Characterization of efflux pumps genes involved in second-line drug resistance of tuberculosis

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CHARACTERIZATION OF EFFLUX PUMPS GENES INVOLVED IN SECOND-LINE DRUG RESISTANCE OF TUBERCULOSIS

by

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Submitted in partial fulfilment of the requirements for the degree

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DECLARATION

I, **Lesibana Anthony Malinga**, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted by me in respect of a degree at any other University or tertiary institution.

……

Signature of candidate

……

Date

"It is through education that the daughter of a peasant can become a doctor, that the son of a mine worker can become the head of the mine".

Nelson Mandela

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Contents

LIST OF ABBREVIATIONS AND ACRONYMS

ºC	Degrees Celsius
α	Alpha
γ	Gamma
µg	Microgram
µl	Microlitre
Mb	Megabase
ml	Millilitre
mM	Millimolar
v/v	Volume per volume
7H9	Middelbrook Broth Medium
ABC	ATP-binding cassette
AMK	Amikacin
AG	Arabinogalactan
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCG	Bacillus Calmette-Guérin
BDQ	Bedaquiline
bp	Basepairs
BWA	Burrons-Wheeler Aligner
CI	Confidence interval
CAP	Capreomycin
CCPI	Cell processes and information pathway
CCCP	Carbon cyanide m-chlorophenylhydrazone
CFZ	Clofazimine
Cov	Coverage
DEG	Differentially expressed genes
Da	Dalton
dH_2O	Distilled water
DMSO	Dimethylsulfoxide

DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
dNTP	Deoxynucleotide triphosphate
DR	Direct repeat
DST	Drug susceptibility testing
EDTA	Ethylenediamine tetraacetic acid
EMB	Ethambutol
EP	Efflux pump
EPI	Efflux pump inhibitor
ESAT-6	Early secreted antigenic target genes
ESX	ESAT-6 secretion systems
ETO	Ethionamide
ETOH	Ethanol
FLQ	Fluoroquinolone
FDA	US Food and Drug Administration
GC	Guanine-Cytosine
Hz	Hertz
HIV	Human immuno-deficiency virus
IFN-γ	Interferon gamma
IL	Interleukin
IS	Insertion sequences
INH	Isoniazid
KAN	Kanamycin
Kb	Kilobase
kV	Kilovolt
LAM	Lipoarabinomannan
LJ	Löwenstein-Jensen
LPA	Line probe assay
LED	Light emitting diode
LM	Lipid metabolism
MA	Mycolic acids
MABA	Microplate AlamarBlue® assay
MATE	Multidrug and toxic compound extrusion
MDR	Multidrug-resistant

MFS	Major facilitator superfamily
$MgCl_2$	Magnesium chloride
MGIT	Mycobacterial growth ndicator tube
MHC	Major histocompitability complex
MIC	Minimum inhibitory concentration
Min	Minute
MmpL	Mycobacterium membrane protein: Large
MOX	Moxifloxacin
M. tuberculosis	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MW	Molecular weight
NaCl	Sodium chloride
NAAT	Nucleic acid amplification assay
NBD	Nucleotide-binding domain
ND	Not done
NPV	Negative predictive value
nsSNP	Non-synonymous single nucleotide polymorphism
nt	Nucleotide
OADC	Oleic albumin-dextrose-catalase
OD	Optical density
OFX	Ofloxacin
PAS	*p*-Amino salicyclic acid
PCR	Polymerase chain reaction
PDIM	Phthiocerol dimycocerosates
PE	Proline–glutamic acid
PG	Peptidoglycan
PIP	Piperine
POC	Point-of-care
PPE	Proline-proline-glutamic acid
PPV	Positive predictive value
PROVEAN	Protein variation effect analyser
QRDR	Quinolone resistance determining region
RIF	Rifampicin
RNA	Ribonucleic acid

rRNA	Ribosomal ribonucleic acid
RND	Resistance nodulation cell division
RP	Regulatory proteins
rpm	Revolutions per minute
RPKM	Reads per kb per million mapped reads
Sec	Seconds
SLD	Second-line drug
SLID	Second-line injectable drug
SMR	Small multidrug resistance
SNP	Single nucleotide polymorphism
STR	Streptomycin
STARD	Standard for reporting diagnostic accuracy studies
STRING	Search tool for retrieval of intercalating genes
tRNA	transfer ribosomal nucleic acid
T7S	Type VII secretion
TB	Tuberculosis
TDZ	Thriodazine
TE	Tris/EDTA
TNF	Tumor Necrotic Factor
TST	Tuberculin skin test
U	Unit
V	Volt
VER	Verapamil
VAC	Vancomycin
VDA	Virulence detoxification and adaptation
WGS	Whole genome sequencing
WHO	World Health Organization
XDR	Extensively drug-resistant

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LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

Publications (2012-2016)

Malinga, L.A., Abeel, T., Desjardins, C., Dlamini, T., Cassell, G., Chapman, S., Birren, B., Earl, A., and Van der Walt, M. (2016) Draft genome sequence of two extensively drug-resistant strains of *Mycobacterium tuberculosis* belonging to the Euro-American S lineage. *Genome Announcement* 4(2), e01771-15

Malinga, L.A., Brand, J., Olorunju, S., Stoltz, A., and Van der Walt, M. (2016) Molecular analysis of genetic mutations among cross-resistant second-line injectable drugs reveals a new resistant mutation in *Mycobacterium tuberculosis. Diagnostic Microbiology and Infectious Disease* 81(4), 433-7.

Malinga, L.A., Stoltz, A., and Van der Walt, M. Efflux pump mediated second-line tuberculosis drug resistance. *Mycobacterial Diseases* 6, 222. (In press).

Conference Presentations (2012-2016)

2016: Nardell E.A, Williams C.M, Bell AJ, Malinga L, Ramsheh MY, Bakir A, Garton NJ, Stoltz A, DeKock E, Waddell S, Hinton J, Fennelly K, Barer M. TB airborne transmission: first gene expression signatures of captured, uncultured *M. tuberculosis* from human source aerosol. Poster abstract presented at American Thoracic Society International Conference, San Francisco, USA- May 15-18 2016.

2016: Malinga L, Tshireledzo R, Sibandze B, Makhado N, Maluleka C, Magazi B. Performance evaluation of Anyplex II MTB/MDR/XDR for detection of first and second-line drug resistance in *Mycobacterium tuberculosis*. Oral abstract to be presented at 17th ICID - Hyderabad, India - March 2-5, 2016

2015: Malinga L, Brand J, Abeel T, Earl A, Van der Walt M. Whole genome sequencing reveals accumulation of single nucleotide polymorphisms (SNPs) associated with treatment failure. Electronic poster presentation at 46th Union World Conference on Lung Health in Cape Town, South Africa.

2014: Malinga L, Brand J, Stoltz A, van der Walt M. Association of *eis* and *rrs* gene mutations with phenotypic second-line injectable drug resistant patterns of *Mycobacterium tuberculosis*. Poster presentation at 45th Union World Conference on Lung Health in Barcelona, Spain.

2014: Malinga LA, Mpanyane DM, Maguga NT, Van der Walt M. Evaluation of Anyplex plus assay for rapid detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* isolates. Oral presentation at the 4th South African TB Conference June 2014.

2012: Malinga LA, van der Walt M, Bapela NB. Evaluation of GenoType® MTBDR*sl* DNA strip assay for rapid detection of fluoroquinolone, aminoglycoside and ethambutol resistance in *Mycobacterium* isolates. Oral presentation at the 3rd South African TB Conference June 2012.

Characterization of efflux pumps genes involved in second-line drug resistance of tuberculosis

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SUMMARY

In this study, we used molecular and proteomic methods to detect novel changes within drug-resistant tuberculosis (TB) strains. Our goal was to detect changes within efflux pump (EP) genes of extensively drug resistant (XDR-TB) strains using genomic and transcriptomic methods. We firstly sequenced multiple genes in discordant samples that lacked molecular markers present on the GenoType® MTBDR*sl* assay. Further analysis by whole genome sequencing was done on XDR-TB strains. Transcriptomic changes of EP genes were detected by RNA sequencing strains. The minimum inhibitory concentration (MIC) of second-line drugs in the presence and absence of efflux pump inhibitors was also measured. Proteomic expression by cloning and expression of three efflux pump genes (Rv1258c, Rv1634 and Rv0194) was done and effect on MICs of second-line drugs measured in the presence and absence of efflux pump inhibitors.

