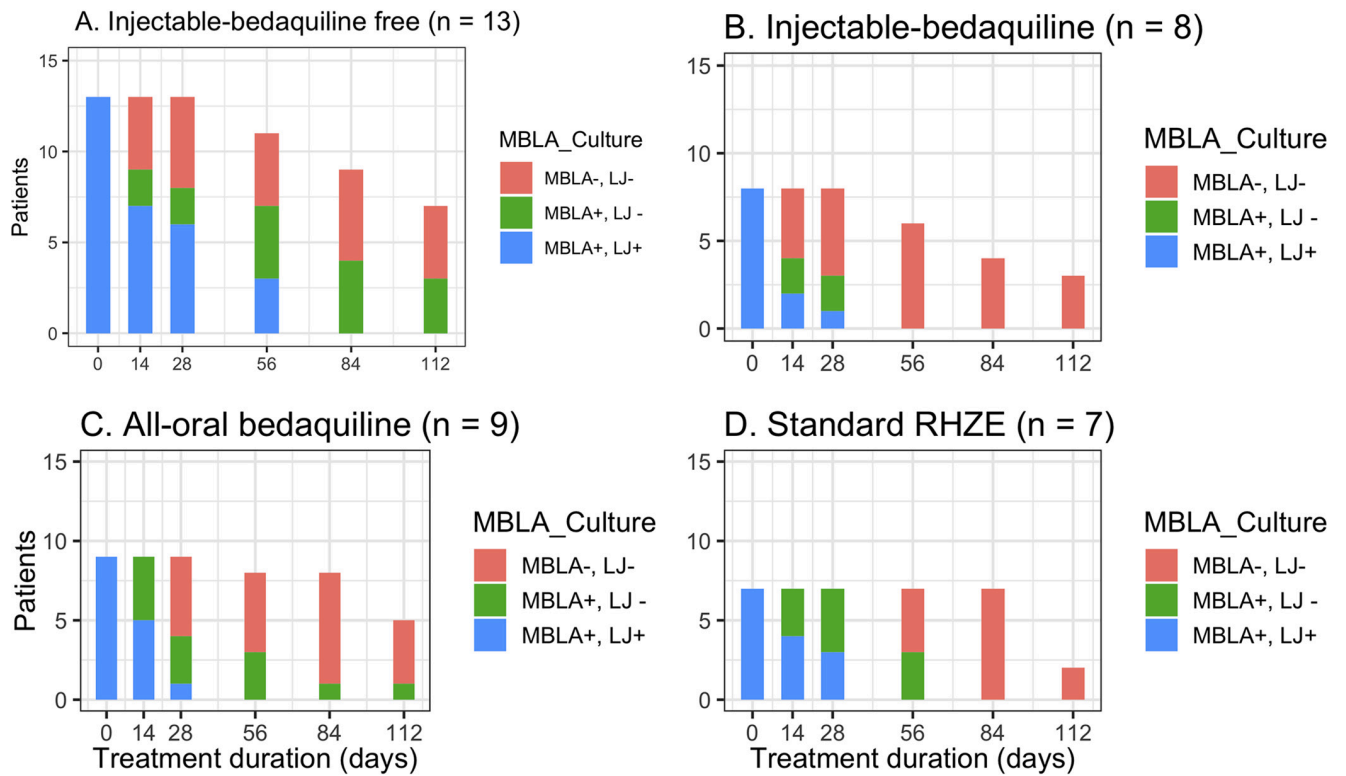
Figure 12: Number of patients who converted to negative by TB-MBLA and Lowenstein-Jensen culture during the first 4 months of treatment with different anti-TB regimens

The overall sputum conversion from positive to negative TB-MBLA and LJ culture results had the same trend in four different regimens. At recruitment (day 0), all 37 patients had positive results for TB by TB-MBLA and culture (MBLA+, LJ+). Both TB-MBLA and culture tests were negative (MBLA-, LJ) at days 56 and 84, respectively, in all patients on either injectable plus bedaquiline (B) or standard RHZE (D) composed of rifampin, isoniazid, PZA, and ethambutol. A total of 3 patients who received the injectable bedaquiline-free regimen (A), together with 1 patient on the all-oral bedaquiline regimen (C), remained TB-MBLA positive but culture negative (MBLA+, LJ-).

