Transmission of bedaquiline-resistant tuberculosis in the Western Cape Province, South Africa

by

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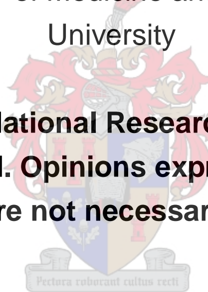

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Abstract

Bedaquiline (BDQ) is the first novel drug to be approved for the treatment of multi-drug resistant TB (MDR-TB) in 40 years. Reduced susceptibility to BDQ commonly occurs through variants in the genes *Rv0678*, *atpE* and *pepQ*.

The aim of this study was to determine the prevalence of BDQ genotypic resistance in the Western Cape, using a high throughput screening method to investigate the acquisition of variants in the three candidate genes and associated phenotypic resistance, as well as potential transmission network of BDQ-resistance.

A total of 326 patient isolates with rifampicin-resistant TB (RR-TB) at baseline were selected between January 2018 to February 2019 using the STATA software for sample processing from the longitudinal drug-resistant strain bank at the Division of Molecular Biology and Human Genetics at Stellenbosch University (SU).

Crude DNA was used for library preparation and targeted deep sequencing (TDS) of the BDQ-resistance candidate genes (*Rv0678*, *atpE* and *pepQ*) using a protocol designed by the translational genomics research institute (TGen). The first TDS run was performed at TGen in Flagstaff, USA on an Illumina MiSeq platform and 146 patient isolates were sequenced. The expertise was then transferred to SU and the protocol was optimised. The second TDS run was done at the South African Medical Research Council (SAMRC) on an Illumina MiniSeq platform and 180 patient isolates were sequenced.

Data obtained from collective TDS runs were analysed using the TB-specific Amplicon Sequencing Analysis Pipeline and yielded 15 isolates with variants suitable for further phenotypic drug susceptibility testing (pDST) using the BACTEC MGIT 960 system. Of the variants detected; six were *Rv0678* variants, eight were *pepQ* variants and one was an *atpE* variant. Two of the isolates with *Rv0678* indels at high frequencies (>97%) were BDQ-resistant, suggesting a phenotypic BDQ-resistance frequency of 0.61% (2/362).

Spoligotyping revealed that 9/13 isolates belonged to lineage 2 (Beijing genotype) and three isolates belonged to lineage 4 (one T1 strain, one LAM3 strain and one LAM1-LAM4 strain).

A subset of six isolates, from the first TDS run, was selected for minimum inhibitory concentration (MIC) testing using the CRyPTIC UKMYC6 plates and for whole-genome sequencing (WGS) on the MiniSeq platform. The UKMYC6 plate data and the WGS data for the subset identified the drug-resistant profiles, which ranged from rifampicin mono-resistant to extensively drug-resistant TB. The two isolates presented with identical sub-lineages (2.2.2) and the phylogeny indicated the close relatedness of the patient isolates.

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The findings from this study suggest that it is possible to use NGS to screen hundreds of patient isolates to identify BDQ resistance-associated variants (RAVs) in the community. However, this approach is dependent on the availability of a comprehensive catalogue of BDQ RAVs with the accompanying pDST and MIC data to support the genotype-phenotype.

In the future NGS surveillance methods could be used in clinical decision-making for designing effective treatment regimens containing BDQ, as well as to investigate transmission within the community. Additionally, this could help to preserve this novel antibiotic to ensure its longstanding use in successful drug-resistant TB therapy in the future.

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Opsomming

Bedaquiline (BDQ) is die eerste nuwe middel wat goedgekeur is vir die behandeling van multi-middel weerstandbiedende TB (MDR-TB) in 40 jaar. Verminderde vatbaarheid vir BDQ kom algemeen voor deur variante in die gene Rv0678, atpE en pepQ.

Die doel van hierdie studie was om die voorkoms van BDQ genotipiese weerstand in die Wes-Kaap te bepaal, deur gebruik te maak van 'n hoë deurvloei siftingsmetode om die verkryging van variante in die drie kandidaatgene en gepaardgaande fenotipiese weerstand te ondersoek, asook potensiële oordragnetwerk van BDQ- weerstand.

Altesaam 326 pasiënt-isolate met rifampisienweerstandige TB (RR-TB) by die basislyn is tussen Januarie 2018 tot Februarie 2019 geselekteer deur gebruik te maak van die STATA-sagteware vir monsterverwerking van die longitudinale geneesmiddel-weerstandige stambank by die Afdeling Molekulêre Biologie en Mensgenetika by Universiteit Stellenbosch (US).

Ru-DNS is gebruik vir biblioteekvoorbereiding en geteikende diepvolgordebepaling (TDS) van die BDQ-weerstandkandidaatgene (Rv0678, atpE en pepQ) met behulp van 'n protokol wat deur die translasiegenomika-navorsingsinstituut (TGen) ontwerp is. Die eerste TDS-lopie is by TGen in Flagstaff, VSA op 'n Illumina MiSeq-platform uitgevoer en 146 pasiënt-isolate is georden. Die kundigheid is toe na die US-universiteit oorgedra en die protokol is geoptimaliseer. Die tweede TDS-lopie is by die Suid-Afrikaanse Mediese Navorsingsraad (SAMRC) op 'n Illumina MiniSeq-platform gedoen en 180 pasiënt-isolate is in volgorde geplaas.

Kollektiewe TDS-lopies volgordebepalingdata is ontleed deur gebruik te maak van die TB-spesifieke Amplicon Sequencing Analysis Pipeline en het 15 isolate gelewer met variante vir verdere fenotipiese geneesmiddel vatbaarheidstoetsing (pDST) deur gebruik te maak van die BACTEC MGIT 960 stelsel. Van die variante wat opgespoor is; ses was *Rv0678* variante, ag was *pepQ* variante en een was 'n atpE variant. Twee van die isolate met Rv0678 indels by hoë frekwensies (>97%) was BDQ-bestand.

Spoligotipering het aan die lig gebring dat 9/13 isolate aan geslag 2 (Beijing genotipe) behoort het en vier isolate aan geslag 4 behoort (een T1-stam, een LAM3-stam en een LAM1-LAM4-stam).

'n Subset van ses isolate, vanaf die eerste TDS-lopie, is geselekteer vir minimum inhiberende konsentrasie (MIC) toetsing deur gebruik te maak van die CRyPTIC UKMYC6 plate en vir

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heelgenoom volgordebepaling (WGS) op die MiniSeq platform. Die UKMYC6-plaatdata en die WGS-data vir die subset het die middelweerstandige profiele geïdentifiseer, wat gewissel het van rifampisien mono-weerstandige tot ekstensief middelweerstandige TB. Die twee isolate wat met identiese sub-afstammelinge aangebied is (2.2.2) en die filogenie het die noue verwantskap van die pasiënt-isolate aangedui.

Die bevindinge van hierdie studie dui daarop dat dit moontlik is om NGS te gebruik om honderde pasiënt-isolate te skerm om BDQ-weerstand-geassosieerde variante (RAVs) in die gemeenskap te identifiseer. Hierdie benadering is egter afhanklik van die beskikbaarheid van 'n omvattende katalogus van BDQ RAV's met die gepaardgaande pDST en MIC data om die genotipe-fenotipe te ondersteun.

In die toekoms kan NGS-toesigmetodes gebruik word in kliniese besluitneming vir die ontwerp van effektiewe behandelingsregimes wat BDQ bevat, asook om oordrag binne die gemeenskap te ondersoek. Daarbenewens kan dit help om hierdie nuwe antibiotika te bewaar om die langdurige gebruik daarvan in suksesvolle middelweerstandige TB-terapie in die toekoms te verseker.

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List of abbreviations

%	Percent
°C	Degrees Celsius
~	Approximately
A	Adenine
AE	Adverse event
AMI	Amikacin
BA	Blood agar
BAM	Binary Alignment Map
BDQ	Bedaquiline
BMD	Broth microdilution
bp	Base pairs
BPaL	Bedaquiline, Pretomanid and Linezolid regimen
BSL3	Biosafety Level 3
BWA	Burrows-Wheeler aligner
C	Cytosine
CC	Critical concentration
CFZ	Clofazimine
CPR	Capreomycin
CRyPTIC	Comprehensive Resistance Prediction for Tuberculosis: An International Consortium
CTAB	Hexadecyltrimethylammonium bromide
dH2O	Distilled water
DLM	Delamanid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DR	Direct repeat
dsDNA	double stranded DNA
DST	Drug susceptibility testing
DS-TB	Drug-susceptible TB
ECOFF	Epidemiological cut-off value
EDTA	Ethylenediaminetetraacetate
EMB	Ethambutol
et al	And others
ETH	Ethionamide
FDA	Food and drug association
FQ	Fluoroquinolone
G	Guanine
Gb	Gigabases

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GC	Growth control
GU	Growth Unit
H_2O	Water
HIV	Human immunodeficiency virus
Indel	Insertion/deletion
INH	Isoniazid
iTOL	Interactive Tree of Life
JSON	JavaScript Object Notation
KAN	Kanamycin
kb	Kilobases
LEVO	Levofloxacin
LPA	Line Probe Assay
M	Molar
MDR-TB	Multi-drug resistant tuberculosis
mg	Milligram
MGIT	Mycobacteria growth indicator tube
MIC	Minimum inhibitory concentration
mL	Millilitre
mM	Millimolar
MM	Master-mix
MOXI	Moxifloxacin
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis complex*
NFW	Nuclease-free water
ng	Nanogram
NGS	Next Generation Sequencing
NHLS	National Health Laboratory Service (South Africa)
nM	Nanomolar
NTC	No-template control
OADC	Oleic albumin dextrose catalase
OFX	Ofloxacin
PCR	polymerase chain reaction
pDST	Phenotypic drug susceptibility testing
pH	Potential of hydrogen
pM	Picomolar
PMD	Pretomanid
PZA	Pyrazinamide
qPCR	quantitative polymerase chain reaction
RAV	Resistance associated variant
RCF	Relative centrifugal force
RIF	Rifampicin
SA	South Africa
SAM	Sequence Alignment Map

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SAMRC	South African Medical Research Council
SDS	sodium dodecyl sulphate
SMOR	Single Molecule Overlapping Reads
SNP	Single Nucleotide Polymorphism
T	Thymine
TAE	Tris-Acid-EDTA buffer
TB	Tuberculosis
TB-ASAP	TB-specific Amplicon Sequencing Analysis Pipeline
TDS	Targeted deep sequencing
TE	Tris-EDTA buffer
TGen	Translational Genomics Research Institute
Tm	Melting temperature
Tris-HCl	Tris-Hydrochloride
UK	United Kingdom
USA	United States of America
USAP	universal sequence analysis sequence analysis pipeline
UT	Universally tailed
UV	Ultraviolet
V	Volts
WGS	Whole-genome sequencing
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis
ZN	Ziehl-Neelsen
µg	Microgram
µL	Microliter
µM	Micromolar

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Chapter 1: General Introduction

1.1 Background and motivation

Effective control of tuberculosis (TB) is threatened by the rise of antibiotic drug resistance[1]. Drug-resistant *Mycobacterium tuberculosis* (MTB) is more difficult to treat than susceptible strains, requiring a cocktail of anti-TB drugs with prolonged treatment periods[2]. The overall success of TB treatment outcomes is much lower for multidrug-resistant (MDR-TB) and extensively drug-resistant TB (XDR-TB) compared to susceptible TB[3]. Rapid identification of drug resistance profiles of MTB infection is essential for determining optimal patient treatment regimens[4].

Drug-resistant MTB strains arise largely due to the selection of chromosomal gene mutations[71]. A number of resistance-conferring mutations in clinically relevant genetic loci have been well characterised and can serve as genetic determinants of MTB phenotypic drug-resistance[5]. However, the number of mutations needed to fully diagnose drug resistance to multiple anti-TB agents is substantial. Mutations associated with resistance are still under investigation for newer drugs such as bedaquiline (BDQ)[6,7].

BDQ was the first novel anti-TB drug in four decades to gain the Food and Drug Administration (FDA) approval to be used for the treatment of MDR-TB[8]. Several studies have found that variants in a handful of genes are linked to raised minimum inhibitory concentrations (MICs) for BDQ and confer BDQ-resistance[9–12].

The *Rv0678* gene codes for the MarR-like transcriptional regulator of the MmpS5-MmpL5 efflux system[13], which is responsible for the efflux of azoles, clofazimine (CFZ), BDQ and ionophores[14]. Studies have shown that variants can be acquired across the entire length of the *Rv0678* gene (498bp)[15–17], making the design of novel molecular diagnostics challenging. The *pepQ* gene encodes a putative Xaa-Pro aminopeptidase[18] and in 2016 it was found to confer low-level resistance to BDQ[11]. The underlying mechanism of resistance to BDQ as conferred by *pepQ* variants is still unclear, however, experimental evidence supports the acquisition of variants during drug exposure[19]. The *atpE* gene encodes the subunit C of the ATP synthase proton pump[20]. Variants in the BDQ-binding region of the *atpE* gene have been previously linked to BDQ-resistance[21].

In June of 2018, South Africa became the first country to announce the roll-out of BDQ for the routine treatment of rifampicin-resistant TB (RR-TB)[22]. However, a review of patients that received BDQ showed a treatment failure rate of 30%[23]. Treatment failure may be explained by the addition of BDQ to an already failing regimen, infection with an already BDQ resistant

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strain or resistance that was acquired during treatment. Furthermore, for newer drugs like BDQ, phenotypic drug susceptibility testing (DST) has not been entirely standardised due to insufficient data for epidemiological cut-off values (ECOFFs)[24].

A study investigating the emergence of BDQ and CFZ resistance-associated variants in South Africa found that resistance variants emerged in multiple locations with evidence of onward transmission largely due to *Rv0678* variants[17]. Concerningly, studies have identified the spontaneous emergence of BDQ-resistance in the absence of prior exposure to BDQ[25,26]. Another study found the emergence of *Rv0678* variants following the withdrawal of BDQ from the regimen (after 6 months of treatment): a period where BDQ persists in the serum for months due to its long half-life, but the emergence of resistance was not suspected[27].

Next-generation sequencing (NGS) is rapidly becoming a popular method for drug susceptibility testing (DST) in MTB and the surveillance of drug resistance[4,28–33]. Studies have shown that targeted deep sequencing (TDS) can be used as a successful method for detecting variants in minor subpopulations, which may lead to drug resistance[4,31,33,34].

At present, there is no surveillance method established for BDQ-resistant TB. We hypothesise that the rollout of BDQ in South Africa will select BDQ-resistant clones, thereby providing an opportunity for transmission, which is undetected by the routine standard of care (diagnosis).

1.2 Problem Statement

As the first novel drug in many years[8], the inclusion of BDQ in current treatment regimens needs to be carefully monitored to ensure its success in current and future therapeutic interventions. In 2018, BDQ was rolled out in South Africa for routine use, without the implementation of routine DST[33]. This raises concerns about the efficacy of the drug, the evolution of drug resistance, as well as the transmission of BDQ resistant strains within the community. The recent roll-out presents an opportunity to establish a novel method to screen for BDQ-resistance in South Africa, as well as to set up surveillance for possible transmission of BDQ-resistant MTB strains.

1.3 Hypothesis

In this study, we hypothesize that variants in *Rv0678*, *atpE* and *pepQ* can serve as a proxy for BDQ-resistance and that TDS can be used to identify BDQ resistance-associated variants in clinical isolates. Furthermore, we hypothesise that BDQ-resistance is transmitted within the Western Cape province in South Africa and that WGS data can be used to identify possible transmission networks within the community, by analysing the single nucleotide polymorphism (SNP) distances between the isolates.

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1.4 Aims and objectives

Aim 1:

Determine the frequency of BDQ-resistant TB amongst drug-resistant TB patients within the Western Cape.

Objective 1: We will use TDS to identify variants in the genes *Rv0678*, *atpE* and *pepQ* in the first and last available MTB isolate from patients collected in the Western Cape province of South Africa from January 2018 to February 2019. Additionally, we will optimise and adapt a TDS protocol within the local setting to screen for BDQ genotypic resistance.

Objective 2: We will use phenotypic drug susceptibility testing to investigate the association between variants in *Rv0678*, *atpE* and *pepQ* and BDQ-resistance.

Aim 2:

Investigate potential transmission networks of BDQ-resistance.

Objective 1: We will use WGS and phylogenetic analysis to infer the transmission networks of BDQ resistant isolates identified in Aim 1.

1.5 Experimental approach

We identified patient isolates with RR-TB between the period of January 2018 to February 2019.The first and last available isolate per patient was selected, with the sampling period between the first and last available isolate occurring 60 days apart or more.

Thereafter, crude DNA from the first and last available isolates was used for targeted deep sequencing *of Rv0678*, *atpE* and *pepQ* genes as designed by the Translational Genomics Research Institute (TGen) North (Flagstaff, Arizona). The sequencing data were analysed using the TB-specific Amplicon Sequencing Analysis Pipeline (TB-ASAP)[35].

The BACTEC™ Mycobacteria Growth Indicator Tube (MGIT)™ 960 system and the EpiCentre software equipped with the TB eXist module for DST[36] was used to determine the phenotypic DSTs of BDQ for isolates containing variants in the above-mentioned genes

Lastly, WGS was done on the isolates showing variants in the target genes using the Illumina MiniSeq platform.

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Chapter 2: Acquisition of bedaquiline resistance as a means of monitoring DR-TB transmission

2.1 Introduction

For more than a decade, tuberculosis (TB) has been the leading cause of death globally due to a single infectious agent, with an estimated 1.3 million TB-related deaths occurring in 2020[37]. TB is a communicable, airborne disease caused by *Mycobacterium tuberculosis* (MTB) that primarily affects the lungs, resulting in pulmonary TB[38]. There are an estimated 2 billion people infected with MTB worldwide[37] although, only a small proportion (5 - 10%) of infected people develop active TB disease[39]. Furthermore, TB is a treatable and curable disease, provided patients are diagnosed timeously and are given adequate and uninterrupted treatment[40]. While the global rate of TB has declined, the emergence of drug-resistant TB threatens TB control strategies[41].

2.1.1 Emergence of drug resistance

One of the means of evolution of drug resistance in MTB is the acquisition of chromosomal mutations in the bacterial genome[3]. Drug-resistant TB (DR-TB) remains the primary obstacle for effective treatment and control of the disease[42]. The world health organisation (WHO) reported that in 2019 there were approximately 465 000 new cases of multidrug-resistant TB (MDR-TB)/ rifampicin-resistant TB (RR-TB) worldwide[37]. The burden of TB is increased by the emergence of resistance to anti-TB drugs[43], as well as the transmission of DR-TB[44]. Drug resistance complicates treatment due to a limited number of advantageous drugs available for treatment, prolonged treatment times and relatively poor adherence partly due to drug side effects, often leading to poor treatment outcomes[45].

TB bacilli that are fully susceptible to treatment with currently available anti-TB drugs are defined as drug-susceptible whereas there are two main types of drug-resistant TB: MDR-TB and extremely drug-resistant TB (XDR-TB). MDR-TB is resistant to both rifampicin (RIF) and isoniazid (INH), the key first-line anti-TB drugs. XDR-TB is resistant to the first-line drugs, RIF and INH, as well as second-line injectable (SLI) agents and fluoroquinolone (FQ) antibiotics[46], as determined by phenotypic or genotypic drug susceptibility testing (DST)[47,48].

2.1.2 Diagnostic strategies

Rapid, reliable and standardised DST is vital for accurate TB diagnosis, design of appropriate treatment regimens as well as the prevention of transmission[49,50]. Culture remains the diagnostic gold standard for TB confirmation[51] and forms the basis for phenotypic DST[52]. Culturing mycobacteria can be done on solid (e.g. Lowenstein-Jensen or Middlebrook 7H10

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and 7H11) or liquid (e.g. Middlebrook 7H9) media however, the MTB yield isolated from a liquid medium is greater than that of solid media[53].

The Mycobacteria Growth Indicator Tube (MGIT) in combination with the BD BACTEC 960 system is a rapid liquid broth (modified Middlebrook 7H9), non-radiometric, automated method for phenotypic DST used for MDR-TB detection[54,55]. The MGIT media utilises a fluorescent compound that fluoresces during oxygen consumption, indicating the presence of growing and respiring organisms[56]. The MGIT 960 instrument is used for the detection of mycobacteria in various clinical specimens[50]. MGIT DST is based on critical concentrations (CC)[57]; the lowest anti-TB drug concentration that inhibits 99% of wild-type (WT) MTB strains[58]. Various studies have demonstrated the reproducibility of MGIT results for first- and second-line anti-TB drugs[54,59].

However, when determining interim CC values, the WHO noted inter-laboratory differences in minimum inhibitory concentration (MIC) values for some drugs (such as bedaquiline), including a limited number of strains tested. The inclusion of more susceptible and resistant strains for MIC testing was recommended in the future[60]. Incorrect DST results are most commonly linked to poorly defined breakpoints for newer drugs and can result in misclassification of resistant strains as susceptible, resulting in suboptimal treatment regimens[61].

The Sensititre MYCOTB MIC plate is another commercial phenotypic DST assay for MTB developed by TREK Diagnostic Systems (Thermo Fisher)[62]. The MYCOTB plate is a broth microdilution (BMD) 96-well plate used for the simultaneous investigation of the MICs for twelve first- and second-line drugs[62,63]. The BMD plate is currently not WHO-endorsed, as it is under evaluation by the WHO for the epidemiological cut-off values (ECOFFs) and drug concentrations used[64]. However, the latest WHO catalogue of mutations in *Mycobacterium tuberculosis* complex (MTBC) used the BMD DST results to determine the mutation resistance-association for some anti-TB drugs[7]. Therefore, this DST method is increasingly being used and several studies have evaluated this BMD method with promising results[62,65,66].

The slow growth rate of MTB makes phenotypic DST a time-consuming method[67], thus, highlighting the need for alternative rapid diagnostic tools. Molecular diagnostic methods offer rapid TB diagnosis and genotypic DST and include molecular assays such as the GenoType MTBDR*plus* Line Probe Assay (Hain, Nehren, Germany) and the Xpert MTB/RIF and Xpert MTB/RIF Ultra (Cepheid, Sunnyvale, CA) assays[51,68].

The Xpert MTB/RIF assay is an automated cartridge-based molecular test[69] that can be used to detect MTB DNA in clinical specimens[41]. It is a WHO endorsed[70] polymerase chain reaction (PCR)-based assay that extracts, amplifies and detects mutations in the RIF resistance

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determining region (RRDR) in the *rpoB* gene in real time[41,55]. Despite displaying high sensitivity and specificity for smear-positive sputum samples, the Xpert MTB/RIF assay demonstrated lower sensitivity when testing smear-negative sputum[71]. The Xpert MTB/RIF Ultra assay is a next-generation molecular test designed to resolve some of the limitations associated with the Xpert MTB/RIF assay: it has superior sensitivity, with improved TB detection capabilities and is better able to identify and differentiate between RIF susceptible and resistant MTB[69,71].

The GenoType MTBDR*plus* Line Probe Assay (LPA) is another molecular DST method endorsed by the WHO[70]. This molecular test uses reverse hybridisation of PCR products to the specific oligo-nucleotide probes to target the RRDR for the identification of RIF-resistance and the *katG* and *inhA* genes for INH-resistance[72]. However, MTBDR*plus* is not recommended for use on smear-negative specimens and thus, many specimens require culture-based drug susceptibility testing[73].

These molecular assays have high sensitivities for detecting anti-TB drug resistance based on established targets[74]. However, these technologies are limited to specific loci being examined and, in most cases, are unable to differentiate between silent mutations and mutations impacting drug efficacy[75].

The advancement in next-generation sequencing (NGS) technologies has made sequencing approaches a viable option for characterising infections and drug resistance[76], with the potential to address the limitations of traditional molecular assays[75]. Whole-genome sequencing (WGS) allows for broad drug resistance characterisation[75]. A number of studies have investigated the use of WGS for DST and have highlighted its potential for improving detection sensitivity and predicting drug resistance in clinical isolates[75,77,78]. WGS has been shown to have a faster turnaround time in detecting anti-TB drug resistance compared to phenotypic testing[79,80]. Furthermore, WGS data can be valuable for informing clinical decision-making by identifying resistance markers, as well as in contact tracing and providing information on likely transmission events[80].

The increased affordability and accessibility of WGS[81], combined with the comprehensive cataloguing of mutations associated with DR-TB, highlights the potential of WGS as a revolutionary tool for TB diagnostics[78]. For instance, in the absence of definitive breakpoints for newer drugs like bedaquiline (BDQ), WGS can assist in detecting new resistance-associated variants (RAVs) and predicting phenotypic resistance[10]. Lastly, integrating WGS approaches with DST for routine TB diagnostics will allow for the comprehensive discovery of variants associated with resistance[10]. There has been a global effort to investigate and document genetic mutations in MTB that are associated with resistance to establish a

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catalogue of reliable genotype-phenotype correlations[5,7,82,83]. Identifying these correlations has allowed for the use of targeted NGS approaches as a method for the identification of resistant clinical isolates[4,35].

Targeted deep sequencing (TDS) allows for sequencing of large genetic targets with a considerable sequencing depth, which is essential for accurate detection of mixed or hetero-resistant populations[35]. This high sequencing depth makes TDS approaches highly sensitive and able to generate important data, identifying distinct resistance-associated variants (RAVs) and potentially predicting MICs[35]. Studies conducted by Colman *et al.* demonstrated the capability and clinical surveillance applications of their rapid DST solution (the Next Gen-RDST assay) to detect low levels of resistant variants and the clinical or surveillance applications of this tool[35,75].

A new molecular diagnostic tool based on TDS is the all-in-one Deeplex-MycTB assay (Genoscreen, Lille, France), which covers 18 genetic regions associated with TB drug-resistance. The assay covers larger genetic regions, as well as assessing drug-resistance regions for all WHO group A and B drugs[84]. This molecular DST method has had promising results[84,85], illustrating the promising future of NGS technologies in the diagnosis of TB drug-resistance.

2.2 Drug treatment regimens

Effective treatment regimens for MDR- and XDR-TB are based on the inclusion of second-line anti-TB drugs, as determined by DST[86]. Within the last 10 years, there has been a considerable evolution in the treatment landscape for DR-TB. Some of these key developments include earlier DR-TB diagnosis due to the implementation of new diagnostic tests (e.g. Xpert MTB/RIF and LPAs)[87,88], a more comprehensive understanding of the pharmacology and bactericidal activity of various drugs[60], the advent of new anti-TB drugs, as well as several pivotal observational and clinical trials[89–92]. The developments of the last decade are largely reflected in the evolving WHO guidance on managing DR-TB and are evident in the DR-TB policy updates and communications[93–95]. Each update has seen key changes in the groupings and rankings of the available anti-TB drugs. These changes are highlighted in Section 2.4.

The availability of effective anti-TB drugs is fundamental to implementing successful MDR-TB treatment regimens and access to new drugs brings a sense of hope to combatting DR-TB. The vast majority of the drugs effective against MTB were discovered in over a 20-year period, starting with streptomycin in 1943 and ending with RIF in 1963[96]. This was followed by a near-five-decade-long dearth in novel anti-TB agent discovery and development[97]. In late 2012, BDQ became the first anti-TB drug with a novel mechanism of action in more than 40 years

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to receive accelerated approval by the U.S. Food and Drug Administration (FDA)[8]. This milestone served as a turning point for the development of new anti-TB drugs, as an assortment of new drugs has since become available for clinical research and TB treatment regimens.

2.2.1 Repurposed and new anti-TB drugs

The drug development process for new drugs generally follows three major phases: (1) discovery, (2) preclinical development and (3) clinical trials[98]. The drug discovery process is lengthy and laborious, requiring various phases before a new drug is approved and can be brought to market[99]. Given the costly and lengthy process of drug discovery, combined with the demand for new TB treatment options, the repurposing of existing drugs as anti-TB agents has become a promising alternative strategy[100].

2.2.1.1 Repurposed drugs

The main goal of repurposing drugs is to use established drug treatments to treat a different disease[101], which results in a reduction of time and money for drug development as toxicity profiles are known, obviating the need for extensive safety trials[102]. The repurposing process involves selecting pre-existing drugs and investigating novel drug-target interactions[102]. Over the years, a number of antibiotic agents have been repurposed for use in anti-TB therapy, including many of the current second-line drugs; FQs, kanamycin, amikacin, carbapenems, amoxicillin/clavulanic acid, clofazimine (CFZ) and linezolid (LZD)[103]. CFZ and LZD are two of the key repurposed drugs gaining attention, with various systematic reviews and clinical trials evaluating their use for MDR-TB treatment[87,104]. Over the last decade, CFZ (group B) and LZD (group A) have been identified by the WHO to be reprioritised for treating RR- and MDR-TB[95].

CFZ belongs to the riminophenazine antibiotic class and has commonly been used as an anti-leprosy agent[105]. Although initially synthesized as an anti-TB drug in the 1950s, CFZ was not used to treat TB due to the inconsistent results observed in animal models[106] and a lack of success when used as monotherapy for primates and humans[107,108]. In addition, one of the adverse effects of CFZ is the skin discolouration associated with treatment, which contributed to some patients depressive and suicidal states[106]. However, after a 2010 clinical study demonstrated that the inclusion of CFZ in a 9-month treatment regimen for MDR-TB patients resulted in a high relapse-free cure rate (87.9%)[100], there has been renewed interest in CFZ for MDR- and XDR-TB treatment[105].

