Faculty of Pharmaceutical, Biomedical and Veterinary Sciences

Department of Biomedical Sciences

Identifying and addressing the gaps impeding fast and accurate phenotypic drug-susceptibility testing of *Mycobacterium tuberculosis*

Het identificeren en oplossen van lacunes die een snelle en nauwkeurige fenotypische antibiotica gevoeligheidstest voor *Mycobacterium tuberculosis* belemmeren

Dissertation for the degree of doctor in Biomedical Sciences at the University of Antwerp to be defended by Rupasinghe Arachchige Praharshinie Rupasinghe

Promotors

Prof. Dr. Leen Rigouts

Prof. Dr. Bouke de Jong

Antwerp 2024

Self-published by Rupasinghe Arachchige Praharshinie Rupasinghe, Antwerp, Belgium.

Members of the Jury

Chair

Prof. Dr. Erik Fransen	University of Antwerp, Belgium

Promoters

Prof. Dr. Leen Rigouts	University of Antwerp, Belgium
Prof. Dr. Bouke de Jong	Institute of Tropical Medicine, Antwerp, Belgium

Members

Prof. Dr. Paul Cos	University of Antwerp, Belgium
Dr. Vanessa Mathys	Sciensano, Brussels, Belgium
Dr. Véronique Fontaine	Vrije Universiteit Brussel, Belgium
Prof. Dr. Frank Kooy	University of Antwerp, Belgium

Table of Contents

List of tables

List of figures

Abbreviations

7H9	Middlebrook 7H9
AFB	Acid fast bacilli
AIDS	Acquired immunodeficiency syndrome
Amk	Amikacin
AST	Antimicrobial susceptibility testing
ATU	Area of technical uncertainty
BCCM	Belgian Coordinated Collections of Microorganisms
BCG	Bacillus Calmette–Guérin
Bdq	Bedaquiline
BMD	Broth microdilution
BPaL	Bedaquiline, pretomanid and linezolid
BPaLM	Bedaquiline, pretomanid, linezolid and moxifloxacin
C	Capreomycin
CB	Clinical breakpoint
CC	Critical concentration
CFU	Colony forming units
Cfz	Clofazimine
CLSI	Clinical & Laboratory Standards Institute
CO_2	Carbon dioxide
CRA	Colorimetric reduction assay
CRyPTIC	Comprehensive Resistance Prediction for Tuberculosis
Cs	Cycloserine
Dlm	Delamanid
DMSO	Dimethyl sulfoxide
DS	Drug-susceptible
DR	Drug-resistant
DR-TB	Drug-resistant tuberculosis
DST	Drug-susceptibility testing

E	Ethambutol
ECOFF	Epidemiological cut-off value
EPTB	Extra-pulmonary tuberculosis
Eth	Ethionamide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FQ	Fluoroquinolones
GC1	100% growth control
GC2	1% growth control
gDST	Genotypic drug-susceptibility testing
Gfx	Gatifloxacin
GU	Growth units
H	Isoniazid
H37Rv	*Mycobacterium tuberculosis* H37Rv reference strain
HIV	Human immunodeficiency virus
IGRA	Interferon-gamma release assay
ITM	Institute of Tropical Medicine
K	Kanamycin
L	Lineage
LAMP	Loop-mediated isothermal amplification
Lfx	Levofloxacin
LJ	Löwenstein-Jensen
LPA	Line Probe Assay
Lzd	Linezolid
M	*Mycobacterium*
M7H10	Middlebrook 7H10
M7H11	Middlebrook 7H11
MDR	Multidrug-resistant tuberculosis
Mfx	Moxifloxacin
MGIT	Mycobacteria Growth Indicator Tube

MIC	Minimum inhibitory concentration
MODS	Microscopic observation drug-susceptibility
MTBc	*Mycobacterium tuberculosis* complex
NAAT	Nucleic acid amplification test
NALC	N-acetyl L-cysteine
NaOH	Sodium hydroxide
NRA	Nitrate reduction assay
NRL	National reference laboratory
NTM	Non-tuberculous mycobacteria
OADC	Oleic acid, albumin, dextrose, catalase
Ofx	Ofloxacin
OR	Odds ratio
Pa/PA	Pretomanid
PANTA	Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin
PAS	Para aminosalicylic acid
PCR	Polymerase chain reaction
PD	Pharmacodynamic
pDST	phenotypic drug-susceptibility testing
PK	Pharmacokinetics
Pre-XDR	Pre-extensively drug-resistant tuberculosis
PTB	Pulmonary tuberculosis
QC	Quality control
R	Resistant/Resistance
REMA	Resazurin microtiter plate assay
Rif	Rifampicin
Rpt	Rifapentine
RR	Rifampicin-resistant
S	Susceptible/Susceptibility
SRL	Supranational reference laboratory

Str	Streptomycin
TAT	Turnaround time
TB	Tuberculosis
TLA	Thin layer agar
tNGS	Targeted next generation sequencing
TST	Tuberculin skin test
W	Week
WGS	Whole genome sequencing
WHO	World Health Organization
WRD	WHO-recommended rapid diagnostic
XDR	Extensively drug-resistant tuberculosis
Z	Pyrazinamide

Definitions

Critical concentration (CC) = the critical concentration is the lowest concentration of an anti-tuberculosis (TB) agent that will inhibit *in vitro* growth of 99% of phenotypically wild-type strains of the *M. tuberculosis* complex (MTBc) (*Reference: 62, Chapter 1*).

Clinical Breakpoint (CB) = the concentration of an antimicrobial agent, which defines a minimal inhibitory concentration (MIC) above the critical concentration that separates strains that will likely respond to treatment from those that will likely not respond to treatment (*Reference: 52, Chapter 1*).

Epidemiological cut-off (ECOFF) = within a species it is the highest concentration of the drug lacking phenotypically expressed resistance. The wild type is presented as WT ≤ z µg/ml and non-wild type as NWT > z µg/ml (*Reference: European committee on antimicrobial susceptibility testing*)

Multi drug resistant tuberculosis (MDR-TB) = TB disease caused by an MTBc strain that is resistant to rifampicin and isoniazid (*Reference: 52, Chapter 1*).

Pre-extensively drug resistant tuberculosis (pre-XDR-TB) = TB disease caused by an MTBc strain that is resistant to rifampicin (and may also be resistant to isoniazid), and that is also resistant to at least one fluoroquinolone (either levofloxacin or moxifloxacin) (*Reference: 52, Chapter 1*).

Rifampicin-resistant tuberculosis (RR-TB) = TB disease caused by an MTBc strain that is resistant to rifampicin. These strains may be susceptible or resistant to isoniazid (MDR-TB), or resistant to other first-line or second-line TB medicines (*Reference: 52, Chapter 1*).

Extensively drug-resistant tuberculosis (XDR-TB) = TB disease caused by a MTBc strain that is resistant to rifampicin (and may also be resistant to isoniazid), and that is also resistant to at least one fluoroquinolone (levofloxacin or moxifloxacin) and to at least one other “Group A” drug (bedaquiline or linezolid) (*Reference: 52, Chapter 1*).

Summary

In 2022, nearly half a million people worldwide were infected with multi-drug-resistant (MDR) or rifampicin-resistant (RR) tuberculosis (TB) resulting in an estimated 160,000 fatalities. Approximately 18% of these MDR/RRTB cases exhibited additional resistance to fluoroquinolone (pre-XDR TB). In 2022, estimated treatment coverage for MDR/RRTB was far below optimal, with just 43% of individuals who developed the disease receiving proper treatment. If proper surveillance, diagnosis, and treatment are not accelerated, nearly 75 million people will develop drug-resistant (DR) TB by 2050, posing a significant amount of human suffering and a burden on the global economy.

To date, for the majority of anti-TB drugs, phenotypic drug-susceptibility testing (pDST) remains the gold standard. However, owing to the time-consuming pDST methods that require sophisticated infrastructure and skilled labor, genotypic DST (gDST) methods are increasingly used. The most accessible rapid gDST methods only rule in resistance as they have a limited sensitivity except for rifampicin and do not cover the novel and re-purposed drugs. More advanced versions of the WHO-endorsed targeted next generation sequencing (tNGS) assays such as Genoscreen Deeplex Myc-TB XL assay are being evaluated to include more resistance associated genes linked to novel and re-purposed drugs. However, regardless of the sequencing technique with which polymorphisms are detected, the interpretation of gDST results will remain a persistent challenge, particularly for drugs targeting non-essential genes, such as nitroimidazoles, for which thousands of different loss-of-function mutations can theoretically confer resistance. Whole-genome sequencing (WGS) provides a more comprehensive view of the entire genome, yet still relies on *Mycobacterium tuberculosis* (MTB) culture isolation. The specialized bioinformatics tools compare each WGS against a WHO catalogue to interpret the results. For classic TB drugs WGS analysis has high accuracy for the detection of resistance, much less so for newer and repurposed drugs with a more diverse and less well-defined genetic basis of resistance. Thus, even countries that have implemented routine WGS cannot entirely abandon pDST.

During my PhD research, we investigated the factors impeding the fast and accurate detection of phenotypic drug resistance, and whether the broth microdilution-based minimum inhibitory

concentration (MIC) testing, recently endorsed by the World Health Organization (WHO), provides advantages relative to traditional pDST.

pDST relies on growth inhibition of MTBc in/on drug-containing culture media. The WHO recommends several culture media for performing pDST of MTBc. Each of these media has its specific critical concentration (CC) for distinct anti-TB drugs. DST failure or false-susceptible results due to lack of growth/slow growth, particularly in fastidious MTBc isolates, is one factor impeding fast and accurate pDST results in MTBc, more or less across all recommended media. However, there is no consensus on which medium should be preferred for such isolates. In **Chapter 3** We compared two commonly used agar media for pDST in MTBc, Middlebrook 7H10 and Middlebrook 7H11 for the occurrence of invalid results and their turnaround time (TAT) to yield interpretable results. Our data suggests that Middlebrook 7H11 reduces both the occurrence of DST failure due to lack of growth and TAT compared to Middlebrook 7H10 medium, while maintaining DST accuracy. Such comparisons of media allow laboratories to make educated decisions regarding the choice of media.

MTBc strains can exhibit varying degrees of resistance to anti-TB drugs, ranging from low- to high-level resistance. However, conventional culture-based DST methods typically categorize strains as either resistant or susceptible based on predefined drug- and medium-specific CCs. This binary classification may overlook strains with borderline or low-level resistance, which are nevertheless important to identify for guiding appropriate treatment regimens. In **Chapters 4 and 5,** we assessed the current WHO-recommended CCs for two key anti-TB drugs (classes), fluoroquinolones and rifampicin, in the Mycobacterial Growth Indicator Tube (MGIT960) system, the most used pDST medium worldwide, including low and middle-income settings. Targetted sanger sequencing of the canonical genes or WGS was used as the reference standard in both analyses. For both rifampicin and fluoroquinolones, the current MGIT-CCs misclassified the mutants associated with borderline resistance or low-level MIC increase, emphasizing the necessity of MIC testing to properly detect such resistance around the CC. However, MIC testing in MGIT or solid media requires a separate slant or tube for each concentration and the drug used making the process labor intensive and costly.

In addition, current knowledge on the CCs for anti-TB drugs is largely biased toward globally dominant MTBc lineages L2 and L4 and not based on sufficient data to accurately define the epidemiological cut-off value (ECOFF) for each drug. In **Chapter 6**, we demonstrated the MTBc

lineage-dependent differences in intrinsic susceptibility to pretomanid, a novel anti-TB drug, used in the now-preferred all-oral regimen for treating RR-TB comprising bedaquiline, pretomanid and linezolid (complemented with moxifloxacin) (BPal(M). This study further underlines the importance of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) requirements to consider MIC data from phylogenetically diverse MTBc strains when defining the cut-offs.

The current gaps in CC-based pDST for the MTBc underscore the urgent need for its standardization ensuring consistency and reliability in DST results across different laboratories and settings. The endorsement by the WHO in 2022 for MIC testing of the MTBc using the 7H9 medium-based broth microdilution (BMD) method, along with interim cut-offs for key anti-TB drugs, represents a significant step towards standardization in pDST for TB. In **Chapter 7**, we validated this method, integrating it with an automated digital dispenser for filling plates with serial dilutions of the drugs, normalized for the solvent. Our results show the method can be successfully used to determine the MICs of the MTBc and quantify the level of resistance, however, further optimization of some areas, such as standardizing inoculum preparation, plate reading and interpretation of pinpoint growth, is crucial for refining the method and ensuring its robustness and reliability in different laboratory settings. Additionally, exploring the use of freshly grown MGIT cultures as a basis for BMD inoculum, as opposed to standard inoculum preparation from cultures grown on solid medium, could lead to faster TATs and increased field-friendliness, and hence addressing some of the challenges associated with traditional pDST methods. Our preliminary data (**Chapter 8**) are encouraging in this regard.

Moving forward, continued efforts to promote the widespread adoption of MIC testing using standardized BMD methods, as endorsed by the WHO, will be essential for enhancing the quality and reliability of pDST for MTBc and ultimately improving TB treatment outcomes globally.

Samenvatting

In 2022 werden wereldwijd bijna een half miljoen mensen geïnfecteerd met multiresistente (MDR) of rifampicineresistente (RR) tuberculose (tbc/TB), met naar schatting 160.000 dodelijke slachtoffers tot gevolg. Ongeveer 18% van deze MDR/RRTB-gevallen vertoonde daarbovenop fluoroquinolonen resistentie (pre-XDR TB). Naar schatting was de behandelingsgraad voor MDR/RRTB ver onder het optimum: slechts 43% van de personen die de ziekte ontwikkelden, kreeg de juiste behandeling. Als de tijdige detectie van tuberculose, de diagnose van resistentie en de juiste behandeling niet worden versneld, zullen tegen 2050 bijna 75 miljoen mensen geneesmiddelenresistente tbc ontwikkelen.. Dit zal leiden tot veel menselijk leed en een zware belasting voor de wereldeconomie.Tot op heden zijn fenotypische antibioticagevoeligheidstesten (pDST) nog steeds de gouden standaard voor de meeste medicijnen tegen tbc. Vanwege de tijdrovende pDST-methoden die een aangepaste infrastructuur en opgeleid personeel vereisen, worden genotypische DST-methoden (gDST) steeds vaker gebruikt. De meest toegankelijke en snelle gDST-methoden kunnen alleen resistentie bevestigen, omdat ze, met uitzondering van rifampicine, niet gevoelig genoeg zijn om resistentie uit te sluiten. Daarnaast zijn deze genetische technieken nog niet geschikt voor de nieuwe geneesmiddelen of de oudere geneesmiddelen die opnieuw worden ingezet voor de behandeling van MDR-TB.Meer geavanceerde versies van door de Wereldgezondheidsorganisatie (WGO) goedgekeurde doelwitgerichte sequencingstests (tNGS), zoals de Genoscreen Deeplex Myc-TB XL-test, worden momenteel geëvalueerd. Deze versies zijn bedoeld om meer genen te detecteren die in verband worden gebracht met resistentie tegen nieuwe en opnieuw gebruikte geneesmiddelen. Ongeacht de sequentietechniek waarmee genetische varianten worden gedetecteerd, blijft de interpretatie van gDST-resultaten een grote uitdaging. Dit voornamelijk voor geneesmiddelen zoals nitroimidazolen die gericht zijn tegen niet-essentiële genen waarin theoretisch duizenden verschillende mutaties met functieverlies van de gecodeerde eiwitten kunnen voorkomen. Sequentiebepalingen van het volledige genoom (WGS) geven een vollediger beeld van genetische varianten, maar is tot op heden enkel mogelijk vanaf in vitro gekweekte *Mycobacterium tuberculosis* (MTB) bacillen. Gespecialiseerde bio-informaticatools vergelijken de bekomen WGS data met een WGO-catalogus om de resultaten te interpreteren. Voor klassieke tbc-medicijnen heeft WGS-analyse een hoge nauwkeurigheid voor de detectie van

resistentie, maar veel minder voor nieuwere en hergebruikte geneesmiddelen die een meer diverse en minder duidelijk gedefinieerde genetische basis van resistentie vertonen. Dus zelfs landen die routinematig WGS hebben geïmplementeerd, kunnen pDST niet helemaal weglaten.

Tijdens mijn onderzoek onderzochten we de factoren die een snelle en nauwkeurige detectie van fenotypische geneesmiddelenresistentie in de weg staan, en of de op vloeibaar medium (7H9) gebaseerde microverdunningstest voor de bepaling van de minimaal inhiberende concentratie (MIC), die onlangs werd goedgekeurd door de WGO, voordelen biedt ten opzichte van de traditionele pDST.

pDST is gebaseerd op groeiremming van MTBc in/op antibioticabevattende kweekmedia. De WGO beveelt verschillende kweekmedia aan voor het uitvoeren van pDST van MTBc. Elk van deze media heeft een specifieke kritische concentratie (CC) voor verschillende anti-tbc medicijnen. Het mislukken van DST's of het voorkomen van vals-gevoelige resultaten door gebrek aan groei of een te lage groei, is één de factoren die snelle en nauwkeurige pDST-resultaten bij MTBc in de weg staan, vooral bij moeilijk groeiende MTBc-isolaten, en dit voor de meeste van de aanbevolen media. Er is echter geen consensus over welk medium de voorkeur verdient voor dergelijke isolaten. In **Hoofdstuk 3 hebben** we twee veelgebruikte agar-media voor pDST van MTBc, namelijk Middlebrook 7H10 en Middlebrook 7H11, vergeleken op het voorkomen van ongeldige resultaten en hun doorlooptijd (turn-around-time, TAT) om interpreteerbare resultaten op te leveren. Onze gegevens suggereren dat 7H11 zowel het optreden van DST-falen door gebrek aan groei als de TAT vermindert in vergelijking tot 7H10, terwijl de DST-nauwkeurigheid behouden blijft. Dergelijke vergelijkingen van media stellen laboratoria in staat om weloverwogen beslissingen te nemen over de keuze van het te gebruiken medium.

MTBc-stammen kunnen verschillende niveaus van resistentie tegen anti-tbc medicijnen vertonen, variërend van een laag tot een hoog niveau van resistentie. Conventionele DST-methoden op basis van kweek categoriseren stammen echter meestal als resistent of gevoelig op basis van vooraf gedefinieerde geneesmiddel- en mediumspecifieke CC's. Deze binaire classificatie kan stammen met grenswaarden of een laag niveau van resistentie over het hoofd zien. Desalniettemin is het belangrijk om deze te identificeren om een aangepast behandelingsschema's te kunnen voorstellen. In **Hoofdstukken 4 en 5** hebben we de huidige door de WGO aanbevolen CC's voor twee belangrijke (klassen van) anti-tbc middelen, namelijk

de fluoroquinolonen en rifampicine, geëvalueerd in het Mycobacterial Growth Indicator Tube (MGIT960)-systeem, het meest gebruikte pDST-medium wereldwijd, zowel in hoge- als lage- en middelinkomst landen. Gerichte Sanger sequentiebepaling van de canonieke genen of WGS data werden gebruikt als referentiestandaard in beide analyses. De huidige MGIT-CC's voor zowel rifampicine als fluoroquinolonen classificeerden de mutanten die geassocieerd werden met borderline resistentie of een kleine verhoging van de MIC-waarde verkeerd, wat de noodzaak van MIC-testen benadrukt om dergelijke resistentie rond de CC-waarde goed te detecteren. Voor het bepalen van de MIC-waarde in het MGIT960 systeem of in vaste voedingsbodems is het echter nodig om voor elke concentratie van het te testen geneesmiddel een extra buis toe te voegen, waardoor het proces arbeidsintensief en kostbaar is.

Bovendien is de huidige kennis over de concentraties die effectief zijn tegen tuberculosemiddelen voornamelijk gebaseerd op de wereldwijd dominante MTBc-stammen L2 en L4. Er is echter onvoldoende gevarieerde data van de verschillen MTBc-stammen om de epidemiologische grenswaarde (ECOFF) voor elk geneesmiddel nauwkeurig vast te stellen. In **Hoofdstuk 6** hebben we laten zien dat er verschillen zijn in de natuurlijke gevoeligheid voor pretomanide tussen verschillende stammen van MTBc, afhankelijk van de MTBc-stam waartoe ze behoren. Pretomanide is een nieuw anti-tbc middel dat gebruikt wordt in het aanbevolen volledig orale behandelregime voor MDR/RR-TB, namelijk BPal(M), dat bestaat uit bedaquiline, pretomanide, linezolid en eventueel moxifloxacineDit onderzoek benadrukt verder het belang van de vereisten van het European Committee on Antimicrobial Susceptibility Testing (EUCAST) om MIC-gegeves van fylogenetisch diverse MTBc-stammen in overweging te nemen bij het bepalen van de cut-offs.

De huidige tekortkomingen in de concentratiegebaseerde fenotypische geneesmiddelenresistentietests (pDST) voor Mycobacterium tuberculosis complex (MTBc) benadrukken de nood om deze methoden te standaardiseren. Dit is essentieel om de consistentie en betrouwbaarheid van de DST-resultaten in verschillende laboratoria en omstandigheden te waarborgen. De goedkeuring door de Wereldgezondheidsorganisatie (WGO) in 2022 voor het gebruik van MIC-tests op MTBc met de op 7H9-gebaseerde bulkmicroverdunningsmethode (BMD), samen met tijdelijk voorgestelde afkapwaarden voor de belangrijkste anti-tuberculosemedicijnen, is een belangrijke stap in de richting van standaardisatie van pDST voor tuberculose. In **Hoofdstuk 7** hebben we de BMD-methode

gevalideerd en geïntegreerd met een geautomatiseerde digitale dispenser, waarmee platen worden gevuld met seriële verdunningen van de geneesmiddelen, inclusief normalisatie voor de oplosmiddelen van de geneesmiddelen. Onze resultaten tonen aan dat de methode succesvol kan worden gebruikt om de minimale inhiberende concentraties (MIC-waarden) van MTBc-isolaten te bepalen en het resistentieniveau te kwantificeren. Toch is verdere optimalisatie van enkele aspecten, zoals de standaardisatie van de inoculumbereiding, plaataflezing en interpretatie van puntgroei, essentieel om de methode te verfijnen en de robuustheid en betrouwbaarheid ervan in verschillende laboratoriumomgevingen te waarborgen. Bovendien zou onderzoek naar het gebruik van vers gekweekte MGIT-culturen als basis voor het enten van BMD-platen, in plaats van de standaard inoculumvoorbereiding van culturen gekweekt op vast medium, kunnen bijdragen aan snellere doorlooptijden en een betere toepasbaarheid in veldomstandigheden. Dit zou enkele van de uitdagingen van traditionele pDST-methoden kunnen verhelpen. Onze voorlopige gegevens (**Hoofdstuk 8**) zijn in dit opzicht veelbelovend.

In de toekomst zullen verdere inspanningen nodig zijn om de brede implementatie van MIC-tests met gestandaardiseerde BMD-methoden, zoals aanbevolen door de WGO, te bevorderen. Dit is essentieel om de kwaliteit en betrouwbaarheid van pDST voor MTBc te verbeteren en uiteindelijk wereldwijd betere resultaten in de behandeling van tuberculose te bereiken.

CHAPTER 1

Introduction

1.1 Tuberculosis

Tuberculosis (TB), historically known as consumption, wasting disease and the white plague, has long been a significant public health concern (1-3). While estimates of the time and geographical origin of the *M. tuberculosis* complex (MTBc) remain uncertain, one of the oldest known TB cases was identified in the remains of a woman found on the Atlit-Yam archaeological site, located on the northern coast of Israel, which dates back to the Neolithic era, approximately 9,000 years ago (4, 84).

In 2022, 7.5 million newly confirmed TB cases were recorded, the highest number for a single year since the World Health Organization (WHO) started worldwide TB monitoring in the mid-1990s (5) (**Figure 1.1**). TB caused 1.30 million fatalities in 2022, a decrease compared to the TB mortality during the SARS-CoV-2 pandemic, yet returning to pre-pandemic levels and far removed from the endTB strategy's 2025 milestone of reducing 75% of TB fatalities between 2015 and 2025 (5, 6) (**Figure 1.2**). Despite decades of research, and ongoing vaccine trials such as the M72/AS01E vaccine in phase 3 testing, a vaccine that efficaciously protects adults from getting TB does not exist yet. Thus, TB remains a prominent cause of mortality and morbidity in the world, ranking second only to SARS-CoV-2 in 2022 as the highest cause of death from a single infectious agent (5, 7, 8).

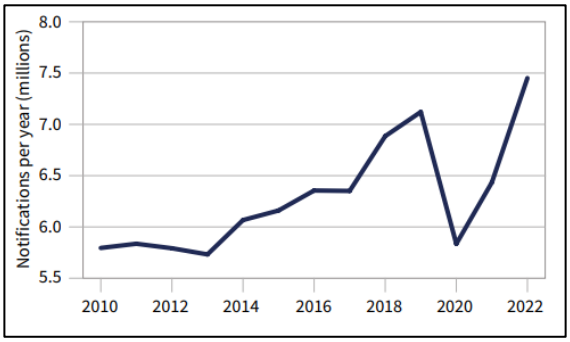


Figure 1.1: Global trend in case notifications of number of people newly diagnosed with TB, 2010–2022

Source: Global Tuberculosis Report 2023 (5)
(5)

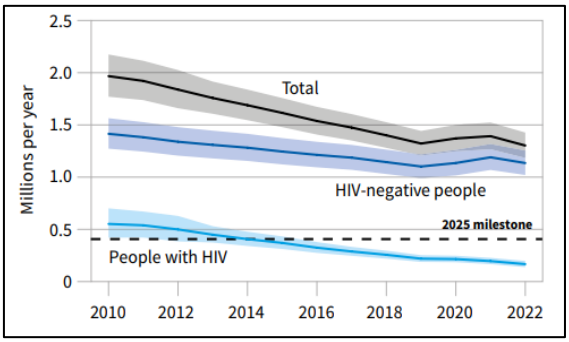


Figure 1.2: Global trend in the estimated deaths caused by TB, 2010-2022.

Source: Global Tuberculosis Report 2023

Shaded areas represent 95% uncertainty levels.

1.2 Etiology of TB

On March 24, 1882, Dr. Robert Koch, a German physician and microbiologist, announced *Mycobacterium tuberculosis* (MTB), as the bacterium that causes TB, to the Physiological Society of Berlin (9). MTB is a member of the MTBc, that comprises nine other members of the genus *Mycobacterium*, namely *M. bovis*, *M. microti*, *M. africanum*, *M. pinnipedii*, *M. caprae*, *M. canettii*, and three newly described members, *M. orygis*, *M. mungi* and *M. suricattae* (10-13). While all MTBc members have been found to cause TB in humans (14-18), MTB and *M. africanum* are the primary cause of TB in humans, with MTB being the most common globally (19, 20).

1.3 Phylogeography of the MTBc

Among the MTBc members, there are ten human-adapted lineages: MTB lineages 1-4 and 7-8, *M. africanum* lineages 5,6,9 and 10 (21-23). These lineages are obligate pathogens of humans and humans are their primary reservoir with occasional spill over to animals such as ferrets and cats, etc. (24).

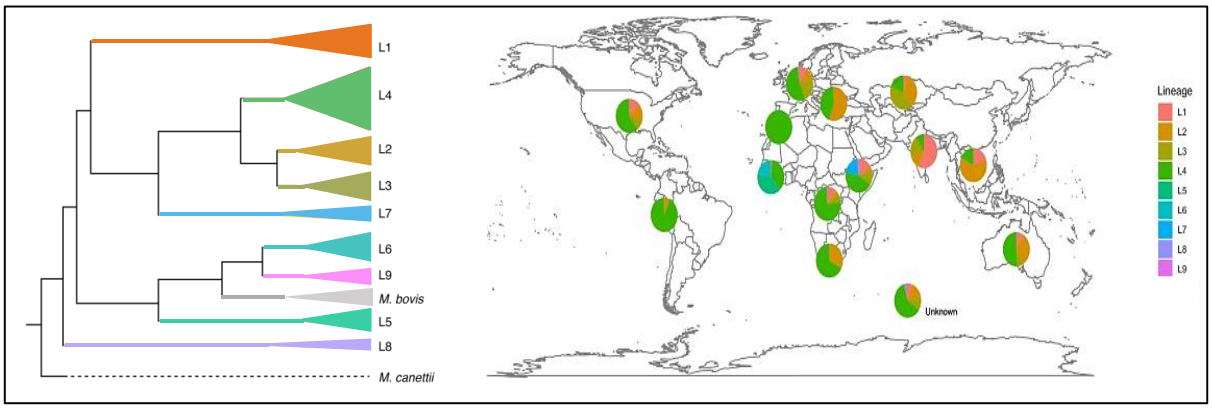


Figure 1.3: Phylogeny and global distribution of the MTBc lineages 1-9 (22, 25)

The geographical distribution of the ten human-adapted lineages varies significantly, with some lineages showing a global distribution while others are geographically restricted (**Figure 1.3**). Africa is the only continent where all ten lineages are present.

Lineage 2 (also known as the East-Asian lineage) and lineage 4 (also known as the Euro-American lineage) occur worldwide. Lineage 1, an 'ancestral' MTB lineage (also known as the

Indo-Oceanic lineage) occurs around the Indian Ocean, and, lineage 3 (also known as Central-Asian Strain) is found in parts of East Africa, Central and South Asia (26). Lineages 5 (also known as West African 1 lineage) and 6 (also known as West African 2 lineage) are highly restricted to West Africa while lineage 7 occurs almost exclusively in Ethiopia (27, 28). The more recently identified lineage 8 was found in Africa's Great Lakes region, lineage 9 in Djibouti and Somalia, and lineage 10 in Central Africa (21-23), with only occasional occurrence of strains from lineages 8, 9 and 10.

1.4 Transmission and pathogenesis of tuberculosis

TB is transmitted from person to person by breathing infected aerosol droplets generated by an individual with active pulmonary TB. In approximately 70% of people, when inhaled bacteria reach the alveoli, alveolar macrophages engulf and break them down without leading to an infection, while in 20-30% of exposed people, bacteria can reproduce and cause an infection (25) (**Figure 1.4**). It is estimated that nearly 20-25% of the world population is infected with *M. tuberculosis*, however, ~90% of people infected with MTBc never develop active disease (29). Instead, their immune system controls the infection, they remain immunoreactive to anti-TB antigens but show no clinical or radiological evidence of the disease. Until recently, it was believed that the bacteria remain in the body of these individuals resulting in latent TB and approximately, 10% of those with latent TB develop active TB during their lifetime, usually within the first five years of infection (30) and for those with a weakened immune system due to HIV-AIDS, malnutrition, immunosuppressive medications, etc, this risk is ~7-10% each year (31-33). However, more recent studies suggest these individuals' immune system clears out the infection retaining only the immunological memory and the minority who develop active TB, do so mostly during the first two years following the infection (34, 35).

TB can affect various parts of the human body, leading to different clinical presentations. TB is classified into two types based on the place of infection: pulmonary TB (PTB) and extrapulmonary TB (EPTB). PTB, which primarily affects the lungs, is the most frequent, accounting for approximately 80% of reported TB cases globally. EPTB, on the other hand, refers to cases where TB affects organs or sites other than the lungs. EPTB can occur almost anywhere in the human body, most commonly found in lymph nodes, pleura, bones, genitourinary

system, meninges, gastrointestinal system, etc. About one out of every five TB cases presents as EPTB (36, 37). Independent risk factors for EPTB include young age, HIV infection, female gender, and Asian-African ethnicity (38). Patients can have both PTB and EPTB; such cases are classified as PTB.

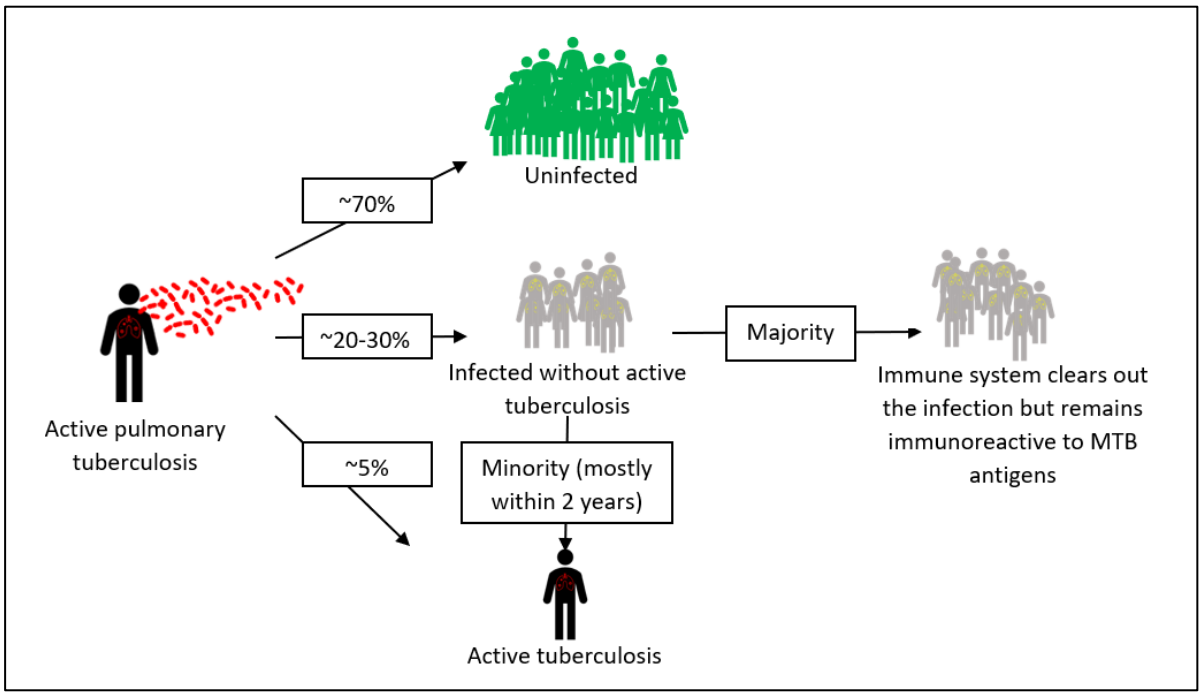


Figure 1.4: TB transmission, infection, and progression to disease

1.5 Treatment for TB

In 1943, Waksman and his team isolated streptomycin from the soil bacterium *Streptomyces griseus*. Streptomycin was the first effective antibiotic for the treatment of TB (39). Prior to the availability of streptomycin, the main treatment for TB involved rest and isolation in sanatoriums, where patients were encouraged to spend time in fresh air and sunlight (40, 41). Patients who had streptomycin injections had their sputum temporarily cleared of TB bacilli, but despite ongoing treatment, they began excreting the bacilli again, this time a more hazardous type that was streptomycin resistant (42). Within the next few decades, with the advent of various other anti-TB drugs, combination therapy became the norm for TB treatment (43). Despite the fact that combination therapy significantly improved TB treatment, the emergence of resistance to two key first-line drugs, isoniazid, and rifampicin, in the 1980s marked the onset of multidrug-resistant TB (MDR-TB) and posed a significant challenge to TB control efforts (44). To treat MDR-TB, second-line drugs such as fluoroquinolones, thioamides

and the injectable drugs of aminoglycosides and cyclic peptides were required. Second-line drugs often have more side effects, and their use requires careful monitoring. The prolonged treatment duration and complexity of the regimens make adherence challenging for patients. In 2010, *Van Deun et.al* published the first highly effective short course regimen against MDR-TB, ushering in a new age of shorter, more effective treatments with less severe side effects (45).

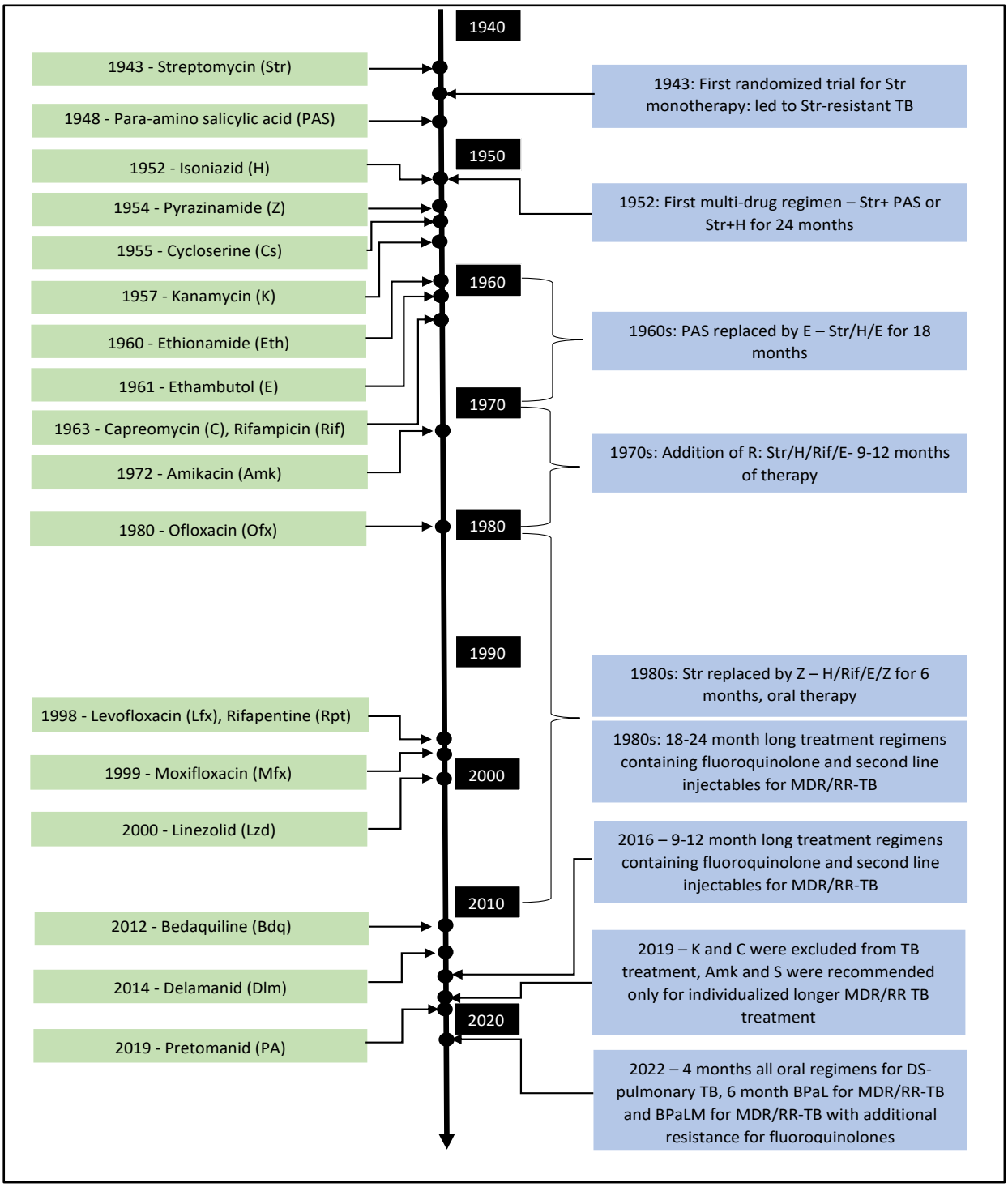


Figure 1.5: Evolution of anti-TB drugs and treatment regimens (54, 55)

In the early 2000, reports began to surface about extensively drug-resistant MTBc (XDR-TB) strains, in those days defined as resistant to isoniazid, rifampicin, fluoroquinolones, and aminoglycosides (46, 47). Treating XDR-TB was even more challenging due to the limited availability of effective drugs, the increased risk of adverse effects, and the need for prolonged treatment regimens. In 2012, marking the end of a four-decade-long TB drug discovery void, a new drug called bedaquiline was developed. Subsequently, in 2014 delamanid was approved, followed in 2019 by pretomanid, providing new hopes to treat drug-resistant TB (DR-TB) (48-50).

Historically, TB treatment required prolonged regimens, often lasting six to nine months for drug-susceptible (DS-) TB and as long as 24 months for DR-TB. With advancements in drug development and treatment optimization, shorter-course all-oral regimens have reduced DS-TB treatment to four months (not yet widely implemented) and MDR/Pre-XDR TB to six months (**Figure 1.5**). The 2019, WHO recommendation to exclude the second line injectables such as kanamycin and capreomycin from short-course MDR/RR-TB treatment as well as classifying amikacin and streptomycin as group C drugs necessitated a re-definition of XDR-TB. Thus in 2020, the WHO redefined XDR-TB as resistance to fluoroquinolones and at least to one group A drug in addition to rifampicin and isoniazid (51) (**Table 1.1**). Treatment options for XDR-TB are still limited, requiring prolonged use of toxic drug combinations, often without DST results, and success is far from guaranteed (52, 53).

Drug group	Drug included
Group A	Levofloxacin/Moxifloxacin, Bedaquiline, Linezolid, Pretomanid*
Group B	Clofazimine, Cycloserine/Terizidone
Group C	Ethambutol, Delamanid, Pyrazinamide, Imipenem/Meropenem, Ethionamide/Protionamide, Amikacin/Streptomycin, Para-amino salicylic acid

Table 1.1: Current list of drugs recommended by the WHO for treating MDR-TB (52)

Group A = Include all drugs in the regimen
Group B = Add one or both drugs
Group C = Add to complete the regimen and when medicines from Groups A and B cannot be used
Group B and C drugs are not applicable for the BPal(M) regimens

* Pretomanid is currently not included in any of the drug groups, however, as Pa is mandatory in BPaL(M), here we grouped it under Group A. BPaL(M) = Bedaquiline, Pretomanid, Linezolid (Moxifloxacin)

Different anti-tuberculosis drugs target various aspects of *Mycobacterium tuberculosis* biology, including protein synthesis, nucleic acid synthesis and inhibition of cell wall synthesis (**Figure 1.6**). For some drugs, the mechanisms of action have not been fully identified.

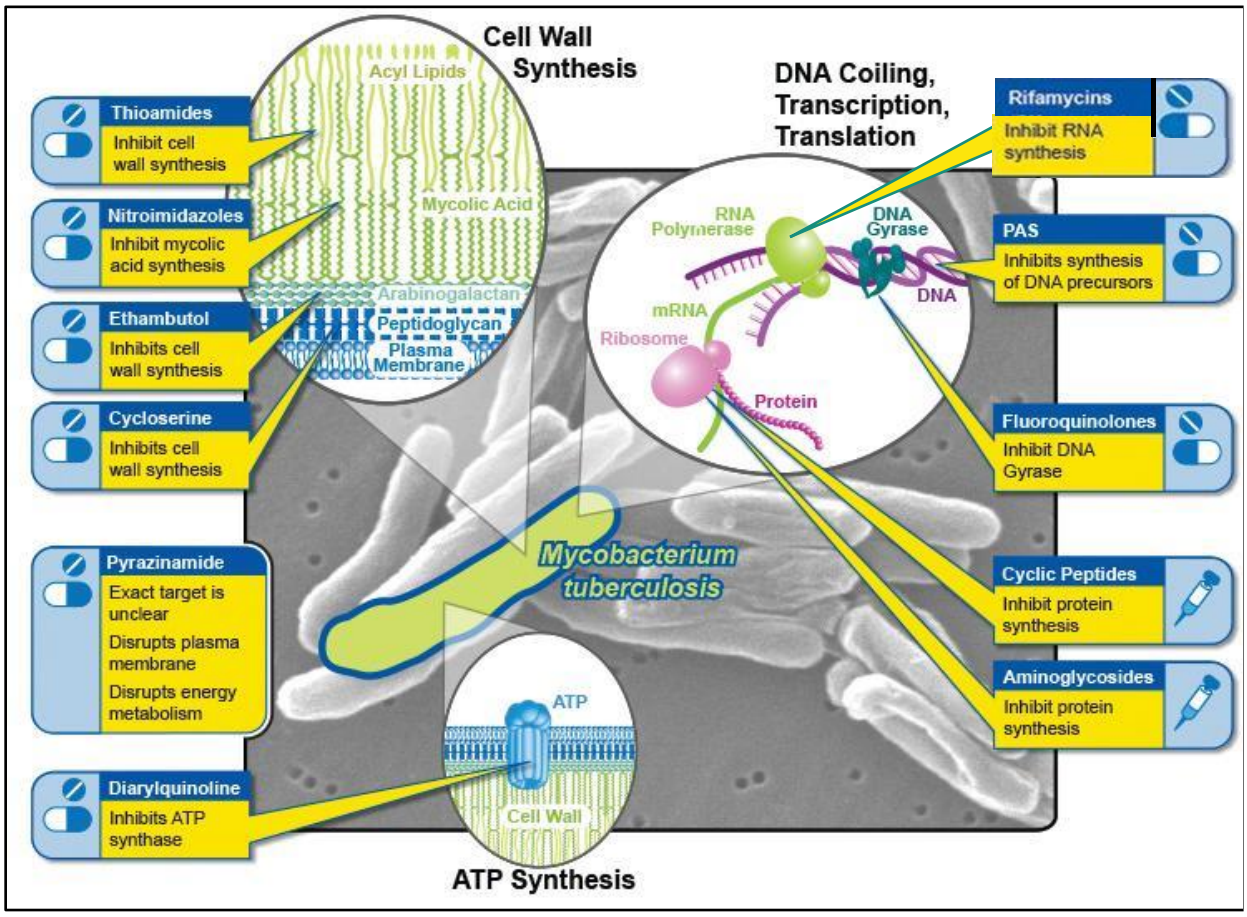


Figure 1.6: Mechanisms of actions of some anti-TB drugs

Source: National Institute of Allergy and Infectious Diseases

1.6 Diagnosis of TB and its drug susceptibility

1.6.1 Clinical diagnosis

The diagnostic cascade for TB begins with the identification of presumptive TB cases, patients who exhibit signs and symptoms compatible with TB. Possible PTB symptoms include cough, typically lasting more than two weeks, possibly with hemoptysis, and constitutional symptoms like fever, significant weight loss, and night sweats. Patients with EPTB have constitutional symptoms and may have infection site-specific symptoms (**Figure 1.7**) (56, 57).

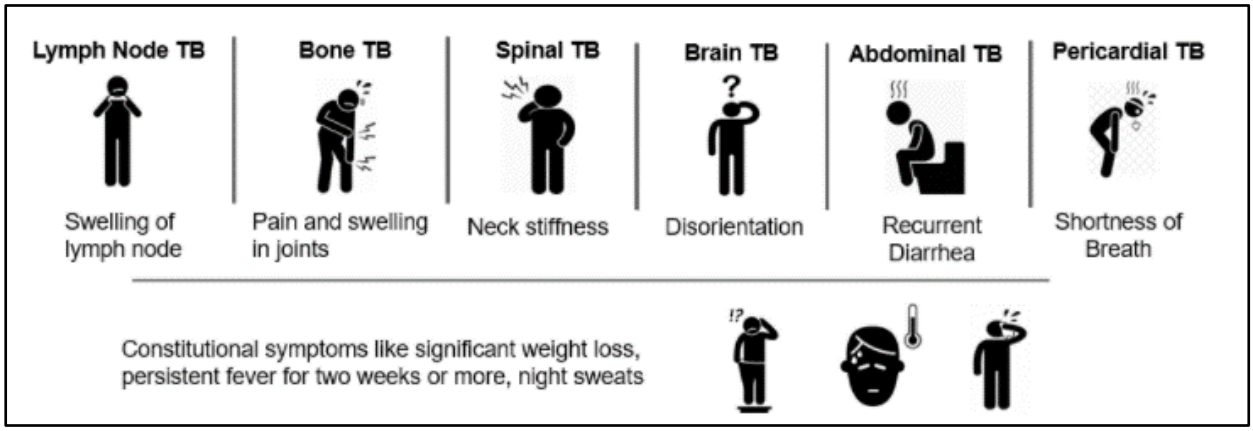


Figure 1.7: Site-specific symptoms of EPTB.

Source: https://ntep.in/node/540/CP-presumptive-ep-tb (58)

1.6.2 Radiological diagnosis

Imaging studies, particularly chest X-rays, are commonly used for TB diagnosis. Typical findings include pulmonary infiltrates, cavities, and lymphadenopathy. However, chest X-rays alone may not provide a definitive diagnosis and require correlation with clinical and laboratory findings (57).

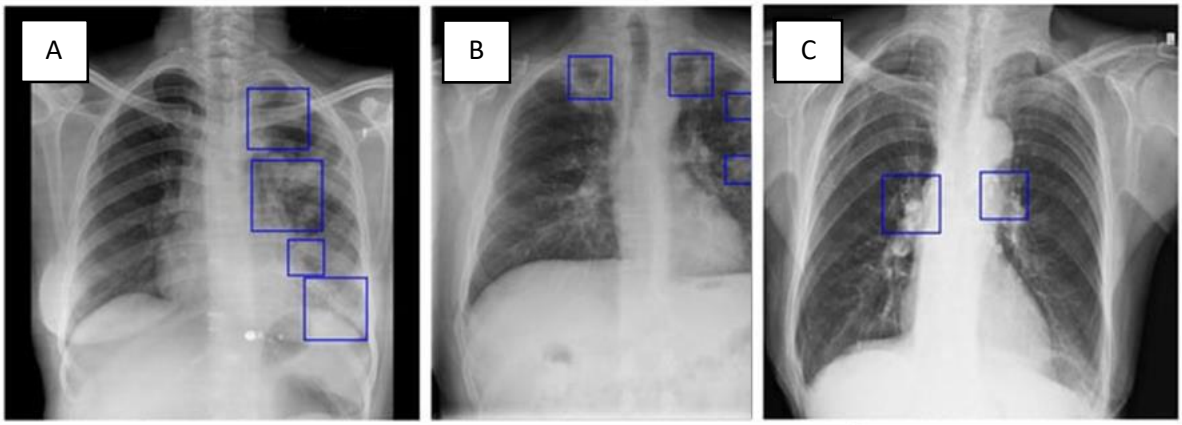


Figure 1.8: Examples of chest X-ray findings suggesting pulmonary tuberculosis.

The following are indicated by the blue boxes: A- Enlarged hilar and mediastinal lymph nodes, B- A thick-walled cavitary lesion in the left upper lobe, C- Bilateral apical thick-walled cavities and multifocal satellite air space opacities.

1.6.3 Immunological assays

The tuberculin skin test (TST) and interferon-gamma release assays (IGRA) are the only immunological procedures approved by the WHO for TB diagnosis. However, because individuals whose infection was cured after treatment have been reported to remain immunoreactive to TB antigens for years, responsiveness to TB antigens does not imply an ongoing TB infection or persistent TB, but rather signals being infected with TB without reflecting an outcome (34, 35). Thus these tests are not recommended for use in settings with high TB burden (59).

IGRA measures the host-immune response to TB-specific antigens using blood samples, while the TST involves injecting a small amount of TB antigen under the skin and measuring the immune response. One of the main advantages of the IGRA is that it does not cross-react with the Bacillus Calmette-Guerin (BCG) vaccine or some NTM infections, which may cause false-positive results with the TST, particularly in high-TB burden countries. Furthermore, the IGRA test requires only one visit, while the TST requires multiple visits to read and interpret the results.

1.6.4 Microbiological diagnostic methods

Rapid and accurate diagnosis of MTBc and its drug susceptibility is key for effective patient-centered management. In recent years, traditional methods for detecting mycobacteria have been continuously improved, and considerable efforts have been made for the development of new methods. Despite these advances, TB and its drug susceptibility are still missed or diagnosed late. In 2022, an estimated 30% of people with tuberculosis were undiagnosed, thus untreated and only 47% of the patients diagnosed received a rapid molecular test as the initial test, as recommended by the WHO, while the rest were diagnosed by sputum smear microscopy (5).

1.6.4.1 Sputum smear microscopy

Smear microscopy is one of the oldest and simplest procedures for detecting TB bacilli, and it is still employed as an initial diagnostic test, especially in resource-limited settings, as well as for

treatment monitoring across the world. Expectorated sputum is stained using different acid-fast staining methods such as Ziehl-Neelson, Kinyoun, or auramine-rhodamine fluorochrome staining. The staining procedure involves a phenol-containing primary stain that, when heated, penetrates the mycolic acid-rich mycobacterial cell wall, and remains even after acid-alcohol decolorization (**Figure 1.9**).

Even though sputum smear microscopy is fast, cost-effective, and simple enough to be performed, even in settings with rudimentary facilities, it has relatively a low sensitivity with a limit of detection of 5000-10,000 bacilli/ml and a 60% to 70% detection rate for pulmonary TB compared to culture (44). This technique detects all members of the genus *Mycobacterium*, thus the presence of acid-fast bacilli (AFB) in a sputum smear does not confirm presence of MTBc, even though very likely in TB-high endemic settings. In addition, smear microscopy cannot distinguish drug-resistant from drug-susceptible bacteria, nor live from dead bacteria.

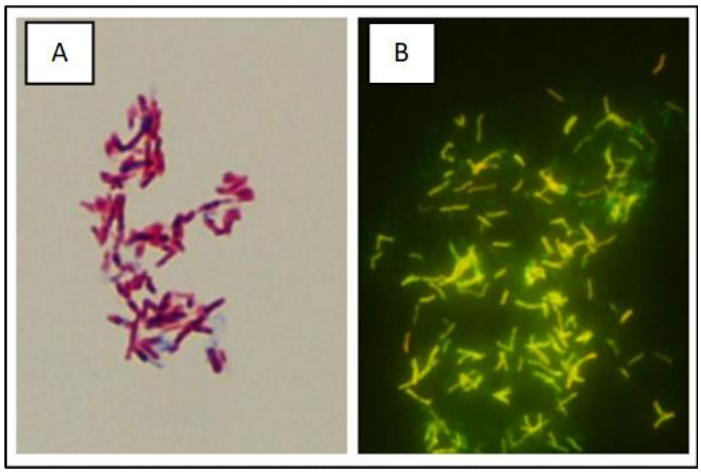


Figure 1.9: A- Acid-fast bacilli stained with Kinyoun stain; B- Auramine-rhodamine staining (60).

1.6.4.2 Culture

Culturing MTBc is the gold standard for bacteriological confirmation of TB. Clinical samples decontaminated with N-acetyl-L-cysteine (NALC) and sodium hydroxide (NaOH) are inoculated on culture media that support the growth of mycobacteria (60). Culture requires only 10-100 bacilli/ml to detect MTBc thus much more sensitive compared to sputum smear microscopy (60). In addition, culturing MTBc has several other advantages such as confirming the presence

of viable bacilli, which is useful for treatment monitoring, identifying the specific species within the MTBc, and performing drug-susceptibility testing (DST) (60).

There are several types of such culture media: solid media such as egg-based Löwenstein-Jensen (LJ) medium, agar-based Middlebrook 7H10 and Middlebrook 7H11 and liquid media such as Middlebrook 7H9, semi-automated commercial MGIT 960 (Becton and Dickinson), Versa TREK (Trek Diagnostic Systems) and, MB/BacT Alert 3D (bioMérieux). Like most mycobacterial species, MTBc grows best at 35-37 °C, and growth is stimulated by the presence of 5-10% CO_2 in air, particularly on agar media. MTBc often grows faster in liquid media compared to solid media, nevertheless, solid media facilitates detection of mixed cultures containing MTBc and non-tuberculous mycobacteria (NTM) by observing colony morphology and macroscopic growth. In addition, liquid media are more prone to contamination by non-mycobacteria compared to solid media (60, 61).

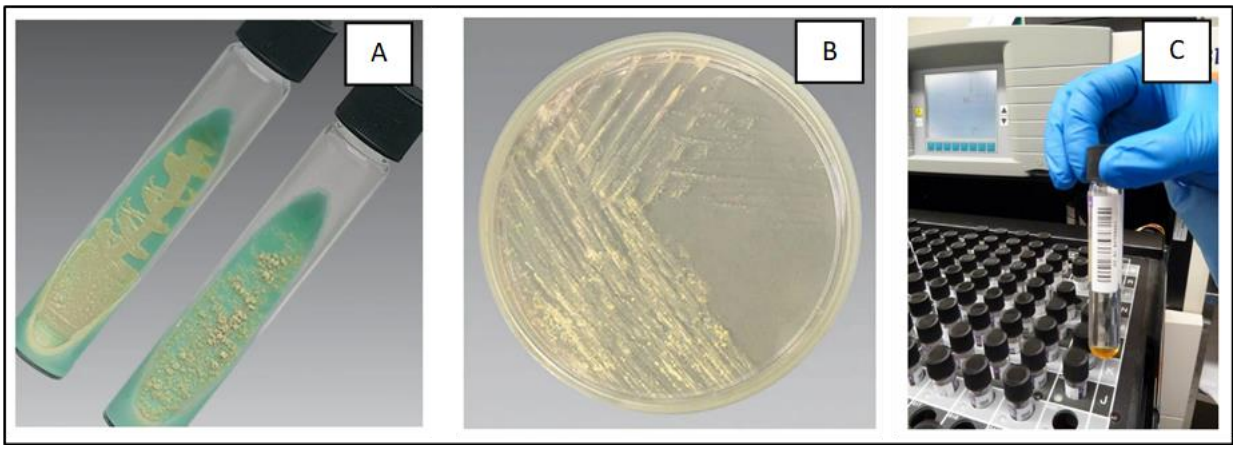


Figure 1.10: A - Cream color, rough and buff MTBc colonies on LJ medium, B - MTBc colonies on Middlebrook 7H11 (Source for A and B: https://www.fishersci.ca) and, C - MGIT 960 automated liquid culture system, the mostly used commercial liquid culture system for MTBc (60).

On solid media, the development of bacterial colonies over time indicates a positive culture; in commercial liquid media systems, such as the MGIT 960, oxygen consumption in the tube; for the Versa TREK, pressure differences in the tube; and for BacT/Alert, CO_2 production in the tube indicate culture positivity. To confirm a pure MTBc culture, particularly liquid positive cultures require further testing, such as the MPT64 antigen test, smear microscopy, and blood agar culture (60).

Culture of MTBc is not used as an initial diagnostic test in most countries due to the long turnaround time (TAT), cost, and infrastructure requirements. However, culture plays an important role in diagnosing pediatric and EPTB, and treatment monitoring.

Culture media are essential components in phenotypic DST (pDST) for MTBc, which involves assessing the growth of MTBc in the presence of a specific anti-TB drugs as compared to drug-free media, particularly for new and repurposed drugs.

1.6.4.3 Phenotypic drug-susceptibility testing methods

pDST methods are currently the gold standard for drug-resistance detection for the majority of anti-TB drugs. Traditionally, pDST methods for MTBc are performed after obtaining a pure culture from a clinical specimen and rely on testing a single concentration of the anti-TB drug known as the critical concentration (CC), which is the lowest concentration of the drug that will inhibit the *in vitro* growth of 99% of phenotypically wild type strains of MTBc to determine susceptibility or resistance (62). Historically, determining the CC for anti-TB drugs relied heavily on expert advice and consensus within the scientific communities, however, the introduction of the epidemiological cut-off values (ECOFFs) by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) marks a shift towards a more data-driven approach to determining susceptibility breakpoints. ECOFFs are essentially minimum inhibitory concentrations (MICs) below which wild-type isolates of a microorganism are expected to be found. In the TB context, ECOFFs help to distinguish between wild-type strains and those with acquired resistance associated genetic variants (63, 64).

In addition, for some drugs such as fluoroquinolones, there is also a clinical breakpoint (CB) established to differentiate between mutant isolates that may still respond to the drug at higher concentrations (low-level resistance) and those that are unlikely to respond to the drug at any concentration (high-level resistance). Current WHO-recommended pDST methods include amongst others the indirect proportion method on solid media, MGIT-960-based commercial liquid DST, and MIC testing using broth microdilution (BMD) (62, 64).

- **Indirect proportion method on solid media**

In this method, a standardized inoculum (ex: a bacterial suspension of an optical density of McFarland 1 or 1 mg/ml of bacteria density) is inoculated onto the medium containing a known concentration of the drug tested and two 10-fold serial dilutions (10^{-1} and 10^{-3} or 10^{-2} and 10^{-4} dilutions) of the inoculum are inoculated onto the drug-free control media (**Figure 1.11**). The visual growth, which is the number of colonies corrected for the dilution factor on a control medium without the drug, is compared with the growth present on medium containing the CC of the drug. Resistance is defined when at least 1% of growth is observed at the critical concentration of drug in the culture medium. Due to slow growth of MTBc on solid medium it can take 28-42 days to obtain results using this method.