Table 10: Hazard ratio of *M. tuberculosis* killing in Cox Proportion-Hazard model

Predictor Variable	Unadjusted model		Adjusted model	
	HR (95% CI)	p-value	HR (95% CI)	p-value
Male gender	0.86 (0.40 – 1.85)	0.705	2.44 (0.82 – 7.24)	0.109
TB/Silicosis	0.20 (0.10- 0.88)	0.028	0.12 (0.03 – 0.49)	0.003
TB/HIV	2.26 (1.07 -4.77)	0.033	0.88 (0.31 – 2.50)	0.813
Cavitary disease	0.38 (0.17 - 0.86)	0.021	0.85 (0.17 – 2.70)	0.790
Positive chest x-ray	0.57 (0.17 – 1.88)	0.354	0.23 (0.03 – 1.62)	0.790
High *Mtb* load	0.72 (0.54 -0.97)	0.033	0.26 (0.13 – 0.54)	< 0.001
Retreatment	1.02 (0.51 - 2.05)	0.958	0.59 (0.24 – 1.44)	0.248
All-oral bedaquiline	1.58 (0.61 - 4.04)	0.344	12.37 (2.87 – 53.30)	0.001
Injectable-bedaquiline	4.63 (1.64 – 13.09)	0.004	14.31 (3.49 – 58.65)	< 0.001
Standard RHZE	1.43 (0.53 – 3.89)	0.482	3.25 (0.90 – 11.73)	0.072
High initial Mtb killing rate	5.96 (2.03 – 17.48)	0.009	4.81 (1.39 – 16.65)	0.013

All-oral bedaquiline regimen was comprised of Bedaquiline (BDQ), levofloxacin (LFX), linezolid (LZD), pyrazinamide (PZA) and ethionamide (ETH). Injectable-bedaquiline is a modified regimen comprised of kanamycin (KAN), BDQ, LFX, PZA and ETH. Standard RHZE included rifampicin (H), isoniazid (H), PZA and ethambutol (E). HR is hazard ratio

4.2   Discussion

This study deployed molecular technologies including the genotype MTBC, MTBDRplus/sl and WGS in clinical settings to identify the *M. tuberculosis* complex species and lineage, describe drug resistance associated mutations and compared to minimum inhibitory concentration of these drugs. Moreover, TB-MBLA was used to assess mycobactericidal effect of different anti-TB regimens among people treated for multidrug-resistant TB. Using these technologies, the study generated potential evidences that are useful in guiding the strategy for shortening and simplifying multidrug-resistant TB treatment. Importantly, a meta-narrative systematic review underscored that these technologies require minimal training skills, and infrastructures such as laboratory biosafety level 2 or 3 containment with biosafety cabinet class II (Mbelele *et al*., 2018). These attributes would argue for feasible application even in resource-poor settings like Tanzania. Subsequently, this will maximize health and well-being of patients.

When compared to genotype MTBDRplus reference method, the genotype MTBC achieved a 97% sensitivity in detecting *M. tuberculosis* complex from patient's direct sputa (Alipanah *et al*., 2019). This result complements an existing 93% sensitivity in detecting *M. tuberculosis* complex from smear-positive sputum and cultured isolates reported 10 years ago (Somoskovi *et al*., 2008). The genotype MTBC and other molecular methods like sequencing technologies have been validated for testing cultured isolates and smear-positive samples. Detecting *M. tuberculosis* complex from patient's sputa in this study provides a new insight into evading laborious work of culturing *M. tuberculosis* and culture-related chance of contamination (Hoza *et al*., 2015; Reddy *et al*., 2014). This approach would shorten duration of characterizing *M. tuberculosis* complex. Nevertheless, there was high discordant between line probe assays (genotype MTBC, MTBDRplus, and MTBDRsl) with Xpert® MTB/RIF assay. For example, about 1 in 3 patients with positive Xpert® MTB/RIF results tested negative for *M. tuberculosis complex* by any of the line probe assays and culture, compared to 20% from other studies (Bhardwaj *et al*., 2019). Under normal circumstances, culture should be more sensitive than Xpert® MTB/RIF assay as it was found in the current study and others (Rasheed *et al*., 2019). This high discordance between Xpert® MTB/RIF and other tests was also reposted in a clinical trial in which 34% of samples re-tested by Xpert® MTB/RIF had no TB (Ngabonziza *et al*., 2020; Variava *et al*., 2020). In keeping with previous studies, the performance of genotype MTBC improved in patients with high bacterial burden measured by Xpert® MTB/RIF at quantification cycle of ≤ 22, predominantly in patients with cavitary disease on chest radiograph, HIV negative and in smear positive samples (Murthy *et al*., 2018; Ong *et al*., 2014; Theron *et al*., 2016, 2018). These findings support the previous report that paucibacillary patients are associated with false-positive Xpert® MTB/RIF, and requiring a repeat test in this population (Ngabonziza *et al*., 2020; Variava *et al*., 2020).