LZD belongs to the oxazolidinone class[109] and was originally approved by the FDA in 2000 for the treatment of gram-positive bacterial infections[110]. The drug has been found to be highly active against MTB both *in vitro*[111,112] and *in vivo*[113]. Furthermore, in clinical trials, LZD has been shown to improve the outcomes of DR-TB[114,115]. One of the difficulties associated with

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LZD is gaining access to the drug due to its high price, especially in resource-constrained settings[116]. Furthermore, adverse effects such as neurotoxicity and hematologic toxicity have frequently been reported[117], as well as various case reports of peripheral neuropathy[118–121]. Despite these concerning reports, an individual patient data meta-analysis study indicated that the odds of treatment success increased 3-fold with LZD use[122]. Therefore, LZD remains an important anti-TB drug that will continue to be introduced into national TB programmes[123].

2.2.1.2 New drugs

Currently, three new anti-TB drugs are in phase 2-3 trials: delamanid (DLM), pretomanid (PMD) and BDQ[124].

DLM, developed by Otsuka Pharmaceutical, first received approval in the European Union (EU) in 2014 for the treatment of MDR-TB[125]. DLM is a nitroimidazole derivative that inhibits mycolic acid biosynthesis, thereby preventing the formation of the mycobacterial cell wall[126]. The first clinical studies investigating DLM reported positive outcomes[127,128]. Several ongoing clinical trials are investigating DLM in combination with other anti-TB drugs[124].

Another new anti-TB agent in the drug pipeline, pretomanid (also known as PA-824 or PMD), belongs to the nitroimidazole drug class[129]. This new therapeutic agent is currently being assessed for its potential use in treating both drug-susceptible and MDR-TB[124]. After a PMD-containing regimen (BPaL regimen of BDQ, PMD and LZD) was shown to be effective during a clinical trial[130] (Discussed in Section 2.5), the regimen was approved by the FDA[131] for the treatment of patients with pulmonary XDR-TB or patients with MDR-TB that are either treatment-intolerant or non-responsive to treatment[129]. One of the clinical trials currently underway (NC-005) has reported distinct bactericidal activity with the drug combination of PMD, BDQ and pyrazinamide (PZA)[124].

BDQ first gained attention after the promising results of two-phase 2b clinical trials[132]. The new anti-TB drug has since been used in various compassionate and expanded use programs[133,134] and received more widespread approval after the WHO recommendations in 2018[86]. This review will focus on BDQ as a novel anti-TB agent, the use of BDQ in clinical trials and the emergence of BDQ-resistance.

2.3 BDQ

BDQ (formerly TMC207 or R207910) was first discovered by Andries and colleagues in 2005, where the compound was shown to potently inhibit drug-susceptible and DR-MTB, and demonstrated antimycobacterial activity on replicating bacilli both *in vitro* and *in vivo*[135]. The drug belongs to a new class of anti-TB drugs known as diarylquinolines[136]. The mechanism of action for this drug is the inhibition of the proton pump of mycobacterial ATP synthase, a

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crucial enzyme for energy generation and consequently, the growth and survival of the bacterium[135,137]. BDQ has also been shown to inhibit actively replicating and non-replicating mycobacteria[138].

Shortly after the compound's discovery, various pre-clinical trials began, demonstrating the sterilising and bactericidal activity of BDQ. Following the discovery of BDQ, an *in vitro* study was conducted using a large number of drug-susceptible and MDR-TB clinical isolates[139]. The study aimed to fully characterise the antimycobacterial spectrum of BDQ, as well as determine the BDQ MICs of various nontuberculous mycobacteria (NTM). The results demonstrated the strong antimycobacterial activity of BDQ against both susceptible and DR-MTB strains, as well as a variety of NTMs[139].

2.3.1 Murine models

Preclinical animal models have served as the cornerstone for evaluating new treatment options for TB control[140]. One of the first BDQ studies in a murine model evaluated the sterilising activity of a variety of BDQ-containing regimens compared to the standard WHO regimen. They found that four months of a standard regimen, where BDQ was either substituted for INH or added to the standard regimen, was as effective as a standard six-month treatment regimen[141]. This finding suggested that shorter BDQ-containing regimens could be possible in the future.

For TB research, guinea pig models are superior to murine models for simulating the pathogenesis of human TB, as they exhibit similar features of infection with humans, such as primary necrotic granuloma formation[140,142]. Two studies have used guinea pig TB models to investigate BDQ. These studies demonstrated that the combination of BDQ with core first-line (RIF, PZA and INH, or RIF and PZA) drugs results in a rapid reduction in bacterial load and the rapid clearance of secondary lesions[143,144], further demonstrating the potent sterilising activity of the drug.

2.3.2 Early bactericidal activity

After conducting pre-clinical animal studies for a new drug candidate, it is essential to consider the early bactericidal activity (EBA) of the new drug candidate before clinical studies can commence. EBA refers to the drug's ability to rapidly reduce bacterial load[142].

The results of an EBA study were published in 2008, where a single daily 400 mg dose of BDQ was administered as monotherapy over the course of seven days for 75 treatment-naïve pulmonary TB patients. The study found that BDQ displayed bactericidal activity, with a similar magnitude to RIF and INH and no serious adverse events were linked to the drug. However,

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the study findings also noted a lack of bactericidal activity during the first two to four days of treatment, which they suggested was the result of BDQ's unique mechanism of action[145].

2.3.3 Metabolism and half-life

The pharmacokinetics and pharmacodynamics of anti-TB drugs may play a vital role in improving TB treatment and preventing the emergence of drug resistance[146]. The pharmacokinetic factors, such as drug metabolism and elimination, are important for establishing drug mechanisms and clinical benefits[147].

The primary metabolism of BDQ occurs in the liver by the cytochrome P450 isoenzyme 3A4 (CYP3A4)[148] (Figure 2.1). The primary metabolite, *N*-monodesmethyl (M2), is less active but with a similar terminal half-life to BDQ[149]. M2, like BDQ, contains a cationic amphiphilic structure, giving it the characteristics of a cationic amphiphilic drug (CAD)[150]. Factors that interfere with the pharmacokinetics of BDQ may affect the drug's concentrations[151]. For example, the co-administration of enzyme inhibitors and/or inducers may influence BDQ plasma concentrations[152]. This is concerning since low anti-TB drug concentrations in plasma have been linked to a delay in treatment response or the risk of treatment failure[146], whereas high concentrations could increase the risk of toxicity[153]. Cellular phospholipidosis may, furthermore, be induced by both BDQ and M2, resulting in neuropathy, QT interval prolongation and hepatotoxicity (discussed below)[142].

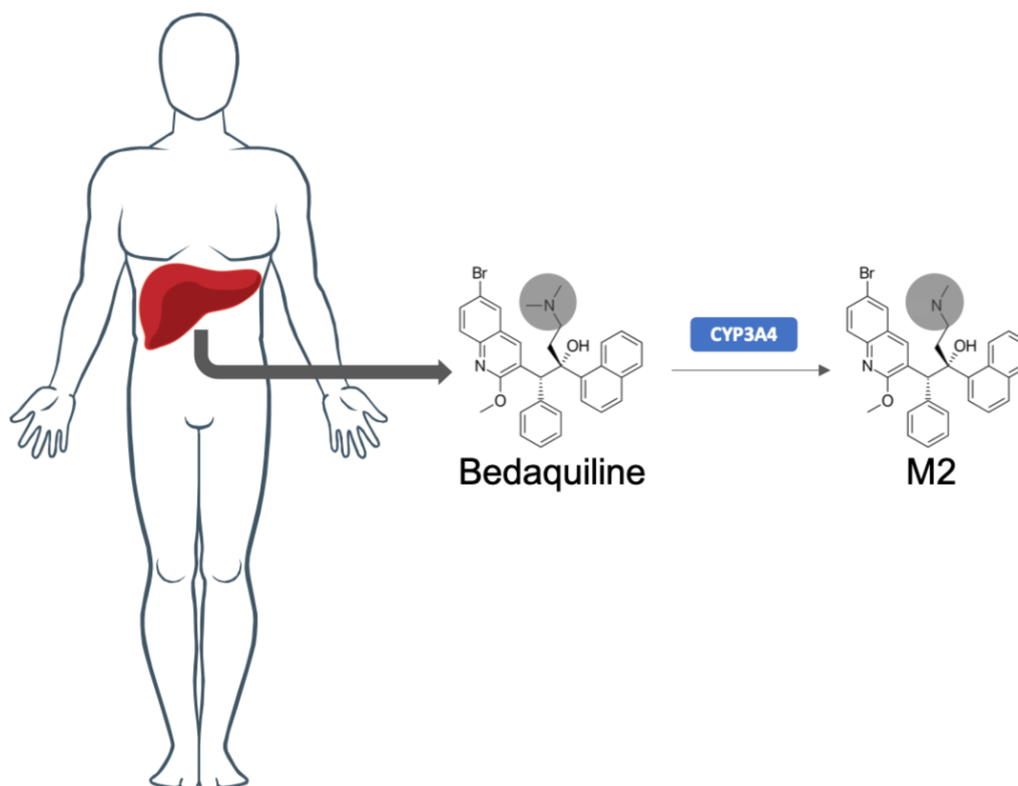

Figure 2.1 Bedaquiline metabolism in the liver by CYP3A4. Bedaquiline is metabolised in the liver of the body, by the cytochrome P450 isoenzyme 3A4 (CYP3A4) to the primary metabolite M2.

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BDQ displays a multiphasic distribution and elimination profile[154]. BDQ has a long terminal elimination half-life [148] of around four to five months[155], which is owed to the long plasma half-life, high tissue penetration, and long half-life in tissues[154]. Since BDQ is highly lipophilic[156], its intracellular accumulation via drug-induced phospholipidosis (binding of compounds with characteristics such as CADs, to the intracellular phospholipids resulting in accumulation in the tissues[150]) could account for its long terminal half-life[157]. This long terminal half-life of BDQ could result in acquired resistance to the drug[46] as well as the acquisition of additional variants after BDQ cessation[27]. For example, cases of non-adherence to treatment in BDQ-containing regimens could subsequently result in BDQ monotherapy, since its half-life exceeds that of other drugs[155]. With BDQ being the only efficacious drug in circulation for these cases, there is a greater likelihood for treatment failure, relapse, or acquired resistance[155].

2.3.4 Tissue penetration

For a drug to employ its desired effect, the drug must first be able to reach its therapeutic target at the necessary concentration[158]. Specifically, drug access to the mycobacterial infection site becomes gradually more problematic owing to the progression of disease during the formation of granulomas with inadequate blood supply[159]. The variability in drug exposure at TB lesion sites could result in drug resistance based on the increased drug pressure caused by periods of drug monotherapy[159]. However, BDQ has high tissue penetration[154] and is mainly distributed in macrophage-rich tissues such as the lungs[107].

2.3.5 Adverse events

The treatment of RR- and MDR-TB is commonly associated with the occurrence of serious adverse events (AEs)[160]. Of concern, new anti-TB drugs such as BDQ have also been linked with AEs in patients[161]. These AEs include nausea, bilateral hearing impairment, extremity pain and non-cardiac chest pain[157]. Of the recorded side effects, there are two potentially fatal AEs from BDQ use that have been identified: cardiac QT (measure between Q wave and T wave in the heart's electrical cycle) prolongation (Figure 2.1) and liver injury[137,161].

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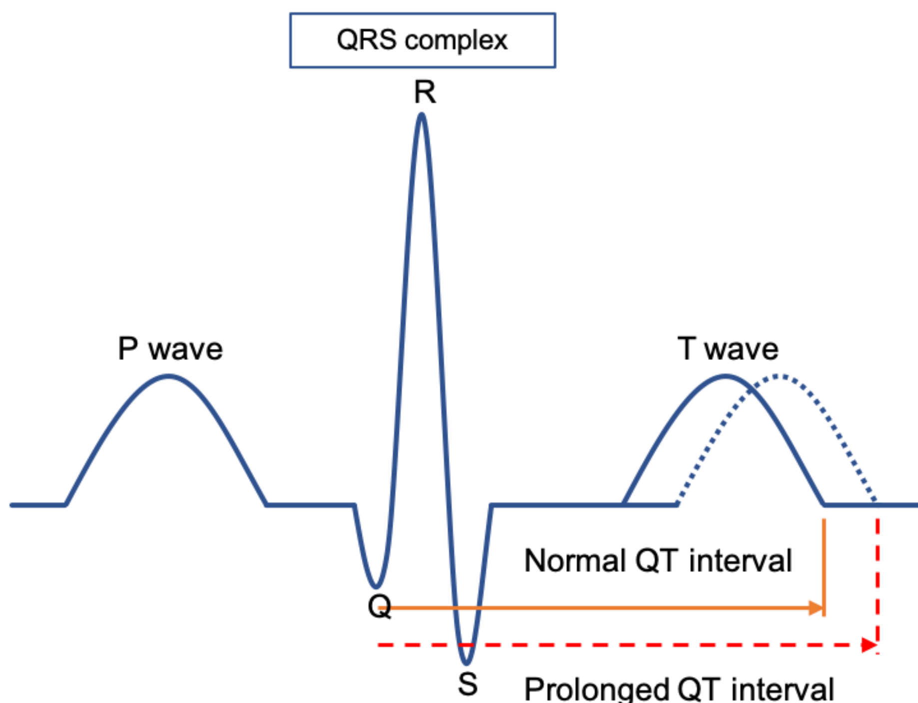

Figure 2.2 Drug-induced prolonged cardiac QT interval, of an electrocardiogram (ECG) (adapted from Brody *et al.* 2016[162]). The QRS complex represents ventricular depolarisation. The QT interval, depicted by the orange line, is defined from the start of the QRS complex to the end of the T wave and represents the time taken for ventricular depolarisation and repolarisation (period of ventricular systole). A prolonged QT interval is depicted by the red dashed line and is longer due to the delayed T wave (dotted blue line).

The prolongation of the QT interval is concerning as it may result in fatal arrhythmias[163]. BDQ's effect in prolonging the QTc interval among patients is an important consideration when designing MDR-TB treatment regimens[46], given that several other anti-TB medications have the capacity to prolong the QT interval, including FQs, CFZ, DLM, PMD and macrolides[164].

Hepatotoxicity is one of the most common and clinically relevant AEs linked to BDQ treatment[149]. The safety data available for the first and second-phase 2 studies indicated a higher prevalence of drug-related hepatic disorders in patients taking BDQ compared to the placebo group[165].

2.3.6 Resistance

The DR-TB epidemic is driven by: (1) primary, or transmitted drug resistance, due to the transmission of DR strains between individuals and (2) secondary or acquired drug resistance, which occurs due to the acquisition of genetic mutations conferring resistance to anti-TB drugs[166]. Even though MTB has been shown to readily acquire mutations that lead to drug resistance in response to drug exposure[166], various studies have shown that most DR-TB cases are due to primary drug resistance[167]. Drug resistance poses a serious threat to controlling the spread of the disease, as effective treatment becomes more complex[168].

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As with all antibiotics, the looming threat of resistant mycobacterial strains threatens the efficacy of anti-TB drugs and BDQ is no exception. The first mechanism of BDQ-resistance was proposed by Andries *et al*[135]. Using complementation studies, they found that mutations in the *atpE* gene, which encodes subunit C of ATP synthase[169], were responsible for target-based resistance to BDQ. However, a subsequent *in vitro* study discovered that mutations in *atpE* were found in 30% of the BDQ resistant mutants, suggesting that there are other non-target-based resistance mechanisms[170].

An *in vitro* mutant study investigating resistance mechanisms of the repurposed drug CFZ discovered the first case of cross-resistance between CFZ and BDQ[171]. By subjecting the mutants to WGS, non-synonymous variants in the *Rv0678* gene were discovered. These variants accounted for the non-target-based cross-resistance and raised MICs[171].

The *Rv0678* gene encodes a transcriptional repressor that regulates the expression of the MmpS5-MmpL5 efflux pump[155]. Andries *et al.* found that mutations in *Rv0678* result in overexpression of the efflux pump and can lead to a 2- to 8-fold increase in BDQ MICs[12]. Another study found that RAVs occurred more frequently in MDR-TB isolates than was previously thought. Concerningly, *Rv0678* mutants were found in both MDR-TB and drug-susceptible TB patients that were BDQ and CFZ-naïve[25].

An additional non-target-based BDQ-resistance mechanism was identified by Almeida and colleagues[11]. They found that loss-of-function mutations in *pepQ*, a putative cytoplasmic peptidase, conferred low-level BDQ and CFZ resistance in a murine model[11].

Despite the high hopes for using BDQ as a novel drug to combat the MDR and XDR-TB epidemic, reports of a BDQ resistant clinical isolate appeared as early as 2014[172], only a few years after its implementation in DR-TB regimens. This patient was diagnosed with MDR-TB in 2011 and BDQ was administered as part of a six-month compassionate use treatment regimen. The patient experienced a relapse five months after treatment termination and DST confirmed additional resistance to CFZ and BDQ. A mutation (nucleotide T2C) in the *Rv0678* gene previously linked to cross-resistance between CFZ and BDQ was identified[171 173].

BDQ-resistance due to variants in *Rv0678* has since been reported after BDQ exposure[12,174,175], with a recent study reporting a greater than four-fold increase associated with a *Rv0678* variant in 4.3% of patients[176]. This study has also suggested that the presence of *Rv0678* variants at baseline in patients could be an indication of community BDQ or CFZ resistance transmission[176].

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2.4 WHO recommended treatment strategies

2.4.1 Period 2011 - 2015

The WHO recommendations published in 2011 encouraged the widespread use of rapid DST with molecular techniques for the detection of RR-TB[93]. The guidelines further state that the MDR/XDR-TB regimen should consist of at least PZA and four second-line drugs considered to be effective based on DST, previous use and/or surveillance data on drug resistance[177]. For example, the recommended regimen should be composed of a group 2 drug (SLI agent), a group 3 drug (FQ) and two bacteriostatic group 4 drugs (preferably prothionamide or ethionamide plus cycloserine or *p*-aminosalicylic)[93]. The WHO suggested that group 5 agents could be used, but were not recommended for inclusion in standard MDR-TB regimens[93]. Following the drug's approval in 2012, the 2013 WHO interim guidelines for BDQ recommended the drug's use in combination with or in place of a group 5 drug[177].

2.4.1.1 BDQ compassionate use and South African context

Clinical trials paved the way for the compassionate use of BDQ in 2011, before its regulatory approval for widespread use in various countries[157]. The WHO estimated in a 2017 report that around 46 countries had used BDQ under various mechanisms of compassionate use, including South Africa[178]. A systematic review conducted by the WHO found that for five cohorts that received BDQ from different countries, microbiological cure was achieved in 79.7% of patients at 6 months, based on sputum culture conversion[178].

In January 2013, the South African National Tuberculosis Programme established the Bedaquiline Clinical Access Programme (BCAP), granting pre-XDR or XDR-TB patients access to the drug[91]. The data obtained from a BCAP cohort of 200 patients, between March 2013 and July 2014, indicated 73.0% of the patients had favourable outcomes (69.5% of patients were cured and 3.5% successfully completed treatment)[179]. Of the 87 serious AEs reported in the patients, investigators attributed 4.6% to BDQ[179].

BDQ was registered in South Africa in 2014[179] and in 2015 it was officially introduced into the South African TB treatment programme, where it was used to strengthen the existing RR-TB regimens for selective MDR, pre-XDR and XDR-TB cases[91,161]. With the introduction of this new anti-TB agent to the national TB programme, there was a need for active reporting by health care workers to the Pharmacovigilance Programme in the case of any suspected AEs or deaths in patients receiving BDQ[161].

2.4.2 Period 2016 – 2017

In 2016, the WHO released an update on the treatment guidelines for DR-TB[94]. The recommendations consisted of a shorter standardised MDR-TB treatment regimen, limited to

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use under specific conditions and a regrouping of anti-TB drugs for the treatment of RR- and MDR-TB in longer regimens[94]. Recommendations for RR- and MDR-TB regimens were to administer at least five effective anti-TB drugs, consisting of PZA and four core second-line drugs. The core second-line drugs were selected in the following manner: one drug from group A, one drug from group B and at least two from group C.

The changes to the group rankings placed later-generation FQs (levofloxacin and moxifloxacin) as core drugs in group A, followed by SLI agents in group B. The update also saw a new ranking for the thionamides and the new and repurposed drugs, LZD and CFZ. Finally, the add-on agents in group D included drugs that did not form part of the core second-line drugs and were recommended for cases where the minimum amount of core drugs could not be composed of the other groups due to resistance[94].

2.4.2.1 WHO short-course regimen

Based on the complexities of designing effective treatment regimens, it is evident that there is a need for shortened and simplified treatment regimens for the effective sterilization and reduction in TB incidence and mortality[180]. Novel drugs and shorter treatment regimens form part of the WHO's End TB Strategy for a 95% reduction in global TB deaths by 2035[181].

Accordingly, in 2016 the WHO announced guidelines for a standardised short-course treatment regimen for RR- and MDR-TB. The alternative regimen proposed a 9 – 12-month treatment duration, split into an intensive and continuation phase[182]. The eligibility and treatment phases for the regimen are summarised in Table 2.1.

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Table 2.1 WHO short-course patient eligibility and medications administered[94].

Criteria for patient eligibility	• Included patients: Confirmed RR- or MDR-TB with pulmonary TB Considered highly unlikely to have FQ or SLI drug resistance • Excluded patients: Pregnant Extrapulmonary TB FQ or SLI drug resistance Documented or likely resistance to drugs used in the regimen Previous treatment with second-line drugs for >1 month Risk of toxicity or intolerance to ≥1 drugs used in the regimen
Intensive phase: 4 - 6 months	• Kanamycin* • Moxifloxacin • Prothionamide • Clofazimine • Pyrazinamide • High-dose isoniazid
Continuation phase: 5 months	• Moxifloxacin • Clofazimine • Pyrazinamide • Ethambutol

TB=tuberculosis; RR-TB=rifampicin resistant TB; MDR-TB=multi drug-resistant TB; FQ = fluoroquinolone; SLI = second-line injectable
*In 2019 a WHO revised update was released which included the recommendation of replacing the injectable agent kanamycin with amikacin[86]

The shortened WHO regimen is currently being investigated in the Standard Treatment Regimen of Anti-tuberculosis Drugs for Patients with MDR-TB (STREAM) trial. The first published results of the trial have shown that the shorter regimen is marginally less efficacious than the longer regimen (78.1% versus 80.6%)[183]. However, the WHO position on the shorter MDR-TB regimen remained the same[184].

2.4.3 Period 2018 – 2020

The WHO released a rapid communication on the key changes to MDR and RR-TB treatment regimens in August 2018. These guidelines replaced all previous WHO guidelines for RR-TB/MDR-TB treatment and saw a regrouping and prioritisation of several anti-TB drugs[95]. In this communication, they recommended the use of BDQ and LZD (a novel and a repurposed drug, respectively) as group A drugs (Table 2.2) in longer MDR-TB treatment regimens. The key changes stemmed from observational studies, clinical trial results and an individual patient data meta-analysis[122,185–187]. However, no routine BDQ DST was available as yet for the majority of countries to impose this regimen change[188,189]. The lack of reliable BDQ DST had further downstream consequences such as the lack of detection of resistance and the increase in reported clinical failures[15,190].

The key changes suggested that all group A drugs should be used, if possible, as well as both group B drugs. Group C drugs consist of older drugs, downgraded due to their side-effect

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profiles and reduced efficacy[191]. These drugs are designated as add-on drugs and are to be supplemented when group A or B drugs cannot be used.

Based on this new guidance, importance was placed on the group A drugs; the later-generation FQs and new and repurposed drugs, BDQ and LZD (highlighted in red in Table 2.2). This announcement served as a significant shift in the treatment regimen guidelines for RR- and MDR-TB treatments, highlighting the important function of new and repurposed anti-TB drugs.

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Table 2.2 The key changes and updates to the WHO-recommended groupings for the treatment and regulation of RR- and MDR-TB from select years (2011[192], 2016[94] and 2018[95])

2011 WHO anti-TB drug groupings to design MDR-TB regimens[193]		**2016 WHO anti-TB drug groupings for RR- and MDR-TB regimens**		**2018 WHO anti-TB drug groupings for RR- and MDR-TB regimens**	
First-line anti-TB drugs*					
Group 1: First-line oral anti-TB drugs	Isoniazid Rifampicin Ethambutol Pyrazinamide	Isoniazid Rifampicin Ethambutol Pyrazinamide		Isoniazid Rifampicin Ethambutol Pyrazinamide	
Second-line anti-TB drugs					
Group 2: SLI agents	Streptomycin** Kanamycin Amikacin Capreomycin	**Group A:** FQs	Levofloxacin Moxifloxacin Gatifloxacin	**Group A:**	Levofloxacin/ moxifloxacin Bedaquiline Linezolid
Group 3: FQs	Levofloxacin Moxifloxacin Gatifloxacin Ofloxacin	**Group B:** SLI agents	Amikacin Capreomycin Kanamycin Streptomycin	**Group B:**	Clofazimine Cycloserine/terizidone
Group 4: Oral bacteriostatic second-line anti-TB drugs	Ethionamide/ protionamide Cycloserine/ terizidone *p*-Aminosalicylic acid	**Group C:** Other second-line agents	Ethionamide/ protionamide Cycloserine/ terizidone Linezolid Clofazimine	**Group C:**	Ethambutol Delamanid Pyrazinamide Imipenem-Cilastrin/ Meropenem Amoxicillin[†]/streptomycin Ethionamide/protionamide *p*-Aminosalicylic acid

Group 5: Anti-TB drugs with limited data	Clofazimine Linezolid Amoxicillin/ clavulanate Meropenem Thioacetazone Clarithromycin Imipenem/ cilastrin	**Group D:** Add on agents	**D1**: Pyrazinamide Ethambutol HD-isoniazid **D2**: Bedaquiline Delamanid **D3**: *p*-Aminosalicylic acid Imipenem/cilastrin Meropenem Amoxicillin-clavulanate (Thioacetazone)		

WHO = World Health Organisation; TB = tuberculosis; RR-TB = rifampicin-resistant-TB; MDR-TB = multi drug-resistant TB; FQs = fluoroquinolones; SLI = second line injectables.
New and repurposed drugs are highlighted in red.
* To be included in a drug-resistance treatment regimen only if there is sufficient laboratory evidence and clinical history suggesting the efficacy of the agent.
** Is a first-line anti-TB agent but is grouped with the other injectable agents.
† The 2020 WHO anti-TB drug groupings are the same as 2018 except amoxicillin is replaced with amikacin

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In June of 2020, the WHO released updated guidelines for the treatment of MDR/RR-TB. The update provided new guidelines for the WHO short course (9 - 11 month) regimen (described in Section 2.4.2.1), recommending the inclusion of BDQ instead of an injectable drug, thus making the regimen all oral[194]. The guidelines also provided support for the use of the all-oral BPaL regimen under operational research conditions (6 - 9 months), as well as a longer (18 – 20 month) all-oral individualised treatment regimen for select cases with severe disease. Lastly, the WHO concluded that BDQ treatment for more than six months was not associated with any major safety concerns[194].

These new WHO recommendations highlight that MDR/RR-TB treatment strategies are moving towards all-oral regimens and the elimination of SLIs due to their associated toxicity and therefore limited adherence. as well as reductions in the length of treatment. The guidelines also demonstrate the importance placed on BDQ in newer drug-resistant treatment strategies.

2.4.3.1 BDQ approval and conditional use

Following the positive preclinical results, BDQ entered the clinical trials phase of drug development. In 2011, whilst undergoing phase 2 clinical trials (expanded on in Section 2.5), Janssen, the manufacturers, introduced a pre-approval access program for BDQ[116]. This was shortly followed by the 2012 FDA approval of BDQ[8]. This accelerated approval could be explained by the promising results of past clinical studies[157,195], as well as the urgent need for new anti-TB drug options to combat MDR- and XDR-TB[196].

Initially, the use of BDQ was limited to conditional use in combination therapy for MDR-TB under the WHO interim guidelines[197]. Briefly, the five conditions to be met for the inclusion of BDQ in an adult MDR-TB treatment regimen were:

1. Closely monitored treatment administration
2. Appropriate patient inclusion (e.g. consideration of age, pulmonary disease, co-infection, etc.)
3. Patient informed consent
4. Adherence to the WHO-recommended guidelines for designing MDR-TB regimens
5. Pharmacovigilance and suitable management of AEs and prevention of drug-drug interactions[177]

The conditional recommendations placed on BDQ use by the WHO were based on the lack of data on clinical outcomes, the lack of information on optimal drug use, as well as an unexplained higher mortality rate in the BDQ arm of a prominent clinical trial[177,198]. Therefore, the WHO interim policy guidelines on BDQ use strongly recommended the development of

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accurate and reproducible DST for BDQ and other second-line anti-TB drugs, to provide comprehensive data for informing prospective policy recommendations[177].