We analysed molecular markers responsible for resistance to second-line drugs of ofloxacin (OFX), amikacin (AMK), kanamycin (KAN) and capreomycin (CAP) in 636 drug-resistant strains using GenoType® MTBDR*sl*. We compared GenoType® MTBDR*sl* with phenotypic second-line drug susceptibility testing. After comparison, 102 (14.8%) strains were discordant between the two methods. In the discordant population, genetic regions of *gyrA*, *gyrB*, *rrs*, *eis*, *tlyA* and EP genes (Rv1634, Rv1258c and Rv0194) were sequenced and analysed in search of mutations. Combining sequencing and GenoType® MTBDR*sl* significantly improved the diagnosis of XDR-TB and second-line drugs. The Rv0194 belongs to the ATP (adenosine triphosphate) binding cassette (ABC) family while Rv1258c and Rv1634 belong to the major facilitator superfamily (MFS) transporters. Since these genes are implicated in multiple drug resistance, our hypothesis was that possible mutations in these genes could confer cross-resistance. Our analysis revealed the appearance of Rv1258c and Rv0194 mutations in strains with cross-resistance to second-line injectable drugs. Further analysis revealed *rrs* G878A mutation that was specific to EuroAmerican X3 lineage (P<0.001) and linked to CAP resistance. The inclusion of G878A in new rapid assays might be beneficial for rapid CAP resistance detection.

Whole genome sequencing with increased resolution and depth was used to study two XDR-TB strains. Drug resistant mutations were detected for all other drugs except for OFX in one strain. We further analysed EP genes for mutations. Bioinformatic prediction tools detected protein changes related to EP gene mutations belonging to Rv0987, Rv2039 and Rv0402. Two of the efflux pump genes (Rv0987, Rv2039) belong to the ABC family, while Rv0402 is of resistance nodulation-cell division (RND) family. Mutations within lipid metabolism and secretion pathways were also detected.

To fully understand the role of EP gene mechanisms at a transcriptional level, we sequenced RNA molecules of 11 XDR-TB, five MDR and two susceptible strains. The RNA signatures of EP and lipid metabolism genes detected in XDR-TB strains were characterized. Further analysis of four XDR strains with MIC data with or without efflux pump inhibitors (EPIs) was performed in relation to RNA sequenced data. The ABC Rv2686/87/88c operon was significantly over-expressed in the background on strains with *gyrA* mutations causing OFX resistance. The Rv1258c, Rv0194 and Rv1634 EP genes were consistently over-expressed in XDR–TB strains. Efflux pump inhibitor of piperine was effective in reducing MICs to hydrophilic drugs of AMK and OFX whereas verapamil reduced MIC of hydrophobic CAP drug. Protein-protein interaction pathways revealed novel associations between ABC, RND and type VII secretion (T7S) proteins.

Finally, we cloned and expressed Rv1258c, Rv0194 and Rv1634 EP genes in *Mycobacterium* vectors. These genes have the potential to cause multidrug resistance. We did not detect increased MIC levels of second-line drugs in the presence of the clones. However, a reduction of MICs in the presence of the EPI, piperine, was observed. Bioinformatic approaches also revealed transmembrane motifs, domains and loops. Since EP genes are implicated in transport of substrates across cell membrane, we used biofilm formation assays to determine the role of each clone. Both Rv1258c and Rv0194 clones showed biofilm formation. Such discovery highlights the secretion of lipid bodies on the cell wall of the bacteria through EP genes/proteins. This information will allow us to develop novel strategies to treat drug-resistant TB.

Our study emphasises the importance of EP gene mechanisms in causing drug resistance. The combination of EP and target genes is important in the detection of second-line drugs. Furthermore, it is suggested that EPIs combined with second-line drugs might be effective in the treatment of XDR-TB.

Chapter 1: Introduction

1.1 Background

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M. tuberculosis*) is a serious problem worldwide, with about 10.4 and 14 million incident and prevalent cases respectively (Karim et al., 2009; WHO, 2009; WHO, 2016a). TB continues to be a global threat and has shown to be difficult to control in regions with high prevalence to human immune deficiency virus (HIV) infection (Louw et al., 2011). Sub-saharan Africa is currently experiencing dual epidemics of both TB and HIV. TB disease in South Africa is hyperendemic, with incidence rates of up to 948 cases per 100 000 population (Bekker and Wood, 2010). Other parts of southern Africa have more than 50% of TB cases co-infected with HIV (WHO, 2014).

Drug resistance continues to threaten global TB control and remains a major public health concern in many countries. Globally in 2014, there were an estimated 480 000 (range: 360 000 to 600 000) incident cases of MDR-TB and among them cases with XDR-TB was highest in Belarus (29% in 2014), Georgia (15% in 2014), Latvia (19% in 2014) and Lithuania (25% in 2013). (WHO, 2015). The global resurgence of TB and the progression of antibiotic resistance *M. tuberculosis* from mono-resistant through multi-drug resistant (MDR), extensively drug-resistant (XDR) and now totally drug-resistant forms is worrisome (Amaral and Viveiros, 2012; Amaral et al., 2014). MDR-TB is defined as resistance to at least rifampicin (RIF) and isoniazid (INH) the two most effective TB drugs whereas XDR-TB is MDR-TB with further resistance to fluoroquinolones (FLQ) and at least one of the three second-line injectable drugs, i.e. amikacin (AMK), kanamycin (KAN) and capreomycin (CAP) (Lobue and Theron, 2010).

Resistance to second-line drugs (SLDs) leading to XDR-TB is emerging as a major threat to global TB control. Few African countries reported XDR-TB cases and this could be underestimated due to lack of laboratory capacity to diagnose the disease (WHO, 2014). Testing coverage among previously treated TB cases also improved considerably in most regions, notably from 5.8% to 67% in the South-East Asia Region and from 9.6% to 33% in the African Region (WHO, 2015). Thus, it is important to improve laboratory infrastructure that will enable rapid and accurate detection of XDR-TB. Molecular diagnostic assays could

be used for rapid detection of XDR-TB, but cannot replace phenotypic tests due to an incomplete understanding of drug-resistant mechanisms towards SLDs.

Conventional drug susceptibility testing (DST) used to detect XDR-TB strains has a long turnaround time, requires technical expertise and involves high biosafety levels. In contrast, molecular methods such as GenoType® MTBDR*sl* (Hain Lifescience, GmbH, Germany) assay detect drug resistant mutations associated with XDR-TB within hours and require less biosafety levels. Performance of Genotype® MTBDR*sl* assay varies from different settings and cannot completely replace conventional DST. GenoType® MTBDR*sl* version 1 assay has few mutational probes (*gyrA* and *rrs*) to detect resistance in SLDs. Our observations with GenoType® MTBDR*sl* assay indicate that this limitation causes discordance when compared to conventional DST in certain XDR-TB strains. The new GenoType® MTBDR*sl* version (Hain Lifescience, GmbH, Germany), version 2, has been modified to include probes within *eis* and *gyrB* genes. The assay has been recommended by World Health Organization as a rule-out test for resistance to second-line drugs (WHO, 2016b). The discordance could be due to uncharacterized mutations in other drug resistant genes (i.e *eis*, *tlyA*, *gyrB*) or other mechanisms such as efflux pump (EP) proteins associated with SLDs. More studies are needed to enhance the rapid detection of SLDs and improve molecular detection of XDR-TB. Newer diagnostic assays based on the detection of drug resistant mutations and EP gene expression could improve the rapid detection of XDR-TB.

Treatment of XDR-TB is long and new drugs that could potentially improve the treatment outcomes of patients are needed. Due to non-effective drugs, non-genetic factors such as drug efflux proteins could be responsible for resistance in strains without genetic mutations. The activity of EPs should be explored, especially in strains without genetic mutations having high minimum inhibitory concentration (MIC) levels. A combination of EP activity and genetic mutations in different bacterial populations could lead to clinical drug resistance. Increased understanding of drug-resistant mechanisms could lead to novel diagnostic markers and new anti-TB drugs.

Resistance mechanisms in *M. tuberculosis* are not fully understood but drug mutations and overexpression of EPs may play a major role (Song and Wu, 2016). Efflux pump activity can be inhibited by depleting the energy source used by EPs for transporting drugs (De Rossi et al.,

2002). An efflux pump inhibitor (EPI), thioridazine was shown to have a theurapeutic activity against MDR and XDR-TB (Amaral et al., 2008). Inhibiting EPs could be used as a possible alternative or adjuvant in anti-TB therapy and thus shorten the treatment duration. Moreover, such compounds which are capable of blocking EPs and enable antibiotics to gain access to the bacteria are very important (Viveiros et al., 2008). Due to limited number of antimicrobials available for TB treatment, it is important to further our understanding on drug efflux and evaluate compounds that could reverse resistance.

1.2 Hypothesis

Inhibition of specific expression targets on second-line drug efflux pump genes leads to drug susceptibility in extensively resistant *Mycobacterium tuberculosis.*

1.3 Aim

To describe the role of efflux pump genes and associated mutations as determinants of second-line drug resistance in *M. tuberculosis.*

1.4 Objectives

a) To correlate assay results between GenoType® MTBDR*sl* assay and MGIT DST as the gold standard, and to sequence *gyrA/B, eis*, *tlyA* and *rrs* genes in discordant samples.
b) To characterize discordant samples by spoligotyping to determine different families of *M. tuberculosis* isolates.
c) To perform minimum inhibitory concentration (MIC) determinations of ofloxacin, amikacin, kanamycin, capreomycin and correlate these to specific genetic mutations in discordant samples.
d) To investigate the expression levels of efflux pump genes in drug resistant and drug susceptible *Mycobacterium tuberculosis* isolates.
e) To determine the effect of efflux pump inhibitors on drug-resistant samples and efflux pump clones.
f) To determine the effect of drug efflux proteins of interest by cloning and expression in transformation vectors.