Importantly, false-positive Xpert® MTB/RIF results may be a biomarker for the presence of a considerable proportion of patients with post-TB lung disease, being treated for MDR-TB. These two conditions need a different algorithm for diagnosis and treatment. Would supportive evidences be available, adding TB-MBLA into the testing algorithm may help to differentiate false-positive from true MDR-TB measured by Xpert® MTB/RIF assay. Furthermore, a differential diagnosis of non-tuberculous mycobacteria is considered in this post TB lung disease group and need to be worked-out. In this study, two species of non-tuberculous mycobacteria, the *M. intracellulare* and *M. kansansii,* were detected from two patients who were living with HIV/AIDS and histories of multiple episodes of TB treatment. *M.*

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intracellulare is considered an otherwise rare non-tuberculous mycobacteria species in Tanzania and sub-Sahara Africa. For instance, Mpagama *et al*., 2013, reported non-tuberculous mycobacterial cases in 6%, including the *M. intracellulare* in patients referred for treatment of MDR-TB in Tanzania (Mpagama *et al*., 2013). In a similar setting, application of whole-genome sequencing technology led to detection of *M. yongonese,* one of non-tuberculous mycobacteria species, in a patients treated for MDR-TB (Mnyambwa *et al*., 2018). Likewise, Fredrick *et al.* (2012*)* detected *M. intracellulare* from a cultured isolate of a non-HIV infected patient treated for drug-susceptible TB in Tanzania. These historical non-tuberculous mycobacteria cases are infrequently reported partly due to resource constraints or being neither a notifiable disease nor a priority condition under national TB programs. The previous cases and those report in this study do however suggest that the burden of non-tuberculous mycobacteria is larger than previously considered (Donohue, 2018; Hoza *et al*., 2016; Stout *et al*., 2016). In Tanzania, a survey of 65 patients treated for TB adenitis in rural areas reported infection with non-tuberculous mycobacteria in 50% of patients (Mfinanga *et al*., 2004) compared to 15% of cases reported in Zambia, one of the countries bordering Tanzania (Chanda-kapata *et al*., 2015). Together, these findings would argue and support for implementing population-survey to estimate its burden in these settings (Shahraki *et al*., 2015). Procedures undertaken for the first time in Africa to identify non-tuberculous mycobacteria species like *M. intracellulare* in this report from the patient's direct sputa as it was from Iran can be further scaled up to support population-surveys (Ahmed, 2020).

The *M. tuberculosis/canettii* was the only member of *M. tuberculosis* complex identified using the genotype MTBC assay from patients presumed for MDR-TB. Because of the close genetic relatedness, the genotype MTBC assay does not differentiate *M. tuberculosis* from *M. canettii*. Even with the recent 894 diverse genomes of *M. canettii* and major phylogenetic groups, the assay cannot address this assay's limitation (Loiseau *et al*., 2019). Using whole genome sequencing in this report, all *M. tuberculosis* complex were identified as *M. tuberculosis*, and therefore addressing the genotype MTBC technical limitations. This supports the argument that Whole genome sequencing has high discriminatory power compared to genotype MTBC and other genotyping methods (Loiseau *et al*., 2019; Somoskovi *et al*., 2008; Wyllie *et a*l., 2018). On the other hands, *M. tuberculosis* predominance in this study is similar to a prevalence surveys in sub-Sahara Africa, that at least 95% of TB was due to *M. tuberculosis*, and 2% due to *M. africanum* (Addo *et al*., 2017). These findings confirm prior epidemiological studies that had suggested the geographic distribution of *M. africanum* to be an important cause of TB in

West Africa, and rarely detected in East African countries, including Tanzania (Zumla *et al*., 2017).

The WGS compared favorably to phenotypic drug susceptibility testing using MIC values from the MycoTB assay for predicting resistance in MTBC, with notable exceptions in some drugs. For drugs such as rifampicin, rifabutin and isoniazid, genotypically non-wildtype had higher MIC values compared to wildtype isolates. Importantly, there were no mutations in genomic regions that confer resistance to bedaquiline, linezolid, fluoroquinolones, clofazimine and cycloserine which comprised the bulk of the R/MDR-TB treatment regimens (Coll *et al.,* 2018; Ramirez *et al.,* 2020). This finding supports the high WGS prediction rate of susceptible phenotypes measured by MycoTB or MGIT that were reported from Romania (Ruesen *et al.,* 2018) and Germany (Heyckendorf *et al.,* 2018). Moreover, previous reports from the same setting in Tanzania showed that genotypic and phenotypic resistance for fluoroquinolones is less common (Lyakurwa *et al.,* 2019; Mpagama *et al.,* 2013). For example, since 2009, there has been only one patient report with extensively drug resistant TB (Lyakurwa *et al.,* 2019). In contrast, the mutations in *gyrA* and *gyrB* genes previously known to confer phenotypic fluoroquinolone resistance in MTBC appear more common in other part of the world, particularly in countries like South Africa (Dheda *et al.,* 2017). Additionally, and given the use of a customized plate with a lower range of MIC values, this study shows that MTBC isolates frequently had lower MICs, often below the breakpoints for key drugs in the current empiric RR/MDR-TB treatment regimen.