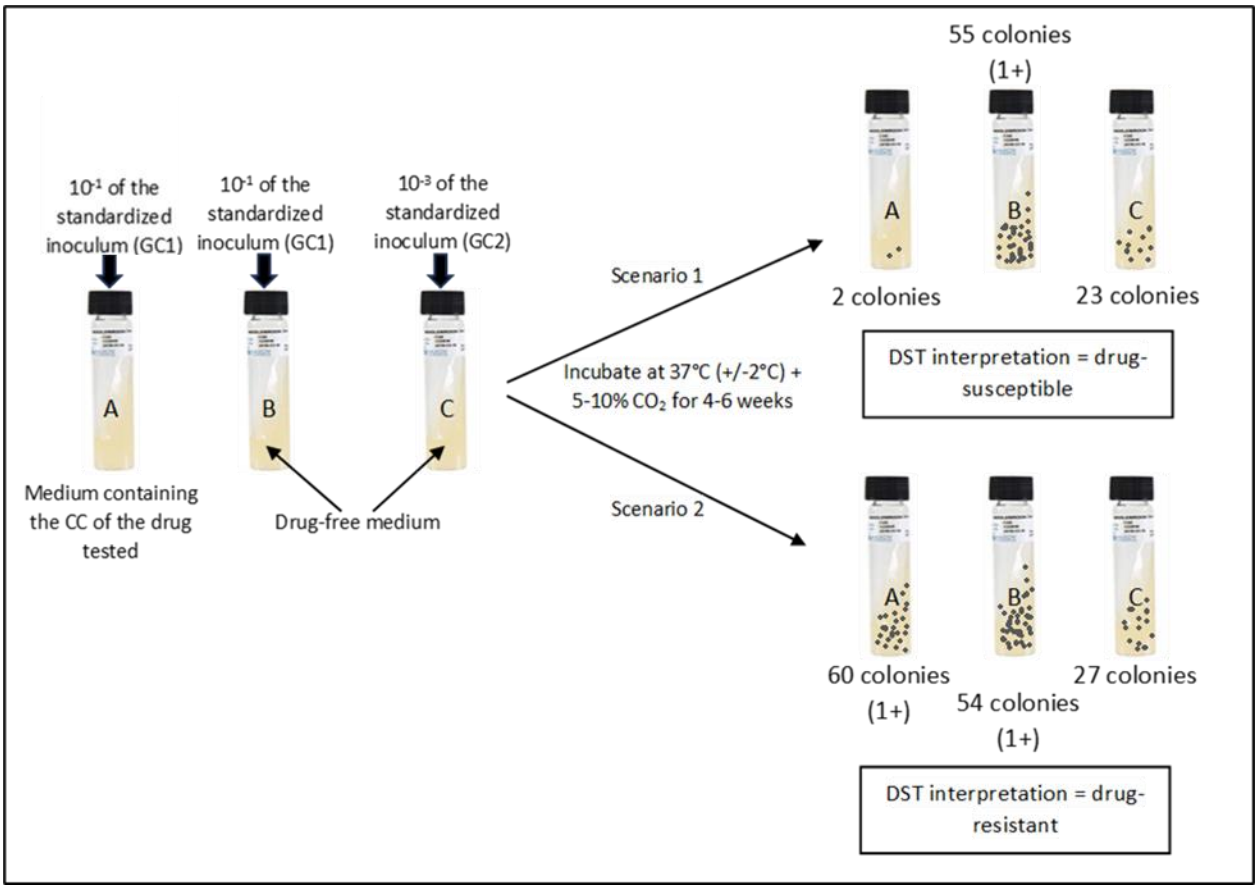


Figure 1.11: An example of inoculation and interpretation of an indirect DST on solid

- **Indirect proportion method on commercial MGIT-960 system**

A standardized inoculum from a pure MTBc culture is added to a MGIT tube containing the CC of the drug, and 1% of the standard inoculum is added to a drug-free MGIT tube. Unlike on solid

media where visual growth on drug-containing and drug-free media is compared, here the MGIT machine through hourly monitoring compares the increase in fluorescence, due to oxygen consumption by the MTBc, in the drug-containing and drug-free tubes, and automatically interprets the results as either susceptible or resistant. Compared to solid media, MTBc grows faster in liquid media, resulting in a shorter TAT time of 4-14 days compared to 28-42 days on solid media. However, faster interpretation of the results in MGIT has been reported to result in false-susceptible results in slow growing MTBc isolates, such as those with borderline *rpoB* mutations conferring resistance to rifampicin yet tend to be missed in MGIT based pDST (65).

- **Broth microdilution (BMD)**

  Employing the classical pDST methods such as the proportion method on solid or in liquid medium for MIC determination is labor-intensive and costly. As an alternative approach, in 2022, the WHO endorsed 96-well plate-based BMD to determine the MICs of MTBc.

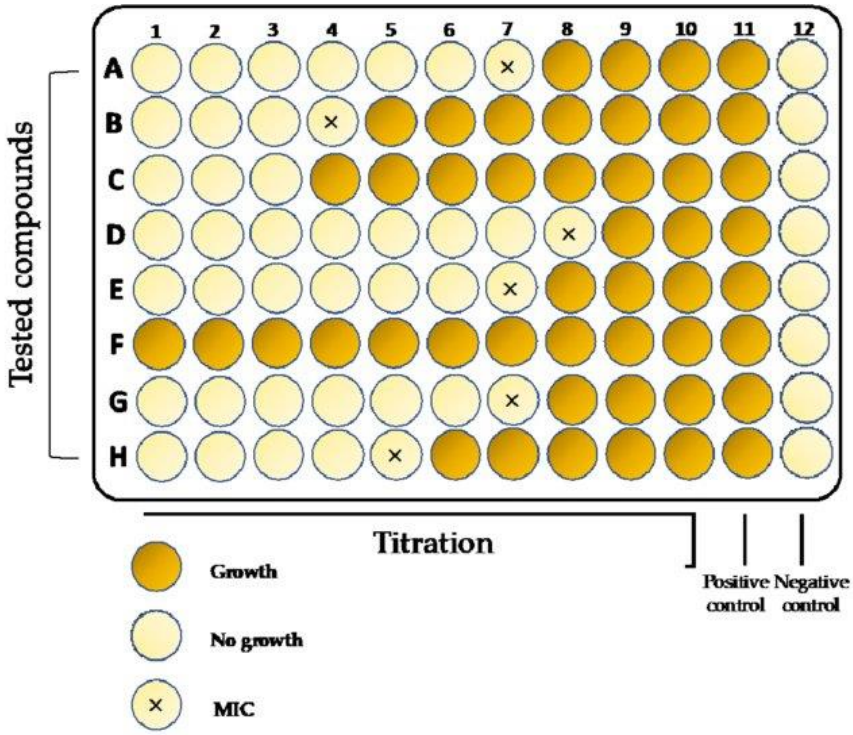


Figure 1.12: An example of a microtiter plate and interpretation of MIC results

Source: https://www.mdpi.com/2079-6382/8/4/174 (84)

MIC-minimum inhibitory concentration.

MIC testing offers several advantages such as,

- MIC values can quantify the *level of* resistance, which is not (always) possible by classic pDST or rapid molecular tests and is particularly important for new drugs.
- MIC testing allows determining the ECOFF, which is the upper end of the wildtype MIC distribution and is used as the surrogate critical concentration in case molecular mechanisms remain unknown. This is particularly important for novel anti-TB drugs as for such drugs a knowledge gap remains on the association of potential resistance-conferring mutations and the MICs.
- MIC testing helps in resolving discordant gDST/pDST results for those mutations causing a MIC around the CC.
- MIC testing allows laboratories to detect systematic/technical errors more rapidly.
- MIC testing of serial isolates from the same patient may be a useful tool for treatment outcome monitoring; a serial MIC increase in patients microbiologically failing treatment (i.e. cultures remain positive) may indicate drug resistance amplification.

The current WHO-recommended pDST methods require sophisticated infrastructure such as biosafety level 3 laboratory facilities to manipulate MTBc-positive cultures and trained personnel, and thus are often restricted to the central or regional level in low- and middle-income countries, if at all available. Furthermore, pDST also is time-consuming, thus gDST approaches for the MTBc are gaining popularity. However, pDST offers several advantages over gDST methods such as,

- pDSTs have superior sensitivity in identifying minority-resistant populations, often detecting resistance at levels as low as 1%, the 'clinically relevant cut-off of resistant proportion'. Only deep sequencing analysis by targeted NGS may reach the same limit of detection.
- pDSTs can rule in and out resistance, particularly for novel and repurposed anti-TB medicines.

- pDSTs reflect the interactions between multiple mutations, which may be missed when investigating only selected genes, such as in the case of epistasis at the level of mmp5 efflux pump mechanism for bedaquiline resistance.

In addition to the indirect methods, the WHO has recommended three non-commercial DST methods with the potential to be applied directly to sputum reducing the TAT, a major limitation of indirect pDST,

- Microscopic observation of drug susceptibility (MODS), a broth-microtiter plate-based method recommended as a direct or indirect test for rapid screening of patients suspected of having MDR-TB. Has never been widely rolled out, mostly because of biosafety concerns and the need for microscopic reading (67).
- Colorimetric reduction assays (CRA) , broth-microtiter plate-based method relying on color changes on day 7 after inoculation, recommended as a direct or indirect test for rapid screening of patients suspected of having MDR-TB. Has never been widely rolled out, mostly because of biosafety concerns and the observation of invalid results for fastidious growing MTBc strains (67).
- Nitrate reduction assay (NRA) - recommended as a direct or indirect test for rapid screening of patients suspected of having MDR-TB. This assay requires high-bacillary load sputa to be applied directly and does not work for nitrate reductase negative MTBc, however such strains are rare (67).

Furthermore, like the above direct methods, the thin layer agar (TLA) approach, another direct DST method that has not yet been recommended by the WHO, provides a faster TAT and is less expensive than commercial methods. However, these technologies have not yet been substantially verified for novel and repurposed medications, and standardization of reading remains a concern (62, 66, 67).

1.6.5 Molecular diagnostics

Molecular diagnostic tools, often referred to as nucleic acid amplification tests (NAATs), play a crucial role in the rapid detection of MTBc and its drug susceptibility. Most NAATs are designed to amplify specific genes associated with MTBc and drug resistance to anti-TB drugs, typically by polymerase chain reaction (PCR).

1.6.5.1 GeneXpert MTB/Rif, GeneXpert MTB/Rif Ultra and GeneXpert XDR

The first WHO-recommended rapid diagnostic test (WRD) for TB, GeneXpert MTB/Rif (Cepheid, USA) is the most widely used NAAT for TB. It is a semi-nested real-time PCR that can simultaneously detect MTBc and resistance to rifampicin. In 2017, this assay was upgraded to GeneXpert MTB/Rif Ultra (Ultra), which incorporates two different multicopy amplification targets, IS*6110* and IS*1081*, and has a bigger DNA reaction chamber than Xpert MTB/RIF cartridges, hence has a lower limit of detection of 15.6 CFU/ml compared to 112.6 CFU/ml for GeneXpert MTB/RIF (68, 69). While these assays has a good sensitivity (~90%) and specificity (>95%) for respiratory specimens, their limit of detection for minority-resistant populations can vary from 20% to 80% depending on the specific *rpoB* mutations (70). Furthermore, Xpert MTB/RIF has been reported to generate false rifampicin-resistant results for the samples with low bacillary load (21, 71). The Xpert Ultra assay is anticipated to address this issue, as it uses melting curve analysis for the *rpoB* gene, while the classical Xpert MTB/RIF relied on the absence of probe binding to detect RR. Nevertheless, it appears from recent field reports that the Xpert Ultra test is still producing false rifampicin-resistant results in samples with low bacillary burden. (*Cuella et al*, unpublished data).

In 2021, Cepheid developed a new GeneXpert cartridge, GeneXpert XDR, to detect resistance to isoniazid, fluoroquinolones, ethionamide, and second-line injectable TB medicines. This assay can also distinguish between fluoroquinolone resistance at low and high levels and resistance to second-line injectable anti-TB medicines. When compared to gene sequencing, GeneXpert XDR is reported to have a sensitivity of 94% to 100%, and a specificity of 100% for all drugs, except ethionamide. Currently, this assay is intended to be used as a reflex assay for specimens that have been diagnosed as positive for MTBc (72).

The operational advantages of the GeneXpert platform include shorter TATs, a streamlined cartridge-based workflow that reduces biohazardous risk, cross-contamination, and the requirement for technical expertise. It also has operational constraints, such as a high initial implementation cost, the demand for a continuous power supply, maintenance and calibration, dust-free temperature and humidity-controlled environment, and limited flexibility.

1.6.5.2 Line Probe Assays (LPA)

LPA is another WRD NAAT that utilizes nitrocellulose membrane strips embedded with MTBc-specific and drug-resistance-related probes. Commercially available WHO-endorsed LPAs include Genotype MTBDR*plus* and Genotype MTBDR*sl* assays (HAIN Life Sciences, Germany), INNO-LiPA Rif.TB assay (Innogenetics, Belgium) and Nipro NTM+MDRTB assay (Nipro, Japan). The Genotype MTBDR*plus* assay, which can detect MTBc as well as resistance to rifampicin and isoniazid simultaneously, and the Genotype MTBDR*sl* assay, which can detect MTBc as well as resistance to fluoroquinolones and second-line injectables, are the most commonly used LPAs in low- and middle-income countries (73). Although LPAs are technically more complex compared to GeneXpert and can only be done in district or regional level labs, they offer similar advantages such as rapid results, usually available within 1-2 days (unless batched testing is done), good sensitivity (98% for rifampicin, 86% for fluoroquinolones) and specificity (99%) and better limit of detection for minority resistant populations compared to GeneXpert MTB/ultra assays (70). However, LPAs are being phased out in most settings due to its higher complexity in comparison to GeneXpert and the introduction of GeneXpert XDR.

1.6.5.3 Loop Mediated Isothermal Amplification (LAMP)

LAMP was endorsed by the WHO in 2016 as a replacement to smear microscopy to diagnose PTB in symptomatic adults or as a follow-on test for smear-negative adults with symptoms of PTB (74). This assay offers many operational advantages such as temperature-independent DNA amplification without the need for a thermal cycler, being self-contained without requiring complex biosafety requirements or laboratory infrastructure, requiring minimal technical expertise, and having a TAT of less than one hour (74). The main operational drawbacks of this test are that it requires a continuous power supply like for many other WRDs, is only applicable

to sputum samples, is more prone to carry-over contamination and, cannot detect drug resistance (75).

1.6.5.4 Truenat MTB, Truenat MTBPlus and Truenat MTB MTB-Rif Dx assays

Truenat MTB and MTBPlus assays are chip-based real-time PCR tests for the semi-quantitative detection MTBc while Truenat MTB-Rif Dx is a reflex test to detect rifampicin resistance in samples found positive for MTBc (by the Truenat MTB and MTBPlus assays). In 2020, the WHO recommended the Truenat MTB and MTBPlus assays as initial diagnostic test to diagnose TB in adults and children with symptoms of PTB, and Truenat MTB Rif Dx as an initial diagnostic test for rifampicin resistance rather than pDST. The sensitivities of the Truenat MTB and MTBPlus assays are 83% and 89% respectively while specificity is reported to be 99% for the MTB and 98% for the MTBPlus assay (76).

All three Truenat assays are battery operated, thus unlike GeneXpert, LPA or LAMP assays, this assay does not require stable power supply and could be used as a (near) point of care test in resource limited settings. In addition, Truenat assays require minimal laboratory infrastructure and provide results in less than one hour. On the other hand, Truenat assays include several hands-on steps including micro-pipetting thus requiring some level of technical expertise.

1.6.5.5 Targeted next generation sequencing (tNGS)

tNGS utilizes next generation sequencing to a set of selected genes to detect drug resistance, usually to multiple drugs at the same time. Unlike the WRDs, which often target the hotspot regions of specific genes, tNGS can interrogate the entire gene of interest, improving the diagnostic accuracy, and is able to detect mutations to several new and repurposed anti-TB drugs. In addition to drug resistance profiling, currently used tNGS assays offer MTBc species and lineage identification. The ability of tNGS to be used directly on smear-positive clinical specimens improves the TAT. However, tNGS methods are more expensive and technically demanding compared to the WRDs (77, 78).

Three commercial tNGS methods have been endorsed by the WHO so far (79),

- Deeplex Myc-TB (Genoscreen, France) can detect resistance to streptomycin, isoniazid, rifampicin, ethambutol, fluoroquinolones, second line injectables, linezolid, clofazimine, and bedaquiline.
- NanoTB (Oxford Nanopore Technologies, UK) can detect resistance to isoniazid, rifampicin, fluoroquinolones, amikacin, linezolid, and streptomycin.
- TBSeq (ShengTing Biotech, China) is designed to detect resistance to ethambutol.

There is currently no commercial tNGS assay that can detect resistance to the nitroimidazoles delamanid and pretomanid.

1.6.5.6 Whole genome sequencing (WGS)

WGS is a sophisticated molecular technology that provides a genome-wide analysis of MTBc, offering a comprehensive and thorough perspective of the genetic makeup, including drug resistance, genomic diversity, and transmission dynamics. Compared to WRDs and tNGS, WGS offers higher resolution, and the ability to analyze the entire genome making it a valuable tool for research, surveillance, and clinical applications. However, factors such as high cost, the need for concentrated pure DNA (and hence culture isolates), creation of massive datasets, and required bioinformatics expertise for analyzing and interpreting data hamper the widespread implementation of WGS for clinical decision making. Furthermore, in the latest version of the WHO mutation catalogue for MTBc, only a limited number of high-confidence mutations are listed, particularly for linezolid and nitroimidazoles, and the sensitivity and specificity for bedaquiline resistance detection is still not optimal, thus WGS alone may not be a reliable diagnostic tool for these drugs (80).

1.7 References

1. Perrin P. 2015. Human and tuberculosis co-evolution: An integrative view. Tuberculosis (Edinb) 95 Suppl 1:S112-6.
2. Houston M. 1999. The white death: A history of tuberculosis. Bmj 318:1705.
3. Barberis I, Bragazzi NL, Galluzzo L, Martini M. 2017. The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus. J Prev Med Hyg 58:E9-e12.
4. Hershkovitz I, Donoghue HD, Minnikin DE, Besra GS, Lee OY, Gernaey AM, Galili E, Eshed V, Greenblatt CL, Lemma E, Bar-Gal GK, Spigelman M. 2008. Detection and molecular characterization of 9,000-year-old *Mycobacterium tuberculosis* from a Neolithic settlement in the Eastern Mediterranean. PLoS One 3:e3426.
5. WHO. 2023. Global tuberculosis report 2023. World Health Organization,
6. WHO. 2015. The End TB Strategy. World Health Organization,
7. Kaufmann SHE. 2021. Vaccine Development Against Tuberculosis Over the Last 140 Years: Failure as Part of Success. Frontiers in Microbiology 12.
8. Tait DR, Hatherill M, Van Der Meeren O, Ginsberg AM, Van Brakel E, Salaun B, Scriba TJ, Akite EJ, Ayles HM, Bollaerts A, Demoitié MA, Diacon A, Evans TG, Gillard P, Hellström E, Innes JC, Lempicki M, Malahleha M, Martinson N, Mesia Vela D, Muyoyeta M, Nduba V, Pascal TG, Tameris M, Thienemann F, Wilkinson RJ, Roman F. 2019. Final Analysis of a Trial of M72/AS01(E) Vaccine to Prevent Tuberculosis. N Engl J Med 381:2429-2439.
9. Gradmann C. 2001. Robert Koch and the pressures of scientific research: tuberculosis and tuberculin. Med Hist 45:1-32.
10. Brites D, Gagneux S. 2015. Co-evolution of *Mycobacterium tuberculosis* and Homo sapiens. Immunol Rev 264:6-24.
11. van Ingen J, Rahim Z, Mulder A, Boeree MJ, Simeone R, Brosch R, van Soolingen D. 2012. Characterization of *Mycobacterium orygis* as *M. tuberculosis* complex subspecies. Emerg Infect Dis 18:653-5.
12. Alexander KA, Laver PN, Michel AL, Williams M, van Helden PD, Warren RM, Gey van Pittius NC. 2010. Novel *Mycobacterium tuberculosis* complex pathogen, M. mungi. Emerg Infect Dis 16:1296-9.
13. Parsons SD, Drewe JA, Gey van Pittius NC, Warren RM, van Helden PD. 2013. Novel cause of tuberculosis in meerkats, South Africa. Emerg Infect Dis 19:2004-7.
14. Macedo R, Isidro J, Gomes MC, Botelho A, Albuquerque T, Sogorb A, Bernardino R, Fernandes TL, Mourato T, Durval M, Gomes JP. 2020. Animal-to-human transmission of *Mycobacterium pinnipedii*. European Respiratory Journal 56:2000371.
15. Kiers A, Klarenbeek A, Mendelts B, Van Soolingen D, Koëter G. 2008. Transmission of *Mycobacterium pinnipedii* to humans in a zoo with marine mammals. Int J Tuberc Lung Dis 12:1469-73.
16. Briquet A, Vong R, Roseau JB, Javelle E, Cazes N, Rivière F, Aletti M, Otto MP, Ficko C, Duron S, Fabre M, Pourcel C, Simon F, Soler C. 2019. Clinical Features of *Mycobacterium canettii* Infection: A Retrospective Study of 20 Cases Among French Soldiers and Relatives. Clin Infect Dis 69:2003-2010.
17. van Soolingen D, van der Zanden AG, de Haas PE, Noordhoek GT, Kiers A, Foudraine NA, Portaels F, Kolk AH, Kremer K, van Embden JD. 1998. Diagnosis of *Mycobacterium microti* infections among humans by using novel genetic markers. J Clin Microbiol 36:1840-5.

18. Kozińska M, Krajewska-Wędzina M, Augustynowicz-Kopeć E. 2020. *Mycobacterium caprae* - the first case of the human infection in Poland. Ann Agric Environ Med 27:151-153.
19. Taye H, Alemu K, Mihret A, Wood JLN, Shkedy Z, Berg S, Aseffa A. 2021. Global prevalence of *Mycobacterium bovis* infections among human tuberculosis cases: Systematic review and meta-analysis. Zoonoses Public Health 68:704-718.
20. Becken BA, Bula Rudas FJ, Chatterjee A. 2023. Chapter 43 - An update on tuberculosis, p 515-524. *In* Bagchi D, Das A, Downs BW (ed), Viral, Parasitic, Bacterial, and Fungal Infections doi:https://doi.org/10.1016/B978-0-323-85730-7.00020-5. Academic Press.
21. Ngabonziza JCS, Loiseau C, Marceau M, Jouet A, Menardo F, Tzfadia O, Antoine R, Niyigena EB, Mulders W, Fissette K, Diels M, Gaudin C, Duthoy S, Ssengooba W, André E, Kaswa MK, Habimana YM, Brites D, Affolabi D, Mazarati JB, de Jong BC, Rigouts L, Gagneux S, Meehan CJ, Supply P. 2020. A sister lineage of the *Mycobacterium tuberculosis* complex discovered in the African Great Lakes region. Nature Communications 11:2917.
22. Coscolla M, Gagneux S, Menardo F, Loiseau C, Ruiz-Rodriguez P, Borrell S, Otchere ID, Asante-Poku A, Asare P, Sánchez-Busó L, Gehre F, Sanoussi CN, Antonio M, Affolabi D, Fyfe J, Beckert P, Niemann S, Alabi AS, Grobusch MP, Kobbe R, Parkhill J, Beisel C, Fenner L, Böttger EC, Meehan CJ, Harris SR, de Jong BC, Yeboah-Manu D, Brites D. 2021. Phylogenomics of *Mycobacterium africanum* reveals a new lineage and a complex evolutionary history. Microb Genom 7.
23. Guyeux C, Senelle G, Le Meur A, Supply P, Gaudin C, Phelan J, Clark TG, Rigouts L, de Jong B, Sola C, Refrégier G. 2024. Newly Identified *Mycobacterium africanum* Lineage 10, Central Africa. Emerging Infectious Disease journal 30:560.
24. Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, Garnier T, Gutierrez C, Hewinson G, Kremer K, Parsons LM, Pym AS, Samper S, van Soolingen D, Cole ST. 2002. A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci U S A 99:3684-9.
25. Napier G, Campino S, Merid Y, Abebe M, Woldeamanuel Y, Aseffa A, Hibberd ML, Phelan J, Clark TG. 2020. Robust barcoding and identification of *Mycobacterium tuberculosis* lineages for epidemiological and clinical studies. Genome Medicine 12:114.
26. Hershberg R, Lipatov M, Small PM, Sheffer H, Niemann S, Homolka S, Roach JC, Kremer K, Petrov DA, Feldman MW, Gagneux S. 2008. High Functional Diversity in *Mycobacterium tuberculosis* Driven by Genetic Drift and Human Demography. PLOS Biology 6:e311.
27. Firdessa R, Berg S, Hailu E, Schelling E, Gumi B, Erenso G, Gadisa E, Kiros T, Habtamu M, Hussein J, Zinsstag J, Robertson BD, Ameni G, Lohan AJ, Loftus B, Comas I, Gagneux S, Tschopp R, Yamuah L, Hewinson G, Gordon SV, Young DB, Aseffa A. 2013. Mycobacterial lineages causing pulmonary and extrapulmonary tuberculosis, Ethiopia. Emerg Infect Dis 19:460-3.
28. de Jong BC, Antonio M, Gagneux S. 2010. *Mycobacterium africanum*—Review of an Important Cause of Human Tuberculosis in West Africa. PLOS Neglected Tropical Diseases 4:e744.
29. Bloom BR, Murray CJ. 1992. Tuberculosis: commentary on a reemergent killer. Science 257:1055-64.
30. Comstock GW, Livesay VT, Woolpert SF. 1974. The prognosis of a positive tuberculin reaction in childhood and adolescence. Am J Epidemiol 99:131-8.

31. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. 1999. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. Jama 282:677-86.
32. Houben RM, Dodd PJ. 2016. The Global Burden of Latent Tuberculosis Infection: A Re-estimation Using Mathematical Modelling. PLoS Med 13:e1002152.
33. Lin PL, Flynn JL. 2010. Understanding latent tuberculosis: a moving target. J Immunol 185:15-22.
34. Behr MA, Edelstein PH, Ramakrishnan L. 2019. Is *Mycobacterium tuberculosis* infection life long? Bmj 367:l5770.
35. Behr MA, Edelstein PH, Ramakrishnan L. 2018. Revisiting the timetable of tuberculosis. BMJ 362:k2738.
36. Lee JY. 2015. Diagnosis and treatment of extrapulmonary tuberculosis. Tuberc Respir Dis (Seoul) 78:47-55.
37. Gambhir S, Ravina M, Rangan K, Dixit M, Barai S, Bomanji J. 2017. Imaging in extrapulmonary tuberculosis. International Journal of Infectious Diseases 56:237-247.
38. Kruijshaar ME, Abubakar I. 2009. Increase in extrapulmonary tuberculosis in England and Wales 1999-2006. Thorax 64:1090-5.
39. Woodruff HB. 2014. Selman A. Waksman, winner of the 1952 Nobel Prize for physiology or medicine. Appl Environ Microbiol 80:2-8.
40. Martini M, Gazzaniga V, Behzadifar M, Bragazzi NL, Barberis I. 2018. The history of tuberculosis: the social role of sanatoria for the treatment of tuberculosis in Italy between the end of the 19(th) century and the middle of the 20(th). J Prev Med Hyg 59:E323-e327.
41. Murray JF, Schraufnagel DE, Hopewell PC. 2015. Treatment of Tuberculosis. A Historical Perspective. Ann Am Thorac Soc 12:1749-59.
42. Pyle MM. 1947. Relative numbers of resistant tubercle bacilli in sputa of patients before and during treatment with streptomycin. Proc Staff Meet Mayo Clin 22:465-73.
43. Chakraborty S, Rhee KY. 2015. Tuberculosis Drug Development: History and Evolution of the Mechanism-Based Paradigm. Cold Spring Harb Perspect Med 5:a021147.
44. Reves R, Blakey D, Snider DE, Jr., Farer LS. 1981. Transmission of multiple drug-resistant tuberculosis: report of a school and community outbreak. Am J Epidemiol 113:423-35.
45. Deun AV, Maug AKJ, Salim MAH, Das PK, Sarker MR, Daru P, Rieder HL. 2010. Short, Highly Effective, and Inexpensive Standardized Treatment of Multidrug-resistant Tuberculosis. American Journal of Respiratory and Critical Care Medicine 182:684-692.
46. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G. 2006. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 368:1575-80.
47. Kherabi Y, Fréchet-Jachym M, Rioux C, Yazdanpanah Y, Méchaï F, Pourcher V, Robert J, Guglielmetti L. 2022. Revised Definitions of Tuberculosis Resistance and Treatment Outcomes, France, 2006-2019. Emerg Infect Dis 28:1796-1804.
48. Mahajan R. 2013. Bedaquiline: First FDA-approved tuberculosis drug in 40 years. Int J Appl Basic Med Res 3:1-2.
49. Gler MT, Skripconoka V, Sanchez-Garavito E, Xiao H, Cabrera-Rivero JL, Vargas-Vasquez DE, Gao M, Awad M, Park S-K, Shim TS, Suh GY, Danilovits M, Ogata H, Kurve A, Chang J, Suzuki K, Tupasi T, Koh W-J, Seaworth B, Geiter LJ, Wells CD. 2012. Delamanid for

Multidrug-Resistant Pulmonary Tuberculosis. New England Journal of Medicine 366:2151-2160.
50. Occhineri S, Matucci T, Rindi L, Tiseo G, Falcone M, Riccardi N, Besozzi G. 2022. Pretomanid for tuberculosis treatment: an update for clinical purposes. Curr Res Pharmacol Drug Discov 3:100128.
51. Viney K, Linh NN, Gegia M, Zignol M, Glaziou P, Ismail N, Kasaeva T, Mirzayev F. 2021. New definitions of pre-extensively and extensively drug-resistant tuberculosis: update from the World Health Organization. Eur Respir J 57.
52. WHO. 2022. WHO consolidated guidelines on tuberculosis: module 4: treatment: drug-susceptible tuberculosis treatment.
53. WHO. 2022. Rapid communication: Key changes to the treatment of drug-resistant tuberculosis.
54. Dookie N, Ngema SL, Perumal R, Naicker N, Padayatchi N, Naidoo K. 2022. The Changing Paradigm of Drug-Resistant Tuberculosis Treatment: Successes, Pitfalls, and Future Perspectives. Clin Microbiol Rev 35:e0018019.
55. Keshavjee S, Farmer PE. 2012. Tuberculosis, Drug Resistance, and the History of Modern Medicine. New England Journal of Medicine 367:931-936.
56. WHO. 2023. Universal access to rapid tuberculosis diagnostics.
57. Gordin FM, Slutkin G, Schecter G, Goodman PC, Hopewell PC. 1989. Presumptive diagnosis and treatment of pulmonary tuberculosis based on radiographic findings. Am Rev Respir Dis 139:1090-3.
58. NTEP KB-ftNTEP-. Presumptive EP-TB. https://ntep.in/node/540/CP-presumptive-ep-tb. Accessed 8 April.
59. WHO. 2020. WHO consolidated guidelines on tuberculosis Module 3: Diagnosis - Rapid diagnostics for tuberculosis detection.
60. Caulfield AJ, Wengenack NL. 2016. Diagnosis of active tuberculosis disease: From microscopy to molecular techniques. Journal of Clinical Tuberculosis and Other Mycobacterial Diseases 4:33-43.
61. Pfyffer GE, Welscher HM, Kissling P, Cieslak C, Casal MJ, Gutierrez J, Rüsch-Gerdes S. 1997. Comparison of the Mycobacteria Growth Indicator Tube (MGIT) with radiometric and solid culture for recovery of acid-fast bacilli. Journal of Clinical Microbiology 35:364-368.
62. WHO. 2018. Technical Report on critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis. https://www.who.int/tb/publications/2018/WHO_technical_report_concentrations_TB_drug_susceptibility/en/.
63. Schön T, Miotto P, Köser CU, Viveiros M, Böttger E, Cambau E. 2017. *Mycobacterium tuberculosis* drug-resistance testing: challenges, recent developments and perspectives. Clin Microbiol Infect 23:154-160.
64. Schön T, Werngren J, Machado D, Borroni E, Wijkander M, Lina G, Mouton J, Matuschek E, Kahlmeter G, Giske C, Santin M, Cirillo DM, Viveiros M, Cambau E. 2020. Antimicrobial susceptibility testing of *Mycobacterium tuberculosis* complex isolates - the EUCAST broth microdilution reference method for MIC determination. Clin Microbiol Infect 26:1488-1492.
65. Van Deun A, Aung KJ, Bola V, Lebeke R, Hossain MA, de Rijk WB, Rigouts L, Gumusboga A, Torrea G, de Jong BC. 2013. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. J Clin Microbiol 51:2633-40.

66. Miotto P, Zhang Y, Cirillo DM, Yam WC. 2018. Drug resistance mechanisms and drug susceptibility testing for tuberculosis. Respirology 23:1098-1113.
67. WHO. 2011. Noncommercial culture and drug-susceptibility testing methods for screening patients at risk for multidrug-resistant tuberculosis: policy statement.
68. Chakravorty S, Simmons AM, Rowneki M, Parmar H, Cao Y, Ryan J, Banada PP, Deshpande S, Shenai S, Gall A, Glass J, Krieswirth B, Schumacher SG, Nabeta P, Tukvadze N, Rodrigues C, Skrahina A, Tagliani E, Cirillo DM, Davidow A, Denkinger CM, Persing D, Kwiatkowski R, Jones M, Alland D. 2017. The New Xpert MTB/RIF Ultra: Improving Detection of *Mycobacterium tuberculosis* and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. mBio 8:10.1128/mbio.00812-17.
69. Organization WH. 2017. WHO meeting report of a technical expert consultation: non-inferiority analysis of Xpert MTB/RIF Ultra compared to Xpert MTB/RIF. World Health Organization,
70. Ng KCS, Supply P, Cobelens FGJ, Gaudin C, Gonzalez-Martin J, Jong BCd, Rigouts L. 2019. How Well Do Routine Molecular Diagnostics Detect Rifampin Heteroresistance in *Mycobacterium tuberculosis*? Journal of Clinical Microbiology 57:10.1128/jcm.00717-19.
71. Ssengooba W, de Dieu Iragena J, Komakech K, Okello I, Nalunjogi J, Katagira W, Kimuli I, Adakun S, Joloba ML, Torrea G, Kirenga BJ. 2021. Discordance of the Repeat GeneXpert MTB/RIF Test for Rifampicin Resistance Detection Among Patients Initiating MDR-TB Treatment in Uganda. Open Forum Infect Dis 8:ofab173.
72. Cao Y, Parmar H, Gaur RL, Lieu D, Raghunath S, Via N, Battaglia S, Cirillo DM, Denkinger C, Georghiou S, Kwiatkowski R, Persing D, Alland D, Chakravorty S. 2021. Xpert MTB/XDR: a 10-Color Reflex Assay Suitable for Point-of-Care Settings To Detect Isoniazid, Fluoroquinolone, and Second-Line-Injectable-Drug Resistance Directly from *Mycobacterium tuberculosis*-Positive Sputum. Journal of clinical microbiology 59:e02314-20.
73. Günther G, Saathoff E, Rachow A, Ekandjo H, Diergaardt A, Marais N, Lange C, Nepolo E. 2022. Clinical Evaluation of a Line-Probe Assay for Tuberculosis Detection and Drug-Resistance Prediction in Namibia. Microbiol Spectr 10:e0025922.
74. WHO. 2016. The Use of Loop-Mediated Isothermal Amplification (TB-LAMP) for the Diagnosis of Pulmonary Tuberculosis: Policy Guidance.
75. Neshani A, Zare H, Sadeghian H, Safdari H, Riahi-Zanjani B, Aryan E. 2023. A Comparative Study on Visual Detection of *Mycobacterium tuberculosis* by Closed Tube Loop-Mediated Isothermal Amplification: Shedding Light on the Use of Eriochrome Black T. Diagnostics (Basel) 13.
76. WHO. 2020. Rapid Communication: Molecular assays as initial tests for the diagnosis of tuberculosis and rifampicin resistance.
77. WHO. 2023. Use of targeted next-generation sequencing to detect drug-resistant tuberculosis. World Health Organization,
78. Murphy SG, Smith C, Lapierre P, Shea J, Patel K, Halse TA, Dickinson M, Escuyer V, Rowlinson MC, Musser KA. 2023. Direct detection of drug-resistant *Mycobacterium tuberculosis* using targeted next generation sequencing. Front Public Health 11:1206056.
79. WHO. 2023. The use of next-generation sequencing for the surveillance of drug-resistant tuberculosis: an implementation manual.

80. WHO. 2023. Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance.
81. Rupasinghe P, Reenaers R, Vereecken J, Mulders W, Cogneau S, Merker M, Niemann S, Omar SV, Rigouts L, Köser CU, Decroo T, Jong BCd. Refined understanding of the impact of the *Mycobacterium tuberculosis* complex diversity on the intrinsic susceptibility to pretomanid. Microbiology Spectrum 0:e00070-24.
82. Bateson A, Ortiz Canseco J, McHugh TD, Witney AA, Feuerriegel S, Merker M, Kohl TA, Utpatel C, Niemann S, Andres S, Kranzer K, Maurer FP, Ghodousi A, Borroni E, Cirillo DM, Wijkander M, Toro JC, Groenheit R, Werngren J, Machado D, Viveiros M, Warren RM, Sirgel F, Dippenaar A, Köser CU, Sun E, Timm J. 2022. Ancient and recent differences in the intrinsic susceptibility of *Mycobacterium tuberculosis* complex to pretomanid. Journal of Antimicrobial Chemotherapy 77:1685-1693.
83. Jaśkiewicz, M., Janczura, A., Nowicka, J. & Kamysz, W. Methods Used for the Eradication of Staphylococcal Biofilms. *Antibiotics* **8**, 174 (2019).
84. Cardona P-J, Català M, Prats C. 2020. Origin of tuberculosis in the Paleolithic predicts unprecedented population growth and female resistance. Scientific Reports 10:42.

CHAPTER 2

Problem statement and Research Objectives

2.1 Rationale and problem statement

In 2022, nearly half a million people worldwide were infected with multi-drug-resistant (MDR) or rifampicin-resistant (RR) tuberculosis (TB) resulting in an estimated 160,000 fatalities. Approximately 18% of these MDR/RRTB cases exhibited additional resistance to fluoroquinolone (pre-XDR TB). In 2022, estimated treatment coverage for MDR/RRTB was far below optimal, with just 43% of individuals who developed the disease receiving proper treatment (1).

Even though access to the WHO recommended rapid diagnostics (WRD) tests is increasing significant variations have been reported in the availability of testing for drug resistance among different countries (Figure 2.1). In 2022, only 47% of people who were newly diagnosed with tuberculosis, received a WRD (1).

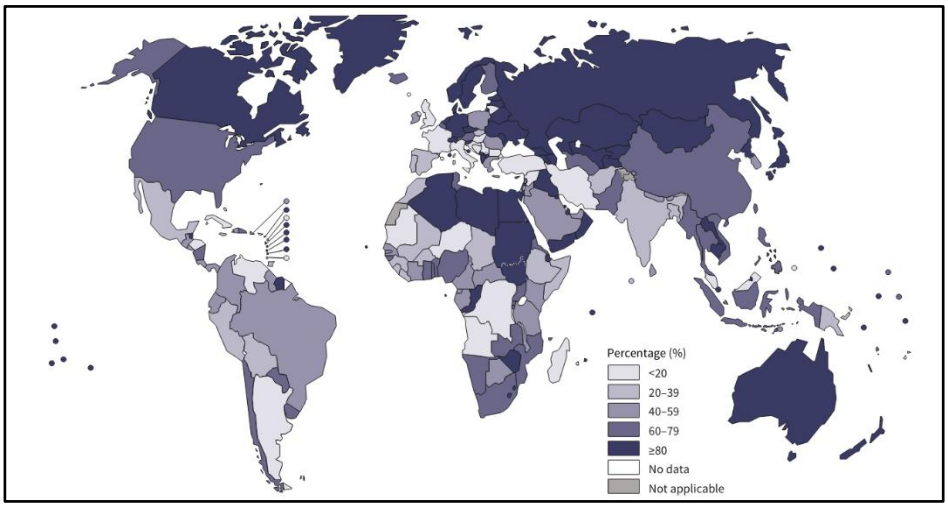


Figure 2.1: Percentage of people newly diagnosed with TB who were initially tested with a WRD in the world, 2022

The global coverage of testing for fluoroquinolone resistance was significantly lower, at slightly over 50% (2). While worldwide data is limited, in the WHO-Europe region, nearly half of countries that had access BPaL and BPAL(M) regimens had access to the DSTs for the drugs used in these regimens (3).

Reports of resistance to new and repurposed anti-TB medications are emerging from different parts of the world. A recent Moldovan study found that more than 15% of the MDR patients who received bedaquiline amplified resistance to it (4). In South Africa and Taiwan, around 3%

of the MDR/RR-TB population had already been exposed to bedaquiline or clofazimine is reported to be resistant to bedaquiline (5). A meta-analysis including 25 studies from 14 different countries reports 4.2% pooled frequency of linezolid resistance among MDR isolates (6). Lastly, worrisome results have been reported concerning pretomanid, another key drug used in the BPaL(M) regimens. Most notably, MTBc lineage 1, which accounts for 28% of tuberculosis cases globally, is reported to be intrinsically less susceptible than the other major MTBC lineages, raising the question whether L1 responds equally well to BPaL(M) compared with other lineages (7). In addition, clinical strains with pretomanid resistance due to mutations in known canonical resistance genes have been identified without known nitroimidazole exposure, suggesting genetic drift or yet unknown selective pressures (8, 9)

Given these results, expanding access to DST is a crucial component of efforts to end the global TB epidemic by 2030. Timely and accurate diagnosis of drug-resistant TB is essential for initiating appropriate treatment and preventing the further spread of resistant strains.

For most anti-TB drugs, the reference standard for determining drug susceptibility is pDST, which mostly involves testing at the CC, to differentiate likely drug-sensitive strains from likely drug-resistant strains. For certain drugs, there is also a clinical breakpoint (CB) established to differentiate between mutant isolates that may still respond to the drug at higher concentrations (low-level resistance) and those that are unlikely to respond to the drug at any concentration (high-level resistance). This binary classification in susceptible versus resistant strains without intermediate classification contrasts with other bacterial pathogens, for which usually quantitative DSTs are performed by determining minimal inhibitory concentrations (MICs), and is fraught with challenges such as,

1. As pDST relies on growth inhibition in/on drug-containing media, fastidious MTBc strains may result in invalid results due to lack of growth in/on the drug-free control tubes/wells, or in unreliable (false-susceptible) results due to too slow growth in/on drug-containing media. Also, the current WHO-recommended BMD method relies on the MTBc inoculum prepared from a solid culture, which requires long incubation, limiting its use for making clinical decisions.

2. Only a single CC value is used for most drugs, yielding a binary classification of resistance or susceptibility which may not always capture nuances in the level of resistance, thus not effectively guiding treatment decision-making. In addition, the current BMD cut-offs recommended by the WHO as an alternate approach for MIC testing of MTBc are provisional.

3. Current knowledge on in-vitro susceptibility to anti-TB drugs has been largely biased toward the globally dominant MTBc lineages L2 and L4. However, other MTBc lineages, such as L1, L3, L5, and L6, are prevalent in specific geographic areas where they can co-circulate with L2 and L4. Some recent evidence suggests lineage-specific drug susceptibility and even treatment outcomes (7, 10).

It is essential to expand research efforts on understanding the current gaps in DST and to study *in vitro* susceptibility across all lineages, to gain a more complete understanding of the drug-resistance landscape, identify lineage-specific variations in susceptibility to key drugs used for rifampicin-resistant TB (RR-TB) treatment and to develop effective TB control and treatment strategies tailored to the local epidemiological context.

2.2 Research objectives

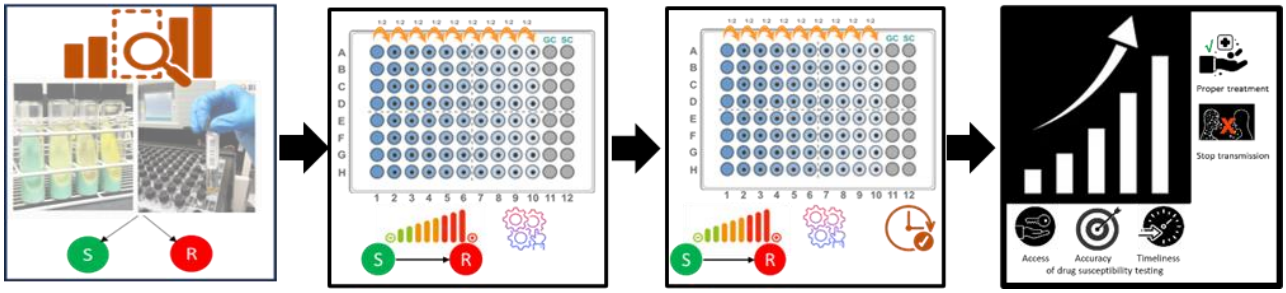


Figure 2:2 Research objectives

This PhD project has three main objectives.

1. To investigate the current limitations impeding speedy and accurate CC-based pDST for MTBc isolates.

   Under this objective, we investigated the effect of,

   - nutritional conditions in the culture medium (**Chapter 3**)
   - binary categorization of resistance or susceptibility (**Chapters 4 and 5**)
   - genetic diversity within the MTBc (**Chapter 6**)

   on the timeliness and accuracy of pDST results for MTBc isolates.

2. To validate a semi-automated MIC-testing method based on BMD for quantifying drug resistance in MTBc isolates.

   Under this objective, we established the,

   - Quality control ranges
   - Wildtype MIC distribution
   - Preliminary epidemiological cut-off (ECOFF) values
   - Sensitivity and specificity of the provisional cut offs
   - Repeatability, reproducibility of the semi-automated BMD method

   for fluoroquinolones, repurposed and new anti-tuberculosis drugs (**Chapter 7).**

3. To develop a protocol for faster, semi-automated MIC testing from freshly positive, actively growing MGIT cultures with a turnaround time similar to MGIT-based DST (**Chapter 8**).

2.3 References

1. WHO. Global tuberculosis report 2023. World Health Organization; 2023.
2. WHO. Global Tuberculosis Report. 2021.
3. Günther G, Guglielmetti L, Kherabi Y, Duarte R, Lange C. Availability of drugs and resistance testing for bedaquiline, pretomanid, linezolid, and moxifloxacin (BPaL(M)) regimen for rifampicin-resistant tuberculosis in Europe. Clin Microbiol Infect. 2024.
4. Chesov E, Chesov D, Maurer FP, Andres S, Utpatel C, Barilar I, et al. Emergence of bedaquiline resistance in a high tuberculosis burden country. Eur Respir J. 2022;59(3).
5. Kaniga K, Hasan R, Jou R, Vasiliauskienė E, Chuchottaworn C, Ismail N, et al. Bedaquiline Drug Resistance Emergence Assessment in Multidrug-Resistant Tuberculosis (MDR-TB): a 5-Year Prospective In Vitro Surveillance Study of Bedaquiline and Other Second-Line Drug Susceptibility Testing in MDR-TB Isolates. J Clin Microbiol. 2022;60(1):e0291920.
6. Azimi T, Khoshnood S, Asadi A, Heidary M, Mahmoudi H, Kaviar VH, et al. Linezolid resistance in multidrug-resistant *Mycobacterium tuberculosis*: A systematic review and meta-analysis. Front Pharmacol. 2022;13:955050.
7. Bateson A, Ortiz Canseco J, McHugh TD, Witney AA, Feuerriegel S, Merker M, et al. Ancient and recent differences in the intrinsic susceptibility of *Mycobacterium tuberculosis* complex to pretomanid. Journal of Antimicrobial Chemotherapy. 2022;77(6):1685-93.
8. Lee BM, Harold LK, Almeida DV, Afriat-Jurnou L, Aung HL, Forde BM, et al. Predicting nitroimidazole antibiotic resistance mutations in *Mycobacterium tuberculosis* with protein engineering. PLOS Pathogens. 2020;16(2):e1008287.
9. Timm J, Bateson A, Solanki P, Paleckyte A, Witney AA, Rofael SAD, et al. Baseline and acquired resistance to bedaquiline, linezolid and pretomanid, and impact on treatment outcomes in four tuberculosis clinical trials containing pretomanid. PLOS Global Public Health. 2023;3(10):e0002283.
10. Rupasinghe P, Reenaers R, Vereecken J, Mulders W, Cogneau S, Merker M, et al. Refined understanding of the impact of the *Mycobacterium tuberculosis* complex diversity on the intrinsic susceptibility to pretomanid. Microbiology Spectrum.0(0):e00070-24.

CHAPTER 3

Middlebrook 7H11 Reduces Invalid Results and Turnaround Time of Phenotypic Drug-Susceptibility Testing of *M. tuberculosis.*

Middlebrook 7H11 Reduces Invalid Results and Turnaround Time of Phenotypic Drug-Susceptibility Testing of *M. tuberculosis*

International Journal of Mycobacteriology 11(4):p 407-411, Oct–Dec 2022.

DOI: 10.4103/ijmy.ijmy_159_22

Praharshinie Rupasinghe[1*], Jens Vereecken[1], Pieter Graulus[1], Tom Decroo[2,3], Elisa Ardizzoni[1], Cathy Hewison[4], Dimitri Donchuk[5], Helena Huerga[4], Anita Mesic[6], Leen Rigouts[1,7], Bouke C. de Jong[1]

[1] Unit of Mycobacteriology, Department of Biomedical Sciences, Institute of Tropical Medicine, 2000, Antwerp, Belgium
[2] Unit of HIV and TB, Department of Clinical Sciences, Institute of Tropical Medicine, 2000, Antwerp, Belgium
[3] Research Foundation Flanders, 1000, Brussels, Belgium
[4] Médecins Sans Frontières, 34 Avenue Jean Jaurès, 75019 Paris, France
[5] Médecins Sans Frontières, Rue de l'Arbre Bénit 46, 1050 Brussels, Belgium
[6] Médecins Sans Frontières, Plantage Middenlaan 14, 1018 DD Amsterdam, Netherlands
[7] University of Antwerp, Antwerp, Belgium

Abstract

Background

Phenotypic drug-susceptibility testing (pDST), which relies on growth inhibition in the drug-containing media, remains a challenge for fastidious *Mycobacterium tuberculosis* complex (MTBc) isolates due to insufficient growth on the growth controls (GC). Middlebrook 7H11 (M7H11) medium contains casein hydrolysate, which may favor the growth of such strains.

Method

In this study, we tested whether M7H11 reduces invalid results due to insufficient growth on the GCs and the turnaround time (TAT) of pDST for MTBc compared to Middlebrook 7H10 (M7H10) without affecting the accuracy of the pDST results and how it differs between rifampicin- and isoniazid-susceptible non multi-drug resistant (non-MDR), MDR and MDR with additional resistance to fluoroquinolones (Pre-XDR) MTBc isolates. We compared the proportions of invalid pDST results due to lack of growth on the GCs, TATs of valid parallel drug-susceptibility testing as an indicator of speed of MTBc growth, and colony-forming unit (CFU) count on the most diluted GC of the parallel pDSTs after equal incubation periods as an indicator of growth abundance on M7H11 and M7H10. We also analyzed the agreement between the pDST results of the same drug or drugs in the same drug class, tested in parallel on both media.

Results

For MDR and pre-XDR isolates, relative to M7H10, M7H11 significantly reduced the occurrence of invalid pDST results due to insufficient growth on the GCs (odds ratio [OR] = ∞ [95% confidence interval (CI) 1.9–∞], P = 0.004 for MDR, OR = ∞ [95% CI 3.3–∞], P = 0.0001 for pre-XDR) and the TAT of pDSTs (OR = 17 [95% CI 2.6–710.4], P = 0.0001 for MDR, OR = 9.3 [95% CI 4.0–26.5], P < 0.0001 for pre-XDR). The growth abundance of MTBc on M7H11 was significantly higher compared to M7H10 (17 CFU on M7H10 vs. 28 on M7H11), irrespective of drug-resistance profiles. The agreement between the pDST results between the two media was high (Cohen's k > 0.98).

Conclusion

Our study findings suggest that M7H11 is preferred over M7H10 for pDSTs of MTBc isolates.

3.1 Introduction

The prompt and accurate diagnosis of drug resistance is key to assign an effective treatment regimen, which minimizes further development and transmission of drug-resistant tuberculosis (TB). Due to the slow-growing nature of the *Mycobacterium tuberculosis* complex (MTBC), as well as the cost and the demand for the sophisticated infrastructure of conventional phenotypic DST (pDST), rapid genotypic DST (gDST) represents the most convenient option to obtain drug-susceptibility data for the clinical management of the patients. However, for most anti-TB drugs, culture-based pDST remains the reference standard, also for resistance conferred by mutations outside the "hotspot" regions targeted by World Health Organization (WHO)-endorsed rapid molecular tests, such as GeneXpert MTB/RIF (Cepheid, USA), Genotype MTBDR*plus* and MTBDR*sl* (Hain Life Sciences, Germany) (1-4). More importantly, rapid molecular tests are not yet available for novel anti-TB drugs, for which a knowledge gap remains on the correlation of resistance-conferring mutations and MICs, and the clinical breakpoint is not yet known (5). Therefore, pDST remains important for accurate diagnosis of drug resistance.

The indirect proportion method is the most used pDST method to determine the drug susceptibility of MTBc starting from a pure culture. This method compares the colony forming unit (CFU) count of a known inoculum on a drug-free medium known as the growth control (GC) versus the CFU count on the drug-containing media containing the critical concentration (CC) of a drug (6). Lowenstein-Jensen (LJ), Middlebrook 7H10 (M7H10), and Middlebrook (M7H11) are commonly used solid media for this method (7,8). Agar-based, M7H11, and M7H10 have advantages over egg-based LJ medium as growth appears earlier and it is easy to visualize colonies on transparent agar-based media (7). M7H11 is considered an improved version of M7H10 due to the presence of casein hydrolysate in M7H11; that provides nitrogen, vitamins, and amino acids and is reported to favor the growth of fastidious, drug resistant MTBc that grow poorly on M7H10 (7,9).

The most complex treatment decisions pertain to patients whose strain is already resistant to rifampicin. Such strains have mutations in the essential rpoB gene that encodes the β sub-unit of RNA polymerase in MTBc, resulting in variable degrees of growth defects (10). Since pDST methods measure growth inhibition in drug-containing media, such mutations may lead to higher proportions of failed pDSTs due to insufficient growth on the growth controls (GCs) or result in false susceptibility if the GC yields a valid result but the lower fitness strain requires

longer incubation to grow in the presence of rifampicin (11). We, therefore, analyzed if M7H11 could reduce the occurrence of invalid results due to insufficient growth on the growth controls, and increase growth speed, and growth abundance compared to M7H10 without affecting the accuracy of the pDST results. We also assessed whether results differed between rifampicin and isoniazid susceptible (non-MDR), multi-drug resistant (MDR), and MDR with additional resistance to fluoroquinolones (Pre-XDR) MTBc isolates.

3.2 Materials and method

3.2.1 Sample size and inclusion criteria

A total of 401 MTBc isolates, originating from Afghanistan, Armenia, Belarus, Georgia, India, Kenya, Kyrgyzstan, Mozambique, Myanmar, and Ukraine, with known resistance profiles for rifampicin, isoniazid, and fluoroquinolones, were included in this study. Based on resistance/susceptibility to rifampicin, isoniazid, and fluoroquinolones; we categorized these isolates into three drug resistance (DR) groups: non-MDR (23 isolates susceptible to rifampicin, isoniazid, and fluoroquinolones), MDR (102 isolates resistant to rifampicin, isoniazid but susceptible to fluoroquinolones) and Pre-XDR (276 MDR with additional resistance to fluoroquinolones). The study design and the workflow are summarized in **Figure 3.1**.

3.2.2 Media and antibiotics

M7H10 and M7H11 media were prepared as per the manufacturer's recommendations (12) and stored at 2–8°C for six months maximum. Stock solutions were prepared for ofloxacin (Sigma-Aldrich, O8757) and levofloxacin (Sigma–Aldrich, 28266) at 10000 mg/L in 0.1 N sterile NaOH, and for linezolid (Sigma–Aldrich, PZ0014) at 10000 mg/L in dimethyl sulfoxide [DMSO (Sigma–Aldrich, D5879)]. All stock solutions were stored in aliquots at below -18 °C for 12 months maximum.

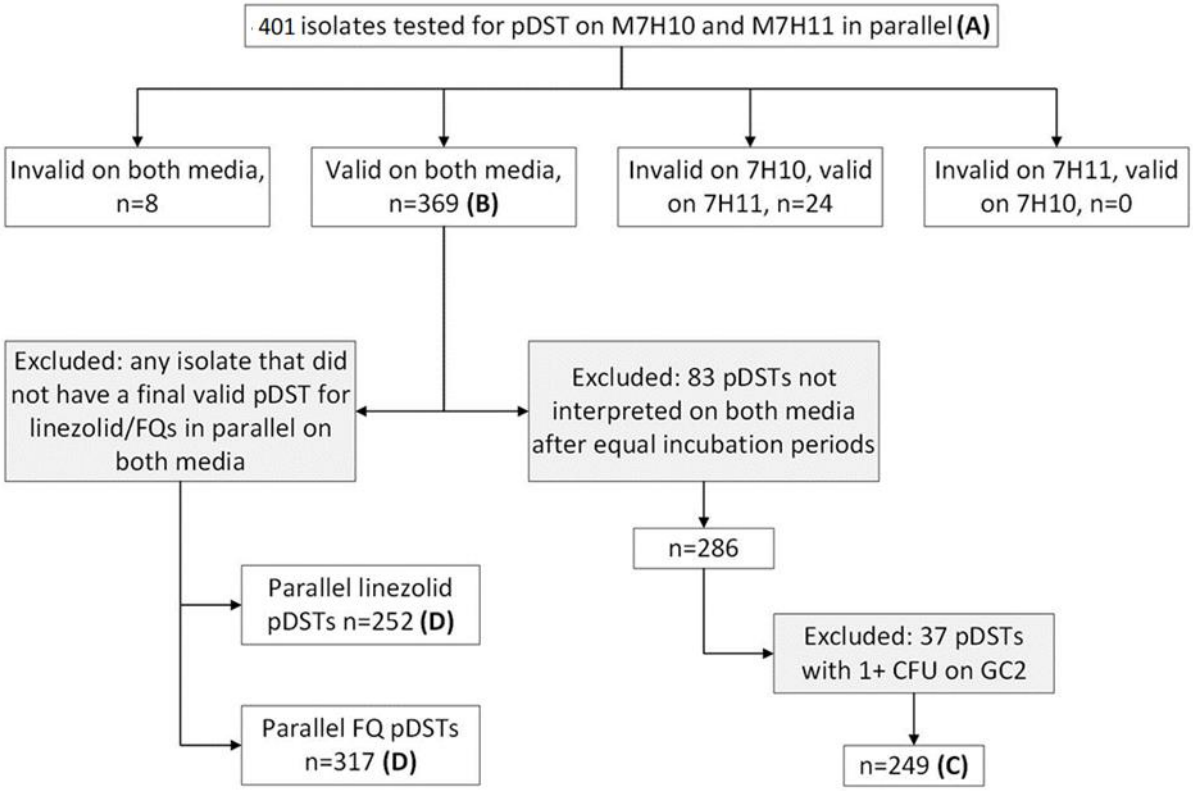


Figure 3.1: Flow diagram for the use of isolates for different analyses.

A = Used to compare the proportion of invalid results for the pDSTs performed in parallel on M7H10 and M7H11
B = Used to compare the growth speed between the two media
C = Used to compare growth abundance on GC2 between M7H10 and M7H11
D = Used to analyze the agreement between pDSTs between the two media
CFU = Colony Forming Units
FQ = Fluoroquinolones
GC2 = The most diluted growth control (1%)
pDST = phenotypic drug susceptibility testing

pDSTs for linezolid (1 µg/ml) on both M7H11 and M7H10, levofloxacin (1 µg/ml) on M7H10, and ofloxacin (2 µg/ml) on M7H11 were performed in parallel, by the same operator on the same day using the same bacterial suspension, using the proportion method. Bacterial colonies were scraped from fresh MTBc cultures on LJ slants, not older than two weeks after the first colonies were visible, and thoroughly homogenized in sterile water with glass beads. The density of the suspension was visually adjusted to McFarland 1. An inoculum of 10^{-1} of McFarland 1 was used for the drug-containing tubes and the least diluted growth control (GC1), while the most diluted growth control (GC2) was inoculated with a 10^{-3} dilution. CFUs were enumerated after four weeks of incubation at 34-38 °C with 5-10% CO_2, using the quantitative scale shown in **Table 3.1**.