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Chapter 2: Literature review

2.1 History of tuberculosis

Tuberculosis (TB) is an ancient disease that has plagued humankind throughout history and continues to cause great epidemics. History suggests that TB appeared about 70 000 years ago and that it remained sporadic up to the 18th century (Daniel, 2006; Banuls et al., 2015). Indeed, the discovery of *M. tuberculosis* by Robert Koch (1843–1910) came into a framework of previous popular and scientific knowledge regarding TB who announced the discovery on March 24, 1882 (Anquetin et al., 2006; Lawn and Zumla, 2011). Koch's work on the anthrax bacillus grown in pure culture paved the way for understanding the fundamental and underlying etiology of infectious diseases, including TB (Weyer et al., 2011). It then became epidemic during the industrial revolution but was soon reduced by the introduction of the Bacillus Calmette–Guérin (BCG) vaccine in 1921 and the use of antimicrobial drugs, such as streptomycin (STR) (1943), isoniazid (INH) (1952) and rifampicin (RIF) (1963) (Banuls et al., 2015). TB incidence increased again in the 1980s, due to the deterioration of health conditions in large cities (Banuls et al., 2015). About one-quarter of the world's population, or approximately 1.7 billion individuals, is infected with TB. Of these, almost 10 million people have active TB and 1.8 million die from this disease each year (WHO, 2016). Substantial geographical and age variation occur (Houben et al., 2016). Human populations act as the natural reservoir of the pathogen (Salgame et al., 2015). More than 90 % of TB cases occur in developing countries and the regions most affected by this disease are Africa, South-East Asia and East Europe (Banuls et al., 2015).

2.2 The tubercle bacillus

The bacterium *M. tuberculosis* belongs to genera named "mycolata" of the *Corynebacterineae* suborder (Pawelczyk and Kremer, 2014). Corynebacteriales have high guanine and cytosine (GC) content and composition of the mycolic acid-containing membrane makes it different to other bacteria (Houben et al., 2014). It is an aerobic, acid-fast, non-motile, non-encapsulated,

non-spore forming bacillus and grows successfully in tissues with high oxygen content, such as the lungs (Lawn and Zumla, 2011).

2.2.1 Cell wall and envelope

Compared with the cell walls of other bacteria, the lipid-rich cell wall is relatively impermeable to basic dyes unless combined with alcohol i.e phenol. Thus, *M. tuberculosis* is neither gram positive nor gram negative but is instead described as acid-fast, since once stained it resists decolourisation with acidified organic solvents (Lawn and Zumla, 2011). The cell envelope of *M. tuberculosis* contains unique lipids and lipopolysaccharides important from the perspective of host pathogen interactions and as potential targets for new drug development against TB (Slayden et al., 2013). The envelope consists of (i) the plasma-membrane (ii) a complex wall of peptidoglycan (PG) and arabinogalactan (AG) (iii) an outer membrane and (iv) a capsule of polysaccharides and proteins (**Figure 1**) (Gutsmann, 2016). The long mycolic acid (MA) chain in *M. tuberculosis* consists of 50 to 60 carbons and the α-branch is typically 24-carbons long (Gutsmann, 2016). Its unique architecture renders the cell envelope very rigid and extremely impermeable, protecting the pathogen from dehydration and rendering it resistant to conventional antibiotics (Gengenbacher and Kaufmann, 2012).

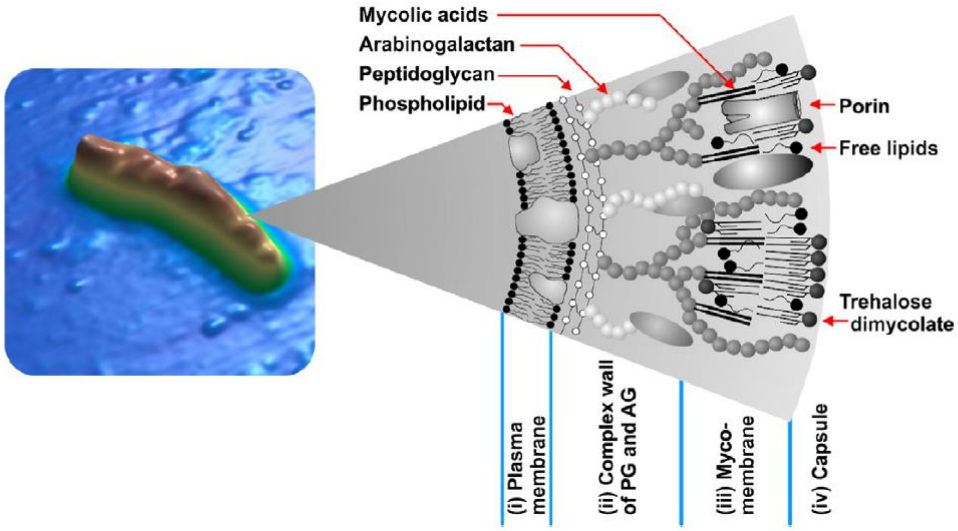

Figure 1: The cell envelope of mycobacteria

Left side: Atomic force microscopic image of a bacille. *Right side*: Model of mycobacterial cell wall composed of four layers:(i) the plasma membrane is composed of phospholipids, (ii) the complex wall links the PG and AG to the (iii) myco-membrane and (iv) capsule (Gutsmann, 2016).

2.2.2 Phylogeny

Modern techniques of molecular genetics and the sequencing of the genome of several strains of *M. tuberculosis* allow a more rigorous estimation of the time of origin of mycobacteria (Daniel, 2006). The complex includes several Mycobacterium species with nearly identical nucleotide sequences and totally identical 16S rRNA sequences and have a common ancestor but differ in terms of host tropism, phenotypes and pathogenicity (Ernst et al., 2007; Banuls et al., 2015). Indeed, some of these species belonging to the *Mycobacterium tuberculosis* complex (MTBC) are human pathogens (*M. tuberculosis, M. africanum, M. canettii*), rodent pathogens (*M. microti*), seals (*M. pinnipedi*), or sheep (*M. caprae*), while *M. bovis* has a larger spectrum of host species, including bovidae and humans (Banuls et al., 2015). Even among the *Mycobacterium* species very different genomic, phenotypic, clinical and epidemiological features can be observed, *M. tuberculosis* and *M. africanum*, for instance, have circular genomes ranging between 4.38 and 4.42 Mb, while the *M. canettii* genome is 10–115 kb larger, representing the tubercle bacillus with the biggest genome (Banuls et al., 2015). Phylogenetic studies have brought some insights into how the MTBC developed (Banuls et al., 2015).

Mycobacterium tuberculosis is divided into lineages L1 to L4 and L7, that together with the *M. africanum* lineages L5 and L6, are human-adapted. The L8 lineage, which includes *M. bovis* and the TB vaccine strain BCG, contains the animal-adapted pathogens (Warner, 2014). *Mycobacterium tuberculosis* complex comprises various bacterial species and sub-species sharing 99.9% DNA sequence identity but differing in their primary host range (Brites and Gagneux, 2015). It is notorious that certain lineages, but also sub-groups within the main MTBC lineages are geographically more widespread than others (Comas et al., 2013) (**Figure 2**). Geographically lineages L5, L6, and L7 are restricted and lineages 2 and 4 are widespread, i.e Haarlem and the LAM families (Brites and Gagneux, 2015). Lineage 2 appeared with the arrival of modern human beings in east Asia about 32 000–42 000 years ago, and the Beijing family expanded 3000–5000 years ago, coinciding with the spread of agriculture in the region (Takiff and Feo, 2015). Global exploration, trade, conquest and migration, coupled with the rapid growth in human population in modern times, would then have resulted in the worldwide spread of these initial lineages and led to the current phylogeographical distribution of TB (Galagan, 2014).