The reasons for poor prediction of drug resistance in MTBC for certain drugs may be related to any of the nature of isolates tested, laboratory methods used to predict resistance including consensus definitions for MIC breakpoints, and the approaches to bioinformatics analysis. First, there were neither enough phenotypically nor genotypically resistant isolates to test for drugs such as levofloxacin, moxifloxacin, clofazimine and p-aminosalicylic acid in which there were no mutations to explain the elevated MICs.

Secondly; in keeping with previous studies, the resistance-associated mutations predicted by WGS and the level of phenotypic resistance set by MIC values on MycoTB assay in this study were often discordant for drugs such as moxifloxacin, ethionamide, aminoglycosides/cyclic peptides and ethambutol (Chen *et al.,* 2019; Foongladda *et al.,* 2016). Certainly, these discrepancies could be due to limitations related to the MycoTB assay (Schön *et al.,* 2019), given the absence of optimal and standardized criteria for interpreting and consensus definition

for MIC breakpoints (Faksri *et al.,* 2019; Ismail *et al.,* 2020). This assertion described by Schon *et al.* (2019) argues that the MycoTB assay contains MIC ranges that are unacceptable for these anti-TB drugs. The MIC ranges are either truncated at their lower-end relative to wild-type distributions or not defined for these drugs at all. Because of this suboptimal definition, prior MIC breakpoints used by Ruesen *et al.* (2018) in the MycoTB plate were likely too high for moxifloxacin (1.0 μg/mL) and amikacin (4.0 μg/mL) compared to ECOFF values published by Ismail *et al.* (2020) and which were used to interpret the MIC values in this study. Heyckendorf *et al.* (2018) employed phenotypic susceptibility in MGIT liquid and Löwenstein Jensen solid media over a more narrow range of concentrations. Higher breakpoints are likely to underestimate MTBC resistance phenotypes in drugs such as fluoroquinolones (Ängeby *et al.,* 2012; Schön *et al.,* 2019). Moreover, presence of hetero-resistance MTBC strains have been also reported to account for discrepancies (Nonghanphithak *et al.,* 2020). Deep amplicon sequencing which confers higher coverage of sequence data than the approach used here has been proposed to address discrepancies related to hetero-resistance strains (Jouet *et al.,* 2021; Operario *et al.,* 2017). While the WHO and others are considering endorsing the MIC testing in patients treated for MDR-TB, the variations in the breakpoints used by Ruesen *et al.* (2018). Heyckendorf *et al.* (2018) and those in the current findings for moxifloxacin continue to stress the importance of defining a consensus for breakpoints in all nature of MTBC isolates on the microdilution assays including MycoTB plate .

Thirdly, even in a situation where a consensus definition for MIC breakpoints is determined, discordances may also be explained by an incomplete catalogue of drug resistant mutations currently used to predict resistance from the genotype as has been shown before (Ngo & Teo, 2019; van Beek *et al.,* 2019), particularly the catalogues employed in semi-automated bioinformatics tools including the MTBSeq (Iketleng *et al.*, 2018; Kohl *et al.*, 2018). The tools have been mainly designed to capture high fidelity mutations (Feuerriegel *et al.*, 2015; Kohl *et al.*, 2018). Fortunately, collaborative databases are needed and initiatives like ReSeqTB or CRyPTIC have been formed to overcome these limitations (Starks *et al.,* 2015; The CRyPTIC Consortium and the 100, 2018). Moreover, the notable discrepancies could partially be attributed to the resistance associated variant discovery cutoff for WGS which was set at 5%. This threshold setting leads to missing all potential mutations present at lower frequencies. As sequencing technologies become more widely available, bioinformatics algorithms for tools such as MTBSeq and others need to be updated in real-time to capture all high and low-fidelity SNPs (Iketleng *et al.*, 2018; Kohl *et al.*, 2018).