CFU count	Recording method
0-50	The exact number of colonies
51-100	1+
101-200	2+
>201	3+
Confluent growth	4+

Table 3.1: Quantitative scale used for the growth of *Mycobacterium tuberculosis* on solid media.

CFU = Colony forming units

If both GC1 and GC2 had sufficient growth at this point, i.e. GC1 ≥ 1+ (51-100 CFUs) and ≤ 1+ GC2 > 3 CFUs, CFU counts were recorded accordingly and pDST results were interpreted. An isolate was considered resistant to the drug tested if the drug-containing tube had equal or more growth than the GC2. If GC1 and/or GC2 had insufficient CFU counts at four weeks, tubes were incubated at 37 °C for two more weeks. Any test that had insufficient CFU counts on GC1 and/or GC2 after six weeks of incubation or that had more than 1+ growth on GC2 was considered invalid.

Targeted deep sequencing (*gyrA* and *gyrB* for fluoroquinolones, *rplC,* and *rrl* for linezolid) was performed using the Deeplex-MycTB assay (Genoscreen, Lille, France) described elsewhere (13) on any isolate that had discrepant pDST results between the two media.

3.2.3 Quality control

The pan susceptible MTBc strain H37Rv (ATCC 27294, BCCM/ITM 2008-03715) and reference strains for each drug (BCCM/ITM 102197 for levofloxacin and ofloxacin, and 130318 for linezolid) were included as quality control strains for each new batch of drug stock solutions and media.

3.2.4 Statistical analysis

Statistical data analysis was performed using Stata/SE 17.0 software (Stata Corp, USA). The Exact McNemar's test was used to compare paired categorical data such as the occurrence of an invalid result and the TAT of the pDSTs between the two media. The TAT of the pDSTs was used as an indicator of the growth speed and all isolates with a valid pDST result on both media in parallel were included in this data set. CFU count on the GC2 of a sub-set of these isolates, whose pDST results were interpreted after equal incubation time (e.g., pDSTs that were interpreted after four weeks of incubation on both media) and with countable colonies (0-50 colonies) on the GC2 of both media was compared as an indicator of growth abundance using Wilcoxon matched-pairs signed-rank test. The occurrence of invalid pDST results due to insufficient growth on the GCs, TAT, and growth abundance between the two media were compared irrespective of the DR profile (overall) and separately for the three DR groups. The difference in proportion or mean difference was calculated with a 95% confidence interval (CI) and p-value, which was considered significant at <0.05. Cohen's Kappa coefficient was used to analyze the extent of agreement between pDST results for the same drug/drugs in the same drug class on M7H11 and M7H10.

3.3 Results

3.3.1 Percentage of invalid results

Of 401 parallel pDSTs, 32 (7.9%) were invalid at least on one medium: 8 (2.0%) were invalid on both media, 24 (6.0%) were invalid only on M7H10, and none was invalid only on M7H11. All 32 pDSTs were invalid due to less than three CFUs on the GC2 after six weeks of incubation.

Overall, there was a statistically significant reduction [OR = ∞ (95% CI 6.01-∞), $p < 0.0001$, **Table 3.2**] of invalid pDST results due to lack of growth on the GC2 on M7H11 compared to M7H10 (**Figure 3.1**). When stratified by the DR profile, in both MDR [OR=∞ (95% CI 1.9-∞), p=0.004] and Pre-XDR groups [OR=∞ (95% CI 3.3-∞), p=0.0001], the occurrence of invalid pDST results due to lack of growth on the GC2 was significantly higher on M7H10 but not in the non-MDR group [OR=∞ (95% CI 0.025-∞), p=1.0, **Table 3.2**].

Overall		M7H11		
		Valid	Invalid	Total
M7H10	Valid	369	0	370
	Invalid	24	8	32
	Total	393	8	401
OR=∞ (95% CI 6.01-∞) McNemar's p<0.0001				

Non-MDR		M7H11		
		Valid	Invalid	Total
M7H10	Valid	21	0	21
	Invalid	1	1	2
	Total	22	1	23
OR=∞ (95% CI 0.025-∞) McNemar's p=1				

MDR		M7H11		
		Valid	Invalid	Total
M7H10	Valid	91	0	91
	Invalid	9	2	11
	Total	100	2	102
OR=∞ (95% CI 1.9-∞) McNemar's p=0.004				

Pre-XDR		M7H11		
		Valid	Invalid	Total
M7H10	Valid	257	0	257
	Invalid	14	5	19
	Total	271	5	276
OR=∞ (95% CI 3.3-∞) McNemar's p=0.0001				

Table 3.2: Occurrence of initially invalid pDST results due to <3 CFU on the most diluted growth control (GC2)

CFU = Colony Forming Units, GC2 = Most diluted growth control (10^{-3}), pDST = Phenotypic Drug Susceptibility Testing, MDR = Multi-Drug Resistant, XDR = Extensively Drug-Resistant

3.3.2 Growth speed

A total of 369 isolates had a valid pDST result in parallel on both media, using the same bacterial suspension. Overall, there was a significant reduction of the TATs of the pDSTs on M7H11 compared to M7H10 [OR=10.8 (95% CI 5.0-27.9), p<0.0001], thus the growth speed of MTBc was significantly higher on M7H11 (**Table 3.3**). When stratified by the DR profile, the TATs of the pDSTs on M7H11 were significantly lower compared to M7H10 in the MDR [OR=17 (95% CI 2.6-710.4), p=0.0001] and Pre-XDR groups [OR=9.3 (95% CI 4.0-26.5), p<0.0001] but not in the non-MDR group [OR=∞ (95% CI 0.41-∞), p=0.25] (**Table 3.3**).

Overall		M7H11		
		4W	6W	Total
M7H10	4W	265	7	272
	6W	76	21	97
	Total	341	28	369
OR=10.8 (95% CI 5.0-27.9) McNemar's p=0.0001				

Non-MDR		M7H11		
		4W	6W	Total
M7H10	4W	15	0	15
	6W	3	3	6
	Total	18	3	21
OR=∞ (95% CI 0.41-∞) McNemar's p=0.25				

MDR		M7H11		
		4W	6W	Total
M7H10	4W	66	1	67
	6W	17	7	24
	Total	83	8	91
OR=17 (95% CI 2.6-710.4) McNemar's p=0.0001				

Pre-XDR		M7H11		
		4W	6W	Total
M7H10	4W	184	6	190
	6W	56	11	67
	Total	240	17	257
OR=9.3 (95% CI 4.0-26.5) McNemar's p<0.0001				

Table 3.3: Turnaround time of the parallel pDSTs with a valid result

pDST = Phenotypic Drug Susceptibility Testing, 4W = four weeks, 6W = six weeks, MDR = Multi-Drug Resistant, XDR = Extensively Drug-Resistant

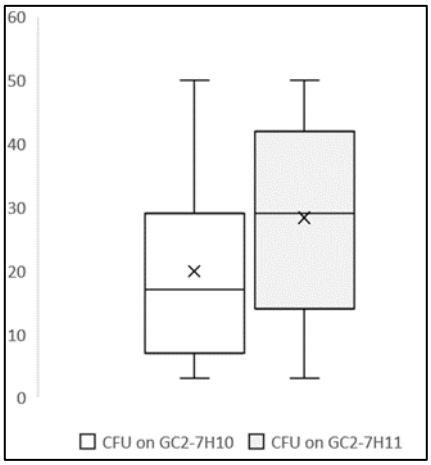
3.3.3 Growth abundance

Of 401 pDSTs inoculated on both media using the same bacterial suspension, 286 (71.3%) were interpreted after equal incubation periods. From these, we excluded 37 pDSTs with 1+ growth on the GC2 of both/any pDSTs and compared the CFU count on GC2 of the remaining 249. On M7H11 growth abundance was significantly higher (Wilcoxon signed-rank test $p<0.0001$) with a median of 28 CFUs (IQR 27) compared to 17 CFUs (IQR 23) on M7H10 (**Figure 3.2**). The difference was significant across all DR groups [(Wilcoxon signed-rank test $p=0.03$ (non-MDR), $p=0.004$ (MDR), $p<0.0001$ (Pre-XDR)] (**Table 3.4**).

	Median CFU count on GC2		Wilcoxon signed-rank *p-value*
	M7H10	M7H11	
Overall	17 (IQR=23)	28 (IQR=27)	$p<0.0001$
Non-MDR	17 (IQR=22)	30 (IQR=27)	$p=0.03$
MDR	17 (IQR=24)	28 (IQR=27.5)	$p=0.004$
Pre-XDR	17 (IQR=24)	28 (IQR=27)	$p<0.0001$

Table 3.4: Median CFUs on GC2 of parallel pDSTs interpreted after equal incubation periods

CFU = Colony Forming Units, GC2 = Most diluted growth control (10^{-3}), pDST = Phenotypic Drug Susceptibility Testing, MDR = Multi-Drug Resistant, XDR = Extensively Drug-Resistant



X= mean of the plotted colony counts

Figure 3.2: Distribution of the colony forming units (CFU) count on growth control 2 (GC2) of 249 phenotypic drug-susceptibility tests inoculated on M7H11 and M7H10 agar in parallel using the same bacterial suspension.

Y axis represents the absolute number of colonies observed in the most diluted growth control (GC2)

3.3.4 Agreement between the pDST results

A total of 250 isolates had valid pDST results for linezolid in parallel on both media. All but one pDST results were concordant (99.6% agreement, Cohen's k: 0.98). One isolate was resistant to linezolid on M7H10 but susceptible on M7H11. Targeted deep sequencing of this isolate did not detect any drug-resistance conferring mutations in the *rplC* and *rrl* genes. A total of 314 isolates had valid pDST results for levofloxacin (1 µg/ml) on M7H10 and ofloxacin (2 µg/ml) on M7H11 in parallel. All pDST results for levofloxacin and ofloxacin had an excellent agreement of 100%.

3.4 Discussion

In this retrospective study, we tested if M7H11 reduces the occurrence of invalid results due to lack of growth on the GCs and increases the growth speed and abundance of MTBc isolates compared to M7H10. Our results showed that, for non-MDR-TB isolates, the differences between the proportions of invalid pDST results due to lack of growth on the GCs or TATs between the two media were not significant. However, for MDR and pre-XDR isolates, compared to M7H10, the M7H11 medium not only significantly lowered the occurrence of invalid results due to lack of growth on the GCs, thus reducing the need to repeat the tests but also significantly reduced the TAT of the pDST results, (~19% of pDSTs had a net gain of two weeks in TAT) without affecting the accuracy of the pDST results. Any decrease in TAT of pDSTs is precious for the clinical management of the patients. Even though some researchers have suggested that the addition of casein hydrolysate makes no difference to the growth of MTBc (14), our study results suggest casein hydrolysate improves the growth of MTBc, especially for isolates with drug resistance-conferring mutations, which are known to affect in vitro fitness to different degrees (15).

This study has limitations. While most rifampicin-resistance conferring mutations carry a significant fitness cost, we did not study the impact of casein hydrolysate on the growth of different rpoB mutants and different lineages of MTBc. In addition, in this study, we did not study if M7H11 provides an advantage for the growth of MTBc already exposed to anti-TB drugs as suggested by Joloba *et al* (16). Another limitation is the comparison of levofloxacin (1 µg/ml) on M7H10 with ofloxacin (2 µg/ml) pDST results on M7H11. Even though in our data set, there

was a perfect agreement between the results, these two drugs have different levels of in-vitro activity against MTBc.

Finally, for non-MDR isolates, M7H11 showed no statistically significant advantages over M7H10 to reduce the proportion of invalid pDSTs due to lack of growth on the GCs or the TAT of the pDSTs. However, this should be generalized with caution as the sample size of non-MDR isolates included in this analysis was smaller compared to the other two DR groups and it might have impacted the power of statistical analyses performed for non-MDR isolates. Moreover, we did not find a detrimental effect of using M7H11 for non-MDR isolates. Patients infected with RR-TB often require additional drug resistance testing for new/repurposed anti-TB drugs for which genotypic drug susceptibility testing is not yet well established, based on our study findings we recommend M7H11 medium over M7H10, for pDSTs of not only MDR or pre-XDR but also for non-MDR isolates.

3.5 References

1. Casela, M. et al. Rapid molecular test for tuberculosis: impact of its routine use at a referral hospital. Jornal brasileiro de pneumologia : publicacao oficial da Sociedade Brasileira de Pneumologia e Tisilogia 44, 112-117 (2018).
2. Amicosante, M. et al. Current use and acceptability of novel diagnostic tests for active tuberculosis: a worldwide survey. Jornal brasileiro de pneumologia : publicacao oficial da Sociedade Brasileira de Pneumologia e Tisilogia 43, 380-392 (2017).
3. Ling, D.I., Zwerling, A.A. & Pai, M. Rapid diagnosis of drug-resistant TB using line probe assays: from evidence to policy. Expert Rev Respir Med 2, 583-8 (2008).
4. WHO. The use of molecular line probe assays for the detection of resistance to second-line anti-tuberculosis drugs. (2016).
5. WHO. Technical Report on critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis. (https://www.who.int/tb/publications/2018/WHO_technical_report_concentrations_TB_drug_susceptibility/en/, 2018).
6. Canetti, G. et al. Mycobacteria: Laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ 29, 565-78 (1963).
7. Rastogi, N., Goh, K.S. & David, H.L. Drug susceptibility testing in tuberculosis: a comparison of the proportion methods using Lowenstein-Jensen, Middlebrook 7H10 and 7H11 agar media and a radiometric method. Res Microbiol 140, 405-17 (1989).
8. Nguyen, T.N.A., Anton-Le Berre, V., Bañuls, A.-L. & Nguyen, T.V.A. Molecular Diagnosis of Drug-Resistant Tuberculosis; A Literature Review. Frontiers in microbiology 10, 794-794 (2019).
9. Cohn, M.L., Waggoner, R.F. & McClatchy, J.K. The 7H11 medium for the cultivation of mycobacteria. Am Rev Respir Dis 98, 295-6 (1968).
10. Ma, P. et al. Compensatory effects of *M. tuberculosis* rpoB mutations outside the rifampicin resistance-determining region. Emerging microbes & infections 10, 743-752 (2021).
11. Torrea, G. et al. Variable ability of rapid tests to detect *Mycobacterium tuberculosis* rpoB mutations conferring phenotypically occult rifampicin resistance. Scientific Reports 9, 11826 (2019).
12. BD, L. Difco™ & BBL™ Manual, 2nd Edition.
13. Ng, K.C.S. et al. How Well Do Routine Molecular Diagnostics Detect Rifampin Heteroresistance in *Mycobacterium tuberculosis*. Journal of Clinical Microbiology 57, e00717-19 (2019).
14. Kaniga, K. et al. A Multi-laboratory, Multi-country Study To Determine Bedaquiline MIC Quality Control Ranges for Phenotypic Drug Susceptibility Testing. Journal of clinical microbiology 54, 2956-2962 (2016).
15. Gagneux, S. Fitness cost of drug resistance in *Mycobacterium tuberculosis*. Clin Microbiol Infect 15(2009).
16. Joloba, M.L. et al. What is the most reliable solid culture medium for tuberculosis treatment trials? Tuberculosis (Edinb) 94, 311-6 (2014).

3.6 Supplementary material

Lab ID	DST profile	DST valid/Invalid		TAT		CFU count		DST FQ		DST LZD	
		M7H10	M7H11	M7H10	M7H11	M7H10	M7H10	LFX-M7H10	OFX-M7H11	LZD-M7H10	LZD-M7H11
2015-02524	Pre-XDR	valid	valid	4	4	16	29	S	S	S	S
2015-02555	Pre-XDR	valid	valid	4	4	5	17	R	R	NA	NA
2015-02558	Pre-XDR	valid	valid	4	4	26	29	R	R	NA	NA
2015-02559	Pre-XDR	valid	valid	4	4	17	40	R	R	NA	NA
2015-02682	Pre-XDR	valid	valid	4	4	3	4	R	R	NA	NA
2015-02756	MDR	valid	valid	4	4	3	22	S	S	NA	NA
2016-00153	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2016-00347	Pre-XDR	Invalid	Invalid	NA	NA	NA	NA	NA	NA	NA	NA
2016-00402	MDR	valid	valid	4	4	24	10	S	S	S	S
2016-00592	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	S	NA
2016-00594	Pre-XDR	valid	valid	4	4	9	26	R	R	NA	NA
2016-00635	Pre-XDR	valid	valid	4	4	15	50	S	S	S	S
2016-00639	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-00641	MDR	valid	valid	4	4	5	8	S	S	NA	NA
2016-00655	Pre-XDR	valid	valid	4	4	10	28	R	R	NA	NA
2016-00686	Pre-XDR	valid	valid	4	4	6	14	R	R	NA	NA
2016-00687	Pre-XDR	valid	valid	4	4	1+	45	R	R	NA	NA
2016-00696	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-00697	Pre-XDR	valid	valid	4	4	6	7	S	S	S	S
2016-00703	Pre-XDR	valid	valid	4	4	38	41	S	NA	NA	S
2016-00704	Pre-XDR	valid	valid	4	4	4	6	R	R	NA	NA
2016-00705	MDR	valid	valid	4	4	45	45	NA	S	NA	NA
2016-00733	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2016-00770	MDR	valid	valid	4	4	20	30	S	S	NA	NA

2016-00771	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-00772	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2016-00774	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2016-00775	Pre-XDR	valid	valid	4	4	9	29	R	R	NA	NA
2016-00776	Pre-XDR	valid	valid	4	4	10	1+	R	R	NA	NA
2016-00777	MDR	valid	valid	4	4	30	11	S	S	NA	NA
2016-00778	MDR	valid	valid	6	4	NA	NA	S	S	NA	NA
2016-00827	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2016-00829	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2016-00830	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2016-00831	MDR	valid	valid	4	4	9	50	S	S	NA	NA
2016-00841	Pre-XDR	valid	valid	4	4	29	23	S	S	S	S
2016-00868	Pre-XDR	valid	valid	4	4	50	24	R	R	NA	NA
2016-00869	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-00870	Pre-XDR	valid	valid	4	4	40	1+	R	R	NA	NA
2016-00871	Pre-XDR	valid	valid	4	4	21	16	R	R	NA	NA
2016-00872	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2016-00874	MDR	valid	valid	4	4	45	25	S	S	NA	NA
2016-00927	MDR	valid	valid	6	4	NA	NA	S	S	NA	NA
2016-01029	Pre-XDR	valid	valid	4	4	3	11	R	R	NA	NA
2016-01037	Pre-XDR	valid	valid	4	4	50	50	S	S	S	S
2016-01038	MDR	valid	valid	4	4	40	34	S	S	NA	NA
2016-01039	MDR	valid	valid	4	4	5	19	S	S	NA	NA
2016-01040	MDR	valid	valid	4	4	30	50	S	S	NA	NA
2016-01041	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-01042	Pre-XDR	valid	valid	4	4	1+	1+	R	R	NA	NA
2016-01050	Pre-XDR	valid	valid	4	6	NA	NA	R	R	NA	NA
2016-01051	MDR	valid	valid	4	4	4	6	S	S	NA	NA
2016-01052	Pre-XDR	valid	valid	4	4	9	19	S	S	S	S
2016-01053	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-01078	Pre-XDR	valid	valid	4	4	30	1+	R	R	NA	NA

2016-01079	MDR	valid	valid	4	4	50	40	S	S	NA	NA
2016-01088	MDR	valid	valid	6	4	NA	NA	S	S	NA	NA
2016-01089	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-01093	Pre-XDR	valid	valid	4	4	40	50	R	R	NA	NA
2016-01095	Pre-XDR	valid	valid	4	4	1+	11	R	R	NA	NA
2016-01141	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2016-01142	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2016-01160	MDR	valid	valid	4	4	12	31	S	S	NA	NA
2016-01161	Pre-XDR	valid	valid	4	4	1+	1+	R	R	NA	NA
2016-01174	MDR	valid	valid	4	4	7	19	S	S	NA	NA
2016-01178	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-01298	Pre-XDR	valid	valid	4	4	29	28	NA	R	NA	NA
2016-01465	MDR	valid	valid	4	4	34	30	S	S	NA	NA
2016-01481	MDR	valid	valid	6	6	13	5	S	S	NA	NA
2016-01483	Non.MDR	valid	valid	4	4	28	28	S	S	NA	NA
2016-01484	MDR	valid	valid	4	4	50	50	S	S	NA	NA
2016-01485	Non.MDR	valid	valid	4	4	35	28	S	S	NA	NA
2016-01486	Non.MDR	valid	valid	6	6	24	35	S	S	NA	NA
2016-01491	Pre-XDR	valid	valid	4	4	20	12	R	R	NA	NA
2016-01493	Pre-XDR	valid	valid	6	6	3	4	R	R	NA	NA
2016-01494	Pre-XDR	valid	valid	4	4	12	6	R	R	NA	NA
2016-01501	Pre-XDR	valid	valid	4	4	1+	1+	R	R	NA	NA
2016-01502	MDR	valid	valid	6	4	NA	NA	S	S	NA	NA
2016-01503	Pre-XDR	valid	valid	4	4	3	12	R	R	NA	NA
2016-01637	Pre-XDR	valid	valid	4	4	5	3	R	R	NA	NA
2016-01638	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2016-01639	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2016-01697	MDR	valid	valid	6	4	NA	NA	S	S	NA	NA
2016-01698	MDR	valid	valid	4	4	29	14	S	S	NA	NA
2016-01699	MDR	valid	valid	6	4	NA	NA	S	S	NA	NA
2017-00012	Pre-XDR	valid	valid	4	4	24	23	S	S	S	S

2017-00138	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2017-00139	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2017-00265	MDR	valid	valid	6	4	NA	NA	NA	NA	NA	NA
2017-00269	Pre-XDR	valid	valid	6	4	NA	NA	R	R	NA	NA
2017-00271	MDR	valid	valid	6	4	NA	NA	S	S	NA	NA
2017-00272	MDR	valid	valid	4	4	34	37	NA	NA	NA	NA
2017-00347	MDR	valid	valid	4	4	40	42	NA	NA	NA	NA
2017-00350	MDR	valid	valid	6	6	9	11	S	S	S	S
2017-00351	MDR	valid	valid	4	4	25	1+	NA	NA	NA	NA
2017-00366	Pre-XDR	valid	valid	4	4	46	44	S	S	S	S
2017-00367	Pre-XDR	valid	valid	4	4	7	30	S	S	S	S
2017-00382	Non.MDR	valid	valid	6	4	NA	NA	NA	NA	NA	NA
2017-00391	MDR	valid	valid	6	4	NA	NA	NA	NA	NA	NA
2017-00392	MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00407	Non.MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00419	MDR	valid	valid	6	4	NA	NA	NA	NA	NA	NA
2017-00442	Pre-XDR	valid	valid	4	4	20	1+	NA	NA	NA	NA
2017-00463	Pre-XDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00609	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2017-00610	MDR	valid	valid	4	4	36	39	NA	NA	NA	NA
2017-00611	Pre-XDR	valid	valid	4	4	50	36	NA	NA	NA	NA
2017-00612	MDR	valid	valid	4	4	8	23	NA	NA	NA	NA
2017-00613	MDR	valid	valid	6	6	5	4	NA	NA	NA	NA
2017-00620	Pre-XDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00674	MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00675	Pre-XDR	valid	valid	4	4	50	1+	NA	NA	NA	NA
2017-00708	MDR	valid	valid	4	4	11	1+	NA	NA	NA	NA
2017-00810	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2017-00838	MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00839	Pre-XDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00840	Pre-XDR	valid	valid	4	4	50	1+	NA	NA	NA	NA

2017-00841	MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00844	MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00845	MDR	valid	valid	6	4	NA	NA	NA	NA	NA	NA
2017-00863	Pre-XDR	valid	valid	6	6	3	3	NA	NA	NA	NA
2017-00864	Pre-XDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00865	Pre-XDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00870	MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2017-00891	MDR	valid	valid	4	4	11	1+	NA	NA	NA	NA
2017-00968	Pre-XDR	valid	valid	6	4	NA	NA	NA	NA	NA	NA
2017-01082	Non.MDR	valid	valid	4	4	1+	1+	NA	NA	NA	NA
2018-00275	Pre-XDR	valid	valid	4	4	27	50	S	NA	NA	S
2018-00292	Pre-XDR	valid	valid	6	6	44	50	S	S	S	S
2018-00293	Pre-XDR	valid	valid	4	4	26	41	S	S	S	S
2018-00295	Pre-XDR	valid	valid	4	4	7	42	NA	S	S	NA
2018-00306	Pre-XDR	valid	valid	4	4	36	47	S	S	S	S
2018-00307	Non.MDR	valid	valid	4	4	27	37	S	S	S	S
2018-00310	Pre-XDR	valid	valid	4	4	45	50	S	S	S	S
2018-00312	Pre-XDR	valid	valid	4	4	40	50	S	NA	NA	S
2018-00320	Pre-XDR	valid	valid	4	4	50	40	S	S	S	S
2018-00323	MDR	valid	valid	4	4	24	19	S	S	S	S
2018-00325	MDR	valid	valid	6	6	44	46	S	S	S	S
2018-00326	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2018-00327	Pre-XDR	valid	valid	6	6	41	42	S	S	S	S
2018-00329	Pre-XDR	valid	valid	4	4	50	45	S	S	S	S
2018-00332	Pre-XDR	valid	valid	4	4	42	42	S	S	S	S
2018-00333	Pre-XDR	valid	valid	4	4	27	36	S	S	S	S
2018-00335	MDR	valid	valid	4	4	16	26	S	S	S	S
2018-00343	Pre-XDR	valid	valid	4	4	50	32	S	S	S	S
2018-00943	Pre-XDR	valid	valid	4	4	45	50	NA	S	S	NA
2018-01031	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2018-01041	Pre-XDR	valid	valid	4	4	40	50	NA	NA	NA	NA

2018-01058	Pre-XDR	valid	valid	4	4	32	50	R	R	R	R
2018-01075	Pre-XDR	valid	valid	4	4	4	7	NA	NA	NA	NA
2018-01082	Pre-XDR	valid	valid	4	4	40	50	NA	NA	NA	NA
2018-01265	MDR	valid	valid	4	4	27	40	NA	NA	NA	NA
2018-01269	MDR	valid	valid	4	4	33	41	NA	S	S	NA
2018-01459	Pre-XDR	valid	valid	4	4	5	39	NA	NA	NA	NA
2018-01512	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2018-02849	Non.MDR	valid	valid	6	4	NA	NA	NA	S	S	NA
2018-03227	Pre-XDR	valid	valid	4	4	1+	1+	S	S	S	S
2018-03296	Pre-XDR	valid	valid	6	4	NA	NA	R	R	R	R
2018-03407	Pre-XDR	valid	valid	4	4	22	50	S	S	S	S
2018-03412	MDR	valid	valid	4	4	5	46	S	S	S	S
2018-03428	Pre-XDR	valid	valid	4	4	25	50	S	S	S	S
2018-03741	Pre-XDR	valid	valid	4	4	1+	47	R	R	R	R
2018-03823	Pre-XDR	valid	valid	4	4	37	28	S	S	S	S
2018-03833	Pre-XDR	valid	valid	4	4	6	37	S	S	S	S
2018-03835	Pre-XDR	valid	valid	6	6	5	15	S	S	S	S
2019-00236	Pre-XDR	valid	valid	4	4	19	43	S	S	S	S
2019-00238	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00240	Pre-XDR	valid	valid	4	4	17	33	S	S	S	S
2019-00377	Pre-XDR	valid	valid	4	4	36	42	S	S	S	S
2019-00449	Pre-XDR	valid	valid	4	4	4	26	S	S	S	S
2019-00450	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2019-00452	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	S	S	NA
2019-00458	Pre-XDR	valid	valid	4	4	4	11	S	S	S	S
2019-00495	MDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00497	Pre-XDR	valid	valid	4	4	10	17	S	S	S	S
2019-00504	Pre-XDR	valid	valid	6	6	4	41	S	S	S	S
2019-00505	Pre-XDR	valid	valid	4	4	26	33	S	S	S	S
2019-00513	Pre-XDR	valid	valid	4	4	26	32	R	R	R	R
2019-00522	Pre-XDR	valid	valid	4	4	10	6	S	S	S	S

2019-00541	MDR	valid	valid	4	4	4	13	S	S	S	S
2019-00544	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00545	Pre-XDR	valid	valid	4	4	8	8	S	S	S	S
2019-00546	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00547	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2019-00548	Pre-XDR	valid	valid	4	4	26	36	S	S	S	S
2019-00549	Pre-XDR	valid	valid	4	4	13	50	S	S	S	S
2019-00551	Pre-XDR	valid	valid	4	4	5	6	S	S	S	S
2019-00552	Pre-XDR	valid	valid	4	4	16	16	S	S	S	S
2019-00553	Pre-XDR	valid	valid	4	4	7	11	S	S	S	S
2019-00555	Pre-XDR	valid	valid	4	6	NA	NA	S	S	S	S
2019-00556	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00557	Pre-XDR	valid	valid	4	4	6	22	S	S	S	S
2019-00558	MDR	valid	valid	4	4	9	40	S	S	S	S
2019-00559	MDR	valid	valid	4	6	NA	NA	S	S	S	S
2019-00560	MDR	valid	valid	4	4	24	50	S	S	S	S
2019-00561	MDR	valid	valid	4	4	5	17	S	S	S	S
2019-00562	Pre-XDR	valid	valid	4	4	37	37	S	S	S	S
2019-00563	Pre-XDR	valid	valid	4	4	23	41	S	S	S	S
2019-00564	Pre-XDR	valid	valid	4	4	4	5	S	S	S	S
2019-00565	Pre-XDR	Invalid	valid	NA	NA	NA	NA	S	S	S	S
2019-00566	Pre-XDR	valid	valid	4	4	15	31	S	S	S	S
2019-00567	Pre-XDR	valid	valid	4	4	8	15	S	S	S	S
2019-00568	MDR	valid	valid	4	4	5	7	S	S	S	S
2019-00569	Pre-XDR	valid	valid	4	4	4	10	S	S	S	S
2019-00570	Pre-XDR	valid	valid	4	4	13	17	S	S	S	S
2019-00571	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00572	Pre-XDR	valid	valid	4	4	7	19	S	S	S	S
2019-00573	Pre-XDR	valid	valid	4	6	NA	NA	S	S	S	S
2019-00574	Pre-XDR	valid	valid	4	6	NA	NA	S	S	S	S
2019-00575	Pre-XDR	valid	valid	4	4	6	7	S	S	S	S

2019-00576	MDR	valid	valid	4	4	24	30	S	S	S	S
2019-00577	Pre-XDR	valid	valid	4	4	5	15	S	S	S	S
2019-00578	Pre-XDR	valid	valid	4	4	49	47	S	S	S	S
2019-00579	Pre-XDR	valid	valid	4	4	9	40	S	S	S	S
2019-00580	Pre-XDR	valid	valid	4	4	1+	1+	S	S	S	S
2019-00581	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2019-00582	Pre-XDR	valid	valid	4	4	7	14	R	R	R	R
2019-00583	Pre-XDR	valid	valid	4	4	31	33	S	S	S	S
2019-00585	MDR	valid	valid	4	4	16	13	S	S	S	S
2019-00586	MDR	valid	valid	4	4	33	40	S	S	S	S
2019-00587	Pre-XDR	valid	valid	6	4	NA	NA	S	S	R	S
2019-00588	Pre-XDR	valid	valid	4	4	11	40	S	S	S	S
2019-00589	Pre-XDR	valid	valid	4	4	35	35	S	S	S	S
2019-00590	MDR	valid	valid	4	4	32	23	S	S	S	S
2019-00591	Pre-XDR	valid	valid	4	4	4	14	S	S	S	S
2019-00592	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00593	Pre-XDR	valid	valid	4	4	15	19	S	S	S	S
2019-00595	Pre-XDR	valid	valid	6	4	NA	NA	R	R	R	R
2019-00596	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00597	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00598	MDR	valid	valid	4	4	6	8	S	S	S	S
2019-00599	Pre-XDR	valid	valid	4	4	7	26	S	S	S	S
2019-00600	Pre-XDR	valid	valid	4	4	5	15	S	S	S	S
2019-00601	Pre-XDR	valid	valid	4	4	29	11	S	S	S	S
2019-00602	Pre-XDR	valid	valid	4	4	5	4	S	S	S	S
2019-00603	Pre-XDR	valid	valid	4	4	9	41	S	S	S	S
2019-00604	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00605	Pre-XDR	valid	valid	4	4	5	34	S	S	S	S
2019-00606	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00614	Pre-XDR	valid	valid	4	4	16	36	S	S	S	S
2019-00621	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S

2019-00622	Pre-XDR	valid	valid	4	4	42	46	S	S	S	S
2019-00837	MDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00840	Pre-XDR	valid	valid	4	4	5	46	S	S	S	S
2019-00842	Pre-XDR	valid	valid	4	6	NA	NA	S	S	S	S
2019-00843	Pre-XDR	valid	valid	4	4	17	21	R	R	R	R
2019-00844	Pre-XDR	valid	valid	4	4	7	11	R	R	R	R
2019-00845	Pre-XDR	valid	valid	4	4	6	19	R	R	R	R
2019-00846	MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2019-00847	MDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00848	Pre-XDR	valid	valid	4	6	NA	NA	S	S	S	S
2019-00849	Pre-XDR	valid	valid	4	4	14	41	S	S	S	S
2019-00851	MDR	valid	valid	4	4	5	48	S	S	S	S
2019-00853	MDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00854	MDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00855	MDR	valid	valid	6	6	5	11	S	S	S	S
2019-00857	MDR	valid	valid	4	4	6	6	S	S	S	S
2019-00858	MDR	valid	valid	4	4	6	29	S	S	S	S
2019-00861	MDR	valid	valid	6	6	16	9	S	S	S	S
2019-00863	Pre-XDR	valid	valid	4	4	8	4	S	S	S	S
2019-00864	Pre-XDR	valid	valid	4	4	19	8	S	S	S	S
2019-00865	Pre-XDR	valid	valid	4	4	28	49	S	S	S	S
2019-00867	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00868	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00869	Non.MDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00870	MDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00871	MDR	valid	valid	4	4	10	19	S	S	S	S
2019-00872	Pre-XDR	valid	valid	4	4	5	4	S	S	S	S
2019-00874	Pre-XDR	valid	valid	4	4	5	6	S	S	S	S
2019-00875	Pre-XDR	valid	valid	6	6	6	4	S	S	S	S
2019-00876	Pre-XDR	valid	valid	4	4	15	38	R	R	R	R
2019-00877	Pre-XDR	valid	valid	4	4	6	9	R	R	R	R

2019-00878	Pre-XDR	valid	valid	4	4	40	50	S	S	S	S
2019-00879	Pre-XDR	valid	valid	6	6	8	4	S	S	S	S
2019-00880	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-00881	MDR	valid	valid	4	4	8	6	S	S	S	S
2019-00928	Pre-XDR	valid	valid	4	4	11	1+	S	S	S	S
2019-00930	Pre-XDR	valid	valid	4	4	12	1+	S	S	S	S
2019-00931	MDR	valid	valid	4	4	1+	1+	S	S	S	S
2019-00933	Pre-XDR	valid	valid	4	4	10	25	S	S	S	S
2019-00955	MDR	valid	valid	4	4	13	23	S	S	S	S
2019-00956	Pre-XDR	valid	valid	4	4	43	27	S	S	S	S
2019-00966	Pre-XDR	valid	valid	4	4	11	42	S	S	S	S
2019-01102	Pre-XDR	valid	valid	4	4	4	30	S	S	S	S
2019-01411	Pre-XDR	Invalid	valid	NA	NA	NA	NA	S	S	S	S
2019-01447	Pre-XDR	valid	valid	4	4	8	38	R	R	R	R
2019-01450	MDR	valid	valid	4	4	19	22	S	S	S	S
2019-01461	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01572	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	S	S	NA
2019-01573	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2019-01584	MDR	valid	valid	4	4	21	23	S	S	S	S
2019-01593	Pre-XDR	valid	valid	6	6	20	28	S	S	S	S
2019-01594	MDR	valid	valid	4	4	8	22	S	S	S	S
2019-01595	MDR	valid	valid	4	4	9	4	S	S	S	S
2019-01597	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2019-01598	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01599	MDR	valid	valid	4	4	32	27	S	S	S	S
2019-01600	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2019-01601	Pre-XDR	valid	valid	4	4	7	22	S	S	S	S
2019-01628	Non.MDR	valid	valid	4	4	5	7	S	S	S	S
2019-01629	Pre-XDR	valid	valid	4	4	8	26	R	R	R	R
2019-01630	Pre-XDR	valid	valid	4	4	17	38	S	S	S	S
2019-01631	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S

2019-01632	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01633	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01634	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01637	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01638	Pre-XDR	valid	valid	4	4	17	22	S	S	S	S
2019-01639	Pre-XDR	valid	valid	4	4	20	7	S	S	S	S
2019-01640	Pre-XDR	valid	valid	4	4	16	31	S	S	S	S
2019-01641	Pre-XDR	valid	valid	4	4	22	24	R	R	R	R
2019-01642	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01643	Pre-XDR	valid	valid	4	4	4	5	S	S	S	S
2019-01644	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01645	Pre-XDR	valid	valid	6	4	NA	NA	R	R	R	R
2019-01646	Pre-XDR	valid	valid	4	4	6	5	R	R	R	R
2019-01647	MDR	valid	valid	4	4	23	44	S	S	S	S
2019-01648	MDR	valid	valid	4	4	31	25	S	S	S	S
2019-01649	Pre-XDR	valid	valid	4	4	23	45	S	S	S	S
2019-01650	Pre-XDR	valid	valid	4	4	5	5	S	S	S	S
2019-01651	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01652	MDR	valid	valid	4	4	33	34	S	S	S	S
2019-01653	MDR	valid	valid	4	4	25	7	S	S	S	S
2019-01654	Pre-XDR	valid	valid	4	4	45	43	S	S	S	S
2019-01655	Pre-XDR	valid	valid	4	4	18	40	S	S	S	S
2019-01656	Pre-XDR	valid	valid	4	4	48	50	S	S	S	S
2019-01657	Pre-XDR	valid	valid	4	4	48	50	S	S	S	S
2019-01658	Pre-XDR	valid	valid	4	4	27	18	S	S	S	S
2019-01659	Pre-XDR	valid	valid	4	4	50	50	S	S	S	S
2019-01660	Pre-XDR	valid	valid	4	4	19	36	S	S	S	S
2019-01661	Pre-XDR	valid	valid	4	4	23	26	S	S	S	S
2019-01662	Pre-XDR	valid	valid	4	4	28	39	S	S	S	S
2019-01663	Pre-XDR	valid	valid	4	4	21	40	S	S	S	S
2019-01664	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S

2019-01665	Pre-XDR	valid	valid	4	4	19	38	S	S	S	S
2019-01666	Pre-XDR	valid	valid	4	4	36	43	S	S	S	S
2019-01668	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-01672	Pre-XDR	valid	valid	4	4	47	44	S	S	S	S
2019-01673	Pre-XDR	valid	valid	4	4	32	44	S	S	S	S
2019-01677	Pre-XDR	valid	valid	4	4	21	26	S	S	S	S
2019-01679	Pre-XDR	valid	valid	4	4	31	37	S	S	S	S
2019-01682	Pre-XDR	valid	valid	4	4	7	18	S	S	S	S
2019-01688	Pre-XDR	valid	valid	4	4	48	36	S	S	S	S
2019-01690	Pre-XDR	valid	valid	4	4	12	24	S	S	S	S
2019-01714	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	S	S	NA
2019-02047	Pre-XDR	Invalid	valid	NA	NA	NA	NA	NA	S	S	NA
2019-02149	Pre-XDR	valid	valid	4	4	1+	32	R	R	R	R
2019-02150	MDR	Invalid	valid	NA	NA	NA	NA	NA	S	S	NA
2019-02455	Pre-XDR	valid	valid	4	4	12	5	S	S	S	S
2019-02750	Pre-XDR	valid	valid	4	4	26	38	S	S	S	S
2019-02833	Pre-XDR	valid	valid	4	4	13	31	R	R	R	R
2019-02836	Pre-XDR	valid	valid	4	4	18	48	R	R	R	R
2019-02865	Pre-XDR	valid	valid	4	4	4	22	R	R	R	R
2019-04083	Pre-XDR	valid	valid	4	4	5	19	S	S	S	S
2019-04306	Pre-XDR	valid	valid	4	4	9	14	R	R	R	R
2019-04438	Pre-XDR	valid	valid	4	4	18	47	R	R	R	R
2019-04904	Pre-XDR	valid	valid	6	6	17	13	S	S	S	S
2019-04905	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04906	Pre-XDR	valid	valid	4	4	50	50	S	S	S	S
2019-04907	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04908	MDR	Invalid	valid	NA	NA	NA	NA	NA	S	S	NA
2019-04909	MDR	valid	valid	6	6	5	4	S	S	S	S
2019-04910	Pre-XDR	valid	valid	4	4	47	47	S	S	S	S
2019-04911	MDR	valid	valid	4	4	38	50	S	S	S	S
2019-04912	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S

2019-04913	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04914	Pre-XDR	valid	valid	4	4	14	8	S	S	S	S
2019-04915	Pre-XDR	valid	valid	4	4	22	19	S	S	S	S
2019-04916	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04917	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04918	Pre-XDR	valid	valid	4	4	19	12	S	S	S	S
2019-04919	Pre-XDR	valid	valid	4	4	4	11	S	S	S	S
2019-04920	Pre-XDR	valid	valid	4	4	10	41	S	S	S	S
2019-04921	Pre-XDR	valid	valid	4	4	42	48	S	S	S	S
2019-04922	Pre-XDR	valid	valid	4	4	22	26	S	S	S	S
2019-04923	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04924	Pre-XDR	valid	valid	4	4	27	47	S	S	S	S
2019-04925	Pre-XDR	valid	valid	4	4	41	50	S	S	S	S
2019-04926	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04927	Pre-XDR	valid	valid	4	4	19	43	S	S	S	S
2019-04928	Pre-XDR	valid	valid	4	4	42	27	S	S	S	S
2019-04929	Pre-XDR	valid	valid	6	6	7	10	S	S	S	S
2019-04930	Pre-XDR	valid	valid	4	4	4	4	S	S	S	S
2019-04931	MDR	valid	valid	4	4	6	11	S	S	S	S
2019-04932	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2019-04933	Pre-XDR	valid	valid	6	4	NA	NA	S	S	S	S
2020-00269	MDR	valid	valid	4	4	29	1+	R	R	R	R
2020-00290	MDR	valid	valid	4	4	14	48	R	R	R	R
2019-03938	Non.MDR	valid	valid	6	6	28	33	NA	NA	NA	NA
2020-01888	Non.MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA
2020-01889	Non.MDR	valid	valid	4	4	27	32	NA	NA	NA	NA
2020-01890	Non.MDR	valid	valid	4	4	16	16	NA	NA	NA	NA
2021-00461	Non.MDR	valid	valid	4	4	42	1+	NA	NA	NA	NA
2021-00498	Non.MDR	valid	valid	4	4	10	32	NA	NA	NA	NA
2021-00716	Non.MDR	valid	valid	4	4	14	36	NA	NA	NA	NA
2021-00805	Non.MDR	valid	valid	4	4	9	8	NA	NA	NA	NA

2021-00813	Non.MDR	valid	valid	4	4	26	46	NA	NA	NA	NA
2021-01320	Non.MDR	valid	valid	4	4	13	33	NA	NA	NA	NA
2021-00472	Non.MDR	valid	valid	4	4	33	1+	NA	NA	NA	NA
2021-02247	Non.MDR	valid	valid	6	6	15	42	NA	NA	NA	NA
2020-00799	Non.MDR	Invalid	valid	NA	NA	NA	NA	NA	NA	NA	NA

Table 3.S1: Supplementary data

CHAPTER 4

Re-evaluation of Critical Concentrations of Anti-tuberculosis Fluoroquinolones in the Mycobacteria Growth Indicator Tube 960 System

Re-evaluation of Critical Concentrations of Anti-tuberculosis Fluoroquinolones in the Mycobacteria Growth Indicator Tube 960 System

International Journal of Mycobacteriology 2023 Jul-Sep;12(3):316-323

Doi: 10.4103/ijmy.ijmy_144_23.

Praharshinie Rupasinghe[1*], Michele Driesen[2], Jens Vereecken[1], Bouke C de Jong[1], Leen Rigouts[1,3]

[1]Unit of Mycobacteriology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium.
[2] Department of Infectious Diseases in Animals, Sciensano, Brussels, Belgium[3]Department of Biomedical Sciences, University of Antwerp, Belgium.
[1] Unit of Mycobacteriology, Department of Biomedical Sciences, Institute of Tropical Medicine, 2000, Antwerp, Belgium.

* Corresponding author

Abstract

Background

Fluoroquinolones (FQs) have substantial activity against the *Mycobacterium tuberculosis* complex (MTBc) by preventing bacterial DNA synthesis through DNA gyrase inhibition. The reference standard for FQ-resistance testing is phenotypic drug-susceptibility testing (pDST) based on growth inhibition of MTBc in drug-containing Mycobacteria Growth Indicator Tube system (MGIT) media at a critical concentration (CC) that differentiates phenotypically wild-type from nonwild-type MTBc and at a clinical breakpoint that identifies strains that will likely still respond to treatment at higher doses. Despite the recent introduction of powerful new TB drugs, highly sensitive detection of clinically defined FQ resistance remains key.

Method

In this study, we re-evaluated the current WHO-recommended CCs of Lfx (1.0 mg/ml), Mfx (0.25 mg/ml), Gfx (0.25 µg/ml), and the nowadays, obsolete CC of Ofx (2.0 mg/ml) for MGIT, using 147 MTBc isolates with known *gyrA* and *gyrB* sequences including both high-and low-level FQ resistance-conferring mutants. We tested a wide range of drug concentrations covering the current and former/obsolete WHO-recommended CCs for FQs and some intermediate concentrations to challenge the current WHO-recommended CCs.

Results

The specificity of all four CCs was 100%. The sensitivities varied: 92.4% for Ofx and Lfx, 85.7% for Mfx, and 83.2% for Gfx. Lowering the CC of Mfx to 0.125 mg/ml would allow us to correctly classify all wild-type and mutant isolates while lowering the CC of Gfx to 0.125 mg/ml would still misclassify some *gyrA/gyrB* mutants as susceptible.

Conclusion

Based on our findings, a minimal inhibitory concentration of 0.125 mg/ml on MGIT medium is a more appropriate CC for Mfx and probably also as a surrogate for overall FQ resistance in the MTBc.

4.1 Introduction

The nalidixic acid derivatives fluoroquinolones (FQs) have substantial in vitro activity against the *Mycobacterium tuberculosis* complex (MTBc) by preventing bacterial DNA synthesis through DNA gyrase inhibition (1,2). Multiple FQs have various in vitro and in vivo activity levels against the MTBc: fourth-generation FQs such as moxifloxacin (Mfx) and gatifloxacin (Gfx) have lower minimal inhibitory concentration (MICs) compared to the second- and third-generation FQs such as ofloxacin (Ofx) and levofloxacin (Lfx) (1-4). FQs are classified as group A drugs for the treatment of multidrug-resistant tuberculosis (MDR-TB), defined as resistant to the first-line drugs rifampicin and isoniazid (5). Despite the recent widespread use of the other group A drugs, bedaquiline, and linezolid, Mfx, and Lfx remain key in most regimens to treat rifampicin-resistant (RR)/MDR-TB (6,7). Thus, sensitively detecting the (level of) FQ resistance remains crucial for proper RR/MDR-TB patient management.

FQ resistance is mostly associated with mutations in the quinolone-resistance determining region (QRDR) of the *gyrA* gene (codons 74–113) and to a lesser extent in the *gyrB* gene (codons 461–499) (8-11). However, previous studies have shown that not all phenotypically FQ-resistant isolates carried mutations in the QRDR (9,12). This compromises the sensitivity and specificity of rapid molecular diagnostic methods that target only the QRDR of *gyrA*/*gyrB* genes such as the Genotype MTBDR*sl* (Bruker, Germany) and the GeneXpert XDR (Cepheid, USA). Thus, the reference standard for FQ-resistance testing remains phenotypic drug-susceptibility testing (pDST) based on the growth inhibition of MTB. The automated Mycobacteria Growth Indicator Tube system (MGIT; Becton Dickinson, USA) has been proposed as the reference method for performing pDST for second-line anti-TB drugs, by exposing the bacilli to a critical concentration (CC) of the drugs (9,12-16). A reference standard represents the highest level of reference and is the best available method for determining the presence or absence of a condition of interest, thus MGIT960-based pDST for FQs should be able to correctly differentiate phenotypically *gyrA*/*gyrB* wild-type and clinically relevant nonwild-type MTBc strains (17). In the context of MTBc, the current definition for the CC is the lowest concentration of an anti-TB agent that will inhibit the in vitro growth of 99% of phenotypically wild-type isolates (18). CC values for FQs have been defined for various testing media (16).

In this study, we re-evaluated the current WHO-recommended CCs for Lfx (1.0 µg/ml) and Mfx (0.25 µg/ml), as well as the now-obsolete CCs for Ofx (2.0 µg/ml) and Gfx (0.25 µg/ml) for the

MGIT960 system. Furthermore, we re-evaluated if any of these four FQs may be used as a proxy for MTBc susceptibility to all FQs previously served by ofloxacin.

4.2 Materials and Method

4.2.1 Isolates

A total of 147 MTBc isolates with previously published *gyrA* and *gyrB* sequences (1) were included in this study. Among these, 40 were *gyrA/gyrB* wild-type or carrying mutations that do not confer resistance to FQs as per the WHO catalog of mutations (Version 1) (19). Of 147, 107 isolates had mutations in the QRDR of *gyrA/gyrB* associated with both low and high levels of FQ resistance, all of them resulting in amino acid substitutions, predominantly in codon 94 (62/107, 58%) and codon 90 (28/107, 26%). Of 147 isolates, 90 had data on their lineage (L): 17 L1, 27 L2,24 L3, and 22 L4.

4.2.2 FQ powders and stock solutions

Stock solutions were prepared for Ofx (Sigma-Aldrich, O8757), Lfx (Sigma-Aldrich, 28266), Mfx (Molekula, 85126158), and Gfx (Sigma-Aldrich, G7298) at 10000 µg/ml in 0.1 N sterile NaOH and stored in aliquots at or below-20°C for 6 months maximum. Aliquots were thawed on the day of use; leftovers were not refrozen. Subsequent working dilutions were made in sterile reverse-osmotic/distilled water.

4.2.3 FQ test concentrations and breakpoints

Initially, we tested two-fold dilutions around the CCs suggested in the WHO guidelines on drug-susceptibility testing from 2008 and 2014 (20) and, some intermediate concentrations to challenge these CCs (**Table 4.1**). In 2018, WHO revised the CC of Lfx from 1.5 µg/ml to 1.0 µg/ml, and the revised CC was not included in our initial concentrations tested for Lfx (16). Therefore, we retested the isolates with an initial Lfx-MIC of 1.12 µg/ml (n = 8) at a single concentration of 1 µg/ml in MGIT 960.

Mycobacteria Growth Indicator Tube (MGIT) system inoculation and reading One hundred microliter of the appropriate drug solution was added to the drug-containing MGIT tubes to achieve the desired final concentrations as described in **Table 4.1**. These tubes were supplemented with 800 µl of OADC (oleic acid, albumin, dextrose, and catalase) and inoculated with 500 µl from an initial MGIT broth culture after 1–2 days of showing positive by the instrument (day 1 to day 2) or diluted 1:5 for day 3 to 5 positive tubes. For each strain, a drug-free control vial was inoculated with a 1:100 dilution of the inoculum to represent 1% of the bacterial population. MGIT tubes were then loaded into the MGIT 960 system for incubation and automated reading. The MIC was determined to be the lowest concentration at which the growth value was <100 growth units (GU), at the moment, the growth control had reached 400 GU. An invalid test (code ×200 or ×400) was repeated once for that strain and drug. Translation from MIC results to resistant (R) versus susceptible (S) was done as follows: if the MIC >set CC, a strain was declared R and if the MIC ≤set CC, it was considered S.

Drug	**Tested concentrations (µg/ml)**								
Ofx			0,5	1.0	1,5	2.0[a]	4.0	6.0	8.0
Lfx	0,37	0,75	**1.0**[b]	1,12	1,5[c]	3.0	4,5	6.0	
Mfx	0,125	0,187	**0,25**[b]	0,5	1.0	1,5	2.0		
Gfx	0,125	0,187	**0,25**[b]	0,5	1.0	1,5	2.0		

Table 4.1: Tested concentrations in MGIT960 for each of the fluoroquinolones

[a]Obsolete WHO recommended CC for Ofx (20)

[b]Latest WHO recommended CCs (16)

[c]Former WHO recommended CC for Lfx (20)

Ofx = ofloxacin; Lfx = levofloxacin; Mfx = moxifloxacin; Gfx = gatifloxacin, CC = critical concentration

4.2.4 Quality control

As an internal quality control, the pan-susceptible MTB H37Rv reference strain (ITM 2008–03715) was included in the runs monthly (~every two runs). MIC range of 0.06–0.25 µg/ml was considered acceptable for H37Rv for Mfx and Gfx while an MIC of ≤1 µg/ml was considered acceptable for Lfx (21,22). In addition, an FQ mono-resistant (ITM number 102197, TB Pannet in vitro selected, MIC expected to be ≥1 µg/ml) QC strain, as well as the FQ-susceptible MDR

(ITM number 2002–01617) and kanamycin-capreomycin-resistant (ITM number 1999–01856) strains were tested for every new batch of FQ stock solution.

4.3 Results

4.3.1 MIC results for quality control strain

For H37Rv, the MICs ranged from 0,5–1 µg/ml for Ofx, ≤0,37 µg/ml for Lfx, ≤0,125–0,187 µg/ml for Mfx, and ≤0,125 µg/ml for Gfx, thus meeting the predefined criteria, albeit data are truncated at the lower end. The FQ-susceptible control strains were found susceptible for all FQs, with a MIC within the range of H37Rv. The in vitro selected Ofx-resistant control strain was found resistant with ≥1 µg/ml MICs for all four FQs.

4.3.2 MIC results for quality control strains

4.3.2.1 Ofloxacin

The MIC distributions for the four FQs are shown in **Table 4.2** and **Figure 4.1**. Of 147 clinical isolates tested, 145 (98.6%) had a valid final MIC result for Ofx. At 2.0 µg/ml CC (20), all (40/40) wild-type isolates were correctly identified as susceptible with a MIC range of ≤0.5–1.0 µg/ml, while eight resistance-associated *gyrA*/*gyrB* mutants (8/105, 7.6%) would be misclassified as susceptible to Ofx. Five of these have a mutation in *gyrB* (*Asn499Thr*, *Asn499Arg*, *Thr500Ala*, *Thr500Asn*, and *Thr500Ile*) and three in *gyrA* (*Asp89Asn*, *Ala90Val*, and *Asp94Ala*) (**Table 4.2**). The remaining 97 (97/105, 92.4%) *gyrA*/*gyrB* mutants with a valid MIC result were correctly classified as resistant, with overall higher MICs for *Asp94Asn*/ *Asp94Gly*/ *Asp94His*/ *Asp94*Tyr mutants (combined 48/48 (100%) with MIC ≥4.0 µg/ml; 45/48 (94%) with MIC ≥6.0 µg/ml; and 26/48 (54%) with MIC ≥8.0 µg/ml) compared to MIC values for *Ala90Val*/*Ser91Pro*/*Asp94Ala* mutants (combined 49/49 (100%) with MIC ≥4.0 µg/ml, 11/49 (22.4%) with MIC ≥6.0 µg/ml; and only 2/49 (4.1%) with MIC ≥8.0 µg/ml). The sensitivity for the obsolete CC of 2 µg/ml of Ofx to detect FQ resistance was 92.4%, with 100% specificity (**Table 4.3**).

4.3.2.2 Levofloxacin

Of 147 clinical isolates tested, 143 (97.3%) had a valid final MIC result for Lfx. At 1.0 µg/ml, the current WHO-recommended CC (16), all (38/38) wild-type isolates were correctly identified as susceptible while eight resistance-associated *gyrA*/*gyrB* mutants (8/105, 7.6%) would be misclassified as susceptible to Lfx (**Table 4.2**). Only six of these eight isolates were the same as the missed Ofx-resistance ones. The remaining *gyrA*/*gyrB* mutants with a valid MIC result (97/105, 92.4%) were correctly classified as resistant, with overall higher MICs for *Asp94Asn*/*Asp94Gly*/*Asp94His*/*Asp94Tyr* mutants (combined 49/49 (100%) with MIC ≥3.0 µg/ml, 28/49 (57%) with MIC ≥4.5 µg/ml, 9/49 (18.3%) with MIC ≥6.0 µg/ml,) compared to MIC values for other mutants (combined 43/48 (89.5%) with MIC ≥1.5 µg/ml, 23/48 (47.9%) with MIC ≥3.0 µg/ml, and 2/48 (4.2%) with MIC ≥4.5 µg/ml). The sensitivity for the current CC of Lfx to detect FQ resistance was 92.4%, with 100% specificity (**Table 4.3**), the same as for Ofx.

4.3.2.3 Moxifloxacin

Of 147 clinical isolates tested, 143 (97.3%) had a valid final MIC result for Mfx. At 0.25 µg/ml CC (16), all (38/38) wild-type isolates were correctly identified as "susceptible" while 15 resistance-associated *gyrA*/*gyrB* mutants (15/105, 14.3%) would be misclassified as "susceptible" to Mfx (**Table 4.2**), which is more than for Ofx and Lfx. Two of these have a mutation in *gyrB* (*Thr500Ile* and *Thr500Asn*) and 13 in *gyrA* (*Ala90Val* and *Asp94Ala*). The remaining 90 (90/105, 85.7%) *gyrA*/*gyrB* mutants with a valid MIC result were correctly classified as resistant, with overall higher MICs for *Asp94Asn*/*Asp94Gly*/ *Asp94His*/*Asp94Tyr* mutants (combined 47/47 (100%) with MIC ≥0.5 µg/ml, 46/47 (98%) with MIC ≥1.0 µg/ml, 24/47 (51%) with MIC ≥1.5 µg/ml and 8/47 (17%) with MIC ≥2.0 µg/ml) compared to MIC values for other mutants (combined 43/43 (100%) with MIC ≥0.5 µg/ml, 18/43 (41.8%) with MIC ≥1.0 µg/ml, and 2/43 (4.6%) with MIC ≥1.5 µg/ml). The sensitivity of the current CC of Mfx to detect FQ resistance (85.7%) was lower compared to Ofx and Lfx, with 100% specificity (**Table 4.3**).