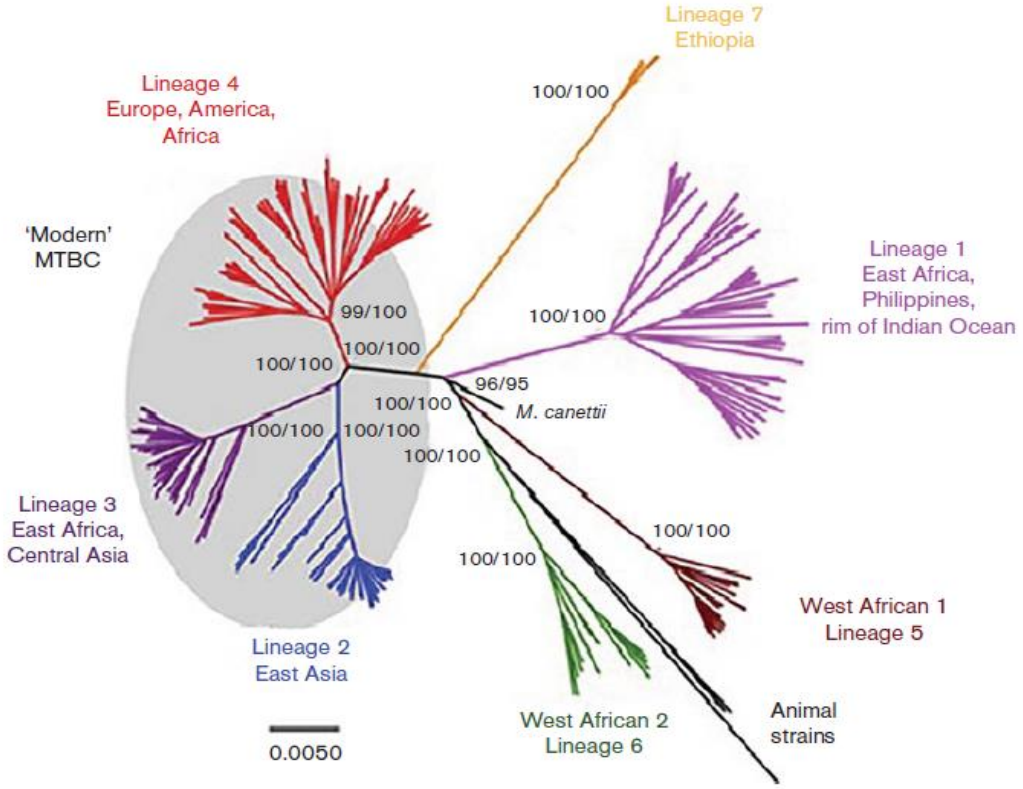

Figure 2: The five *Mycobacterium tuberculosis* lineages (1, 2, 3, 4 and 7)

The figure highlights the two individualized lines of *M. africanum* (lineages 5 and 6) and the ancestral position of *M. canettii*. The animal lineages represent a monophyletic branch in the complex. The lineages in the grey oval are the so-called 'modern' lineages, as opposed to all the other lineages, which are called 'ancestral' (Comas et al., 2013).

2.2.3 *Mycobacterium tuberculosis* genome and transcriptome

Genomic information is now an integral part of the research workflow, and impacts all aspects of TB research including drug discovery, diagnostics, vaccine development and epidemiology (Daniel, 2006). A little more than 100 years after the bacterium was isolated by Robert Koch, was revealed to have a GC-rich genome of 4.4 Mb that contains ~4,000 genes (Houben et al., 2014) (**Figure 3**).

In the late 90's whole genome sequencing (WGS) of bacteria was slow, extremely costly and labour-intensive but now are widely available and WGS is used intensively to identify single nucleotide polymorphisms (SNPs) or other mutations in the genome of spontaneous drug-

resistant mutants allowing for target identification of novel compounds (Gutsmann, 2016). The combination of genomics with other 'omics' technologies — such as transcriptiomics, proteomics, metabolomics and lipidomics — allows for assaying cells with unprecedented breadth and depth (Houben et al., 2014). Beyond mapping genome sequence and structure, which is rapidly becoming routine, sequencing-based technologies are now also enabling global profiling of genome function (Houben et al., 2014). The 204 structurally characterized proteins from *M. tuberculosis* H37Rv are assigned into 5 functional groups: 130 (64%) to "house-keeping metabolism" and "resistance/survival mechanism", 31 (15%) to "genetic information processing", 15 (7%) to "cellular information processing and substrate transport", 11 (6%) "virulence factors", while 17 (8%) are of unknown function mostly annotated as "hypothetical"(Ernst et al., 2007).

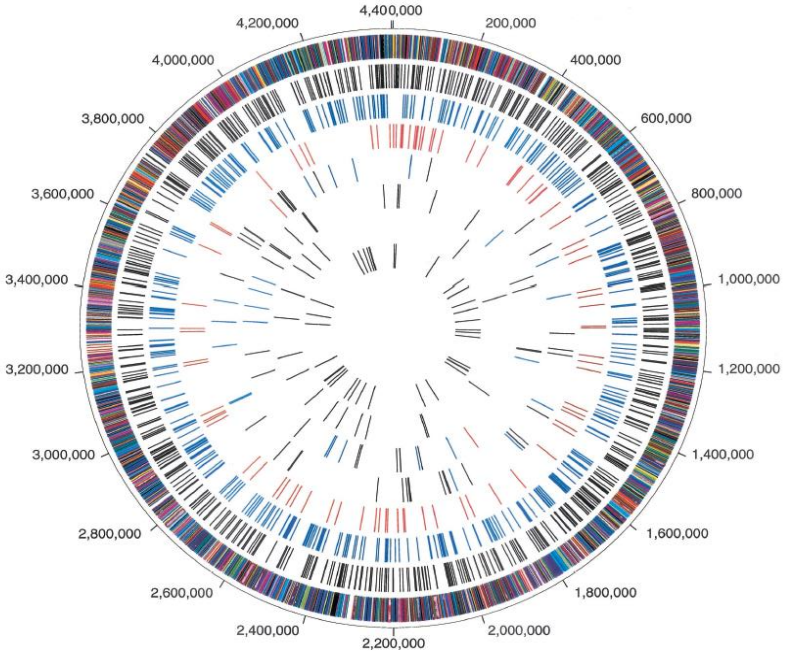

Figure 3: Structure of the *Mycobacterium tuberculosis* genome

Outer concentric circle shows predicted protein-coding regions on both strands, color coded according to role category. The second concentric circle shows the location of nonsynonymous substitutions (black). The third concentric circle shows the location of synonymous substitutions (blue). The fourth concentric circle shows the location of substitutions in noncoding regions (red). The fifth concentric circle shows the location of insertions in strain CDC1551, including coding (black) and noncoding (blue) regions, and the location of phage phiRv1 (red). The sixth concentric circle shows the location of insertions in strain H37Rv, including coding (black) and noncoding (blue) regions, and the location of phage phiRv1 (red). The seventh concentric circle shows the location of IS*6110* insertion elements in strains CDC1551 (blue) and H37Rv (red). The eighth (innermost) concentric circle shows the location of tRNAs (blue) and rRNA (red) (Fleischmann et al., 2002).

The 4.4 Mb genome of *M. tuberculosis* encodes 13 sigma factors and more than 100 annotated transcriptional regulators in response to environmental stresses imposed *in vitro*, during macrophage infection, and in human disease (Arnvig and Young, 2012). RNA sequencing is particularly useful for genome-wide studies of small regulatory RNAs as the expression of non-coding RNAs and other novel transcripts can be easily detected (Comas and Gagneux, 2009). Non coding RNAs are small transcripts, mostly in the range of 50 to 250 nucleotides, and they can be encoded opposite open reading frames (antisense or cis-encoded) or between open reading frames (intergenic or trans encoded) (Arnvig and Young, 2012). RNA sequencing is an excellent approach for transcriptome profiling, which uses deep-sequencing technologies to directly determine the cDNA sequence.

2.3 Infection, transmission and immunology

Tuberculosis is a contagious infectious disease spread essentially through air when an infectious person coughs, sneezes, talks or spits, saliva droplets containing tubercle bacilli are projected into the air and can be inhaled by a nearby person (Banuls et al., 2015). People worldwide became more aware of this disease, which is transmitted from the ill to the healthy through a minute droplet of aerosol that contains *M. tuberculosis* (Cheon et al., 2016).

The natural history of TB begins with the exposure of a susceptible host to an infectious case of pulmonary TB (Salgame et al., 2015) (**Figure 4**). Once in the lungs the *M. tuberculosis* droplet nuclei measuring 1–2 µm containing micro-organisms from infectious patients are inhaled and interaction of *M. tuberculosis* with the host begins (Meya and McAdam, 2007). After inhalation, *M. tuberculosis* enters the alveoli where it is taken up by macrophages and dendritic cells which transport the bacteria to draining lymph nodes where antigen-specific T lymphocytes are stimulated (Kaufmann, 2016).