Additionally, and given the use of a customized plate with a lower range of MIC values, this study shows that *M. tuberculosis* isolates frequently had lower MICs, often below the breakpoints for key drugs in the RR/MDR-TB treatment regimen. In Tanzania, and perhaps in other low MDR-TB burden countries where second-line drug-susceptibility testing capacity is currently limited, findings from the present and previous studies argue for a RR/MDR-TB regimen comprised of bedaquiline, clofazimine, levofloxacin with or without linezolid. The regimen can be modified when drug susceptibility testing results are made available for clinical decision. However, while MIC values for bedaquiline, delamanid and linezolid were not available to quantify potential phenotypic resistance, the *mmpL5* V193A & V222F (possible novel mutations for bedaquiline), *fbiC* G646R (possible novel mutation for delamanid), and *rplC* R38C (possible novel mutation for linezolid) were detected. Delamanid mutations, such as *fbiA* Arg321Ser and *fbiC* Trp678Gly in *M. tuberculosis* were absent in the present study. In settings where East-Asian lineage 2 is dominant such as in countries like China, India and South Africa (Freschi *et al.,* 2021; Rutaihwa *et al.,* 2019; Wu *et al.,* 2021), these mutations had previously been reported among patients with drug resistant and susceptible TB, respectively (Kardan-Yamchi *et al*., 2020). Similar to other findings, predominance of East-African-Indian lineage 3 and Euro-American lineage 4 in this report (Freschi *et al.,* 2021; Senghore *et al.,* 2020) which have low transmissibility, virulence, and drug resistance compared to East-Asian lineage 2 (Beijing family) may partly support the low RR/MDR-TB burden in Tanzania (Freschi *et al.,* 2021; Rutaihwa *et al.,* 2019; Wu *et al.,* 2021). Collectively, these demonstrate the need for continuous anti-drug resistance surveillance by WGS and phenotypic methods including the MycoTB assay (Suthar *et al*., 2018; World Health Organization, 2015a).

In a quest to address a challenge of monitoring treatment response, the current study has shown for the first time that the killing rates of *M. tuberculosis* in patients treated for RR/MDR-TB as well as those with concomitant TB/silicosis varies with treatment regimens. As measured by TB-MBLA, *M. tuberculosis* decreased significantly over time on treatment, and this kinetic correlated with what was observed using solid culture medium. Overall, there was rapid and prominent killing of *M. tuberculosis* at day 14 for patients who received kanamycin regardless of receipt of bedaquiline. However, superior activity of kanamycin containing regimens at day 14 had no long term-bactericidal effect. As a result, 3 patients on injectable containing but bedaquiline free regimen remained positive by TB-MBLA but negative culture after 4 months of treatment. On the other hand, patients who received an all-oral bedaquiline containing regimen achieved these rates of killing at or after 1 month of treatment. This observation

concurs with previous reports that the bactericidal activity of bedaquiline in patients treated for MDR-TB is delayed at the beginning, but accelerates later in therapy (Nguyen *et al.*, 2016). Usually, recovery of *M. tuberculosis* by TB-MBLA correlates better with MGIT liquid than Lowenstein-Jensen solid culture, which may partially explain the discrepancy between the two tests at month 4 of treatment (Sabiiti *et al.*, 2020). This argument supports previous findings that culturing *M. tuberculosis* on Lowenstein-Jensen solid media recovers a lower yield than in MGIT liquid culture (Diriba *et al.*, 2017). High yield on MGIT liquid culture and TB-MBLA is partially related to its low detection limit of *M. tuberculosis* at ≤ 10 CFU/mL compared to Lowenstein-Jensen solid medium which requires 10-100 CFU/mL bacilli (Honeyborne *et al.*, 2011a; Sabiiti *et al.*, 2020; van Zyl-Smit *et al.*, 2011). For decades, culture has been used as a routine microbiological tool for monitoring drug-resistant TB treatment response (Goletti *et al.*, 2018; Rockwood *et al.*, 2016). However, in many TB endemic settings, culture is unavailable or limited to specialized centers. Importantly, culture results can take up to 8 weeks from the time of sputum collection, which delays patient care if a treatment decision is made based on a result from a specimen collected two months earlier. Given the continued decentralization of RR/MDR-TB services in Tanzania and elsewhere, monitoring treatment response in laboratories capable of performing qPCR, such as with Xpert MTB/RIF, will allow laboratory assays to impact treatment decisions closer to the point-of-care. Moreover, these findings as measured by TB-MBLA in the present study fit with the pharmacodynamical understanding that kanamycin and other aminoglycoside/polypeptides if active against mycobacteria, primarily exert their effect against those extracellular organisms that are rapidly dividing and may be more abundant early in the treatment course (Krause *et al.*, 2016; Motta *et al.*, 2018) . Therefore, this study in RR/MDR-TB compliments the growing evidence base for the application of TB-MBLA in routine clinical management (Honeyborne *et al.*, 2011a, 2014; Sabiiti *et al.*, 2020).