4.3.2.4 Gatifloxacin

Of 147 clinical isolates tested, 145 (98.6%) had a valid final MIC result for Gfx. At 0.25 µg/ml CC (16), 100% of (38/38) wild-type isolates were correctly identified as "susceptible" while 18 resistance-associated *gyrA/gyrB* mutants (18/107, 16.8%) would be misclassified as "susceptible" to Gfx (**Table 4.2**), which is more than for Ofx, Lfx, and Mfx. Four of these have a mutation in *gyrB* (*Thr500Ala*, *Asn499Arg*, *Thr500Asn*, and *Thr500Ile*), 13 in *gyrA* (*Ala90Val* and *Asp94Ala*), and one had both *gyrA* and *gyrB* mutations (*Ala90Val* + *Gly512Arg*). The latter showed low-level resistance for Ofx, Lfx, and Mfx (**Table 4.2**). The remaining 89 (89/105, 84.8%) *gyrA*/*gyrB* mutants with a valid MIC result were correctly classified as resistant, with overall higher MICs for *Asp94Asn*/*Asp94Gly*/*Asp94His*/*Asp94Tyr* mutants (combined 49/49 (100%) with MIC ≥0.5 µg/ml, 38/49 (77.5%) with MIC ≥1 µg/ml, and 4/49 (8.2%) with MIC 1.5 µg/ml and none above 1.5 µg/ml) compared to MIC values for other mutants (combined 40/40 (100%) with MIC ≥0.5 µg/ml, 8/40 (20.0%) with MIC ≥1.0 µg/ml, and 1/40 (2.5%) with MIC 1.5 µg/ml). The sensitivity of the CC of Gfx to detect FQ resistance (83.2%) was the lowest of all four FQs tested, with 100% specificity (**Table 4.3**).

gyrAB sequence	Total tested	MIC Ofloxacin (µg/ml)									MIC Levofloxacin (µg/ml)										MIC Moxifloxacin (µg/ml)									MIC Gatifloxacin (µg/ml)								
		≤0.5	1.0	1.5	**2.0 (CC)**	4.0	6.0	8.0	>8	Invalid	≤0.37	0.75	**1.0 (CC)**	1.12	1.5	3.0	4.5	6.0	>6	Invalid	≤0.125	0.187	**0.25 (CC)**	0.5	1.0	1.5	2.0	>2	Invalid	≤0.125	0.187	**0.25 (CC)[16]**	0.5	1.0	1.5	2.0	>2	Invalid
Wildtype*	40	27	13	-	-	-	-	-	-	-	36	2	-	-	-	-	-	-	-	2	38	-	-	-	-	-	-	-	2	38	-	-	-	-	-	-	-	2
gyrA mutants	101																																					
Asp89Asn	2	-	-	-	**1**	1	-	-	-	-	-	-	**1**	1	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	1	1	-	-	-	-
Ala90Val	27	-	-	-	**1**	17	7	1	-	1	-	**1**	**1**	3	8	11	1	-	-	2	-	**1**	**9**	10	7	-	-	-	-	**1**	**1**	**7**	18	-	-	-	-	-
Ala90Val+Gly512Arg	1	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-
Ser91Pro	7	-	-	-	-	6	1	-	-	-	-	-	-	1	1	5	-	-	-	-	-	-	-	3	3	-	-	-	1	-	-	-	4	3	-	-	-	-
Ser91Pro+Gly512Arg	2	-	-	-	-	2	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	-	-	1	1	-	-	-	-
Asp94Ala	13	-	-	-	**1**	11	1	-	-	-	-	-	-	-	8	5	-	-	-	-	-	-	**3**	7	3	-	-	-	-	-	-	**4**	8	1	-	-	-	-
Asp94Asn	7	-	-	-	-	-	-	5	2	-	-	-	-	-	-	1	4	1	1	-	-	-	-	-	1	3	1	2	-	-	-	-	-	4	3	-	-	-	-
Asp94Gly	34	-	-	-	-	3	18	6	7	-	-	-	-	-	-	18	12	3	1	-	-	-	-	1	19	12	1	1	-	-	-	-	11	22	1	-	-	-
Asp94Gly+Gly512Arg	2	-	-	-	-	-	1	1	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	2	-	-	-	-
Asp94His	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	1	-	-	-	-
Asp94Tyr	5	-	-	-	-	-	-	3	1	1	-	-	-	-	-	1	2	2	-	-	-	-	-	-	2	1	2	-	-	-	-	-	-	5	-	-	-	-
gyrB mutants	6																																					
Asn499Thr	1	-	-	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	-	-
Asn499Arg	1	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	**1**	-	-	-	-	-	-	-	-
Asn499Ser	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	1	-
Thr500Ala	1	-	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	**1**	-	-	-	-	-	-
Thr500Asn	1	-	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	**1**	-	-	-	-	-
Thr500Ile	1	**1**	-	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-	**1**	-	-	-	-	-	-	-
Total	147	28	14	2	4	41	28	16	12	2	38	5	3	8	20	42	20	8	2	4	38	2	13	26	38	18	5	3	4	41	2	13	43	41	4	-	1	2

Table 4.2: MIC distribution of the *Mycobacterium tuberculosis* isolates with final valid phenotypic MGIT960 results, stratified per drug and *gyrA/gyrB* mutation.

*Strains with wild type *gyrA* and *gyrB* genes or *gyrA/gyrB* mutations that are not associated with resistance to fluoroquinolones (WHO Mutation Catalogue, Version 1.0) (3). *gyrA/gyrB* mutants misclassified as S by the current WHO recommended CCs for Lfx, Mfx and Gfx and the obsolete CC for Ofx are boxed with bold text.

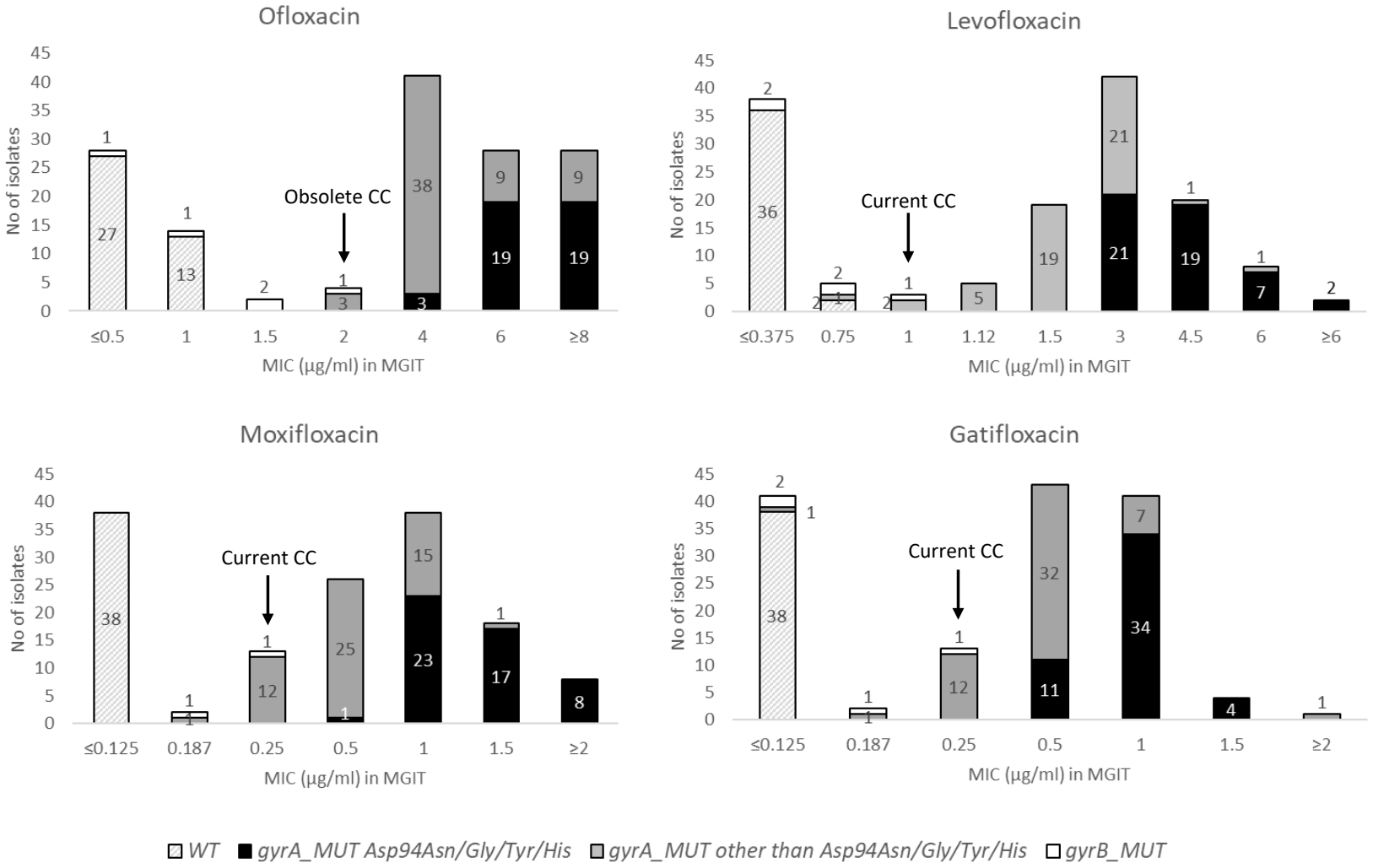


Figure 4.1: Minimal inhibitory concentration (MIC) distribution of the drugs tested in relation to the *gyrA/gyrB* mutations.

CC = critical concentration, MIC = minimal inhibitory concentration, MUT = mutation/s

CC (µg/ml)		Mutants showing resistance to the drug at the depicted critical concentration (number of isolates)				
		Ofx 2.0 µg/ml	Lfx 1.0 µg/ml	Mfx 0.25 µg/ml	Gfx 0.25 µg/ml	None of the four CCs
Mutants found susceptible to the drug and critical concentration depicted (number of isolates)	Ofx 2.0	NA	*gyrA_Asp94Ala* (1) *gyrA_Asp89Asn* (1)	*gyrA_Asp89Asn* (1) *gyrB_Thr500Ala* (1) *gyrB_Asn499Thr* (1)	*gyrA_Asp89Asn* (1) *gyrB_Asn499Thr* (1)	NA
	Lfx 1.0	*gyrA_Ala90Val* (1) *gyrA_Asp89Asn* (1)	NA	*gyrA_Asp89Asn* (1) *gyrB_Thr500Ala* (1) *gyrB_Asn499Thr* (1)	*gyrA_Asp89Asn* (1) *gyrB_Asn499Thr* (1)	NA
	Mfx 0.25	*gyrA_Ala90Val* (9) *gyrA_Asp94Ala* (2)	*gyrA_Ala90Val* (8) *gyrA_Asp94Ala* (3)	NA	*gyrA_Ala90Val* (3)	NA
	Gfx 0.25	*gyrA*_Ala90Val (8) *gyrA*_Asp94Ala (3) *gyrA_ Ala90Val gyrB_Gly512Arg* (1)	*gyrA_Ala90Val* (7) *gyrA_Asp94Ala* (4) *gyrA_Ala90Val + gyrB_Gly512Arg* (1)	*gyrA_Ala90Val* (2) *gyrA_Asp94Ala* (1) *gyrA_ Ala90Val + gyrB_Gly512Arg* (1) *gyrB_Thr500Ala* (1)	NA	NA
	All four CCs	NA	NA	NA	NA	*gyrA_Ala90Val* (1) *gyrB_Thr500Asn* (1) *gyrB_Thr500Ile* (1)

Table 4.3: Overview of *gyrA/gyrB* mutants classified as susceptible to one or more of the FQs tested at the current critical concentrations

At the lowest concentrations evaluated in this study, Ofx (0.5 µg/ml) and Lfx (0.375 µg/ml) exhibited overlapping MICs of wild-type and *gyrB* mutants, Gfx (0.125 µg/ml) exhibited overlapping MICs of both *gyrA* and *gyrB* mutants with wild types, whereas at 0.125 µg/ml of Mfx, *gyrA*/*gyrB* wild types could be distinguished from the mutants, as the MICs of the *gyrA/gyrB* mutants were ≥0.187 µg/ml. Overall, *gyrB* mutants exhibited lower MICs to all four FQs, with the exception of *Asn499Ser* which showed MIC values at the upper end for all four FQs. The *gyrB_Asn499Thr* and *Thr500Ala* were only detected by Mfx testing, two (*Thr500Asn* and *Thr500Ile*) were missed by all four CCs, and *Asn499Arg*, which did not have a valid MIC for Mfx-was missed by the other three FQs (**Tables 4.2 and 4.4**).

Drug and CC	Resistance associated mutations	R/S by MIC testing in MGIT				
		S	R	Total	**Sensitivity** [95% CI]	**Specificity** [95% CI]
Ofx	Absent*	40	8	**48**	**92.4%**	**100.0%**
	Present	0	97	**97**		
2.0 µg/ml	Total	**40**	**105**	**145**	[85.5% to 96.7%]	[91.2% to 100.0%]
Mfx	Absent*	38	15	**53**	**85.7%**	**100.0%**
	Present	0	90	**90**		
0.25 µg/ml	Total	**38**	**105**	**143**	[77.5% to 91.8%]	[90.8% to 100.0%]
Lfx	Absent*	38	8	**46**	**92.4%**	**100.0%**
	Present	0	97	**97**		
1.0 µg/ml	Total	**38**	**105**	**143**	[85.5% to 96.6%]	[90.8% to 100.0%]
Gfx	Absent*	38	18	**56**	**83.2%**	**100.0%**
	Present	0	89	**89**		
0.25 µg/ml	Total	**38**	**81**	**145**	[74.7% to 89.7%]	[90.8% to 100.0%]

Table 4.4: Sensitivity and specificity of the obsolete CC for Ofx and the current CCs for Lfx, Mfx and Gfx along with their 95% confidence intervals (CI).

*Includes trains with wild type *gyrA* and *gyrB* genes or *gyrA*/*gyrB* mutations that are not associated with resistance to fluoroquinolones (WHO Mutation Catalogue, version 1.0) (19).

CC = critical concentration; Ofx = ofloxacin; Lfx = levofloxacin; Mfx = moxifloxacin; Gfx = gatifloxacin; S = susceptible; R = resistant; MIC = minimal inhibitory concentration

4.4 Discussion

FQs are recommended by the WHO for use in the 4-month short-course therapy of drug-sensitive TB, in the treatment of RR TB without FQ resistance, and in salvage regimens with low-level FQ resistance (6,23). Thus, there is an important need for accurate detection of FQ resistance. The WHO recommends testing the specific FQs used in treatment regimens proposing the MGIT960-based pDST as the reference method (16). In this study, we re-evaluated the current WHO-recommended CCs for Lfx (1.0 µg/ml) and Mfx (0.25 µg/ml), as well as the now-obsolete CCs for Ofx (2.0 µg/ml) and Gfx (0.25 µg/ml) for the MGIT960 system. The specificity of the CCs of all four FQs tested was 100%. However, the sensitivity of the CCs varied, with Ofx and Lfx showing the highest sensitivity (92.4%), followed by Mfx 0.25 µg/ml (85.7%) and Gfx 0.25 µg/ml (83.2%).

Discordance between phenotypic and genotypic DSTs for FQs has previously been observed, particularly for low-level FQ resistance-conferring *gyrA/gyrB* mutations, and breakpoint artifacts can be a key contributor to such discordances (24,25). Based on previously published MIC data of clinical isolates, the WHO lowered the CCs of Mfx and Lfx in 2018 to correspond to the epidemiological cutoff (ECOFF), the upper end of the wild-type MIC distribution (16,26). Our findings suggest that the current CC of Mfx may be above the highest end of the wild-type MIC distribution, with the isolates included in this investigation all falling at least one dilution below the current CC. However, our truncated data in the lower concentration ranges do not allow a clear view of the wild-type MIC distribution. In addition, the isolates included in this study represented only two South Asian countries, Bangladesh, and Pakistan, and 90 isolates with lineage data represent only four MTBc lineages, thus their wild-type MIC distribution may not represent the global wild-type MIC distribution of MTBc for FQs. Further, in this study, we did not test at least 100 wild-type isolates as per CLSI guidelines. Thus, we were not able to conclude if the current CCs represent the ECOFF.

In line with previous studies, our data also suggest that different mutations in *gyrA* and *gyrB* cause variable levels and patterns of resistance to different FQs (27-30). While non-Ala codon 94 mutations in the *gyrA* gene showed overall higher MIC increases to all the FQs, the other commonly found *gyrA* mutations such as *Ala90Val* and *Asp94Ala* mutations showed a low-to-moderate increase of the MICs, notably for Mfx and Gfx, leading to false-susceptibility. In addition, three of the five *gyrB* mutants with a valid MIC to Mfx showed resistance while

only one of them showed resistance to all four FQs, implying that *gyrB* mutations may have a greater impact on Mfx than the other FQs and highlights the importance of testing the specific FQs used in treatment regimens and further investigating the possible use of Lfx for such mutants.

In MGIT, the *gyrB* mutation *Thr500Asn* has previously been reported to exhibit variable patterns of FQ resistance, however, in our study, this was classified as susceptible by all four FQs with MICs one dilution lower than the current CCs, probably underscoring the lack of reproducibility of pDSTs for the low-level FQ resistance-conferring mutations (10,27,31).

Despite the fact that the now obsolete CC of Ofx and the current CC of Lfx showed the highest sensitivity in this study, they still misclassified mutants, notably the *gyrB* mutations, which may cause clinically relevant resistance at least for Mfx, thus may not be suitable as a surrogate for FQ susceptibility in MTBc.

On the other hand, lowering the CC of Mfx to 0.125 µg/ml would allow us to correctly classify all wild-type and mutant isolates while lowering the CC of Gfx to 0.125 µg/ml would still misclassify some *gyrA/gyrB* mutants. Based on our findings, 0.125 µg/ml in MGIT medium may be an appropriate CC for Mfx as well as a surrogate for FQ resistance in MTBc.

4.5 Ethics Considerations

Ethics approval was not required for this laboratory-based study, as anonymized stored clinical isolates were used.

4.6 Funding

This study was funded by internal funding from the Unit of Mycobacteriology, Institute of Tropical Medicine, Antwerp.

4.7 Acknowledgements

An abstract based on the presented data was accepted for the Forty-second European Society of Mycobacteriology conference, Bologna, Italy (June 2022) (P-09).

4.8 References

1. Rigouts L, Coeck N, Gumusboga M, de Rijk WB, Aung KJ, Hossain MA, *et al.* Specific gyrA gene mutations predict poor treatment outcome in MDR-TB. J Antimicrob Chemother 2016;71:314-23.
2. Wang JY, Lee LN, Lai HC, Wang SK, Jan IS, Yu CJ, *et al.* Fluoroquinolone resistance in *Mycobacterium tuberculosis* isolates: Associated genetic mutations and relationship to antimicrobial exposure. J Antimicrob Chemother 2007;59:860-5.
3. Ji B, Lounis N, Maslo C, Truffot-Pernot C, Bonnafous P, Grosset J. *In vitro* and *in vivo* activities of moxifloxacin and clinafloxacin against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 1998;42:2066-9.
4. Alvirez-Freites EJ, Carter JL, Cynamon MH. *In vitro* and *in vivo* activities of gatifloxacin against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2002;46:1022-5.
5. World Health Organization. Meeting Report of the WHO Expert Consultation on the Definition of Extensively Drug-Resistant Tuberculosis; 2021.
6. World Health Organization. Rapid Communication: Key Changes to the Treatment of Drug-Resistant Tuberculosis. Geneva, Switzerland: World Health Organization; 2022.
7. World Health Organization. WHO Consolidated Guidelines on Tuberculosis. Module 4: Treatment – Drug-Resistant Tuberculosis Treatment. Geneva, Switzerland: World Health Organization; 2020.
8. Mayer C, Takiff H. The molecular genetics of fluoroquinolone resistance in *Mycobacterium tuberculosis*. Microbiol Spectr 2014;2:M2-2013.
9. Maruri F, Sterling TR, Kaiga AW, Blackman A, van der Heijden YF, Mayer C, *et al*. A systematic review of gyrase mutations associated with fluoroquinolone-resistant *Mycobacterium tuberculosis* and a proposed gyrase numbering system. J Antimicrob Chemother 2012;67:819-31.
10. Avalos E, Catanzaro D, Catanzaro A, Ganiats T, Brodine S, Alcaraz J, *et al.* Frequency and geographic distribution of gyrA and gyrB mutations associated with fluoroquinolone resistance in clinical *Mycobacterium tuberculosis* isolates: A systematic review. PLoS One 2015;10:e0120470.
11. Soudani A, Hadjfredj S, Zribi M, Messaoud T, Masmoudi A, Majed B, *et al.* First report of molecular characterization of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates from a Tunisian hospital. Clin Microbiol Infect 2010;16:1454-7.
12. Devasia R, Blackman A, Eden S, Li H, Maruri F, Shintani A, *et al.* High proportion of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates with novel gyrase polymorphisms and a gyrA region associated with fluoroquinolone susceptibility. J Clin Microbiol 2012;50:1390-6.
13. Chien JY, Chiu WY, Chien ST, Chiang CJ, Yu CJ, Hsueh PR. Mutations in gyrA and gyrB among fluoroquinolone- and multidrug-resistant *Mycobacterium tuberculosis* isolates. Antimicrob Agents Chemother 2016;60:2090-6.
14. Singh P, Jain A, Dixit P, Prakash S, Jaiswal I, Venkatesh V, *et al.* Prevalence of gyrA and B gene mutations in fluoroquinolone-resistant and -sensitive clinical isolates of *Mycobacterium tuberculosis* and their relationship with MIC of ofloxacin. J Antibiot (Tokyo) 2015;68:63-6.
15. Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W. Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant *Mycobacterium tuberculosis* complex isolates. J Clin Microbiol 2010;48:1683-9.

16. World Health Organization. Technical Report on Critical Concentrations for Drug Susceptibility Testing of Medicines used in the Treatment of Drug-Resistant Tuberculosis. Geneva, Switzerland: World Health Organization; 2018. Available from: https://www.who.int/publications/i/ item/WHO-CDS-TB-2018.5. [Last accessed on 2023 Aug 29].
17. Cook C. Challenges with diagnoses: Sketchy reference standards. J Man Manip Ther 2012;20:111-2.
18. World Health Organization. Technical Report on Critical Concentrations for Drug Susceptibility Testing of Isoniazid and the Rifamycins (Rifampicin, Rifabutin and Rifapentine. Geneva, Switzerland: World Health Organization; 2021.
19. World Health Organization. Catalogue of Mutations in *Mycobacterium tuberculosis* Complex and Their Association with Drug Resistance. Geneva, Switzerland: World Health Organization; 2021.
20. World Health Organization. Companion Handbook to the WHO Guidelines for the Programmatic Management of Drug-Resistant Tuberculosis. Geneva, Switzerland: World Health Organization; 2014.
21. Isaeva Y, Bukatina A, Krylova L, Nosova E, Makarova M, Moroz A. Determination of critical concentrations of moxifloxacin and gatifloxacin for drug susceptibility testing of *Mycobacterium tuberculosis* in the BACTEC MGIT 960 system. J Antimicrob Chemother 2013;68:2274-81.
22. Sturegård E, Ängeby KA, Werngren J, Juréen P, Kronvall G, Giske CG, *et al.* Little difference between minimum inhibitory concentrations of *Mycobacterium tuberculosis* wild-type organisms determined with BACTEC MGIT 960 and Middlebrook 7H10. Clin Microbiol Infect 2015;21:7.e5-7.
23. World Health Organization. WHO Consolidated Guidelines on Tuberculosis: Module 4: Treatment: Drug-Susceptible Tuberculosis Treatment. Geneva, Switzerland: World Health Organization; 2022.
24. Heyckendorf J, Andres S, Köser CU, Olaru ID, Schön T, Sturegård E, *et al.* What is resistance? Impact of phenotypic versus molecular drug resistance testing on therapy for multi- and extensively drug-resistant tuberculosis. Antimicrob Agents Chemother 2018;62:e01550-17.
25. Schön T, Matuschek E, Mohamed S, Utukuri M, Heysell S, Alffenaar JW, *et al.* Standards for MIC testing that apply to the majority of bacterial pathogens should also be enforced for *Mycobacterium tuberculosis* complex. Clin Microbiol Infect 2019;25:403-5.
26. Kahlmeter G, Turnidge J. How to: ECOFFs-the why, the how, and the don'ts of EUCAST epidemiological cutoff values. Clin Microbiol Infect 2022;28:952-4.
27. Finci I, Albertini A, Merker M, Andres S, Bablishvili N, Barilar I, *et al.* Investigating resistance in clinical *Mycobacterium tuberculosis* complex isolates with genomic and phenotypic antimicrobial susceptibility testing: A multicentre observational study. Lancet Microbe 2022;3:e672-82.
28. Willby M, Chopra P, Lemmer D, Klein K, Dalton TL, Engelthaler DM, *et al.* Molecular evaluation of fluoroquinolone resistance in serial *Mycobacterium tuberculosis* isolates from individuals diagnosed with multidrug-resistant tuberculosis. Antimicrob Agents Chemother 2020;65:e01663-20.
29. Zhang Z, Lu J, Wang Y, Pang Y, Zhao Y. Prevalence and molecular characterization of fluoroquinolone-resistant *Mycobacterium tuberculosis* isolates in China. Antimicrob Agents Chemother 2014;58:364-9.

30. Huo F, Ma Y, Li S, Xue Y, Shang Y, Dong L, *et al.* Specific gyrA gene mutations correlate with high prevalence of discordant levofloxacin resistance in *Mycobacterium tuberculosis* isolates from Beijing, China. J Mol Diagn 2020;22:1199-204.
31. Long Q, Li W, Du Q, Fu Y, Liang Q, Huang H, *et al.* gyrA/B fluoroquinolone resistance allele profiles amongst *Mycobacterium tuberculosis*

4.9 Supplementary material

Lab nr	Origin	Lineage	PCR gyrase	MIC in MGIT (µg/ml)			
				OFX	**LVX**	**MFX**	**GFX**
72228	Bangladesh	NA	94Ala	4	1.5	0.5	0.25
90829	Bangladesh	1.1.3.1	539Ala	1.5	0.75	0.5	0.187
111485	Bangladesh	NA	90Val, 551Arg	4	1.5	0.5	0.25
120583	Bangladesh	NA	90Val	4	1.5	0.5	0.25
131127	Pakistan	1.2.2	90Val	4	1.12	0.5	0.25
140938	Pakistan	4.1.3	538Arg	1	≤0,37	X	≤0,125
1077	Bangladesh	4.3.4.2	90Val	6	3	1	0.5
22934	Bangladesh	3	90Val	4	1,5	0.25	0.25
51552	Bangladesh	4.3.4.2	94Gly	6	3	1	1
51580	Bangladesh	1	539Asn	1.5	0.75	0.25	0.25
52144	Bangladesh	2.2.1	WT	≤0,5	≤0,37	≤0,125	≤0,125
52169	Bangladesh	4.8	WT	1	≤0,37	≤0,125	≤0,125
52398	Bangladesh	4.3.4.2	90Val	4	3	0.5	0.5
52427	Bangladesh	4	90Val	6	4.5	0.5	0.5
52879	Bangladesh	NA	WT	1	0.75	≤0,125	≤0,125
60626	Bangladesh	1.1.3	90Val	6	3	1	0.5
61618	Bangladesh	2.2.1	90Val	4	3	0.5	0.5
61934	Bangladesh	3.1	WT	≤0,5	≤0,37	≤0,125	≤0,125
63450	Bangladesh	1.1.3.3	WT	≤0,5	X	≤0,125	X
63452	Bangladesh	4.3.4.2	90Val	X	X	0.5	0.5
64048	Bangladesh	2.2.1	WT	≤0,5	≤0,37	≤0,125	≤0,125
70053	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
71338	Bangladesh	4.3.4.2	WT	≤0,5	≤0,37	≤0,125	≤0,125
72225	Bangladesh	4.1.3	538Ser	>8	6	1.5	>2
72422	Bangladesh	3	WT	≤0,5	≤0,37	≤0,125	≤0,125
73097	Bangladesh	2.2.1	94Ala	4	1.5	0.5	0.5
73311	Bangladesh	1.2.2.2	94Ala	4	3	0.5	0.5
80072	Bangladesh	NA	90Val	4	1.5	0.25	0.5
82030	Bangladesh	3	90Val	4	1.5	0.25	0.25
82038	Bangladesh	4.3.4.2	90Val	4	3	0.5	0.5
140348	Bangladesh	NA	94Gly	6	3	1	0.5
141128	Bangladesh	3.1.2.1	94Gly	4	3	0.5	0.5
141201	Bangladesh	4.1.3	91Pro	4	3	1	1
141969	Bangladesh	1.2.2.2	WT	≤0,5	≤0,37	≤0,125	≤0,125
83358	Bangladesh	2	94Gly, 551Arg	8	4.5	1.5	1
83113	Bangladesh	NA	94Gly	6	3	1	0.5
83145	Bangladesh	2.2.1	94Ala	4	1.5	0.5	0.5
83694	Bangladesh	4.1.3	94His	>8	6	2	1
84277	Bangladesh	NA	94Ala	4	3	0.5	0.5
90102	Bangladesh	NA	94Asn	>8	>6	>2	1.5
90490	Bangladesh	4.3.4.2	94Gly	8	4.5	1.5	1
90798	Bangladesh	NA	90Val	4	1.5	0.25	0.5
91126	Bangladesh	NA	94Gly	6	3	1.5	1
91130	Bangladesh	NA	94Gly	>8	6	1.5	1
92032	Bangladesh	NA	94Ala	4	1.5	0.25	0.25

92333	Bangladesh	3	91Pro	4	3	0.5	0.5
93573	Bangladesh	4	94Ala	2	1.5	0.25	0.25
100380	Bangladesh	NA	94Gly	6	4.5	1.5	1
100271	Bangladesh	4.1.3	94Ala	4	1.5	1	0.5
101140	Bangladesh	2	91Pro, 551Arg	4	1.5	1	0.5
101142	Bangladesh	NA	90Val	6	3	1	0.5
102091	Bangladesh	1.2.2.2	94Ala	4	3	1	1
101905	Bangladesh	4	94Ala	6	3	1	0.5
102106	Bangladesh	3	90Val	4	1.5	0.187	0.25
102627	Bangladesh	NA	94Gly	4	3	1	0.5
110113	Bangladesh	NA	89Asn	2	1.12	0.5	1
110339	Bangladesh	NA	90Val	4	1.5	0.25	0.5
110632	Bangladesh	4	94Gly	>8	6	1.5	1
110617	Bangladesh	3	94Gly	8	4.5	1	0.5
110911	Bangladesh	4	94Ala	4	1.5	0.25	0.25
111209	Bangladesh	NA	90Val	6	3	1	0.5
111160	Bangladesh	4.1.1.1	94Gly	>8	4.5	1	1
111502	Bangladesh	NA	94Ala	4	3	0.5	0.5
112382	Bangladesh	NA	90Val	6	3	1	0.5
120036	Bangladesh	2.2.1	94Gly	6	4.5	1.5	1
120065	Bangladesh	NA	94Gly, 551Arg	6	3	1	1
120375	Bangladesh	2	91Pro, 551Arg	4	1.5	1	1
120718	Bangladesh	2	539Ile	≤0,5	≤0,37	0.187	≤0,125
121038	Bangladesh	2.2.1	90Val	4	0.75	0.25	≤0,125
121808	Bangladesh	NA	94Gly	8	4.5	1.5	1
130235	Bangladesh	NA	94Gly	4	3	1	0.5
131004	Bangladesh	NA	94Tyr	8	4.5	2	1
131010	Bangladesh	NA	91Pro	4	1.12	1	1
131011	Bangladesh	4.1.1.1	90Val	4	1.5	0.5	0.5
131039	Bangladesh	2.2.1	94Gly	6	3	1.5	1
131041	Bangladesh	2.2.1	94Gly	6	3	1	0.5
131781	Bangladesh	NA	94Gly	>8	4.5	1	1
132224	Bangladesh	1.1.2	90Val	4	3	0.5	0.5
130233	Bangladesh	2.2.1	94Gly	6	4.5	1	1
130549	Bangladesh	2.2.1	94Gly	6	3	1.5	1
51554	Bangladesh	NA	WT	1	≤0,37	X	≤0,125
51852	Bangladesh	NA	WT	1	≤0,37	≤0,125	≤0,125
52088	Bangladesh	NA	WT	1	≤0,37	≤0,125	≤0,125
60639	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
61597	Bangladesh	NA	WT	≤0,5	X	≤0,125	≤0,125
62401	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
62518	Bangladesh	NA	WT	1	≤0,37	≤0,125	≤0,125
64063	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
70097	Bangladesh	NA	WT	1	0.75	≤0,125	≤0,125
70235	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
71769	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
71851	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
72209	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
72503	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
72506	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125

73296	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
73309	Bangladesh	NA	WT	≤0,5	≤0,37	≤0,125	≤0,125
52675	Bangladesh	2.2.1	WT	≤0,5	≤0,37	≤0,125	≤0,125
52873	Bangladesh	NA	WT	1	≤0,37	≤0,125	≤0,125
60633	Bangladesh	4.3.4.2	WT	≤0,5	≤0,37	≤0,125	≤0,125
111257	Pakistan	NA	94Tyr	8	6	1.5	1
131057	Pakistan	1.1.3	94Tyr	>8	6	2	1
131062	Pakistan	NA	94Tyr	8	4.5	1	1
131157	Pakistan	3	94Gly	6	3	1	0.5
131188	Pakistan	3	94Gly	6	3	1	0.5
131223	Pakistan	NA	Asn538Thr	2	1	0.5	1
131228	Pakistan	2.2.1.1	90Val	2	1	0.25	0.187
131240	Pakistan	3	94Gly	6	3	1	1
131498	Pakistan	1.1.3	91Pro	6	3	1	1
131569	Pakistan	2.2.1	94Asn	8	3	2	1.5
131579	Pakistan	2.2.1	94Asn	8	4.5	1.5	1
131580	Pakistan	2.2.1	94Asn	8	4.5	1.5	1
132271	Pakistan	2.2.1	90Val	4	1.12	0.25	0.25
132301	Pakistan	NA	94Gly	>8	6	2	1
132317	Pakistan	2.2.1	94Gly	6	3	1	0.5
132393	Pakistan	1.1.3	90Val	8	3	1	0.5
132396	Pakistan	1.1.3	90Val	6	3	1	0.5
132401	Pakistan	NA	94Gly	8	4.5	1.5	1
132402	Pakistan	3	90Val	4	1.12	0.25	0.25
132435	Pakistan	NA	90Val	4	X	0.5	0.5
132671	Pakistan	2.2.1.2	94Ala	4	1.5	0.5	0.5
132746	Pakistan	NA	94Gly	>8	>6	>2	1.5
140112	Pakistan	4.1.2.1	94Asn	>8	6	>2	1.5
130687	Pakistan	3	89Asn	4	1	0.5	0.5
130704	Pakistan	2.2.1	94Gly	8	3	1.5	1
130751	Pakistan	3	94Asn	8	4.5	1.5	1
130769	Pakistan	2.2.1	94Gly	6	3	1	1
130789	Pakistan	2.2.1	94Gly	8	4.5	1.5	1
132059	Pakistan	NA	94Tyr	X	3	1	1
132139	Pakistan	3	94Gly	6	3	1	1
132143	Pakistan	NA	94Gly	6	3	1	1
132149	Pakistan	3	94Gly	6	4.5	1	0.5
132151	Pakistan	3	94Gly	>8	4.5	1	1
140009	Pakistan	3.1.2	94Asn	8	4.5	1	1
140015	Pakistan	3	91Pro	4	3	0.5	0.5
140097	Pakistan	3	91Pro	4	1.5	0.5	0.5
141502	Pakistan	NA	91Pro	4	3	X	0.5
132284	Pakistan	2.2.1	WT	≤0,5	≤0,37	≤0,125	≤0,125
132312	Pakistan	1.1.3	WT	1	≤0,37	≤0,125	≤0,125
132323	Pakistan	1.1.3	WT	1	≤0,37	≤0,125	≤0,125
132338	Pakistan	NA	WT	1	≤0,37	≤0,125	≤0,125
132349	Pakistan	1.1.3	WT	1	≤0,37	≤0,125	≤0,125
130637	Pakistan	3	WT	≤0,5	≤0,37	≤0,125	≤0,125
130707	Pakistan	3	WT	1	≤0,37	≤0,125	≤0,125
132109	Pakistan	3	WT	≤0,5	≤0,37	≤0,125	≤0,125

140045	Pakistan	1.1.2	WT	≤0,5	≤0,37	≤0,125	≤0,125
140071	Pakistan	3	WT	≤0,5	≤0,37	X	X
083715	Quality control	4	H37Rv replicate	1	≤0,37	≤0,125	≤0,125
083715	Quality control	4	H37Rv replicate	1	≤0,37	≤0,125	≤0,125
083715	Quality control	4	H37Rv replicate	1	≤0,37	≤0,125	≤0,125
083715	Quality control	4	H37Rv replicate	1	≤0,37	≤0,125	≤0,125
083715	Quality control	4	H37Rv replicate	1	≤0,37	0.187	≤0,125
083715	Quality control	4	H37Rv replicate	≤0,5	≤0,37	≤0,125	≤0,125
083715	Quality control	4	H37Rv replicate	1	≤0,37	≤0,125	≤0,125
083715	Quality control	4	H37Rv replicate	1	≤0,37	≤0,125	≤0,125
083715	Quality control	4	H37Rv replicate	1	≤0,37	X	≤0,125
083715	Quality control	4	H37Rv replicate	≤0,5	≤0,37	≤0,125	X

Table 4.S1: Supplementary data

CHAPTER 5

Reduced critical concentration might not have improved MGIT-based DST's sensitivity to rifampicin

Reduced critical concentration might not have improved MGIT-based DST's sensitivity to rifampicin

Antimicrobial Agents and Chemotherapy

DOI: https://doi.org/10.1128/aac.01701-23

Praharshinie Rupasinghe[1,9]*, Azka Ashraf[2], Nadia Barreda[3], Shafiqua Parveen[2], Muhammad Zubair[2], Roger Calderon[3], Sunil Asif[2], Nilma Hirani[4], Layila Chingisova[5], Atang Bulane[6], Pham Thu Hang[7], Doan Thu Ha[8], Elisa Ardizzoni[1], Nazia Kursheed[2], Willem Bram De Rijk[1], Leen Rigouts[1,9], Lorenzo Guglielmetti[10,11,12], Carol Mitnick[13,14,15], Bouke C. de Jong[1]

[1] Unit of Mycobacteriology, Institute of Tropical Medicine, Antwerp, Belgium
[2] The Indus Hospital laboratory, Karachi, Pakistan
[3] Socios en Salud, Lima, Peru
[4] Department of Microbiology, Sir JJ Hospital, Mumbai, India
[5] National Tuberculosis Reference Lab, Almaty, Kazakhstan
[6] Center for tuberculosis, National Institute of communicable diseases, South Africa
[7] Regional Tuberculosis Reference Lab, Ho Chi Minh, Vietnam
[8] National Tuberculosis Reference Lab, Hanoi, Vietnam
[9] Department of Biomedical Sciences, University of Antwerp, Wilrijk, Antwerp, Belgium
[10] Medical Department, MSF, France
[11] Sorbonne University, Centre d'Immunologie et des Maladies Infectieuses (Cimi-Paris)
[12] AP-HP, Bactériologie-Hygiène, Hôpital Pitié-Salpêtrière, Centre National de Référence des Mycobactéries, Paris, France
[13] Brigham and Women's Hospital, Boston, Massachusetts, USA.
[14] Partners In Health, Boston, Massachusetts
[15] Department of Global Health and Social Medicine, Harvard Medical School, Boston, Massachusetts.

*Corresponding author

Abstract

Recent studies have shown that phenotypic drug susceptibility testing (pDST) is not a reliable reference as it frequently misses *rpoB* mutations associated with borderline phenotypic resistance, especially in the Mycobacteria Growth Indicator Tube (MGIT), due to impaired bacterial growth.

Consequently, WHO now recommends sequencing the entire *rpoB* gene as the new reference standard and revised the CC in MGIT from 1.0 µg/ml to 0.5 µg/ml to boost its ability to detect borderline *rpoB* mutations.

We assessed the ability of the revised CC for rifampicin in MGIT using 37 isolates found genotypically rifampicin-resistant but susceptible at 1.0µg/ml in MGIT.

We performed indirect *rpoB* sequencing on the isolates and direct *rpoB* sequencing on the sediments from which the isolates originated. Meanwhile, the isolates were retested in MGIT at the revised CC (0.5 µg/mL) in single replicates.

All, 37 isolates carried *rpoB* mutations: 32 Group-1, four Group-2, and one Group-5. In total, 28 of the 32 isolates with Group-1 *rpoB* mutations had a borderline *rpoB* mutation, alone or together with a Group-1 or Group-2 *rpoB* mutation. At the revised CC, 2/32 (6.2%) of Group-1 *rpoB* mutants were classified as resistant, with only 1/28 (3.6%) Group-1-borderline *rpoB* mutations; this is much less than the WHO's predicted 21% reduction in misclassifying borderline *rpoB* mutations by reducing the rifampicin CC in MGIT.

Our data suggest that despite revising the CC in MGIT, genotypic tests are better at detecting these mutations.

5.1 Introduction

Rifampicin plays a pivotal role in the first-line treatment of tuberculosis, and resistance to rifampicin is a significant concern, often indicating multi-drug resistant tuberculosis (MDR-TB). Rifampicin inhibits the elongation of messenger RNA of the *Mycobacterium tuberculosis* complex (MTBc) by binding to the β-subunit of the RNA polymerase, thus the majority of rifampicin-resistant MTBc isolates harbor mutations in the 81-base pair long 'hotspot' region of the *rpoB* gene that codes for the β-subunit of the RNA polymerase (1).

Historically, phenotypic drug susceptibility testing (pDST) based on bacilli growth in media containing the critical concentration (CC) of rifampicin was considered the gold standard for detecting rifampicin resistance. CC is the lowest concentration of an anti-tuberculosis drug that inhibits 99% phenotypically wildtype MTBc in vitro and is specific to the culture medium used (2). However, recent studies have underscored its limitations, particularly in Mycobacteria Growth Indicator Tube (MGIT), which frequently misses borderline rpoB mutations associated with impaired bacterial growth (3-5). As a result, WHO now recommends sequencing the entire rpoB gene as the new reference standard (6, 7) and revised the CC in MGIT from 1.0 µg/ml to 0.5 µg/ml to boost its ability to detect the borderline rpoB mutations (6, 8). This study assesses whether the revised CC increases MGIT's ability to detect borderline rpoB mutations.

5.2 Method

Thirty-seven pre-treatment isolates from participants of the Unitaid-funded endTB and endTB-Q clinical trials in Pakistan (n=33), and Peru (n=4) were included in this analysis. These isolates were genotypically rifampicin-resistant by the MTBDR*plus* assay and phenotypically susceptible at 1.0 µg/ml in MGIT (9). We sequenced the *rpoB* gene of the isolates (indirect sequencing) using Sanger-sequencing or whole genome sequencing (WGS) and direct *rpoB* sequencing using Sanger-sequencing or the Genoscreen Deeplex® Myc-TB test on the sediments from which the isolates were isolated. Meanwhile, the isolates were retested in MGIT at the revised CC in single replicates.

5.3 Results

All 37 isolates carried rpoB mutations: 32 Group-1, four Group-2, and one Group-5 (7) (**Table 5.1**). Twenty-eight of the 32 isolates with Group-1 rpoB mutations, had a borderline *rpoB* mutation, alone or together with a Group-1 or Group-2 *rpoB* mutation. Thirty of the 37 sediments had valid direct *rpoB* sequences: all were concordant with the indirect *rpoB* sequences. At the revised CC, 2/32 (6.2%) of Group-1 *rpoB* mutants were classified as resistant, with only 1/28 (3.6%, 95% CI = 0.09%-18.3 95% CI) Group-1-borderline *rpoB* mutations.

5.4 Discussion

In 2021, with the revised CC, the WHO estimated a 21% decrease in misclassifying borderline *rpoB* mutations (8). However, our results show an overall decrease of only 6.2% in misclassification of group 1 *rpoB* mutations. Moreover, the revised CC picked up only 1/28 (3.6%, 95% CI = 0.09%-18.3 95% CI) Group-1-borderline *rpoB* mutations, much less than the 21% reduction by the WHO, indicating that lowering the CC in MGIT has not resolved the impaired sensitivity of MGIT-based pDST for rifampicin.

In the endTB/endTBQ clinical trials, patients with rifampicin resistance detected by genotypic testing received second-line anti-tuberculosis treatment, therefore we were unable to assess the clinical response of different borderline *rpoB* mutations to rifampicin-based treatment. However, it has been reported that borderline *rpoB* mutations may have the same clinical significance and transmission potential as high-confidence *rpoB* mutations, thus misdiagnosing them can lead to treatment failure and further transmission of rifampicin-resistant tuberculosis (8, 9). Our data suggest that despite revising the CC in MGIT genotypic tests are better at detecting these mutations. However, we tested only a limited number of isolates from just two distinct geographical locations and did not include MIC or replicate testing. A larger study including MIC and replicate testing would provide more comprehensive assessment of the MIC distribution of the borderline rpoB mutations and whether further reduction of the CC is required.

Isolate ID	Direct *rpoB* sequencing using the sputum sediment	Indirect *rpoB* sequencing using the culture isolate	WHO grouping of the rpoB mutation (7)	pDST results at the revised CC for rifampicin (0.5 µg/ml) in MGIT
PE-1	Asp435Val	Asp435Val	Group-1	S
PK-1	His445Arg	His445Arg	Group-1	S
PE-2	His445Cys	His445Cys	Group-1	R
PK-2	NA	His445Tyr	Group-1	S
PK-3	Asp435Tyr	Asp435Tyr	Group-1-borderline	S
PK-4	Asp435Tyr*	Asp435Tyr	Group-1-borderline	S
PK-5	Asp435Tyr	Asp435Tyr	Group-1-borderline	S
PK-6	Asp435Tyr	Asp435Tyr	Group-1-borderline	S
PK-7	Asp435Tyr	Asp435Tyr	Group-1-borderline	S
PK-8	Asp435Tyr	Asp435Tyr	Group-1-borderline	R
PK-9	Asp435Tyr	Asp435Tyr[#]	Group-1-borderline	S
PK-10	His445Asn	His445Asn	Group-1-borderline	S
PK-11	His445Asn	His445Asn	Group-1-borderline	S
PK-12	His445Asn*	His445Asn	Group-1-borderline	S
PK-13	His445Asn	His445Asn	Group-1-borderline	S
PK-14	His445Asn*	His445Asn	Group-1-borderline	S
PK-15	His445Asn	His445Asn	Group-1-borderline	S
PE-3	His445Asn	His445Asn	Group-1-borderline	S
PK-16	Leu430Pro	Leu430Pro	Group-1-borderline	S
PK-17	Leu430Pro	Leu430Pro	Group-1-borderline	S
PK-18	Leu430Pro*	Leu430Pro	Group-1-borderline	S
PK-19	Leu430Pro	Leu430Pro	Group-1-borderline	S
PK-20	Leu430Pro	Leu430Pro	Group-1-borderline	S
PK-21	Leu430Pro	Leu430Pro	Group-1-borderline	S
PK-22	Leu452Pro	Leu452Pro	Group-1-borderline	S
PK-23	NA	Leu452Pro	Group-1-borderline	S
PK-24	NA	His445Leu	Group-1-borderline	S
PK-25	NA	His445Leu[#]	Group-1-borderline	S

PK-26	His445Leu	His445Leu[#]	Group-1-borderline	S
PK-27	Asp435Tyr; Met434Ile	Asp435Tyr; Met434Ile	Group-1-borderline + Group-2	S
PK-28	NA	Asp435Tyr; Met434Ile	Group-1-borderline + Group-2	S
PK-29	Leu430Pro;Met434Ile	Leu430Pro;Met434Ile	Group-1-borderline + Group-2	S
PK-30	NA	Asp435Gly	Group-2	S
PK-31	NA	Asp435Gly	Group-2	S
PE-4	His445Gln	His445Gln	Group-2	S
PK-32	Lys446Arg	Lys446Arg	Group-2	S
PK-33	Thr444Thr	Thr444Thr	Group-5	S

Table 5.1: Overview of the results from the 37 clinical isolates included in this analysis.

Direct *rpoB* sequencing using the sediments was done using the GenoScreen Deeplex® Myc-TB assay or targeted Sanger sequencing - **rpoB* sequences by Sanger sequencing

Indirect *rpoB* sequencing using the isolates was done using the targeted Sanger sequencing or WGS - [#]*rpoB* sequences by WGS

R= Resistant, CC= Critical Concentration, pDST = Phenotypic Drug Susceptibility Testing, NA = interpretable direct *rpoB* sequencing results not available

Group 1 – Associated with resistance
Group 2 – Associated with resistance - interim
Group 3 – Uncertain significance in resistance
Group 4 – Not associated with resistance - interim
Group 5 – Not associated with resistance
This analysis did not include His445Ser, the sixth Group-1-borderline rpoB mutation (7).

5.5 Ethics considerations

The endTB and endTBQ trials were approved by institutional review/ethics boards of Harvard Medical School, Interactive Research and Development, Institute of Tropical Medicine, Médecins Sans Frontières, and each participating site.

5.6 References

1. Xu G, Liu H, Jia X, Wang X, Xu P. 2021. Mechanisms and detection methods of *Mycobacterium tuberculosis* rifampicin resistance: The phenomenon of drug resistance is complex. Tuberculosis 128:102083.
2. WHO. 2018. Technical manual for drug susceptibility testing of medicines used in the treatment of tuberculosis.
3. Van Deun A, Aung KJ, Bola V, Lebeke R, Hossain MA, de Rijk WB, Rigouts L, Gumusboga A, Torrea G, de Jong BC. 2013. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. J Clin Microbiol 51:2633-40.
4. Ocheretina O, Escuyer VE, Mabou M-M, Royal-Mardi G, Collins S, Vilbrun SC, Pape JW, Fitzgerald DW. 2014. Correlation between Genotypic and Phenotypic Testing for Resistance to Rifampin in *Mycobacterium tuberculosis* Clinical Isolates in Haiti: Investigation of Cases with Discrepant Susceptibility Results. PLOS ONE 9:e90569.
5. Rafael LL, Raquel MS, Rogelio FA, Miroslava FP, Alejandra-Isabel JG, Paola RS. 2021. Discordant results between genotypic and phenotypic assays (Xpert MTB/RIF vs. BACTEC MGIT 960 system) for detection of RIF-resistant *Mycobacterium tuberculosis* isolates in a high burden region. Infect Genet Evol 96:105142.
6. WHO. 2021. Technical Report on critical concentrations for drug susceptibility testing of isoniazid and the rifamycins (rifampicin, rifabutin and rifapentine).
7. WHO. 2023. Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance.
8. Deun AV, Decroo T, Aung KJM, Hossain MA, Gumusboga M, Rijk WBD, Tahseen S, Jong BCd, Rigouts L. 2021. *Mycobacterium tuberculosis* borderline *rpoB* mutations: emerging from the unknown. European Respiratory Journal 58:2100783.
9. Lempens P, Van Deun A, Aung KJM, Hossain MA, Behruznia M, Decroo T, Rigouts L, de Jong BC, Meehan CJ. 2023. Borderline rpoB mutations transmit at the same rate as common *rpoB* mutations in a tuberculosis cohort in Bangladesh. Microb Genom 9.

CHAPTER 6

Refined understanding of the impact of the *Mycobacterium tuberculosis* complex diversity on the intrinsic susceptibility to pretomanid

Refined understanding of the impact of the *Mycobacterium tuberculosis* complex diversity on the intrinsic susceptibility to pretomanid

Microbiology Spectrum, March 2024 Volume 12 Issue 3 e00070-24

DOI: https://doi.org/10.1128/spectrum.00070-24

Praharshinie Rupasinghe[1,2*], Rabab Reenaers[1], Jens Vereecken[1], Wim Mulders[1], Sari Cogneau[1], Matthias Merker,[3,4,5], Stefan Niemann[3,5], Shaheed Vally Omar[6], Leen Rigouts[1,2], Claudio U. Köser[7], Tom Decroo[8], Bouke C de Jong[1]

[1] Unit of Mycobacteriology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[2]Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium
[3]Molecular and Experimental Mycobacteriology, Research Center Borstel, Borstel, Germany
[4]Evolution of the Resistome, Research Center Borstel, Borstel, Germany
[5]German Center for Infection Research, Partner site Hamburg-Lübeck-Borstel-Riems, Parkallee, Borstel, Germany
[6]Center for Tuberculosis, National Institute of Communicable Diseases, a division of the National Health Laboratory Service, Johannesburg, South Africa
[7]Department of Genetics, University of Cambridge, Cambridge, United Kingdom
[8]Unit of HIV and TB, Department of Clinical Sciences, Institute of Tropical Medicine, Antwerp, Belgium

* Corresponding author

Abstract

Previous work reported unprecedented differences in the intrinsic in vitro susceptibility of the *Mycobacterium tuberculosis* complex (MTBC) to pretomanid (Pa) using the Mycobacteria Growth Indicator Tube (MGIT) system.

We tested 125 phylogenetically diverse strains from all known MTBC lineages (1–9) without known Pa resistance mutations and four strains with known resistance mutations as controls.

This confirmed that MTBC, unlike most bacteria-antimicrobial combinations, displayed substantial differences in the intrinsic susceptibility relative to the technical variation of Pa MIC testing. This was also the case for the Middlebrook 7H11 (7H11) medium, demonstrating that these differences were not specific to MGIT.

Notably, lineage 1 was confirmed to have intrinsically elevated MICs compared with lineages 2, 3, 4, and 7 (L2–4/7), underlining the urgent need for WHO to publish its decision of whether lineage 1 should be deemed treatable by BPaL(M), the now preferred all-oral regimen for treating rifampin-resistant tuberculosis.

Lineages 5 and 6, which are most frequent in West Africa, responded differently to Pa, with lineage 5 being more similar to L2–4/7 and lineage 6 being more susceptible. More data are needed to determine whether 7H11 MICs are systematically lower than those in MGIT.

This study confirmed that the *Mycobacterium tuberculosis* complex lineage 1, responsible for 28% of global tuberculosis cases, is less susceptible to pretomanid (Pa). It also refined the understanding of the intrinsic susceptibilities of lineages 5 and 6, most frequent in West Africa, and lineages 8 and 9.

Regulators must review whether these in vitro differences affect the clinical efficacy of the WHO-recommended BPaL(M) regimen and set breakpoints for antimicrobial susceptibility testing accordingly. Notably, regulators should provide detailed justifications for their decisions to facilitate public scrutiny.

6.1 Introduction

Pretomanid is a prodrug that goes through bio reductive activation through deazaflavin-dependent nitroreductase (ddn) enzyme. The all-oral BPaL(M) regimen, consisting of bedaquiline, pretomanid (Pa), linezolid, and moxifloxacin (moxifloxacin is stopped if fluoroquinolone resistance is detected) is becoming the preferred option for treating rifampin-resistant tuberculosis (TB) (1, 2). Pa poses two challenges in this context. First, Bateson et al. described unprecedented differences in the intrinsic susceptibility of different *Mycobacterium tuberculosis* complex (MTBc) lineages to Pa using the Mycobacteria Growth Indicator Tube (MGIT) system (3). Most notably, lineage 1 (L1), which accounts for 28% of TB cases globally, was found to be intrinsically less susceptible than the other major MTBC lineages [lineage 2 (L2), lineage 3 (L3) and lineage 4 (L4)], raising the question whether L1 responds equally well to BPaL(M) compared with L2-4 (3).

Second, clinical strains with high Pa MICs due to mutations in known Pa resistance genes were identified without known nitroimidazole exposure, suggesting genetic drift or yet unknown selective pressures (3-5). In the few settings with good surveillance or routine antimicrobial susceptibility (AST) results, these mutants are rare (5, 6). However, because these mutants are known to be transmissible, it is plausible that some settings exist in which an intrinsically Pa-resistant cluster is frequent, underlining the need for routine AST (4, 7). Yet, rapid AST directly from clinical samples is currently impossible as no commercial genotypic AST assay exists that interrogates *ddn* (*Rv3547*), *fbiA* (*Rv3261*), *fbiB* (*Rv3262*), *fbiC* (*Rv1173*), *fbiD* (*Rv2983*), and *fgd1* (*Rv0407*), the six genes required for the activation of the pro-drug Pa (no resistance mutations have been described in *dprE2* (*Rv3791*), the target of Pa, to date) (8-10). Although efforts are underway to address this diagnostic gap (e.g. Genoscreen is evaluating Deeplex Myc-TB XL, an updated version of its WHO-endorsed targeted next-generation sequencing assay), the interpretation of genotypic AST results will remain a persistent challenge as the aforementioned resistance genes are non-essential and, consequently, thousands of different loss-of-function mutations can theoretically confer resistance (9, 11, 12).

The goal of this study was two-fold. First, we used MGIT to refine the current understanding of the effect of the MTBC diversity on susceptibility, with a particular focus on the less frequent lineage 5 (L5), lineage 6 (L6), lineage 7 (L7), lineage 8 (L8), and lineage 9 (L9) that were not tested or were underrepresented in the literature (3, 13, 14). Second, we used Middlebrook

7H11 (7H11) as an alternative medium to investigate whether the differences observed with MGIT were media specific.

6.2 Methods

6.2.1 Strains

We tested 125 MTBc strains from L1-9 from patients who had never received nitroimidazole treatment originating from 45 different countries in five different continents. Of these, 118 lacked known resistance mutations in the six canonical Pa-resistance genes and the remaining seven did not have whole genome sequencing data but were selected based only on the treatment naivety to nitroimidazoles to augment L5 and L6. Of 125 strains, 49% (n=61) were drug-susceptible (DS), 27% (n=34) mono-/poly-resistant (mono/PDR) to other TB drugs other than Pa, 23% (n=29) were multidrug-resistant (MDR) and 1% (n=1) was pre-extensively drug-resistant (pre-XDR) (**Table 6.S1**) (15). All 125 strains were tested on 7H11 whereas a subset of 41 isolates were tested in MGIT. In addition, four Pa-resistant strains with known resistance mutations were included for both methods (**Table 6.1**). Ten of the total 129 strains had also tested in Bateson *et al*. (**Table 6.S2**) (3). As per the ITM-IRB consultation, the fully anonymized use of clinical isolates for test validation did not require ethical review.

6.2.2 7H11 MIC testing

Pa powder (Sigma-Aldrich SML-1290) was dissolved in DMSO (Sigma D5879) to prepare a stock solution of 4000 µg/mL and stored in 600 µL aliquots at -80 °C. Standard 7H11 base was supplemented with 10% (v/v) oleic acid-albumin-dextrose-casein (OADC) enrichment and 0.5% (v/v) glycerol to prepare the 7H11 solid medium. A two-fold dilution series of Pa ranging from 1 to 0.002 µg/mL plus 0.75 µg/mL (i.e. 11 concentrations in total) were tested for all strains, except for the four Pa-resistant strains, for which 0.25-8 µg/mL were used instead. Bacterial colonies were scraped from Löwenstein-Jensen slants and thoroughly homogenized in sterile water with glass beads. The density of the suspension was adjusted visually to McFarland 1. The least diluted growth control (GC1) and the drug-containing media in polypropylene tubes were inoculated with a 10^{-1} dilution of the McFarland 1 suspension, while the most diluted growth

control (GC2) was inoculated with a 10^{-3} dilution. Colony forming units (CFU) were enumerated after four weeks of incubation at 34-38 °C with 5-10% CO_2. If both growth controls had sufficient growth at this point (i.e., at least 1+ (51-100 CFUs) on GC1 and 3 CFUs on GC2), CFU counts were recorded accordingly, and MIC results were interpreted. If GC1 and/or GC2 had insufficient CFUs at four weeks, tubes were incubated for two more weeks. Any test with insufficient CFUs on GC1 and/or GC2 after six weeks of incubation or more than 1+ growth on the GC2 was considered invalid and repeated once. The MIC was defined as the lowest drug concentration that inhibits the growth of more than 99% of the MTBC population.

Since MIC testing of Pa on 7H11 had not yet been established in our laboratory at the Unit of Mycobacteriology, Institute of Tropical Medicine, we first tested 30 replicates of the pan-susceptible H37Rv reference strain (BCCM/ITM CT2008-03715/ITM500735), using three different batches of Pa-containing medium, with repeated testing on different days over ten weeks. Subsequently, H37Rv was included as control in every batch of clinical strains.

6.2.3 MGIT MIC testing

Pa working solutions prepared from the same stock solution used for 7H11 testing were added to MGIT tubes (100 µL each) to achieve ten two-fold Pa dilutions from 0.002 to 1 µg/mL, whereas higher concentrations (0.25-4 µg/mL) were tested for Pa-resistant strains. An inoculum was prepared directly from a positive MGIT tube that had flagged within 1-2 day or after a 1 in 5 dilution of a positive MGIT tube that had flagged within 3-5 days, and 500 µL of inoculum was added to the Pa-containing tubes supplemented with 800 µL of OADC. The drug-free control vial was inoculated with a 1:100 dilution of the inoculum. MICs were determined using MGIT 960 TBeXIST extended protocol according to the manufacturer's instructions. The MIC was determined to be the lowest concentration at which the growth value of the drug-containing tube was <100 when the growth control had reached 400 growth units. A test resulting in an invalid code (X200 or X400) was repeated once. Based on Bateson *et al.,* 0.06-0.5 µg/mL was used as a tentative quality control (QC) range and a corresponding QC target of 0.125-0.25 µg/mL for H37Rv, which was included in every batch of clinical strains (3).

6.3 Results

6.3.1 Technical reproducibility of Pa MIC testing

A good technical reproducibility was observed for both methods. Pa MICs in MGIT were 0.125-0.25 µg/mL and on 7H11 0.06-0.125 µg/ml for H37Rv.

6.3.2 MICs for strains with Pa resistance mutations

The four strains with known Pa resistance mutations had MICs of >4 µg/mL in MGIT, which was in line with earlier results, and >8 µg/mL using 7H11 (**Table 6.1**) (3).