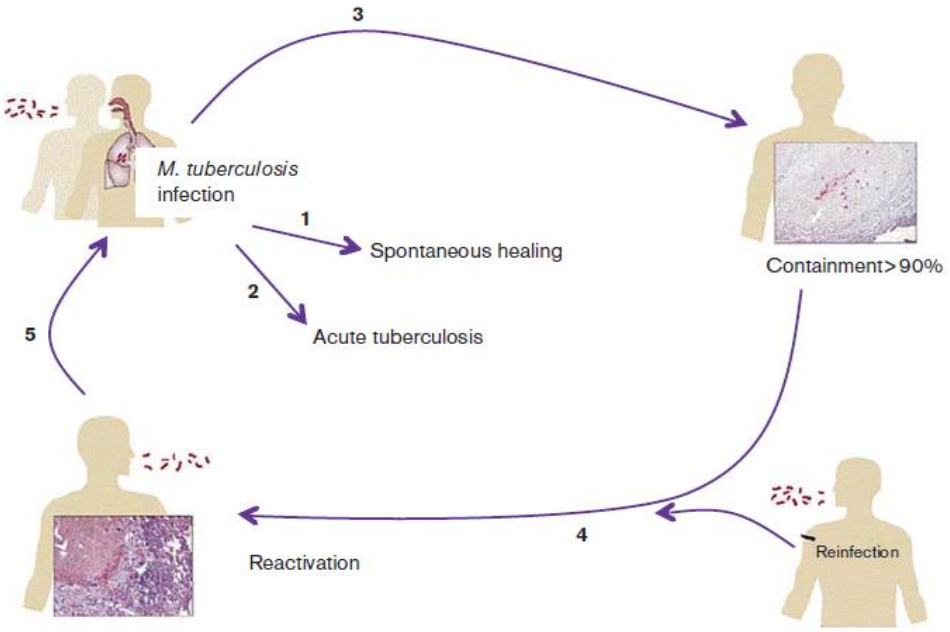

Figure 4: *Mycobacterium tuberculosis* enters the host by inhalation of aerosols

Different scenarios are possible: (1) immediate elimination of *M. tuberculosis* by the pulmonary immune system; (2) infection progresses to active tuberculosis; (3) infection does not progress to active disease and *M. tuberculosis* enters a latency phase; (4) after the latency phase, *M. tuberculosis* can become active following endogenous reactivation or a new exogenous infection or both; (5) at this stage, there is *M. tuberculosis* dissemination and transmission (Banuls et al., 2015).

The T-cells finally arrive at the site of infection they function to contain the bacteria, and play a role in granuloma formation, positioning themselves around infected macrophages processed through the major histocompatibility complex class II (MHC II) and MHC I pathways (Andersen and Urdahl, 2015; Kaufmann, 2016) (**Figure 5**). Both the CD4 and CD8 effector T cells sense inflammatory signals emitted by macrophages which have accumulated around *M. tuberculosis* and a solid granuloma with a fibrous wall is formed (Kaufmann, 2016). The predominant T cell population required for protection are T helper (Th) 1 cells producing cytokines such as interferon gamma (IFN-γ), tumor necrosis factor (TNF) and interleukin (IL)-2 (Kaufmann and Parida, 2008; Kaufmann, 2016). In addition to Th1 cells, Th17 cells are generated during the early inflammatory stage of TB, which seem to transiently pave the way for the build-up of protective immunity (Kaufmann, 2016).

Solid granulomas can persist over years in healthy individuals with latent TB and disturbances in the tight immune regulation can lead to cell death and necrosis that releases the organisms

into the environment and access to the blood stream promotes dissemination to other organ sites (Kaufmann, 2016). The causative agent *M. tuberculosis* is slow growing bacterium with a lung portal of entry that manipulates the host response to delay the onset of adaptive immunity (Salgame et al., 2015).

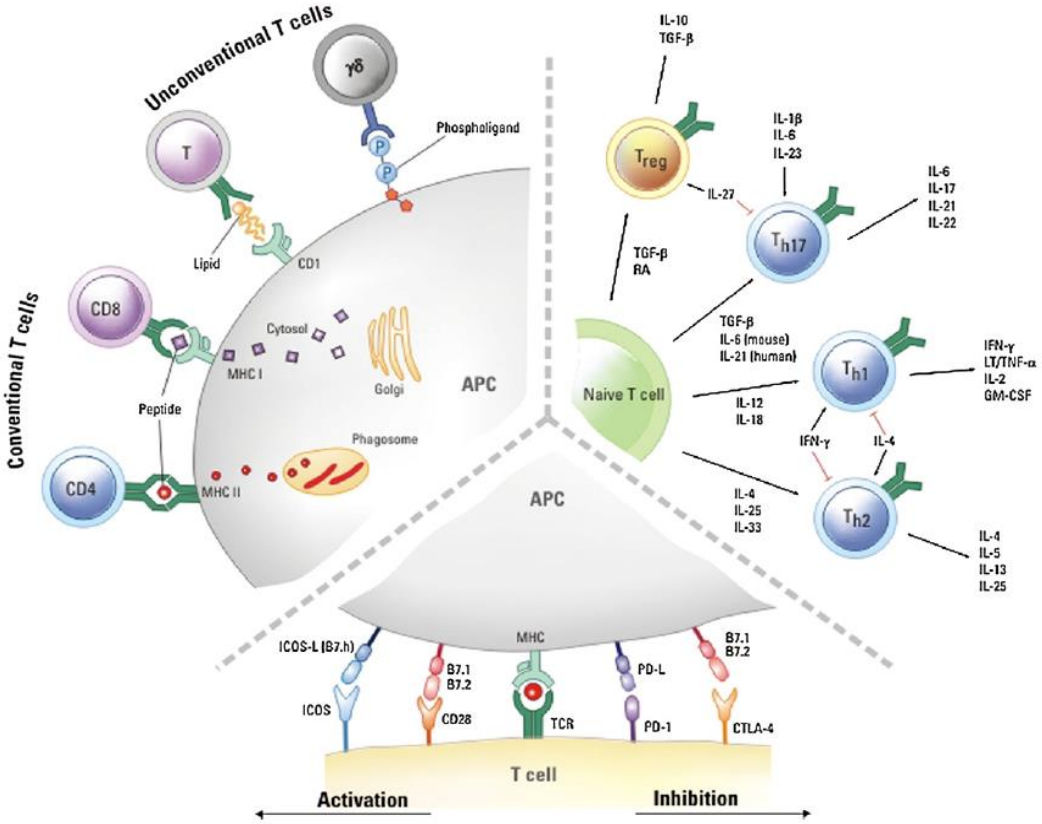

Figure 5: Critical steps toward T-cell activation and polarization

(1) T cells recognize antigen not in its natural form; rather the antigen is presented by reference structures encoded by the major histocompatibility complex; (2) CD4 T-cells produce interferon gamma (IFN-g), tumor necrosis factor alpha (TNF-a), lymphotoxin (LT), interleukin (IL)-2, and granulocyte-macrophage colony-stimulating factor; (3) Complete activation of T-cells by surface molecules (Kaufmann and Parida, 2008).

2.4 Tuberculosis detection

2.4.1 X-rays

The development of scientific equipment, such as X-rays, facilitated the radiographic visualization of changes caused by TB in a healthy person (Cheon et al., 2016). Chest

radiography played a major role in reducing the prevalence of TB in Europe and North America in the 1950s and 1960s, with mobile radiograph units used for mass screening of communities (Golub, 2005). The introduction of digital radiography has improved image quality and facilitates the storage and sharing of images and, if required, a second opinion may be sought by remote (electronic) access (Schito et al., 2015). Compared to film-based radiographs, running costs are reduced and reagent stock outs avoided, but the cost of buying/leasing and maintaining the equipment remains high (Schito et al., 2015). A further development in application of computer-aided image analysis to provide an automated imaging service would be ideal at peripheral areas. Chest X-rays have a sensitivity of 55.6% [95% confidence interval (CI) 30.8–78.5%] and a specificity of 98.3% (CI 98.1–98.5%) for detection of one case of active TB (Weinrich et al., 2016).

2.4.2 Microscopy

Microscopy is the primary method of examination for people with TB symptoms, but it has limitations such as laborious and time- consuming procedures for the laboratory staff (Cheon et al., 2016). However, this technique has a limited sensitivity, and up to 50% of cases are routinely missed (Comas and Gagneux, 2011). The limitations of microscopy are well known, with low sensitivity (40%–60% when compared with culture) being the most pressing (Weyer et al., 2011). Microscopy for acid fast bacilli (AFB) cannot distinguish *M. tuberculosis* complex from nontuberculous mycobacteria; it cannot distinguish viable from nonviable organisms and has reduced sensitivity in HIV positive individuals (WHO, 2015). The effectiveness of microscopy is further diminished by the conditions in which laboratory staff work with low resolution microscopes, variable electricity, insufficient time to examine slides properly, and little value placed on their work (Weyer et al., 2011). Infectious cases therefore remain undetected and thus untreated, and must in part have contributed to the size of the epidemic seen today (Weyer et al., 2011). Advances in physics led to the development of light emitting diodes (LED), with appropriate fluorescent light output coupled with low power consumption, creating cheaper robust LED fluorescent microscopes, requiring minimum mains or battery power (Drobniewski et al., 2013).