Despite the lack of routine DST available for BDQ being commonplace in programmatic settings[199], the drug has been widely used[91] and was recently promoted to a group A drug by the WHO[95]. In June 2018 the South African Department of Health announced the use of BDQ for all eligible RR-TB patients, thus replacing injectable agents[200]. However, there are still substantial gaps in the data available for BDQ, including the lack of robust breakpoints[201] and the long-term safety of BDQ[161]. This emphasises the importance of the previous and current clinical trials investigating BDQ and the insights these trials will provide for the future of BDQ.

2.5 Clinical trials investigating BDQ

Clinical trials, specifically randomised controlled trials (RCTs), have traditionally served as the gold standard for clinical research, evaluating the safety and efficacy of a therapeutic agent before marketing authorisation[202]. A total of 11 phase 1 studies have been conducted, including 265 individuals collectively, during the 2005–2012 period to assess BDQ's pharmacokinetic and pharmacodynamic parameters, dosing regimens and drug-drug interactions[177]. Thereafter, the BDQ phase 2 programme began, which included two pivotal phase 2b clinical trials: TMC207-C208 and TMC207-C209[177]. It was soon after these phase 2b studies that BDQ received accelerated approval[203].

2.5.1 Completed trials.

2.5.1.1 TMC207-C208 randomised trial (NCT00449644)

One of the first clinical trials to take an in-depth look at the efficacy and effects of BDQ was the TMC207-C208 clinical trial. This trial consisted of a two-stage, phase 2, randomised, placebo-controlled trial.

The first exploratory stage of the trial was conducted in South Africa over an eight-week period to assess the overall safety of BDQ; determine the AE profile of the drug; and determine the pharmacokinetic and antibacterial activity during the prolonged course of administration. Recruitment started in June of 2007 and patients included in this study were between the ages of 18 to 65 with a new diagnosis of pulmonary MDR-TB. A total of 47 patients were included in the first stage and were randomly assigned at an almost 1:1 ratio to receive the placebo (24 patients), or BDQ (23 patients) in combination with a standard five-drug, second-line anti-TB regimen. Once the eight weeks of double-blind treatment were completed, patients continued treatment with the background regimen and received 96 weeks of follow-up[195].

The results of the first stage indicate that the addition of BDQ to standard MDR-TB therapy (a five-drug regimen, preferably ethionamide, PZA, ofloxacin, kanamycin and cycloserine)

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demonstrates safety and tolerability, as well as reduced culture conversion time. Furthermore, during the follow-up period, the risk of accumulating additional drug resistance was lower for the BDQ treated group compared to the placebo group (4.8% versus 21.7%). Lastly, the adverse events reported by patients seemed to be of similar frequencies ($p > 0.05$) for both the BDQ and placebo group, with the exception of a higher proportion of patients reporting nausea in the BDQ group (26% versus 4%; $p = 0.04$)[157].

After assessing that the serum drug levels were attained in the patients, the second stage of the trial began. For this proof-of-efficacy stage, BDQ was incorporated with the background regimen for 24 weeks in a new group of 160 patients from sites located in Brazil, India, Latvia, Peru, the Philippines, Russia, South Africa and Thailand. As with the first stage, patients received either BDQ or a placebo at a 1:1 ratio, in combination with a second-line anti-TB background regimen. The total trial duration was 30 months. This consisted of six months of BDQ administration during an 18 to 24-month treatment period, followed by six months of follow-up.

Table 2.3 Comparison of the results from Stage 1[195] and Stage 2[204] of the TMC207-208 trial

Key trial information and findings	**Stage 1:** ***exploratory***		**Stage 2:** ***proof-of-efficacy***	
	BDQ + BR	**Placebo + BR**	**BDQ + BR**	**Placebo + BR**
No. of patients	23	24	66	66
BDQ Treatment period	8 weeks	8 weeks	24 weeks	24 weeks
Total treatment period	96 weeks	96 weeks	96 weeks	96 weeks
No. of patients completed treatment	13/23 (56.5%)	11/24 (45.8%)	50/66 (75.7%)	49/66 (74.2%)
Culture conversion rate	48%	9%	79%	58%
Serious AEs	4.35%	4.17%	22.78%	18.52%
Patient deaths	1	0	10	2

BDQ = bedaquiline; BR = standard background regimen (five-drugs); AEs = adverse events

Both trials reported a reduction in time to sputum culture conversion and an increased rate of culture conversion[195,204]. However, the second stage results displayed pronounced QT prolongation in the BDQ group (mean increase of 15.4 msec at week 24) compared to the placebo arm (mean increase of 3.3 msec at week 24) of the study, although only one of the patients had a QT prolongation greater than 500 msec[204]. Furthermore, there was an increased mortality rate observed in the BDQ group (12.7%) compared to the placebo group

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(2.5%)[174]. However, none of the deaths were reportedly linked to BDQ and there was no association between these deaths and BDQ plasma concentrations[204].

After the interim results of the second stage of this study, BDQ was made available for compassionate use in 2011 and not long after, the drug received regulatory approval in the USA (2012) and Europe (2014)[174].

2.5.1.2 TMC207-C209 phase 2, open-label, single-arm trial (NCT00910871)

The second phase 2 study for BDQ had an open-label, single-arm design[205]. The study was undertaken to evaluate the safety, tolerability and effectiveness of the drug, in combination with a background regimen, for the treatment of adults with pulmonary MDR-TB, pre-XDR-TB and XDR-TB[205]. The study began enrolling patients from August 2009 to September 2010, at 31 sites across several countries including Peru, South Africa and various Asian and Eastern European countries. The study enrolled a total of 233 adult patients, of which 179 patients completed the study[174]. The primary efficacy endpoint for this study was the time to confirmed sputum culture conversion after 24 weeks of treatment[205]. The median time to culture conversion was 57 days and the conversion rate was 79.5%[174]. The culture conversion rate for this study was similar to the culture conversion rate observed in the C208 study (Table 2.4). Of the 54 patients that discontinued the study, 7.3% discontinued due to AEs[174].

Overall, the study reported that the AEs were mostly grade 1 or 2 and were comparable with other MDR-TB cohorts[199]. The frequency of serious AEs reported for this study were similar to that of the TMC207-C208 study (Table 2.4). A key finding of this phase 2 study was the overall mortality rate of 6.9%, a much lower mortality rate than the previous phase 2 study (Table 2.4), as well as more recent MDR-TB treatment mortality reports[206].

Table 2.4 Comparison of the key results found for the TMC207-C208 and TMC207-C209 clinical trials

Trial name	TMC207-C208	TMC207-C209
Culture conversion at week 24	78.8%	79.5%
Serious AEs at 120 weeks	23%	20.2%
Mortality	12%	6.9%

AEs = adverse events

Targeted sequencing of clinical isolates from the TMC207-C208 and TMC207-C209 trials revealed that *Rv0678* RAVs were present in 6.3% of MDR-TB isolates at baseline, with BDQ MICs ranging from high (>0.24 mg/L) to low (<0.03 mg/L) on 7H11 media[25]. The variable effect of these RAVs on the MICs highlights the importance of phenotypic DST in assessing BDQ susceptibility[25].

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The TMC207-C208 and TMC207-C209 trials served as the basis for the efficacy and safety of BDQ, informing the 2013 WHO interim guidelines on BDQ use for the treatment of MDR-TB[177].

2.5.1.3 NC-005 (NCT02193776)

The NC-005 clinical trial was a multicentre, phase 2b open-label, partially randomised study conducted at ten sites across Africa; seven sites in South Africa, two sites in Tanzania and one site in Uganda[207]. The aim of the study was to investigate the safety and efficacy of a regimen consisting of BDQ, PMD and PZA (BPaZ) compared to the standard TB regimen in patients, during an 8-week treatment period. The study included two patient cohorts: newly diagnosed drug-susceptible TB (DS-TB) and RR-TB[207]. Additionally, the study also assessed the response of RR-TB over eight weeks to BPaZ plus moxifloxacin (BPaMZ) as a means of compensating for potential PZA resistance[207]. Patient enrolment occurred between October 2014 and May 2016 and 180 DS-TB and 60 RR-TB patients were enrolled[208]. Participants were aged 18 to 75 years and were randomly assigned to receive one of the four therapeutic regimens for 56 days. The four different regimens consisted of:

1. DS-TB: BDQ (loading dose) + PMD-824 + PZA
2. DS-TB: BDQ (200 mg) + PMD + PZA
3. DS-TB: HRZE (INH + RIF + PZA + ethambutol)
4. MDR-TB: BDQ (200 mg) + moxifloxacin + PMD + PZA[207].

The primary efficacy endpoint was the daily percentage change in time to sputum culture positivity in liquid medium[207].

The first results for the NC-005 study were reported in 2017 at the Conference on Retroviruses and Opportunistic Infections (CROI). The results indicated that the BPaMZ regimen exhibited the highest bactericidal activity of the three regimens[208]. The 2017 results also indicated that the BPaMZ regimen was well tolerated[209]. The final results were published by Tweed and colleagues in 2019. They found that there was higher bactericidal activity in the BPaZ group compared to the standard regimen[207].

The trial indicated that PZA-susceptible RR-TB patients on the BPaMZ regimen displayed considerably faster sputum culture conversion compared to DS-TB patients receiving the standard regimen[210]. The BPaZ treatment group noted a higher proportion of individuals discontinuing the study due to AEs compared to the standard regimen[207]. The study reported 4% of DS-TB patients and 7% of RR-TB patients died, however, none of the deaths were considered to be associated with treatment[207].

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The promising results of both the BPaZ and the BPaMZ regimens in this study highlight the important role that newer drugs, such as BDQ and PMD, can have on treatment outcomes and therefore the design of shorter and more efficacious treatment regimens for both DS-TB and RR-TB.

2.5.1.4 Janssen Japan trial (NCT02365623)

Another principal phase 2 clinical trial investigating BDQ was the Janssen Japan trial. This open-label, single-arm, multicentre study was conducted in Japan at two sites and aimed to evaluate the safety, efficacy and pharmacokinetics of BDQ in the treatment of Japanese patients with pulmonary MDR-TB[87]. The study enrolled patients aged ≥20 years to receive BDQ for 24 weeks (with the option to extend the BDQ treatment period as per the investigator's judgement) in combination with an individualised background regimen[211]. The safety of BDQ was evaluated based on reports of treatment-related AEs. Efficacy was assessed based on time to sputum culture conversion after BDQ treatment was initiated[211].

A total of six patients were enrolled in the study. Of the six patients, five (83.3%) experienced treatment-related AEs during the investigational phase of the study[211]. This study reported no deaths and no discontinuation of BDQ treatment due to AEs. For the four patients evaluated as part of the full analysis set, the time to sputum culture (MGIT or Ogawa media) ranged from 14 to 15 days and their microbiological status was 'culture converted'[211]. This study reported no new safety concerns in the interim analysis, including the patients who received BDQ treatment beyond the initial 24-week treatment period[211].

2.5.1.5 NiX-TB trial (NCT02333799)

The Nix-TB trial was an open-label, single-group multisite study being conducted within South Africa, investigating the efficacy of an all oral six-month BPaL regimen in XDR-TB and treatment-intolerant or non-responsive MDR-TB patients. The phase 3 trial was conducted at three different study sites throughout South Africa: the Sizwe Tropical Disease Hospital in Johannesburg, the Task Applied Science group at Brooklyn Chest Hospital in Cape Town and the King DiniZulu Hospital Complex in Durban. They enrolled: patients >14 years old that phenotypically or genotypically tested with pulmonary MDR/XDR-TB, that were recorded on culture or molecular testing within three months before screening[175]. The trial also included HIV co-infected patients. Patients enrolled in the trial received six months of the BPaL treatment, with the option to continue the treatment regimen for an additional three months if patients were sputum culture-positive after four months[209]. After the exclusion process during screening, 109 patients were enrolled in the study in the period between April 2015 and November 2017 and included for safety and efficacy analysis[212].

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The first results for the Nix-TB trial were presented at the Conference on Retroviruses and Opportunistic Infections[130]. The results indicated that 61 patients were enrolled in the trial, four patients died during the initial treatment phase, 27% of patients experienced serious adverse events, 100% of patients achieved culture conversion after four months and one patient had microbiological relapse[130].

The trial reported good safety and efficacy for the short all-oral BPaL regimen and the preliminary data resulted in the FDA approval of DR-TB treatment using the BPaL, all-oral drug combination, for six-months[156].

The most recent results were published by Conradie *et al.* in 2020[175]. The results indicated that 90% of the patients had a favourable outcome (i.e. Resolution of clinical disease, negative culture status) at six months after treatment and 88% had a favourable outcome at 24 months after treatment follow-up[213]. The expected LZD toxicities were common (81% of patients experience neuropathy and 48% experienced myelosuppression) but manageable. Overall, the trial demonstrated that the BPaL regimen is a viable option for highly drug-resistant TB in the presence of adequate safety management[175].

2.5.1.6 NExT trial (NCT02454205)

The NExT trial was an open-label trial evaluating and comparing a 6 – 9-month all-oral regimen containing BDQ, ethionamide/high-dose INH, LZD, levofloxacin and PZA, to the standard short course MDR-TB WHO regimen (described in Section 2.4.2.1). The trials primary endpoint was a WHO-defined favourable treatment outcome 24-month post treatment initiation[214].

The trial was prematurely stopped in 2019 after the standard of care in South Africa became BDQ-based and thus randomisation amongst the treatment arms was no longer ethical[214]. The results of the study found that participants receiving the all-oral BDQ-containing regimen were 2.2 times more likely to experience a favourable outcome 24-month post treatment initiation compared to those receiving the standard-of-care regimen. However, drug toxicity was frequent in both study arms. Overall, the study provided support for shorter, all-oral regimens for MDR/RR-TB[214].

2.5.1.7 DELIBERATE ACTG 5343 (NCT02583048)

The “DELamanId BEdaquiline for ResistAnt TubErculosis (DELIBERATE)” or AIDS trial was an open-label phase 2 trial that assessed the safety, tolerability and pharmacokinetics of receiving BDQ, DLM or both[215,216]. Eighty-four RR-TB patients were recruited between August 2016 and July 2018 and were randomly assigned in a 1:1:1 ratio to the treatment groups. The

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primary end point of the study was to determine the QTc effects of the treatment groups over six months of MDR-TB treatment[216].

The results indicated that no participants experienced grade 3 or 4 QTc prolongation AEs and there was tolerability to the anti-TB drugs administered, including for HIV co-infected patients. The study observed cardiac safety with the co-administration of BDQ and DLM with clinically modest effects to the QTcF interval and therefore advocated the use of combined BDQ and DLM treatment[216].

2.5.2 Ongoing clinical trials

As part of the 2013 interim policy guidelines on BDQ use, the WHO strongly recommended the acceleration of phase 3 trials, to accumulate comprehensive evidence that can inform future policy guidelines updates[177]. Conducting phase 3 clinical trials in a short time frame in the 21st century is challenging[217]. For the most recent phase 3 trials, the period between the start of patient enrolment and the first publicly available results can range from 4.6 to 8.4 years[218–220].

These trials often require larger cohort sizes to demonstrate the benefits and the start of the trial is often delayed due to the need for independent ethical review(s), national regulatory body approval and training as well as Good Clinical Practice (GCP) compliance[217]. Furthermore, there may be cumulative trial costs relative to research and development funding shortfalls[221]. These challenges and time constraints make it difficult to rapidly conduct clinical trials that can address critical public health questions[217]. The phase 3 trials, amongst other ongoing phase 2 clinical trials, are summarised in Table 2.5.

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Table 2.5 Summary of current clinical trials investigating BDQ

Trial Name	Brief description	Phase	Study start date	Type of study	No. of enrolled patients	Status	Preliminary results available	Results	Estimated study completion date	Trial Registry identifier
STREAM stage II	An international, multi-centre, open-label, controlled trial in patients with MDR-TB including patients with RIF-resistant and INH-sensitive TB	3	2016	Interventional; Randomised; Parallel Assignment	588	Active, not recruiting	No	-	2022	NCT02409290
ZeNiX	Partially-blinded, trial assessing the safety and efficacy of various doses and treatment durations of BPaL in patients with pulmonary infection of XDR-TB, pre-XDR-TB or treatment intolerant or non-responsive MDR-TB	3	2017	Interventional; Randomised; Parallel Assignment	181	Active, not recruiting	Yes (Mishra 2021, Conradie *et al.* 2021)[213,222]	BPaL regimen effective against highly DR-TB Reduced dosage/duration of LZD similar efficacy and improved safety High relapse-free cure rate with BPaL	2021	NCT03086486
endTB	Open-label, non-inferiority, multi-country trial evaluating the efficacy and safety of 5 new, all-oral, shortened regimens for MDR-TB	3	2016	Interventional; Randomised; Parallel Assignment	750 (estimated)	Recruiting	Yes (Franke *et al.* 2021)[223]	Frequent culture conversion with a BDQ and/or DLM-containing regimen	2023	NCT02754765

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TB-PRACTECAL	Multi-centre, open-label, multi-arm, controlled trial; evaluating short treatment regimens containing BDQ and PMD in combination with existing and re-purposed anti-TB drugs for the treatment of biologically confirmed pulmonary MDR-TB	2/3	2017	Interventional; Randomised; Parallel Assignment	552	Active, not recruiting	No	-	2022	NCT02589782
SimpliciTB	Open-label, partially randomised trial to evaluate the efficacy, safety and tolerability of a 4-month treatment of BPaMZ compared to a 6-month treatment of HRZE/HR (Control) in adult participants with DS-TB and a 6-month treatment of BPaMZ in adult participants with smear-positive pulmonary DR-TB	2/3	2018	Interventional; Randomised; Parallel Assignment	455	Active, not recruiting	No	-	2022	NCT03338621

TRUNCATE TB	To evaluate 2-month regimens composed of first-line drugs in combination with new and repurposed second-line drugs for the treatment of DS-TB	2/3	2018	Interventional; Randomised; Parallel Assignment	900 (estimated)	Recruiting	No	-	2022	NCT03474198
Janssen C211	Open-label, multicentre, single-arm study evaluating the pharmacokinetics, safety, tolerability and anti-mycobacterial activity of BDQ in combination with a background regimen of MDR-TB drugs for the treatment of children and adolescents 0 months to <18 years of age who have confirmed or probable pulmonary MDR-TB	2	2016	Interventional; N/A; Single Group Assignment	60 (estimated)	Recruiting	No	-	2025	NCT02354014
IMPAACT P1108	Open-label trial to evaluate the pharmacokinetics, safety and tolerability of BDQ in Combination with optimized	1/2	2017	Interventional; N/A; Single Group Assignment	72 (estimated)	Recruiting	No	-	2023	NCT02906007

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	individualized MDR-TB therapy in HIV-infected and HIV-uninfected infants, children and adolescents with MDR-TB									
BEAT TB	Open-label trial to establish the efficacy and safety of 6 months of BDQ, DLM and LZD, with levofloxacin and CFZ compared to the current South African standard of care for 9 Months for the treatment of RR-TB	3	2019	Interventional; Randomised; Parallel Assignment	400 (estimated)	Recruiting	No	-	2023	NCT04062201
InDEX	Open-label, controlled trial comparing individualised treatment based on WGS to control standard of care TB treatment	4	2017	Interventional; Randomised; Parallel Assignment	448 (estimated)	Recruiting	No	-	2022	NCT03237182

BDQ = bedaquiline; PMD = pretomanid; LZD = linezolid; DLM = delamanid; RIF = rifampicin; CFZ = clofazimine; TB = tuberculosis; DS-TB = drug-susceptible TB; RR-TB = rifampicin-resistant-TB; MDR-TB = multi drug-resistant TB; XDR-TB = extensively drug-resistant TB; PK = pharmacokinetics; BPaL = regimen consisting of BDQ, PMD and LZD; HRZE/HR = first-line TB regimen (isoniazid, rifampicin, pyrazinamide, ethambutol/isoniazid, rifampicin); HIV = human immunodeficiency virus

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2.6 New regimens containing BDQ

2.6.1 BPaMZ and BPaL TB treatment regimens

The advent of novel anti-TB medications has made it possible to investigate and devise new regimens with the possibility of shortening TB treatment durations and effectively treating both drug-susceptible and DR strains[181]. A study using a murine model showed that a combination of BDQ, PMD, moxifloxacin and PZA (referred to as BPaMZ) had superior bactericidal and sterilizing activity compared to a combination of RIF, INH and PZA[224]. Further evidence from a clinical trial (discussed in Section 2.5.1) demonstrated faster sputum culture conversion in MDR-TB patients receiving the BPaMZ regimen compared to drug-susceptible patients undergoing the standard HRZE regimen[210].

Similarly, the BPaL (BDQ, PMD and LZD) regimen, consisting of two novel anti-TB drugs and a repurposed anti-TB drug, has shown superior bactericidal and sterilising efficacy compared to a first-line drug combination, when studied in a murine model[225]. The NiX-TB clinical trial was founded on the promising results of this study[156] and is discussed in Section 2.5.1.

Based on the WHO's re-prioritisation of anti-TB drugs and the promising results of novel treatment regimens, it is evident that newer drugs, such as BDQ, are pivotal for TB treatment regimens and should be considered as a priority.

2.7 Transmission of BDQ-resistance

The effective diagnosis and treatment of DR-TB is fundamental to the reduction in transmission[49]. Currently, with the lack of a standard genotypic diagnostic tool for BDQ-resistance, conducting phenotypic DST is essential where BDQ is being used to treat MDR- or XDR-TB on a programmatic level[226].

The extent of transmission of BDQ-resistance within communities is currently unknown, hence the systematic surveillance of BDQ-resistance is crucial. WGS and TDS data can be used to gain insight into transmission networks and the evolution of genomic mutations for TB[45]. Furthermore, the use of WGS for the analysis of the emergence of drug resistance in clinical isolates can provide scope on the efficacy and vulnerabilities of the current TB programme[227]. Lastly, monitoring the emergence of BDQ-resistance and identifying clustered networks indicative of transmission will provide an indication of the performance of BDQ in the national TB control programme, as well as the ability of the background regimen to protect BDQ as a new anti-TB agent.

South Africa's announcement in 2018 to institute the use of BDQ for all eligible RR-TB patients, thus replacing injectable agents[200], made it the first country to roll out BDQ for routine use. BDQ has played a critical role in the last decade in changing the treatment landscape for

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DR-TB[228], which is further illustrated in the most recent adjustments in the recommended treatment strategies by the WHO.

2.8 Concluding remarks

Combatting the DR-TB landscape requires new anti-TB drugs and novel regimens to improve clinical outcomes and bolster the standard treatment regimens that are currently available. Various clinical trials have demonstrated the safety and efficacy of BDQ in the treatment of MDR-TB and XDR-TB. Although resistance to BDQ and the frequency with which it is observed in recent studies is concerning, the therapeutic advantage it provides in designing DR-TB regimens makes it a promising anti-TB agent. However, with the ongoing evolution and transmission of DR MTB strains, there is a need for a more comprehensive understanding of the BDQ-resistance mechanisms and the possible transmission of this resistance within the community. Therefore, an efficient surveillance method is required to assess the performance of this anti-TB drug in the programmatic treatment of DR-TB.

NGS of amplicons is fast becoming a popular DST method for *Mycobacterium tuberculosis* (MTB)[4,28–32]. Furthermore, NGS can be used to detect and evaluate low levels of hetero-resistance: the presence of both WT and resistance-associated variants in the same isolate[229].

The Division of Molecular Biology and Human Genetics at Stellenbosch University is ideally positioned to address this question, given the archive of all DR-TB isolates cultured from patients residing in the Western Cape. With more than 45,000 MTB isolates already bio-banked, this is one of the largest global repositories with an ongoing collection period stemming from 2002. The drug-resistant MTB strains are amassed from an ongoing collaboration with the National Health Laboratory Services (NHLS) in Green Point, Cape Town, which serves as the TB reference laboratory for the Western Cape.

The NHLS processes all specimens of patients undergoing treatment at primary health care facilities in the Western Cape, performing analysis such as smear microscopy, Xpert MTB/RIF Ultra, Line Probe Assays (LPAs) and routine phenotypic DST (pDST). This archive provides an ideal resource where BDQ-resistance-conferring variants could be identified. We propose to rapidly identify variants in baseline and final isolates from all patients with DR-TB using targeted deep sequencing. Potential transmission networks will be identified through WGS and review of clinical records.

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Chapter 3: Methods and materials

This chapter will be divided into two sections. The first section will describe the methodology we followed at the translational genomics research institute (TGen), Flagstaff and the second section will focus on the implementation of the methodology transfer to Stellenbosch University (SU), Cape Town.

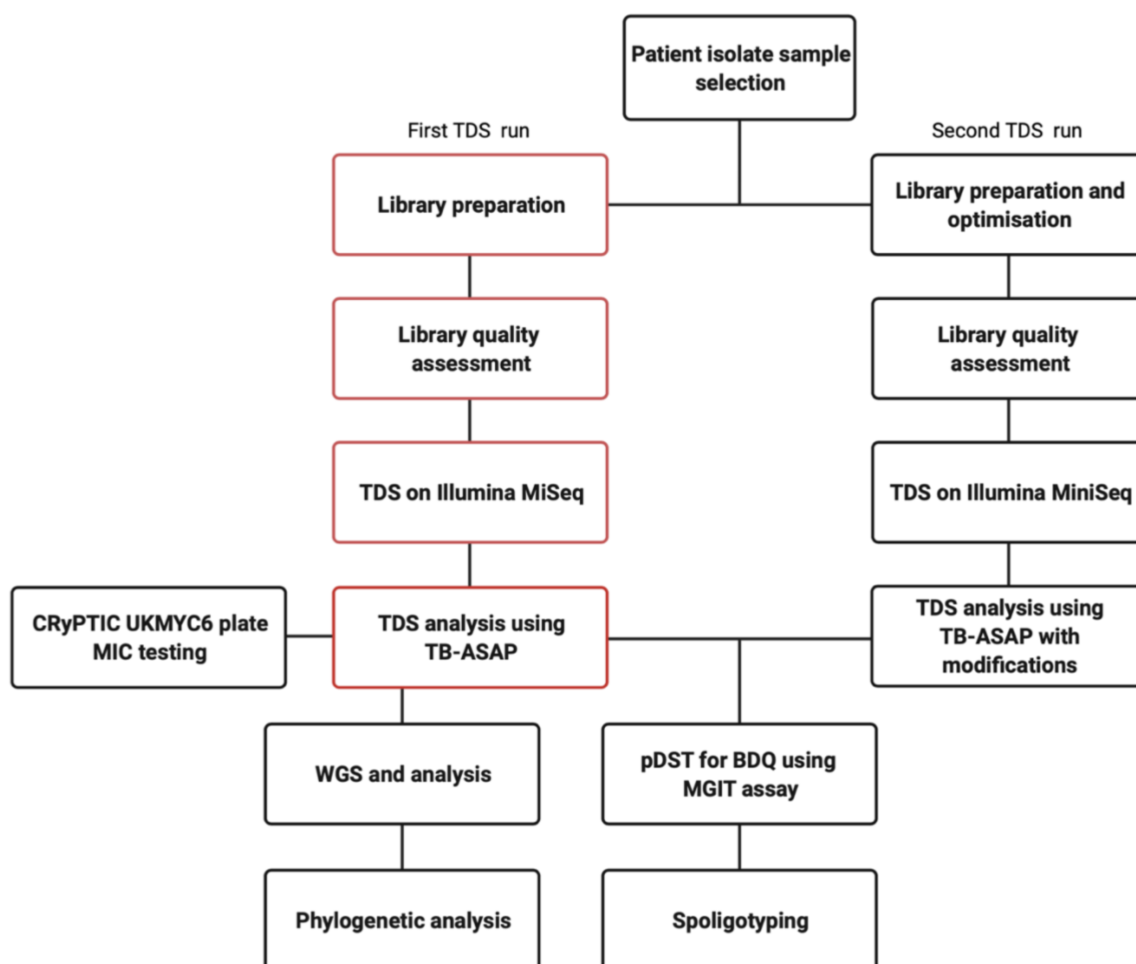

Figure 3.1 Flow diagram overview of methods. The red boxes indicate methods done at the translational genomic research institute (TGen) in Flagstaff, USA, whereas the black boxes indicate the methods done at the Stellenbosch University. TDS = targeted deep sequencing; TB-ASAP = TB-specific Amplicon Sequencing Analysis Pipeline; CRyPTIC = Comprehensive Resistance Prediction for Tuberculosis: An International Consortium; MIC = minimum inhibitory concentration; WGS = whole genome sequencing; pDST = phenotypic drug susceptibility testing, BDQ = bedaquiline; MGIT = mycobacterial growth indicator tube. Created by Amy Steinhobel using BioRender.com.

3.1 Patient isolate sample selection

Patient clinical isolates were selected from a reference database of drug-resistant *Mycobacterium tuberculosis* (MTB) strains housed at the Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences at Stellenbosch University.

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With more than 45,000 isolates already biobanked, this is one of the largest global repositories with an ongoing collection period stemming from 2002. The drug-resistant MTB strains are amassed from an ongoing collaboration with the National Health Laboratory Service (NHLS) in Green Point, Cape Town, which serves as the TB reference laboratory for the Western Cape. The NHLS processes all specimens of patients undergoing treatment at primary health care facilities in the Western Cape, performing analysis such as smear microscopy, Xpert MTB/RIF Ultra, Line Probe Assays (LPAs) and routine phenotypic drug susceptibility testing (pDST). The database houses patient information, including all isolates received per patient, clinic where the patient isolates were collected, NHLS drug resistance status of the isolates, etc.