Interestingly, the current findings suggest that bactericidal activity at day 14 may not be a suitable predictor for long-term efficacy of a regimen, particularly when that regimen is bedaquiline containing. In this cohort at day 14, more than 75% of people had a positive TB-MBLA and more than half had a positive culture result. Whereas between 14-56 days there was substantial *M. tuberculosis* killing in those treated with a bedaquiline containing regimens, suggesting that evaluation of bactericidal activity be performed later, such as at day 56, for modern RR/MDR-TB regimens. Using culture, one previous phase 2b clinical trial reported high bactericidal activity of a bedaquiline containing regimen in patients with drug-susceptible

and RR/MDR-TB (Tweed *et al*., 2019). However, detectable *M. tuberculosis* beyond day 56 in this study supports this trial's argument that day 56 is unreliable indicator of a regimen's ability to either predict a long term treatment outcomes or a shorten treatment duration (Tweed *et al*., 2019). This further raises the question of whether TB-MBLA may in fact be a superior predictor to culture.

The shorter overall time to sputum conversion to negative, as measured by TB-MBLA and conventional culture, for all patients who received bedaquiline regardless of kanamycin further supports arguments that bedaquiline should be a cornerstone of regimens designed to shorten MDR-TB treatment duration (Doan *et al*., 2018). The conventional injectable-containing but bedaquiline free regimen has been in practice for decades, even though more than 40% of patients treated with this regimen had unfavorable outcomes in TB endemic settings (WHO, 2019a). Aminoglycosides such as kanamycin are no longer part of the current MDR-TB treatment regimens not because of its lack of bactericidal activity, as this data would suggest the contrary in the early treatment period, but rather because of the significant toxicity and patient intolerances that led to treatment interruption (WHO, 2018a, 2019b). From a microbiological perspective alone, as demonstrated in this study and others (Mpagama *et al*., 2014), and in a more patient-centered approach however, these results demonstrate the potential importance of finding a tolerable substitutes for kanamycin that can match the early bactericidal effect.

This thesis has key strengths for policy implications in clinical practices. Foremost, it reports for the first-time clinical application of molecular methods such as genotype MTBC and Mycobacterium CM VER 2.0 assays in Africa to test and identify *M. tuberculosis* complex and non-tuberculous mycobacteria to the species level from direct sputa, respectively. Importantly, detection of *M. intracellulare* species in a patient who was treated multiple times as for MDR- and XDR-TB would have not been possible in absence of this study. Ultimately, the patient received appropriate treatment. Therefore, it supports integration of these assays into testing algorithms for screening non-tuberculous mycobacteria and optimal care. Secondly, not only that the study has added knowledge that WGS resistance associated mutations correlates with the MIC values of anti-TB drugs measured by MycoTB Sensitire plate, but also it updated the list of curated mutations in biological databases. This list is important to bioinformatic pipeline developers and scientists working in molecular TB diagnostics. Lastly, for the first time, the study reports application of TB-MBLA to model killing rates of *M. tuberculosis* among patients

with RR/MDR-TB and those with TB/silicosis. In sub-Sahara Africa, TB and Silicosis trends are increasing. For example in Tanzania, exposure to dust silica among miners presenting at health facility with symptoms and signs of TB plus radiological features suggestive of TB/Silicosis accounted for 24% in 2015 (Mpagama *et al.,* 2015) compared to 34% in the current study. This study has shown that patients with TB/silicosis had slower *M. tuberculosis* killing rates measured by TB-MBLA compared to those with TB but without silicosis. This slow rate of killing could partially be attributed to the underlying pulmonary pathophysiology which can include progressive massive fibrosis (Konečný *et al*., 2019; Skowroński *et al*., 2018), and a blunted local host immune response to *M. tuberculosis* infection (Konečný *et al*., 2019). A similarly slower rate of *M. tuberculosis* killing was observed among patients with RR/MDR-TB who had high initial bacterial load, which supplements previous studies of TB-MBLA kinetics from patients with drug sensitive TB (Honeyborne *et al*., 2011a, 2014; Sabiiti *et al*., 2020). In this study, approximately 1 and 4 out of 10 patients had respectively positive LJ-culture and TB-MBLA at day 56. This supports the previous argument that TB-MBLA is more sensitive compared to agar based Loewenstein Jensen culture, in which *M. tuberculosis* population gets lost due to decontamination procedures in the later (Mtafya *et al*., 2019).