2.4.3 Culture

The culture method provides the most accurate diagnosis method available, and it is a more sensitive than sputum smears. In a conventional laboratory setting, susceptibility strains are

identified with indirect drug susceptibility testing (DST) on solid agar medium or in liquid culture (Mani et al., 2014). Thus, *M. tuberculosis* culture is still the gold standard technique for clinical and research diagnosis of active TB (Cheon et al., 2016). Automated liquid culture systems are now the gold standard for the diagnosis of TB; they are substantially faster and have a 10% greater yield than solid media (Lawn and Zumla, 2011). Capacity to perform culture and DST is crucial because MDR and XDR-TB are defined based on susceptibility results. Conventional laboratory capacity is generally inadequate in the 30 countries regarded by WHO as carrying high-MDR-TB burdens (defined by at least 4000 new MDR-TB cases annually or >10% MDR among newly registered cases) (Walter et al., 2012). Conventional phenotypic DST should generally be used to confirm results from other assays, as the correlative phenotypic effects of many identified target gene mutations are not absolute. Confirmatory phenotypic testing, however, has a variety of limitations reliability of breakpoints (Wilson and Tsukayama, 2016). The newer DST methods include the microscopic observation drug susceptibility test, thin layer agar method, nitrate reductase assay, colorimetric redox indicator methods, and phage-based assays (Ahmad and Mokaddas, 2014). Alternative methods have been reported to provide rapid indirect drug susceptibility test results, such as the microplate AlamarBlue® colourimetric (MABA) method (Franzblau et al., 1998). Minimum inhibitory concentration (MIC) is the concentration of compound required to arrest bacterial growth (stasis). Determination of MIC plays a critical role during the process of screening, prioritizing, and optimizing a chemical series during early drug discovery (Franzblau et al., 2012). Bactec Mycobacterial Growth Indicator Tube (MGIT) 960 is expensive and lacks a high-throughput format. Alternatively, a higher sensitivity for resazurin and MABA would be desirable, along with a shorter detection time (Sharma et al, 2014). The MABA method is cheap and rapid, however, it requires a greater level of technical expertise to provide reliable results (Huang et al, 2004). Alamar blue or resazurin reduction is the most frequently used endpoint for those performing microplate assays, with about half using a fluorometric readout and half using a visual readout (Franzblau et al., 2012).

2.4.4 Immunoassays

These tests measure the immunological response of the host to specific TB proteins include the tuberculin skin test (TST) and two different assays that measure IFN-γ from the patient's lymphocytes after exposure to specific TB antigens (Horvat, 2015). The two new diagnostic assays, both termed IFN-γ release assays, differ mainly in the technique for detecting responses

to *M. tuberculosis* derived antigens this include QuantiFERON–TB Gold (Cellestis, Melbourne, Australia) uses ELISA and T-SPOT.TB while (Oxford Immunotec, Abington, UK) uses ELISPOT to detect secretion of IFN-γ released from ESAT-6 and/ or CFP-10–specific T-lymphocytes (Lalvani and Millington, 2008). Both are *in vitro* assays that use fresh blood samples, offer the advantage over TST by requiring two visits for every subject (first to have purified protein derivative injected and then to have a skilled reader measure and record the result) (Ernst et al., 2007). These two assays have now become the gold standard for identifying individuals whose immune system has previously encountered *M. tuberculosis* (Lawn and Zumla, 2011). The performance of QuantiFERON®TB Gold In-Tube (QFT-GIT, Qiagen, Germantown, USA) is as accurate as the two above-mentioned assays, despite a lack of TB7.7 antigen and decrease in quantitative values for individuals with intact immunity (Yi et al, 2016).

2.4.5 Molecular

Molecular diagnosis using a nucleic acid amplification test (NAAT) for TB is rapid, highly specific, and can predict drug resistance from clinical specimens. Generally, semi-automated non-integrated NAAT uses PCR to amplify and detect mycobacterial rRNA or DNA directly from any clinical sample (blood, sputum, bone marrow, tissue, etc.) but have not been widely used due to the expensive resource setting (Cheon et al., 2016). Commercial tests perform better than in-house developed tests, and some have been approved by the US Food and Drug Administration (FDA) of the USA and the WHO for routine diagnosis of TB, include the Cobas TaqMan MTB test, Amplified Mycobacterium Tuberculosis Direct test, BDProbeTec ET *M. tuberculosis* complex direct detection assay, and GeneXpert MTB/RIF (Xpert MTB/RIF) assay (Cepheid, Sunnydale, CA)(Ahmad and Mokaddas, 2014). Line probe assays (LPA) hybridize amplified DNA product to immobilized oligonucleotide probes fixed to a nitrocellulose strip. The binding of the target probes correlating with select gene mutations can be determined by the appearance of visible colored bands on the strips (Wilson and Tsukayama, 2016). The LPAs brought the first significant break-through, detecting RIF and INH resistance within 24–48 hours. Finally, a rapid, high throughput technology to detect MDR-TB was available, albeit suitable at reference laboratory level, and in smear-positive specimens only (Weyer et al., 2011). The Genotype® MTBDR*sl* used in combination with the GenoType® MTBDR*plus* assay, has the potential to provide a means of rapid detection of XDR-TB. These and similar molecular assays reduce the time to diagnosis of MDR and XDR-TB from weeks or months

to a matter of days (Lawn and Zumla, 2011). The Genotype® MTBDR*sl* version 1 contains 22 probes, including 16 probes for gene mutation detection and 6 probes (includes conjugate, amplification, a *M. tuberculosis* complex and locus probes (*gyrA*, *rrs* and *embB*) controls for test procedure. The remaining probes detect fluoroquinolones (FLQ) resistance (*gyrA*), second-line injectable drugs (SLIDs) resistance (*rrs*) and ethambutol (EMB) resistance (*embB*). The probes contained in the assay do not detect all mutations in these genes but are targeted to the most commonly occurring mutations (Kiet et al. 2010). Conventional DNA sequencing is a non-commercial assay for detection of mutations associated with *M. tuberculosis* resistance is not routinely available in the commercial setting due to expense, necessary expertise, and time-consuming nature, it is available in some research and public health laboratories (Kalokhe et al., 2011). Conventional DNA sequencing utilizes a "chain-termination method" to sequence DNA fragments and remains the gold-standard of DNA sequencing, is highly accurate, and offers the advantage of being able to read larger amounts of DNA (Kalokhe et al., 2011). Pyrosequencing shows similarity to DNA sequencing and can be used as an effective alternative to the Genotype® MTBDR*sl* assay in reference clinical laboratories because it identifies any mutation in the observed region and can be utilized for other analyses requiring short sequencing reads (Kotsevaya et al., 2011).

The development of Xpert MTB/RIF has, however, set the stage for moving from widely outdated to 21st century TB diagnostics (Weyer et al., 2011). The WGS of clinical drug-resistant strains of *M. tuberculosis* should be combined with the analysis of clinical information from patients infected by these strains to yield valuable new insights into the biology of drug resistance (Trauner et al., 2014).

2.4.6 Point-of-care

There is a great need for rapid point-of-care (POC) tests that can be readily used at all levels of the health system and in the community (Lawn and Zumla, 2011). Ideally, a POC test should also screen for drug resistance and positively impact clinical decision-making, reduce health care staff workload, improve patient outcomes, and change health economics (Weyer et al., 2011). Thus, new diagnostic tests that are cheap, rapid and sensitive and capable of working with direct clinical samples such as sputum, blood, or urine are necessary for detecting and controlling TB diseases (Cheon et al., 2016). The POC tests based on nanoscience and nanotechnology have been intensively developed in sample preparation and detection for

simple, inexpensive, sensitive, specific and rapid diagnosis of TB. Sample preparation is one of the challenging problems and is necessary for reducing false-positive signals from clinical specimens containing the target biomarker and unwanted cellular molecules. Most POC tests usually have lower sensitivity and specificity than laboratory based equivalents, and tests aimed at measuring host response have been most disappointing (Weyer et al., 2011). An alternative approach to culture is *M. tuberculosis* antigen detection, best illustrated by the use of lipoarabinomannan (LAM) in urine as a POC diagnostic test (Haas et al., 2016). The lateral flow urine LAM assay is a commercially available POC test for active TB. The test detects LAM, a lipopolysaccharide present in mycobacterial cell walls, which is released from metabolically active or degenerating bacterial cells and appears to be present only in people with active TB disease (Shah et al., 2016). High on the TB research agenda is the discovery of host and pathogen biomarkers of active TB for diagnosis, monitoring treatment, and assessing outcomes (including cure and relapse). Thus a biomarker or set of biomarkers might serve as a diagnostic test (Lawn and Zumla, 2011). The developmental pipeline of assays is summarised in **Figure 6** below.