For this study, the criteria for patient isolate selection were (a) isolates received by the NHLS between January 2018 and February 2019; (b) isolates presenting as rifampicin-resistant TB (RR-TB) at baseline (as determined by routine diagnostic testing by the NHLS); (c) only the first and last available isolate per patient (where possible); and (d) the first and last available patient isolate should be more than 60 days apart.

At the commencement of this study, the goal was to include patient isolates from the first few years that bedaquiline (BDQ) was rolled out in South Africa for routine use and thus extract additional patient isolate data after February 2019; the date of the initial patient isolate data extraction from the database. The RR-TB isolate selection included all isolates with at least rifampicin resistance and the drug resistance profiles ranged from rifampicin mono-resistant to extensively drug resistant TB (XDR-TB). The time period between the serial isolates was based on the median time to culture conversion in treatment of multi-drug resistant TB (MDR-TB) patients[230].

The database was filtered using the statistical software STATA (version 16.0)[231] to select all patients with isolates meeting the criteria. The subsequent patient isolate list was further curated, removing duplications and additional discrepancies (described in Appendix B).

3.2 Targeted deep sequencing on Illumina MiSeq (TGen, Flagstaff)

Targeted deep sequencing (TDS) was used to determine whether variants serve as a proxy for BDQ-resistance within the community, based on raised minimum inhibitory concentrations (MICs)[9,10,232,233]. The TDS was done at TGen, Flagstaff and consisted of library preparation, library quality assessment, setting up the TDS run on the Illumina MiSeq platform and bioinformatic analysis.

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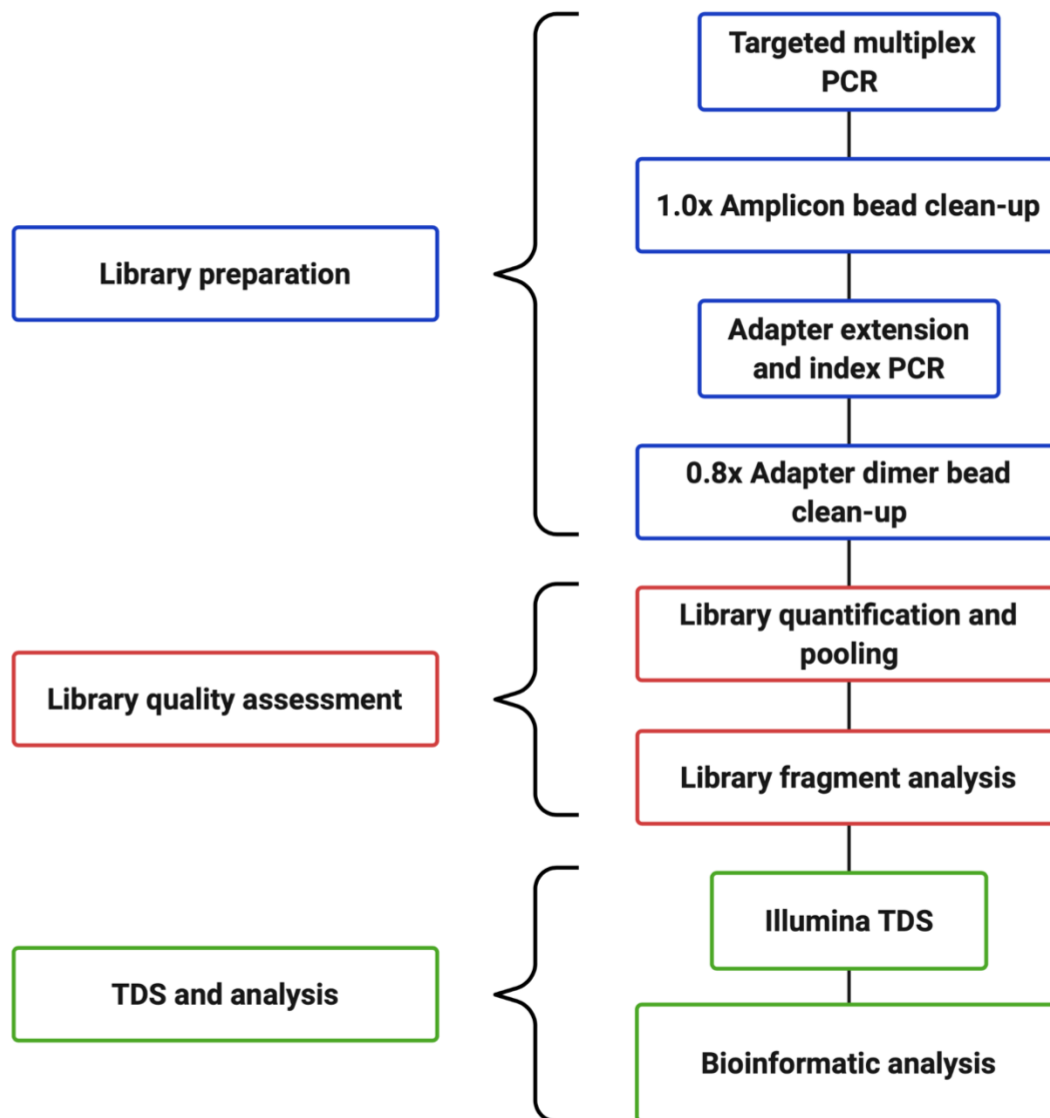

Figure 3.2 Flow diagram of the targeted deep sequencing protocol designed by TGen. TDS = targeted deep sequencing. Created by Amy Steinhobel using BioRender.com.

3.2.1 Library preparation for targeted deep sequencing

Library preparation consisted of creating amplicons for the three BDQ-resistance candidate genes *atpE*, *Rv0678* and *pepQ* (Figure 3.3) by multiplexing, an amplicon bead clean-up, an adapter extension and indexing PCR followed by an additional amplicon bead clean-up. The amplicon products span the full length of each genes. However, the primer trimming software used by the bioinformatic pipeline often trims the last few bases of the *Rv0678* and *pepQ* genes.

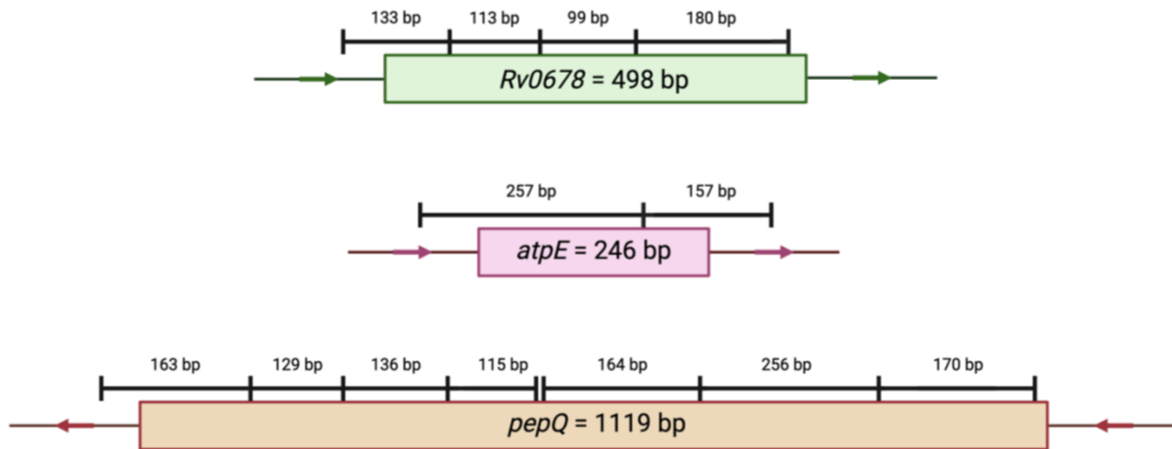

Figure 3.3 The three genes investigated for BDQ resistance-associated variants: *Rv0678*, *atpE* and *pepQ*. The genes and their corresponding sizes are shown and the arrows indicate the gene orientation. The amplicon products sizes created by library preparation for this protocol are depicted above the genes. Created by Amy Steinhobel using BioRender.com.

3.2.1.1 Targeted multiplex Polymerase Chain Reaction (PCR)

Multiplex polymerase chain reaction (PCR) for the gene targets were performed in a two master-mix setup, using a 96-well plate in a clinical array setup (Figure 3.4). Each master-mix contained several primer sets targeting different regions of the three genes, using previously designed primers (Table 3.1 and 3.2).

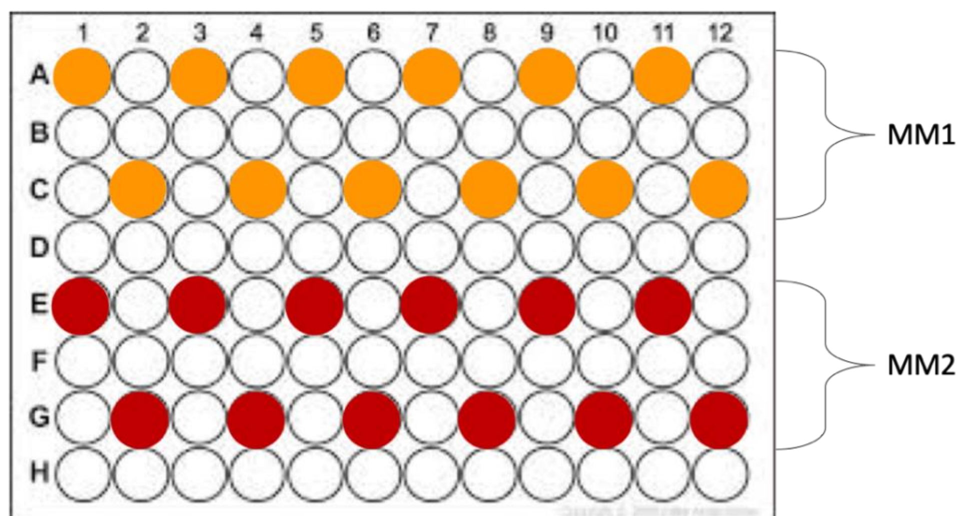

Figure 3.4 Master-mix 1 and 2 setup for a 96-well plate using a clinical array format. The clinical array format allowed for 10 isolates and two controls to be processed at a time on a 96-well plate, in a two master-mix setup. The clinical array format is indicated by the empty wells (white circles) surrounding the wells containing PCR reactions (orange and red circles) and is intended to prevent contamination across wells. The orange circles represent the master-mix 1 PCR reactions and the red circles represent the master-mix 2 PCR reactions. MM1 = master-mix 1; MM2 = master-mix 2.

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The PCR reactions were carried out in 30 µL reaction volumes. Master-mix 1 (MM1) had a total of 17 primers, whereas master-mix 2 (MM2) had 13 primers. The complete PCR master-mix compositions are described in Tables 3.1 and 3.2.

Table 3.1 PCR reaction composition for master-mix 1

Final Concentration	Reagent	Reagent volume (µL) per reaction
1×	NEB Q5 Hotstart	15.00
1 M	Betaine solution	6.00
200 nM	*Rv0678f*-57 + *Rv0678*r124	0.30
400 nM	*Rv0678*f115-1 + *Rv0678*r336-1 + *Rv0678*f115-2 + *Rv0678*r336-2	0.60
400 nM	*pepQ*f-60 + *pepQ*r152	0.60
200 nM	*pepQ*f199 + *pepQ*r421	0.30
100 nM	*pepQ*f496 + *pepQ*r713-1 + *pepQ*r713-2	0.15
100 nM	*pepQ*f899 + *pepQ*r+19	0.15
200 nM	*atpE*f-84 + *atpE*r222	0.30
-	Nuclease-free water	4.60
-	DNA template	2.00
	Total	**30.00**

Table 3.2 PCR reaction composition for master-mix 2

Final Concentration	Reagent	Reagent volume (µL) per reaction
1×	NEB Q5 Hotstart	15.00
1 M	Betaine solution	6.00
200 nM	*Rv0678*f64 + *Rv0678*r255	0.30
400 nM	*Rv0678*f291-1 + *Rv0678*f291-2 + *Rv0678*r+24	0.30
400 nM	*pepQ*f74 + *pepQ*r324	0.23
200 nM	*pepQ*f351 + *pepQ*r535	0.30
100 nM	*pepQ*f640 + *pepQ*r968	0.60
100 nM	*atpE*f130 + *atpE*r+96	0.30
-	DNA template	2.00
-	Nuclease-free water	4.90
	Total	**30.00**

NEB = New England Biolabs Inc.

Crude DNA templates for each isolate were centrifuged at 4000 relative centrifugal force (RCF) for 10 minutes. Thereafter, 2 µL was taken from the top of the solution and added to the PCR master-mix in a 96-well plate. For each master-mix, a no template control (NTC) and positive control were included: nuclease-free water and H37Ra genomic DNA, respectively. The PCR master-mix was briefly vortexed and spun down before PCR cycling in a standard thermal cycler. PCR reactions were set up in a one-way system, consisting of separate rooms (a pre-PCR room was used to prepare the master-mix setup and another room was dedicated

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to template addition) to avoid cross-contamination of amplicons. The PCRs were also set up in the mornings to prevent further cross-contamination from potential amplicon exposure. The thermocycling conditions for the PCR reactions are described in Table 3.3.

Table 3.3 Thermocycling conditions for gene-targeted multiplex PCR

Step	Cycles	Temperature (°C)	Time
Initial denaturation	1	98	2 min
Denaturation	25	98	30 sec
Annealing		60	20 sec
Extension		72	30 sec
Final extension	1	72	5 min
Cooling	1	10	∞

3.2.1.2 1.0× Amplicon bead clean-up

A 1.0× magnetic bead-based amplicon clean-up was performed on the PCR products to remove salts, primers, primer-dimers and deoxynucleoside triphosphates (dNTPs). Room temperature Agencourt AMPure XP beads (Beckman Coulter, Massachusetts) were resuspended thoroughly, vortexing for 10 seconds (or until all beads were thoroughly resuspended) and then 30 µL added to the PCR products. The beads were mixed with the PCR products by aspirating 10× and incubating for five minutes at room temperature. The PCR products were then incubated for a further five minutes on a magnetic plate to allow for separation. The liquid not bound to the beads was aspirated and discarded. Two 80% ethanol washes were conducted without disturbing the beads, before resuspending the PCR products in 20 µL of pre-heated (50°C) elution buffer (containing 10mM Tris-Hydrochloride (Tris-HCl) [pH 8.0] and 0.05% Tween 20) and transferring the cleaned-up products to a new plate for storage at -20°C. If the beads were disturbed the mixture was incubated for an additional two minutes on the magnetic plate before transferring the re-suspended amplicons to a new plate.

3.2.1.3 Adapter extension and index PCR

A total of 199 i5 and 199 i7 universally tailed (UT) 8-bp index primers designed by TGen were used to create unique index primer combinations for each library. The UT index primers were added to the individual libraries using an extension PCR step. The PCR reaction for the adapter and index PCR consisted of KAPA HiFi, Betaine solution, UT primer mixes (described in Appendix A), nuclease-free water (NFW) and an aliquot of the targeted multiplex PCR product in a 50 µL reaction (Table 3.4).

The PCR reactions were briefly vortexed and spun before thermocycling in a standard thermal cycler. The thermocycling conditions are described in Table 3.5.

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Table 3.4 PCR reaction calculations for adapter and index extension PCR

Final Concentration	Reagent name	Reagent volume (µL) per sample well
1×	KAPA HiFi	25
1M	Betaine solution	10
0.4 µM	UT Index primer mix (i5 and i7)	2
-	Targeted PCR product (post 1X bead clean-up)	2
-	Nuclease-free water	11
	Total	**50**

KAPA = KAPA Biosystems, Wilmington, USA; UT = Universally tailed; PCR = polymerase chain reaction

Table 3.5 Thermocycling conditions for adapter extension and index PCR

Step:	Cycles:	Temperature (°C):	Time:
Initial denaturation	1	98	2 min
Denaturation	6	98	30 sec
Annealing		60	20 sec
Extension		72	30 sec
Final extension	1	72	5 min
Cooling	1	10	∞

3.2.1.4 0.8× Adapter dimer bead clean-up

A 0.8× magnetic bead-based amplicon clean-up was performed on the PCR products as described in section 3.2.1.2, using 40 µL of room temperature Agencourt AMPure XP. Agarose gel electrophoresis was used to visualise the individual libraries and to identify adapter dimers. A Thermo Fisher Scientific E-Gel 48 Agarose Gel (2%) in conjunction with 5 µL Thermo Fisher Scientific E-Gel 50 bp DNA Ladder was used to visualise the adapter and index extension PCR products. The E-Gel was run for 5 minutes using a Thermo Fisher Scientific Mother E-Base Device and visualised using an iBright™ CL1000 Imaging System (Thermo Fisher Scientific Inc., Waltham, USA).

3.2.2 Library quality assessment

The quality of the libraries was assessed prior to setting up the TDS run. The quality assessment consisted of library quantification and fragment analysis.

3.2.2.1 Library quantification and pooling

Individual libraries were quantified using quantitative PCR (qPCR). A 1:100,000 dilution for each library was prepared in elution buffer (containing 10mM Tris-HCl pH 8.0 and 0.05% Tween 20) and the final dilution was used for qPCR with the KAPA Library Quantification kit (KK4873) for Illumina platforms (KAPA Biosystems, Wilmington, USA). The diluted libraries,

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as well as the six standards provided with the kit, were run in triplicate. The 10 µL qPCR reaction consisted of the following reagents: 6 µL KAPA SYBR® FAST qPCR Master Mix, 2 µL of NFW and 2 µL of the diluted library. The thermocycling conditions were as follows: initial denaturation at 95°C for 5 minutes (ramp rate of 1.9°C/second), followed by 40 cycles of denaturing at 95°C for 30 seconds (ramp rate of 1.9°C/second), annealing/extension at 60°C for 20 seconds (ramp rate of 1.6°C/second).

The linear range was computed for each qPCR run using the results from the standards and the quantity for each library was deduced relative to where the library was located on the linear plot. The plot was considered acceptable if the slope was around -3,3 and the R^2 value was about 0.99. The qPCR results were used to verify the individual library concentrations as well as confirm that the libraries had been successfully indexed during the adapter extension and index PCR step since the KAPA library quantification kits contain primers that are complementary to the P5 and P7 flow cell sequence motifs used for Illumina sequencing.

The individual libraries were subsequently pooled in equimolar concentrations based on the library concentrations determined during qPCR quantification. Two individual library pools were prepared, which were once more quantified using qPCR as described. These two library pools were pooled together equally based on the qPCR results to create the final pool of pools. The final pool was again quantified using qPCR and if the pool had a very high concentration (>10 000 pM), then it was diluted in Tris-HCl buffer and quantified again before proceeding.

3.2.2.2 Library fragment analysis

An Agilent DNA 2100 Bioanalyzer system with an Agilent DNA 1000 kit (Agilent Technologies Inc., Santa Clara, USA) was used for fragment analysis. The libraries were first pooled and quantified before running on the chip. The libraries were pooled by adjusting the volumes of each library in the pool based on the individual concentrations to obtain a final pooled concentration of 10.5 pM, containing equimolar concentrations of the individual libraries. Once the final library pool was created, a Bioanalyzer chip was run to check for the presence of adapter dimers (approximately 150 – 170 bp in length) in the final pool. The Bioanalyzer chip was run as per the manufacturer's instructions[234]. If adapter dimers were identified in the pool based on the Bioanalyzer chip electropherogram result, an additional 0.8× bead clean-up was performed on the final library pool and the steps from 3.2.2.1 were repeated. Subsequently, the final library pool was run on the Agilent chip.

3.2.3 Targeted deep sequencing on an Illumina MiSeq platform

An Illumina MiSeq platform was used to perform the TDS of the prepared libraries, with the 2 × 300 bp version 3 sequencing chemistry (Illumina, California, USA). The pooled libraries were diluted, pooled and denatured according to the guidelines provided by the Illumina MiSeq

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System Denature and Dilute libraries Guide[235]. These guidelines were also used to prepare the denatured PhiX Control and combine the denatured sample libraries with the denatured PhiX control for the final loading on the thawed reagent cartridge. The custom sequencing primers (Read 1, Read 2 and Index 1) were spiked into the Illumina sequencing primer wells (24, 25 and 28) at a final concentration of 0.5 µM, using the guidelines on the Illumina website[236].

3.2.4 Targeted deep sequencing run bioinformatic analysis

The TDS data were analysed using the TB-specific Amplicon Sequencing Analysis Pipeline (TB-ASAP). The pipeline yields a single nucleotide polymorphism (SNP) report for each isolate using the FASTQ files from the sequencer as well as a JavaScript Object Notation (JSON) file[35]. Briefly, the adapter sequences of the reads are trimmed and the quality assessed (average quality of below 20 was cut) using Trimmomatic (version 0.32)[237]. The trimmed reads are then aligned to the MTB reference sequence (accession no. NC_000962; National Center for Biotechnology Information (NCBI)) using Novoalign (Novocraft, Selangor, Malaysia) (version 3.02.13) mapping software with the default parameters. The binary alignment map (BAM) files alongside the JSON files are analysed using the Single-Molecule Overlapping Read (SMOR) analysis script (https://github.com/TGenNorth/SMOR). The script is automated to detect the presence and frequency of any SNP within the sequenced amplicons and outputs an excel sheet with a summary of all the variants.

3.3 Targeted deep sequencing on Illumina MiniSeq (SU, Cape Town)

The second TDS run was done at SU, Cape Town and consisted of library preparation and optimisation, library quality assessment, setting up the TDS run on the Illumina MiniSeq platform and TDS run bioinformatic analysis.

3.3.1 Library preparation and optimisation steps for the protocol

The second TDS run was conducted at the South African Medical Research Council (SAMRC) in Cape Town. To ensure the TDS run would be successful, various methods were used to optimise the library preparation steps.

Due to differences in equipment and discrepancies between reagents available at the different institutions, some optimisation was needed for TDS library preparation.

3.3.1.1 Maximising sample processing for a 96-well plate

The clinical array setup for the PCR plates was removed and all available wells of the 96-well plate were utilised (Figure 3.5). This allowed for the inclusion of 46 patient isolates per plate in a two MM setup, in addition to the NTC and H37Rv control. This allowed for maximising on

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space for targeted multiplex PCR and adapter extension PCR plate setup, as well as the time for sample processing.

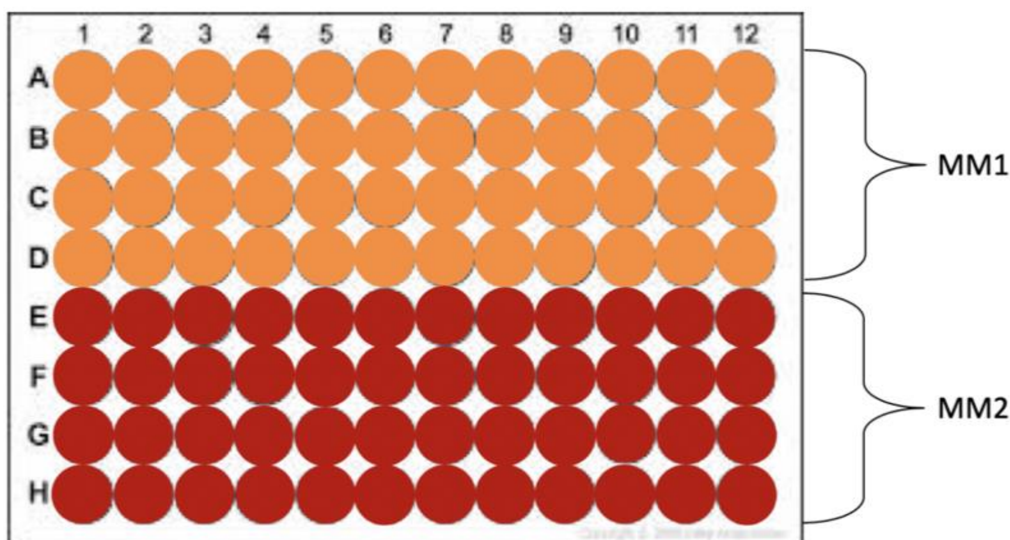

Figure 3.5 Master-mix 1 and 2 setups for a 96-well plate using a non-clinical array format. The non-clinical array format allowed for 46 isolates and two controls to be processed at a time on a 96-well plate, in a two master-mix setup thus maximising the number of sample isolates processed per plate. The orange circles represent the master-mix 1 PCR reactions and the red circles represent the master-mix 2 PCR reactions. MM1 = master-mix 1; MM2 = master-mix 2.

3.3.1.2 Testing various cycle numbers for the targeted multiplex PCR

First, the optimal cycle number for the BDQ gene target multiplex PCR step was determined. A gradient PCR was setup to investigate a range of 25 - 40 cycles. Briefly, the PCR reaction was setup as described in section 3.2.1.1 and run in a standard thermocycler with different cycle numbers.

A Qubit® dsDNA High Sensitivity assay (Thermo Fisher Scientific Inc., Waltham, USA) was used to determine the yield.

The PCR products size and intensity were evaluated for the various cycle numbers using agarose gel electrophoresis. A 2% agarose gel was prepared with 100 ml 1X tris base acetic acid ethylene diamine tetra acetic acid (TAE) buffer (pH 8.0) (Appendix A), was used for the visualisation and evaluation of the PCR products.

3.3.1.3 Testing various annealing temperatures for the targeted multiplex PCR

A range of temperatures (58°C - 66°C) were tested to determine the optimal annealing temperature that produced quality libraries using a gradient PCR. Briefly, a PCR reaction was set up as described in section 3.2.1.1. The product yield of the various annealing temperatures was assessed by agarose gel visualisation as described in Section 3.3.1.2.

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3.3.1.4 Testing various cycle numbers for the adapter extension PCR

The number of cycles for the adapter extension and indexing PCR step was optimised by testing a range of cycles (4× – 10×) using a gradient PCR. Briefly, the PCR was setup as previously described in Section 3.2.1.3 and run in a standard thermocycler.

The PCR products post 0.8× bead clean-up were compared on an agarose gel (as described in Section 3.3.1.2) and using the Qubit HS DNA assay (as described in Section 3.3.1.2) to determine number of cycles with the best library yield.

3.3.1.5 Adjusting the volume of amplicon product to be used for the adapter extension PCR

The template volume to be added to the adapter extension and indexing PCR was assessed. PCR reactions were setup as described in Section 3.2.1.3 to compare the original template volume of 2 µL to a template volume of 4 µL. The PCR products were compared on an agarose gel (as described in Section 3.3.1.2) and using the Qubit HS DNA assay (as described in Section 3.3.1.2).

3.3.1.6 Adjusting the bead ratio for the 0.8× bead clean-up

The volumes of the adapter extension PCR products were measured using a micropipette prior to performing the 0.8× AMPure XP bead clean-up to ensure an accurate volume was used to obtain the correct 0.8× bead ratio. The volume of the beads to be added was adjusted based on the actual volume of the PCR products and a 0.8× magnetic bead-based amplicon clean-up was performed on the PCR products as described in section 3.2.1.4.

The cleaned-up products were visualised with agarose gel electrophoresis as described in Section 3.3.1.2.

3.3.2 Library quality assessment

The quality of the libraries was assessed prior to TDS. The quality assessment consisted of library quantification and library fragment analysis.

3.3.2.1 Library quantification and pooling

The Qubit High Sensitivity assay (Invitrogen, Thermo Fisher Scientific Inc., Waltham, USA) was used to quantify the concentration of the individual libraries using a Qubit 4 fluorometer (Invitrogen, Thermo Fisher Scientific Inc., Waltham, USA). The Qubit reagents were prepared and the concentrations of the libraries were tested on the fluorometer as described in Section 3.3.1.2. The library concentrations were recorded and any libraries that were outside the range of quantification for the High Sensitivity assay were diluted in the storage buffer (10mM Tris-HCl pH 8.0; and 0.05% Tween 20) before re-quantifying.

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The individual libraries were subsequently pooled in equimolar concentrations based on the library concentrations determined by the Qubit High Sensitivity assay. Eight library pools were prepared, which were once more quantified using the Qubit High Sensitivity assay as described. An equimolar solution of the eight pools was created based on the Qubit results. The concentration of this final pool was determined by Qubit High Sensitivity assay.

In addition to Qubit analysis, the concentration of the final library pool was confirmed using the universal KAPA Library Quantification kit (KK4824) for Illumina platforms (KAPA Biosystems, Wilmington, USA). A 1:100,000 dilution for each library was prepared in 10mM Tris-HCl pH 8.0; 0.05% Tween 20 buffer and the 1:10,000 and 1:100,000 dilutions were assessed with qPCR. The diluted libraries as well as the standards provided with the kit were run in triplicate. The 10.2 µL qPCR reaction consisted of the following reagents: 6 µl KAPA SYBR® FAST qPCR Master Mix, 0.2 µL of 50× ROX Low and 4 µL of the diluted library. The thermocycling conditions were as follows: initial denaturation at 95°C for 5 minutes (ramp rate of 1.9°C/second), followed by 40 cycles of denaturing at 95°C for 30 seconds (ramp rate of 1.9°C/second), annealing/extension at 60°C for 20 seconds (ramp rate of 1.6°C/second). A melt curve was included to determine the presence of adapter dimers in the pools. The linear range was computed for each qPCR run using the results from the standards as is described in Section 3.2.2.1.