Strain ID	Lineage	DR profile	Genome accession	Pa resistance mutation		Study	Pa MIC (µg/mL)	
				ddn	*fbiC*		7H11	MGIT
2013-02481[a]	1.1.3	PDR	ERR8025345	-	Arg536Leu	Current	>8	>4
						Bateson	NT	>16
2020-00011	1.1.3	MDR	ERR12115304	Trp27Stop[c]	-	Current	>8	>4
						Bateson	NT	NT
2020-03565	1.1.3	DS	SAMN11179707	Trp27Stop[c]	-	Current	>8	>4
						Bateson	NT	>8
2020-03568[b]	2.2.1	DS	ERR7361928	Gln58Stop[c]	-	Current	>8	>4
						Bateson	NT	>16

Table 6.1: MICs for Pa-resistant strains

NT = not tested, a = In vitro mutant, b = Selected by WHO as resistant control strain for delamanid and Pa in the forthcoming AST manual, c = Recognized as conferring cross-resistance to delamanid and Pa in the second edition of the WHO mutation catalogue (12).

6.3.3 MICs for strains without Pa resistance mutations

Bateson *et al*. reported a mode of 1 µg/mL and 99th percentile of 2 µg/mL for L1 MICs in MGIT, which was elevated compared with the mode of 0.125 µg/mL and 99th percentile of 0.5 µg/mL for L2-4/7 (3). The MGIT MIC data from this study agreed with these findings (**Table 6.2**). L6 strains were even more susceptible with MICs ≤0.016 µg/mL in both studies. L5 MICs were 0.03-0.06 µg/mL in both studies, but we tested a greater number of, demonstrating that the susceptibility of L5 was more similar to that of L2-4/7 than that of L6. Based on a single replicate for one strain each, it was unclear whether L8 was more similar to L1 or L2-4/7, whereas L9 most resembled L6.

Lineage	Medium	Study	Pa MIC range (µg/mL)	7H11 MICs (µg/mL)														Total
				Invalid	≤0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	0.75	1	2	4	
1[a]	7H11	Current	0.25-1	-	-	-	-	-	-	-	-	4	**9**	6	2	NT	NT	21
	MGIT	Current	0.5-1	-	-	-	-	-	-	-	-	-	1	NT	**4**	NT	NT	5
		Bateson	0.125-2	-	NT	NT	NT	-	-	-	2	3	39	NT	**76**	7	-	127
2[b]	7H11	Current	0.03-0.25	-	-	-	-	-	2	**21**	5	2	-	-	-	NT	NT	30
	MGIT	Current	0.125-0.25	-	-	-	-	-	-	-	1	**3**	-	NT	-	NT	NT	4
		Bateson	0.06-1	-	NT	NT	NT	-	-	1	22	**24**	15	NT	1	NT	NT	63
3	7H11	Current	0.06-0.125	-	-	-	-	-	2	**6**	3	-	-	-	-	NT	NT	11
	MGIT	Current	0.125	-	-	-	-	-	-	-	**5**	-	-	NT	-	NT	NT	5
		Bateson	0.06-0.125	-	-	NT	NT	-	-	4	**8**	-	-	NT	-	NT	NT	12
4[c]	7H11	Current	0.03-0.25	-	-	-	-	-	**17**	15	5	1	-	-	-	NT	NT	38
	MGIT	Current	0.125-0.25	-	-	-	-	-	-	-	**5**	2	-	NT	-	NT	NT	7
		Bateson	0.03-0.5	-	-	-	-	-	3	25	**52**	31	2	NT	-	NT	NT	113
4-H37Rv[d]	7H11	Current	0.06-0.125	-	-	-	-	-	-	2	**36**	-	-	-	-	NT	NT	38
	MGIT	Current	0.125-0.25	-	-	-	-	-	-	-	2	**3**	-	NT	-	NT	NT	5
		Bateson	0.06-0.5	-	NT	NT	NT	-	-	8[e]	47[e,f]	**60**[e,f]	2[e]	NT	NT	NT	NT	117
5	7H11	Current	0.03-0.125	-	-	-	-	-	4	**6**	1	-	-	-	-	NT	NT	11
	MGIT	Current	0.03-0.06	-	-	-	-	-	2	**6**	-	-	-	NT	-	NT	NT	8
		Bateson	0.03-0.06	-	NT	NT	NT	-	**1**	**1**	-	-	-	NT	-	NT	NT	2
6	7H11	Current	≤0.002-0.004	1	1	**9**	-	-	-	-	-	-	-	-	-	NT	NT	11
	MGIT	Current	≤0.002-0.008	-	1	**7**	1	-	-	-	-	-	-	NT	-	NT	NT	9
		Bateson	0.004-0.016	-	-	1	1	1	-	-	-	-	-	NT	-	NT	NT	3
7[c]	7H11	Current	0.125	-	-	-	-	-	-	-	**1**	-	-	-	-	NT	NT	1
	MGIT	Current	0.125	-	-	-	-	-	-	-	**1**	-	-	NT	-	NT	NT	1
		Bateson	0.25	-	NT	NT	NT	-	-	-	-	**3**	-	NT	-	NT	NT	3
8	7H11	Current	0.25	-	-	-	-	-	-	-	-	**1**	-	-	-	NT	NT	1
	MGIT	Current	1.0	-	-	-	-	-	-	-	-	-	-	NT	1	NT	NT	1
		Bateson	NT															0
9	7H11	Current		1	-	-	-	-	-	-	-	-	-	-	-	NT	NT	0
	MGIT	Current	≤0.002	-	**1**	-	-	-	-	-	-	-	-	NT	-	NT	NT	1
		Bateson	NT															0
M. bovis	7H11	Current	NT															0
	MGIT	Current	NT															0
		Bateson	0.03	-	NT	NT	NT	-	**3**	-	-	-	-	NT	-	NT	NT	3
M. caprae	7H11	Current	NT															0
	MGIT	Current	NT															0
		Bateson	0.03	-	NT	NT	NT	-	**1**	-	-	-	-	NT	-	NT	NT	1
M. microti	7H11	Current	NT															0
	MGIT	Current	NT															0
		Bateson	0.125	-	NT	NT	NT	-	-	-	**1**	-	-	NT	-	NT	NT	1
M. pinnipedii	7H11	Current	NT															0
	MGIT	Current	NT															0
		Bateson	0.06	-	NT	NT	NT	-	-	**1**	-	-	-	NT	-	NT	NT	1
M. canetti	7H11	Current	NT															0
	MGIT	Current	NT															0
		Bateson	2-4	-	NT	NT	NT	-	-	-	-	-	-	NT	-	**18**	3	21

Table 6.2: MICs for H37Rv and clinical strains without known Pa resistance mutations.

NT= not tested. Modes of MIC distributions are highlighted in bold text. [a] Two strains were tested in both studies (**Table 6.S2**).[b] Three strains were tested in both studies (**Table 6.S2**). [c] One strain was tested in both studies (**Table 6.S2**). [d] Replicates of H37Rv. [e] Tentative QC range based on Bateson et al. (3). [f] Tentative QC target based on Bateson *et al.* (3).

Similar relative susceptibilities of the different lineages were observed on 7H11 (**Table 6.2**). For example, the 99th percentile of the L1 distribution at 1 µg/mL was two doubling dilutions higher than for L2-4. MGIT MICs for L1-4 were approximately twice as high as the corresponding 7H11 MICs (**Table 6.3**), which was also apparent when comparing the modes of their MIC distributions (**Table 6.2**). In contrast, the absolute MICs for L5 and L6 were similar for both media. As only a single strain was tested for L7-8, no meaningful comparison was possible for these lineages.

Lineage	No of stains with their MIC_{MGIT}/MIC_{7H11} ratio				
	0.5	1	2	4	8
1	-	2	2	1	-
2	-	-	1	3	-
3	-	-	4	1	-
4	-	-	4	2	1
4-H37Rv	-	1	4	-	-
5	1	5	2	-	-
6	-	7	-	-	-
7	-	1	-	-	-
8	-	-	-	1	-

Table 6.3: MGIT to 7H11 MIC ratio for H37Rv and clinical strains without Pa resistance mutations

L9 was excluded as no 7H11 MIC was available, and the ratio was not calculated for strains with at least one truncated MIC (see **Table 6.S2** for more details).

6.4 Discussion

This study confirmed that Pa MICs were elevated in L1, regardless of medium used and phenotype measured (7H11 MIC testing relies on visual growth inhibition on solid medium, whereas MGIT measures oxygen consumption in liquid medium (16)). The fundamental question for regulators is whether BPaL(M) should be used for L1, even though the clinical outcome data demonstrating good outcomes are more limited for L1 compared with L2-4, therefore remains pressing in light of the ongoing adoption of this regimen globally (3). In January 2023, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) set a "provisional screen value" of 2 µg/mL for MGIT, which was reaffirmed in 2024, without an

accompanying explanation of the meaning or intended use of this concentration (17, 18). Given a history of mistakes when setting breakpoints for MTBC by multiple regulators, we call on EUCAST to publish a justification for its decision to enable external scrutiny (19-22). Moreover, EUCAST should engage with the European Medicines Agency to review its breakpoint for MGIT given that the current choice of 1 mg/L for MGIT is too high for L2-4/7 and too low for L1 (23). WHO reviewed these questions independently and is due to publish its decision shortly.

L6, which causes up to half of TB in some West African countries yet appears underrepresented among rifampicin-resistant strains, was more susceptible than L5 and L2-4/7 and should, therefore, respond better to BPaL(M) (3, 24). More MICs are needed for L8, L9, and the different animal-adapted MTBC genotypes (e.g., M. bovis) would be desirable to clarify their likely response to BPaL(M), although this is not a priority as these are much rarer than L1-7 (3, 14, 24).

MICs for L1-4 appeared to be systematically lower in 7H11 than in MGIT in this single-site study, requiring confirmation in other laboratories (i.e., technical variability may account for this apparent difference, as previously observed for H37Rv tested in different media in MGIT (3, 25-27)).

Our findings further underline the importance of the EUCAST requirements to consider MIC data from multiple laboratories and from phylogenetically diverse MTBC strains (3, 25, 26). Accordingly, the TB Alliance is preparing a study to define the L1 and non-L1 MIC distributions using the EUCAST reference method (26). Commercial phenotypic AST devices (e.g. a lyophilised Pa product for MGIT or a lyophilised broth microdilution assay, which would be preferable to manually weighing Pa for MGIT testing as is the only option currently) will have to be calibrated against the reference method to ensure that any MIC differences are fully systematic (e.g. that the technical variability is not excessive, resulting in wider MIC distributions and, thus, increasing the likelihood of very major diagnostic errors) (26, 28, 29). In the future, such quality-assured and comprehensively validated commercial AST assays should be co-developed with novel relevant antimicrobials given that empiric use risks their long-term utility (30-32).

6.5 Funding

This study was supported by an EWI (Economy, Science, and Innovation) grant to the Institute of Tropical Medicine, Antwerp, Belgium. P.R is employed by the ITM under UNITAIND endTB grant. C.U.K. is a visiting scientist at the Department of Genetics, University of Cambridge, and a research associate at Wolfson College, University of Cambridge.

6.6 Conflicts of interest

C.U.K. is a consultant for Becton Dickinson, the Foundation for Innovative New Diagnostics, and the TB Alliance. C.U.K.'s consulting for Becton Dickinson involves a collaboration with Janssen and Thermo Fisher Scientific. C.U.K. is an unpaid advisor to Cepheid and GenoScreen (GenoScreen covered related travel and accommodation expenses only). C.U.K. worked as a consultant for the Stop TB Partnership, the WHO Global TB Program, and the WHO Regional Office for Europe. C.U.K. gave a paid educational talk for Oxford Immunotec. C.U.K. collaborated with PZA Innovation and was an unpaid advisor to BioVersys.

6.7 References

1. World Health Organization. 2023. Global tuberculosis report 2023. (https://iris.who.int/handle/10665/373828, accessed 7 November 2023).
2. Bateson A, Ortiz Canseco J, McHugh TD, Witney AA, Feuerriegel S, Merker M, Kohl TA, Utpatel C, Niemann S, Andres S, Kranzer K, Maurer FP, Ghodousi A, Borroni E, Cirillo DM, Wijkander M, Toro JC, Groenheit R, Werngren J, Machado D, Viveiros M, Warren RM, Sirgel F, Dippenaar A, Köser CU, Sun E, Timm J. 2022. Ancient and recent differences in the intrinsic susceptibility of *Mycobacterium tuberculosis* complex to pretomanid. J Antimicrob Chemother 77:1685-1693.
3. Lee BM, Harold LK, Almeida DV, Afriat-Jurnou L, Aung HL, Forde BM, Hards K, Pidot SJ, Ahmed FH, Mohamed AE, Taylor MC, West NP, Stinear TP, Greening C, Beatson SA, Nuermberger EL, Cook GM, Jackson CJ. 2020. Predicting nitroimidazole antibiotic resistance mutations in *Mycobacterium tuberculosis* with protein engineering. PLoS Pathog 16:e1008287.
4. Timm J, Bateson A, Solanki P, Paleckyte A, Witney AA, Rofael SAD, Fabiane S, Olugbosi M, McHugh TD, Sun E. 2023. Baseline and acquired resistance to bedaquiline, linezolid and pretomanid, and impact on treatment outcomes in four tuberculosis clinical trials containing pretomanid. PLOS Glob Public Health 3:e0002283.
5. Battaglia S, Spitaleri A, Cabibbe AM, Meehan CJ, Utpatel C, Ismail N, Tahseen S, Skrahina A, Alikhanova N, Mostofa Kamal SM, Barbova A, Niemann S, Groenheit R, Dean AS, Zignol M, Rigouts L, Cirillo DM. 2020. Characterization of genomic variants associated with resistance to bedaquiline and delamanid in naive *Mycobacterium tuberculosis* clinical strains. J Clin Microbiol 58:e01304-20.
6. Merker M, Kohl TA, Barilar I, Andres S, Fowler PW, Chryssanthou E, Ängeby K, Jureen P, Moradigaravand D, Parkhill J, Peacock SJ, Schön T, Maurer FP, Walker T, Köser C, Niemann S. 2020. Phylogenetically informative mutations in genes implicated in antibiotic resistance in *Mycobacterium tuberculosis* complex. Genome Med 12:27.
7. Kadura S, King N, Nakhoul M, Zhu H, Theron G, Köser CU, Farhat M. 2020. Systematic review of mutations associated with resistance to the new and repurposed *Mycobacterium tuberculosis* drugs bedaquiline, clofazimine, linezolid, delamanid and pretomanid. J Antimicrob Chemother 75:2031-2043.
8. Mohamed S, Köser CU, Salfinger M, Sougakoff W, Heysell SK. 2021. Targeted next-generation sequencing: a Swiss army knife for mycobacterial diagnostics? Eur Respir J 57:2004077.
9. Abrahams KA, Batt SM, Gurcha SS, Veerapen N, Bashiri G, Besra GS. 2023. DprE2 is a molecular target of the anti-tubercular nitroimidazole compounds pretomanid and delamanid. Nat Commun 14:3828.
10. World Health Organization. 2023. Use of targeted next-generation sequencing to detect drug-resistant tuberculosis: rapid communication, July 2023, (https://apps.who.int/iris/handle/10665/371687, accessed 4 August 2023).
11. World Health Organization. 2023. Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance, second edition. (https://iris.who.int/handle/10665/374061, accessed 15 November 2023).
12. Coscolla M, Gagneux S, Menardo F, Loiseau C, Ruiz-Rodriguez P, Borrell S, Otchere ID, Asante-Poku A, Asare P, Sánchez-Busó L, Gehre F, Sanoussi CN, Antonio M, Affolabi D, Fyfe J, Beckert P, Niemann S, Alabi AS, Grobusch MP, Kobbe R, Parkhill J, Beisel C, Fenner

L, Böttger EC, Meehan CJ, Harris SR, de Jong BC, Yeboah-Manu D, Brites D. 2021. Phylogenomics of Mycobacterium africanum reveals a new lineage and a complex evolutionary history. Microb Genom 7:000477.
13. Koleske BN, Jacobs WR, Jr., Bishai WR. 2023. The *Mycobacterium tuberculosis* genome at 25 years: lessons and lingering questions. J Clin Invest 133:e173156.
14. World Health Organization. 2021. Meeting report of the WHO expert consultation on the definition of extensively drug-resistant tuberculosis. 27-29 October 2020. (https://apps.who.int/iris/handle/10665/338776, accessed 26 January 2021).
15. Pfyffer GE, Palicova F, Rüsch-Gerdes S. 2002. Testing of susceptibility of *Mycobacterium tuberculosis* to pyrazinamide with the nonradiometric BACTEC MGIT 960 system. J Clin Microbiol 40:1670-4.
16. European Committee on Antimicrobial Susceptibility Testing. 2023. Breakpoint tables for interpretation of MICs and zone diameters. Version 13.0, valid from 2023-01-01. (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_13.0_Breakpoint_Tables.xlsx, accessed 2 January 2024).
17. European Committee on Antimicrobial Susceptibility Testing. 2024. Breakpoint tables for interpretation of MICs and zone diameters. Version 14.0, valid from 2024-01-01. (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_14.0_Breakpoint_Tables.xlsx. accessed 6 January 2024).
18. World Health Organization. 2018. Technical report on critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis. (https://apps.who.int/iris/handle/10665/260470, accessed 13 August 2021).
19. World Health Organization. 2021. Technical report on critical concentrations for drug susceptibility testing of isoniazid and the rifamycins (rifampicin, rifabutin and rifapentine). (https://apps.who.int/iris/handle/10665/339275, accessed 13 August 2021).
20. Antimycobacterial Susceptibility Testing Group. 2022. Updating the approaches to define susceptibility and resistance to anti-tuberculosis agents: implications for diagnosis and treatment. Eur Respir J 59:2200166.
21. Köser CU, Georghiou SB, Schön T, Salfinger M. 2021. On the consequences of poorly defined breakpoints for rifampin susceptibility testing of *Mycobacterium tuberculosis* complex. J Clin Microbiol 59:e02328-20.
22. European Medicines Agency. 2021. Dovprela: EPAR – product information. (https://www.ema.europa.eu/en/documents/product-information/dovprela-epar-product-information_en.pdf, accessed 6 June 2021).
23. Gagneux S. 2018. Ecology and evolution of *Mycobacterium tuberculosis*. Nat Rev Microbiol 16:202-213.
24. European Committee on Antimicrobial Susceptibility Testing. 2019. SOP for calibrating surrogate MIC methods for *M. tuberculosis* against the EUCAST reference MIC method. Version 1.0. (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Mycobacteria/Methods_in_AMST/CalibrationSOP_Mtb_190718.pdf, accessed 9 September 2019).
25. Schön T, Köser CU, Werngren J, Viveiros M, Georghiou S, Kahlmeter G, Giske C, Maurer F, Lina G, Turnidge J, van Ingen J, Jankovic M, Goletti D, Cirillo DM, Santin M, Cambau E, ESGMYC. 2020. What is the role of the EUCAST reference method for MIC testing of the *Mycobacterium tuberculosis* complex? Clin Microbiol Infect 26:1453-1455.

26. Kahlmeter G, Turnidge J. 2023. Wild-type distributions of minimum inhibitory concentrations and epidemiological cut-off values-laboratory and clinical utility. Clin Microbiol Rev doi:10.1128/cmr.00100-22:ahead of print.
27. World Health Organization. 2022. Optimized broth microdilution plate methodology for drug susceptibility testing of *Mycobacterium tuberculosis* complex. (https://apps.who.int/iris/handle/10665/353066, accessed 12 April 2022).
28. Köser CU, Maurer FP. 2023. Minimum inhibitory concentrations and sequencing data have to be analysed in more detail to set provisional epidemiological cut-off values for *Mycobacterium tuberculosis* complex. Eur Respir J 61:2202397.
29. Köser CU, Javid B, Liddell K, Ellington MJ, Feuerriegel S, Niemann S, Brown NM, Burman WJ, Abubakar I, Ismail NA, Moore D, Peacock SJ, Török ME. 2015. Drug-resistance mechanisms and tuberculosis drugs. Lancet 385:305-307.
30. Köser CU, Maurer FP, Kranzer K. 2019. `Those who cannot remember the past are condemned to repeat it´: Drug-susceptibility testing for bedaquiline and delamanid. Int J Infect Dis 80S:S32-S35.
31. Ness T, Van LH, Petermane I, Duarte R, Lange C, Menzies D, Cirillo DM. 2023. Rolling out new anti-tuberculosis drugs without diagnostic capacity. Breathe (Sheff) 19:230084.

6.8 Supplementary material

Lineage	Medium	Total tested	Proportion of tested strains	No of strains tested by drug resistance profile				Geographical origin based on WHO regions					
				DS	Mono/PDR	MDR	Pre-XDR	African	Americas	South-East Asian	European	Eastern-Mediterranean	Western-Pacific
1	7H11	21	17%	11	7	3	-	1	0	12	2	0	6
	MGIT	5	12%	5	-	-	-	0	0	2	1	0	2
2	7H11	30	24%	5	12	13	-	1	0	10	5	0	14
	MGIT	4	10%	2	-	2	-	0	0	0	2	0	2
3	7H11	11	9%	9	2	-	-	4	0	2	3	1	1
	MGIT	5	12%	4	1	-	-	0	0	2	2	0	1
4	7H11	38	30%	14	12	11	1	13	8	3	8	1	5
	MGIT	7	17%	6	-	1	-	2	1	1	3	0	0
5	7H11	11	9%	10	-	1	-	10	1	0	0	0	0
	MGIT	8	19%	7	-	1	-	7	1	0	0	0	0
6	7H11	11	8%	10	-	1	-	9	1	0	1	0	0
	MGIT	9	22%	8	-	1	-	7	1	0	1	0	0
7	7H11	1	1%	1	-	-	-	1	0	0	0	0	0
	MGIT	1	2%	1	-	-	-	1	0	0	0	0	0
8	7H11	1	1%	-	1	-	-	1	0	0	0	0	0
	MGIT	1	2%	-	1	-	-	1	0	0	0	0	0
9	7H11	1	1%	1	-	-	-	1	0	0	0	0	0
	MGIT	1	2%	1	-	-	-	1	0	0	0	0	0

Table 6.S1: Overview of the 125 MTBC strains without Pa resistance mutations from **Table 6.2** included in this study

DS= drug-susceptible, MDR= multi-drug resistant, PDR= poly-drug resistant, Pre-XDR= MDR+ resistant to a fluoroquinolone.

Strain ID	Genome accession	Lineage	Origin	7H11 MIC (µg/mL)	MGIT MIC (µg/mL)	MGIT MIC batch Nr	MIC MGIT/7H11 ratio	BCCM ID	If included in Bateson et al., strain ID used; MGIT MIC (µg/mL)
1968-01344	ERR12115306	5.2	United States	0.03	0.06	1	2	ITM-500032	-
1973-03608	ERR12115307	5.1.3	Central African Republic	0.06	0.06	2	1	ITM-500084	-
1982-08470	ERR12115308	6.1.1	France	0.004	0.004	1	1	ITM-500120	-
1991-07531	ERR12115309	5	Burkina Faso	0.06	0.03	3	2	ITM-500255	-
1992-08557	WGS not available	5	Cote D'Ivoire	0.06	-	-	-	ITM-500259	-
1996-00683	ERR3132183	1.1.3	Bangladesh	0.5	1	1	2	ITM-500321	-
1996-00723	ERR3132226	1.1.3	Bangladesh	0.5	-	-	-	ITM-500322	-
1996-01650	ERR3132259	4.3.4.2	Bangladesh	0.03	0.125	1	4	ITM-500338	-
1996-01863	ERR3132281	2.2.1	Bangladesh	0.06	-	-	-	ITM-500340	-
1997-00472	ERR3132292	1.1.2	Bangladesh	0.75	-	-	-	ITM-500342	-
1997-00991	ERR3132073	2.2.2	Bangladesh	0.03	-	-	-	ITM-500345	-
1997-01510	ERR3132084	1.1.2	Bangladesh	0.25	-	-	-	ITM-500350	-
1998-00166	ERR3132106	4.4	Bangladesh	0.06	-	-	-	ITM-500352	-
1998-00277	ERR12115310	1.1.3.1	Bangladesh	0.25	-	-	-	ITM-500354	-
1998-01164	ERR3132117	1.2.2	Bangladesh	0.5	-	-	-	ITM-500357	TB-TDR-0014; 2
1999-00020	ERR12115311	2.2.1	Russian Fed	0.125	-	-	-	ITM-500364	-
1999-00168	ERR3132128	2.2.1	Bangladesh	0.125	-	-	-	ITM-500365	-
1999-01202	ERR3132139	4.6.1.2	DR Congo	0.03	0.125	4	4	ITM-500367	-
1999-01341	ERR3132172	2.2.1	Azerbaidjan	0.06	0.25	2	4	ITM-500370	TB-TDR-0019; 0.25
1999-01545	ERR3132212	4.3.3	DR Congo	0.06	-	-	-	ITM-500374	-
1999-01548	ERR3132220	4.6.1.2	DR Congo	0.06	-	-	-	ITM-500375	-
1999-01994	ERR3132221	1.2.2	Bangladesh	0.5	-	-	-	ITM-500378	-
2000-00092	ERR12115312	1.1.3.2	Malawi	0.75	-	-	-	ITM-500385	-
2000-00288	ERR3132225	4.4.1.1	Azerbaidjan	0.06	-	-	-	ITM-500386	-
2000-00440	ERR3132228	2.2.1	Kazakstan	0.06	-	-	-	ITM-500388	-
2000-00446	ERR3132229	2.2.1	Bangladesh	0.06	-	-	-	ITM-500389	TB-TDR-0032; 0.25

2000-00448	ERR3132230	4.7	Belgium	0.03	-	-	-	ITM-500390	-
2000-00611	ERR3132232	2.2.1	Bangladesh	0.06	-	-	-	ITM-500394	-
2000-01353	ERR3132234	1.2.2	Bangladesh	1	-	-	-	ITM-500404	-
2000-01699	ERR3132238	2.2.1	Bangladesh	0.06	-	-	-	ITM-500408	-
2001-00204	WGS not available	1	Bangladesh	0.5	-	-	-	ITM-500414	-
2001-00294	ERR3132239	3	Bangladesh	0.03	0.125	1	4	ITM-500416	-
2001-00299	ERR3132240	1.1.2	Bangladesh	1	-	-	-	ITM-500417	-
2001-00966	ERR3132242	2.2.1	Bangladesh	0.06	-	-	-	ITM-500432	-
2001-01466	WGS not available	1	Bangladesh	0.75	-	-	-	ITM-500447	-
2002-02478	ERR12115313	2.2.1	Bangladesh	0.06	-	-	-	ITM-500477	-
2003-01714	ERR3132257	4.6.1.2	Burundi	0.125	-	-	-	ITM-500488	-
2003-01801	ERR3132260	4.3.4.2.1	Burundi	0.06	-	-	-	ITM-500490	-
2004-00851	ERR3132266	4.1.1.3	South Africa	0.06	-	-	-	ITM-500504	-
2004-01086	ERR3132089	4.6.1.2	Rwanda	0.125	-	-	-	ITM-500506	-
2004-01195	ERR3132279	2.2.1	South Korea	0.06	0.25	2	4	ITM-500517	-
2004-01198	ERR3132283	2.2.1	South Korea	0.06	-	-	-	ITM-500520	-
2004-01199	ERR3132284	2.2.1	South Korea	0.06	-	-	-	ITM-500521	-
2004-01200	ERR3132285	3	South Korea	0.06	0.125	2	2	ITM-500522	-
2004-01202	ERR3132286	2.2.1	South Korea	0.125	-	-	-	ITM-500523	-
2004-01203	ERR3132287	2.2.1	South Korea	0.06	-	-	-	ITM-500524	-
2004-01204	ERR3132288	2.2.1	South Korea	0.06	-	-	-	ITM-500525	-
2004-01205	ERR3132289	4.9	South Korea	0.125	-	-	-	ITM-500526	-
2004-01207	ERR3132291	4.5	South Korea	0.03	-	-	-	ITM-500528	-
2004-01212	ERR3132296	4	South Korea	0.03	-	-	-	ITM-500532	-
2004-01219	ERR3132302	2.2.2	South Korea	0.06	-	-	-	ITM-500538	-
2004-01220	ERR3132074	2.2.2	South Korea	0.06	-	-	-	ITM-500539	-
2004-01237	ERR3132268	2.2.2	South Korea	0.03	-	-	-	ITM-500550	-
2004-01239	ERR3132087	2.2.2	South Korea	0.06	-	-	-	ITM-500551	-
2004-01241	ERR3132112	2.2.1	South Korea	0.06	-	-	-	ITM-500553	-
2004-01244	ERR3132092	2.2.2	South Korea	0.06	-	-	-	ITM-500556	-
2004-01248	ERR3132094	4	South Korea	0.06	-	-	-	ITM-500558	-
2004-01259	ERR3132100	2.2.2	South Korea	0.25	-	-	-	ITM-500563	-
2004-01285	ERR3132105	4	Brazil	0.06	-	-	-	ITM-500568	-

2004-01286	ERR3132107	4.3.4.2	Brazil	0.03	0.25	2	8	ITM-500569	-
2004-01289	ERR3132108	4.3.4.2	Brazil	0.03	-	-	-	ITM-500570	-
2004-01458	ERR3132111	4.3.4.2.1	Rwanda	0.06	-	-	-	ITM-500574	-
2004-01659	ERR3132116	4.6.1.1	Germany	0.03	-	-	-	ITM-500582	-
2004-01670	ERR3132123	4.8	Ukraine	0.03	-	-	-	ITM-500589	-
2004-01679	ERR3132126	2.2.1	Nepal	0.25	-	-	-	ITM-500592	-
2004-02160	ERR3132142	4.6.1.2	Rwanda	0.125	-	-	-	ITM-500599	-
2004-02613	ERR3132151	4.1.2.1	Peru	0.03	-	-	-	ITM-500618	-
2004-02614	ERR3132152	4	Peru	0.25	-	-	-	ITM-500619	-
2004-02760	ERR3132154	1.2.1	Philippines	0.25	-	-	-	ITM-500621	-
2004-02763	ERR3132156	1.2.1	Philippines	0.5	-	-	-	ITM-500623	-
2004-02765	ERR3132157	1.2.1	Philippines	0.75	-	-	-	ITM-500624	-
2004-02911	ERR3132162	4.1.2	Germany	0.03	-	-	-	ITM-500630	-
2004-02915	ERR3132166	3	Pakistan	0.06	0.125	1	2	ITM-500634	-
2004-02916	ERR3132167	4.6.2.2	Nigeria	0.03	-	-	-	ITM-500635	-
2004-02921	ERR3132171	1.1.2	China (Tibet)	0.5	-	-	-	ITM-500639	TB-TDR-0189; 1
2004-02924	ERR3132174	4.3	Peru	0.06	-	-	-	ITM-500641	-
2004-02926	ERR3132176	4.3.4.2	Portugal	0.03	-	-	-	ITM-500643	-
2004-02927	ERR3132177	4.3.4.2	Rep. Domin.	0.06	-	-	-	ITM-500644	-
2005-00691	ERR3132186	4.3	Peru	0.03	-	-	-	ITM-500652	-
2005-00706	ERR3132200	4.1.2.1	Peru	0.03	-	-	-	ITM-500665	-
2005-02460	ERR439931	4.1.2.1	Cameroon	0.06	0.125	2	2	ITM-501055	-
2006-00789	ERR439937	5.1.2	Benin	0.03	-	-	-	ITM-501069	-
2006-00790	ERR439938	6.2.1	Benin	≤0.002	≤0.002	3	NC	ITM-501070	-
2006-00795	ERR439941	5.1.4	Benin	0.06	0.06	3	1	ITM-500691	-
2006-00800	ERR439945	5.3	Benin	0.03	0.03	1	1	ITM-500692	-
2006-02410	ERR12115314	5.1.1	Guinea	0.06	0.06	3	1	ITM-500699	-
2006-02732	ERR3132210	2.2.1	Georgia	0.125	-	-	-	ITM-500701	-
2006-02951	ERR12115315	3.1.1	Zambia	0.06	-	-	-	ITM-500703	-
2008-00618	WGS not available	6	Senegal	0.004	0.004	1	1	ITM-500722	-
2008-01456	ERR439971	6.3.1	Guinea	0.004	0.004	1	1	ITM-501058	-
2008-04431	ERR3132213	2.2.1	DR Congo	0.06	-	-	-	ITM-500737	-
2009-02073	ERR3132215	4.6.2.2	Niger	0.03	-	-	-	ITM-500745	-

2010-01231	WGS not available	6	Senegal	0.004	0.004	3	1	ITM-500758	-
2011-02171	ERR12115303	6	Gambia	0.004	0.004	1	1	ITM-500782	-
2012-00445	ERR12115316	3	Sweden	0.125	-	-	-	ITM-500785	-
2012-00752	ERR12115317	5.1.2	Niger	0.06	0.06	2	1	ITM-501059	-
2013-00036	ERR12115318	6.1.1	United States	0.004	0.004	2	1	ITM-500811	-
2013-02158	ERR12115319	3.1.1	Kenya	0.06	-	-	-	ITM-500854	-
2013-02481	ERR8025345	1.1.3	Belgium	>8	>4	-	-	ITM-500859	ITM 500859; >16
2013-03157	WGS not available	6	Gambia	0.004	0.004	3	1	ITM-500873	-
2013-03158	WGS not available	6	Gambia	0.004	-	-	-	ITM-500874	-
2014-01606	ERR12115320	1.2.2.2	Belgium	0.75	-	-	-	ITM-500884	-
2018-00082	ERR2704704	1.2.1	Philippines	0.75	1	3	1	ITM-500941	-
2018-00083	ERR2704680	1.1.2	India	0.5	1	2	2	ITM-500942	-
2018-00089	ERR2704675	3.1	India	0.06	0.125	3	2	ITM-500947	-
2018-00090	ERR2704693	3.1.3	Afghanistan	0.125	-	-	-	ITM-500948	-
2018-00091	ERR2704678	3	Ethiopia	0.125	-	-	-	ITM-500949	-
2018-00092	ERR2704705	4.6.2.2	Ghana	0.06	-	-	-	ITM-500950	-
2018-00095	ERR2704686	5.1.1	Ghana	0.125	0.06	3	0.5	ITM-500953	-
2018-00097	ERR2704706	5.1	Sierra Leone	0.03	-	-	-	ITM-500955	-
2018-00098	ERR2704687	6.3.1	Ghana	invalid	0.008	5	NC	ITM-500956	-
2018-00099	ERR2704681	6.1.1	Gambia	0.004	-	-	-	ITM-500957	-
2018-00101	ERR2704711	7	Ethiopia	0.125	0.125	3	1	ITM-500959	12195/18; 0.25
2018-00102	ERR2704679	1.1.1	China	0.5	0.5	2	1	ITM-500960	-
2018-01172	ERR12115321	8	Rwanda	0.25	1	5	4	ITM-500961	-
2018-03241	ERR2704677	2.2.2	China	0.06	0.125	2	2	ITM-500976	-
2019-03960	ERR12115322	3.1.1	Tanzania	0.03	-	-	-	ITM-501067	-
2020-03563	ERR7361924	1	United Kingdom	0.25	1	5	4	ITM-501090	-
2020-03564	ERR7361925	3	United Kingdom	0.06	0.125	4	2	ITM-501091	-
2020-03565	SAMN11179707	1.1.3	United Kingdom	>8	>4	5	NC	ITM-501092	PRE_004, (TN34503), ITM 501092; >8
2020-03567	ERR7361927	4	United Kingdom	0.06	0.125	5	2	ITM-501094	-
2020-03568	ERR7361928	2.2.1	Ukraine	>8	>4	5	NC	ITM-501095	14A1; >16

2020-03572	SAMN11179714	4.8	United Kingdom	0.125	0.25	4	2	ITM-501099	PRE_012 (TN33547), ITM 501099; 0.5
2021-00504	ERR12115323	9	Rwanda	invalid	≤0.002	5	NC	ITM-501138	-
2020-03571	SAMN11179698	2.2.1	United Kingdom	0.06	0.25	5	4	ITM-501098	PRE_011 (TN11265), ITM 501098; 0.25
2020-03570	ERR7361929	4.3.3	United Kingdom	0.06	0.125	4	2	ITM-501097	-
2020-00018	ERR12115305	2	India	0.125	-	-	-	NA	-
2020-00011	ERR12115304	1.1.3	Bangladesh	>8	>4	2	NC	NA	-
1996-01852	ERR3132270	4	Bangladesh	0.03	-	-	-	ITM-500339	-
H37Rv	-	4	BCCM/ITM	0.06	0.125	1	2	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	0.125	2	1	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	0.25	3	2	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	0.25	4	2	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	0.25	5	2	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.06	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-

H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-
H37Rv	-	4	BCCM/ITM	0.125	-	-	-	ITM-500735	-

Table 6.S2: Pa MICs for all strains included in this study.

NC= Not calculated due to truncated/invalid MIC for at least on one medium, W = Week, B= 7H11-Pa batch nr.

Lineage was determined using WGS data for strains with WGS data and Spoligotyping for strains without WGS data. All strains with a BCCM ID can be purchased from the BCCM/ITM Mycobacteria Collection (https://bccm.belspo.be/about-us/bccm-itm).

To measure the technical variability of MIC testing, 30 replicates of H37Rv (BCCM/ITM, ITM 500735/CT2008-03715) were tested weekly for ten weeks using three different batches of 7H11-Pa media.

CHAPTER 7

Implementation validation of the WHO-endorsed broth microdilution testing for the minimal inhibitory concentration of MTBc

Implementation validation of the WHO-endorsed broth microdilution testing for the minimal inhibitory concentration of MTBc

Unpublished data

Praharshinie Rupasinghe[1,2*], Jens Vereecken[1], Christel Dezmaretz[1], Ramata Balde[1], Rabab Reenaers[1], Bouke C de Jong[1], Leen Rigouts[1,2]

[1] Unit of Mycobacteriology, Department of Biomedical Sciences, Institute of Tropical Medicine, Antwerp, Belgium
[2]Department of Biomedical Sciences, University of Antwerp, Antwerp, Belgium
* Corresponding author

Abstract

Background

The World Health Organization (WHO) has endorsed 96-well-plate broth microdilution testing (BMD) for *Mycobacterium tuberculosis* complex MICs. The Institute of Tropical Medicine in Antwerp integrated this method with an automated drug-dispenser system. This study determined the quality control range (QC range) and preliminary epidemiological cut-off (ECOFF) values for clofazimine, levofloxacin, moxifloxacin, bedaquiline, linezolid, and delamanid, assessed the sensitivity and specificity of the current interim cut-off values for these drugs, and the reproducibility and repeatability of the semi-automated BMD procedure integrated with an automated dispenser system.

Method

The H37Rv reference strain was used to assess the QC range, reproducibility, and repeatability; ECOFF was determined using probable drug-susceptible isolates, while the sensitivity and specificity of the interim cut-off values were assessed using both probable susceptible and probable resistance isolates for each drug. Each parameter was assessed for both full (MIC_{100}) and "99%" ($MIC_{classic}$) inhibition.

Results

All tested drugs had acceptable maximum 3-dilution QC ranges, and good technical reproducibility and repeatability for both $MIC_{classic}$ and MIC_{100}. At the tentative cut-offs, all tested drugs had 100% sensitivity and all but clofazimine had >95% specificity for both MICclassic and MIC_{100}. For clofazimine, MIC_{100} did not meet the pre-defined >95% specificity criterium.

Conclusions

Considering both sensitivity and specificity, $MIC_{classic}$ provides more accurate MICs for all drugs tested, making it the ultimate result to report with reading at day 14.

7.1 Introduction

In 2022, globally 410,000 people were infected with multi-drug-resistant (MDR) or rifampicin-resistant (RR) tuberculosis (TB) (1). It is anticipated that without proper surveillance and diagnosis, nearly 75 million individuals will develop drug-resistant (DR) TB by 2050, costing the world economy $16.7 trillion (2). Owing to the time-consuming phenotypic drug-susceptibility testing (DST) methods that require sophisticated infrastructure and skilled labor, genotypic DST methods for the *Mycobacterium tuberculosis* complex (MTBc) have become increasingly popular (3). However, phenotypic DST of MTBc remains indispensable, especially for the novel and re-purposed anti-TB drugs for which often multiple genes are associated with resistance, complicating the development of rapid molecular tests, and the mechanisms of resistance which are not fully understood (4-6).

In most laboratories, phenotypic DST for MTBc relies on the proportion method on solid medium or in the liquid BACTEC MGIT 960 system. Traditionally, DST for MTBc is done by testing only the breakpoints to separate probably susceptible (wild type) from probably resistant (mutant) isolates, known as critical concentrations (CC) and, if applicable in some cases, the clinical breakpoints (CB) to separate low- from high-level resistance (7, 8). Minimal inhibitory concentration (MIC) testing is not widely used for MTBc as both solid medium or MGIT960 for quantitative DST are labor-intensive and costly. However, MIC testing offers several advantages, such as quantifying the resistance level - which is not always possible by classic phenotypic DST or rapid molecular tests alone -, resolving discordances between genotypic and phenotypic DST results for mutations resulting in a MIC increase around the CCs, allowing laboratories to detect systematic errors in a more sensitive manner, and for defining DST breakpoints for novel anti-TB drugs for which a knowledge gap remains on the correlation of potential resistance-conferring mutations and their resistance phenotype. As an alternative approach, the World Health Organization (WHO) has endorsed 96-well-plate-based broth microdilution testing (BMD) to determine the MICs of MTBc (9). At the Institute of Tropical Medicine, Antwerp, this method was integrated with an automated dispenser system (HP D300e digital dispenser instrument), which was already validated for plate based MIC testing using the colorimetric resazurin microtiter assay (REMA). The HP D300e digital dispenser enables dispensing antibiotic solutions in dimethyl-sulfoxide (DMSO) or surfactant-containing aqueous solutions in different plate formats, directly from the stock solution. The system makes

use of a single-use dispense head to dispense solutions directly and contact-free in the assay plate in the picolitre to microliter range. The system is also able to normalize the matrix concentration in the plate (e.g., equal DMSO concentration in the entire plate). The use of the D300e system eliminates the need of working solutions, serial dilutions, and laborious manual workflows to fill the (normalized) plates and should facilitate standardization of BMD testing. In addition, the dispenser system allows printing large batches of plates in a short time.

The objectives of this analysis included (i) determining the quality control (CQ) range and preliminary epidemiological cut-off (ECOFF) values for clofazimine, levofloxacin, moxifloxacin, bedaquiline, linezolid, and delamanid, (ii) assessing the sensitivity and specificity of the current interim cut-off values for these drugs, as well as (iii) determining the reproducibility and repeatability of the semi-automated BMD procedure integrated with the automated drug dispenser.

7.2 Method

7.2.1 Plate layout and drug concentrations tested

Stock solutions of bedaquiline, clofazimine, linezolid, and delamanid were dissolved in dimethyl sulfoxide (DMSO) whereas stock solutions of moxifloxacin and levofloxacin were dissolved in 0.1N sodium hydroxide (NaOH). Inner wells of the sterile, 96-well, round bottom polystyrene microtiter plates were filled manually with 100 µl of 7H9-S medium (7H9 broth + 10% OADC + 0.5% glycerol + 0.1% casitone), and outer wells with sterile distilled water (SDW) or 7H9-S (**Figure 7.1**). Serial two-fold dilutions of levofloxacin, moxifloxacin, clofazimine, bedaquiline, linezolid and delamanid were distributed directly in the 7H9-S wells using the HP D300e dispenser. The final DMSO concentration was normalized to 0.2% across all wells containing DMSO-dissolved drugs, as well as DMSO-containing positive and negative controls, except for the well with 4 µg/ml clofazimine, which contained 0.4% DMSO.

For water-based stock solutions of levofloxacin and moxifloxacin, 0.03% Tween 20 was added as surfactant according to the dispenser procedure. The Tween 20 concentration was normalized to 0.3% across all wells containing water-dissolved drugs, as well as Tween-containing positive and negative controls.

Stability of the drugs in dispensed plates was assessed prior to this study by REMA; all drugs but delamanid assessed in that study were stable up to 10 weeks of storage in plastic sealed bags at -20°C. Therefore, delamanid was added freshly using the dispenser just before the inoculation of the plates.

	1	2	3	4	5	6	7	8	9	10	11	12
A		H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20
B	H_20	BDQ 2,0	BDQ 1,0	BDQ 0,5	BDQ 0,25	BDQ 0,125	BDQ 0,06	BDQ 0,03	BDQ 0,015	BDQ 0,008	GC 100% DMSO	NC DMSO
C	H_20	LNZ 8,0	LNZ 4,0	LNZ 2,0	LNZ 1,0	LNZ 0,5	LNZ 0,25	LNZ 0,125	LNZ 0,06	LNZ 0,03	GC 1% DMSO	NC DMSO
D	H_20	DLM 0,5	DLM 0,25	DLM 0,125	DLM 0,06	DLM 0,03	DLM 0,015	DLM 0,008	DLM 0,004	DLM 0,002	GC 100%	NC
E	H_20	CFZ 4,0	CFZ 2,0	CFZ 1,0	CFZ 0,5	CFZ 0,25	CFZ 0,125	CFZ 0,06	CFZ 0,03	CFZ 0,015	GC 1%	NC
F	H_20	MFX 4,0	MFX 2,0	MFX 1,0	MFX 0,5	MFX 0,25	MFX 0,125	MFX 0,06	MFX 0,03	MFX 0,015	GC 100% a+0,3%TW	NC a+0,3%TW
G	H_20	LFX 8,0	LFX 4,0	LFX 2,0	LFX 1,0	LFX 0,5	LFX 0,25	LFX 0,125	LFX 0,06	LFX 0,03	GC 1% a+0,3%TW	NC a+0,3%TW
H	primer DLM	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20

Figure 7.1: Plate layout with serial diluted drug concentrations tested during this broth microdilution validation.

GC = growth control, NC = negative control, BDQ= bedaquiline, LNZ = linezolid, DLM = delamanid, CFZ = clofazimine, MFX = moxifloxacin, LFX = levofloxacin, TW=Tween 20

7.2.2 Inoculum preparation

A sterile loop was used to transfer bacterial colonies from 2-3 weeks old MTBc cultures grown on Löwenstein-Jensen (LJ) medium into a 15 ml sterile screw-cap glass tube containing 5-10 sterile glass beads. About three drops of SDW were added to this tube, which was then firmly closed and vortexed vigorously for one minute, paying attention to the beads rolling down the glass tube's wall, until the clumps were well dispersed. After allowing the closed tube to stand for 5 minutes to settle aerosols, 5 ml of SDW was added, the tube was tightly closed, and the contents were vigorously vortexed for 15 seconds until the tube's content was homogenized. After letting the closed tube stand for 30 minutes for the clumps to settle, the supernatant was transferred into a new sterile glass tube. Using the densitometer, the turbidity of the supernatant was adjusted to McFarland standard (McF) 0.5 using SDW, and then further diluted

1:100 in 7H9-S broth to prepare a 10^{-2} of the McF 0.5 bacterial suspension. If the undiluted supernatant's density was below McF 0.5, a new suspension was prepared.

Using sterile filter tips, the drug-containing wells and the 100% growth controls (B11, D11 and F11, **Figure 7.1**), were inoculated with 100 µl of the 10^{-2} of McF 0.5 bacterial suspension. The 10^{-2} McF bacterial suspension was further diluted 1:100 using 7H9-S broth to prepare the 10^{-4} McF bacterial suspension, which was used to inoculate the 1% growth controls (C11, E11 and G11, **Figure 7.1**). After inoculation, the plates were incubated at 36°C (±2°C) for a maximum of 21 days.

7.2.3 Reading and interpretation of the MICs

The plates were read by visual inspection using an inverted mirror to detect growth in the wells. Systematic reading was done at day 7 and day 14 of incubation. If there was still no growth of the GC1% after day 14, the incubation was extended to a maximum of 21 days. As soon as the growth GC100% and the GC1% were positive, or when at least 2/3rd of the GC1% positive controls of the same isolate were positive, the $MIC_{classic}$ and MIC_{100} values were interpreted.

$MIC_{classic}$ = the highest concentration of the serial drug dilution that did not have MTBc growth, disregarding pinpoint MTBc growth (see **Figure 7.2** for pinpoint growth) when trailing pinpoint growth was observed.

MIC_{100} = the highest concentration of the serial drug dilution that did not have any MTBc growth at all.

When a single skipped well or a single well with pinpoint growth bordered by wells with clear growth was observed, MICs were interpreted disregarding the single skipped/pinpoint well. When multiple skipped wells or wells with pinpoint growth bordered by wells with growth were observed, MICs were considered invalid, and the test was repeated (10).

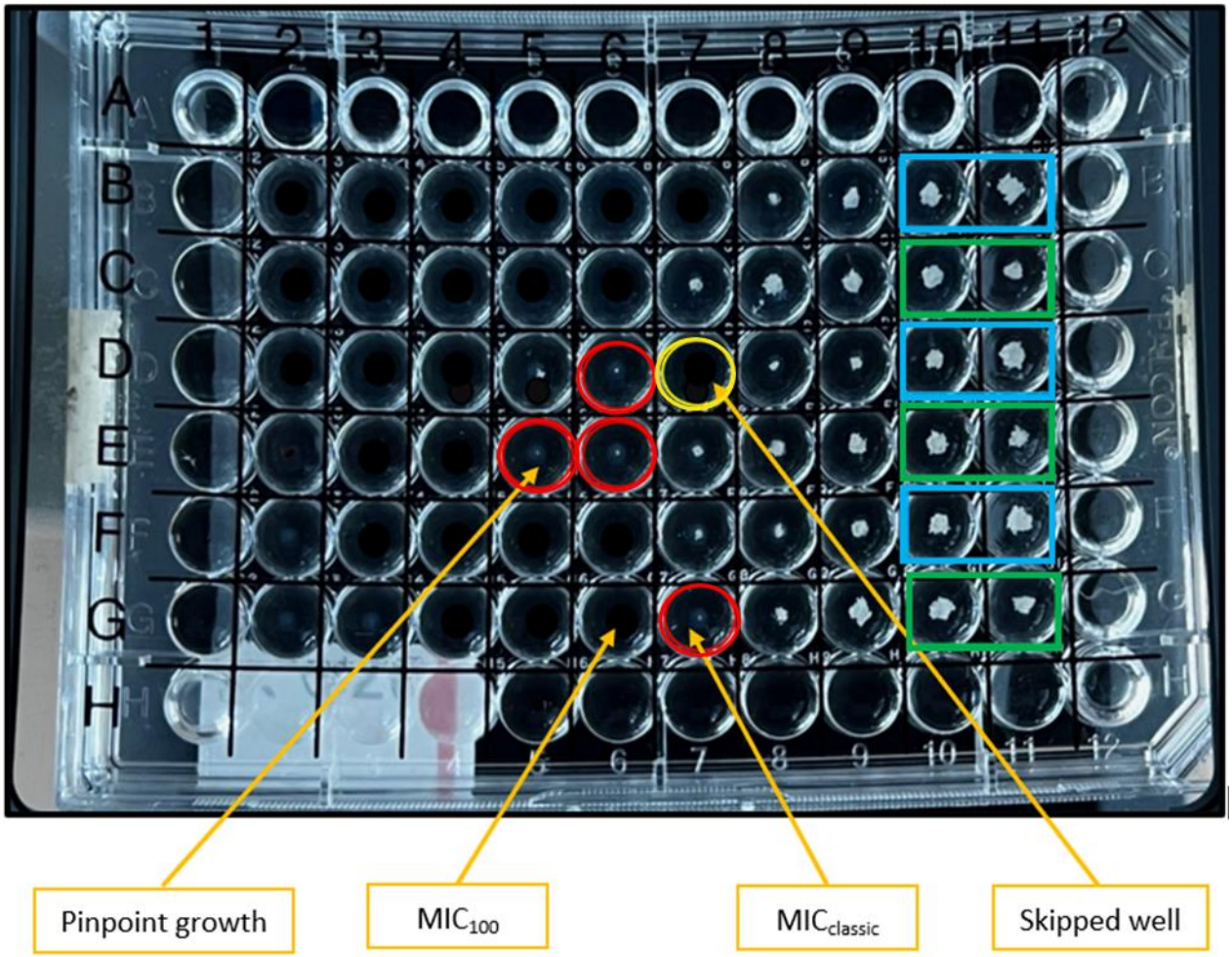


Figure 7.2: Examples of pinpoint growth and skipped wells

Circled in yellow is a skipped well, circled in red are pinpoint growth, tiny buttons of bacterial growth significantly smaller than the 1% growth control (marked in green). Marked in blue are 100% growth controls.

7.2.4 Strain selection and pre-defined acceptance criteria for different test categories

Test category	No of isolates	Selection criteria	Test procedure and acceptance criterium
Quality control range and reproducibility	1	Pan-susceptible H37Rv strain	H37Rv was tested using three different batches of printed BMD plates with repeated testing on different days by different operators to evaluate inter-day variability on each of three different media lots. Tests were scheduled twice a week for 5 weeks. In total 30 different MIC values per drug were generated, i.e., 10 values per lot tested. Each replicate must use individually prepared inoculum suspensions. Plate layout used as in **Figure 7.1**. **Acceptance criterium: ≥95% of the replicate MICs for each drug should fall below the tentative cut-off values, differ by max 1 drug dilution, and fall within a maximum of a three-dilution MIC range.**
Repeatability	1	Pan-susceptible H37Rv strain	Five replicates of the H37Rv strain were tested by the same lab technician on the same day using the same bacterial suspension and BMD plates from the same batch. Plate layout used is in **Figure 7.1**.

			≥95% of the replicate MICs for each drug should fall below the tentative cut-off values, differ by max 1 drug dilution.
Wildtype MIC distribution Preliminary ECOFF Specificity of provisional cut-offs		Selection criteria for MTBc WT isolates*	Isolates were tested using the plate layout shown in **Figure 7.1.** **Acceptance criterium: The wild-type MICs follow a normal (Gaussian) distribution** **≥95% specificity**
Levofloxacin	304	No group 1-2 *gyrA*/*gyrB* mutations present	
Moxifloxacin	304	No group 1-2 *gyrA*/*gyrB* mutations present	
Bedaquiline	275	No group 1-3 *mmpS5*/*mmpL5*/*mmpR5*/ *atpE*/*pepQ*/*Rv1979c* mutations present	
Clofazimine	285	No group 1-3 *mmpL5*/*mmpR5*/*pepQ* mutations present	
Linezolid	297	No group 1-3 *rrl*/*rplC* mutations present	
Delamanid	265	No group 1-3 *ddn*/*fgd1*/*fbiA*/*fbiB*/*fbiC*/*fbiD* mutations present	
Sensitivity of the tentative cut-offs		Selection criteria for MTBc MUT isolates**	Isolates were tested using the plate layout shown in **Figure 7.1.**

Levofloxacin	34	At least one group 1-2 *gyrA*/*gyrB* mutation present	**Tentative cut-off values applied (9)** Levofloxacin – 1 µg/ml
Moxifloxacin	34	At least one group 1-2 *gyrA*/*gyrB* mutation present	Moxifloxacin – 0.25 µg/ml Bedaquiline – 0.25 µg/ml
Bedaquiline	9	At least one group 1-2 *mmpR5*/*atpE*/*pepQ* mutation present	Clofazimine – 0.5 µg/ml Linezolid – 1.0 µg/ml
Clofazimine	9	At least one group 1-2 *mmpR5* /*pepQ* mutation present	Delamanid – 0.125 µg/ml
Linezolid	2	At least one group 1-2 *rrl*/*rplC* mutation present	**Acceptance criterium: ≥95% of the probable resistant isolates should have MICs higher than the tentative cut-offs applied**
Delamanid	12	Delamanid resistant reference strains/ clinical isolates with *fbiB*/*fbiC* or *ddn* mutations amplified while receiving delamanid	

Table 7.1: Overview of the strains used and acceptance criterium applied for each test category.

Based on the WHO Mutation Catalogue Version 2 (2023): Group 1: Associated with resistance, Group 2: Associated with resistance interim, Group 3: Unknown significant in resistance, Group 4: Not associated with resistance interim, Group 5: Not associated with resistance (5)

All clinical isolates used to determine the wildtype MIC distribution and specificity and sensitivity of the interim cut-offs were multi-drug resistant *M. tuberculosis* complex (MTBc) strains with or without additional resistance to fluoroquinolones.

7.3 Results

7.3.1 Quality control ranges and reproducibility

For all tested drugs, 100% of the MIC_{100} and $MIC_{classic}$ for H37Rv fell within a maximum three dilution range (**Figure 7.3**), thus met the pre-defined acceptance criteria. For all but clofazimine the mode of MIC_{100} and $MIC_{classic}$ were the same. For clofazimine the MIC_{100} mode was one dilution more than the mode of $MIC_{classic}$. Both our observed MIC_{100} and $MIC_{classic}$ for clofazimine were one dilution higher than the ones proposed by CLSI and Jansen, while the ranges for the other drugs fell within the expected values (9) (**Table 7.2**).

Of the 30 H37Rv replicates, 20 (67%) had interpretable growth at day 7, whereas the remaining 10 took 14 days to have interpretable growth.

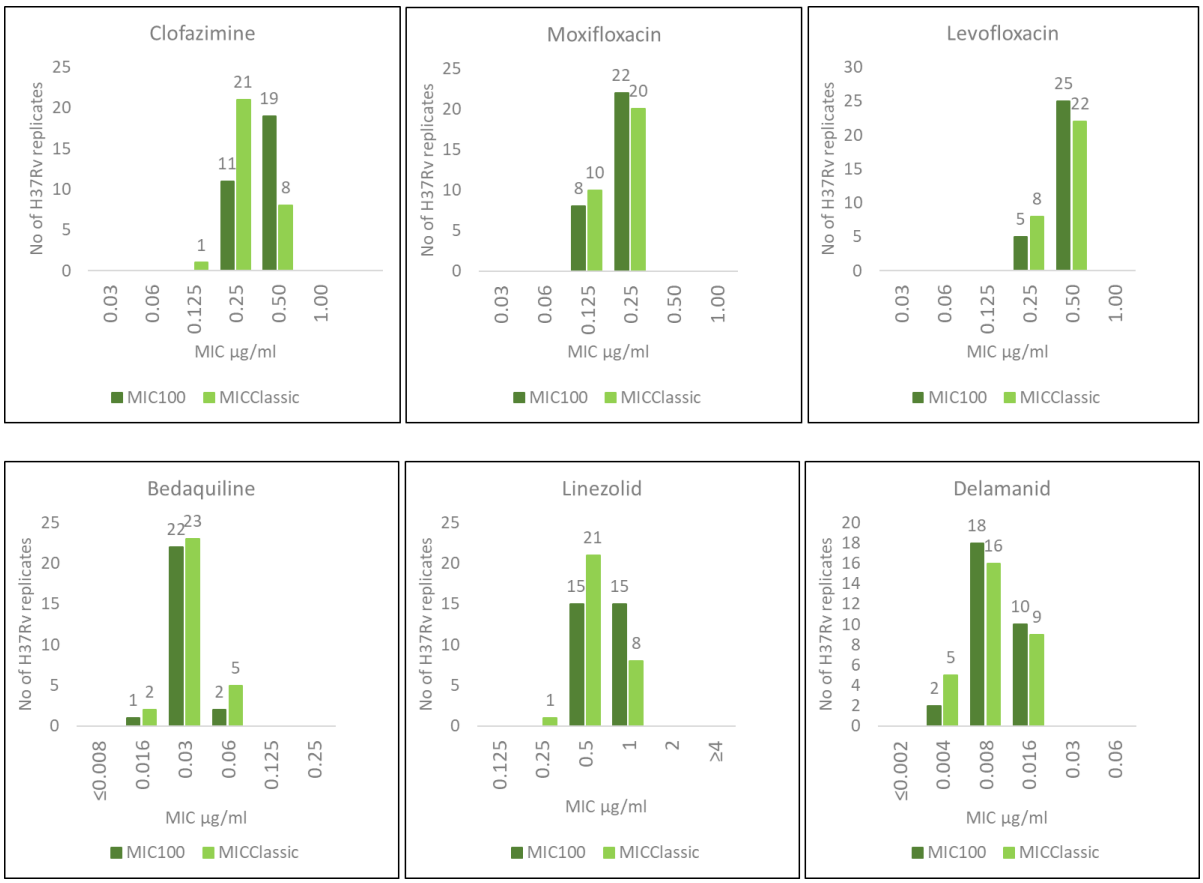


Figure 7.3: Quality control ranges of the H37Rv reference strain for clofazimine, moxifloxacin, levofloxacin, bedaquiline, linezolid and delamanid

Drug	QC ranges - ITM-BMD method		QC ranges - other frozen format methods (9)		
	$MIC_{classic}$ (µg/ml)	MIC_{100} (µg/ml)	CLSI (µg/ml)	Jansen - Tier 2 (µg/ml)	Jansen - Tier 3 (µg/ml)
Cfz	0.125-0.5	0.25-0.5	≤0.06-0.25	≤0.06-0.25	≤0.03-0.25
Mfx	0.125-0.25	0.125-0.25	-	-	-
Lfx	0.25-0.5	0.25-0.5	≤0.125-1.0	≤0.125-1.0	0.25-1.0
Bdq	0.016-0.06	0.016-0.06	0.016-0.06	0.016-0.06	0.016-0.125
Lzd	0.25-1.0	0.5-1.0	0.25-2.0	0.25-2.0	0.25-2.0
Dlm	0.004-0.016	0.004-0.016	-	-	-

Table 7.2: Quality control ranges of the H37Rv reference strain for clofazimine, moxifloxacin, levofloxacin, bedaquiline, linezolid and delamanid

Cfz= clofazimine, Mfx= moxifloxacin, Lfx= levofloxacin, Bdq= bedaquiline, Lzd= linezolid, Dlm= delamanid

7.3.2 Repeatability

For all drugs evaluated, both $MIC_{classic}$ and MIC_{100} met the pre-defined acceptance criteria for repeatability, with 100% of the MIC values of the H37Rv replicates for each drug falling below the proposed cut-off values and varied by no more than one drug dilution (**Table 7.3**).