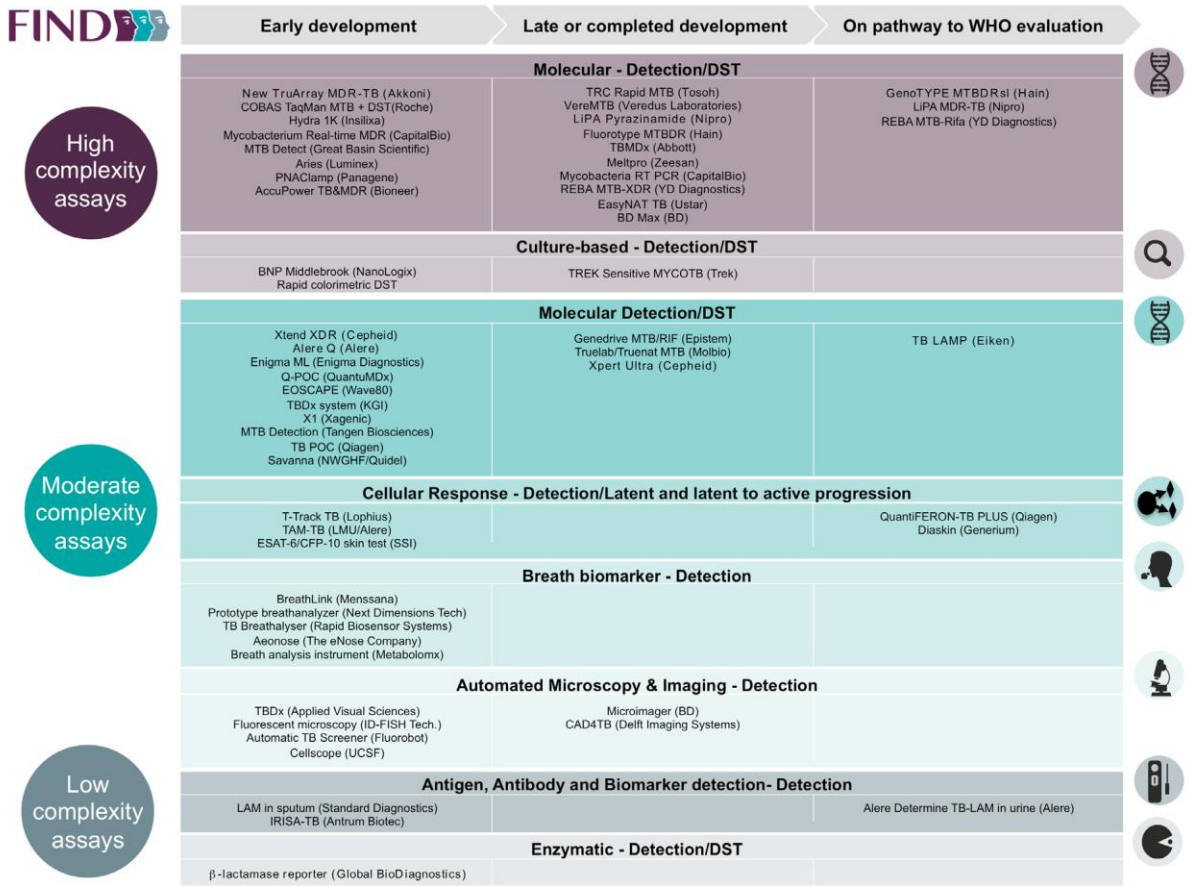

Figure 6: The tuberculosis diagnostics pipeline
(UNITAID, 2015)

2.5 TB treatment

Successful treatment (conversion of sputum from culture-positive to culture-negative within 2 months) depends on the duration and combination of drugs being prescribed, the patient's adherence to treatment, and adverse drug reactions (Ahmad and Mokaddas, 2014). The major goals of anti-TB treatment are to cure the patient by eliminating most of the bacilli, prevent the development of resistance by using a drug combination regimen (Berti et al., 2014).

2.5.1 First-line treatment

First-line drugs INH, RIF, ethambutol (EMB), and pyrazinamide (PZA) are highly effective, relatively less toxic than other anti-TB drugs, and mostly bactericidal oral agents suitable for combination (Ahmad and Mokaddas, 2014). To avoid antibiotic resistance, it is mandatory to administer combination therapy comprising RIF, INH, PZA and EMB for 2 months followed by 4 months of RIF and INH to treat active TB (Lechartier et al., 2014).

2.5.2 Second-line treatment

Most of the treatment regimens against drug-resistant-TB require the use of SLDs which are less effective and toxic. Several factors are considered when choosing the appropriate drug, including availability, rationale, the cost of the drug, and the possibility of toxic adverse events (Caminero et al., 2010).

In recent years, the treatment of drug-resistant TB has been modified from longer to shorter treatment options. Group A is composed of fluoroquinolones i.e levofloxacin, moxifloxacin, gatifloxacin), groups B include bactericidal SLDs including the aminoglycosides (STR, AMK and KAN) and CAP, while group C include other core second-line agents of ethionamide (ETO), cycloserine, linezolid and clofazimine (CFZ) (WHO, 2016a). Group D consists of pyrazinamide (PZA), EMB, bedaquiline (BDQ), delamanid, *p* aminosalicyclic acid and agents such as meropenem and thioacetazone (WHO, 2016b).

Table 1 below summarizes the classification for treatment of drug-resistant TB.

Table 1: Drugs recommended for the treatment of multidrug drug-resistant tuberculosis (WHO, 2016a)

Group	Class	Drug
A	Fluoroquinolones	Levofloxacin Moxifloxacin Gantifloxacin
B	Second-line injectable agents	Amikacin Capreomycin Kanamycin (Streptomycin)
C	Other core second-line agents	Ethionamide/prothionamide Cycloserine/terizidone Linezolid Clofazimine
D	Add on agents (not part of the core MDR-TB	**D1** Pyrazinamide Ethambutol High-dose isoniazid
		D2 Bedaquiline Delamanid
		D3 p-aminosalicyclic acid Imipinem-cilastatin Meropenem Amoxicillin-clavulanate (Thioacetazone)

Drugs used for treatment of MDR-TB are classified into four groups found in most programmatic conditions (WHO, 2016b). The FLQ drugs are classified under group A and deliver better clinical outcomes than do the drugs in the other groups (Caminero et al., 2010).

The third group of drugs are also mainstay in the treatment of MDR-TB and XDR-TB and includes the aminoglycosides and polypeptides (Caminero et al., 2010). Linezolid and CFZ classified under group C may be an important option for the treatment of XDR-TB; however, it is associated with adverse events (Anger et al., 2010; Lee et al., 2012).

2.5.3 New drugs for tuberculosis treatment

An important aim for TB drug discovery is to identify, characterize, and prioritize compounds that can eradicate *M. tuberculosis* populations that contribute to relapse in human disease. (Nathan and Barry, 2015). The goals of these resurgent efforts in TB drug discovery are primarily to: a) shorten and simplify treatment of drug-sensitive, active TB; b) improve treatment of drug-resistant (MDR-TB and XDR-TB) active TB; c) facilitate treatment of HIV-

coinfected TB patients; and d) shorten and simplify treatment of latent TB infection , both drug-sensitive and drug-resistant (Ginsberg, 2010).

Advances in new and repurposed drugs continuously update WHO guidelines for use in designing treatment regimens for MDR-TB and XDR-TB. There is an increasingly impressive portfolio of compounds being evaluated in the clinic with an aim to register them for a TB indication, and many more programs in discovery and preclinical development being pursued in both the public and private sectors, and through public-private partnerships [see also the Stop TB Partnership Working Group on New Drugs website portfolio of TB drug projects underway: www.newtbdrugs.org (**Figure 7**) (Ginsberg, 2010).

The introduction of new, potent drugs is a welcome development in drug-resistant TB management. Bedaquiline (BDQ), a diarylquinoline, was approved by United States Food and Drug Administration (FDA) in December 2012 for the treatment of MDR and XDR-TB in combination therapy with at least three other active drugs and when an effective treatment regimen cannot otherwise be composed (Wilson and Tsukayama, 2016).

Delamanid (a nitroimidazole) received accelerated approvals based on small trials showing accelerated sputum culture conversion (Wallis et al., 2016). A new and more potent ethylenediamine derivative (SQ109) is active against EMB-resistant *M. tuberculosis* strains, it is an ETO analogue and targets MmpL3, a membrane transporter involved in mycolic acid synthesis and cell wall assembly (Cole and Riccardi, 2011) (Ahmad and Mokaddas, 2014). Currently SQ109 is in phase two clinical trials and showing great promise.

Ongoing studies of rifamycins (rifapentine and RIF) and FLQ seek mainly to optimise or define their roles in drug-susceptible TB (Wallis et al., 2016). Regimens comprising entirely new drugs would be an important therapeutic advance, because they would reduce the present requirement for DST, thus simplifying patient care (Wallis et al., 2016).