If the pool had a very high concentration (>10 000 pM), then it was diluted in Tris-HCl buffer and quantified again before proceeding.

3.3.2.2 Library fragment analysis

The libraries were pooled by adjusting the volumes of each library in the pool based on the individual concentrations as determined by the Qubit High Sensitivity dsDNA assay. The Perkin Elmer LabChip GX/GXII Touch was used with a HT DNA High Sensitivity Assay and LabChip (Perkin Elmer, Waltham, MA, USA) for fragment analysis and determining the presence of adapter dimers (approximately 150 – 170 bp in length) in the final pool.

The reagent and chip preparation were carried out as per the manufacturer's instructions [238] with adaptations to the ladder preparation step. The ladder was prepared in a two-step dilution process. First, a 1× dilution was prepared by adding 12 µL of DNA ladder to 108 µl of 10mM Tris-HCl [pH 8.0], 0.05% Tween 20 buffer in a PCR tube and mixing gently by aspirating 10 times. A 0.2× dilution was then prepared in another PCR tube by transferring 24 µL of the 1× dilution to 96 µL of NFW and gently mixing by aspirating 10 times. The entirety of the 0.2× dilution ladder was then transferred to the ladder tube. Both the prepared ladder tube and the buffer tube were then loaded onto the LabChip GX/GXII Touch.

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The LabChip run was then set up on the LabChip GX/GXII Touch using machine guidelines [239], allowing for an initial priming step before the run commenced.

If adapter dimers were identified on the electropherogram of the final library pool, an additional 0.8× bead clean-up was performed on the pooled library and the steps from 3.3.2.1 were repeated. Subsequently, another LabChip run was performed for the final library pool.

3.3.3 Targeted deep sequencing on an Illumina MiniSeq platform

An Illumina MiniSeq platform (Illumina, California, USA) was used to perform the TDS of the prepared libraries with a Mid Output 300 cycle reagent cartridge. The pooled libraries were diluted, pooled and denatured according to the guidelines provided by the Illumina MiniSeq System Denature and Dilute Libraries Guide [240]. These guidelines were also used to prepare the denatured PhiX Control and combine the denatured sample libraries with the denatured PhiX control for the final loading on the thawed reagent cartridge. The cartridge loading, flow-cell preparation and setting up of the sequencing run were done according to the Illumina MiniSeq System Guide [241]. The custom sequencing primers (Read 1, Read 2, Index 1 and Index 2) were spiked into the Illumina sequencing primer wells (24, 25 and 28) at a final concentration of 0.3 µM, using the guidelines on the Illumina website[236].

3.3.4 Targeted deep sequencing run bioinformatic analysis

The TDS data were analysed as described in Section 3.2.4. However, since the sequencing was done on a MiniSeq, which generates shorter reads (150 vs 300), the TB-ASAP pipeline was not set up to analyse these reads with SMOR analysis and adjustments had to be made: The TB-ASAP pipeline was run as described previously, without the SMOR analysis command and the cut-off was set to 5% for single nucleotide variants (SNVs) and indels to exclude as much sequencing error as possible. From there, any variants detected in the H37Rv controls were excluded in the analysis of patient isolates.

The remaining variants were manually investigated using the alignment viewer Tablet[242] and an online translation program Expasy (SIB Swiss Institute of Bioinformatics, Switzerland)[243] excluding all false variants and all synonymous variants from the final list of isolates with variants.

3.4 Bedaquiline phenotypic drug susceptibility testing (pDST)

The BDQ phenotypic drug resistance profile was determined for isolates with genetic variants in BDQ-resistance-associated genes using phenotypic drug susceptibility testing (pDST). The BACTEC™ Mycobacteria Growth Indicator Tube (MGIT)™ 960 instrument was used to determine the BDQ-resistance profiles of the isolates and the CRyPTIC UKMYC6 plate was used to determine the MICs for a subset of the isolates.

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3.4.1 Bedaquiline drug preparation

A concentration of 1.0 µg/mL was used to define phenotypic BDQ susceptibility using the MGIT assay. This was based on the technical manual for DST by the WHO[244], as well as other studies[58,188,245]. A 1.0 mL stock solution of BDQ was prepared in DMSO-H_2O (Merck group, Sigma-Aldrich) at a final concentration of 10 mg/mL in a 1.5mL polystyrene tube (Thermo Fisher Scientific Inc., Waltham, USA). A working solution of 1,000 µg/mL was prepared by diluting the 10 mg/mL stock solution 1:10 in dH_2O. The 1,000 µg/mL solution was used to prepare an 84 µg/mL working stock solution (calculations in Appendix A). This 84 µg/mL solution was used to achieve the 1.0 µg/mL concentration for pDST in MGIT. The stock and working stock solutions were stored at -20°C for up to six months. The BDQ working solution of 84 µg/mL was freshly prepared for each DST experiment.

3.4.2 Sub-culturing for BDQ DST in MGIT

Sub-cultures in MGIT medium were prepared for each of the patient isolates selected, using the standard recommended procedures[246,247]. The inoculated MGIT tubes were incubated in the MGIT™ 960 instrument at 37°C (±1°C) until a growth indicator was obtained. The first day that the MGIT™ tube indicated positive was considered day 0. The positive cultures were incubated for an additional 48 hours to ensure optimal growth for pDST testing. Sub-cultures on day 1 or 2 of positivity could be used directly for setting up BDQ pDST. However, if the MGIT tube positivity was between days 3–5, a 1:5 culture dilution was prepared using sterile 0.85% saline (Appendix A). A contamination check for each sample was done by inoculating a drop of each positive MGIT tube onto a blood agar culture plate and incubating at 37°C for 48 hours[248], as well as using Ziel-Neelsen (ZN) gram staining[249].

3.4.3 Setting up phenotypic DST in MGIT

The pDST for BDQ was performed using the BACTEC™ MGIT 960 instrument (Becton Dickinson, Flanklin Lakes, NJ, USA) in combination with the EpiCentre software equipped with the TBeXist module, as described previously. The interim critical concentration (CC) for BDQ of 1.0 µg/mL was used. The MGIT 960 DST methodology recommended for DST first-line drugs was used, including the minor modifications for BDQ[250]. The drug-free growth control tube inoculum was prepared by diluting the positive sub-cultures (1:100) in sterile 0.85% saline (Appendix A). Once inoculated into the growth control tube, this represented 1% of the bacterial population.

The growth control and drug-containing MGIT tubes for each sample were allowed to incubate in the BACTEC MGIT™ 960 system until the growth control MGIT tube reached a growth unit of ≥400 as determined by the TBeXIST software (a maximum of 28 days). Once this growth unit was reached, the growth unit (GU) of the drug-containing MGIT tube could be interpreted.

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A GU of ≥100 for the drug-containing tube confirmed BDQ-resistance, whereas a GU of ≤100 confirmed BDQ-susceptibility.

3.5 Isolate drug resistance profile and lineage determination

3.5.1 CRyPTIC UKMYC6 MIC determination

The first subset of isolates with variants that were identified by TDS underwent minimum inhibitory concentration (MIC) testing using the UKMYC6 (Thermo Fisher Scientific Inc., UK) plate developed by the CRyPTIC Consortium (Comprehensive Resistance Prediction for Tuberculosis: An International Consortium)[251]. The UKMYC6 plate is based on the UKMYC5 plate previously designed by the CRyPTIC Consortium which was used to measure the MICs of 14 different anti-TB drugs, including first- and second line drugs[252].

The UKMYC6 plates were subsequently modified, adjusting the concentrations of certain drugs, removing the anti-TB agent para-aminosalicylic acid and thus testing the MICs of 13 drugs[251] including isoniazid, rifampicin, ethambutol, amikacin, kanamycin, moxifloxacin, levofloxacin, ethionamide, rifabutin, linezolid, clofazimine, bedaquiline and delamanid.

The plates were prepared and evaluated as previously described by Rancoita and colleagues[252]. Briefly, patient isolates were sub-cultured using MGIT tubes to prepare starter cultures as previously described (Section 3.4.2). The positive cultures were adjusted to the turbidity of a McFarland 0.5 standard using saline-tween with glass beads (Thermo Fisher Scientific Inc., Waltham, USA) and a Sensititre™ Nephelometer (Thermo Fisher Scientific Inc., Waltham, USA). The adjusted culture was then inoculated into BD Difco™ Dehydrated Culture Media: Middlebrook 7H9 Broth (Becton Dickinson, Flanklin Lakes, NJ, USA) supplemented with 10% OADC, 0.2% glycerol and 0.05% Tween 80 (Appendix A) in filtered screw cap tissue culture flasks (Greiner Bio-one, Maybachstreet, Germany), before aliquoting 100 µL of the inoculum into each well of the UKMYC6 microtiter plate. The plate was then sealed properly and incubated at 37°C for 14 – 21 days.

The sealed microtiter plates were read by capturing an image of the plate using a digital camera. The plate images were then analysed, assessing the growth controls (H11 and H12) first for acceptable growth, followed by the growth in the drug-containing wells. The first drug dilution with no growth detected was recorded for each of the anti-TB agents.

3.5.2 Spoligotype lineage determination

The PCR-based genotyping method, spacer oligonucleotide typing (spoligotyping)[253], was used for identifying and differentiating the patient isolate strains. This method is based on the unique structure and preservation of the direct repeat (DR) locus of the genome and the

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neighbouring spacers, in combination, they are known as the direct variable repeat (DVR) sequences[254].

Spoligotyping was performed as per the internationally standardised PCR protocol[255], using the DR primers DRa (GGTTTTGGGTCTGACGAC) and DRb (CCGAGAGGGGACGGAAAC). The PCR-amplified spacer DNA was perpendicularly hybridised to oligonucleotides covalently bound to a membrane. Since one of the DR primers is biotinylated, hybridisation was detected by chemiluminescence and exposure to X-ray film or UV light using a Chemidoc XRS+ Gel Imaging System (Bio-Rad Laboratories, Inc., CA, USA). The distinct hybridisation patterns caused by the absence or presence of the DVRs observed were used to differentiate between the strains and the hybridisation pattern was analysed using the online genotyping database SITVIT2 (http://www.pasteur-guadeloupe.fr:8081/SITVIT2)[256].

3.6 Whole-genome sequencing (WGS) and analysis

3.6.1 Culture of *M. tuberculosis* isolates with confirmed phenotypic BDQ-resistance

A 5mL culture for each phenotypically BDQ resistant isolate was set up for DNA extraction. Briefly, primary cultures were prepared by inoculating 2 - 3 beads of frozen culture glycerol stock into 5mL 7H9 Middlebrook liquid media (Becton Dickinson, Flanklin Lakes, NJ, USA) supplemented with 10% OADC, 0.2% glycerol and 0.05% Tween 80 in filtered screw cap tissue culture flasks (Greiner Bio-one, Maybachstreet, Germany). The cultures were incubated at 37°C for 2 – 3 weeks. The cultures were inspected for contamination using ZN gram staining[249] and inoculation on blood agar culture plates[248]. After confirming the absence of contamination, glycerol freezer stocks were prepared for each culture and subsequently stored at -80°C.

3.6.2 DNA extraction from *M. tuberculosis* cultures and quantification

Once the cultures displayed sufficient turbidity, each 5mL culture was transferred to a labelled 15mL polypropylene tube and heat-killed in an oven at ~80°C for two hours. Subsequently, the tubes were centrifuged at 4,000 RCF for 30 minutes at room temperature and the supernatant was discarded leaving only the pellet in the tube. The pellet was resuspended in 400 µL Tris-ethylenediaminetetraacetic acid (TE) buffer (Appendix A); pH 8.00. Fresh lysozyme (10 mg/ml) was prepared and 50 µL was added to the re-suspended pellet, followed by gently flicking to evenly distribute the enzyme for bacterial cell lysis. The lysing efficiency of the enzyme was increased by overnight incubation of the tubes at 37°C.

The following day, 70 µL of 10% (v/v) sodium dodecyl sulphate (SDS) (Sigma-Aldrich, St. Louis, Germany) and 5 µL of proteinase K (10 mg/mL) was added to each tube of cell lysate and mixed by inversion. The tubes were incubated at 65°C for 10 minutes with intermittent

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mixing. Following the 10-minute incubation, 100 µL 5M sodium chloride (NaCl) was added to the tubes and thoroughly mixed before adding 100 µL cetyl trimethylammonium bromide (CTAB)/NaCl (10% w/v CTAB, 0.7 M NaCl) (Appendix A) solution. The CTAB/NaCl solution was prewarmed at 65°C to ensure accurate pipetting of the viscous liquid. After the incubation step was completed, the tubes were mixed for ~20 seconds using a vortex until the solution appeared milky and then incubated at 65°C once again for 10 minutes. Subsequently, 750 µL of room temperature chloroform/isoamyl alcohol (24:1 v/v) (Merck Laboratories, New Jersey, USA) was added to each tube and vortexed for 10 seconds or more, followed by centrifugation at room temperature, 12,000 RCF for 10 minutes. The aqueous phase was carefully aspirated, without disturbing the interphase and transferred into a sterile 1.5 mL low-binding Eppendorf tube (Hamburg, Germany). Thereafter, 450 - 500 µL ice-cold isopropanol (Merck Laboratories, New Jersey, USA) was added to each tube, followed by mixing by inversion and incubation at -20°C for one hour.

Following incubation, the tubes were centrifuged at room temperature, 12,000 RCF for 30 minutes and the supernatant was aspirated and discarded, without disturbing the pellet. The pellet was washed using 1.0 mL of cold 70% ethanol and then centrifuged again at room temperature for 30 minutes at 12,000 RCF. The ethanol was aspirated without disturbing the pellet and allowed to air-dry at room temperature overnight. The following day, the dried pellet was resuspended in 25 µL of TE buffer and incubated at 4°C overnight.

Following overnight incubation, the extracted DNA for each isolate was quantified and the quality was assessed. The DNA concentration was measured using the Thermo Scientific NanoDrop 2000/2000 C spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). Briefly, the NanoDrop was calibrated using 1.0 µL of TE buffer as the blank, followed by measuring 1.0 µL of each isolate at 260 nm. Furthermore, the quality of the extracted DNA was assessed using the A260/280 and A260/230 ratios. An isolate with an A260/280 ratio of 1.8 and an A260/230 ratio between 2.0 and 2.2 was considered to be high-quality DNA[257].

3.6.3 Illumina WGS

Whole-genome sequencing (WGS) was done at the South African Medical Research Council (SAMRC) Genomics Centre using the Illumina MiniSeq platform (San Diego, Ca, USA). The DNA concentration of the genomic DNA was confirmed using Qubit High Sensitivity dsDNA assay prior to library preparation for WGS.

The WGS libraries were prepared using the Nextera DNA Flex Library Preparation Kit (San Diego, Ca, USA) according to the manufacturer's instructions. Each library was normalised to 1 ng/µL by diluting in 10 mM Tris-HCl (Appendix A). The concentrations of the normalised

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libraries were confirmed using the Qubit High Sensitivity dsDNA assay (Thermo Fisher Scientific Inc., Waltham, USA).

The normalised sample libraries then underwent On-Bead Tagmentation, which combines the fragmentation of the genomic DNA and the adapter ligation into a single reaction. This was followed by library preparation where the tagmented libraries were indexed using the Nextera XT Index Kit (San Diego, Ca, USA) and the manufacturer's guidelines. Indexed libraries then underwent library clean up using Agencourt AMPure XP beads, which was followed by another quantification step using the Qubit High Sensitivity dsDNA assay. Subsequently, fragment analysis is done to determine the fragment sizes of the libraries using the Perkin Elmer LabChip GX/GXII Touch with a HT DNA High Sensitivity Assay and LabChip.

Finally, the libraries were diluted, pooled and denatured according to the guidelines provided by the Illumina MiniSeq System Denature and Dilute libraries Guide[240]. These guidelines were also used to prepare the denatured PhiX Control and combine the denatured sample libraries with the denatured PhiX control for the final loading on the thawed reagent cartridge. Depending on the number of samples available to set up a WGS run, either a Mid or High Output Illumina MiniSeq 300 cycle cartridge was used (San Diego, Ca, USA). The cartridge loading, flow-cell preparation and setting up of the sequencing run were done according to the Illumina MiniSeq System Guide[241].

3.6.4 Computational analysis of WGS

The whole-genome sequences for the isolates were analysed using an in-house custom automated NGS pipeline, USAP (universal sequence analysis pipeline), as well as TB-Profiler[258]. The in-house automated pipeline (USAP) was used for rapid analysis of the MTB WGS data as previously described[259]. Briefly, the quality of the raw data (in FASTQ format) was assessed using FastQC software (Babraham Institute, UK) followed by trimming of the reads by Trimmomatic (version 0.32)[237]. The trimmed reads were aligned to the MTB reference genome H37Rv (GenBank accession no. NC_000962.3) using three alignment algorithms: Burrows-Wheeler Aligner (BWA) (version 0.6.2), Novoalign (Novocraft, Selangor, Malaysia) (version 3.02.13) and SMALT (version 0.75)[260,261]. The alignments were validated and improved upon using various open-source software tools, prior to variant identification by means of two variant callers: The Genome Analysis Toolkit (GATK) and SAMTools (version 1.3). Lastly, the sequence data was annotated and the lineage classification was determined[262].

TB-profiler is a pipeline that uses WGS data, in FASTQ format, for lineage and drug resistance prediction for 14 anti-TB drugs[258]. The TB-profiler tool was used in the command line. After

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installing the latest TB-profiler software (version 3.0.3), all FASTQ files for the WGS patient isolates were run through the software and subsequently the results were collated.

3.7 Phylogenetic analysis

A phylogenetic analysis was done to determine whether any of the isolates were closely related (within a variant distance that is regarded as epidemiologically indicative of transmission events) and the potential transmission networks of the closely related strains will be determined.

A maximum-likelihood phylogenetic tree was created for the isolates using the WGS data analysed by the Universal Sequence Analysis Pipeline (USAP) and IQtree software (version 2.1.3). First, the relevant annotated variants were filtered to exclude heterogenous variants (using an allele frequency of 1.0 as the cut-off), low-quality variants and variants found in hard-to-map regions (PE/PPE, insertion sequences and phages, as well as repeat regions), by only including variants identified by two variant callers, in all three alignment algorithms. A SNP string consisting of all high confidence variable sites (SNPs only) were used for phylogenetic analysis as previously described[259]. The IQTree 2 software was used to build the maximum likelihood phylogenetic tree[263] and the online tool Interactive Tree of Life (iTOL) was used to visualise and adjust the tree[264].

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Chapter 4: Results

This chapter will be divided into 2 sections. The first section will describe the results obtained at the translational genomics research institute (TGen), Flagstaff and the second section will focus on the implementation and optimisation of the methodology transferred to Stellenbosch University (SU), Cape Town, as well as the subsequent investigation of the patient isolates' phenotypic and genotypic profiles

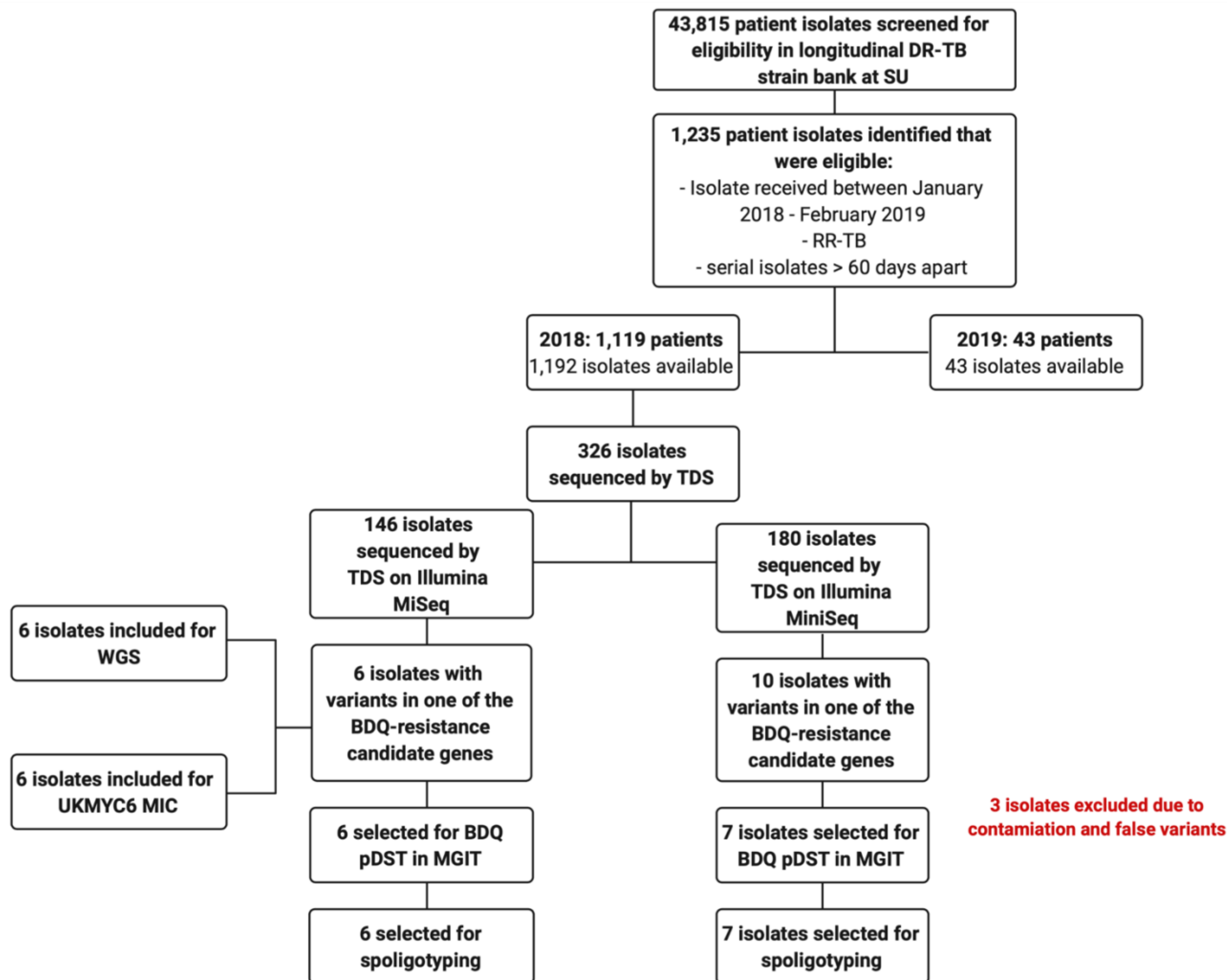

Figure 4.1 A flow chart of the patient isolate selection process. DR-TB = drug-resistant TB; RR-TB = rifampicin-resistant TB; BDQ = bedaquiline. Created by Amy Steinhobel using BioRender.com.

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4.1 Patient isolate sample selection

A total of 1,235 patient isolates were identified for 1,162 patients, of which 1,119 patients were received in 2018 and 43 patients were received in 2019. Limited samples were received for 2019 due to a delay in processing between the National Health Laboratory Service (NHLS) and SU. This delay was amplified due to the COVID-19 pandemic. The isolates were selected in chronological order for sequencing based on the NHLS registration date. No serial isolates were sequenced during this study. The NHLS drug resistant status of the patient isolates can be found in Appendix B.

4.2 Targeted deep sequencing on Illumina MiSeq (TGen, Flagstaff)

4.2.1 Targeted deep sequencing

A total of 160 patient sample isolates were processed for targeted deep sequencing (TDS). After analysing the prepared libraries, 14 patient isolates were excluded from sequencing due to a lack of product detected during agarose gel visualization or displaying a low library concentration during quantification (below 10 nM). In total, 146 patient isolates (first available) were sequenced (178 including the no template control (NTC) and positive H37Ra control per plate).

Using the TB-specific Amplicon Sequencing Analysis Pipeline (TB-ASAP), 49 isolates were identified with variants in the target genes. Forty-seven (47/146; 32.19%) isolates had *Rv0678* variants. Of the 47 isolates with *Rv0678* variants, 43 isolates had a -11C>A variant, two isolates had A152C variants and two isolates had indels (T141TC and T138TG). One of the isolates with a A152C *Rv0678* variant also had a -11C>A variant. Two isolates had a *pepQ* variant: one had a T196C and the other a G540A. No *atpE* variants were detected.

A literature search was done to determine if any of the identified variants were novel to this study and whether any previous association with phenotypic BDQ-resistance had been observed before. The -11C>A variant was excluded from subsequent analysis and investigation as previous studies indicated a BDQ-susceptible phenotypic association with this variant.

After the exclusion of the isolates with -11C>A variants, six (6/146; 4.1%) isolates were identified with a variant that could be associated with BDQ-resistance and were selected for further investigation. These included the four isolates with *Rv0678* variants (R39661, R39673, R40435 and R40711) and the two isolates with *pepQ* variants (R40593 and R40778).

The sequencing depth of the isolate variants from the first TDS run ranged from 878 to 23569 total reads and the variant frequencies ranged from 5.13% - 98.49% (Appendix B), indicating

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some degree of heterogeneity among the isolates. However, 4/6 (66.67%) had variants at a frequency >96%.

4.3 Targeted deep sequencing on Illumina MiniSeq (SU, Cape Town)

4.3.1 Assessing the quality of the library preparation protocol

Initially, the first plate (Plate 1 consisting of 46 patient isolates in a two master-mix (MM) setup) was set up as per the protocol designed by the TGen. To improve the quality of the libraries for downstream sequencing, the library preparation steps required optimisation: the multiplex gene target polymerase chain reaction (PCR) was optimised, followed by the adapter extension and index PCR steps.

The subset of isolates to be used for optimisation was selected based on the agarose gel visualisation of Plate 1. The following patient isolates were selected: R40830, R40882, R40898, R40956. Nuclease-free water (NFW) and H37Rv genomic DNA were included as the no-template control (NTC) and positive control.

The agarose gels showed that some patient isolates were not successfully amplified by the protocol (Figure 4.2). For MM1, 8/46 (17.39%) patient isolates showed no amplification product on the agarose gel (R40830, R40884, R40894, R40896, R40903, R40924, R40950 and R40964). For MM2, 8/46 (17.39%) patient isolates showed no amplification product on the agarose gel (R40830, R40884, R40894, R40903, R40935, R40950, R40964 and R40965). Aside from the patient isolates that failed to amplify, many of the isolates presented faint amplification bands, suggesting that the PCR reaction and thermocycling conditions were sub-optimal.

This was also evident in the concentrations of the indexed amplicon libraries, with the concentrations ranging from 0.54 –1 74.67 ng/µL (Appendix B) as determined by Qubit High Sensitivity dsDNA assay (Thermo Fisher Scientific Inc., Waltham, USA). The average concentration for the libraries in MM1 were low (6.34 ng/µL) when compared to MM2 (29.07 ng/µL) and almost half the libraries had a concentration below 3 ng/µL.