H37Rv replicate	**$MIC_{classic}$**						**MIC_{100}**					
	Cfz	**Mfx**	**Lfx**	**Bdq**	**Lzd**	**Dlm**	**Cfz**	**Mfx**	**Lfx**	**Bdq**	**Lzd**	**Dlm**
1	0.25	0.25	0.5	0.06	0.5	0.008	0.25	0.25	0.5	0.06	0.5	0.008
2	0.25	0.125	0.5	0.06	0.5	0.008	0.25	0.125	0.5	0.06	0.5	0.008
3	0.25	0.25	0.5	0.06	0.5	0.008	0.25	0.25	0.5	0.06	0.5	0.008
4	0.25	0.25	0.5	0.06	0.5	0.008	0.25	0.25	0.5	0.06	0.5	0.008
5	0.5	0.25	0.5	0.06	0.5	0.008	0.5	0.25	0.5	0.06	0.5	0.008

Table 7.3: Repeatability of the H37Rv MIC results

Cfz= clofazimine, Mfx= moxifloxacin, Lfx= levofloxacin, Bdq= bedaquiline, Lzd= linezolid, Dlm= delamanid

7.3.3 Wildtype distribution and tentative ECOFF using probable drug susceptible MTBc isolates

The majority of the clinical isolates (>95%) had interpretable growth in both the GC100% and GC1% growth controls only after 14 days of incubation. For all the drugs, the wild type MIC distribution followed a Gaussian distribution (**Figure 7.4**). The tentative ECOFFs for each drug are shown in **Table 7.4**. Our data is based on a single site testing and did not include MTBc lineages 5-10, thus our ECOFF is considered "tentative".

Drug	Tentative ECOFF[1]	ECOFF from other methods (9)		
	ITM-BMD for both $MIC_{classic}/MIC_{100}$	Dry format CRyPTIC	Dry format Janssen	Frozen format Janssen
Cfz	1.0 µg/ml/1.0 µg/ml	0.25	-	0.5
Mfx	0.5 µg/ml/1.0 µg/ml	-	-	-
Lfx	1.0 µg/ml/2.0 µg/ml	1	-	1
Bdq	0.25 µg/ml/0.25 µg/ml	0.25	0.125	0.125
Lzd	>2.0 µg/ml/>2.0 µg/ml	1	-	2
Dlm	0.125 µg/ml/0.125 µg/ml	0.125	-	-

Table 7.4: Tentative ECOFFs

Cfz= clofazimine, Mfx= moxifloxacin, Lfx= levofloxacin, Bdq= bedaquiline, Lzd= linezolid, Dlm= delamanid. [1]Definition of ECOFF (EUCAST, ECDC): within a species it is the highest concentration of the drug lacking phenotypically expressed resistance". The wild type is presented as WT ≤ z µg/ml and non-wild type as NWT > z µg/ml (EUCAST. Definitions of clinical breakpoints and epidemiological cut-off value. Växjö: EUCAST; 2012. Available from: http//www. Srga.org/Eucastwt/eucastdefinitions.html).

The tentative ECOFFs from our study are higher than the ECOFFs reported from other BMD methods for clofazimine (both MIC_{100} and $MIC_{classic}$), levofloxacin (only MIC_{100}) and linezolid (both MIC_{100} and $MIC_{classic}$) (**Table 7.4**). In addition, although our QC range for bedaquiline was consistent with both the CRYPTIC and Janssen Tier 2 studies, our bedaquiline tentative ECOFF is consistent with the CRYPTIC study but one dilution higher than the Janssen study.

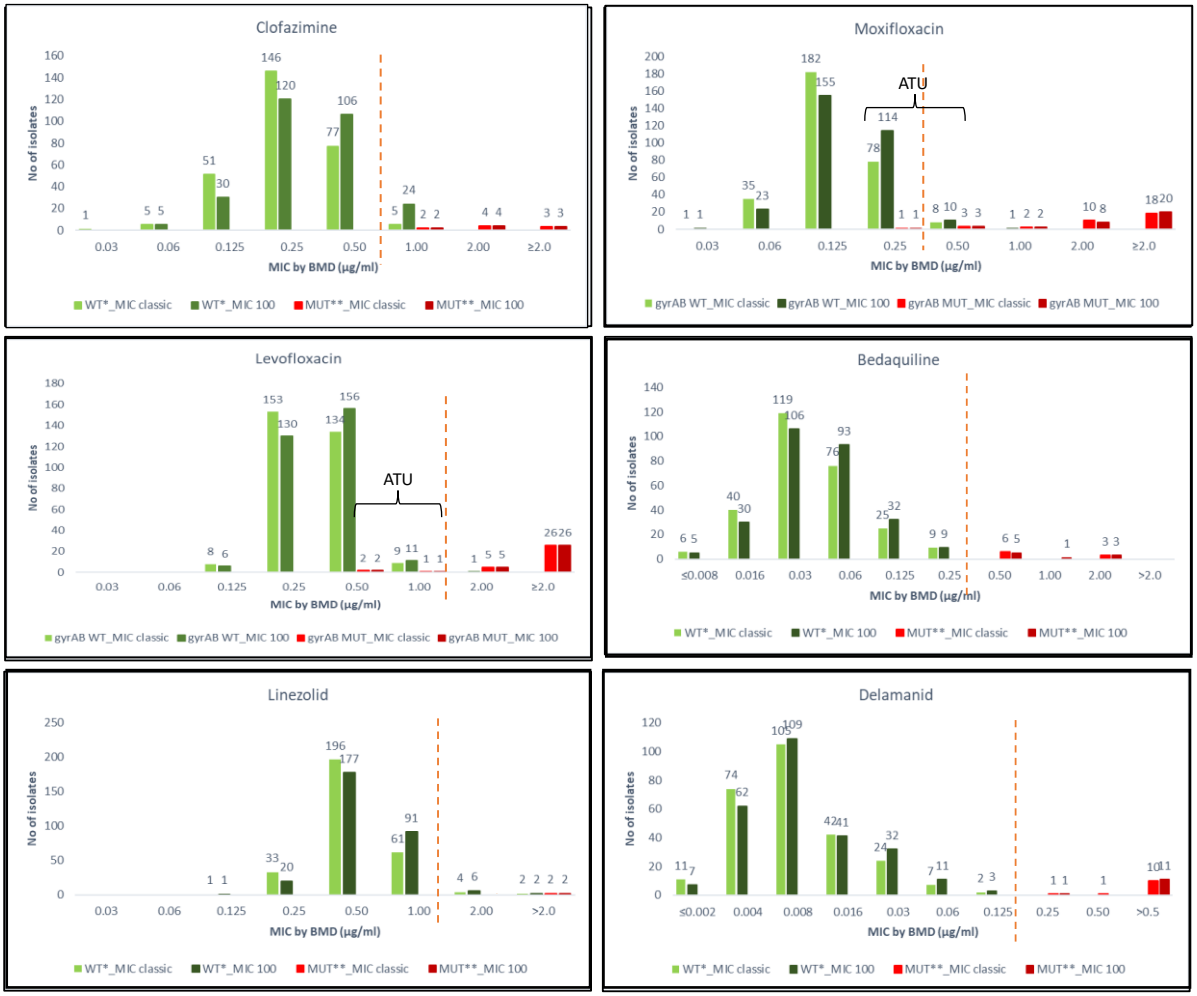


Figure 7.4: MIC distribution of probable drug-susceptible and drug-resistant clinical strains

WT = Wildtype, MUT = Mutant, ATU= Area of technical uncertainty

*Refer to selection criteria for WT isolates in **Table 7.1**, **Refer to selection criteria for MUT isolates in **Table 7.1**

Marked in red dashed lines are the tentative cut-offs applied (9).

7.3.4 Sensitivity and specificity of the current tentative cut-off values

Sensitivity and specificity for the drugs tested applying the current tentative cut-off values were excellent (100%) for bedaquiline and delamanid regardless of $MIC_{classic}$ or MIC_{100} interpretation (**Table 7.5**). For clofazimine and linezolid, 100% sensitivity was reached when using the $MIC_{classic}$ with a good respective specificity of 98.3% and 97.9% respectively. Applying the MIC_{100} interpretation, maintained the 100% sensitivity but at a minor cost in specificity for linezolid (97.3%) and a high cost in specificity for clofazimine (91.6%). For moxifloxacin, the $MIC_{classic}$ resulted in a good sensitivity of 97.1% and a similar specificity. MIC_{100} interpretation resulted in a 96.4% sensitivity and a specificity of 97.4%. The lowest sensitivity was observed

for levofloxacin regardless of the applied interpretation (91.2% at $MIC_{classic}$ and MIC_{100}), with a slight loss in specificity when applying MIC_{100} (99.7% compared to 100% for $MIC_{classic}$).

To investigate potential causes for false-R results, we analyzed targeted next-generation sequencing data (Deeplex Myc/TB) on the sediments of the sputum specimens the MTBc isolates originated from to check for presence of relevant (minority) variants, and we assessed likely exposure to the drug(s) of concern.

From the 11 MTBc isolates with probable false-R results for moxifloxacin, eight had Deeplex results available, of which seven had WT *gyrA/gyrB* genes and one had a *gyrA_Ala74Ser* mutation, known to be not associated with fluoroquinolone resistance (**Table 7.6**). Three of them were baseline isolates from patients before starting MDR-TB treatment. The isolates belonged to lineages 2 and 4.

The two isolates with probable false-linezolid-R results had WT *rrl* or *rplC* genes and belonged to lineage 2 or 3. Previous exposure to linezolid was unlikely, as both were baseline isolates (**Table 7.6**).

The five isolates that had false-clofazimine-R results at $MIC_{classic}$, had WT *Rv0678* genes by Deeplex and belonged to lineage 4. Two of these patients had been exposed to Cfz/Bdq for 2-12 weeks. In addition, from the 19 isolates that were only false-clofazimine-R at MIC_{100}, 11 had Deeplex results available. Ten of them had WT *Rv0678* genes, while one showed minority variant *Val150Ala* of uncertain significance in gene *Rv0678*. Nine of the 19 patients had been exposed to Cfz/Bdq for 2 to 43 weeks (**Table 7.6**).

We detected pinpoint growth for all drugs at varied frequencies, most notably for clofazimine, where pinpoint growth was more common and had a greater influence on resulting in probable false-R results by MIC_{100} than for the other drugs (**Table 7.7**).

Drug and the tentative CC (4)	Resistance associated mutations*, **	R/S by $MIC_{classic}$					R/S by MIC_{100}				
		S	R	Total	**Sensitivity** [95% CI]	**Specificity** [95% CI]	S	R	Total	**Sensitivity** [95% CI]	**Specificity** [95% CI]
Clofazimine 0.5 µg/ml	Absent	280	5	**285**	**100.0%** [69.15% to 100.0%]	**98.3%** [95.9% to 99.4%]	261	24	**285**	**100.0%** [59.04% to 100%]	**91.6%** [87.7% to 94.5%]
	Present	0	9	**9**			0	9	**9**		
	Total	**280**	**14**	**294**			**261**	**33**	**294**		
Moxifloxacin 0.25 µg/ml	Absent	296	8	**304**	**97.1%** [84.7% to 99.9%]	**97.4%** [94.9% to 98.9%]	293	11	**304**	**97.1%** [84.6% to 99.9%]	**96.4%** [93.6% to 98.1%]
	Present	1	33	**34**			1	33	**34**		
	Total	**297**	**41**	**338**			**294**	**44**	**338**		
Levofloxacin 1.0 µg/ml	Absent	304	0	**304**	**91.2%** [76.3% to 98.1%]	**100.0%** [98.7% to 100.0%]	303	1	**304**	**91.2%** [76.3% to 98.1%]	**99.7%** [98.2% to 99.99]
	Present	3	31	**34**			3	31	**34**		
	Total	**306**	**33**	**338**			**306**	**33**	**338**		
Bedaquiline 0.25 µg/ml	Absent	275	0	**275**	**100.0%** [66.3% to 100.0%]	**100.0%** [98.7% to 100.0%]	275	0	**275**	**100.0%** [66.3% to 100.0%]	**100.0%** [98.7% to 100.0%]
	Present	0	9	**9**			0	9	**9**		
	Total	**275**	**9**	**284**			**275**	**9**	**284**		
Linezolid 2.0 µg/ml	Absent	291	6	**297**	**100.0%** [15.8% to 100.0%]	**97.9%** [95.6% to 99.3%]	289	8	**297**	**100.0%** [15.8% to 100.0%]	**97.3%** [94.7% to 98.8%]
	Present	0	2	**2**			0	2	**2**		
	Total	**291**	**8**	**299**			**289**	**10**	**299**		
Delamanid 0.125 µg/ml	Absent	265	0	**265**	**100.0%** [73.5% to 100.0%]	**100.0%** [98.6% to 100.0%]	265	0	**265**	**100.0%** [73.5% to 100.0%]	**100.0%** [98.6% to 100.0%]
	Present	0	12	**12**			0	12	**12**		
	Total	265	12	**277**			**265**	**12**	**277**		

Table 7.5: Sensitivity and specificity for various anti-TB drugs, applying the current tentative cut-offs.

*Refer to selection criteria for WT isolates in **Table 7.1**, **Refer to selection criteria for MUT isolates in **Table 7.1**

R = Resistant, S = Susceptible, CI= Confidence intervals

CT	Probable false-R for drug(s) at		Lineage	Deeplex info for gene(s) relevant to the tested drugs[1]	Exposure to the drug(s) of concern[2]	
	$MIC_{classic}$	MIC_{100}			Duration	Remark
2019-04865	Mfx	Mfx	L4.4.1.1	Not available	Unlikely exposed	Baseline isolate
2019-04853	Mfx	Mfx	L2.2.2	*gyrAB* WT	Unlikely exposed	Baseline isolate
2019-01783	Mfx	Mfx	L4.3.3	*gyrA Ala74Ser*	Unlikely exposed	Baseline isolate
2019-01779	Mfx	Mfx	L4.3.3	*gyrAB* WT	Unlikely exposed	Baseline isolate
2020-03299	Mfx	Mfx	L4.3.3	*gyrAB* WT	Unlikely exposed	Baseline isolate
2019-00479	Mfx	Mfx	L2.2.1	Not available	Unlikely exposed	Baseline isolate
2020-03234	Mfx	Mfx	L4.3.3	*gyrAB* WT	Unlikely exposed	Baseline isolate
2020-03337		Mfx	L4	*gyrAB* WT	Unlikely exposed	Baseline isolate
2023-01688		Mfx	L2.2.1	*gyrAB* WT	Unlikely exposed	Baseline isolate
2023-02198	Mfx	Mfx	L2.2.1	*gyrAB* WT	Unlikely exposed	Baseline isolate
2020-03298		Mfx	L4.3.2	Not available	Unlikely exposed	Baseline isolate
2023-00579	Lzd	Lzd	L3.1.2.1	*rrL/rplC* WT	Unlikely exposed	Baseline isolate
2023-01615	Lzd	Lzd	L2.2.1	*rrL/rplC* WT	Unlikely exposed	Baseline isolate
2020-03239	Cfz	Cfz	L4.3.3	*Rv0678* WT	Unlikely exposed	Baseline isolate
2020-03244	Cfz	Cfz	L4.3.3	*Rv0678* WT	Unlikely exposed	Baseline isolate
2020-03430	Cfz	Cfz	L4.3.3	*Rv0678* WT	Unlikely exposed	Baseline isolate
2023-00732	Cfz	Cfz	L4.3.3	*Rv0678* WT	Cfz/Bdq for 12 weeks	
2023-00791	Cfz	Cfz	L4.8	*Rv0678* WT	Cfz/Bdq for 2 weeks	
2019-02712		Cfz	L2.2.1	Not available	Cfz/Bdq for 43 weeks	
2020-01261		Cfz	L2.2.1	Not available	Cfz/Bdq for 12 weeks	
2020-01266		Cfz	L2.2.1	Not available	Cfz/Bdq for 4 weeks	
2020-03246		Cfz	L4.3.3	*Rv0678* WT	Unlikely exposed	Baseline isolate
2020-03275		Cfz	L4.3.3	*Rv0678* WT	Unlikely exposed	Baseline isolate
2020-03287		Cfz	L4.3.3	*Rv0678* WT	Unlikely exposed	Baseline isolate
2020-03317		Cfz	L4.3.3	Not available	Unlikely exposed	Baseline isolate
2021-02318		Cfz	L4.3.4	Not available	Unlikely exposed	Baseline isolate
2023-00577		Cfz	L4.3.5	Not available	Cfz/Bdq for 28 weeks	

2023-00639		Cfz	L4.3.6	minority *Rv0678*	Unlikely exposed	Baseline isolate
2023-00734		Cfz	L4.3.7	*Rv0678* WT	Unlikely exposed	Pre-secreening
2023-00735		Cfz	L4.3.8	*Rv0678* WT	Cfz/Bdq for 2 weeks	
2023-00737		Cfz	L4.3.9	*Rv0678* WT	Cfz/Bdq for 8 weeks	
2023-00781		Cfz	L4.3.10	*Rv0678* WT	Cfz/Bdq for 4 weeks	
2023-00783		Cfz	L4.3.11	Not available	Cfz/Bdq for 14 weeks	
2023-00786		Cfz	L4.3.12	*Rv0678* WT	Cfz/Bdq for 15 weeks	

Table 7.6: Overview of the MTBc isolates that had likely false-R MIC results to one or more drugs.

[1] Deeplex MycTB assay was performed on the decontaminated sputum sediment from which these isolates were originated

[2] Patients who had more than 30 days exposure to the experimental drugs within the last five years or have had household contact with someone proven to be resistant to an experimental drug were excluded from the endTB trial, thus baseline isolates were considered as 'unlikely' exposed to FQs, clofazimine, bedaquiline, linezolid or delamanid.

Mfx= moxifloxacin, Lfx= levofloxacin, Cfz= clofazimine, Bdq= bedaquiline

Drug	Total number of isolates tested	Number of isolates with pinpoint colonies in any well	Number of wells with pinpoint growth				% switched from S to R due to pinpoint growth (MIC_{100})
			1	2	3	4	
Cfz	294	96 (33%)	89	6	1	0	19 (6%)
Mfx	338	56 (17%)	55	1	0	0	2 (1%)
Lfx	338	34 (10%)	34	0	0	0	1 (0.2%)
Bdq	284	54 (19%)	54	0	0	0	0 (0%)
Lzd	299	53 (18%)	52	1	0	0	0 (0%)
Dlm	277	44 (16%)	37	3	2	2	0 (0%)

Table 7.7: Occurrence of pinpoint growth and its impact on the susceptibility status for different drugs tested.

[1] Deeplex MycTB assay was performed on the decontaminated sputum sediment from which these isolates were originated. Mfx= moxifloxacin, Lfx= levofloxacin, Cfz= clofazimine, Bdq= bedaquiline

7.4 Discussion

Our data shows good technical reproducibility and repeatability for both $MIC_{classic}$ and MIC_{100} for all the drugs tested, indicating the consistence and precision of the semi-quantitative BMD method integrated with the HP D300e digital dispenser. We observed inconsistencies between our QC ranges and tentative ECOFF and those from other methods such as CRYPTIC and Jansens plate formats (9). However, as we did not further investigate the technical details such as inoculum concentration, inoculum preparation method, reading time and, interpretation criteria of the MICs of these methods, we were not able to further investigate the possible causes for the inconsistencies observed (9). In addition, for some drugs, such as linezolid and levofloxacin, the tentative ECOFF values were increased due to one or two probably susceptible isolates having outlier MIC values. Two isolates with no mutations in the *rrL*/*rplC* genes exhibited MICs >8 µg/ml for linezolid. Both isolates had Deeplex done on the sediments of the sputum specimens they originated from, and no mutation was identified in the *rrL*/*rplC* genes. Although these isolates lacked resistance-associated genes, we can't exclude they are actually drug-resistant via alternative mechanisms such as efflux pumps (11) (**Table 7.6**). Furthermore, 11/24 (46%) of the isolates with likely false-R clofazimine by MIC_{100} and 2/5 (40%) of the isolates with likely false-R clofazimine by $MIC_{classic}$ were isolated while the patients were receiving clofazimine or bedaquiline treatment (**Table 7.6**), implying the presence of minority clofazimine-resistant populations or clofazimine resistance via mechanisms other than mutations in the known canonical genes is possible.

We observed overlapping wild-type and mutant MIC distributions for both fluoroquinolones. This has been reported not only for fluoroquinolones, but also for the majority of the anti-TB drugs, where low-level resistance conferring mutations overlap with the MIC distribution of the susceptible strains (8, 12, 13). Testing additional drug concentrations could in theory minimize this overlap. The EUCAST has introduced the term 'area of technical uncertainty' (ATU) which is normally a one dilution where the overlap occurs (9). MICs falling within the ATU should be carefully interpreted, as such isolates may not be equivocally classified as resistant or susceptible based on a single MIC result. In this analysis the ATUs are 0.5-1.0 µg/ml for levofloxacin and 0.25–0.5µg/ml for moxifloxacin. However, at 0.5 µg/ml for levofloxacin and 0.25 µg/ml for moxifloxacin, the percentage of the *gyrAB* mutants overlapping with the wild types is less than 2%, whereas at 1 µg/ml for levofloxacin and 0.5 µg/ml for moxifloxacin, it is

around 10-20% (**Figure 7.4**). Thus, it would be acceptable to report MICs <1.0 µg/ml for levofloxacin as susceptible and MICs <0.5 µg/ml for moxifloxacin as susceptible, MICs >1.0 µg/ml for levofloxacin and >0.5 µg/ml for moxifloxacin as resistant and MICs of 1.0 µg/ml for levofloxacin and 0.5 µg/ml for moxifloxacin as inconclusive. Inconclusive MICs could be further interpreted considering other data available, i.e., if a known drug-resistance conferring mutation is detected in an isolate with inconclusive MIC results, the final results should be considered 'resistant'.

There are currently no EUCAST guidelines for interpreting pinpoint growth in MTBc*,* thus, we continue to record both MIC_{100} and MICclassic, while $MIC_{classic}$ has shown higher sensitivity and specificity overall (**Table 7.5**). Hence, we report $MIC_{classic,}$ amongst others for the endTB and endTBQ clinical trials. Meanwhile, additional research, such as modifying the inoculum preparation method and reading the plates between days 7 and 14 (for example, day 10) should be conducted in an attempt to reduce the occurrence of pinpoint growth and improve the reading and interpretation.

7.5 Conclusions

1. Good technical reproducibility was observed for both $MIC_{classic}$ and MIC_{100} for all the drugs tested.

2. All tested drugs had acceptable maximum 3-dilution quality control ranges of the H37RV reference strain for both $MIC_{classic}$ and MIC_{100}.

3. The tentative cut-offs of all drugs tested except levofloxacin had >95% sensitivity for both $MIC_{classic}$ and MIC_{100}. For clofazimine, bedaquiline, linezolid and delamanid, wide confidence intervals for sensitivity were observed, suggesting that the true sensitivity of the test could vary over a broad range. In practical terms, this means that we are less certain about the accuracy of the sensitivity estimate. This is likely due to the small number of known resistant strains included in this analysis, consequently, while we accept the current sensitivities, they should be recalculated at a later stage using a greater number of known resistant isolates.

4. The tentative cut-offs of all but clofazimine had >95% specificity for both $MIC_{classic}$ and MIC_{100}. For clofazimine, MIC_{100} did not meet the pre-defined >95% specificity criterium.

5. Considering both sensitivity and specificity, $MIC_{classic}$ provides more accurate MICs for all drugs tested, making it the ultimate result to report for all previously tested isolates at day 14.

7.6 References

1. WHO. 2023. Global tuberculosis report 2023. World Health Organization,
2. Foundation TR. 2022. Drug-resistant TB threatens to kill 75 million people by 2050, cost $16.7 trillion. https://www.reuters.com/article/us-health-tuberculosis-economy-idUSKBN0MK00520150324/. Accessed
3. Moga S, Bobosha K, Fikadu D, Zerihun B, Diriba G, Amare M, Kempker RR, Blumberg HM, Abebe T. 2023. Diagnostic performance of the GenoType MTBDRplus VER 2.0 line probe assay for the detection of isoniazid resistant *Mycobacterium tuberculosis* in Ethiopia. PLoS One 18:e0284737.
4. Kadura S, King N, Nakhoul M, Zhu H, Theron G, Köser C, Farhat M. 2020. Systematic review of mutations associated with resistance to the new and repurposed *Mycobacterium tuberculosis* drugs bedaquiline, clofazimine, linezolid, delamanid and pretomanid. The Journal of antimicrobial chemotherapy 75.
5. WHO. 2023. Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance.
6. Mohamed S, Köser CU, Salfinger M, Sougakoff W, Heysell SK. 2021. Targeted next-generation sequencing: a Swiss army knife for mycobacterial diagnostics? Eur Respir J 57.
7. WHO. 2018. Technical manual for drug susceptibility testing of medicines used in the treatment of tuberculosis.
8. WHO. 2018. Technical Report on critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis. https://www.who.int/tb/publications/2018/WHO_technical_report_concentrations_TB_drug_susceptibility/en/.
9. WHO. 2022. Optimized broth microdilution plate methodology for drug susceptibility testing of *Mycobacterium tuberculosis* complex.
10. KARATUNA O. 2021. Phenotypic Methods Used in Antimicrobial Susceptibility Testing.
11. Escribano I, Rodríguez JC, Llorca B, García-Pachon E, Ruiz M, Royo G. 2007. Importance of the efflux pump systems in the resistance of *Mycobacterium tuberculosis* to fluoroquinolones and linezolid. Chemotherapy 53:397-401.
12. Rupasinghe P, Driesen M, Vereecken J, de Jong BC, Rigouts L. 2023. Re-evaluation of Critical Concentrations of Antituberculosis Fluoroquinolones in the Mycobacteria Growth Indicator Tube 960 System. The International Journal of Mycobacteriology 12:316-323.
13. Köser CU, Georghiou SB, Schön T, Salfinger M. 2021. On the Consequences of Poorly Defined Breakpoints for Rifampin Susceptibility Testing of *Mycobacterium tuberculosis* Complex. J Clin Microbiol 59.
14. Drug-susceptibility testing for *Mycobacterium tuberculosis* complex by ITM BMD or REMA, Internal standard operating procedure, ITM (Version 4).

7.7 Supplementary material

CT No	MFX D14 MIC_{100}	LFX D14 MIC_{100}	MFX D14 $MIC_{classic}$	LFX D14 $MIC_{classic}$	Lineage	FQ S/R?	*gyrA* mutations	*gyrB* mutations
2019-00461	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-00463	2	>2	2	>2	2.2.1	R	*Asp94Gly* (1.0)	-
2019-00465	0.125	0.25	0.125	0.25	4.8	S	-	-
2019-00467	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-00469	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-00469	0.25	0.25	0.125	0.25	2.2.1	S	-	-
2019-00471	>2	>2	>2	>2	2.2.1	R	*Asp94Gly* (1.0)	-
2019-00475	0.125	0.25	0.125	0.25	4.1	S	-	-
2019-00475	0.25	0.5	0.125	0.25	4.1	S	-	-
2019-00477	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-00477	0.25	0.25	0.125	0.25	2.2.1	S	-	-
2019-00479	0.25	0.5	0.25	0.5	2.2.1	S	-	-
2019-00479	0.5	0.5	0.5	0.5	2.2.1	S	-	-
2019-00481	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-00483	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-00485	0.25	0.25	0.25	0.25	2.2.1	S	-	-
2019-00487	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-00489	0.125	0.25	0.125	0.25	4.2.1	S	-	-
2019-01715	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01717	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01721	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2019-01723	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01725	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01727	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01729	0.25	0.5	0.25	0.5	4.3.4.1	S	-	-

2019-01731	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01733	0.25	0.25	0.25	0.25	4.8	S	-	-
2019-01735	0.25	0.5	0.25	0.5	4.8	S	-	-
2019-01737	0.125	0.5	0.125	0.25	4.3.3	S	-	-
2019-01739	0.25	0.5	0.125	0.25	4.3.4.1	S	-	-
2019-01741	0.125	0.25	0.125	0.25	4.1.2.1	S	-	-
2019-01743	0.25	0.5	0.25	0.5	4.3.4.2	S	-	-
2019-01745	0.25	0.5	0.25	0.5	4.8	S	-	-
2019-01747	0.125	0.25	0.125	0.25	4.1.2.1	S	-	-
2019-01749	0.25	1	0.25	1	4.3.3	S	-	-
2019-01751	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01753	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01755	0.25	0.5	0.25	0.5	4.8	S	-	-
2019-01757	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01759	0.125	0.25	0.125	0.25	4.3.3	S	-	-
2019-01759	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01761	0.125	0.25	0.125	0.25	4.8	S	-	-
2019-01765	0.125	0.25	0.125	0.25	4.1.2.1	S	-	-
2019-01767	0.25	0.5	0.25	0.5	4.1.2.1	S	-	-
2019-01769	0.125	0.5	0.125	0.5	4.1.2.1	S	-	-
2019-01771	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01775	0.25	0.5	0.25	0.5	4	S	-	-
2019-01777	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01779	0.5	1	0.5	1	4.3.3	S	-	-
2019-01783	0.5	1	0.5	1	4.3.3	S	-	-
2019-01785	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01789	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01791	0.125	0.25	0.125	0.25	4.3.4.2	S	-	-
2019-01793	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01795	0.125	0.5	0.125	0.5	4.3.3	S	-	-

2019-01797	0.25	1	0.125	0.5	4.3.3	S	-	-
2019-01799	0.25	0.5	0.25	0.5	4.1.2.1	S	-	-
2019-01801	0.125	0.25	0.125	0.25	4.3.3	S	-	-
2019-01803	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01805	0.125	0.25	0.125	0.25	4.8	S	-	-
2019-01809	2	2	2	2	4.3.3	R	*Ala74Ser*, *Ala90Val* (1.0)	*Thr500Asn* (1.0)
2019-01811	0.125	0.25	0.125	0.25	4.8	S	-	-
2019-01813	0.06	0.125	0.06	0.125	4.1	S	-	-
2019-01815	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01819	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-01821	0.125	0.25	0.125	0.25	4.3.4.2	S	-	-
2019-01823	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2019-01827	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01831	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2019-01833	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01835	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2019-01837	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01839	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2019-01843	0.125	0.25	0.125	0.25	4.3.2	S	-	-
2019-01845	0.125	0.25	0.125	0.25	4.8	S	-	-
2019-01845	0.25	0.5	0.25	0.5	4.8	S	-	-
2019-01853	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2019-01857	4	8	4	8	4.3.3	R	*Asp94Tyr* (1.0)	-
2019-02066	0.125	0.25	0.125	0.25	4.1.1.3	S	-	-
2019-02068	0.125	0.25	0.125	0.25	4.1.1	S	-	-
2019-02084	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02086	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2019-02089	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2019-02089	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2019-02090	0.25	0.5	0.25	0.5	2.2.1.1	S	-	-

2019-02680	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02682	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02684	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02688	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2019-02696	0.25	0.25	0.25	0.25	2.2.1	S	-	-
2019-02698	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02702	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02704	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02706	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02710	0.5	2	0.5	2	2.2.1	R	*Asp94Ala* (1.0)	-
2019-02712	2	4	2	4	2.2.1	R	*Asp94Gly* (1.0)	-
2019-02716	0.25	0.25	0.25	0.25	2.2.1	S	-	-
2019-02718	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02724	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02726	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02730	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-02734	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-04829	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-04830	0.25	0.5	0.25	0.5	2.2.1	S	-	-
2019-04833	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2019-04836	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2019-04838	0.25	1	0.25	1	4.3.2.1	S	-	-
2019-04841	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2019-04842	1	2	1	2	2.2.1	R	*Ala90Val* (0.97),*Asp94Tyr* (0.01)	*Glu501Asp* (0.03)
2019-04847	0.25	0.5	0.25	0.5	4.4.1.1	S	-	-
2019-04853	0.5	0.5	0.5	0.5	2.2.2	S	-	-
2019-04859	0.125	0.25	0.125	0.25	4.3.4.2.1	S	-	-
2019-04863	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2019-04865	0.5	1	0.5	1	4.4.1.1	S	-	-
2019-04866	0.25	0.25	0.25	0.25	2.2.1	S	-	-

2019-04879	0.25	0.25	0.25	0.25	4.4.1.1.1	S	-	-
2019-04881	0.25	0.5	0.125	0.25	4.4.1.1	S	-	-
2019-04885	0.125	0.25	0.125	0.25	4.1.1.3	S	-	-
2019-04934	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2020-01235	0.125	0.5	0.125	0.5	3, 1.1	S	-	-
2020-01237	0.06	0.25	0.06	0.25	3	S	-	-
2020-01241	0.06	0.25	0.06	0.25	3, 1.1	S	-	-
2020-01243	0.06	0.25	0.06	0.25	3, 1.1	S	-	-
2020-01245	0.125	0.25	0.125	0.25	3.1.2.1, 1.1	S	-	-
2020-01247	0.06	0.25	0.06	0.25	3, 1.1	S	-	-
2020-01249	0.125	0.25	0.06	0.25	3, 1.1	S	-	-
2020-01261	0.25	0.25	0.125	0.25	2.2.1	S	-	-
2020-01266	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2020-01281	0.25	0.25	0.125	0.25	2.2.1	S	-	-
2020-01283	0.25	0.25	0.125	0.25	2.2.1	S	-	-
2020-03226	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03227	0.25	0.5	0.25	0.5	4.1.2.1	S	-	-
2020-03228	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03231	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03234	1	2	0.5	1	4.3.3	S	-	-
2020-03239	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2020-03242	0.06	0.25	0.06	0.25	4.3.3	S	-	-
2020-03244	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03245	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03246	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2020-03246	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03250	0.25	0.5	0.25	0.5	4.8	S	-	-
2020-03252	0.25	0.5	0.25	0.25	4.1.1.3	S	-	-
2020-03254	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03255	0.06	0.125	0.06	0.125	2.2.1	S	-	-

2020-03256	0.03	0.125	0.03	0.125	2.2.1	S	-	-
2020-03258	0.125	0.5	0.125	0.25	4.3.3	S	-	-
2020-03259	0.125	0.25	0.06	0.25	2.2.1	S	-	-
2020-03260	0.125	0.25	0.125	0.25	4.1.1	S	-	-
2020-03262	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03263	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2020-03264	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03265	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03266	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2020-03267	0.125	0.125	0.125	0.125	4.3.3	S	-	-
2020-03268	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03269	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2020-03270	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03272	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03273	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2020-03275	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03276	0.25	0.25	0.125	0.25	4.1.1.3	S	-	-
2020-03278	0.06	0.25	0.06	0.25	4.3.3	S	-	-
2020-03280	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03281	0.25	0.5	0.25	0.5	4.3.4.2	S	-	-
2020-03282	0.25	0.5	0.25	0.5	4.1.1.3	S	-	-
2020-03284	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03285	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03286	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2020-03287	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03288	0.125	0.25	0.125	0.25	4.3.3	S	-	-
2020-03289	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03290	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03294	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03295	4	8	2	8	4.3.3	R	*Ala74Ser* (1.0), *Ala90Val* (1.0)	-

2020-03297	2	8	2	8	4.3.3	R	*Ala74Ser* (1.0); *Ala90Val* (0.38)	*Ala504Thr* (0.63)
2020-03298	0.5	0.5	0.25	0.5	4.3.2	S	-	-
2020-03299	0.5	1	0.5	1	4.3.3	S	-	-
2020-03300	0.125	0.25	0.125	0.25	4.3.4.2	S	-	-
2020-03302	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03303	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03304	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03305	0.125	0.25	0.125	0.25	4.3.3	S	-	-
2020-03306	>4	>8	>4	>8	4.3.3	R	*Asp94Asn* (1.0)	-
2020-03308	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03309	0.06	0.25	0.06	0.25	4.3.3	S	-	-
2020-03310	0.125	0.25	0.125	0.25	4.8	S	-	-
2020-03311	2	2	2	2	4.8	R	*Asp89Asn* (1.0)	-
2020-03312	0.125	0.25	0.125	0.25	2.2	S	-	-
2020-03314	0.06	0.125	0.06	0.125	4.3.4.2	S	-	-
2020-03315	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03316	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2020-03317	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03319	0.06	0.125	0.06	0.125	4.8	S	-	-
2020-03320	0.25	1	0.25	1	4.3.3	S	-	-
2020-03321	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03322	0.125	0.25	0.125	0.25	4.3.4.1	S	-	-
2020-03323	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03324	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03325	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03326	0.125	0.25	0.125	0.25	4	S	-	-
2020-03329	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03330	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03331	0.25	0.5	0.25	0.5	4.8	S	-	-
2020-03332	0.25	0.5	0.25	0.5	4.3.3	S	-	-

2020-03333	0.25	0.5	0.25	0.5	4.1.1	S	-	-
2020-03335	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03336	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03337	0.5	0.5	0.25	0.25	4	S	-	-
2020-03338	0.125	0.25	0.125	0.25	4.3.3	S	-	-
2020-03339	0.25	0.5	0.125	0.5	4.8	S	-	-
2020-03340	0.125	0.25	0.125	0.25	4.3.2	S	-	-
2020-03342	0.25	0.5	0.25	0.25	4.3.2	S	-	-
2020-03343	0.125	0.25	0.06	0.125	2.2.1	S	-	-
2020-03344	0.125	0.25	0.125	0.25	4.1.1	S	-	-
2020-03347	0.125	0.25	0.125	0.25	4.1.1.3	S	-	-
2020-03348	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03349	0.25	0.5	0.25	0.25	4.3.4.2	S	-	-
2020-03351	0.125	0.25	0.06	0.125	2.2.1	S	-	-
2020-03352	0.25	0.25	0.25	0.25	2.2.1	S	-	-
2020-03353	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2020-03355	0.25	0.5	0.125	0.5	4.1.2.1	S	-	-
2020-03356	0.25	0.5	0.125	0.25	4.8	S	-	-
2020-03358	0.25	0.25	0.25	0.25	4.8	S	-	-
2020-03359	0.25	0.5	0.25	0.5	4.3.4.2	S	-	-
2020-03374	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2020-03381	4	8	4	8	4.3.3	R	*Asp94His* (1.0)	-
2020-03430	4	>8	4	>8	4.3.3	R	*Ala74Ser* (1.0), *Ala90Val* (1.0)	-
2021-00873	0.25	0.25	0.25	0.25	4.8	S	-	-
2021-00876	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00879	0.25	0.5	0.125	0.25	4.8	S	-	-
2021-00885	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00889	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00893	0.125	0.25	0.125	0.25	4.4.1.1	S	-	-
2021-00897	0.25	0.25	0.125	0.25	2.2.1.1	S	-	-

2021-00899	0.25	0.5	0.125	0.25	2.2.1.1	S	-	-
2021-00908	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00915	0.125	0.5	0.125	0.5	4.4.1.1	S	-	-
2021-00918	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00922	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00924	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00925	0.25	0.5	0.25	0.5	4.1.1.3	S	-	-
2021-00927	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-00932	0.25	0.25	0.125	0.25	2.2.1	S	-	-
2021-00936	0.125	0.25	0.125	0.25	4.1.1.3	S	-	-
2021-00939	0.25	0.5	0.25	0.5	4.4.1.1	S	-	-
2021-00942	0.25	0.5	0.25	0.5	4.3.2.1	S	-	-
2021-00944	0.25	0.5	0.25	0.5	2.2.1.1	S	-	-
2021-00945	0.5	1	0.5	1	1.2.2.2	R	*Gly88Ala* (1.0)	-
2021-01208	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-01236	0.125	0.25	0.125	0.25	3.1	S	-	-
2021-01240	0.06	0.25	0.06	0.25	3	S	-	-
2021-01244	0.06	0.25	0.06	0.25	3	S	-	-
2021-01246	0.06	0.25	0.06	0.25	3	S	-	-
2021-01248	0.125	0.25	0.125	0.25	3	S	-	-
2021-01715	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2021-01725	0.25	0.5	0.25	0.5	2.2.1	S	-	-
2021-02299	0.125	0.25	0.125	0.25	4.8	S	-	-
2021-02318	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2021-02320	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2021-02340	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2022-00049	0.125	0.5	0.125	0.5	1.2.2.2	S	-	-
2022-00053	0.125	0.25	0.125	0.25	2.2.1.1	S	-	-
2022-00061	0.25	0.5	0.125	0.25	2.2.1	S	-	-
2022-00101	4	8	4	4	2.2.1	R	*Asp94Gly* (1.0)	-

2022-00240	0.06	0.25	0.06	0.25	3	S	-	-
2022-00266	0.06	0.25	0.06	0.25	3.1	S	-	-
2022-00274	0.06	0.25	0.06	0.25	3.1	S	-	-
2022-00306	0.25	0.5	0.25	0.5	1.1.2	S	-	-
2022-00322	0.125	0.5	0.125	0.5	1.1.2	S	-	-
2022-00354	0.25	0.5	0.25	0.5	1.1.2	S	-	-
2022-00356	0.25	0.5	0.25	0.5	1.1.2	S	-	-
2022-00564	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2022-00566	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2022-01169	0.06	0.25	0.06	0.25	3.1	S	-	-
2023-00337	0.125	0.25	0.06	0.25	4.3.3	S	-	-
2023-00347	4	8	4	8	4.3.3	R	*Asp94Asn* (1.0)	-
2023-00397	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2023-00408	4	4	4	4	2.2.1	R	*Asp94Asn* (1.0)	-
2023-00457	0.25	0.5	0.125	0.5	2.2.1	S	-	-
2023-00549	0.125	0.5	0.06	0.25	3.1	S	-	-
2023-00569	0.125	0.5	0.06	0.25	3	S	-	-
2023-00571	0.125	0.25	0.125	0.25	3	S	-	-
2023-00575	0.06	0.25	0.06	0.25	3	S	-	-
2023-00577	0.25	0.5	0.06	0.25	3	S	-	-
2023-00579	0.06	0.25	0.06	0.25	3.1.2.1	S	-	-
2023-00581	0.125	0.5	0.06	0.25	3.1.2.1	S	-	-
2023-00593	0.125	0.5	0.06	0.25	3.1	S	-	-
2023-00595	0.125	0.5	0.06	0.25	3	S	-	-
2023-00599	0.25	0.5	0.25	0.5	2.2.1	R	-	*Thr500Asn* (0.11)
2023-00607	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2023-00615	0.25	0.5	0.25	0.5	1.1.2	S	-	-
2023-00639	0.125	0.25	0.06	0.25	3.1.2	S	-	-
2023-00732	4	8	2	8	4.3.3	R	*Asp94Tyr* (1.0)	-
2023-00733	invalid	invalid	invalid	invalid	4.3.3	R	*Asp94Asn* (1.0)	-

2023-00734	>4	>8	>4	>8	4.3.3	R	*Asp94Asn* (1.0)	-
2023-00735	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2023-00737	0.25	1	0.25	1	4.3.3	S	-	-
2023-00740	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2023-00741	0.125	0.5	0.125	0.5	4.3.3	S	-	-
2023-00744	4	8	4	8	4.3.3	R	*Asp94His* (1.0)	-
2023-00748	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2023-00749	0.125	0.25	0.125	0.25	2.2.1	S	-	-
2023-00750	0.125	0.5	0.125	0.25	2.2.1	S	-	-
2023-00762	0.25	0.5	0.25	0.25	2.2.1	S	-	-
2023-00766	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2023-00767	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2023-00771	2	8	2	8	4.3.3	R	*Ala74Ser* (1.0), *Ala90Val* (0.1)	-
2023-00772	4	8	4	8	4.3.3	R	*Ala74Ser* (1.0)	*Thr500Asn* (0.17)
2023-00773	4	8	4	4	4.3.3	R	*Ala74Ser* (1.0)	*Thr500Asn* (1.0)
2023-00774	4	8	4	4	4.3.3	R	*Ala74Ser* (1.0)	*Thr500Asn* (1.0)
2023-00781	0.25	0.5	0.125	0.5	4.3.3	S	-	-
2023-00783	>4	>8	>4	>8	4.3.3	R	*Asp94Asn* (1.0)	-
2023-00784	>4	>8	>4	>8	4.3.3	R	*Asp94Asn* (1.0)	-
2023-00786	>4	>8	>4	>8	4.3.3	R	*Asp94Asn* (1.0)	-
2023-00789	0.125	0.5	0.125	0.25	4.8	S	-	-
2023-00791	0.125	0.25	0.125	0.25	4.8	S	-	-
2023-00813	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2023-01162	>4	>8	>4	>8	4.3.3	R	*Asp94Asn* (1.0)	-
2023-01181	0.25	0.5	0.25	0.5	4.3.3	S	-	-
2023-01366	0.125	0.5	0.125	0.5	4.9	S	-	-
2023-01366	0.06	0.25	0.06	0.25	4.9	S	-	-
2023-01366	0.06	0.25	0.06	0.25	4.9	S	-	-
2023-01372	0.25	0.5	0.25	0.5	2.2.1	S	-	-
2023-01375	0.06	0.25	0.06	0.25	3.1.2	S	-	-

2023-01383	0.5	2	0.5	2	3.1.2	R	*Ala90Val* (1.0)	-
2023-01604	0.25	0.5	0.125	0.25	2.2.1	S	-	-
2023-01608	4	8	4	8	2.2.1	R	*Asp94Gly* (1.0)	-
2023-01615	0.125	0.5	0.125	0.5	2.2.1	S	-	-
2023-01688	0.5	1	0.25	0.5	2.2.1	S	-	-
2023-01720	0.25	0.5	0.125	0.25	2.2.1	S	-	-
2023-01981	1	0.5	1	0.5	3.1.2.1	R	-	*Glu501Asp* (1.0)
2023-01983	2	8	2	8	3	R	*Asp94Gly* (0.98)	-
2023-02138	0.25	0.5	0.25	0.5	4.3.2.1	S	-	-
2023-02138	0.125	0.5	0.125	0.25	4.3.2.1	S	-	-
2023-02167	0.25	0.5	0.25	0.5	4.3.4.2.1	S	-	-
2023-02171	2	4	2	4	2.2.1	R	*Ala90Val* (0.86), *Asp94Ala* (0.1)	-
2023-02198	0.5	1	0.5	0.5	2.2.1	S	-	-
2023-02199	0.25	0.5	0.25	0.5	2.2.1.1	S	-	-

Table 7.S1: Isolates used for validation of fluoroquinolone MIC testing using BMD

CT No	CFZ D14 MIC_{100}	BDQ D14 MIC_{100}	CFZ D14 $MIC_{classic}$	BDQ D14 $MIC_{classic}$	Lineage	CFZ/BDQ S/R?	*mmpL5* mutations	*mmps5* mutations	*mmpr5 (rv0678)* mutations	*atpE* mutations	*pepQ* mutations	*Rv1979c* mutations
2012-01331	1	.5	1	0.5	1.1.3	R	-	-	*c.343_343del,*	-	-	-
2013-01327	>4	02	>4	2	1.1.3	R	-	-	*c.343_343del*	-	-	-
2013-02481	4	0.5	4	0.5	1.1.3	R	-	-	*c.343_343del*	-	-	-
2019-00461	0.5	invalid	0.5	invalid	2.2.1	Cfz S	-	-	-	-	-	-
2019-00463	0.125	0.25	0.125	0.25	2.2.1	S	-	-	-	-	-	-
2019-00465	0.5	0.125	0.5	0.125	4.8	S	-	-	-	-	-	-
2019-00467	0.06	invalid	0.06	invalid	4.3.3	Cfz S	-	-	-	-	-	-
2019-00469	0.25	invalid	0.25	invalid	2.2.1	Cfz S	-	-	-	-	-	-
2019-00469	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-00471	0.25	0.125	0.25	0.125	2.2.1	S	-	-	-	-	-	-
2019-00475	0.5	invalid	0.5	invalid	4.1	Cfz S	-	-	-	-	-	-
2019-00477	0.25	invalid	0.25	invalid	2.2.1	Cfz S	-	-	-	-	-	-
2019-00477	0.25	0.03	0.125	0.03	2.2.1	S	-	-	-	-	-	-
2019-00479	0.25	invalid	0.25	invalid	2.2.1	Cfz S	-	-	-	-	-	-
2019-00479	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-00481	0.25	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2019-00483	0.5	0.25	0.5	0.25	4.3.3	S	-	-	-	-	-	-
2019-00485	0.25	0.125	0.25	0.125	2.2.1	S	-	-	-	-	-	-
2019-00487	0.25	0.25	0.25	0.25	2.2.1	S	-	-	-	-	-	-
2019-00489	0.5	0.125	0.5	0.125	4.2.1	S	-	-	-	-	-	-
2019-01715	0.25	0.06	0.25	0.015	4.3.3	S	-	-	-	-	-	-
2019-01717	0.125	0.03	0.125	0.03	4.3.3	S	-	-	-	-	-	-
2019-01721	0.25	0.03	0.125	0.03	4.3.3	S	-	-	-	-	-	-
2019-01723	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01727	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2019-01731	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2019-01733	0.25	0.03	0.25	0.03	4.8	S	-	-	-	-	-	-
2019-01735	0.5	0.06	0.25	0.06	4.8	S	-	-	-	-	-	-
2019-01737	0.5	<=0.008	0.125	<=0.008	4.3.3	S	-	-	-	-	-	-
2019-01741	0.25	0.03	0.25	0.03	4.1.2.1	S	-	-	-	-	-	-
2019-01743	0.25	0.03	0.25	0.03	4.3.4.2	S	-	-	-	-	-	-
2019-01745	0.25	0.015	0.25	0.015	4.8	S	-	-	-	-	-	-

2019-01747	0.125	0.015	0.125	0.015	4.1.2.1	S	-	-	-	-	-	-
2019-01749	0.5	0.125	0.5	0.125	4.3.3	S	-	-	-	-	-	-
2019-01751	0.5	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01753	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2019-01755	0.25	0.03	0.25	0.03	4.8	S	-	-	-	-	-	-
2019-01757	0.5	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01759	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01759	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2019-01761	0.25	0.06	0.25	0.06	4.8	S	-	-	-	-	-	-
2019-01765	0.25	0.03	0.25	0.03	4.1.2.1	S	-	-	-	-	-	-
2019-01769	0.5	0.03	0.5	0.03	4.1.2.1	S	-	-	-	-	-	-
2019-01771	0.125	0.06	0.125	0.06	4.3.3	S	-	-	-	-	-	-
2019-01777	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01779	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2019-01783	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2019-01785	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01787	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2019-01789	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01791	0.125	0.03	0.125	0.03	4.3.4.2	S	-	-	-	-	-	-
2019-01793	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01795	0.125	0.03	0.125	0.03	4.3.3	S	-	-	-	-	-	-
2019-01797	0.5	0.25	0.5	0.25	4.3.3	S	-	-	-	-	-	-
2019-01797	0.5	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01799	0.5	0.06	0.5	0.06	4.1.2.1	S	-	-	-	-	-	-
2019-01803	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2019-01805	0.25	0.03	0.25	0.03	4.8	S	-	-	-	-	-	-
2019-01809	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01811	0.25	0.015	0.25	0.015	4.8	S	-	-	-	-	-	-
2019-01815	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2019-01819	0.5	0.06	0.5	0.06	2.2.1	S	-	-	-	-	-	-
2019-01821	0.25	0.03	0.25	0.03	4.3.4.2	S	-	-	-	-	-	-
2019-01823	0.5	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01827	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2019-01831	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2019-01833	0.25	0.125	0.25	0.125	4.3.3	S	-	-	-	-	-	-

2019-01835	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2019-01837	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01837	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2019-01839	0.5	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-01843	0.125	0.03	0.125	0.03	4.3.2	S	-	-	-	-	-	-
2019-01845	0.25	0.03	0.25	0.015	4.8	S	-	-	-	-	-	-
2019-01845	0.25	0.125	0.25	0.06	4.8	S	-	-	-	-	-	-
2019-01849	0.5	0.03	0.25	0.015	4.3.4.2	S	-	-	-	-	-	-
2019-01853	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2019-01857	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2019-02066	0.25	0.06	0.25	0.06	4.1.1.3	S	-	-	-	-	-	-
2019-02084	0.125	0.06	0.125	0.06	2.2.1	S	-	-	-	-	-	-
2019-02680	0.125	0.06	0.125	0.06	2.2.1	S	-	-	-	-	-	-
2019-02682	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-02684	0.25	0.125	0.25	0.125	2.2.1	S	-	-	-	-	-	-
2019-02696	0.5	invalid	0.5	invalid	2.2.1	Cfz S	-	-	-	-	-	-
2019-02698	0.25	0.015	0.25	0.015	2.2.1	S	-	-	-	-	-	-
2019-02704	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-02706	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-02710	0.125	0.03	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2019-02712	1	0.03	0.5	0.03	2.2.1	S	-	-	-	-	-	-
2019-02718	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-02724	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-02726	0.5	0.03	0.125	0.03	2.2.1	S	-	-	-	-	-	-
2019-02730	0.25	0.03	0.125	0.03	2.2.1	S	-	-	-	-	-	-
2019-02734	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2019-04829	0.25	0.25	0.25	0.25	2.2.1	S	-	-	-	-	-	-
2019-04830	0.25	0.015	0.25	0.015	2.2.1	S	-	-	-	-	-	-
2019-04842	0.25	0.015	0.125	0.008	2.2.1	S	-	-	-	-	-	-
2019-04847	0.25	invalid	0.25	invalid	4.4.1.1	Cfz S	-	-	-	-	-	-
2019-04859	0.5	0.25	0.5	0.25	4.3.4.2.1	S	-	-	-	-	-	-
2019-04863	0.125	invalid	0.125	invalid	2.2.1	Cfz S	-	-	-	-	-	-
2019-04866	0.5	0.03	0.5	0.03	2.2.1	S	-	-	-	-	-	-
2019-04879	0.5	0.125	0.5	0.125	4.4.1.1.1	S	-	-	-	-	-	-
2019-04881	0.25	0.015	0.125	0.015	4.4.1.1	S	-	-	-	-	-	-

2019-04885	0.125	0.03	0.125	0.03	4.1.1.3	S	-	-	-	-	-	-
2019-04934	0.25	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2020-00016	NA	2	NA	2	NA	Bdq R	-	-	-	*Ala63Pro*	-	-
2020-00024	2	NA	2	NA	NA	Cfz R	-	-	*Ins140*	-	-	-
2020-01237	0.25	invalid	0.25	invalid	3	Cfz S	-	-	-	-	-	-
2020-01241	0.5	invalid	0.5	invalid	3;La1.1	Cfz S	-	-	-	-	-	-
2020-01243	4	2	4	2	3;La1.1	R	-	-	*Leu117Arg (1.0)*	-	-	-
2020-01249	0.5	0.06	0.25	0.06	3;La1.1	S	-	-	-	-	-	-
2020-01261	1	0.06	0.5	0.03	2.2.1	S	-	-	-	-	-	-
2020-01266	1	0.125	0.5	0.125	2.2.1	S	-	-	-	-	-	-
2020-01281	0.5	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2020-01283	0.25	0.015	0.25	0.015	2.2.1	S	-	-	-	-	-	-
2020-03226	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03228	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03231	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2020-03234	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03239	1	0.06	1	0.06	4.3.3	S	-	-	-	-	-	-
2020-03242	0.25	0.015	0.25	0.015	4.3.3	S	-	-	-	-	-	-
2020-03244	1	0.125	1	0.06	4.3.3	S	-	-	-	-	-	-
2020-03245	0.5	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03246	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2020-03246	1	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03250	0.5	0.06	0.5	0.06	4.8	S	-	-	-	-	-	-
2020-03252	0.5	0.06	0.25	0.06	4.1.1.3	S	-	-	-	-	-	-
2020-03254	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03255	0.125	0.015	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2020-03256	0.125	0.015	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2020-03258	0.25	0.03	0.125	0.03	4.3.3	S	-	-	-	-	-	-
2020-03259	0.5	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2020-03260	0.125	0.015	0.06	0.015	4.1.1	S	-	-	-	-	-	-
2020-03262	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03264	0.25	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03265	0.5	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03266	0.5	0.06	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03267	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-

2020-03268	0.5	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03270	0.25	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03272	0.25	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03273	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03275	1	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03276	0.5	0.03	0.25	0.03	4.1.1.3	S	-	-	-	-	-	-
2020-03278	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03280	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03281	0.5	0.03	0.25	0.03	4.3.4.2	S	-	-	-	-	-	-
2020-03282	0.5	0.03	0.5	0.03	4.1.1.3	S	-	-	-	-	-	-
2020-03284	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03285	0.5	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03286	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03287	1	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2020-03288	0.25	0.03	0.125	0.03	4.3.3	S	-	-	-	-	-	-
2020-03289	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03290	0.25	0.06	0.125	0.06	4.3.3	S	-	-	-	-	-	-
2020-03294	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03295	0.25	0.03	0.125	0.015	4.3.3	S	-	-	-	-	-	-
2020-03297	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03298	1	0.06	0.125	0.06	4.3.2	S	-	-	-	-	-	-
2020-03300	0.25	0.03	0.25	0.015	4.3.4.2	S	-	-	-	-	-	-
2020-03302	0.5	0.125	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2020-03303	0.5	0.125	0.5	0.125	4.3.3	S	-	-	-	-	-	-
2020-03304	0.25	0.015	0.25	0.015	4.3.3	S	-	-	-	-	-	-
2020-03305	0.125	0.03	0.125	0.03	4.3.3	S	-	-	-	-	-	-
2020-03306	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03308	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03309	0.125	0.015	0.125	0.015	4.3.3	S	-	-	-	-	-	-
2020-03310	0.5	0.06	0.5	0.06	4.8	S	-	-	-	-	-	-
2020-03311	0.25	0.03	0.25	0.03	4.8	S	-	-	-	-	-	-
2020-03312	0.125	0.015	0.125	0.015	2.2	S	-	-	-	-	-	-
2020-03314	0.125	0.015	0.125	0.015	4.3.4.2	S	-	-	-	-	-	-
2020-03315	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2020-03316	0.5	0.06	0.5	0.03	4.3.3	S	-	-	-	-	-	-