This study has limitations. Recruiting more patients with multiple history of TB treatment rather that TB naïve may probably impacted discrepancy in detecting *M. tuberculosis* complex to the species level from unprocessed sputa by genotype MTBC as in previous reports (Jaworski *et al.,* 2018). Nonetheless these patients represent an important population at risk for acquiring drug resistance or other circulating strains given their exposure to the healthcare setting and as evidenced by detection of non-tuberculous mycobacteria in this population. Also, the MIC values were derived from samples of patients treated for RR/MDR-TB, that including fully susceptible *M. tuberculosis* isolates would have aided to establish local epidemiological cut-off values. Additionally, this would have helped determination of adequacy MIC range of the custom MycoTB plate. Moreover, there were no MIC testing for bedaquline, delamanid and linezolid to be associated with novel mutations and the level of phenotypic resistance. This limitation represents an important step to inform clinical decision when using these drugs in the new all-oral MDR-TB treatment regimen. Lastly, bactericidal activities of anti-TB regimens were monitored for 4 months only, such that predicting long-term treatment success was not possible. Nevertheless, modelling *M. tuberculosis* killing for 4 months accomplished here has been used as a biomarker for treatment failure and relapse in several observational studies (Ahmad *et al*., 2018; Goletti *et al*., 2018). However, this duration exceeds the follow

up time for monitoring treatment that has been used in other trials of RR/MDR-TB regimens (Tweed *et al*., 2019). Yet, these previous trials have employed conventional culture based techniques, which are more laborious and prone to contamination (Hoza *et al*., 2015; Reddy *et al*., 2014). In addition, the number of patients per treatment regimen was small that findings on bactericidal activities of anti-TB regimens should be cautiously inferred to other RR/MDR-TB populations. Nevertheless, a longitudinal cohort design allowed control of variabilities between patients and as well as tracking within-person regimen's bactericidal activities over time (Guo *et al*., 2013; Schober & Vetter, 2018).

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1   Conclusion

This thesis generated evidence on application of molecular diagnostics to support the design of regimen for optimal clinical management of patients treated for RR/MDR-TB.   Overall findings reported here provide insight into formulating an optimal all-oral regimen for treating RR/MDR-TB at a shorter duration but with optimal outcome. Specific conclusions drawn from this study include the following; First, all forms of TB including the multidrug resistant TB are mainly caused by *M. tuberculosis*, and it mainly belonged to the lineage 3 (East-Africa-Indian, e.g. Delhi-CAS) and 4 (Euro-American: e.g. LAM). This information facilitates clinical decision when designing intervention to simplify treatment compared to if a mixture of species or if the *M. tuberculosis* belonged to lineage 2 (East-Asian, or Beijing family), the highly resistant and pathogenic lineage. Importantly, using directed sputa instead of previous validation in cultured isolates, the *M. tuberculosis* complex was identified to the *M. tuberculosis* by the genotype MTBC, with a high concordance to the genotype MTBDRplus or MTBDRsl and culture.   Use of direct sputum by the genotype MTBC evades technical challenges of culture including delays of results and chance for contamination, which both delays clinical decision. However, the genotype MTBC performed well with samples containing high bacterial load, and it lagged behind the yields by the Xpert® MTB/RIF assay, accounting for 29% discordant with other additional tests

 Secondly; non-tuberculous mycobacteria species, such as *M. intracellulare* and *M. kansasii* are the commonest culprit in patients with multiple history of TB treatment, particularly in people living with HIV/AIDS and may complicate patient's care if it co-exists with RR/MDR-TB. These species and historical cases reported fosters clinician's high suspension index in order to simply patient's care. Co-existence of non-tuberculous mycobacteria and MDR-TB compromise patient's health-outcomes.

Thirdly; WGS drug resistant associated mutation were comparable to the level of resistance measured by MIC. Mutant isolates had high MIC values compared to nonmutants (genotypically wildtype). The tested M. *tuberculosis* isolates were genotypically wildtype to the potential drugs used to treated multidrug resistant TB, and therefore supports their central use in RR/MDR-TB treatment.

Fourthly; *M. tuberculosis* RNA is a potential biomarker for monitoring treatment response in patients with MDR-TB. Bedaquiline containing regimens were efficacious. Patients who received bedaquiline-containing regimens exhibited higher *M. tuberculosis* killing-rates and had shorter time to TB-MBLA and culture conversion to negative. Higher Mycobacterial efficacy was achieved when bedaquiline was combined with injectable aminoglycoside, which is not part of the proposed MDR-TB regimens.

5.2   Recommendations

The high discordances between Xpert® MTB/RIF and other tests such as the genotype MTBC v1.x, and MTBDRplus, MTBDRsl, and culture particularly in patients with low bacterial burden requires further management. Clinical evaluation of this discordance may include repeating the Xpert® MTB/RIF and withholding anti-TB medications while monitoring patient's clinical response. Importantly, integrating TB-MBLA, a biomarker for viable *M. tuberculosis* into TB testing algorithm may discerns active TB from post-tuberculosis complications, thereby avoid misdiagnosis. Further research to identify biomarkers that are capable of delineating active and dead bacilli along with phenotypic TB culture are also recommended.