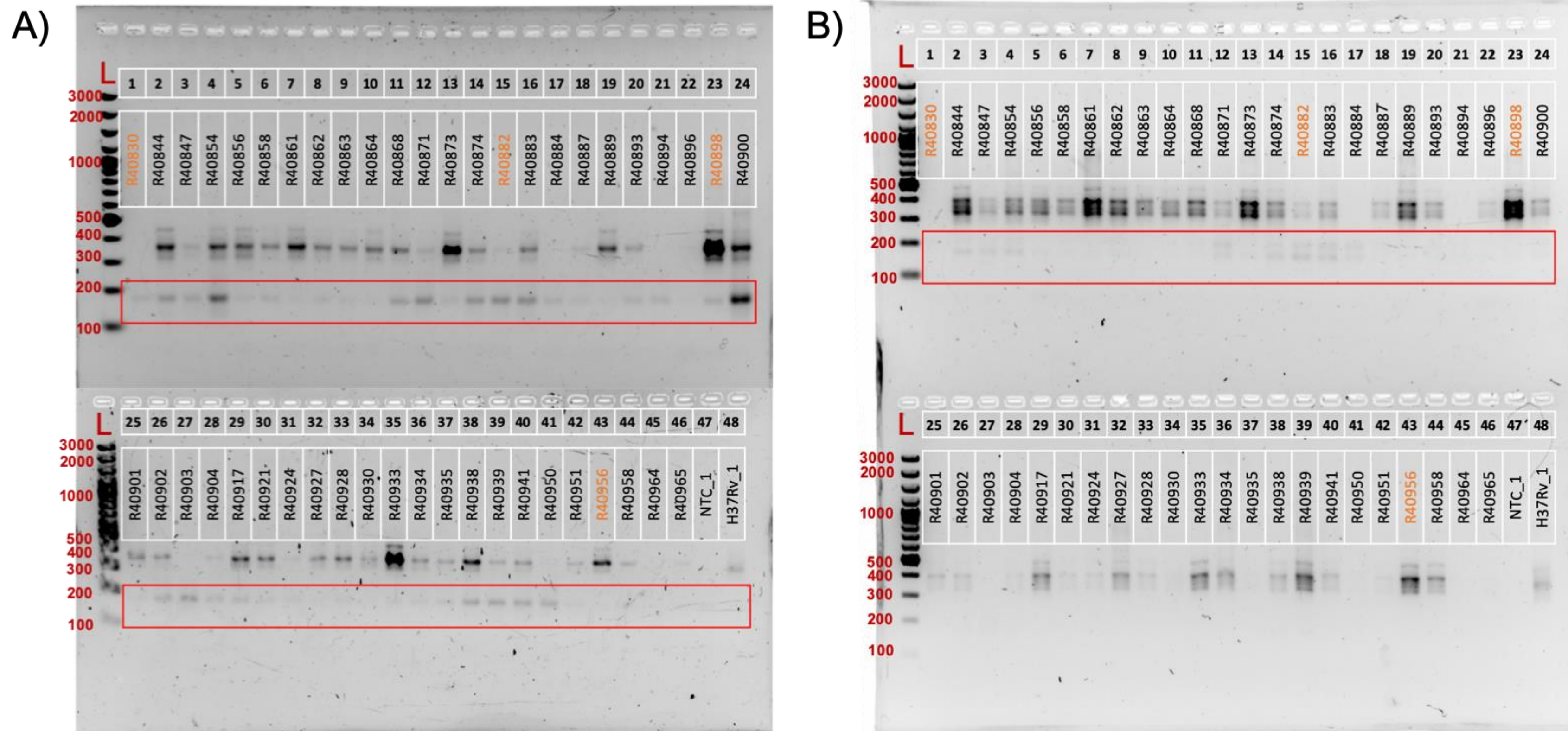

Figure 4.2 Agarose gel visualisation of Plate 1 indexed amplicon libraries, post 0.8× bead clean-up for master-mix (MM) 1 and 2 using the standard library preparation protocol developed by the translational genomics institute (TGen). A) MM1. The expected product sizes of the indexed amplicon libraries for MM 1 were between 346 – 429 bp. Lane L shows the GeneRuler 100 bp Plus DNA ladder (GeneRuler, Scientific, USA) that was used as a molecular weight marker. Lanes 1 – 46 represent amplicon products for patient isolates. Lane 47 shows the no-template control and lane 48 shows the positive control (H37Rv). The red boxes highlight the presence of adapter dimers in the libraries (150 – 170 bp fragments). The four patient isolates in orange were selected as a subset for library preparation optimisation. B) MM2. The expected product sizes of the indexed amplicon libraries for MM 2 were between 317 – 475 bp. Lane L shows the GeneRuler 100 bp Plus DNA ladder (GeneRuler, Scientific, USA) that was used as a molecular weight marker. Lanes 1 – 46 represent amplicon products for patient isolates. Lane 47 shows the no-template control and lane 48 shows the positive control (H37Rv). The red box highlights the presence of adapter dimers in the libraries (150 – 170 bp fragments). The four patient isolates in orange were selected as a subset for library preparation optimisation.

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Furthermore, the agarose gels showed that many of the amplicon libraries had small fragments present (150 –170 bp), besides the expected amplicon fragments, which are indicative of adapter dimers. The fragment sizes in the libraries were assessed by running a LabChip for the subset of isolate libraries. The electropherogram results for isolate R40882 and R40898 are displayed in Figure 4.3 (MM1) and Figure 4.4 (MM2). Isolate R40882 had a low concentration (4.06 ng/µL and 11.33 ng/µL for MM1 and MM2, respectively) and a faint band on the agarose gel image (Figure 4.2), whereas isolate R40898 had a much higher concentration (34.30 ng/µL and 174.67 ng/µL for MM1 and MM2, respectively) and a dark band on the agarose gel image (Figure 4.2). Furthermore, the electropherogram in Figure 4.4 A) indicated that the library preparation protocol was not successful for MM2 for this isolate based on the low fluorescence.

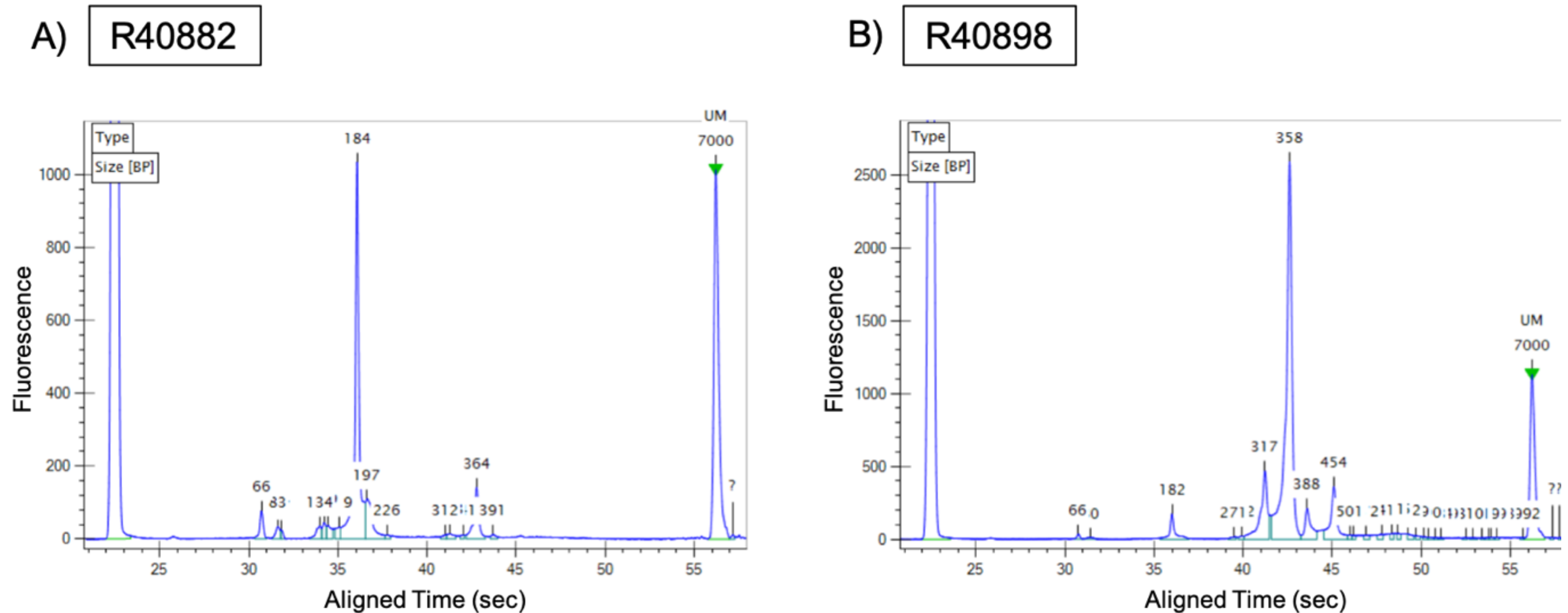

Figure 4.3 LabChip electropherogram results for the library of isolate R40882 and isolate R40898 for MM1. A) The small peaks around ~360 bp are the fragment sizes expected for the amplicons created once the targeted gene multiplex PCR amplicons have been indexed. The tall peak at 184 bp is most likely an indication of adapter dimer in this library. The height of the adapter dimer peak compared to the amplicon peaks indicate the high concentration of the adapter dimer in the library. B) The four peaks ranging from 317 – 454 indicate the various amplicon product size created once the targeted gene multiplex PCR amplicons have been indexed. The peak at 182 bp is a possible indication of adapter dimer in this library.

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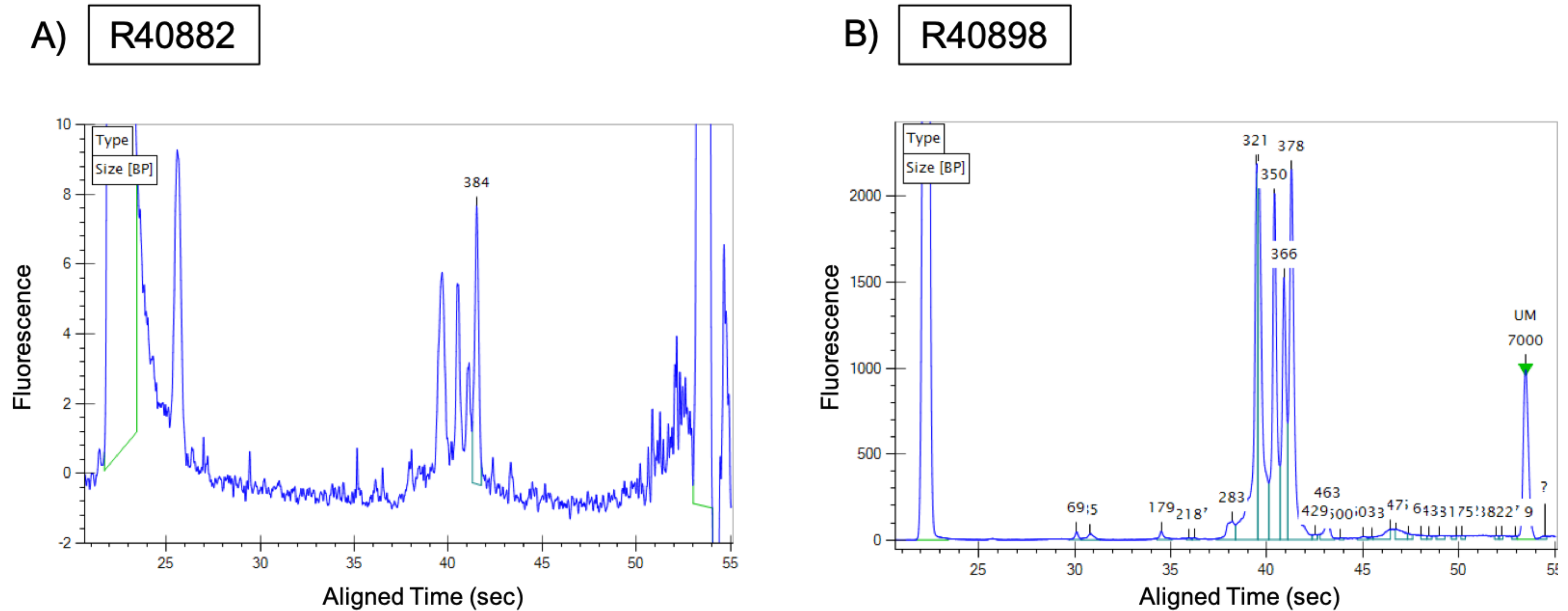

Figure 4.4 LabChip electropherogram results for the library of isolate R40882 and isolate R40898 for MM2. A) The small peaks around ~380 bp are the fragment sizes expected for the amplicons created once the targeted gene multiplex PCR amplicons have been indexed. The small peaks and corresponding fluorescence indicate that library preparation for MM2 was not successful for this isolate. B) The four peaks ranging from 321 – 378 indicate the various amplicon product size created once the targeted gene multiplex PCR amplicons have been indexed. The small peak at 179 bp is a possible indication of adapter dimer in this library.

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The LabChip electropherogram results for a subset of isolates confirmed the presence of the amplicon products ranging from 317 – 475 bp in size after indexing. However, the electropherogram also confirmed the presence of a smaller fragment at 182 - 184 bp which could be indicative of adapter dimer or amplicons that were not successfully indexed during the adapter extension PCR step. Smaller fragments of around ~170 bp were seen in the majority of the isolate libraries of Plate 1, providing further evidence that the isolate library preparation protocol was underperforming.

Thus, to reduce the adapter dimers present in the libraries (as seen in Figure 4.1 and 4.2) and to increase the overall yield of functional libraries, some library preparation optimisation was necessary.

4.3.2 Library preparation optimisation

The PCR plate design without using a clinical array setup allowed for the inclusion of 46 patient isolates on a plate rather than 10. This resulted in a faster turnaround time for sample isolate processing. The various gradient and optimisation PCR results allowed for adjusting the thermocycling conditions to obtain the optimal library preparation conditions for most of the patient isolates. Increasing the number of cycles for the gene-targeted PCR to 38 and the melting temperature to 64°C resulted in the greatest yield of product. Increasing the number of cycles to 8 during the extension PCR step and increasing the template volume to 4 µL resulted in the greatest library yield with minimal adapter dimer formation. The adjustments made are summarised in Table 4.1.

Table 4.1 The thermocycling optimisation results for library preparation that produced the greatest isolate library yield for the majority of the patient isolates

Library preparation step before optimisation	Conditions investigated	Library preparation after optimisation
No. of cycles during gene-targeted multiplex PCR: 25	25 - 40 cycles	No. of cycles during gene-targeted multiplex PCR: 38
Tm of gene-targeted multiplex PCR: 60°C	Tm of 58°C - 64°C	Tm of gene-targeted multiplex PCR: 64°C
No. of cycles during adapter extension PCR: 6	4 – 10 cycles	No. of cycles during adapter extension PCR: 8
Template volume added to adapter extension PCR: 2 µL	Template volume of 2 – 4 µL	Template volume added to adapter extension PCR: 4 µL

Tm = melting temperature

4.3.3 Library preparation results after optimisation

The final results of library preparation optimisation are shown in Figures 4.5 and 4.6. These indicate the before and after agarose gel images of library products. The adapter dimers have been largely reduced (confirmed by library fragment analysis) and the libraries appear more concentrated after optimisation.

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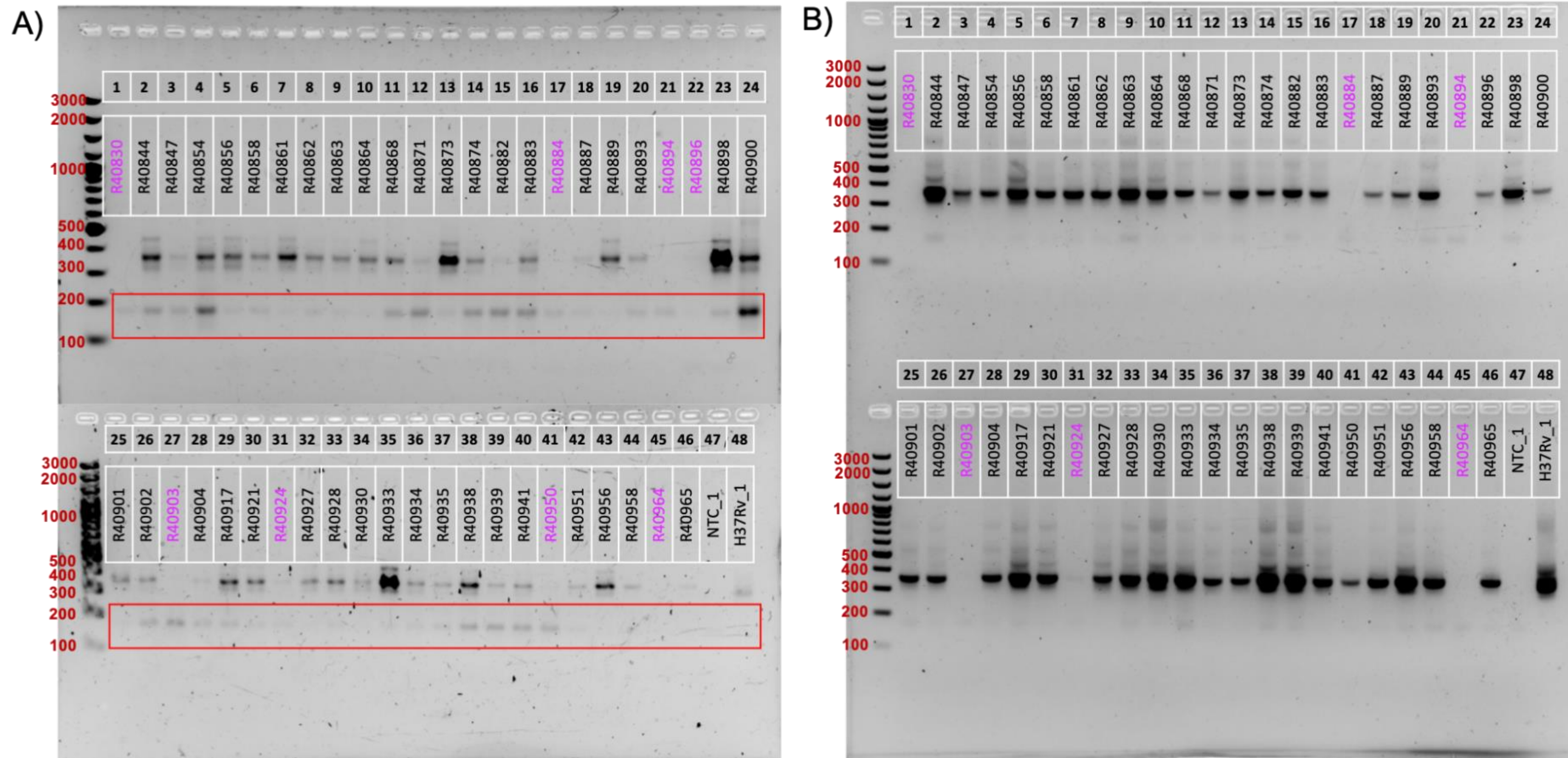

Figure 4.5 Agarose gel visualisation of Plate 1, MM1 library preparation post 0.8× bead clean-up. The isolate names in purple indicate the isolates for which the original library preparation protocol did not produce amplicons. The red boxes highlight the smaller fragments indicative of adapter dimers. A) Before library preparation optimisation B) After library preparation optimisation.

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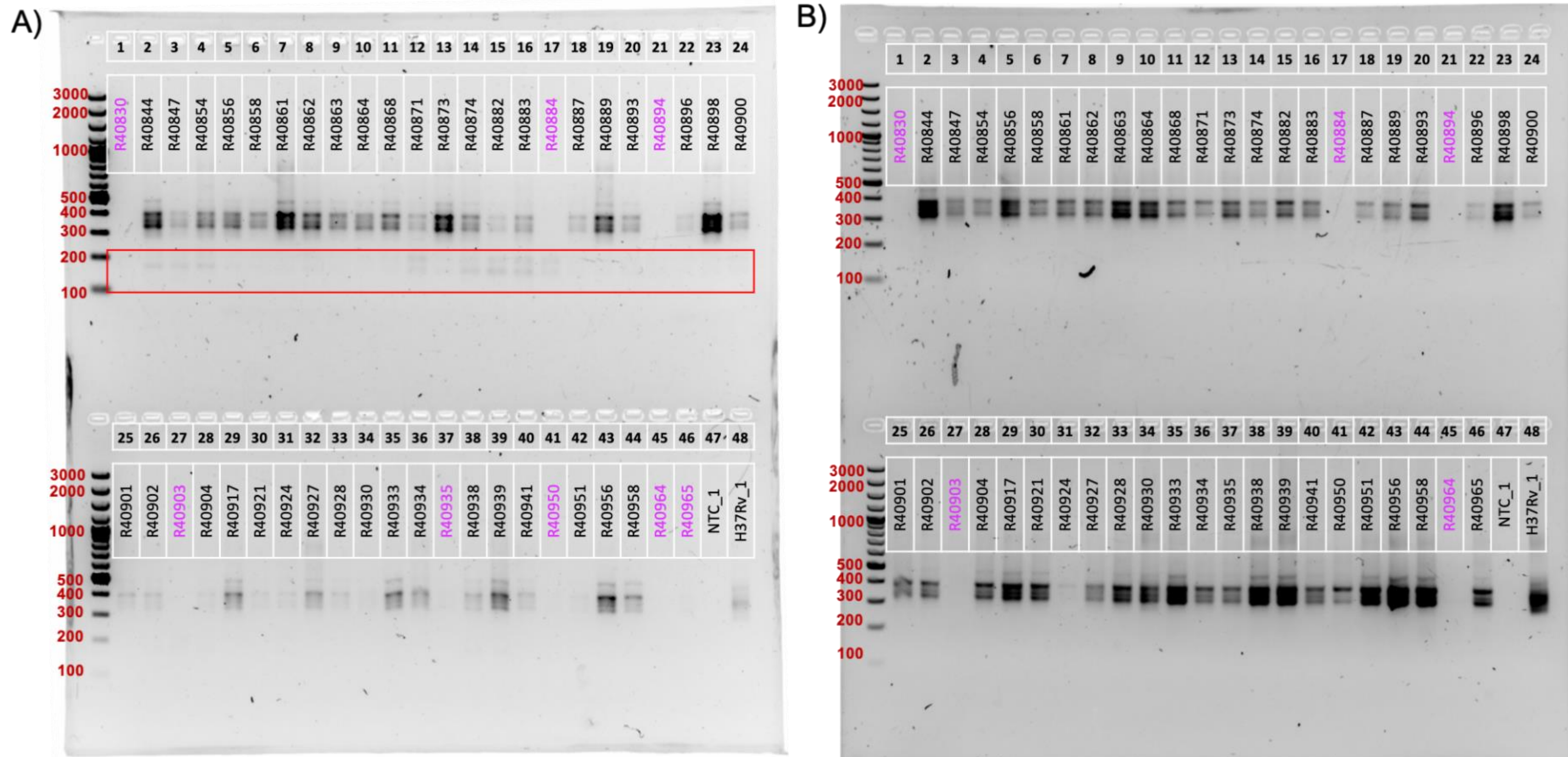

Figure 4.6 Agarose gel visualisation of Plate 1, MM2 library preparation post 0.8× bead clean-up. The isolate names in purple indicate the isolates for which the original library preparation protocol did not produce amplicons. The red box highlights the smaller fragments indicative of adapter dimers A) Before library preparation optimisation B) After library preparation optimisation.

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The libraries were subsequently prepared for 180 patient isolates across four PCR plates, in the two MM setup previously described. The individual libraries were quantified with Qubit analysis (data not shown) and pooled in equimolar concentrations.

The final pool was 4180.83 pM as determined by quantitative PCR (qPCR). The standard curve for the qPCR results indicated a slope of -3.52 and an R^2 value of 0.999 indicating the success of the qPCR run (Appendix B). The concentration of the final pool as determined by qPCR was deemed acceptable for fragment analysis and downstream TDS.

Fragment analysis performed on the LabChip showed four major fragment peaks a 334 bp, 359 bp, 366 bp and 394 bp (Appendix B). The four fragment sizes were weighted equally within the final pool for determining the optimum loading concentration of 1.0 pM.

4.3.4 Targeted deep sequencing results on Illumina MiniSeq platform

After library preparation optimisation the second TDS run was done on the Illumina MiniSeq platform and 180 patient isolates were sequenced. The second TDS run yielded 10 patient isolates with non-synonymous variants in the BDQ candidate genes. Two isolates had the same *Rv0678* variant and two isolates had the same *pepQ* variant. The alignment for the variants were manually analysed and one isolate with three *pepQ* variants was excluded from further analysis due to the alignment indicating false variants. Therefore, nine isolates were identified by the second TDS run for investigation.

Out of the 9 isolates with variants detected in the target genes, *Rv0678* variants were detected in 2/9 (22.2%) of the isolates, *pepQ* variants were detected in 6/9 (66.7%) and an *atpE* variant was detected in 1/9 (11.1%) of the isolates.

A literature search was done to determine if any of the identified variants were novel to this study and whether any previous association with phenotypic BDQ-resistance had been observed before.

Two patient isolates had *Rv0678* variants: R40901 and R41422. These isolates had the same G52T variant present at a low allelic frequency (~5%), which results in a non-sense mutation (E18* stop codon). This variant has not been previously described in the literature and is, therefore, a novel *Rv0678* variant found in two clinical isolates. The variant was investigated with an online translation program and an additional start codon was observed downstream of the amino acid change caused by the variant (Appendix B). Both the isolates with a G52T variant were selected for BDQ MIC testing in MGIT.

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Six patient isolates had *pepQ* variants: R40883, R40964, R41033, R41065, R41082 and R41299. One of the *pepQ* variants had been described previously[7]. The other five variants are novel to this study.

One patient isolate (R41071) had an *atpE* variant. The variant T47A (I16N amino acid change) variant *atpE* was present at 5.92%. This was a novel *atpE* variant in this study.

The second TDS run had variants identified at a lower sequencing depth, ranging from 105 reads to 1214 total reads. The variant frequencies ranged from 5.01% - 99.51%, however the majority were below 6%. The amino acid changes and corresponding number of reads for the isolates can be found in Appendix B.

The first TDS run had a sequencing depth ranging from ~800X to ~23 000X, whereas the second TDS run had ~100X to ~1200X.

A total of 16 patient isolates out of 326 patient isolates sequenced by the collective TDS runs were found to have variants that could be associated with BDQ-resistance. The first TDS run performed on an Illumina MiSeq machine detected six patient isolates with non-synonymous variants in the BDQ candidate genes. The second TDS run performed on an Illumina MiniSeq platform yielded 10 patient isolates with non-synonymous variants (two of which had the same *Rv0678* variant and two of which had the same *pepQ* variant) in the BDQ candidate genes. The variants were manually analysed by assessing the variants in the alignments of the BAM files. Any variants that could not be identified manually in the alignments were considered false variants. One isolate with three false *pepQ* variants was excluded from further analysis, leaving 15 patient isolates for further investigation.

4.4 Isolate drug resistance profile and lineage determination

A total of 15 patient isolates with variants in the BDQ-resistance candidate genes were identified by the collective TDS runs. All isolates were selected for BDQ pDST, however, two isolates (R41299 and R41422) were excluded due to contamination in the primary culture. The remaining 13 isolates underwent BDQ pDST using the mycobacterial growth indicator tube (MGIT) assay and lineage determination by spoligotyping. The six isolates identified by the first TDS run were selected for anti-TB drug MIC determination using the CRyPTIC UKMYC6 plates and whole-genome sequencing (WGS).

4.4.1 BDQ Phenotypic DST using MGIT

The BDQ DST using the MGIT assay revealed that 2/13 isolates were resistant to BDQ: R39661 and R40711. The BDQ-resistant isolates had *Rv0678* indels. The rest of the isolates were phenotypically susceptible to BDQ. The patient isolate with a novel *Rv0678* variant (G52T) was selected for BDQ-MIC testing using the MGIT assay. The results indicated that

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this variant does not confer BDQ-resistance, as the MIC was 0.06 µg/mL which is below the critical concentration (CC) of 1.0 µg/mL (Table 4.2).

Table 4.2 The patient isolates with variants and the BDQ-resistance status as determined by the MGIT assay

	BDQ-resistance	
Isolate	**Variant identified using TB-ASAP (%)***	**MGIT DST result**
R39661	*Rv0678*: T141TC (97.13)	R
R39673	*Rv0678*: A152C (5.13); -11C>A (97.19)	S
R40435	*Rv0678*: A152C (5.94)	S
R40593	*pepQ*: T196C (96.13)	S
R40711	*Rv0678*: T138TG (98.49)	R
R40778	*pepQ*: G540A (20.80)	S
R40883	*pepQ*: A863T (5.01)	S
R40901**	*Rv0678*: G52T (5.26)	S (MIC: 0.06 mg/mL)
R40964	*pepQ*: G317T (7.34)	S
R41033	*pepQ*: A1080T (6.03)	S
R41065	*pepQ*: T71C (99.51)	S
R41071	*atpE*: T47A (5.92)	S
R41082	*pepQ*: G1066T (5.11)	S

BDQ = bedaquiline, TB-ASAP = TB-specific Amplicon Sequencing Analysis Pipeline, MGIT = mycobacterial growth indicator tube, DST = drug susceptibility testing, S = susceptible, R = resistant, GC = growth control, MIC = minimum inhibitory concentration
* Only variants found at a frequency of > 5% are listed, synonymous mutations are not shown
**R40901 was selected for BDQ MIC testing

4.4.2 Spoligotype Results

The lineages of the 13 isolates with variants were determined by spacer oligonucleotide typing (spoligotyping). The spoligotyping patterns indicated that 9/13 (69.23%) isolates were from the Beijing strain family, one isolate was a T1 strain, one was a LAM3 strain and one was a LAM1-LAM4 strain. The lineages of the isolates were distributed between lineage 2 and lineage 4, with 9/13 belonging to lineage 2 and 3/13 isolates belonging to lineage 4 (Table 4.3). One isolate did not successfully amplify and no spoligotype pattern or lineage information was available.

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Table 4.3 Spoligotype lineage and pattern for the 13 patient isolates

Isolate	Lineage	SIT Number	SITVIT Family	Spoligotype Pattern
R39661	2	1	Beijing	
R39673	2	1	Beijing	
R40435	2	1	Beijing	
R40593	4	53	T1	
R40711	2	1	Beijing	
R40778	4	2161	LAM3	
R40883	2	1	Beijing	
R40901	2	1	Beijing	
R40964	-	-	-	-
R41033	2	1	Beijing	
R41065	4	1321	LAM1-LAM4	
R41071	2	1	Beijing	
R41082	2	1	Beijing	

- There was no amplification observed for the spoligotyping PCR, therefore no spoligotyping information available

4.4.3 MIC using the CRyPTIC UKMYC6 plate

The MIC results of the UKMYC6 plates indicated a range of resistance profiles for the isolates. One isolate, R40435, presented as rifampicin mono-resistant TB and one isolate, R40778, presented as multi-drug resistant (MDR-TB). Two isolates had a pre-extensively drug-resistant (XDR) TB phenotype: R39673 and R40593. Lastly, two isolates were XDR-TB: R39661 and R40711. The drug-resistant profiles identified by UKMYC6 MIC testing correlated with the NHLS drug-resistance profiles reported (data not reported).