2020-03317	1	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03319	0.06	<0.008	0.06	<0.008	4.8	S	-	-	-	-	-	-
2020-03320	0.5	0.125	0.5	0.125	4.3.3	S	-	-	-	-	-	-
2020-03321	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03322	0.25	0.03	0.25	0.03	4.3.4.1	S	-	-	-	-	-	-
2020-03323	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03325	0.5	0.125	0.5	0.125	4.3.3	S	-	-	-	-	-	-
2020-03329	0.25	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03330	0.5	0.125	0.5	0.125	4.3.3	S	-	-	-	-	-	-
2020-03331	0.25	0.03	0.25	0.03	4.8	S	-	-	-	-	-	-
2020-03332	0.5	0.03	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2020-03333	0.5	0.03	0.5	0.03	4.1.1	S	-	-	-	-	-	-
2020-03335	0.25	0.015	0.25	0.015	4.3.3	S	-	-	-	-	-	-
2020-03336	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2020-03338	0.125	0.03	0.125	0.015	4.3.3	S	-	-	-	-	-	-
2020-03339	0.5	0.125	0.25	0.125	4.8	S	-	-	-	-	-	-
2020-03340	0.25	0.03	0.25	0.03	4.3.2	S	-	-	-	-	-	-
2020-03342	0.125	0.03	0.125	0.03	4.3.2	S	-	-	-	-	-	-
2020-03344	0.125	0.03	0.125	0.03	4.1.1	S	-	-	-	-	-	-
2020-03347	0.125	0.03	0.125	0.03	4.1.1.3	S	-	-	-	-	-	-
2020-03348	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2020-03349	0.25	0.03	0.25	0.03	4.3.4.2	S	-	-	-	-	-	-
2020-03352	0.25	0.015	0.25	0.015	2.2.1	S	-	-	-	-	-	-
2020-03353	0.5	0.03	0.5	0.015	4.3.3	S	-	-	-	-	-	-
2020-03355	0.5	0.06	0.25	0.06	4.1.2.1	S	-	-	-	-	-	-
2020-03356	0.5	0.125	0.25	0.125	4.8	S	-	-	-	-	-	-
2020-03358	0.25	0.03	0.25	0.03	4.8	S	-	-	-	-	-	-
2020-03359	0.5	0.06	0.25	0.06	4.3.4.2	S	-	-	-	-	-	-
2020-03374	0.5	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03381	0.25	0.03	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2020-03430	1	0.06	1	0.03	4.3.3	S	-	-	-	-	-	-
2021-00873	0.25	0.015	0.25	0.015	4.8	S	-	-	-	-	-	-
2021-00876	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2021-00879	0.06	0.015	0.06	0.015	4.8	S	-	-	-	-	-	-
2021-00885	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-

2021-00889	0.25	0.125	0.25	0.125	2.2.1	S	-	-	-	-	-	-
2021-00893	0.25	0.06	0.25	0.06	4.4.1.1	S	-	-	-	-	-	-
2021-00908	0.5	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2021-00915	0.125	0.015	0.125	0.015	4.4.1.1	S	-	-	-	-	-	-
2021-00922	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2021-00924	0.25	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2021-00925	0.5	0.06	0.25	0.06	4.1.1.3	S	-	-	-	-	-	-
2021-00927	0.125	<0.008	0.125	<0.008	2.2.1	S	-	-	-	-	-	-
2021-00932	0.25	<=0.008	0.25	<=0.008	2.2.1	S	-	-	-	-	-	-
2021-00936	0.25	0.06	0.125	0.06	4.1.1.3	S	-	-	-	-	-	-
2021-00939	0.25	0.03	0.25	0.03	4.4.1.1	S	-	-	-	-	-	-
2021-00942	0.125	0.06	0.125	0.06	4.3.2.1	S	-	-	-	-	-	-
2021-01200	1	0.5	NA	0.5	3	R	-	-	*DelC frameshiift**	-	-	-
2021-01208	0.25	0.015	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2021-01236	0.5	0.03	0.5	0.03	3.1	S	-	-	-	-	-	-
2021-01244	0.25	0.06	0.25	0.06	3	S	-	-	-	-	-	-
2021-01725	0.5	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2021-02299	0.25	0.03	0.25	0.03	4.8	S	-	-	-	-	-	-
2021-02318	1	0.06	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2021-02320	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2021-02340	0.125	0.015	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2022-00053	0.25	0.015	0.25	0.015	2.2.1.1	S	-	-	-	-	-	-
2022-00061	0.5	0.06	0.5	0.03	2.2.1	S	-	-	-	-	-	-
2022-00101	0.125	0.015	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2022-00240	0.5	0.06	0.5	0.06	3	S	-	-	-	-	-	-
2022-00266	0.5	0.125	0.5	0.125	3.1	S	-	-	-	-	-	-
2022-00274	0.25	0.06	0.25	0.03	3.1	S	-	-	-	-	-	-
2022-00564	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2022-00566	0.25	0.03	0.25	0.015	2.2.1	S	-	-	-	-	-	-
2022-01169	0.125	0.03	0.125	0.03	3.1	S	-	-	-	-	-	-
2023-00397	0.125	0.03	0.125	0.03	2.2.1	S	-	-	-	-	-	-
2023-00457	0.5	0.125	0.25	0.125	2.2.1	S	-	-	-	-	-	-
2023-00552	2	0.5	2	0.5	3.1.1	R	-	-	*DelG frameshiift**	-	-	-
2023-00571	0.5	0.03	0.5	0.015	3	S	-	-	-	-	-	-
2023-00575	0.25	0.25	0.25	0.25	3	S	-	-	-	-	-	-

2023-00577	1	0.015	0.5	0.015	3	S	-	-	-	-	-	-
2023-00579	0.25	0.125	0.25	0.125	3.1.2.1	S	-	-	-	-	-	-
2023-00581	0.5	0.25	0.5	0.25	3.1.2.1	S	-	-	-	-	-	-
2023-00593	0.5	0.125	0.25	0.125	3.1	S	-	-	-	-	-	-
2023-00595	0.25	0.125	0.125	0.125	3	S	-	-	-	-	-	-
2023-00599	0.5	0.015	0.5	0.015	2.2.1	S	-	-	-	-	-	-
2023-00607	0.5	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2023-00639	1	0.03	0.5	0.03	3.1.2	S	-	-	-	-	-	-
2023-00732	1	0.03	1	0.03	4.3.3	S	-	-	-	-	-	-
2023-00733	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2023-00734	1	0.25	0.5	0.25	4.3.3	S	-	-	-	-	-	-
2023-00735	1	0.06	0.5	0.03	4.3.3	S	-	-	-	-	-	-
2023-00737	1	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2023-00740	0.5	0.125	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2023-00741	0.5	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2023-00744	0.5	0.125	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2023-00748	0.25	0.03	0.25	0.03	2.2.1	S	-	-	-	-	-	-
2023-00749	0.25	0.03	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2023-00750	0.5	0.015	0.5	0.015	2.2.1	S	-	-	-	-	-	-
2023-00762	0.5	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2023-00766	0.5	0.125	0.5	0.125	4.3.3	S	-	-	-	-	-	-
2023-00767	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2023-00771	0.25	0.125	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2023-00772	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2023-00773	0.5	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2023-00774	0.5	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2023-00780	0.5	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-
2023-00781	1	0.125	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2023-00783	1	0.06	0.5	0.06	4.3.3	S	-	-	-	-	-	-
2023-00784	0.5	0.125	0.25	0.125	4.3.3	S	-	-	-	-	-	-
2023-00786	1	0.125	0.5	0.125	4.3.3	S	-	-	-	-	-	-
2023-00789	0.25	0.06	0.125	0.06	4.8	S	-	-	-	-	-	-
2023-00791	1	0.06	1	0.06	4.8	S	-	-	-	-	-	-
2023-00813	0.25	0.06	0.25	0.03	4.3.3	S	-	-	-	-	-	-
2023-01162	0.25	0.06	0.25	0.06	4.3.3	S	-	-	-	-	-	-

2023-01181	0.25	0.06	0.125	0.06	4.3.3	S	-	-	-	-	-	-
2023-01366	0.25	<=0.008	0.125	<=0.008	4.9	S	-	-	-	-	-	-
2023-01366	0.06	0.015	0.03	0.015	4.9	S	-	-	-	-	-	-
2023-01366	0.06	0.015	0.06	0.015	4.9	S	-	-	-	-	-	-
2023-01372	0.25	0.125	0.25	0.125	2.2.1	S	-	-	-	-	-	-
2023-01375	0.25	0.03	0.25	0.03	3.1.2	S	-	-	-	-	-	-
2023-01383	0.25	0.06	0.25	0.06	3.1.2	S	-	-	-	-	-	-
2023-01394	2	1	2	0.5	2	R	-	-	*DelC frameshiift**	-	-	-
2023-01604	0.25	0.03	0.125	0.03	2.2.1	S	-	-	-	-	-	-
2023-01615	0.5	0.03	0.5	0.03	2.2.1	S	-	-	-	-	-	-
2023-01688	1	0.06	0.25	0.06	2.2.1	S	-	-	-	-	-	-
2023-01720	0.25	0.06	0.125	0.03	2.2.1	S	-	-	-	-	-	-
2023-01981	0.25	0.06	0.125	0.03	3.1.2.1	S	-	-	-	-	-	-
2023-02138	0.5	0.03	0.25	0.03	4.3.2.1	S	-	-	-	-	-	-
2023-02138	0.25	0.03	0.25	0.03	4.3.2.1	S	-	-	-	-	-	-
2023-02167	0.5	0.06	0.5	0.06	4.3.4.2.1	S	-	-	-	-	-	-
2023-02171	0.5	0.03	0.5	0.03	2.2.1	S	-	-	-	-	-	-
2023-02198	1	0.06	0.5	0.03	2.2.1	S	-	-	-	-	-	-
2023-02892	2	0.5	2	0.5	2	R	-	-	*DelC frameshiift**	-	-	-

Table 7.S2: Isolates used for validation of bedaquiline and clofazimine MIC testing using BMD

*deeplex data

CT No	LZD D14 MIC_{100}	LZD D14 $MIC_{classic}$	Lineage	LZD S/R?	*rplC* mutations	*rrL* mutations
2019-00461	0.5	0.5	2.2.1	S	-	-
2019-00463	0.5	0.5	2.2.1	S	-	-
2019-00465	0.5	0.5	4.8	S	-	-
2019-00467	1	1	4.3.3	S	-	-
2019-00469	0.5	0.5	2.2.1	S	-	-
2019-00469	0.5	0.5	2.2.1	S	-	-
2019-00471	0.5	0.5	2.2.1	S	-	-
2019-00477	0.5	0.5	2.2.1	S	-	-
2019-00477	0.5	0.5	2.2.1	S	-	-
2019-00481	0.5	0.5	2.2.1	S	-	-
2019-00483	0.5	0.5	4.3.3	S	-	-
2019-00485	0.5	0.5	2.2.1	S	-	-
2019-00487	0.5	0.5	2.2.1	S	-	-
2019-00489	0.5	0.5	4.2.1	S	-	-
2019-01715	1	1	4.3.3	S	-	-
2019-01717	0.5	0.5	4.3.3	S	-	-
2019-01721	0.5	0.5	4.3.3	S	-	-
2019-01723	1	1	4.3.3	S	-	-
2019-01725	1	1	4.3.3	S	-	-
2019-01729	0.5	0.5	4.3.4.1	S	-	-
2019-01731	0.5	0.5	4.3.3	S	-	-
2019-01733	0.5	0.5	4.8	S	-	-
2019-01735	1	0.5	4.8	S	-	-
2019-01737	0.5	0.5	4.3.3	S	-	-
2019-01739	0.5	0.25	4.3.4.1	S	-	-
2019-01741	0.5	0.5	4.1.2.1	S	-	-
2019-01743	0.5	0.5	4.3.4.2	S	-	-
2019-01745	0.5	0.5	4.8	S	-	-
2019-01747	0.25	0.25	4.1.2.1	S	-	-
2019-01749	1	1	4.3.3	S	-	-
2019-01751	1	1	4.3.3	S	-	-
2019-01753	1	1	4.3.3	S	-	-

2019-01755	0.5	0.5	4.8	S	-	-
2019-01757	2	1	4.3.3	S	-	-
2019-01759	0.5	0.5	4.3.3	S	-	-
2019-01759	0.5	0.5	4.3.3	S	-	-
2019-01765	0.25	0.25	4.1.2.1	S	-	-
2019-01767	0.5	0.5	4.1.2.1	S	-	-
2019-01769	1	1	4.1.2.1	S	-	-
2019-01771	0.5	0.5	4.3.3	S	-	-
2019-01775	0.5	0.5	4	S	-	-
2019-01777	1	1	4.3.3	S	-	-
2019-01783	1	1	4.3.3	S	-	-
2019-01785	0.5	0.5	4.3.3	S	-	-
2019-01787	1	1	4.3.3	S	-	-
2019-01789	0.5	0.5	4.3.3	S	-	-
2019-01791	0.5	0.5	4.3.4.2	S	-	-
2019-01793	1	1	4.3.3	S	-	-
2019-01795	1	0.5	4.3.3	S	-	-
2019-01797	1	1	4.3.3	S	-	-
2019-01797	0.5	0.5	4.3.3	S	-	-
2019-01799	0.5	0.5	4.1.2.1	S	-	-
2019-01801	0.5	0.5	4.3.3	S	-	-
2019-01803	1	1	4.3.3	S	-	-
2019-01805	0.5	0.5	4.8	S	-	-
2019-01809	0.5	0.5	4.3.3	S	-	-
2019-01811	1	1	4.8	S	-	-
2019-01813	0.5	0.5	4.1	S	-	-
2019-01819	1	1	2.2.1	S	-	-
2019-01821	0.5	0.5	4.3.4.2	S	-	-
2019-01823	0.5	0.5	4.3.3	S	-	-
2019-01827	1	1	4.3.3	S	-	-
2019-01831	1	0.5	4.3.3	S	-	-
2019-01833	2	2	4.3.3	S	-	-
2019-01837	0.25	0.25	4.3.3	S	-	-
2019-01837	1	1	4.3.3	S	-	-
2019-01839	1	1	4.3.3	S	-	-

2019-01843	0.5	0.5	4.3.2	S	-	-
2019-01853	0.5	0.5	4.3.3	S	-	-
2019-01857	0.5	0.5	4.3.3	S	-	-
2019-02066	0.5	0.5	4.1.1.3	S	-	-
2019-02068	0.5	0.5	4.1.1	S	-	-
2019-02084	0.5	0.5	2.2.1	S	-	-
2019-02086	0.5	0.5	2.2.1.1	S	-	-
2019-02089	0.5	0.5	2.2.1.1	S	-	-
2019-02089	0.5	0.5	2.2.1.1	S	-	-
2019-02090	1	1	2.2.1.1	S	-	-
2019-02680	1	1	2.2.1	S	-	-
2019-02682	1	0.5	2.2.1	S	-	-
2019-02688	0.25	0.25	2.2.1.1	S	-	-
2019-02688	1	0.25	2.2.1.1	S	-	-
2019-02696	0.5	0.5	2.2.1	S	-	-
2019-02698	0.5	0.5	2.2.1	S	-	-
2019-02704	0.5	0.5	2.2.1	S	-	-
2019-02706	1	1	2.2.1	S	-	-
2019-02710	0.25	0.25	2.2.1	S	-	-
2019-02712	1	0.5	2.2.1	S	-	-
2019-02716	1	1	2.2.1	S	-	-
2019-02718	0.5	0.5	2.2.1	S	-	-
2019-02724	0.5	0.5	2.2.1	S	-	-
2019-02726	0.5	0.5	2.2.1	S	-	-
2019-02730	1	0.5	2.2.1	S	-	-
2019-04829	0.5	0.5	2.2.1	S	-	-
2019-04830	1	0.5	2.2.1	S	-	-
2019-04833	1	1	2.2.1.1	S	-	-
2019-04836	1	1	2.2.1.1	S	-	-
2019-04838	1	1	4.3.2.1	S	-	-
2019-04842	0.5	0.5	2.2.1	S	-	-
2019-04847	0.5	0.5	4.4.1.1	S	-	-
2019-04853	2	2	2.2.2	S	-	-
2019-04859	0.5	0.5	4.3.4.2.1	S	-	-
2019-04863	0.5	0.5	2.2.1	S	-	-

2019-04865	0.5	0.5	4.4.1.1	S	-	-
2019-04866	0.5	0.5	2.2.1	S	-	-
2019-04879	0.5	0.5	4.4.1.1.1	S	-	-
2019-04881	1	0.5	4.4.1.1	S	-	-
2019-04885	0.25	0.25	4.1.1.3	S	-	-
2019-04934	0.5	0.5	2.2.1	S	-	-
2020-00013	>2	>2	4	R	-	*Gly2061Thr*
2020-00027	>2	>2	4	R	-	*Gly2061Thr*
2020-01237	0.5	0.5	3	S	-	-
2020-01241	0.5	0.5	3;La1.1	S	-	-
2020-01245	0.5	0.5	3.1.2.1;La1.1	S	-	-
2020-01261	0.5	0.5	2.2.1	S	-	-
2020-01266	0.5	0.5	2.2.1	S	-	-
2020-01281	0.5	0.5	2.2.1	S	-	-
2020-01283	0.5	0.5	2.2.1	S	-	-
2020-03226	0.5	0.5	4.3.3	S	-	-
2020-03227	1	0.5	4.1.2.1	S	-	-
2020-03228	1	1	4.3.3	S	-	-
2020-03231	0.5	0.5	4.3.3	S	-	-
2020-03234	0.5	0.5	4.3.3	S	-	-
2020-03239	1	1	4.3.3	S	-	-
2020-03242	0.25	0.25	4.3.3	S	-	-
2020-03244	0.5	0.5	4.3.3	S	-	-
2020-03245	0.5	0.5	4.3.3	S	-	-
2020-03246	0.5	0.5	4.3.3	S	-	-
2020-03246	1	0.5	4.3.3	S	-	-
2020-03250	0.5	0.5	4.8	S	-	-
2020-03252	0.5	0.5	4.1.1.3	S	-	-
2020-03254	0.5	0.5	4.3.3	S	-	-
2020-03256	0.5	0.5	2.2.1	S	-	-
2020-03258	0.5	0.25	4.3.3	S	-	-
2020-03260	0.25	0.25	4.1.1	S	-	-
2020-03262	0.5	0.5	4.3.3	S	-	-
2020-03263	0.5	0.5	2.2.1	S	-	-
2020-03264	1	0.5	4.3.3	S	-	-

2020-03265	1	0.5	4.3.3	S	-	-
2020-03266	1	1	4.3.3	S	-	-
2020-03268	0.5	0.5	4.3.3	S	-	-
2020-03270	1	0.5	4.3.3	S	-	-
2020-03272	0.5	0.5	4.3.3	S	-	-
2020-03273	1	1	4.3.3	S	-	-
2020-03275	0.5	0.5	4.3.3	S	-	-
2020-03276	0.5	0.5	4.1.1.3	S	-	-
2020-03278	0.25	0.25	4.3.3	S	-	-
2020-03280	0.5	0.5	4.3.3	S	-	-
2020-03281	0.5	0.5	4.3.4.2	S	-	-
2020-03282	0.5	0.5	4.1.1.3	S	-	-
2020-03284	1	1	4.3.3	S	-	-
2020-03285	0.5	0.5	4.3.3	S	-	-
2020-03286	1	1	4.3.3	S	-	-
2020-03287	1	0.5	4.3.3	S	-	-
2020-03288	0.5	0.5	4.3.3	S	-	-
2020-03290	0.5	0.5	4.3.3	S	-	-
2020-03294	0.5	0.5	4.3.3	S	-	-
2020-03295	0.5	0.5	4.3.3	S	-	-
2020-03297	0.5	0.5	4.3.3	S	-	-
2020-03299	1	0.5	4.3.3	S	-	-
2020-03300	0.5	0.5	4.3.4.2	S	-	-
2020-03302	0.5	0.5	4.3.3	S	-	-
2020-03303	1	0.5	4.3.3	S	-	-
2020-03304	0.5	0.5	4.3.3	S	-	-
2020-03309	0.25	0.25	4.3.3	S	-	-
2020-03312	0.25	0.25	2.2	S	-	-
2020-03314	0.25	0.25	4.3.4.2	S	-	-
2020-03315	0.5	0.5	4.3.3	S	-	-
2020-03316	1	1	4.3.3	S	-	-
2020-03319	0.25	0.25	4.8	S	-	-
2020-03320	1	1	4.3.3	S	-	-
2020-03321	0.5	0.5	4.3.3	S	-	-
2020-03322	0.5	0.25	4.3.4.1	S	-	-

2020-03323	0.5	0.5	4.3.3	S	-	-
2020-03324	0.5	0.5	4.3.3	S	-	-
2020-03325	1	1	4.3.3	S	-	-
2020-03326	0.5	0.25	4	S	-	-
2020-03329	0.5	0.5	4.3.3	S	-	-
2020-03330	2	2	4.3.3	S	-	-
2020-03331	0.5	0.5	4.8	S	-	-
2020-03332	1	1	4.3.3	S	-	-
2020-03333	0.5	0.25	4.1.1	S	-	-
2020-03335	0.5	0.5	4.3.3	S	-	-
2020-03336	0.5	0.5	4.3.3	S	-	-
2020-03337	0.5	0.5	4	S	-	-
2020-03338	0.5	0.5	4.3.3	S	-	-
2020-03339	1	1	4.8	S	-	-
2020-03340	0.5	0.5	4.3.2	S	-	-
2020-03342	0.5	0.5	4.3.2	S	-	-
2020-03343	1	0.5	2.2.1	S	-	-
2020-03344	0.25	0.25	4.1.1	S	-	-
2020-03348	0.5	0.5	4.3.3	S	-	-
2020-03349	0.5	0.5	4.3.4.2	S	-	-
2020-03351	0.25	0.25	2.2.1	S	-	-
2020-03352	0.5	0.5	2.2.1	S	-	-
2020-03353	0.5	0.5	4.3.3	S	-	-
2020-03355	0.5	0.5	4.1.2.1	S	-	-
2020-03356	1	0.5	4.8	S	-	-
2020-03358	0.5	0.5	4.8	S	-	-
2020-03359	0.5	0.5	4.3.4.2	S	-	-
2020-03374	1	0.5	4.3.3	S	-	-
2020-03381	0.5	0.5	4.3.3	S	-	-
2020-03430	1	1	4.3.3	S	-	-
2021-00873	1	1	4.8	S	-	-
2021-00876	0.5	0.5	2.2.1	S	-	-
2021-00879	1	1	4.8	S	-	-
2021-00885	0.25	0.25	2.2.1	S	-	-
2021-00893	0.5	0.5	4.4.1.1	S	-	-

2021-00897	0.5	0.5	2.2.1.1	S	-	-
2021-00899	1	0.5	2.2.1.1	S	-	-
2021-00908	1	0.5	2.2.1	S	-	-
2021-00915	0.5	0.5	4.4.1.1	S	-	-
2021-00918	0.5	0.5	2.2.1	S	-	-
2021-00922	1	1	2.2.1	S	-	-
2021-00924	0.5	0.5	2.2.1	S	-	-
2021-00925	0.5	0.5	4.1.1.3	S	-	-
2021-00927	0.5	0.5	2.2.1	S	-	-
2021-00932	0.5	0.5	2.2.1	S	-	-
2021-00939	0.5	0.25	4.4.1.1	S	-	-
2021-00942	0.5	0.5	4.3.2.1	S	-	-
2021-00944	0.5	0.5	2.2.1.1	S	-	-
2021-00945	0.5	0.5	1.2.2.2	S	-	-
2021-01208	0.25	0.25	2.2.1	S	-	-
2021-01236	0.5	0.5	3.1	S	-	-
2021-01240	0.5	0.5	3	S	-	-
2021-01244	0.5	0.5	3	S	-	-
2021-01246	0.5	0.5	3	S	-	-
2021-01248	0.5	0.5	3	S	-	-
2021-01715	0.5	0.5	2.2.1	S	-	-
2021-01725	1	1	2.2.1	S	-	-
2021-02340	0.5	0.25	2.2.1	S	-	-
2022-00049	0.5	0.5	1.2.2.2	S	-	-
2022-00053	0.5	0.5	2.2.1.1	S	-	-
2022-00061	0.5	0.5	2.2.1	S	-	-
2022-00101	0.5	0.5	2.2.1	S	-	-
2022-00240	1	1	3	S	-	-
2022-00266	1	1	3.1	S	-	-
2022-00274	0.5	0.5	3.1	S	-	-
2022-00306	0.5	0.5	1.1.2	S	-	-
2022-00322	0.5	0.5	1.1.2	S	-	-
2022-00354	0.5	0.5	1.1.2	S	-	-
2022-00356	0.5	0.5	1.1.2	S	-	-
2022-00564	1	1	2.2.1	S	-	-

2022-00566	0.5	0.25	2.2.1	S	-	-
2022-01169	0.25	0.25	3.1	S	-	-
2023-00397	0.25	0.25	2.2.1	S	-	-
2023-00408	0.5	0.5	2.2.1	S	-	-
2023-00457	0.5	0.5	2.2.1	S	-	-
2023-00549	1	0.5	3.1	S	-	-
2023-00569	2	1	3	S	-	-
2023-00571	0.125	0.125	3	S	-	-
2023-00575	1	0.5	3	S	-	-
2023-00577	0.5	0.25	3	S	-	-
2023-00579	>8	>8	3.1.2.1	S	-	-
2023-00581	1	0.5	3.1.2.1	S	-	-
2023-00593	0.5	0.5	3.1	S	-	-
2023-00595	1	0.5	3	S	-	-
2023-00599	0.5	0.5	2.2.1	S	-	-
2023-00607	0.5	0.25	2.2.1	S	-	-
2023-00615	1	1	1.1.2	S	-	-
2023-00639	0.5	0.5	3.1.2	S	-	-
2023-00732	0.5	0.5	4.3.3	S	-	-
2023-00733	0.25	0.25	4.3.3	S	-	-
2023-00734	1	0.5	4.3.3	S	-	-
2023-00735	2	2	4.3.3	S	-	-
2023-00737	1	1	4.3.3	S	-	-
2023-00740	1	0.5	4.3.3	S	-	-
2023-00741	1	0.5	4.3.3	S	-	-
2023-00744	0.5	0.5	4.3.3	S	-	-
2023-00748	0.5	0.5	2.2.1	S	-	-
2023-00749	0.5	0.5	2.2.1	S	-	-
2023-00750	0.5	0.5	2.2.1	S	-	-
2023-00762	0.5	0.5	2.2.1	S	-	-
2023-00766	1	1	4.3.3	S	-	-
2023-00767	1	1	4.3.3	S	-	-
2023-00771	1	0.5	4.3.3	S	-	-
2023-00772	1	1	4.3.3	S	-	-
2023-00773	1	1	4.3.3	S	-	-

2023-00774	1	1	4.3.3	S	-	-
2023-00813	0.5	0.5	4.3.3	S	-	-
2023-01162	0.5	0.5	4.3.3	S	-	-
2023-01181	1	0.5	4.3.3	S	-	-
2023-01366	1	0.5	4.9	S	-	-
2023-01366	1	1	4.9	S	-	-
2023-01366	1	1	4.9	S	-	-
2023-01372	0.5	0.5	2.2.1	S	-	-
2023-01375	1	1	3.1.2	S	-	-
2023-01383	1	1	3.1.2	S	-	-
2023-01604	0.5	0.5	2.2.1	S	-	-
2023-01608	0.5	0.5	2.2.1	S	-	-
2023-01615	>8	>8	2.2.1	S	-	-
2023-01688	1	1	2.2.1	S	-	-
2023-01720	0.5	0.5	2.2.1	S	-	-
2023-01981	0.5	0.25	3.1.2.1	S	-	-
2023-02138	0.5	0.25	4.3.2.1	S	-	-
2023-02138	0.5	0.5	4.3.2.1	S	-	-
2023-02167	0.5	0.5	4.3.4.2.1	S	-	-
2023-02171	1	1	2.2.1	S	-	-
2023-02198	1	1	2.2.1	S	-	-
2023-02199	0.5	0.5	2.2.1.1	S	-	-

Table 7.S3: Isolates used for validation of linezolid MIC testing using BMD

CT No	Dlm D14 MIC_{100}	Dlm D14 $MIC_{classic}$	Lineage	Dlm S/R?	*fgd1* mutations	*fbiC* mutations	*fbiD* mutations	*fbiA* mutations	*fbiB* mutations	*ddn* mutations
2015-01882	>0.5	>0.5	NA	R	-	-	-	-	-	*3987023 Ins*
2015-01887	>0.5	>0.5	NA	R	-	-	-	-	-	*3987149 Del*
2019-00461	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-00463	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-00467	0.06	0.06	4.3.3	S	-	-	-	-	-	-
2019-00469	0.06	0.06	2.2.1	S	-	-	-	-	-	-
2019-00471	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2019-00475	0.008	0.004	4.1	S	-	-	-	-	-	-
2019-00477	0.015	0.008	2.2.1	S	-	-	-	-	-	-
2019-00479	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-00481	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-00485	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2019-00487	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2019-01715	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01717	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2019-01721	0.03	0.015	4.3.3	S	-	-	-	-	-	-
2019-01723	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01727	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2019-01729	0.008	0.008	4.3.4.1	S	-	-	-	-	-	-
2019-01731	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01733	0.008	0.008	4.8	S	-	-	-	-	-	-
2019-01735	0.008	0.008	4.8	S	-	-	-	-	-	-
2019-01737	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01739	0.015	0.008	4.3.4.1	S	-	-	-	-	-	-
2019-01743	0.008	0.008	4.3.4.2	S	-	-	-	-	-	-
2019-01745	0.03	0.03	4.8	S	-	-	-	-	-	-
2019-01749	0.125	0.125	4.3.3	S	-	-	-	-	-	-

2019-01751	<=0.002	<=0.002	4.3.3	S	-	-	-	-	-	-
2019-01753	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01755	0.004	0.004	4.8	S	-	-	-	-	-	-
2019-01757	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2019-01759	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01759	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01761	0.06	0.06	4.8	S	-	-	-	-	-	-
2019-01771	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01775	0.008	0.004	4	S	-	-	-	-	-	-
2019-01777	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01779	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01783	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01785	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01787	0.06	0.06	4.3.3	S	-	-	-	-	-	-
2019-01789	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2019-01791	0.008	0.008	4.3.4.2	S	-	-	-	-	-	-
2019-01793	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01795	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2019-01801	0.004	<=0.002	4.3.3	S	-	-	-	-	-	-
2019-01803	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01805	0.008	0.008	4.8	S	-	-	-	-	-	-
2019-01809	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01811	<=0.002	<=0.002	4.8	S	-	-	-	-	-	-
2019-01813	0.008	0.008	4.1	S	-	-	-	-	-	-
2019-01815	0.008	0.004	4.3.3	S	-	-	-	-	-	-
2019-01821	0.008	0.008	4.3.4.2	S	-	-	-	-	-	-
2019-01827	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2019-01831	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01833	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2019-01835	0.015	0.015	4.3.3	S	-	-	-	-	-	-

2019-01837	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01837	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2019-01839	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2019-01843	0.008	0.008	4.3.2	S	-	-	-	-	-	-
2019-01845	0.015	0.015	4.8	S	-	-	-	-	-	-
2019-01845	0.06	0.06	4.8	S	-	-	-	-	-	-
2019-01849	0.03	0.008	4.3.4.2	S	-	-	-	-	-	-
2019-01853	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2019-02066	0.004	0.004	4.1.1.3	S	-	-	-	-	-	-
2019-02084	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2019-02086	0.125	0.125	2.2.1.1	S	-	-	-	-	-	-
2019-02682	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-02684	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-02688	0.004	0.004	2.2.1.1	S	-	-	-	-	-	-
2019-02688	0.015	0.015	2.2.1.1	S	-	-	-	-	-	-
2019-02698	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2019-02702	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-02710	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-02716	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2019-02718	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-02724	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-02726	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-02734	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-04829	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-04830	0.03	0.015	2.2.1	S	-	-	-	-	-	-
2019-04838	0.03	0.03	4.3.2.1	S	-	-	-	-	-	-
2019-04842	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2019-04847	0.004	0.004	4.4.1.1	S	-	-	-	-	-	-
2019-04853	0.015	0.015	2.2.2	S	-	-	-	-	-	-
2019-04859	0.008	0.008	4.3.4.2.1	S	-	-	-	-	-	-

2019-04865	0.008	0.008	4.4.1.1	S	-	-	-	-	-	-
2019-04866	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2019-04881	0.004	0.004	4.4.1.1	S	-	-	-	-	-	-
2019-04885	<=0.002	<=0.002	4.1.1.3	S	-	-	-	-	-	-
2019-04934	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2020-01235	0.008	0.008	3, 1.1	S	-	-	-	-	-	-
2020-01243	0.015	0.015	3, 1.1	S	-	-	-	-	-	-
2020-01245	0.008	0.008	3.1.2.1, 1.1	S	-	-	-	-	-	-
2020-01247	0.004	0.004	3, 1.1	S	-	-	-	-	-	-
2020-01261	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2020-01266	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2020-01281	0.03	0.015	2.2.1	S	-	-	-	-	-	-
2020-01283	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2020-03228	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03231	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03234	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03242	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03244	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03245	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03246	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03250	0.008	0.008	4.8	S	-	-	-	-	-	-
2020-03252	0.008	0.008	4.1.1.3	S	-	-	-	-	-	-
2020-03254	0.008	<=0.002	4.3.3	S	-	-	-	-	-	-
2020-03255	<=0.002	<=0.002	2.2.1	S	-	-	-	-	-	-
2020-03258	0.004	<=0.002	4.3.3	S	-	-	-	-	-	-
2020-03262	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03263	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2020-03264	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03265	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03266	0.004	0.004	4.3.3	S	-	-	-	-	-	-

2020-03267	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03268	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03269	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03270	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03275	0.008	0.004	4.3.3	S	-	-	-	-	-	-
2020-03276	0.008	0.008	4.1.1.3	S	-	-	-	-	-	-
2020-03278	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03280	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03281	0.06	0.004	4.3.4.2	S	-	-	-	-	-	-
2020-03284	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03285	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2020-03286	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2020-03287	0.008	0.004	4.3.3	S	-	-	-	-	-	-
2020-03288	0.008	0.004	4.3.3	S	-	-	-	-	-	-
2020-03289	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2020-03290	0.008	0.004	4.3.3	S	-	-	-	-	-	-
2020-03294	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03295	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03297	>0.5	>0.5	4.3.3	R	-	*1268dupG***	-	-	-	-
2020-03298	0.03	0.015	4.3.2	S	-	-	-	-	-	-
2020-03299	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03302	0.015	0.008	4.3.3	S	-	-	-	-	-	-
2020-03303	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03304	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03305	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03308	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03309	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03311	0.03	0.03	4.8	S	-	-	-	-	-	-
2020-03312	0.004	0.004	2.2	S	-	-	-	-	-	-
2020-03314	<0.002	<0.002	4.3.4.2	S	-	-	-	-	-	-

2020-03315	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03316	0.03	0.008	4.3.3	S	-	-	-	-	-	-
2020-03317	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03319	0.008	0.008	4.8	S	-	-	-	-	-	-
2020-03321	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2020-03322	0.004	0.004	4.3.4.1	S	-	-	-	-	-	-
2020-03323	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03324	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2020-03325	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03326	0.004	0.002	4	S	-	-	-	-	-	-
2020-03329	0.008	0.004	4.3.3	S	-	-	-	-	-	-
2020-03330	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03331	0.004	0.004	4.8	S	-	-	-	-	-	-
2020-03332	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03333	0.008	0.004	4.1.1	S	-	-	-	-	-	-
2020-03335	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03336	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2020-03337	0.004	0.004	4	S	-	-	-	-	-	-
2020-03338	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03339	0.004	0.004	4.8	S	-	-	-	-	-	-
2020-03340	0.015	0.015	4.3.2	S	-	-	-	-	-	-
2020-03342	0.008	0.008	4.3.2	S	-	-	-	-	-	-
2020-03343	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2020-03344	0.004	0.004	4.1.1	S	-	-	-	-	-	-
2020-03349	0.008	0.008	4.3.4.2	S	-	-	-	-	-	-
2020-03351	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2020-03352	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2020-03353	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2020-03358	0.004	0.004	4.8	S	-	-	-	-	-	-
2020-03374	0.004	0.004	4.3.3	S	-	-	-	-	-	-

2020-03381	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2020-03430	>0.5	>0.5	4.3.3	R	-	*1268dupG (0.68)***	-	-	-	-
2021-00873	0.03	0.03	4.8	S	-	-	-	-	-	-
2021-00876	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2021-00879	0.06	0.06	4.8	S	-	-	-	-	-	-
2021-00885	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2021-00887	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2021-00889	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2021-00893	0.015	0.015	4.4.1.1	S	-	-	-	-	-	-
2021-00897	0.015	0.015	2.2.1.1	S	-	-	-	-	-	-
2021-00899	0.008	0.008	2.2.1.1	S	-	-	-	-	-	-
2021-00908	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2021-00915	0.008	0.008	4.4.1.1	S	-	-	-	-	-	-
2021-00922	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2021-00925	0.008	0.004	4.1.1.3	S	-	-	-	-	-	-
2021-00927	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2021-00932	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2021-00939	0.015	0.008	4.4.1.1	S	-	-	-	-	-	-
2021-00942	0.015	0.015	4.3.2.1	S	-	-	-	-	-	-
2021-00944	>0.5	0.5	2.2.1.1	R	-	-	-	-	*c.-378C>T (1.0)***	-
2021-00945	0.008	0.008	1.2.2.2	S	-	-	-	-	-	-
2021-01208	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2021-01240	0.004	0.004	3	S	-	-	-	-	-	-
2021-01244	0.004	0.004	3	S	-	-	-	-	-	-
2021-01246	0.004	0.004	3	S	-	-	-	-	-	-
2021-01248	0.004	0.004	3	S	-	-	-	-	-	-
2021-01715	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2021-01725	0.125	0.015	2.2.1	S	-	-	-	-	-	-
2021-02299	0.03	0.03	4.8	S	-	-	-	-	-	-

2021-02318	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2021-02320	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2021-02340	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2022-00049	0.004	0.004	1.2.2.2	S	-	-	-	-	-	-
2022-00053	0.004	0.004	2.2.1.1	S	-	-	-	-	-	-
2022-00061	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2022-00306	0.004	0.004	1.1.2	S	-	-	-	-	-	-
2022-00322	0.008	0.008	1.1.2	S	-	-	-	-	-	-
2022-00354	0.008	0.008	1.1.2	S	-	-	-	-	-	-
2022-00356	0.004	0.004	1.1.2	S	-	-	-	-	-	-
2022-00564	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2022-00566	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2023-00337	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00347	>0.5	>0.5	4.3.3	R	-	*598dupG (1.0)***	-	-	-	-
2023-00397	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2023-00408	>0.5	>0.5	2.2.1	R	-	-	-	-	*1070dupC (0.79)***	-
2023-00457	0.06	0.008	2.2.1	S	-	-	-	-	-	-
2023-00575	0.004	0.004	3	S	-	-	-	-	-	-
2023-00577	0.008	0.004	3	S	-	-	-	-	-	-
2023-00579	0.008	0.008	3.1.2.1	S	-	-	-	-	-	-
2023-00581	>0.5	>0.5	3.1.2.1	R	-	-	-	-	-	*113delG (0.9)***
2023-00595	0.008	0.004	3	S	-	-	-	-	-	-
2023-00599	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2023-00607	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2023-00615	0.004	0.004	1.1.2	S	-	-	-	-	-	-
2023-00639	0.015	0.015	3.1.2	S	-	-	-	-	-	-
2023-00732	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2023-00734	>0.5	>0.5	4.3.3	R	-	*1087dupG (0.35); 1884_1885dupGC (0.16)***	-	-	-	*Pro63Leu (0.46)***

2023-00735	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00737	0.015	0.015	4.3.3	S	-	-	-	-	-	-
2023-00740	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00741	0.015	0.008	4.3.3	S	-	-	-	-	-	-
2023-00744	0.03	0.03	4.3.3	S	-	-	-	-	-	-
2023-00748	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2023-00749	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2023-00750	0.008	0.008	2.2.1	S	-	-	-	-	-	-
2023-00762	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2023-00766	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00767	0.008	0.004	4.3.3	S	-	-	-	-	-	-
2023-00771	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00772	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00773	0.03	0.008	4.3.3	S	-	-	-	-	-	-
2023-00774	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00780	0.015	0.008	4.3.3	S	-	-	-	-	-	-
2023-00781	0.015	0.008	4.3.3	S	-	-	-	-	-	-
2023-00783	0.008	0.008	4.3.3	S	-	-	-	-	-	-
2023-00784	0.06	0.03	4.3.3	S	-	-	-	-	-	-
2023-00789	0.03	0.03	4.8	S	-	-	-	-	-	-
2023-00791	0.06	0.06	4.8	S	-	-	-	-	-	-
2023-00813	0.004	0.004	4.3.3	S	-	-	-	-	-	-
2023-01162	>0.5	>0.5	4.3.3	R	-	-	-	-	-	*Pro63Leu (0.97)***
2023-01181	0.03	0.015	4.3.3	S	-	-	-	-	-	-
2023-01366	0.008	0.004	4.9	S	-	-	-	-	-	-
2023-01366	<=0.002	<=0.002	4.9	S	-	-	-	-	-	-
2023-01366	0.008	0.008	4.9	S	-	-	-	-	-	-
2023-01372	>0.5	>0.5	2.2.1	R	-	-	-	-	-	*311delA (1.0)***
2023-01375	0.004	0.004	3.1.2	S	-	-	-	-	-	-

2023-01383	0.004	0.004	3.1.2	S	-	-	-	-	-	-
2023-01604	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2023-01608	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2023-01615	0.004	0.004	2.2.1	S	-	-	-	-	-	-
2023-01688	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2023-01720	0.015	0.015	2.2.1	S	-	-	-	-	-	-
2023-01981	0.004	0.004	3.1.2.1	S	-	-	-	-	-	-
2023-01983	<=0.002	<=0.002	3	S	-	-	-	-	-	-
2023-02138	0.03	0.015	4.3.2.1	S	-	-	-	-	-	-
2023-02138	0.008	0.008	4.3.2.1	S	-	-	-	-	-	-
2023-02167	0.008	0.008	4.3.4.2.1	S	-	-	-	-	-	-
2023-02171	0.03	0.03	2.2.1	S	-	-	-	-	-	-
2023-02198	0.06	0.015	2.2.1	S	-	-	-	-	-	-

Table 7.S4: Isolates used for validation of delamanid MIC testing using BMD

**These mutations amplified while patients were receiving treatment regimens containing delamanid, thus considered as associated with delamanid resistance.

CHAPTER 8

Broth microdilution plate-based minimal inhibitory concentration testing using MGIT-positive cultures

Broth microdilution plate-based minimal inhibitory concentration testing using MGIT-positive cultures

Broth microdilution plate-based minimal inhibitory concentration testing using MGIT-positive cultures

Praharshinie Rupasinghe[1,2*], Nore Van de Straeten[3], Aissatou Diallo[3], Jens Vereecken[1], Bouke C de Jong[1], Leen Rigouts[1,2]

[1] Institute of Tropical Medicine, Antwerp, Belgium
[2] Department of Biomedical Sciences, University of Antwerp, Belgium
[3] Karel de Grote High School, Antwerp
*Corresponding author

Abstract

Background

The current WHO-endorsed 96-well-plate-based broth microdilution testing (BMD) for minimum inhibitory concentration (MIC) testing of *Mycobacterium tuberculosis* complex (MTBc) relies on inoculum prepared from a solid-medium culture, which requires long incubation, limiting its use in clinical settings. We aimed to develop a protocol for BMD-MIC testing from freshly positive, actively growing MGIT cultures.

Method

Bacterial suspensions were prepared by resuspending well-dispersed pellets obtained by centrifuging the contents of purity-confirmed, 3-5 days old positive MGIT cultures in sterile distilled water (SDW). The bacterial suspensions with optical density (OD) ≤McF 0.5, were diluted 1:100 in supplemented 7H9 broth to be used as the inoculum for BMD-MIC testing while those with OD >McF0.5 were diluted in SDW to obtain an OD of McF 0.5 before diluting 1:100 in 7H9. MICs obtained for moxifloxacin, levofloxacin, clofazimine, bedaquiline, linezolid, and delamanid using an MGIT inoculum were compared to the MICs obtained using a solid medium inoculum.

Results

We tested 19 MTBc isolates, yielding 114 MICs: 13/19 MGIT inocula achieved an OD of McF 0.45-0.55, the OD of the remaining six ranged between McF0.26-0.40. Of the 19 MGIT inocula, only eight yielded CFU counts within the acceptable range of 5×10^4 - 5×10^5. However, irrespective of the optical densities/CFU counts, all 19 inocula yielded interpretable MICs for all drugs tested. Among the 114 BMD-MGIT MIC values, 107 (94%) showed a maximum +/- 1 dilution difference from BMD-LJ MIC values, with five (4%) of them shifting from resistant to susceptible or vice versa. Of the remaining seven isolates, 6 exhibited a +/- two-fold change in MICs between the two techniques, with one causing a shift in susceptibility pattern. For all drugs except clofazimine and linezolid, BMD-MGIT MICs were systematically higher than that of BMD-LJ, however the majority had only one dilution increase. The average turnaround time (TAT) from MGIT culture inoculation to BMD-MGIT MIC results were 26 days, roughly half of the 50 days TAT of BMD-LJ method starting from LJ cultures.

Conclusion

Our results suggest that standardized MGIT inocula yield comparable BMD-MICs to that of solid medium inoculum while reducing the turn-around time by half.

8.1 Introduction

In 2022, the World Health Organization (WHO) endorsed 96-well plate-based broth microdilution testing (BMD) to test the minimum inhibitory concentrations (MIC) of the *Mycobacterium tuberculosis* complex (MTBc), which could serve as an alternative to conventional phenotypic drug-susceptibility testing (pDST) assays such as the agar proportion method and the Mycobacterial Growth Indicator Tube (MGIT) system (1). The main advantage of BMD-MIC testing is that it enables all-in-one antibiotic panel testing and provides quantitative (MICs) rather than binary test results, which can be particularly important in the accurate detection of low-level resistance and defining breakpoints to new anti-TB drugs (1).

So far, the BMD method relies on the MTBc inoculum prepared from a solid culture, which requires long incubation, limiting its use for clinical purpose. This study aims to develop a protocol for reliable MIC testing from freshly positive, actively growing MGIT cultures.

8.2 Method

8.2.1 Sample selection

Stored (-20°C) sputum sediments from isolates that had valid BMD-MIC results using the solid-culture-based approach were re-inoculated in MGIT medium supplemented with OADC (Oleic acid, Albumin, Dextrose, Catalase) and PANTA (Polymyxin B, Amphotericin B, Nalidixic Acid, Trimethoprim, Azlocillin) to obtain a new positive MGIT culture that could be utilized to create a standard inoculum for BMD from MGIT, after check of purity on blood agar and presence of acid-fast bacilli by microscopic examination.

8.2.2 Inoculum preparation and inoculation of plates

The entire content of a purity confirmed positive MGIT culture not older than 3-5 days since flagged positive by the MGIT machine, was transferred into a sterile 50 ml falcon tube, and centrifuged for 20 minutes at 3500 g and 4 °C. After letting the tube stand for 5 minutes to settle aerosols, the supernatant was carefully discarded without disturbing the pellet. About 2-3 drops of sterile distilled water (SDW) and 5-10 sterile glass beads (diameter 2 mm) were added

to the tube containing the pellet, which was then firmly closed and vortexed vigorously for one minute, paying attention to the beads rolling down the glass tube's wall, until the clumps were well dispersed. After allowing the closed tube to stand for 5 minutes to settle aerosols, 1 ml of SDW was added, the tube was tightly closed, and vigorously vortexed for 15 seconds until the tube's content was homogenized. After letting the closed tube stand for 30 minutes for the clumps to settle, the supernatant was transferred into a new sterile glass tube. Using the densitometer (Grant Instruments, United Kingdom), the optical density of the supernatant was adjusted to MacFarland standard (McF) 0.5 using SDW, and then further diluted 1:100 in 7H9-S broth (7H9 broth + 10% OADC + 0.5% glycerol + 0.1% casitone) to prepare a 10^{-2} of the McF 0.5 bacterial suspension.

Using sterile filter tips, the drug-containing wells and the 100% growth controls (B11, D11, and F11, **Figure 8.1**), were inoculated with 100 µl of the 10^{-2} of McF 0.5 bacterial suspension. The 10^{-2} McF bacterial suspension was further diluted 1:100 using 7H9-S broth to prepare the 10^{-4} McF bacterial suspension, which was used to inoculate the 1% growth controls (C11, E11, and G11, **Figure 8.1**). Even if the optical density of the undiluted supernatant was less than McF 0.5, it was processed in the same way as a McF 0.5 inoculum and inoculated in the BMD plate. After inoculation, the plates were incubated at 36°C (±2°C) for a maximum of 21 days.

	1	2	3	4	5	6	7	8	9	10	11	12
A		H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20
B	H_20	BDQ 2,0	BDQ 1,0	BDQ 0,5	BDQ 0,25	BDQ 0,125	BDQ 0,06	BDQ 0,03	BDQ 0,015	BDQ 0,008	GC 100% DMSO	NC DMSO
C	H_20	LNZ 8,0	LNZ 4,0	LNZ 2,0	LNZ 1,0	LNZ 0,5	LNZ 0,25	LNZ 0,125	LNZ 0,06	LNZ 0,03	GC 1% DMSO	NC DMSO
D	H_20	DLM 0,5	DLM 0,25	DLM 0,125	DLM 0,06	DLM 0,03	DLM 0,015	DLM 0,008	DLM 0,004	DLM 0,002	GC 100%	NC
E	H_20	CFZ 4,0	CFZ 2,0	CFZ 1,0	CFZ 0,5	CFZ 0,25	CFZ 0,125	CFZ 0,06	CFZ 0,03	CFZ 0,015	GC 1%	NC
F	H_20	MFX 4,0	MFX 2,0	MFX 1,0	MFX 0,5	MFX 0,25	MFX 0,125	MFX 0,06	MFX 0,03	MFX 0,015	GC 100% a+0,3%TW	NC a+0,3%TW
G	H_20	LFX 8,0	LFX 4,0	LFX 2,0	LFX 1,0	LFX 0,5	LFX 0,25	LFX 0,125	LFX 0,06	LFX 0,03	GC 1% a+0,3%TW	NC a+0,3%TW
H	primer DLM	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20	H_20

Figure 8.1: Plate layout, drugs and the serial dilutions tested.

GC = growth control, NC = negative control, BDQ= bedaquiline, LNZ = linezolid, DLM = delamanid, CFZ = clofazimine, MFX = moxifloxacin, LFX = levofloxacin, TW=Tween 20

8.2.3 Reading and interpretation of the MICs

The plates were read by visual inspection using an inverted mirror to detect positive and negative growth in the wells. Systematic reading was done on day 7 and day 14 of incubation. If there was still no growth of the GC1% on day 14, the incubation was extended to a maximum of 21 days. As soon as the GC100% and the GC1% were positive, or when at least 2 of 3GC1% positive controls of the same strain were positive, the $MIC_{classic}$ values were interpreted.

When we observed a single skipped well or a single well with pinpoint growth bordered by wells with clear growth, MICs were interpreted disregarding the single skipped/pinpoint well. When multiple skipped wells or wells with pinpoint growth bordered by wells with growth were observed, MICs were considered invalid, and the test was repeated.

Readers were blinded to the previously obtained MIC results by solid-medium-based BMD.

8.2.4 Quantification of the inoculum

Ten microliters of the 10^{-2}, 10^{-3} and 10^{-4} inoculum dilutions from were plated on Middlebrook 7H11 agar in triplicate. Colony forming units (CFU) counts were enumerated after four, six, and eight weeks of incubation at 36°C ± 2°C. The targeted CFU count for the 10^{-2} of the McF 0.5 suspension was $1x10^5$ CFU/ml, with an acceptable range of $5x10^4$ to $5x10^5$ CFU/ml.

8.2.5 Comparison of the MIC results

BMD-MICs obtained from the previously obtained standard solid-culture-based method (BMD-LJ) were compared with those obtained from the inocula prepared using MGIT cultures (BMD-MGIT). When available, MGIT-DST results and direct genotypic DST results by deeplex/GeneXpert XDR for the drugs under evaluation were also compared with the MICs obtained from the two methods. MICs with a maximum +/- 1 dilution difference between the two methods were deemed acceptable (3).

8.3 Results

So far, we have tested 19 MTBc isolates by BMD-MGIT method, yielding 114 MICs across six different drugs. Most (13/19, 68%) MGIT-based inocula achieved an optical density within the acceptable range of McF 0.45-0.55, while the remaining six had optical densities ranging from McF 0.26 to 0.40. There was no clear correlation between the growth units (GU) in the MGIT culture and the optical density of the inoculum. For example, sample S16 had the highest GUs but the lowest optical density, whereas S1, S4 and S8 had lower GUs but reached the desired optical density. There was also no clear correlation between the smear grade of the sediments and the time to positivity (TTP) of the MGIT cultures or the growth units at TTP (**Table 8.1**).

Of the 19 MGIT-based inocula, only eight yielded CFU counts within the acceptable range of 5 x 10^4 - 5 x 10^5 CFU/ml. However, irrespective of the optical densities/CFU counts, all 19 inocula yielded interpretable MICs for all drugs tested. Only one isolate (S7, **Table 8.1**) had both GC100% and GC1% positive after one week by both LJ- and MGIT-BMD, whereas the remaining 18 had interpretable growth in the GCs only after 14 days by both methods. The TTP of the primary culture was 10 days in MGIT and 34 days on LJ. Including the two additional days required for purity confirmation of the MGIT cultures, the average turnaround time (TAT) from MGIT culture inoculation to BMD-MGIT MIC results were 26 days, roughly half of the 50 days TAT of BMD-LJ method starting from LJ cultures (**Table 8.1**).

Among the 114 BMD-MGIT MIC values, 107 (94%) showed a maximum +/- 1 dilution difference from BMD-LJ MIC values, with five (4%) of them shifting from resistant to susceptible or vice versa. Of the remaining seven isolates, 6 exhibited a +/- two-fold change in MICs between the two techniques, with one causing a shift in susceptibility pattern. Among these six, three isolates' MGIT-based inoculums could not achieve the necessary OD; yet, in all three cases, MICs by BMD-MGIT were greater than MICs by BMD-LJ, indicating that the OD had little effect on the MIC. One isolate (S17), whose MGIT-based inoculum did not reach the desired OD, exhibited a three-fold change in the delamanid MIC compared to the MIC from BMD-LJ method, but no alteration in susceptibility pattern occurred.

ID	MGIT GU	MGIT TTP	BMD-MGIT inoculum		MIC-BMD-MGIT						BMD-MGIT total TAT	LJ TTP	MIC-BMD-LJ						BMD-LJ total TAT	MGIT DST results					Direct gDST results			
			OD	CFU/ml	CFZ	MFX	LFX	BDQ	LNZ	DLM			CFZ	MFX	LFX	BDQ	LNZ	DLM		CFZ	LFX	BDQ	LNZ	DLM	CFZ	LFX	BDQ	LNZ
S1	87	5	0.5	invalid	0.5	0.25	0.5	0.25	0.5	0.015	21	26	0.25	0.125	0.25	0.06	0.5	0.008	41	S	S	S	S	S	NA	NA	NA	NA
S2	420	10	0.5	1.2E+4	0.25	0.125	0.25	0.03	0.25	0.008	26	20	0.25	0.25	0.25	0.06	0.5	0.004	35	S	S	S	S	S	NA	S*	NA	NA
S3	243	4	0.5	1.5E+4	1.0	2.0	2.0	0.25	0.5	0.015	20	41	0.5	1.0	1.0	0.125	0.5	0.004	56	S	R	S	S	S	NA	S*	NA	NA
S4	81	9	0.45	5.0E+4	0.125	0.25	0.25	0.03	0.5	0.004	25	41	0.5	0.125	0.125	0.03	0.5	0.008	56	S	S	S	S	S	NA	S*	NA	NA
S5	148	11	0.54	2.0E+3	1.0	0.25	0.5	0.25	0.5	0.008	27	47	1.0	0.125	0.25	0.125	0.5	0.004	62	R	S	R	S	S	NA	S*	NA	NA
S6	406	12	0.38	1.0E+4	0.25	0.25	0.5	0.125	0.5	0.004	28	36	0.25	0.125	0.25	0.06	0.5	0.002	51	S	S	S	S	S	NA	S*	NA	NA
S7	559	8	0.53	2.5E+5	0.25	1.0	2.0	0.06	0.5	0.004	17	15	0.25	1.0	2.0	0.03	0.5	0.004	22	S	R	S	S	S	S	R	S	S
S8	80	10	0.5	invalid	2.0	0.25	0.5	1.0	0.5	0.008	26	32	2.0	0.125	0.25	0.5	1.0	0.008	47	S	S	S	S	S	NA	S*	NA	NA
S9	120	12	0.4	8E+4	0.25	2.0	4.0	0.06	0.5	0.008	28	36	0.125	2.0	4.0	0.015	0.5	0.004	51	S	R	S	S	S	S	R	S	S
S10	230	17	0.55	1.0E+4	0.125	0.125	0.25	0.03	0.5	0.015	29	28	0.25	0.125	0.25	0.03	0.5	0.008	43	S	S	S	S	S	NA	S*	NA	NA
S11	324	11	0.55	4.6E+4	0.5	0.125	0.25	0.125	0.5	0.008	27	26	1.0	0.06	0.125	0.5	0.5	0.008	41	S	S	S	S	S	NA	S*	NA	NA
S12	198	11	0.47	2.5E+4	0.25	0.125	0.5	0.125	0.5	0.004	27	56	0.125	0.06	0.25	0.03	0.25	0.004	71	S	S	S	S	S	NA	NA	NA	NA
S13	2587	14	0.53	5.0E+4	1.0	2.0	8.0	0.5	0.5	0.015	30	28	0.5	2.0	4.0	0.5	0.5	0.008	43	R	R	R	S	S	R	R	R	S
S14	106	12	0.43	invalid	0.125	0.06	0.125	0.06	0.25	0.008	28	43	0,25	0,25	0.25	0.015	0,5	0,008	58	S	S	S	S	S	S	S	S	S
S15	151	10	0.55	1.1E+5	0.25	0.125	0.25	0.125	0.5	0.008	26	43	0,25	0,25	0,5	0,06	1.0	0,008	58	S	S	S	S	S	NA	S*	NA	NA
S16	3885	7	0.26	5.5E+3	0.25	0.25	0.5	0.03	0.5	0.008	23	43	0,5	0,5	1.0	0,03	0,5	0,008	58	S	S	S	S	S	S	S	S	S
S17	286	16	0.3	2.5E+2	0.125	0.25	0.25	0.25	1.0	0.03	32	28	0.25	0.125	0.25	0.125	2.0	0.004	43	S	R	S	S	R	NA	S*	NA	NA
S18	85	9	0.36	5.0E+4	0.125	0.125	0.25	0.125	1.0	0.008	25	27	0.25	0.125	0.25	0.06	0.5	0.008	42	S	S	S	S	S	NA	S*	NA	NA
S19	1722	4	0.52	1.2E+5	0.25	0.25	0.125	0.03	0.5	0.008	20	49	0.25	0.125	0.25	0.06	0.5	0.008	64	S	S	S	S	S	NA	S*	NA	NA
Average		**10**									**26**	**35**							**50**									

Table 8.1: Inoculum, MIC and DST data of the 19 isolates used.

GU = Growth Units, OD= Optical density, TTP = Time to Positivity in days, CFU = Colony forming units, NA = Not available, Invalid = no growth observed in any of the CFU counting plates, *Fluoroquinolone resistance data by GeneXpert XDR. MIC values discordant with the MGIT DST results are highlighted in red.

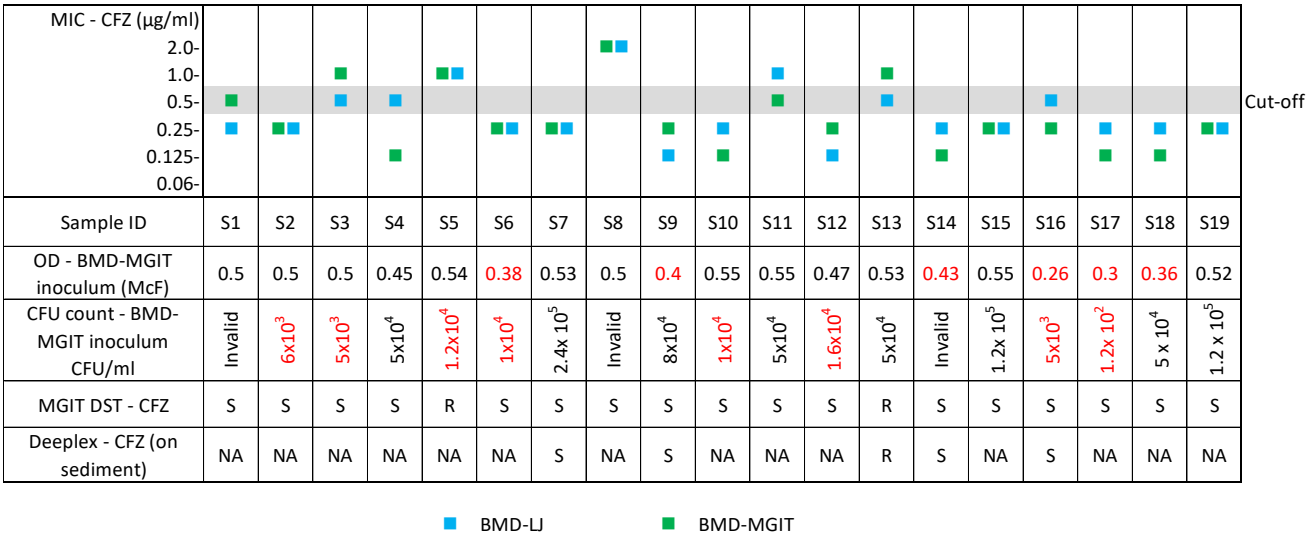
Cut off values used in BMD → clofazimine (CFZ) = 0.5 µg/ml, moxifloxacin (MFX) = O.25 µg/ml, levofloxacin (LFX) = 1.0 µg/ml, Bedaquiline (BDQ) = 0.25 µg/ml, Linezolid (LZD) = 1.0 µg/ml, Delamanid (DLM) = 0.125 µg/ml. Cut off values used in MGIT → CFZ = 1.0 µg/ml, MFX = O.25 µg/ml, LFX = 1.0 µg/ml, BDQ = 0.25 µg/ml, LZD = 1.0 µg/ml, DLM = 0.6 µg/ml

Below we discuss the MIC results by both methods per drug.