Findings from this thesis recommends integration of non-tuberculous mycobacteria testing into the national TB program. The genotypes Mycobacterium CM assay or whole genome sequencing can be used if available. This will avert miss diagnosis and treatment of historical non-tuberculosis mycobacterial cases reported in this study and others in Tanzania, and elsewhere (Mnyambwa *et al.,* 2018; Mpagama *et al.*, 2013; Shahraki *et al.*, 2015) .

These findings recommend use of empirical all-oral MDR-TB regimen comprised of fluoroquinolones, bedaquiline, delamanid, linezolid and clofazimine and the regimen can be modified when MIC testing results are made available for clinical decision. Despite absence of drug-resistance associated mutations on genes conferring resistance to key MDR-TB drugs, routine drug resistant TB surveillance by WGS & MIC is recommended. MIC testing can also be extended to patients who achieve culture conversion to negative beyond 2 months of therapy.

For the high *M. tuberculosis* killing rate of a regimen containing bedaquiline and injectable aminoglycosides, and given that injectables are no longer used, findings from this study recommend evaluation of other candidate drugs which have high early bactericidal activity.

Such drugs include moxifloxacin and high dose levofloxacin which act synergistically with bedaquiline.

Lastly, findings from this study recommend deployment of TB-MBLA for monitoring of RR/MDR-TB treatment response. Together with drug susceptibility testing by MIC for example, TB-MBLA can be used in clinical settings to guide clinician in regimen adjustments. Additionally, TB-MBLA can also be deployed in clinical trials particularly those assessing an all oral bedaquiline based regimens.

Findings of this study have provided an avenue for future research in various areas of diagnosis and monitoring tuberculosis treatment. The proposed research agenda include;

(a) Validating TB testing algorithm for diagnosis, designing regimens and monitoring treatment in patients with low or very low bacterial load using TB-MBLA

(b) Finding & evaluation a drug with early bactericidal activities like Moxifloxacin which will synergy Bedaquiline's efficacy

(c) Application of TB-MBLA beyond 4 months in monitoring response to detect long term MDR-TB treatment outcome, such as failure, and or relapse

(d) To describe *M. tuberculosis* killing kinetics measured by TB-MBLA and compare with other biomarkers such as pharmacokinetics/pharmacodynamic and MIC of anti-TB drugs., with ultimate aim for dose adjustment in presence of anti-TB drug susceptibility testing results.

(e) To compare the WGS resistance associated mutations with MIC of anti-TB drugs including bedaquiline, delamanid, linezolid, clofazimine among patients with drug pan-susceptible and resistant TB.

(f) Validation of genotype MTBC for detecting Mycobacteria to species and genotype Mycobacterium CM for detecting NTM from sputa.

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APPENDICES

Appendix 1: Ethical clearance Certificate

THE UNITED REPUBLIC OF TANZANIA

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NIMR/HQ/R.8a/Vol. IX/2662

Ministry of Health, Community
Development, Gender, Elderly & Children
University of Dodoma,
Faculty of Arts and Social Sciences
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40478 Dodoma

29th December 2017

Dr Peter Masunga Mbelele
Nelson Mandela-African Institution
of Science and Technology (NM-AIST)
P. O. Box 447, Arusha
ARUSHA

RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled:Molecular Diagnostics Approach for Optimizing Clinical Management of Multidrug-Resistant Tuberculosis in Tanzania (Mbele, PM*et al)*hasbeen granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health, Community Development, Gender, Elderly & Children and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Site: Kibong'oto Infectious Diseases Hospital, Siha District,Kilimanjaro Region.

Approval is valid for one year: 29th December 2017 to 28thDecember 2018.

Name: Prof. Yunus DaudMgaya

Signature
**CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE**

CC: RMO of Kilimanjaro Region
       DMOs/DEDs of Siha District

Name: Prof. Muhammad Bakari Kambi

Signature
**CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY
DEVELOPMENT, GENDER, ELDERLY
&CHILDREN**

RESEARCH OUTPUTS

(i) Journal Articles

Mbelele, P. M., Sauli, E., Mpolya, E. A., Mohamed, S. Y., Addo, K. K., Mfinanga, S. G., Heysell, S. K., & Mpagama, S. (2021). TB or not TB? Definitive determination of species within the Mycobacterium tuberculosis complex in unprocessed sputum from adults with presumed multidrug-resistant tuberculosis. *Tropical Medicine & International Health*, 26(9), 1057-1067. doi: 10.1111/tmi.13638

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(ii) Conference Paper

(iii) Poster Presentations