All of the isolates had a BDQ MIC below the epidemiological cut-off value (ECOFF) (0.25 µg/mL). The XDR-TB isolates had raised BDQ MICs (12.0 µg/mL), as well as raised clofazimine (CFZ) MICs (12.0 µg/mL). The BDQ MIC data for each isolate can be found in Table 4.4. The UKMYC6 plate isolate MIC results for the remaining 12 anti-TB drugs can be found in Appendix B.

4.4.4 WGS on Illumina MiniSeq

The DNA for the subset of isolates were successfully extracted and WGS was performed for the purified DNA. The WGS data analysis indicated that all six of the isolates were successfully sequenced and mapped to the reference genome (~99% mapped reads for all isolates). The WGS sequencing quality and metrics can be found in Appendix B.

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WGS analysis identified four of the isolates belonged to lineage 2 (R39661, R39673, R40435 and R40711) and two of the isolates belonged to lineage 4 (R40593 and R40778). The genetic drug resistance profiles as determined by WGS corresponded with the phenotypic drug resistance profiles identified by UKMYC6 MIC testing described in Section 4.4.3 (Table 4.4). The WGS data detected the same variants reported by TDS in the target genes of the isolates except for the *Rv0678* A152C variants identified at ~5% by TDS in two isolates.

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Table 4.4 Summary of genetic and phenotypic results for the isolates with variants identified by the first targeted deep sequencing run

	DR profile		**BDQ-resistance**					**Lineage**		**Reference**
Isolate	**UKMYC6**	**TB profiler**	**Variant identified using TB-ASAP (%)**	**Variant identified using USAP (%)**	**Variant identified using TB profiler (%)**	**UKMYC6 BDQ MIC (µg/mL)**	**MGIT pDST**	**Spoligotyping**	**TB Profiler**	**Variant found in literature**
R39661	XDR	XDR	*Rv0678*: T141TC (97.13)	*Rv0678*: T141TC (99.38)	*Rv0678*: T141TC (97.00)	0.12	R	Beijing	2.2.2	(Andries et al. 2014; Ismail et al. 2018; Zimenkov et al. 2017; Gómez-González et al. 2021) [12,15,20,110]
R39673	Pre-XDR	Pre-XDR	*Rv0678*: A152C (5.13); -11C>A (97.19)	*Rv0678:* -11C>A (100.00)	*Rv0678:* -11C>A (100.00)	<0.008	S	Beijing	2.2.1	(Ismail et al. 2018)[15]
R40435	RIF MONO	RIF MONO	*Rv0678*: A152C (5.94)	-	-	0.03	S	Beijing	2.2.1.1	(Ismail et al. 2018)[15]
R40593	Pre-XDR	Pre-XDR	*pepQ*: T196C (96.13)	*pepQ*: T196C (100.00)	*pepQ:* T196C (100.00)	0.015/ 0.03	S	T1	4.1.1.3	(Gómez-González et al. 2021)[20]
R40711	XDR	XDR	*Rv0678*: T138TG (98.49)	*Rv0678*: T138TG (100.00)	*Rv0678:* 138 del (69.00); T138TG (99.00)	0.12/ 0.25	R	Beijing	2.2.2	(Zimenkov et al. 2017; Gómez-González et al. 2021; Ismail et al. 2018)[20,110,252]
R40778	MDR	MDR	*pepQ*: G540A (20.80)	*pepQ*: G540A (37.67)	*pepQ:* G540A (41.00)	0.015	S	LAM3	4.4.1.1	N/A

DR = drug resistance; XDR = extensively drug resistant; Pre-XDR = pre-extensively drug resistant; RIF MONO = resistance to rifampicin only; MDR = multi-drug resistant; R = resistant (Resistance is defined as growth at a critical concentration of 1 µg/mL); MIC = minimum inhibitory concentration; TB-ASAP = TB-specific Amplicon Sequencing Analysis Pipeline; USAP = Universal Sequence Alignment Pipeline; S = susceptible; N/A = not applicable

4.5 Phylogenetic analysis and transmission

A maximum-likelihood phylogenetic reconstruction (Figure 4.7) was done for the six isolates containing variants in the BDQ target genes based on the WGS data. *Mycobacterium tuberculosis complex* (MTBC) Lineage determination based on phylogenetic markers using TB-profiler revealed that all the patient isolates belonged to either lineage 2 (East-Asian) or 4 (Euro-American), therefore previously published MTB isolates representative of these lineages were included in the reconstruction[259]. Due to time constraints and funding, we were not able to perform WGS for the isolates identified by the second TDS run and therefore the isolates are not included in the phylogenetic reconstruction.

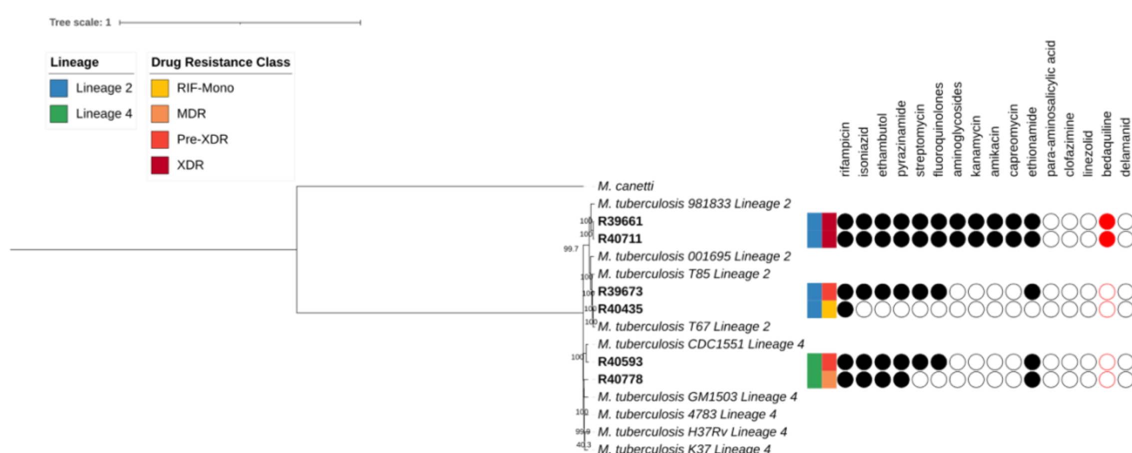

Figure 4.7 Maximum likelihood phylogenetic reconstruction generated by the author. Bootstrap resampling using 1000 replicates was used to assess the reliability of the tree. The tree was produced by RaxML and visualised and annotated in iTOL. The bootstrap values are indicated for each cluster next to the nodes. The patient isolates are represented in bold font and the lineages have been indicated by colour (blue =lineage 2; green = lineage 4). The associated drug resistance profile determined by WGS data is indicated for each isolate (yellow = RIF-mono TB; orange = MDR TB; red = pre-XDR TB). The patient isolates and corresponding resistance to an anti-TB agent (as determined by WGS) are indicated by the solid black circles. The solid red circles indicate the phenotypic BDQ-resistance profile as determined by MGIT.

The phylogeny indicates the close relatedness of patient isolates. Two of the clustered isolates with a bootstrap value of 100%, R39661 and R40711, presented identical sub-lineages (sub-lineage 2.2.2) based on spoligotyping and WGS data (Table 4.4). A pairwise variant comparison between these two isolates found that R39661 had 11 unique variants and R40711 had 8 unique variants.

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Chapter 5: Discussion

This study of bedaquiline (BDQ) resistance within the Western Cape province of South Africa was intended to improve our current understanding of the acquisition of clinical BDQ-resistance within the community and to identify possible routes of transmission.

A targeted deep sequencing (TDS) approach was used to detect variants in BDQ-resistance candidate genes (*Rv0678*, *atpE* and *pepQ*) for the selected rifampicin-resistant TB (RR-TB) patient isolates, including variants occurring in minor sub-populations. RR-TB patient isolates were selected based on eligibility for receiving a BDQ-containing treatment regimen in South Africa in 2018[22,189].

Our study shows that the prevalence of variants in the BDQ-resistance candidate genes in the screened sample set was 4.6% (15/326) and the BDQ-resistance amongst the patient isolates with variants was 15.4% (2/13). Under the assumption that only isolates with variants in one of the BDQ-resistance candidate genes could confer resistance, the BDQ-resistance frequency in our study was 0.61% (2/326). A recent study of BDQ-resistance in South Africa found that 3% of patient isolates were phenotypically resistant[265].

5.1 *Rv0678* variants

In our study, six patient isolates with *Rv0678* variants were identified by the collective TDS runs and selected for further investigation. Three of the *Rv0678* variants (T141TC, A152C and T138TG) identified have been previously described in literature[8,11,28,100,237]. One *Rv0678* variant (G52T), identified in two isolates (R40901 and R41422), was novel to this study.

Although one study found the -11C>A variant is associated with increased BDQ-susceptibility[25], other studies have found that the lacks an association with a phenotypic effect[232,266]and another study found that this variant is exclusively found in the Beijing lineage[267], suggesting that it is most likely a phylogenetic marker. Therefore, patient isolates with only this variant present were excluded from further investigation.

Two A152C *Rv0678* variants were detected at ~5%, suggesting a heterogenous population in these isolates. The A152C (amino acid change: Q51P) *Rv0678* variant has been found previously in a BDQ naïve patient isolate, which displayed intermediate resistance to BDQ and clofazimine (CFZ)[15]. Our study found that this *Rv0678* substitution did not confer BDQ-resistance in the two isolates and only one exhibited a slightly raised minimum inhibitory concentration (MIC) (0.03 µg/mL) on the UKMYC6 broth-microdilution (BMD) plate developed by the CRyPTIC consortium. On the other hand, the low allele frequency of these variants could indicate minor BDQ resistant subpopulations were present in these isolates[4]. It is

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possible that neither the BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system nor the UKMYC6 plate could accurately detect these minor subpopulations and therefore the intermediate BDQ-resistance phenotype was not observed.

A recent study revealed that indels are more commonly found in *Rv0678* than *atpE* or *pepQ*[20]. The first TDS run in this study yielded two isolates with indels in *Rv0678*: T141TC and T138TG. Both indels conferred BDQ-resistance using MGIT pDST. A recent systematic review investigating genetic variants and their association with phenotypic BDQ-resistance found that the only statistically relevant *Rv0678* variant to be associated with BDQ-resistance was a T138TG indel[18]. Our finding of the BDQ resistant T138TG indel provides further evidence that this variant confers BDQ phenotypic resistance.

The *Rv0678* T141TC indel has been previously found in two XDR isolates that displayed high (4-fold increase) BDQ MICs using the MGIT 960 system and the broth microdilution (BMD) method[9]. Our study supported these findings, with both the T141TC and T138TG indels resulting in a raised MIC (0.12 µg/mL) on the UKMYC6 plate and a resistant phenotype with the MGIT 960 system.

On the other hand, the UKMYC6 plate MIC results classed the *Rv0678* indel isolates as BDQ susceptible since the MIC was below the epidemiological cut-off value (ECOFF). The disagreement between the BDQ-resistance profiles using the UKMYC6 plate and the MGIT assay for the *Rv0678* indels is surprising, considering the MICs for the indels are raised and one dilution below the suggested ECOFF. However, the ECOFF of 0.25 µg/mL for BDQ, as recently determined by the CRyPTIC consortium and others, still requires evaluation[251].

With a number of factors proven to have a significant impact on BDQ MICs, BDQ is a challenging drug to work with[268]. Neither BDQ MIC technique used in this study is without drawbacks. The WHO has not provided endorsement for the CRyPTIC UKMYC6 BMD plates[269]. Although studies have shown that MGIT is the preferred method for pDST[58,60,188], a standardised BDQ DST method is yet to be developed and agreed upon by the WHO[177,270,271].

This discordance in our findings further indicates that the 0.25 µg/mL tentative ECOFF for BDQ proposed for the UKMYC6 plates still requires further investigation and it is essential that isolates with confirmed phenotypic BDQ-resistance (as determined by other phenotypic drug susceptibility testing (pDST) methods) be included for further evaluation. Then again, it is worth noting that the T141TC indel has previously been found in both a BDQ-susceptible isolate and BDQ resistant isolates[9], suggesting that the link between BDQ-resistance and this insertion variant is still unclear and requires comprehensive MIC testing. Our phenotypic BDQ-resistant findings for the T141TC indel can add to the growing body of evidence that this variant is a BDQ resistant-associated variant (RAV).

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The novel *Rv0678* variant which caused a non-sense mutation (G52T; amino acid change: E18 stop) was identified in two isolates (R40901 and R41422), but did not confer phenotypic BDQ-resistance. This suggests that this stop mutation did not cause the protein to be aborted and the MmpS5-MmpL5 repressor activity was sustained for these isolates. Upon further investigation of the amino acid changes caused by this variant, an online translation program indicated that although the variant resulted in a stop codon, a few amino acid residues downstream of this variant's location there is another start codon present. This adds to the evidence that the repressor protein was likely still synthesised, thus not conferring BDQ-resistance *via* the upregulation of MmpS5-MmpL5 efflux pump. Then again, it is worth noting that this variant was found in the isolate at a low frequency (~5%). The premature stop codon in this sub-population may cause the repressor protein to be aborted, conferring BDQ-resistance at a level that is undetected by the pDST method. Computational techniques can be used to investigate how point mutations can confer drug-resistance[272,273].

Identifying variants at low frequencies is a potential indication of the presence of sub-populations within the isolates. The simultaneous occurrence of drug-susceptible and drug-resistant sub-populations in a sample results in hetero-resistance[34]. The drug-resistant sub-populations are often undetected by current molecular and phenotypic techniques and can lead to the development of detectable drug-resistance[4,34]. Therefore, the presence of variants at frequencies of ~5% in this study could be representative of BDQ micro-heteroresistance in the patient isolates and could lead to the development of detectable BDQ-resistance further down the line.

The isolate R40901 with the non-sense variant (G52T) was selected for MIC testing in MGIT and R41422 was excluded due to contamination and time constraints. The results indicated that this novel mutation resulted in an MIC of 0.06 µg/mL in R40901, which is a 2-fold increase compared to the H37Rv wild type (WT) control. However, BDQ-MIC in MGIT data published by the WHO in 2018 indicated that BDQ phenotypically-WT clinical isolates from various studies had BDQ-MICs up to and above the suggested EUCAST (European Committee on Antimicrobial Susceptibility Testing) medium-independent clinical breakpoint of 0.25 µg/mL, including H37Rv strains, as well as isolates with varying drug-resistant profiles[60]. This suggests that the 2-fold BDQ-MIC increase observed for this isolate is not a marked increase in an MIC in the context of other study observations.

The genotype-phenotype correlations of the *Rv0678* indel in this study corroborate several of the findings of two recent studies conducted in South Africa. A large-scale BDQ-surveillance study found that all phenotypically BDQ-resistant isolates harboured a RAV in the *Rv0678* locus[265]. A recent systematic review found that the T138TG *Rv0678* indel has a statistically

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relevant genotype-phenotype correlation[18]. In our study only two BDQ RAVs were identified amongst the 13 variants investigated, of which both were *Rv0678* indels and one of indels was a T138TG.

5.2 *pepQ* variants

This study identified nine patient isolates with variants in the *pepQ* gene. Of the two *pepQ* variants identified in the first subset of TDS isolates, one variant was novel (G540A) and the other variant (T196C) has been observed previously[237].

Five out of the seven isolates with variants detected in the second TDS run had *pepQ* variants. Only one of the *pepQ* variants has been reported in literature before: T71C (amino acid change: I24T)[7]. The other four *pepQ* base substitutions identified in the second TDS run appear to be novel to this study.

The *pepQ* variants investigated in this study did not confer BDQ-resistance based on the UKMYC6 plate results and pDST in MGIT. This is unsurprising as *pepQ* variants have been associated with low-level BDQ-resistance[11] and MIC testing is needed to detect these small increases in MICs. Furthermore, the clinical relevance of mutations in *pepQ* is still unknown as some variants have been linked with reduced efficacy of BDQ and CFZ *in vivo* but did not lead to complete resistance[274]. Therefore, further investigation of the role *pepQ* variants play in clinical isolates is needed to assess the effect of variants on BDQ MICs and conferring resistance.

One isolate had three *pepQ* variants. However, this isolate was excluded from further investigation after manual analysis of the alignment suggested these variants were false.

5.3 *atpE* variants

There were no *atpE* variants detected in the first TDS run and only one isolate had an *atpE* variant in the second TDS run[20]. The non-synonymous *atpE* variant T47A (amino acid change: I16N) had not been reported in literature at the time of this study, although an amino acid substitution at the same position (I16V) has been reported previously by Karmakar and colleagues[275].

The study by Karmakar *et al.* (2019) used a computational approach and predicted that the I16V variant could not confer BDQ-resistance as the variant was approximately 10 amino acid residues apart from the BDQ-binding site and thus unlikely to disrupt the mechanism of action of BDQ[239]. Given that the variant identified in this study was located at the same amino acid residue, this provides a plausible explanation for the BDQ-susceptible status of the isolate in question.

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The scarcity of *atpE* variants amongst the sequenced patient isolates was not surprising, since *atpE* variants have been less frequently detected in clinical isolates[32], possibly due to additional fitness costs associated with *in vivo* variants[110,233]. A recent systematic review with one of the largest catalogues of variants in the three genes associated with BDQ-resistance found that only one *atpE* variant has a significant phenotypic-genotypic association with BDQ-resistance, a G187C substitution[18].

The premise of using variants in the three target genes as a proxy for BDQ-resistance is dependent on the availability of robust pDST data to support the genotype-phenotype correlation. Currently, the genes *Rv0678*, *pepQ* and *atpE* are most likely linked to phenotypic BDQ-resistance[276], although, the current World Health Organisation (WHO) catalogue of mutations still does not support the connection between resistance and genetic mutations in these three target genes or any other gene targets[7]. However, the WHO catalogue of mutations (2021) was published before the recent systematic review by Ismail *et al.* (2021) that provided substantial statistical evidence of two RAVs (*Rv0678* indel: T138TG and *atpE* substitution: G187C) and it is likely that the WHO catalogue will be updated once the new evidence is reviewed.

Notably, a study investigating *in vitro* susceptibility to BDQ in China found that half of the BDQ-resistant isolates lacked a variant in *Rv0678*, *atpE* and *pepQ*[19]. This suggests that there are other mechanisms of BDQ-resistance besides these three gene targets. This is further substantiated by a recent study by Saeed and colleagues that indicated the possibility of variants in efflux pump genes serving as an alternative mechanism of BDQ-resistance[277]. Furthermore, a recent study found that *Rv0678* variants may be epistatic in nature when coinciding with loss of function mutations in efflux pump genes (*mmpS5* and *mmpL5*)[278].

RAVs in the BDQ target genes may not be an indication of BDQ exposure. Several studies have reported BDQ RAVs in *Rv0678* in BDQ-naïve isolates[12,25]. More recently, a study investigating the clinical treatment outcomes of drug-resistant patients intended for BDQ-based therapy found that 5.4% (5/92) of baseline patient isolates had *Rv0678* variants prior to BDQ exposure[17]. Although the baseline isolates were not BDQ-resistant, some patient isolates had raised MICs. One patient had an MIC that increased considerably with a treatment regimen containing BDQ in conjunction with the variant allele frequency increasing (72% to 96%). They also observed emergent *Rv0678* variants in 5.7% of the patients during therapy, with variants associated with >8-fold increases in the BDQ MICs[17]. Another study investigating the long-term treatment of a patient, found that BDQ RAVs emerged after treatment cessation, which was attributed to the long half-life of BDQ[27].

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These study findings suggest that BDQ RAVs may already be present in the population without prior BDQ exposure (possibly due to transmission of BDQ-resistant strains within the community), that BDQ RAVs can emerge during BDQ treatment and that there is the added risk of resistance emerging after BDQ cessation as well. Nevertheless, a recent study found that patients with *Rv0678* variants who had received BDQ as part of their treatment regimen still had favourable outcomes[267], suggesting that currently variants in *Rv0678*, as well as *pepQ* and *atpE*, may not serve as robust genetic markers to determine the exclusion of BDQ from treatment regimens for DR-TB patients.

Nevertheless, the presence of a BDQ RAV in a patient isolate is still cause for concern and clinicians should use caution when prescribing BDQ and should do so with the support of relevant phenotypic data.

One of the MTB genotypes that is geographically widespread and highly virulent, is the Beijing genotype family[279]. Lineage 2 (East-Asian lineage) is majorly represented by the Beijing genotype. The increasing global prevalence of the Beijing sub-lineage is concerning as these strains have a propensity for highly virulent phenotypes[280]. Beijing genotypes appear to be associated with higher transmissibility[281] and MDR- and XDR-TB phenotypes[282]. A local study previously found that 55% of the MDR-TB isolates in the Western Cape Province belong to the Beijing genotype[283]. In our study the predominant lineage amongst the sequenced isolates was lineage 2 (Beijing genotype). A recent study noted that *Rv0678* variants are commonly found in MTB isolates belonging to the Beijing genotype[19]. Indeed, 100% of the isolates in this study with a *Rv0678* variant were Beijing strains. There were varying resistance profiles amongst the Beijing genotype isolates identified in this study, including rifampicin-mono-resistant-TB, MDR-TB, pre-XDR-TB and XDR-TB. The resistance profiles identified by the UKMYC6 plates were congruent with WGS results.

In the past there was the erroneous assumption that drug-resistant TB strains were less transmissible than drug-susceptible-TB (DS-TB) strains, based on the impaired fitness associated with drug-resistance mutations, thus focusing the global policy on managing these DS-TB strains[44]. There is a better understanding now, that the acquisition of resistance can occur during TB treatment, and primary drug resistance can occur due to person-to-person transmission of already resistant strains[284]. The drug-resistant TB epidemic in South Africa is believed to be primarily due to MDR-TB strain transmission[283].

Preventing drug-resistant TB transmission requires rapid and accurate diagnosis, administering effective treatment regimens combined with effective infection control programmes [285], as well as the identification of active transmission chains in a population[242]. WGS can be used to predict transmission with relatively high resolution[286,287].

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With WGS of the patient isolates we identified two isolates (R39661 and R40711) with identical lineages and drug resistance profiles. Both isolates displayed BDQ-resistance based on pDST in MGIT. However, these isolates had different indels in the *Rv0678* gene identified by TDS and WGS, which we predict attributed to the phenotypic BDQ-resistance. The variant distance for these isolates was 20, which is greater than the maximum of 12 SNPs often used as the upper threshold of direct transmission events[288]. Then again, determining the appropriate threshold is based on the context of the study and will be influenced by study-specific factors[289]. The upper threshold proposed by Walker *et al.* was based on two studies conducted in a low-incidence TB setting[286] and the threshold was defined using isolates from a country with low-incidence of TB drug-resistance[290]. Given South Africa's high TB incidence rates and high DR-TB burden[37,291], the proposed threshold may not be as relevant in this setting of the current study. There is currently no international standard for SNP distance cut-off to define possible transmission[286], which makes it difficult to clearly define transmission based on SNP distances in the setting. Furthermore, Bryant *et al.* (2013) previously demonstrate that even with the high resolution that WGS provides, the low mutation-rate of MTB makes it difficult to confidently infer transmission events[287].

Limited studies have investigated transmission of BDQ resistance in Southern Africa. A 2020 study found evidence of onward transmission of a *Rv0678* clone in South Africa. They sequenced the whole genomes of 648 isolates, which they used in conjunction with additional publicly available isolate data to assess the phylogenetic relatedness of the isolates. This allowed them to identify the spread of this clone linked to raised BDQ MICs[17]. These findings suggest that in order to identify evidence of transmission events, large-scale WGS data is needed. In our study we only had access to WGS data for six isolates and therefore could not fully investigate potential transmission within the community. In the future, a larger cohort of isolates with WGS data in combination with cluster analysis and SNP distance comparisons could aid in identifying onward transmission events.

In order for NGS methods to be considered for the application of DST in clinical settings, the turnaround-time needs to be quicker than conventional pDST methods[83]. In this study we observed mixed results regarding the turnaround-time for genotypic DST (gDST) compared to pDST results. On the other hand, it is worth noting that this study was retrospective and is therefore not an accurate reflection of the DST turnaround-time that would be observed in a clinical setting.

Library preparation, sequencing and subsequent data analysis conducted at TGen took up to four weeks for a batch of ~140 samples (using a clinical array format). By comparison, conducting pDST for nine isolates with variants using MGIT took up to six weeks or more to

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obtain the final results, based on the various culturing steps required. Using TDS to screen patient isolates for genotypic BDQ-resistance provides a faster turnaround-time, as well as the option to screen large batches of patients isolates at a time. However, the phenotypic BDQ-resistance status for each isolate still requires confirmation using pDST methods due to the lack of a BDQ-resistance mutation catalogue at present. Therefore, despite gDST having a quicker turnaround-time in this study, the need for phenotypic confirmation prolonged the overall time to obtaining the final DST results.

Additionally, the pDST results confirmed that only 2/13 isolates with variants investigated in this study displayed phenotypic BDQ-resistance. If the 11 variants identified in the BDQ-resistance candidate genes were used as a proxy for phenotypic resistance in clinical settings, these patients would likely be excluded from receiving BDQ during treatment, despite having a phenotypically BDQ susceptible MTB infection. This has advantages and disadvantages.

The exclusion of BDQ from the treatment regimens for these patients could help to preserve this important antibiotic, as well as ensure an effective treatment regimen is used. The observed variants, especially the low-frequency variants, may not confer BDQ-resistance, but these variants may confer intermediate resistance[4], with raised MICs that are below the critical concentration (CC). Thus, there is a possibility that the MTB strains causing the infections could develop BDQ-resistance during treatment resulting in a treatment regimen with reduced efficacy. By excluding BDQ before commencing treatment, this could allow for the preservation of the drug and ensure the patients receive therapeutic interventions with the highest chance of successful treatment outcomes.

Conversely, excluding BDQ based on the observed variants reduces the therapeutic options for designing an effective treatment strategy. Without BDQ, patients would likely be put on an injection-containing regimen, depending on their drug-resistance status. This is especially concerning for patients with highly drug-resistant MTB infections for whom optimal treatment regimens are more complex to design[86]. In addition, it is possible that the observed variants confer no genotypic or phenotypic effects on the functionality of BDQ as a therapeutic agent. Currently, there is insufficient data from large-cohort studies to consistently link *Rv0678* RAVs with poor clinical outcomes[292]. This further highlights the need for clinical studies and treatment cohorts with adequate sample sizes, as well as the importance of having the accompanying BDQ-MIC data for corresponding genetic variants to fully understand the role they play in BDQ-resistance.

WGS data for the first subset of isolates was in complete agreement with the drug-resistance profiles for these isolates as determined by UKMYC6 plate MIC. This suggests that WGS data can determine the drug-resistance status of an isolate with near phenotypic accuracy.

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The WGS identified the majority of the variants identified by the first TDS run. However, the WGS data did not identify the *Rv0678* A152C variant in isolate R39673 and isolate R40435. This could be due to the higher resolution of TDS for variants at low frequencies as a result of the sequencing depth compared to that achieved by WGS. The variants were detected at ~5% which could account for the WGS missing the variant. On the other hand, the missed variant by WGS could also be due to the different bioinformatic pipelines used for each NGS technology and the parameters implemented during the analysis.

5.4 Limitations

The transfer of methodology from TGen to SU had some limitations. The equipment and reagents available at SU differed from those at TGen and thus adjustments to the protocol were needed to optimise the protocol in this setting and with the available resources.

It is apparent that the quality of sequencing data is reliant on the quality of the input material being sequenced[293]. Therefore, well-designed NGS library construction is a critical step in the success of a sequencing run.

One of the drawbacks of trying to optimise and tailor the library preparation steps of a pre-existing protocol, as was done in this study, is the possibility of inducing other errors during the optimisation process. For instance, with attempting to increase the PCR-product yield by increasing the amplification cycles during the gene-specific multiplex PCR step, it is possible for there to be a loss of heterogeneity due to PCR bias. This could lead to variable sequencing quality[294].

There was some variation in the concentrations observed by the two quantification methods in this study (Qubit analysis and quantitative PCR (qPCR)). Qubit analysis is a useful tool for checking the quality of DNA prior to NGS methods[295], although this quantification method is unable to distinguish between varying fragment lengths (primer dimers, adapter dimers, genomic DNA or amplicons) as well as library fragments without adapters[296]. Similarly, qPCR cannot detect the difference between adapter dimers and fragments with adapters[297] however it is able to quantify the fragmentation of the DNA whereas Qubit does not[295]. Furthermore, qPCR has been primarily used for quantification of NGS libraries[298] and therefore the qPCR concentrations were used to determine the final pooled library concentration for loading on the Illumina MiniSeq cartridge.

Another limitation encountered during the methodology transfer was the Illumina sequencing platform and the subsequent sequencing data analysis. The protocol was designed by TGen to run on an Illumina MiSeq platform and the bioinformatic pipeline was tailored to analyse the read data produced by this platform. At SU we had access to an Illumina MiniSeq platform,

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which produces shorter reads than the MiSeq platform[299]. Shorter reads lead to less overlap of the forward and reverse reads during analysis, which leads to decreased accuracy in the detection of true variants. The sequencing results were analysed by the same bioinformatic pipeline; however, some adjustments were made to accommodate the shorter reads.