8.3.1 Clofazimine

Seven isolates had the same clofazimine-MIC by both methods, 11 isolates had a +/- one dilution difference and one isolate (S4) had a +/- two dilution difference between the two methods. Five of these 12 isolates exhibiting a MIC difference between the two methods had higher MICs using BMD-MGIT, whereas seven had higher MICs using BMD-LJ (**Figure 8.2**).

Two isolates, S3 and S13, switched from clofazimine-susceptible to clofazimine-resistant by the BMD-MGIT method; S13 was clofazimine-resistant also by MGIT DST and deeplex MTB assay on the sputum sediment favoring the BMD-MGIT method. S11 switched from clofazimine-resistant to clofazimine-susceptible by BMD-MGIT. For S8, MICs from both BMD methods indicated clofazimine resistance, while MGIT DST was susceptible (**Figure 8.2**).



Sample ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19
OD - BMD-MGIT inoculum (McF)	0.5	0.5	0.5	0.45	0.54	0.38	0.53	0.5	0.4	0.55	0.55	0.47	0.53	0.43	0.55	0.26	0.3	0.36	0.52
CFU count - BMD-MGIT inoculum CFU/ml	Invalid	$6x10^3$	$5x10^3$	$5x10^4$	$1.2x10^4$	$1x10^4$	$2.4x\ 10^5$	Invalid	$8x10^4$	$1x10^4$	$5x10^4$	$1.6x10^4$	$5x10^4$	Invalid	$1.2x\ 10^5$	$5x10^3$	$1.2x\ 10^2$	$5\ x\ 10^4$	$1.2\ x\ 10^5$
MGIT DST - CFZ	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S
Deeplex - CFZ (on sediment)	NA	NA	NA	NA	NA	NA	S	NA	S	NA	NA	NA	R	S	NA	S	NA	NA	NA

Figure 8.2: Clofazimine MICs by the two different methods

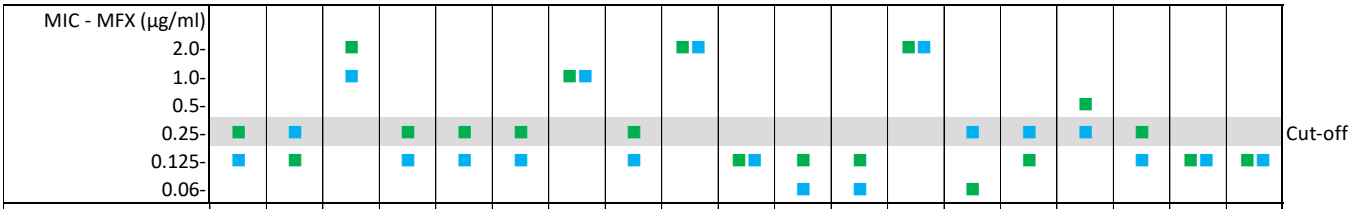
S = Susceptible, R = Resistant, NA = Not available, Cfz = Clofazimine; DST = drug-susceptibility testing, CFU = colony forming units, OD = optical density, MGIT = Mycobacteria Growth Indicator tube, LJ = Löwenstein-Jensen, MIC = minimal inhibitory concentration.

ODs and CFU counts below the desired range are depicted in red text.

8.3.2 Moxifloxacin

Six isolates had the same moxifloxacin-MIC using both methods, 12 isolates had a maximum +/- one dilution difference, and one isolate (S14) had a +/- two dilution difference between the two methods. Ten of the 13 isolates with a MIC difference between the two methods had higher MICs using BMD-MGIT, whereas just three had higher MICs using BMD-LJ, possibly indicating a systematic increase in MICs by the BMD-MGIT approach (**Figure 8.3**).

One isolate S16, switched from moxifloxacin-susceptible to moxifloxacin-resistant by BMD-MGIT method. This isolate was levofloxacin susceptible by MGIT DST and no moxifloxacin DST results were available. GeneXpert XDR performed on the respective processed sputum sediment showed no fluoroquinolone resistance (**Figure 8.3**), favoring the BMD-LJ MIC.



Sample ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19
OD - BMD-MGIT inoculum (McF)	0.5	0.5	0.5	0.45	0.54	0.38	0.53	0.5	0.4	0.55	0.55	0.47	0.53	0.43	0.55	0.26	0.3	0.36	0.52
CFU count - BMD-MGIT inoculum CFU/ml	Invalid	$6x10^3$	$5x10^3$	$5x10^4$	$1.2x10^4$	$1x10^4$	$2.4x\ 10^5$	Invalid	$8x10^4$	$1x10^4$	$5x10^4$	$1.6x10^4$	$5x10^4$	Invalid	$1.2x\ 10^5$	$5x10^3$	$1.2x\ 10^2$	$5\ x\ 10^4$	$1.2\ x\ 10^5$
MGIT DST - LFX	S	S	R	S	S	S	R	S	R	S	S	S	R	S	S	S	R	S	S
Deeplex/Xpert XDR - FQ (on sediment)	NA	S*	S*	S*	S*	S*	R	S*	R	S*	S*	NA	R	S	S*	S	S*	S*	S*

BMD-LJ BMD-MGIT

Figure 8.3: Moxifloxacin MICs by the two different methods

S = Susceptible, R = Resistant, NA = Not available, Cfz = Clofazimine; DST = drug-susceptibility testing, CFU = colony forming units, OD = optical density, MGIT = Mycobacteria Growth Indicator tube, LJ = Löwenstein-Jensen, MIC = minimal inhibitory concentration.

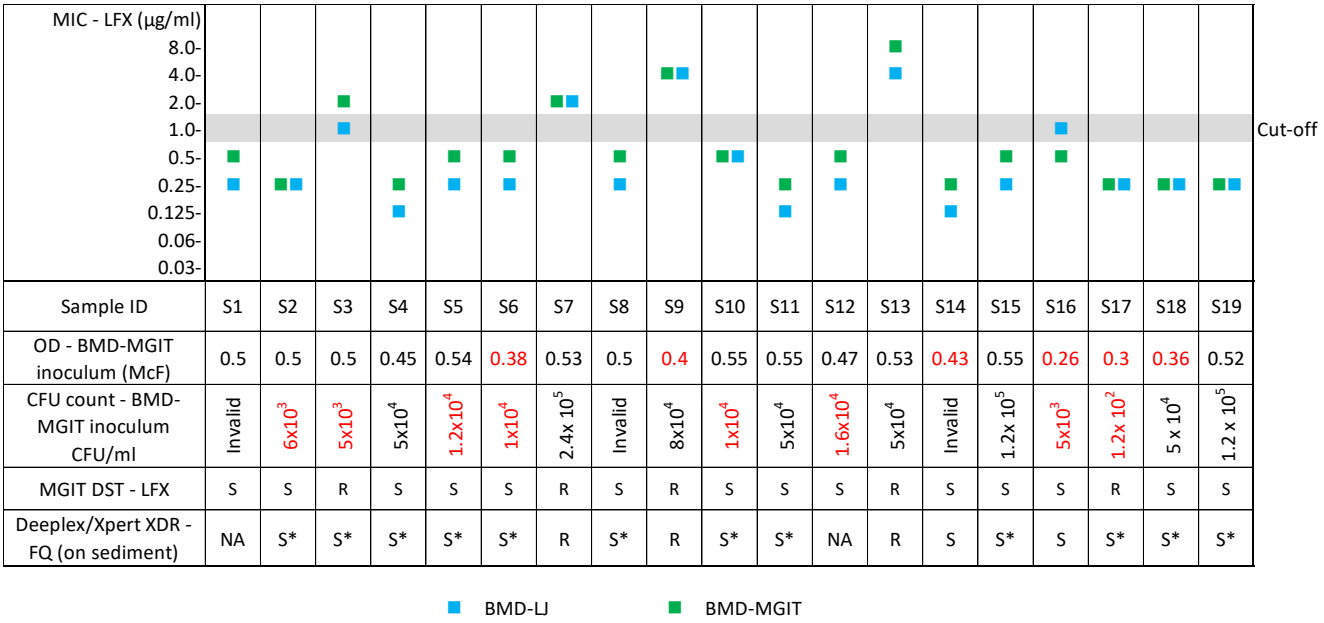
ODs and CFU counts below the desired range are depicted in red text.

*FQ susceptibility data from GeneXpert XDR on sputum sediment

8.3.3 Levofloxacin

Seven isolates had the same levofloxacin-MIC using both methods, 11 isolates had a maximum +/- one dilution difference, and one isolate (S19) had a +/- two dilution difference between the two methods. Ten of the 12 isolates with a MIC difference between the two methods had higher MICs using BMD-MGIT, whereas only two had higher MICs using BMD-LJ, suggesting a systematic increase in MICs by the BMD-MGIT approach. (**Figure 8.4**).

S3 switched from levofloxacin-susceptible to levofloxacin-resistant when the MGIT inoculum was used: this isolate was also levofloxacin resistant by MGIT DST and Xpert XDR on the sputum sediment indicated fluoroquinolone resistance (**Figure 8.4**), favoring the BMD-MGIT-MIC.



Sample ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19
OD - BMD-MGIT inoculum (McF)	0.5	0.5	0.5	0.45	0.54	0.38	0.53	0.5	0.4	0.55	0.55	0.47	0.53	0.43	0.55	0.26	0.3	0.36	0.52
CFU count - BMD-MGIT inoculum CFU/ml	Invalid	$6x10^3$	$5x10^3$	$5x10^4$	$1.2x10^4$	$1x10^4$	$2.4x\ 10^5$	Invalid	$8x10^4$	$1x10^4$	$5x10^4$	$1.6x10^4$	$5x10^4$	Invalid	$1.2x\ 10^5$	$5x10^3$	$1.2x\ 10^2$	$5\ x\ 10^4$	$1.2\ x\ 10^5$
MGIT DST - LFX	S	S	R	S	S	S	R	S	R	S	S	S	R	S	S	S	R	S	S
Deeplex/Xpert XDR - FQ (on sediment)	NA	S*	S*	S*	S*	S*	R	S*	R	S*	S*	NA	R	S	S*	S	S*	S*	S*

Figure 8.4: Levofloxacin MICs by the two different methods

S = Susceptible, R = Resistant, NA = Not available, Cfz = Clofazimine; DST = drug-susceptibility testing, CFU = colony forming units, OD = optical density, MGIT = Mycobacteria Growth Indicator tube, LJ = Löwenstein-Jensen, MIC = minimal inhibitory concentration.

ODs and CFU counts below the desired range are depicted in red text.

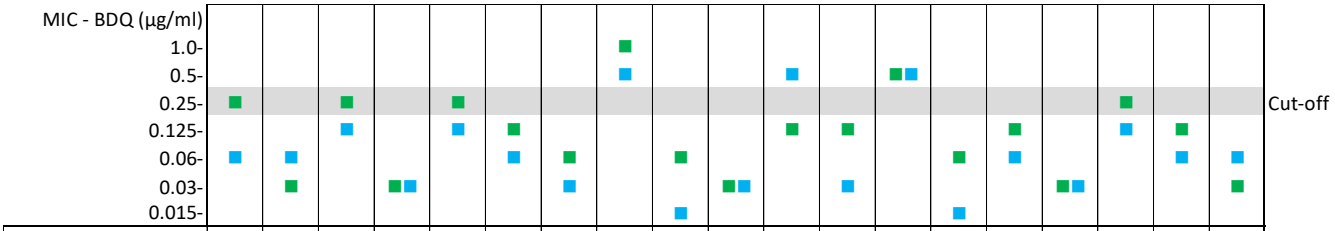
*FQ susceptibility data from GeneXpert XDR on sputum sediment

8.3.4 Bedaquiline

Four isolates had the same bedaquiline-MIC using both methods, 10 isolates had a maximum +/- one dilution difference, and five isolates had a +/- two dilution difference between the two methods. Twelve of the 15 isolates with a MIC difference between the two methods had higher MICs using BMD-MGIT, whereas only three had higher MICs using BMD-LJ, suggesting a systematic increase in MICs by the BMD-MGIT approach. (**Figure 8.5**).

For S1, S9, S12, and S15, MICs with the MGIT-inoculum were two dilutions higher than LJ-based MICs. S11, which was bedaquiline-resistant using the BMD-LJ method, exhibited a two-fold drop in MIC utilizing the BMD-MGIT method, making it bedaquiline-susceptible. This isolate was bedaquiline-susceptible by MGIT DST, favoring the BMD-MGIT MIC.

S5 had bedaquiline MICs on/below the cut-off by both BMD methods, but this isolate was bedaquiline-resistant by MGIT DST, and S8 had MICs above the cut-off by both BMD methods but was bedaquiline-susceptible by MGIT DST (**Figure 8.5**).



Sample ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19
OD - BMD-MGIT inoculum (McF)	0.5	0.5	0.5	0.45	0.54	0.38	0.53	0.5	0.4	0.55	0.55	0.47	0.53	0.43	0.55	0.26	0.3	0.36	0.52
CFU count - BMD-MGIT inoculum CFU/ml	Invalid	$6x10^3$	$5x10^3$	$5x10^4$	$1.2x10^4$	$1x10^4$	$2.4x\ 10^5$	Invalid	$8x10^4$	$1x10^4$	$5x10^4$	$1.6x10^4$	$5x10^4$	Invalid	$1.2x\ 10^5$	$5x10^3$	$1.2x\ 10^2$	$5\ x\ 10^4$	$1.2\ x\ 10^5$
MGIT DST - BDQ	S	S	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S	S
Deeplex - BDQ (on sediment)	NA	NA	NA	NA	NA	NA	S	NA	S	NA	NA	NA	R	S	NA	S	NA	NA	NA

BMD-LJ BMD-MGIT

Figure 8.5: Bedaquiline MICs by the two different methods

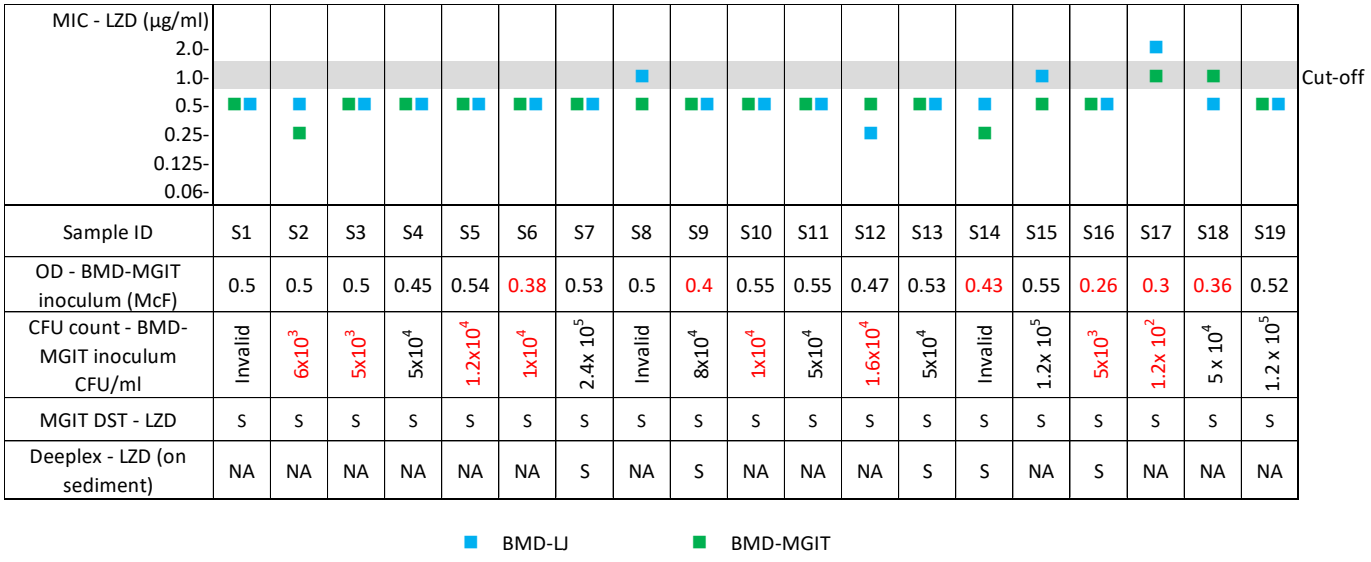
S = Susceptible, R = Resistant, NA = Not available, Cfz = Clofazimine; DST = drug-susceptibility testing, CFU = colony forming units, OD = optical density, MGIT = Mycobacteria Growth Indicator tube, LJ = Löwenstein-Jensen, MIC = minimal inhibitory concentration.

ODs and CFU counts below the desired range are depicted in red text.

8.3.5 Linezolid

Twelve isolates had the same linezolid-MIC using both methods, and seven isolates had a +/- one dilution difference. Four of the seven isolates with a MIC difference between the two techniques had higher MICs using BMD-LJ, whereas three had higher MICs using BMD-MGIT (**Figure 8.6**).

S17, switched from linezolid-resistant to linezolid-susceptible by the BMD-MGIT method; this isolate was linezolid-susceptible also by MGIT DST favoring the BMD-MGIT MIC.



Sample ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19
OD - BMD-MGIT inoculum (McF)	0.5	0.5	0.5	0.45	0.54	0.38	0.53	0.5	0.4	0.55	0.55	0.47	0.53	0.43	0.55	0.26	0.3	0.36	0.52
CFU count - BMD-MGIT inoculum CFU/ml	Invalid	$6x10^3$	$5x10^3$	$5x10^4$	$1.2x10^4$	$1x10^4$	$2.4x\ 10^5$	Invalid	$8x10^4$	$1x10^4$	$5x10^4$	$1.6x10^4$	$5x10^4$	Invalid	$1.2x\ 10^5$	$5x10^3$	$1.2x\ 10^2$	$5\ x\ 10^4$	$1.2\ x\ 10^5$
MGIT DST - LZD	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Deeplex - LZD (on sediment)	NA	NA	NA	NA	NA	NA	S	NA	S	NA	NA	NA	S	S	NA	S	NA	NA	NA

BMD-LJ BMD-MGIT

Figure 8.6: Linezolid MICs by the two different methods

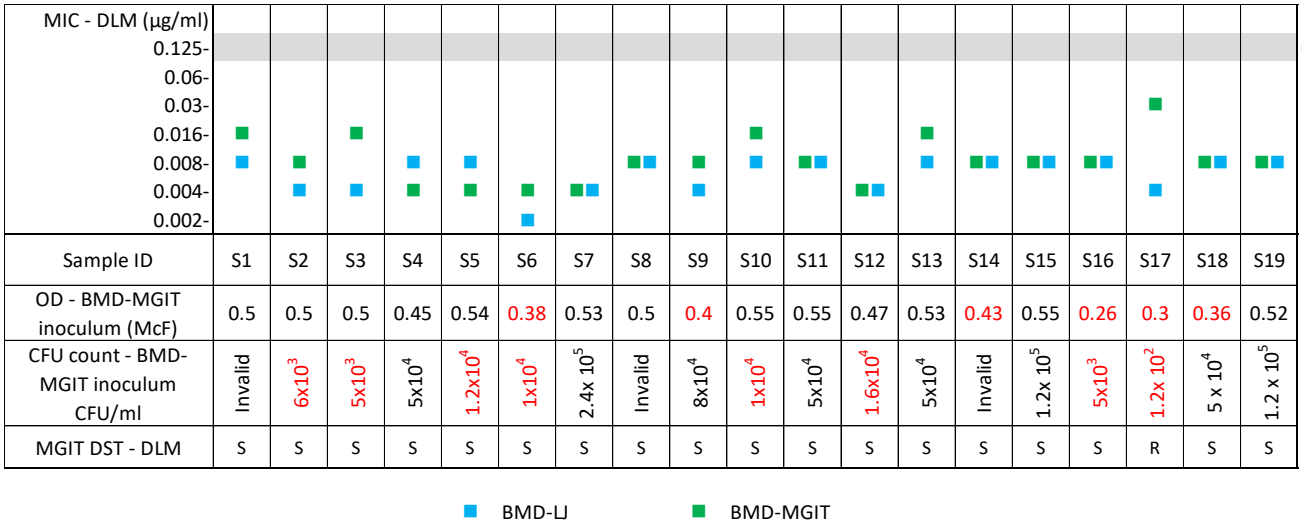
S = Susceptible, R = Resistant, NA = Not available, Cfz = Clofazimine; DST = drug-susceptibility testing, CFU = colony forming units, OD = optical density, MGIT = Mycobacteria Growth Indicator tube, LJ = Löwenstein-Jensen, MIC = minimal inhibitory concentration.

ODs and CFU counts below the desired range are depicted in red text.

8.3.6 Delamanid

Nine isolates had the same delamanid-MIC using both methods; eight isolates had a maximum +/- one dilution difference; one isolate (S3) had a +/- two dilution difference; and one isolate (S17) had a +/- three dilution difference between the two methods. Seven of the 10 isolates with a MIC difference between the two methods had higher MICs using BMD-MGIT, whereas only three had higher MICs using BMD-LJ, suggesting a systematic increase in MICs by the BMD-MGIT approach. (**Figure 8.7**).

S17, which had a three dilution increase in the MICs by BD-MGIT method, was delamanid-resistant by MGIT DST.



Sample ID	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19
OD - BMD-MGIT inoculum (McF)	0.5	0.5	0.5	0.45	0.54	0.38	0.53	0.5	0.4	0.55	0.55	0.47	0.53	0.43	0.55	0.26	0.3	0.36	0.52
CFU count - BMD-MGIT inoculum CFU/ml	Invalid	$6x10^3$	$5x10^3$	$5x10^4$	$1.2x10^4$	$1x10^4$	$2.4x\ 10^5$	Invalid	$8x10^4$	$1x10^4$	$5x10^4$	$1.6x10^4$	$5x10^4$	Invalid	$1.2x\ 10^5$	$5x10^3$	$1.2x\ 10^2$	$5\ x\ 10^4$	$1.2\ x\ 10^5$
MGIT DST - DLM	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S

Figure 8.7: Delamanid MICs by the two different methods

S = Susceptible, R = Resistant, NA = Not available, Cfz = Clofazimine; DST = drug-susceptibility testing, CFU = colony forming units, OD = optical density, MGIT = Mycobacteria Growth Indicator tube, LJ = Löwenstein-Jensen, MIC = minimal inhibitory concentration.

ODs and CFU counts below the desired range are depicted in red text.

8.4 Discussion

Our results suggest that standardized MGIT inocula yield comparable BMD-MICs to that of solid medium inoculum while reducing the turn-around time nearly by half where MGIT is used for primary MTBc isolation.

We used a densitometer to measure the OD of the inocula, reducing potential bias introduced by subjective visual assessments of turbidity. Although the optical density of four inocula did not meet the desired optical density range of McF 0.45 - 0.55, the growth controls of the BMDs inoculated from these inocula exhibited interpretable growth after 14 days of incubation, similar to those that attained the desired OD. Furthermore, we did not observe inocula with lower OD yielding systematically lower or higher MICs. However, the sample size included in this study thus far does not allow an accurate analysis of the impact of the optical density on the MICs. In addition, the densitometer required at least one milliliter of the inoculum to be introduced into the cuvette; consequently, regardless of the size of the pellet obtained by centrifugation of the MGIT culture, all were dissolved in 1 ml of SDW, most likely excessively diluting the smaller pellets. In the future, options for measuring the OD with lower inoculum volumes should be explored.

We found that the CFU count on Middlebrook 7H11 did not always correspond well with an inoculum's OD or capacity to yield an interpretable MIC. In particular, three inocula with ODs ≥ McF 0.5 and did not develop on the CFU plates; yet had interpretable MICs by BMD-MGIT. We did not investigate the probable root reasons for these discrepancies.

For all drugs except clofazimine and linezolid, we observed a systematic increase in the MICs by the BMD-MGIT method, however, in most cases, this increase was restricted to one dilution, thus within the acceptable range of technical variation.

Given the intrinsic variance of BMD, and the accepted variability of one dilution difference on repeat testing, the MICs around the cut-offs may shift from resistant to susceptible or susceptible to resistant, which affected six isolates.

We used retrospective BMD-LJ data in this comparison, hence no CFU counting was done on the inocula prepared using LJ cultures. However, during the validation of the densitometer in our

laboratory, CFU counting was performed for inocula prepared using LJ cultures, and the same inconsistencies between the OD and CFU count as with MGIT-based inocula were observed.

While this preliminary data shows that inocula prepared using MGIT cultures can yield comparable MIC results to that prepared from solid cultures, more testing is required to conclude on its accuracy (including repeatability and reproducibility) and applicability in a routine diagnostic context.

8.5 References

1. WHO. 2022. Optimized broth microdilution plate methodology for drug susceptibility testing of *Mycobacterium tuberculosis* complex.
2. Kahlmeter G, Turnidge J. 2022. How to: ECOFFs—the why, the how, and the don'ts of EUCAST epidemiological cutoff values. Clinical Microbiology and Infection 28:952-954.
3. Clinical and Laboratory Standards Institute. 2001. Development of in vitro susceptibility testing criteria and quality control parameters; approved guideline—2nd ed. CLSI document M23-A2. CLSI, Wayne, PA.

CHAPTER 9

General Discussion

The gap in tuberculosis (TB) diagnostics has been a persistent challenge, spanning decades of efforts to improve the detection of *Mycobacterium tuberculosis* complex (MTBc) and its drug resistance (1). Roll-out of new drugs without availability of accurate DST both for patient care and surveillance risks repeating errors of the past, and rapidly losing these drugs to resistance when patients are 'blindly' treated with already compromised regimens. Expanding research efforts to understand the current gaps in phenotypic drug-susceptibility testing (pDST) in MTBc is crucial for gaining a more comprehensive understanding of the drug-resistance landscape and improving patient-centered TB management strategies.

During my PhD research, we investigated the factors impeding the fast and accurate detection of phenotypic drug resistance, and whether the broth microdilution-based minimum inhibitory concentration (MIC) testing, recently endorsed by the World Health Organization (WHO), provides advantages relative to traditional pDST.

Effect of culture medium on the pDSTs

The most complex treatment decisions pertain to patients who are infected with rifampicin-resistant tuberculosis (RR-TB) strains with additional resistance to novel anti-TB drugs. Such strains have mutations in the *rpoB* gene, which encodes the beta subunit of RNA polymerase in MTBc, causing varying degrees of in vitro growth defects. Since pDST methods measure growth inhibition in drug-containing media, such mutations can lead to higher proportions of failed pDSTs due to insufficient growth on the growth controls (GCs) or result in false susceptibility in case sufficient growth in GCs supports a valid result but the lower fitness of the mutant strains requires longer incubation to grow in the presence of rifampicin. This may be the case for *rpoB* borderline mutations (16). To date, there is no single standard medium universally accepted to assess MTBc's susceptibility to all anti-TB drugs, particularly for fastidious MTBc strains. The choice of culture media for DST can vary depending on factors such as drug to be assessed, laboratory infrastructure, available resources, and local preferences. In **Chapter 3**, we compared two commonly used solid media Middlebrook 7H11 and Middlebrook 7H10 agar. Our results suggest 7H11 being better at reducing the occurrence of invalid results and improving the turn-around time (TAT), particularly in drug-resistant (DR) MTBc. The major difference between these two media is that 7H11 contains an additional nutritional supplement, casein hydrolysate, which

is known to provide essential nutrients and growth factors that support bacterial growth. Even though 7H11 improved pDST outcomes compared to 7H10, indirect pDSTs on solid medium still takes longer than in liquid media. Liquid media such as MGIT are known to promote faster growth in MTBc, however, they are also known to cause false susceptible DST results in fastidious MTBc, particularly borderline *rpoB* mutants (2-4). Although several growth promoters such as nutrient broth, vitamin B12, pyruvate, zinc etc. and resuscitation factors such as antimicrobial peptides have been shown in multiple studies to enhance MTBc recovery in liquid medium from clinical specimens, their potential to improve pDST has not yet received as much attention (5, 6, 17, 18).

Review of critical concentrations (CC) for fluoroquinolones and rifampicin

The CCs have undergone several revisions since the WHO initially introduced them in the 1960s, incorporating data on clinical outcomes, PK/PD, and wildtype distribution where available. However, as such data is still relatively rare for the majority of anti-TB drugs, the current CCs may not always correctly reflect the epidemiological cut-off (ECOFF)(7, 8). In addition, due to the lack of standardization across different pDST methods, the technical variations between different laboratories further complicate comparing data from different laboratories (9). In **Chapters 4 and 5** we evaluated the current CCs recommended by the WHO for fluoroquinolones and rifampicin. Our results from **Chapter 4** suggest that for fluoroquinolones, there is an overlap between the *gyrA/B* wildtypes and low-level fluoroquinolone resistance-conferring mutations. Our findings emphasize the need of quantitative DST rather than binary classification as resistant or susceptible using a single CC and use of an area of technical uncertainty (ATU) as introduced by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (12).

Recognizing that the MGIT 960 system misclassifies rifampicin resistance conferred by borderline *rpoB* mutations, the WHO reduced the CC from 1.0 to 0.5 in MGIT in 2022, anticipating a 21% decrease in misclassifying such mutations (15); however, our results in **Chapter 5** show that, despite this reduction, the vast majority of borderline *rpoB* mutations are still misclassified as susceptible in MGIT. Our study included only a limited number of isolates from just two distinct geographical locations and did not include MIC or replicate testing.

Torrea *et.al* (19) have shown that extending the incubation period beyond the standard maximum of 13 days increased the sensitivity for borderline *rpoB* mutations significantly, from 5.7% using the standard procedure at 1 µg/ml with the pre-set incubation time, to 68.6% using 1µg/ml at 21 days, and to 65.7% using 0.5µg/ml at 15 days. However, extending the incubation time of the MGIT DSTs should be further evaluated on a larger *rpoB* wildtype sample to determine the risk of generating false-resistant results. Additionally, extended incubation requires the TBeXIST software module, which requires manual interpretation of the DST data. Therefore, our data support the recommendation that genotypic DST should overrule MGIT DST for rifampicin, and MGIT should not be used for confirmation of genotypically determined rifampicin resistance despite the updated CC.

Effect of MTBc genetic diversity on the CCs

Increasing evidence on the impact of genetic diversity in MTBc on the in-vitro susceptibility of different drugs has not yet been widely integrated into the CCs (10, 11). The existing knowledge on in-vitro susceptibility to the majority of anti-TB drugs has been largely based on globally dominant lineages 2 and 4. In **Chapter 6** we demonstrated that MTBc lineage 1 (L1) has intrinsically higher and MTBc lineage 6 (L6) has intrinsically lower MICs to the novel anti-TB drug, pretomanid, underscoring the need to expand research to gain a more complete understanding of the drug resistance landscape and identify lineage-specific variations in susceptibility to key drugs used for DR-TB treatment, and to develop effective TB control and treatment strategies tailored to the local epidemiological context.

In 2024, the WHO recommended two cut-off values for pretomanid in MGIT medium, 0.5 and 2.0 µg/ml. If no growth is observed at 0.5 µg/ml, the strain is considered pretomanid-susceptible. If growth is observed at 0.5 µg/ml but not at 2.0 µg/ml, the strain is considered pretomanid-susceptible, with a comment on uncertainty. Any strain with growth at 2.0 µg/ml is considered pretomanid-resistant (14). With this categorization, a subset of MTBc lineage 1 strains will be identified as phenotypically susceptible. The accuracy of these cut-offs should be carefully monitored (more details in **Chapter 10**).

Broth microdilution-based MIC testing as an alternative to CC-based pDST

We assessed the recently approved BMD approach by the WHO in **Chapter 7.** This method may be a substitute for some problems associated with CCs; nevertheless, its implementation in low- and middle-income settings is still complex (12) as manual dispensing of the drugs to the wells, normalization of DMSO across the wells containing drugs dissolved in DMSO and manual interpretation of the results are labor intensive. Thus, cost-effective automation is required to make this approach more field friendly.

While the BMD method could serve as an alternative to the CC-based pDST, certain issues may not be fully resolved by this method either. We observed overlapping wild-type and mutant MIC distributions for both moxifloxacin and levofloxacin. The overlap may theoretically be reduced by evaluating additional intermediate drug concentrations, although two-fold dilutions are usually examined in MIC testing. Future WHO recommendations should include ATU (12), along with a warning that MICs falling within the ATU should be interpreted carefully, as such isolates may not be classified as resistant or susceptible based on a single MIC result.

In addition, more standardized guidance should be provided particularly for the interpretation of pinpoint colonies that are substantially smaller than the growth observed in the 1% growth control.

In **Chapter 8,** we assessed the feasibility of using freshly positive actively growing MGIT cultures to yield accurate MIC results for fluoroquinolones, new and repurposed anti-TB drugs using the BMD method. Since BMD offers semi-quantitative DST results with the same turnaround time as the commercial, more expensive MGIT DST, it could serve as an alternative to MGIT-DST, providing a quantitative result. While our data in Chapter 7 shows promising results, to conclude, further testing including all key anti-TB drugs and testing in multiple laboratories is required.

9.1 References

1. Singh S, Dey B, Sachdeva KS, Kabra SK, Chopra KK, Chaudhary VK, Sharma P, Katoch VM. 2015. Challenges in tuberculosis diagnosis and management: recommendations of the expert panel. J Lab Physicians 7:1-3.
2. Van Deun A, Aung KJ, Bola V, Lebeke R, Hossain MA, de Rijk WB, Rigouts L, Gumusboga A, Torrea G, de Jong BC. 2013. Rifampin drug resistance tests for tuberculosis: challenging the gold standard. J Clin Microbiol 51:2633-40.
3. Ocheretina O, Escuyer VE, Mabou M-M, Royal-Mardi G, Collins S, Vilbrun SC, Pape JW, Fitzgerald DW. 2014. Correlation between Genotypic and Phenotypic Testing for Resistance to Rifampin in *Mycobacterium tuberculosis* Clinical Isolates in Haiti: Investigation of Cases with Discrepant Susceptibility Results. PLOS ONE 9:e90569.
4. Rafael LL, Raquel MS, Rogelio FA, Miroslava FP, Alejandra-Isabel JG, Paola RS. 2021. Discordant results between genotypic and phenotypic assays (Xpert MTB/RIF vs. BACTEC MGIT 960 system) for detection of RIF-resistant *Mycobacterium tuberculosis* isolates in a high burden region. Infect Genet Evol 96:105142.
5. Brittle W, Marais BJ, Hesseling AC, Schaaf HS, Kidd M, Wasserman E, Botha T. 2009. Improvement in Mycobacterial Yield and Reduced Time to Detection in Pediatric Samples by Use of a Nutrient Broth Growth Supplement. Journal of Clinical Microbiology 47:1287-1289.
6. Hilpert K, Munshi T, López-Pérez PM, Sequeira-Garcia J, Hofmann S, Bull TJ. 2023. Discovery of Antimicrobial Peptides That Can Accelerate Culture Diagnostics of Slow-Growing Mycobacteria Including *Mycobacterium tuberculosis*. Microorganisms 11:2225.
7. WHO. 2018. Technical Report on critical concentrations for drug susceptibility testing of medicines used in the treatment of drug-resistant tuberculosis. https://www.who.int/tb/publications/2018/WHO_technical_report_concentrations_TB_drug_susceptibility/en/.
8. CLSI. 2018. CLSI. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes; approved standard. 2nd ed.
9. Schön T, Matuschek E, Mohamed S, Utukuri M, Heysell S, Alffenaar JW, Shin S, Martinez E, Sintchenko V, Maurer FP, Keller PM, Kahlmeter G, Köser CU. 2019. Standards for MIC testing that apply to the majority of bacterial pathogens should also be enforced for *Mycobacterium tuberculosis* complex. Clin Microbiol Infect 25:403-405.
10. Rupasinghe P, Reenaers R, Vereecken J, Mulders W, Cogneau S, Merker M, Niemann S, Omar SV, Rigouts L, Köser CU, Decroo T, Jong BCd. Refined understanding of the impact of the *Mycobacterium tuberculosis* complex diversity on the intrinsic susceptibility to pretomanid. Microbiology Spectrum 0:e00070-24.
11. Battaglia S CM, Stenback Gabro V. 2021. Lineage 1 has elevated pyrazinamide minimum inhibitory concentrations compared with other lineages of *Mycobacterium tuberculosis* complex. , abstr 41st Annual Congress of the European Society of Mycobacteriology.
12. WHO. 2022. Optimized broth microdilution plate methodology for drug susceptibility testing of *Mycobacterium tuberculosis* complex.
13. WHO. 2021. Technical Report on critical concentrations for drug susceptibility testing of isoniazid and the rifamycins (rifampicin, rifabutin and rifapentine).

14. WHO. 2024. Module 3: Diagnosis WHO operational handbook on tuberculosis Rapid diagnostics for tuberculosis detection.
15. Gagneux S. 2018. Ecology and evolution of *Mycobacterium tuberculosis*. Nat Rev Microbiol 16:202–213. Cross ref PubMed. ISI.
16. Deun, A.V. et al. *Mycobacterium tuberculosis* borderline rpoB mutations: emerging from the unknown. European Respiratory Journal 58, 2100783 (2021).
17. Difco Laboratories. 1984. Manual of dehydrated culture media and reagents, 10th ed., p. 108-109 and 622-623. Difco Laboratories Incorporated, Detroit, MI.
18. Power, D. A., and P. J. McCuen (ed.). 1988. BBL manual of products and laboratory procedures, 6th ed., p. 1-9, 26-27, and 178-179. BBL, Division of Becton, Dickinson and Co., Cockeysville, MD.
19. Torrea, G. et al. Variable ability of rapid tests to detect *Mycobacterium tuberculosis* rpoB mutations conferring phenotypically occult rifampicin resistance. Scientific Reports 9, 11826 (2019).

CHAPTER 10

Future Research

10.1 Implementation and clinical application of MIC testing for MTBc at the supranational and national reference laboratory level

During this PhD research work, we have demonstrated the feasibility of using freshly positive actively growing MGIT cultures to yield accurate MIC results for fluoroquinolones, new and repurposed anti-TB drugs using the BMD method. Since BMD offers quantitative DST results with the same turnaround time as the commercial, more expensive MGIT DST, it could serve as an alternative to MGIT DST. However, several further research questions need to be addressed to ensure the field friendliness of this method.

During the implementation validation of BMD at ITM, we noted the occurrence of pinpoint colonies that are substantially smaller than the growth observed in the 1% growth control. While most drugs show no trailing or have only a single pinpoint well at the end of their trailing growth, other drugs, such as delamanid and pretomanid, can show multiple pinpoint wells. The EUCAST multicenter validation study so far experienced little to no pinpoint colonies, and one of the primary differences between the EUCAST and BMD methods is that during inoculum preparation the EUCAST technique requires twice two minutes of vortexing the bacterial suspension, whereas the BMD method employs one minute of vortexing followed by another 15 seconds of vortexing. Thus, it should be investigated if increasing the vortexing period in the BMD inoculum production process reduces the prevalence of pinpoint colonies. Furthermore, it should be explored if the second reading is done between day 7 and day 14 rather than day 14 reduces the occurrence of pinpoint growth.

Then, BMD relies on human interpretation for the presence or lack of macroscopic growth; indeed, plate readings can be subjective to the reader and, in some situations, to lighting conditions. Presence of pinpoint growth increases the risk of inter-reader variability. To minimize the impact of such external factors, further research on automated plate reading that can provide reliable data at a low cost is required.

In addition, since the BMD method involves pipetting of minute volumes, human pipetting is extremely time-consuming and prone to errors, hence automated dispensing of antibiotics should be used in conjunction with this method. Thus, to ensure the implementation of this method is affordable in low- and middle-income settings, further research should be performed

to determine the market availability of low-cost, open-source alternatives for automated drug distribution/DMSO normalization.

Further, a comprehensive multicenter validation including all key anti-TB drugs, all MTBc lineages and both low- and high-level resistance conferring mutations should be conducted, preferably in settings with high prevalence of DR-TB before implementing this method in field level for clinical use.

Since MIC testing is not yet widely used for MTBc, the correlation between treatment outcomes and DST results is often based on categorical DST that relies on testing at the CC or for fluoroquinolones additionally at the clinical breakpoint. Such analysis excludes a refined analysis on the impact of the level of drug resistance on treatment outcome. Thus, efforts should be made to correlate treatment outcomes to MIC data for key drugs, preferably alongside genotypic data to enrich the WHO mutation catalogue.

Furthermore, serial MIC testing of patients who remain culture positive may identify a progressive increase in MICs due to early stages of amplifying drug resistance, even if the MIC remains lower than the CC. Thus, the potential of serial MIC testing as an early treatment response predictor should be investigated.

10.2 Collateral drug resistance/susceptibility in MTBc

During the first phase of in-vitro selection for clofazimine resistance, one isolate had a deletion at codon 344-345 of the *rv0678* gene after being exposed to 4 µg/ml clofazimine. This mutation demonstrated low-level phenotypic resistance to both clofazimine (1 µg/ml by BMD) and bedaquiline (0.5 µg/ml by BMD). A *fbiC* mutation, *Arg536Leu* was amplified during the second round of selection, when the same isolate was exposed to a higher concentration of clofazimine (>4≤8µg/ml), which further increased the MIC of clofazimine (4 µg/ml by BMD). This isolate was tested in the study "Refined understanding of the impact of the *Mycobacterium tuberculosis* complex diversity on the intrinsic susceptibility to pretomanid" (**Chapter 5**) included in this PhD thesis and was found also phenotypically resistant to pretomanid and delamanid. Random

amplification of nitroimidazole resistance during in-vitro selection of clofazimine-resistant strains has also been observed elsewhere (Ismail, N. *et al*, unpublished data).

Collateral drug-resistance is commonly found in drugs or drug classes that share the same mechanism of action however, the above examples warrant further investigation of collateral resistance between unrelated drugs/drug classes, particularly in clinical isolates to design more effective treatment regiments. In addition to collateral drug-resistance, understanding collateral drug-susceptibility among different anti-TB drugs is important in choosing the most optimal treatment combinations.

Further, the isolate that amplified nitroimidazole resistance after being exposed to clofazimine belonged to the MTBc lineage 1. MTBc lineage 1 is reported to have intrinsically high MICs to one of the two nitroimidazoles, pretomanid. Thus, it is worth investigating if the bacterial genetic backbone has an impact on collateral drug-susceptibility or resistance.

Finally, investigating potential past clofazimine exposure in clinical isolates reported resistant to nitroimidazoles without being previously exposed to nitroimidazoles may give greater insight into whether clofazimine exposure might cause nitroimidazole resistance in MTBc.

10.3 Lineage and intrinsically high MICs for different anti-TB drugs

Mycobacterium tuberculosis Lineage 1 (MTBc L1) is a distinct and ancient lineage with unique genetic, epidemiological, and clinical characteristics. L1 is predominant in the Indo-Oceanic region and, compared to the modern lineages, less virulent, and less frequently associated with MDR-TB (4,12,13). In **Chapter 5** of this PhD thesis, we demonstrated that MTBc L1 has intrinsically higher MICs to pretomanid than the other MTBc lineages, which is consistent with prior findings by Bateson *et al* (3). This is not the only instance where MTBc L1 has demonstrated higher MICs to anti-TB medicines. At the 41st European Society of Mycobacteriology meeting in Bologna, Battaglia *et al*. reported that MTBc L1 exhibits higher pyrazinamide MICs than other lineages (2). In addition, at ITM, we evaluated the lineage-specific response to a new ethionamide-boosting drug (brand name withheld until publication of the results at the request of the manufacturer) and discovered that L1 is unresponsive to this molecule.

Understanding the historic genetic background variation in the evolution of drug resistance, between the MTBc lineages, which has not been thoroughly investigated, is critical in the development of innovative anti-TB drugs. Thus, a lineage-specific genome-wide association study to investigate the potential cause behind L1's intrinsically high MICs/unresponsiveness to certain anti-TB drugs should be conducted.

In addition, it is important to monitor if all isolates that belong to the 'uncertain' category by the current WHO-recommended cut-offs for pretomanid are explained by L1, and if not, non-L1 lineages that belong to the 'uncertain' category carry mutations in any of the canonical genes associated with nitroimidazole resistance.

Further as demonstrated in **Chapter 5**, given the intrinsic hyper susceptibility of L6 to pretomanid, it should be investigated if pretomanid-resistance-conferring mutations in the known canonical genes confer the same level of phenotypic resistance as for the other lineages, without misclassifying them when the current WHO-recommended cut-offs are applied.

10.4 Cross resistance between the anti-TB nitroimidazoles

Delamanid and pretomanid are two relatively new anti-TB drugs that belong to a class of compounds known as nitroimidazoles (5). Delamanid is currently recommended as part of the 9-months or longer MDR-TB treatment regimens, whilst pretomanid is recommended as a part of the 6-month BPal(M) regimens. Both drugs have a comparable dual mode of action, inhibiting the synthesis of mycolic acids, essential components of the mycobacterial cell wall and respiratory poisoning (6). Given the similarities in mode of actions, the likelihood of cross resistance between the two drugs is high. However, several studies using pre-clinical or limited clinical isolates have reported varying levels of cross resistance (8-11). While mutations in the genes involved for metabolic activation of both drugs, *fbiA*, *fbiB*, *fbiC*, *fbiD*, *fgd1*, and *ddn*, have been linked to resistance to delamanid and pretomanid, additional genes, such as *ndh*, may also be linked to delamanid resistance (7,8).

To date, there is limited data on the frequency and levels of pretomanid resistance in clinical isolates, that have amplified resistance to delamanid after receiving delamanid-containing treatment regimens, and their eligibility to receive the now-preferred BPal(M) regimen and vice

versa. Our study will include the clinical isolates from the patients who received delamanid-containing treatment regimens in the endTB observational study, as well as the endTB and endTB-Q clinical trials, and who developed delamanid resistance during treatment. We will analyze whole genome sequencing data and MICs for pretomanid and delamanid, aiming to understand,

- the frequency and the level of pretomanid resistance in delamanid-resistant isolates
- the potential gene mutations associated with resistance to both nitroimidazoles, as well as individual resistance to delamanid and pretomanid
- the frequency of bedaquiline and linezolid resistance among delamanid-resistant, pretomanid-susceptible isolates

Currently, only a small number of clinical isolates that amplified pretomanid resistance during BPal(M) regimens are available; however, with the growing use of this regimen over the world, such isolates may become more common. In that case, a similar analysis should be performed on such isolates to understand if delamanid can still be used for certain patients that amplified resistance to pretomanid.

In addition, since MTBc L1 shows intrinsically higher MICs to pretomanid, it is worth investigating if the risk of acquiring pretomanid resistance during BPal(M) regimens is relatively higher for MTBc lineage 1 strains.

10.5 Reference

1. Waller NJE, Cheung C-Y, Cook GM, McNeil MB. 2023. The evolution of antibiotic resistance is associated with collateral drug phenotypes in *Mycobacterium tuberculosis*. Nature Communications 14:1517.
2. Battaglia S CM, Stenback Gabro V. 2021. Lineage 1 has elevated pyrazinamide minimum inhibitory concentrations compared with other lineages of *Mycobacterium tuberculosis* complex. , abstr 41st Annual Congress of the European Society of Mycobacteriology.
3. Bateson A, Ortiz Canseco J, McHugh TD, Witney AA, Feuerriegel S, Merker M, Kohl TA, Utpatel C, Niemann S, Andres S, Kranzer K, Maurer FP, Ghodousi A, Borroni E, Cirillo DM, Wijkander M, Toro JC, Groenheit R, Werngren J, Machado D, Viveiros M, Warren RM, Sirgel F, Dippenaar A, Köser CU, Sun E, Timm J. 2022. Ancient and recent differences in the intrinsic susceptibility of *Mycobacterium tuberculosis* complex to pretomanid. J Antimicrob Chemother 77:1685-1693.
4. Coscolla, M. & Gagneux, S. Consequences of genomic diversity in *Mycobacterium tuberculosis*. Semin Immunol 26, 431-44 (2014)
5. Mudde, S.E., Upton, A.M., Lenaerts, A., Bax, H.I. & De Steenwinkel, J.E.M. Delamanid or pretomanid? A Solomonic judgement! J Antimicrob Chemother 77, 880-902 (2022).
6. Boshoff, H.I. et al. The transcriptional responses of *Mycobacterium tuberculosis* to inhibitors of metabolism: novel insights into drug mechanisms of action. J Biol Chem 279, 40174-84 (2004).
7. WHO. Catalogue of mutations in *Mycobacterium tuberculosis* complex and their association with drug resistance. 2nd Edition edn (2023).
8. Hayashi, M. et al. Adduct Formation of Delamanid with NAD in Mycobacteria. Antimicrob Agents Chemother 64(2020).
9. Rifat, D. et al. Mutations in fbiD (Rv2983) as a Novel Determinant of Resistance to Pretomanid and Delamanid in *Mycobacterium tuberculosis*. Antimicrobial Agents and Chemotherapy 65, e01948-20 (2020).
10. Wen, S.a. et al. Comparison of in vitro activity of the nitroimidazoles delamanid and pretomanid against multidrug-resistant and extensively drug-resistant tuberculosis. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 38, 1293-1296 (2019).
11. Lee, B.M. et al. Predicting nitroimidazole antibiotic resistance mutations in *Mycobacterium tuberculosis* with protein engineering. PLOS Pathogens 16, e1008287 (2020).
12. Ajawatanawong, P. et al. A novel Ancestral Beijing sublineage of *Mycobacterium tuberculosis* suggests the transition site to Modern Beijing sublineages. Scientific Reports 9, 13718 (2019).
13. Buu, T.N. et al. The Beijing genotype is associated with young age and multidrug-resistant tuberculosis in rural Vietnam. Int J Tuberc Lung Dis 13, 900-6 (2009).
14. Lee, B.M. et al. Predicting nitroimidazole antibiotic resistance mutations in *Mycobacterium tuberculosis* with protein engineering. PLOS Pathogens 16, e1008287 (2020).

Acknowledgement

My PhD dream would not have been possibility without the support and guidance of several teams who in one way or another contributed and supported me in preparation and completion of my PhD research within a short time frame.

Words cannot express my gratitude to my PhD supervisors, Prof. Bouke de Jong and Prof. Leen Rigouts for their invaluable guidance, unwavering support and advice that shaped the work compiled in this PhD thesis.

I would also like to thank my internal PhD defense committee members, Prof Erik Fransen and Prof Paul Cos for serving as my committee members.

I am grateful to all my colleagues at ITM and in the endTB project, as well as to everyone I collaborated with externally, for their support and input.

Lastly, I thank my family and friends, my husband who has been solidly behind me throughout this process, keeping my spirits and motivation high, my mother, and my late father for all of the sacrifices that they've made on my behalf.

Curriculum Vitae

Education			
Institution	**Degree**	**Date**	**Field**
Post Graduate Institute of Science, University of Peradeniya, Sri Lanka	Master of Science	5/2008-10/2010	Medical Microbiology
University of Kelaniya, Sri Lanka	Bachelor of Science	4/2004-5/2008	Chemistry, Zoology, Microbiology

Work Experience			
Institution	**Position**	**Date**	**Location**
Mycobacteriology Unit, Institute of Tropical Medicine, Antwerp	Scientific Assistant	11/2017-to date	Belgium
Médecins sans frontières	Laboratory Manager – TB, HIV, Hepatitis C	6/2016-7/2017	Nukus & Tashkent, Uzbekistan
Médecins sans frontières	Laboratory Manager – Ebola response & primary health care	12/2015-2/2016	Magburaka, Tonkolili district, Sierra Leone
Médecins sans frontières	Laboratory Manager – TB and comprehensive health care	2/2015-11/2015	Lashkar Gah, Helmand, Afghanistan
Médecins sans frontières	Laboratory Advisor – TB, HIV	10/2012-10/2014	Mbabane, Eswatini
Duke-Ruhuna collaboration, Faculty of Medicine, University of Ruhuna	Research Coordinator – Respiratory infections	6/2011-6/2012	Galle, Sri Lanka

Training courses completed			
Institution	**Training course**	**Year**	**Location**
Vlaamse Interuniversitaire Raad	Research integrity: online training tool Mind the GAP	2024	Online
Institute of Tropical Medicine, Antwerp	Multivariable analysis using R	2024	Antwerp, Belgium

Institute of Tropical Medicine, Antwerp	General data protection regulation (GDPR)	2023	Antwerp, Belgium
Sunnikan Consulting, Paris, France	Good clinical practices (GCP)	2022	Online
Institute of Tropical Medicine, Antwerp	Good clinical laboratory practices (GCLP)	2021	Antwerp, Belgium
SGS Belgium	International transport of dangerous goods, infectious substances cat A and B	2018	Antwerp, Belgium
Institute of Microbiology & Laboratory Medicine, Gauting	Quality management of TB laboratories – SLIPTA, SLMTA tools	2017	Tashkent, Uzbekistan
University of Washington	Advance didactic and laboratory training for global health	2012	Seattle, USA
University of Washington	Principles of STD/HIV Research	2012	Seattle, USA
PATH and the University of Washington	Point of Care Diagnostics for Global Health	2011	Seattle, USA
University of Peradeniya	Analysis of experimental data	2011	Kandy, Sri Lanka
Genetech Sri Lanka	Comprehensive Polymerase chain reaction technology	2011	Colombo, Sri Lanka
University of Peradeniya	Molecular diagnosis of bacterial infectious diseases	2009	Kandy, Sri Lanka

Professional memberships
Registered medical laboratory technologist of Sri Lanka Medical Council.

List of Publications

1. **Rupasinghe P**, Ashraf A, Barreda N, Parveen S, Zubair M, Calderon R, Asif S, Hirani N, Chingisova L, Bulane A, Hang Pham T, Ha Doan T, Ardizzoni E, Kursheed N, De Rijk Willem B, Rigouts L, Guglielmetti L, Mitnick C, de Jong Bouke C. 2024. Reduced critical concentration might not have improved MGIT-based DST's sensitivity to rifampicin. Antimicrobial Agents and Chemotherapy 0:e01701-23.

2. **Rupasinghe P**, Reenaers R, Vereecken J, Mulders W, Cogneau S, Merker M, Niemann S, Omar SV, Rigouts L, Köser CU, Decroo T, Jong BCd. Refined understanding of the impact of the *Mycobacterium tuberculosis* complex diversity on the intrinsic susceptibility to pretomanid. Microbiology Spectrum 0:e00070-24.

3. **Rupasinghe P**, Driesen M, Vereecken J, de Jong BC, Rigouts L. 2023. Re-evaluation of Critical Concentrations of Antituberculosis Fluoroquinolones in the Mycobacteria Growth Indicator Tube 960 System. The International Journal of Mycobacteriology 12:316-323.

4. **Rupasinghe P**, Vereecken J, Graulus P, Decroo T, Ardizzoni E, Hewison C, Donchuk D, Huerga H, Mesic A, Rigouts L, de Jong BC. 2022. Middlebrook 7h11 reduces invalid results and turnaround time of phenotypic drug-susceptibility testing of *M. tuberculosis*. Int J Mycobacteriol 11:407-411.

5. Patil SB, Tamirat M, Khazhidinov K, Ardizzoni E, Atger M, Austin A, Baudin E, Bekhit M, Bektasov S, Berikova E, Bonnet M, Caboclo R, Chaudhry M, Chavan V, Cloez S, Coit J, Coutisson S, Dakenova Z, De Jong BC, Delifer C, Demaisons S, Do JM, Dos Santos Tozzi D, Ducher V, Ferlazzo G, Gouillou M, Khan U, Kunda M, Lachenal N, LaHood AN, Lecca L, Mazmanian M, McIlleron H, Moreau M, Moschioni M, Nahid P, Osso E, Oyewusi L, Panda S, Pâquet A, Thuong Huu P, Pichon L, Rich ML, **Rupasinghe P**, Salahuddin N, Sanchez Garavito E, Seung KJ, Velásquez GE, Vallet M, Varaine F, et al. 2023. Evaluating newly approved drugs in combination regimens for multidrug-resistant tuberculosis with fluoroquinolone resistance (endTB-Q): study protocol for a multi-country randomized controlled trial. Trials 24:773.

6. Van Rie A, Walker T, de Jong B, **Rupasinghe P**, Rivière E, Dartois V, Sonnenkalb L, Machado D, Gagneux S, Supply P, Dreyer V, Niemann S, Goig G, Meehan C, Tagliani E, Cirillo DM. 2022. Balancing access to BPaLM regimens and risk of resistance. The Lancet Infectious Diseases 22:1411-1412.

7. Guglielmetti L, Ardizzoni E, Atger M, Baudin E, Berikova E, Bonnet M, Chang E, Cloez S, Coit JM, Cox V, de Jong BC, Delifer C, Do JM, Tozzi DDS, Ducher V, Ferlazzo G, Gouillou M, Khan A, Khan U, Lachenal N, LaHood AN, Lecca L, Mazmanian M, McIlleron H, Moschioni M, O'Brien K, Okunbor O, Oyewusi L, Panda S, Patil SB, Phillips PPJ, Pichon L, **Rupasinghe P**, Rich ML, Saluhuddin N, Seung KJ, Tamirat M, Trippa L, Cellamare M, Velásquez GE, Wasserman S, Zimetbaum PJ, Varaine F, Mitnick CD. 2021. Evaluating newly approved drugs for multidrug-resistant tuberculosis (endTB): study protocol for an adaptive, multi-country randomized controlled trial. Trials 22:651.

Conference presentations/abstracts

1. **Rupasinghe P**, Vereecken J, Desmaretz C, MIC distribution of the endTB and endTB Q clinical trial isolates for the new and repurposed anti-TB drugs; Mycodays 2024, Institut Pasteur de Lille, France (Oral presentation).

2. **Rupasinghe P**, Reenaers R, Vereecken J, *et al.* Refined understanding of the impact of the Mycobacterium tuberculosis complex diversity on the intrinsic susceptibility to pretomanid; 4th Asian African congress of Mycobacteriology, Online (Oral presentation).

3. **Rupasinghe P**, Van de Straeten N, Aissatou D, *et al.* Broth microdilution plate-based MIC testing using MGIT-positive cultures; European Society of Mycobacteriology annual conference 2024, Bruges, Belgium (Poster).

4. **Rupasinghe P**, Ashraf A, Parveen S, *et al*. Reduced critical concentration might not have improved MGIT-based DST's sensitivity to rifampicin; International Union Against Tuberculosis and Lung Diseases conference 2023, Paris, France (Oral presentation).

5. **Rupasinghe P**, Reenaers R, Vereecken J, *et al.* Prolonged heating should be avoided during the preparation of Delamanid containing Middlebrook 7H11 medium; European Society of Mycobacteriology annual conference 2023, Tirana, Albania (Poster).

6. **Rupasinghe P**, Ashraf A, Mitnick C, *et al*. Xpert MTB/RIF's higher rate of valid drug-susceptibility results compared to MTBDRsl informs switch to Xpert MTB/XDR; International Union Against Tuberculosis and Lung Diseases conference 2022 (Oral presentation).

7. **Rupasinghe P**, Vereecken J, Graulus P, *et al*. Middlebrook 7h11 reduces invalid results and turnaround time of phenotypic drug-susceptibility testing of *M. tuberculosis*; European Society of Mycobacteriology annual conference 2022, Bologna, Italy (Poster).

8. **Rupasinghe P**, Driesen M, Vereecken J, *et al*. Re-evaluation of critical concentrations of anti-TB fluoroquinolones in the MGIT 960 system; European Society of Mycobacteriology annual conference 2022, Bologna, Italy (Poster).

9. **Rupasinghe P**, Reenaers R, Vereecken J, *et al.* Intrinsic differences in in-vitro susceptibility of *Mycobacterium tuberculosis* to pretomanid; European Society of Mycobacteriology annual conference 2022, Bologna, Italy (Poster).