Analysing the results with a pipeline that is not optimised for the sequencing data could result in inaccurate conclusions due to difficulties discriminating between meaningful variation and insignificant sequencing errors[300]. This raises concerns for the validity of the results from the second TDS run and as a result we cannot place high confidence in these variants.

The accurate detection of hetero-resistant sub-populations by NGS requires a large depth of coverage[35]. The first TDS run had much higher sequencing depth than the second TDS run for the variants identified. The first TDS run had sequencing depth ranging from ~800X to ~23 000X, which is considerably higher than the ~100X to ~1200X observed in the second TDS run. Therefore, there is a higher confidence in the hetero-resistance observed in the isolates of the first TDS run.

Two isolates were found to be phenotypically BDQ-resistant in this study using the MGIT assay. In spite of this, the isolates were not resistant using the UKMYC6 plate, indicating discrepancies in the pDST results. These discrepancies could be resolved by investigation with a third pDST method (such as BMD or the agar proportion method). In this study there was a lack of pDST and MIC data available for anti-TB drugs besides BDQ for the second subset of sequenced isolates. This was due to the unavailability of UKMYC6 plates for anti-TB drug testing, as well as the cost associated with conducting pDST for several anti-TB agents for each isolate.

The BDQ pDST in MGIT for the isolates identified by the second TDS run was done in duplicate to provide higher confidence in the pDST results obtained. However, the growth control (GC) for the second replicate for isolate R41033 failed to grow, thus indicating a failed result and only one replicate was available for this isolate. We were unable to prepare an additional pDST replicate for the isolate due to time constraints. Although, in a clinical setting, pDST is rarely done in duplicate and the results of the first replicate would have been used for clinical decision-making.

In this study a spoligotype result is missing for one of the patient isolates (R40964), with a novel G317T *pepQ* variant. There was a lack of amplification for the isolate during the spoligotyping PCR step. This could be attributed to the use of a crude DNA isolate for amplification. When a PCR reaction is not successful with a crude DNA sample, it is possible to optimise the reaction by sonication, or adjusting the template volume to mitigate the presence of inhibitors in the sample. However, we were unable to repeat the spoligotyping

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method for this isolate due to time constraints and the cost associated with running a spoligotyping blot for a single isolate.

Due to time constraints and funding, we were not able to perform WGS for the isolates identified by the second TDS run. Therefore, the genotypic resistance profiles are unavailable for the second subset of sequenced isolates with variants and the pairwise SNP comparison could not be done. Furthermore, without WGS data available for the isolates, they were excluded from the phylogenetic reconstruction. Thus, the evolutionary relationships between the patient isolate strains are currently unknown.

Another limitation of this study is that 326/1235 isolates were sequenced by the collective TDS runs. We were unable to sequence the remaining 909 isolates due to time constraints and the cost of sequencing. Additionally, only three BDQ-resistance candidate genes were investigated by TDS. Other genes have been linked with cross-resistance to BDQ[269] and were not included in this study.

One of the main limitations encountered in this study is the lack of accompanying clinical patient data, such as treatment regimens received and treatment outcomes, for the investigated isolates other than the RR-TB status. Without clinical data for the isolates, it is currently unknown which of the isolates received BDQ during treatment, or if at all and what the clinical outcomes were for the patients with a BDQ-containing regimen. Thus, no correlations can be made between the clinical data and the variants identified or the BDQ phenotypic resistance status of the isolates.

It is well known that there is cross-resistance between BDQ and clofazimine (CFZ), which are linked with RAVs in the *Rv0678* locus[171,301]. Without clinical data in this study, it is unknown whether the *Rv0678* variants emerged due to the exposure of BDQ or CFZ, if any, during treatment or whether these variants were pre-existing in the MTB strains. In particular, the *Rv0678* RAVs identified that conferred elevated BDQ MICs were not investigated for CFZ-resistance in MGIT either, therefore the phenotypic cross-resistance status to CFZ for these RAVs is unknown in this study.

The BDQ-CFZ cross-resistance status of an isolate has important clinical considerations for designing effective treatment regimens. An *in vitro* study previously found that 97% of CFZ-resistant mutants had an *Rv0678* variant[301]. Therefore, the prior use of CFZ may reduce the efficacy of BDQ in treatment regimens due to the associated *Rv0678* RAVs. However, a recent study found that the majority of CFZ-resistant isolates retained BDQ-susceptibility, suggesting there was no statistical correlation between CFZ and BDQ-MICs[302].

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Clinical patient data could provide greater insight into the association between the clinical treatment and the MTB strains with RAVs observed in this study and help to elucidate the emergence of clinical BDQ- and CFZ-resistance.

5.5 Future studies

BDQ MIC data for all the isolates with variants identified in this study are needed to fully discern the role these variants play in these patient isolates. Although pDST conducted using the MGIT assay is currently considered the most reliable method of pDST for BDQ[58,86], nevertheless, it is worth investigating the BDQ MICs of these isolates using various pDST techniques to provide a congruent understanding of the phenotypic effects of the variants.

A serial isolate is available for the patient with a *Rv0678* T141TC indel that was found to be BDQ resistant in MGIT, as well as the patient isolate with a *pepQ* A863T variant. Investigating the genetic and phenotypic resistance profile of these serial isolates could provide additional valuable information on the nature of the clinical MTB infection and the possible evolution of BDQ-resistance within this patient.

Using the TDS approach described and optimised in this study to investigate the remaining 909 isolates identified in this study could provide further insight on BDQ-resistance within the Western Cape community of South Africa, adding to the growing body of evidence of RAVs for BDQ and the surveillance of the emerging BDQ-resistance and transmission of resistant strains in the population.

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Chapter 6: Conclusion

The emergence of BDQ-resistance in MTB strains circulating in the community so soon after its introduction for routine use is concerning. Especially with the discovery of variants in BDQ-naïve isolates and BDQ-resistance in isolates without variants in one of the three genes, suggesting an unknown mechanism of resistance.

Our study highlights the need for a greater understanding of what drives BDQ-resistance in clinical MTB isolates and what role variants in *Rv0678*, *atpE* and *pepQ* play in conferring phenotypic BDQ-resistance. We identified 13 isolates with variants in the BDQ-resistance candidate genes yet only two of the variants appear to be RAVs. The two *Rv0678* indels add to the growing body of evidence supporting the genotype-phenotype correlation for these RAVs with BDQ-resistance. The other *Rv0678*, *atpE* and *pepQ* variants require further investigation to elucidate their role in the MTB strains.

The study demonstrates the potential of using NGS to screen large numbers of patient isolates for BDQ RAVs with high resolution. The prospective use of the identified RAVs for clinical decision-making relies heavily on the availability of robust pDST and MIC data to consolidate the genotypic and phenotypic data. This data will aid in defining a BDQ-resistance catalogue of mutations which could be used in the future to design a molecular diagnostic tool for the detection of BDQ-resistance.

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Appendices

Appendix A

Preparation of growth media, reagents, and buffers

CULTURE MEDIA:

Middlebrook 7H9 Broth supplemented with 10% OADC, 0.2% glycerol and 0.05% Tween 80

- 4.7 g Middlebrook 7H9
- 900 mL double distilled H_2O
- 2 ml 100% glycerol
- 500 µL 100% Tween 80
- Autoclave for 20 minutes at 121°C
- Add 100 mL OADC
- Filter sterilize and store at 4°C

Sterile 0.85% saline

- Dissolve 8.5 g NaCl in 1000 mL dH_2O
- Autoclave for 20 minutes at 121°C

DRUG PREPARATION:

Bedaquiline drug preparation

- Dissolve 10 mg of bedaquiline in 1.0 mL DMSO in a polystyrene tube (10 mg/mL stock) Working solution of BDQ
- Dilute 10 mg/mL BDQ stock 1:10 to prepare a 1000 µg/mL working solution using DMSO in polystyrene tubes
- Store at -80°C

GEL ELECTROPHORESIS:

10 X TAE buffer (pH 8.0)

- 108 g Tris
- 10.9 g Acetic acid
- 20 mL of 0.5M EDTA (pH 8.0)

2% Agarose gel

- Dissolve 2 g agarose powder in 100 mL 1 X TAE Buffer
- Microwave
- Add 5 µL SYBR Safe Gel Stain

AMPLICON ISOLATION:

80% Ethanol

- 40 mL 100% Absolute Molecular Grade Ethanol
- 10 mL Molecular Biology Water

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10mM Tris-HCL pH 8.0; 0.05% Tween 20

- 0.5 mL 1M Tris-HCl
- 49.5 mL Molecular Biology Water
- 100% Tween 20

DNA EXTRACTION:

CTAB/NaCl (10% w/v CTAB, 0.7 M NaCl)

- Dissolve 4.1 g NaCl in 80 ml distilled water
- While stirring, add 10 g CTAB
- Heat solution in 65°C incubator
- Adjust volume to 100 mL with distilled water

SEQUENCING REAGENT PREPARATION:

200 mM Tris-HCl

- 40 µL Molecular Biology Water
- 10 µL 1M Tris-HCl pH 7.00

20 pM Denatured PhiX

- 5 µL 4 nM PhiX
- 5 µL 0.2N NaOH
- 990 µL pre-chilled HT1 buffer

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Appendix B

Approval Letter
Progress Report

17/09/2021

Project ID: 4113

Ethics Reference No: N09/11/296

Project Title: Longitudinal reference database and M.tuberculosis drug resistance culture bank

Dear Prof RM Warren

We refer to your request for an extension/annual renewal of ethics approval dated 08/09/2021 11:53.

The Health Research Ethics Committee reviewed and approved the annual progress report through an expedited review process.

The approval of this project is extended for a further year.

Approval date: 24 October 2021

Expiry date: 23 October 2022

Kindly be reminded to submit progress reports two (2) months before expiry date.

Where to submit any documentation

Kindly note that the HREC uses an electronic ethics review management system, *Infonetica*, to manage ethics applications and ethics review process. To submit any documentation to HREC, please click on the following link: https://applyethics.sun.ac.za.

Please remember to use your Project Id 4113 and ethics reference number N09/11/296 on any documents or correspondence with the HREC concerning your research protocol.

Please note that for studies involving the use of questionnaires, the final copy should be uploaded on Infonetica.

Yours sincerely,

Ms Brightness Nxumalo
Coordinator: Health Research Ethics Committee 2 (HREC 2)

National Health Research Ethics Council (NHREC) Registration Number:
REC-130408-012 (HREC1)•REC-230208-010 (HREC2)

Federal Wide Assurance Number: 00001372
Office of Human Research Protections (OHRP) Institutional Review Board (IRB) Number:
IRB0005240 (HREC1)•IRB0005239 (HREC2)

The Health Research Ethics Committee (HREC) complies with the SA National Health Act No. 61 of 2003 as it pertains to health research. The HREC abides by the ethical norms and principles for research, established by the World Medical Association (2013). Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects; the South African Department of Health (2006). Guidelines for Good Practice in the Conduct of Clinical Trials with Human Participants in South Africa (2nd edition); as well as the Department of Health (2015). Ethics in Health Research: Principles, Processes and Structures (2nd edition).

The Health Research Ethics Committee reviews research involving human subjects conducted or supported by the Department of Health and Human Services, or other federal departments or agencies that apply the Federal Policy for the Protection of Human Subjects to such research (United States Code of Federal Regulations Title 45 Part 46); and/or clinical investigations regulated by the Food and Drug Administration (FDA) of the Department of Health and Human Services.

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STATA software commands (shown at individual bullets) used to filter patient isolate list extracted from DR-TB strain bank housed at the Division of Molecular Biology and Human Genetics:

- Sort “Patient_ID” and “NHLSregDate” into chronological order
    - order Patient_ID NHLSregDate
    - sort Patient_ID NHLSregDate Ch_num
- Generate a “first” and “last” column based on “Patient_ID”
    - by Patient_ID: gen first = Patient_ID[_n == 1]
    - by Patient_ID: gen last = Patient_ID[_n == _N]
- Generate a “middle” column with dummy value = 1 if the value in first AND last column is missing (.) and then delete the rows that have a “middle” value of 1
    - gen middle = 1 if first == . & last == .
    - drop if middle == 1
- Generate a “dateddiff” column based on “Patient_ID” that shows the difference in date between first and last
    - by Patient_ID: gen datediff = NHLSregDate[_n] - NHLSregDate[_n-1]
- Generate a “less60” column with dummy value = 1 if the value in “datediff” is less than or equal to 59
    - by Patient_ID: gen less60 = 1 if datediff <=59
- Generate a “less60any” column based on “Patient_ID”, with dummy value = 1 for all “Patient_ID”’s with value = 1 in “less60”
    - by Patient_ID: egen less60any = max(less60)
- Generate a “Rif_resitant” column with a dummy value = 1 if there is a “R” in the “Rif” column
    - gen Rif_resistant = 1 if Rif == "R"
- Generate a “rif_S_first” column with a dummy value = 1 if the value in the “first” column is NOT missing AND the “Rif_resistant” value does NOT = 1
    - gen rif_S_first = 1 if first != . & Rif_resistant != 1
    - by Patient_ID: egen rif_S_firstany = max(rif_S_first)
- Delete rows if “rif_S_firstany” has value = 1
    - drop if rif_S_firstany == 1
- Generate a “in2018any” column based on “Patient_ID”, with dummy value = 1 for all “Patient_ID”’s with value = 1 in “in2018”
    - by Patient_ID: egen in2018any = max(in2018)
- Delete rows if “less60any” has the value 1 AND “less60” does NOT have value 1
    - drop if less60any == 1 & less60 != 1
- Delete rows if “in2018any” does NOT have the value 1
    - drop if in2018any != 1
- Generate a column “num_samples” with value = 1 if “first” value is NOT missing AND if “last” is NOT missing, value = 2 if “less60” = 1, value = 3 if “last” is NOT missing AND “first” is missing AND “less60” is missing as well as if “last” is missing AND “first” is NOT missing and “less60” is missing
    - gen num_samples = 1 if first !=. & last != .
    - replace num_samples = 2 if less60 == 1
    - replace num_samples = 3 if last != . & first == . & less60 == .
    - replace num_samples = 3 if last == . & first != . & less60 == .
- Create labels for variable “num_samples” called “Number of Samples”, define labels value 1 = “Baseline only”, value 2 = “First & Last <60 days apart” and value 3 = “First & Last >/=60 days apart”
    - label variable num_samples "Number of Samples"
    - label define Samples 1 "Baseline only" 2 "First & Last <60 days apart" 3 "First & Last >/=60 days apart"
    - label values num_samples Samples

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    - order Patient_ID Ch_num num_samples NHLSregDate
- Order the list starting with "Patient_ID", "Ch_num", "num_samples", "NHLSregDate" followed by all other columns
    - label variable num_samples "Number of Samples"
    - label define Samples 1 "Baseline only" 2 "First & Last <60 days apart" 3 "First & Last >/=60 days apart"
    - label values num_samples Samples
    - order Patient_ID Ch_num num_samples NHLSregDate
- Generate a column "last_dummy" with value = 1 if "last" is NOT missing, generate a column "first_dummy" with value = 1 if "first" is NOT missing, rename "last_dummy" as "last_sample" and reorder columns starting with "Patient_ID" "Ch_num" "NHLSregDate" "last_sample" "num_samples"
    - gen last_dummy = 1 if last != .
    - gen first_dummy = 1 if first != .
    - rename last_dummy last_sample
    - order Patient_ID Ch_num NHLSregDate last_sample num_samples

Table B.1 Summary of selected patient information after filtering with STATA

Patient information	**2018** n (%)	**2019** n (%)
All patients	1119 (100)	43 (100)
Age, years		
Mean age	41	42
≥ 50 years	270 (24.13)	10 (23.26)
Sex		
Male	639 (57.10)	26 (60.47)
Female	479 (42.80)	17 (39.53)
NHLS drug resistance		
MDR-TB	799 (71.40)	38 (88.37)
Pre-XDR	129 (11.53)	11(25.58)
XDR	49 (4.38)	4 (9.30)

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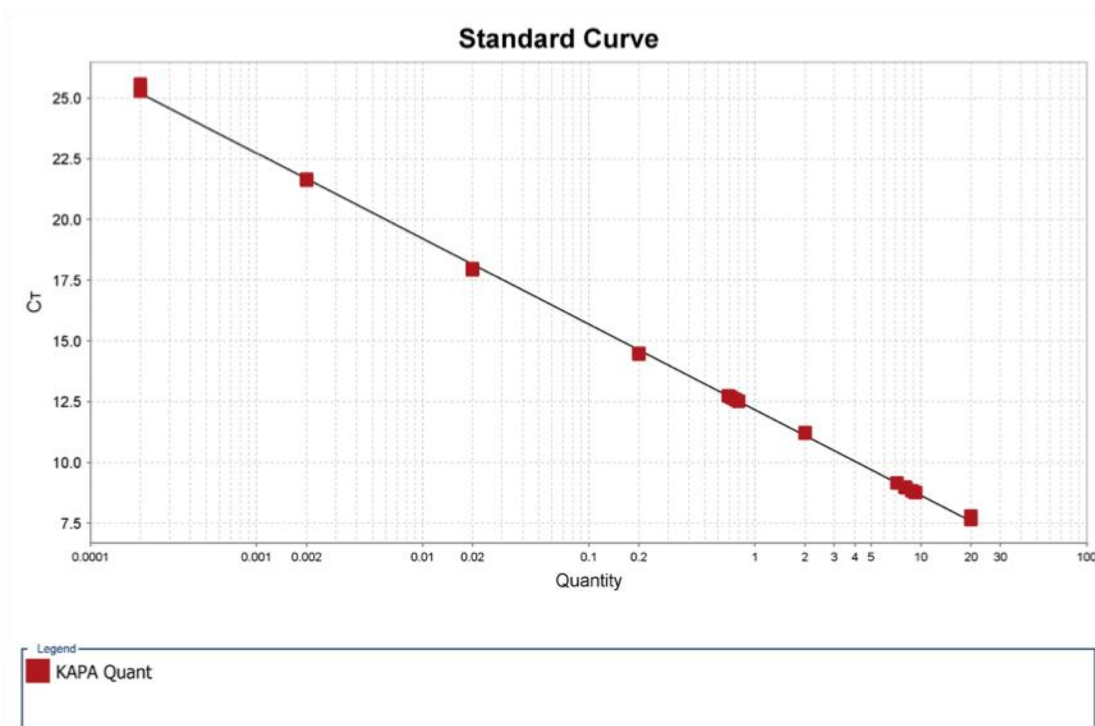
Table B.2 Patient isolate indexed amplicon library concentrations as determined by Qubit High Sensitivity DNA assay for Plate 1 before library preparation optimisation

Plate 1		**MM1**	**MM2**
No.	**Patient Isolate**	**Qubit average (ng/µL)**	**Qubit average (ng/µL)**
1	R40830	2.31	3.49
2	R40844	22.83	43.10
3	R40847	5.61	12.30
4	R40854	2.90	27.90
5	R40856	6.58	25.20
6	R40858	2.51	14.73
7	R40861	8.29	67.20
8	R40862	3.63	34.63
9	R40863	2.76	17.30
10	R40864	3.09	18.46
11	R40868	4.15	36.93
12	R40871	12.00	19.13
13	R40873	33.90	82.13
14	R40874	5.10	24.13
15	R40882	4.06	11.33
16	R40883	3.85	29.77
17	R40884	1.10	5.54
18	R40887	1.32	10.70
19	R40889	5.45	73.30
20	R40893	2.40	15.10
21	R40894	1.25	2.86
22	R40896	1.24	7.48
23	R40898	34.30	174.67
24	R40900	25.17	23.07
25	R40901	4.40	30.37
26	R40902	2.77	20.27
27	R40903	4.18	11.43
28	R40904	9.75	13.43
29	R40917	4.95	44.53
30	R40921	2.87	11.83
31	R40924	1.23	12.30
32	R40927	3.83	38.53
33	R40928	3.56	16.20
34	R40930	2.56	11.07
35	R40933	23.30	48.37
36	R40934	4.68	35.80
37	R40935	2.25	7.93
38	R40938	7.16	18.23
39	R40939	4.04	79.00
40	R40941	6.33	26.57
41	R40950	2.99	7.75
42	R40951	1.60	9.30
43	R40956	6.83	80.95
44	R40958	1.25	37.67
45	R40964	0.54	4.51
46	R40965	1.25	6.86

47	NTC_1	1.16	3.75
48	H37Rv_1	4.97	38.00

MM1 = master-mix 1; MM2 = master-mix 2



Slope: -3.522 **Y-intercept:** 12.171 **R^2:** 0.999

Figure B.2 Standard curve of the pool of pools for the libraries with the standards. The slope was -3.522 and the R2 value was 0.999

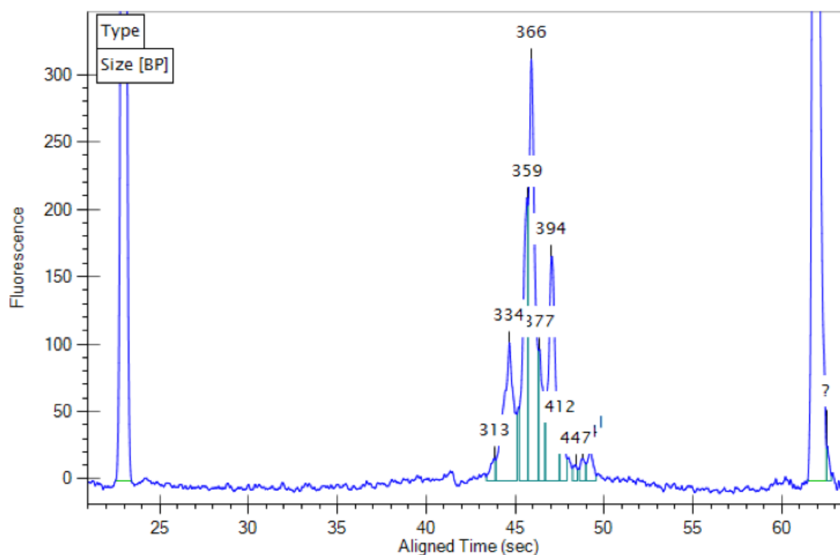


Figure B.1 LabChip electropherogram of the final pool of pools to be used for targeted deep sequencing on the Illumina MiniSeq platform. The peaks were used to determine the weight of the library fragment sizes for the optimum loading concentration on the cartridge.

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Table B.3 Isolate variants and associated information

Isolate	Variant identified (nucleotide change and gene position)	Amino acid change	Variant number of reads	Serial isolate available	NHLS drug resistance status
R39661	*Rv0678*: T141TC	indel	7468/7689 (97.13%)	Yes: R42673	XDR-TB
R39673	*Rv0678*: A152C	Q 51 P	45/878 (5.13%)	No	Pre-XDR-TB
R40435	*Rv0678*: A152C	Q 51 P	125/2104 (5.95%)	No	RIF-Mono-resistant TB
R40593	*pepQ*: *T196C*	S 66 P	7227/7516 (96.13%)	No	Pre-XDR-TB
R40711	*Rv0678*: T138TG	indel	3073/3120 (98.49%)	No	XDR-TB
R40778	*pepQ*: G*540A*	M 180 I	4903/23569 (20.8%)	No	MDR-TB
R40883	*pepQ*: A863T	E 288 V	18/359 (5.01%)	Yes: R43266	MDR-TB
R40901	*Rv0678*: G52T	E 18 * stop codon	11/209 (5.26%)	No	MDR-TB
R40964	*pepQ*: G317T	G 106 V	21/286 (7.34%)	No	MDR-TB
R41033	*pepQ*: A1080T	E359 D	26/431 (6.03%)	No	MDR-TB
R41065	*pepQ*: *T71C*	I 24 T	1208/1214 (99.51%)	No	MDR-TB
R41071	*atpE*: T47A	I 16 N	9/152 (5.92%)	No	MDR-TB
R41082	*pepQ*: G1066T	G 355 W	9/176 (5.11%)	No	MDR-TB
R41299	*pepQ*: G1066T	G 355 W	16/315 (5.08%)	No	MDR-TB
R41422	*Rv0678*: G52T	E 18 * stop codon	6/105 (5.71%)	No	MDR-TB

NHLS = National Health Laboratory Service; TB = tuberculosis; MDR-TB = multi-drug-resistant TB; XDR = extensively-drug-resistant TB; RIF = rifampicin; Q = glutamine; P = proline; S = serine; M = methionine; I = Isoleucine; E = glutamic acid; V = valine; G = glycine; D = aspartic acid; T = Threonine; N = asparagine; W = tryptophan

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gtg agc gtc aac gac ggg gtc gat cag atg ggc gcc gag ccc gac atc atg taa ttc gtc
 V   S   V   N   D   G   V   D   Q   M   G   A   E   P   D   I   M   -   F   V
gaa cag atg ggc ggc tat ttc gag tcc agg agt ttg act cgg ttg gcg ggt cga ttg ttg
 E   Q   M   G   G   Y   F   E   S   R   S   L   T   R   L   A   G   R   L   L
ggc tgg ctg ctg gtg tgt gat ccc gag cgg cag tcc tcg gag gaa ctg gcg acg gcg ctg
 G   W   L   L   V   C   D   P   E   R   Q   S   S   E   E   L   A   T   A   L
gcg gcc agc agc ggg ggg atc agc acc aat gcc cgg atg ctg atc caa ttt ggg ttc att
 A   A   S   S   G   G   I   S   T   N   A   R   M   L   I   Q   F   G   F   I
gag cgg ctc gcg gtc gcc ggg gat cgg cgc acc tat ttc cgg ttg cgg ccc aac gct ttc
 E   R   L   A   V   A   G   D   R   R   T   Y   F   R   L   R   P   N   A   F
gcg gct ggc gag cgt gaa cgc atc cgg gca atg gcc gaa ctg cag gac ctg gct gac gtg
 A   A   G   E   R   E   R   I   R   A   M   A   E   L   Q   D   L   A   D   V
ggg ctg agg gcg ctg ggc gac gcc ccg ccg cag cga agc cga cgg ctg cgg gag atg cgg
 G   L   R   A   L   G   D   A   P   P   Q   R   S   R   R   L   R   E   M   R
gat ctg ttg gca tat atg gag aac gtc gtc tcc gac gcc ctg ggg cga tac agc cag cga
 D   L   L   A   Y   M   E   N   V   V   S   D   A   L   G   R   Y   S   Q   R
acc gga gag gac gac tga
 T   G   E   D   D   -
```

Figure B.3 Translation program results for G52T Rv0678 variant. The black circle indicates the stop codon translation caused by the variant. The red Ms represent the methionine start codons in the Rv0678 gene.

Table B.4 CRyPTIC UKMYC6 MIC plate results for the isolates from the first targeted deep sequencing run.

		Anti-TB drugs (Proposed ECOFFs in mg/L)												
Patient Isolate	**DR Class**	**BDQ (0.25)**	**EMB (4.00)**	**RIF (0.50)**	**KANA (4.00)**	**AMI (1.00)**	**LEVO (1.00)**	**MXF (1.00)**	**DLM (0.12)**	**LZD (1.0)**	**CFZ (0.25)**	**ETHIO (4.00)**	**RIFAB (0.12)**	**INH (0.10)**
R39661	XDR	0.12	4	>8	>16	16	8	>4	<0.008	0.25	0.12	>8	2	12.8
R39673	Pre-XDR	<0.008	8	>8	<1	<0.25	2	2	<0.008	0.25	<0.03	8	>2	0.8
R40435	RIF MONO	0.03	0.5	4	2	<0.25	0.25	0.12	<0.008	0.25	0.06	0.5	>2	<0.025
R40593	Pre-XDR	0.015/ 0.03	8	>8	2	<0.25	2	1	<0.008	0.25/ 0.5	0.06	>8	>2	3.2/6.4
R40711	XDR	0.12/ 0.25	4	>8	>16	>16	8	2//4	<0.008	0.25	0.12/ 0.25	2	0.25	6.4/ 12.8
R40778	MDR	0.015	4//8	8	<1	<0.25	0.25	0.12/ 0.25	<0.008	0.12	<0.03	1	>2	>12.8

ECOFFs = epidemiological cut-off values; DR = drug resistance; XDR = extensively drug resistant; Pre-XDR = pre-extensively drug resistant; RIF MONO = resistance to rifampicin only; MDR = multi drug resistant; BDQ = bedaquiline; EMB = ethambutol; RIF = rifampicin; KANA = kanamycin; AMI = amikacin; LEVO = levofloxacin; MXF = moxifloxacin; DLM = delamanid; LZD = linezolid; CFZ = clofazimine; ETHIO = ethionamide; RIFAB = rifabutin; INH = isoniazid
The ECOFFs for each anti-TB drug are in parenthesis. The MICs that were above the ECOFF are highlighted in red, indicating resistance.

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Table B.5 Summary of quality assessment of whole genome sequences

Patient isolate	Number of reads	Number of mapped reads	% mapped reads	Sequencing depth (mean)	% GC content
R39661	5750945	5653507	99.07	184	48.48
R39673	7258018	7115436	98.98	228.67	48.86
R40435	7708886	7562989	99.01	243.67	48.82
R40593	6066978	5981278	99.18	191	48.86
R40711	2962107	2914037	99.12	95.67	48.31
R40778	3938289	3883502	99.28	128	48.38