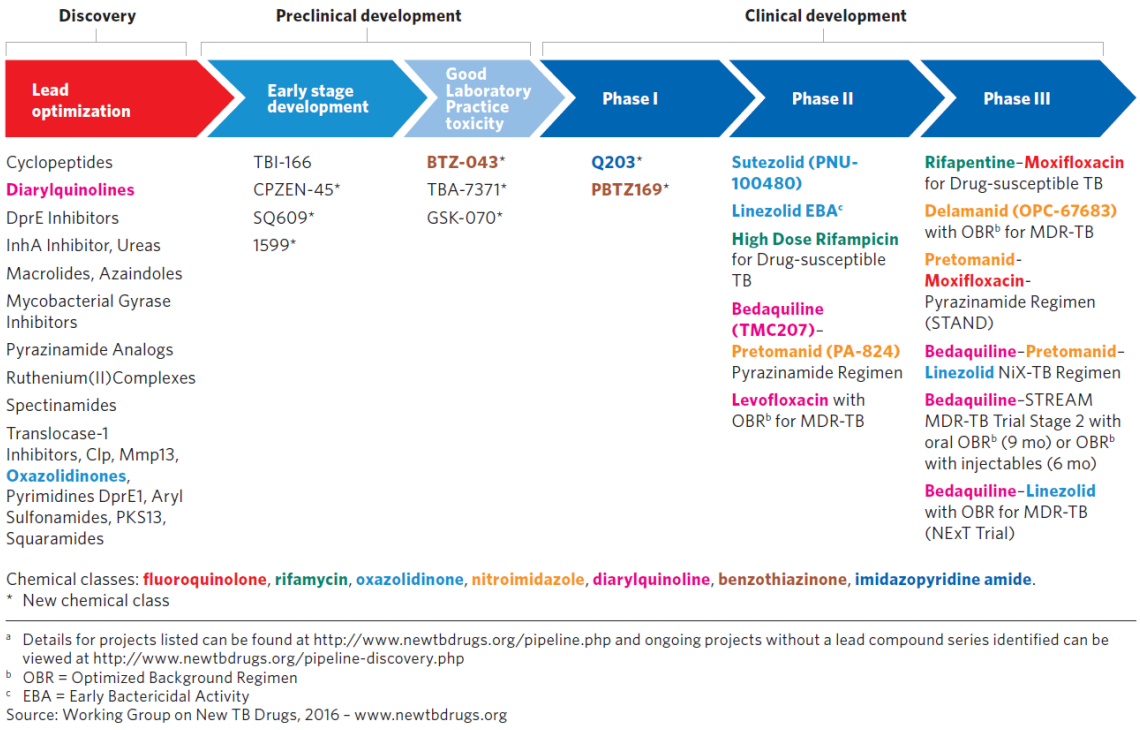

Figure 7: The tuberculosis drug development pipeline

(WHO., 2016b)

2.6 Drug-resistance mechanisms

Drug resistance is a widespread problem in all bacterial pathogens and is typically driven by a combination of low gene transfer, genome rearrangement and nucleotide mutation (Galagan, 2014). Drug resistance in *M. tuberculosis* can be acquired *de novo* in individual patients undergoing TB treatment, either because of lack of patient adherence or an interrupted drug supply or direct transmission of TB strains that are already drug-resistant (Borrell and Gagneux, 2011). The development of drug resistance arises from random spontaneous genetic alterations that propagate under selective environmental pressure and subsequently provide selective advantages for mycobacterial growth. Each mutation is drug specific however resistance to more than one drug could arise spontaneously due to upregulation of EPs that affect more than one drug (Nathan and Barry, 2015). Drug resistance in mycobacteria can either be acquired or intrinsic.

2.6.1 Acquired drug resistance

Acquired antibiotic resistance may occur in bacteria through either mutations or horizontal gene transfer mediated by phages, plasmids or transposon elements. In *M. tuberculosis*, horizontal transfer of drug resistance genes has not been reported; but resistance mostly arises from chromosomal mutations under the selective pressure of antibiotic use. Antibiotic resistance becomes predominant traits in *M. tuberculosis* populations because they bring survival advantages to the arising mutants under selective pressure. The continuous drug exposure during lengthy regimens, together with patients' non-adherence, has pushed evolution to select for resistant mutants that otherwise would never predominate the population because of their reduced fitness. This process occurring during combination therapies, has created a steady evolution of *M. tuberculosis* strains, gradually becoming resistant to most drugs. Acquired resistance is the result of new mutations in the infecting strains evolving under the selective pressure of drugs. The acquired resistance to second-line drug resistance is discussed below.

Second-line drug resistance

The main mechanism of development of FLQ resistance in *M. tuberculosis* is by chromosomal mutations in the quinolone resistance-determining region (QRDR) of *gyrA* or *gyrB* (Laurenzo, 2011). Previously only 60-70% of *M. tuberculosis* strains with FLQ resistance can only be accounted for by these mutations within QRDR (van Doorn et al., 2008). Currently novel assays detect FLQ resistance in the region of 79.2-90.4% of cultured strains (WHO, 2016b). The mutations include those found in the target mutations in position 90, 91, and 94 in the *gyrA* gene. In addition, novel mutations such as Asp89Asn and Asp89Gly substitutions have been detected in FLQ resistant strains (Devasia et al., 2012).

Aminoglycosides and cyclic peptides group referred to as injectables inhibit protein synthesis through modification of ribosomal structures at 16S rRNA and formation of 30S ribosomal subunit respectively (Liou and Tanaka, 1976). Appropriate use of the ''second-line'' injectable drugs, AMK, KAN and/or CAP is critical to the effective treatment of MDR-TB and to the prevention of XDR-TB (Georghiou et al., 2012). Point mutations of A1400G, A1401G and G1484T within 16S rRNA gene (*rrs*) are responsible for high level resistance to AMK and KAN (Suzuki et al., 1998). The KAN resistance is further caused by C14T, G37T and G10A

mutations found within the promoter region of enhanced intracellular survival (*eis*) gene (Zaunbrecher et al., 2009). While CAP resistance is mainly caused by C1402T mutation on the *rrs* gene while amino acid changes in the *tlyA* gene are also involved (Engstrom et al., 2011). However, only 70-80% of global *M. tuberculosis* strains harbour *rrs*, *eis* and *tlyA* mutations (Georghiou et al., 2012). The remaining resistance cannot be explained on the basis of these target site mutations and this can be explained by other mechanisms.

2.6.2 Intrinsic drug resistance

Intrinsic resistance refers to efflux of antibiotics or toxic substances by induced expression of EPs coded by bacterial genes or plasmids (Song and Wu, 2016). The cell structure and physiology of *M. tuberculosis* make it intrinsically resistant to many antibiotics commonly used to treat infections.

Intrinsic resistance is dependent on the unique mycobacterial cell envelope acting in combination with other systems thus limits the rate of antibiotic penetration into the cytoplasm (Burian et al., 2012). These intrinsic resistance mechanisms provide the TB bacillus with a high background of drug resistance, that can limit the use of existing antibiotics and also makes the development of new drugs challenging. Intrinsic resistance to several structural classes of antibiotics (macrolides, lincosamides, tetracyclines and aminoglycosides) is dependent on *whiB7*, encoding a putative redox sensitive transcriptional regulator (Burian et al., 2012). The regulon of *whiB7* includes *eis* (Rv2416c), *tap* (Rv1258c), *ermMT* (Rv1988) genes, as well as other genes with functions seemingly unrelated to antibiotic resistance (Burian et al., 2012).

2.7 Drug resistance in *Mycobacterium tuberculosis*

Drug resistance in *M. tuberculosis*, as in any other bacterium, is an outcome of multiple mechanisms operating simultaneously (Balganesh et al., 2012). Changes within cell permeability, mutations within target genes and drug efflux contribute to drug resistance in *M. tuberculosis* (Drlica and Malik, 2003). Mutations in target genes are the major contributors, but when in combination with EP system they can make organisms hard to treat.

Bacterial EPs are membrane transporters that can extrude a broad range of potentially harmful small molecules from the bacterial cytoplasma or periplasma including drugs (Lu et al., 2014). *Mycobacterium tuberculosis* grows intracellularly in the host cell, particularly in the

macrophage and EPs due to immune or drug pressures could be activated. Once activated they decrease accumulation of antimycobacterial drugs and reduce their cytoplasmic concentration to sub-inhibitory levels (Viveiros et al., 2003). The EP systems can confer resistance to single drug or multiple classes of drugs and contribute to resistance by *M. tuberculosis* (Marquez, 2005).

2.7.1 Activation of efflux pumps and accumulation of mutations

There is substantial variability in the response to TB therapy, even in those patients infected with fully drug-sensitive strains (Wallis et al., 1999). Given the pharmacokinetic (e.g time course of drug levels in body fluids) and pharmacodynamics (e.g, rate and extent of bactericidal action) variability of drug concentrations at the site of infection, optimal microbial killing may not be achieved and resistance may ensues (Levison and Levison, 2009). It is proposed that induction of EPs which transports two or more drugs is the first step to the emergence of resistance (Machado et al., 2012). Schmalstieg *et al* proposed that the induction is not due to the immune system but is specifically in response to subtherapeutic drug stress within the bacteria (Schmalstieg et al., 2012). During treatment with INH, greater than 99 % of the initial sputum bacillary load is killed during the first 2 days of treatment, after which the rate of killing drops off markedly (Szumowski et al., 2013). The residual bacteria are a phenotypically resistant, ‘‘drug-tolerant’’ population; and their minimum inhibitory concentrations (MICs) remain unchanged throughout treatment (Szumowski et al., 2013). Empirical studies have shown that it takes months of therapy to eradicate drug-tolerant bacteria and produce a stable cure (Mitchison and Davies, 2012).

Adams *et al* proposed that EPs induced by macrophages lead to drug-tolerance, an important barrier *in vivo* to shorten TB treatment (Adams et al., 2011). Drug-tolerant bacteria originate in macrophages and their survival is dependent on the activation of bacterial EPs used to transport drugs out of the cytoplasm (Adams et al., 2011). The drug-tolerant bacteria continue to replicate under the protection of EPs and can generate chromosomal mutations associated with high level resistance (Pasipanodya and Gumbo, 2011). Once mutations within drug target genes are acquired high level drug-resistance in mycobacteria is initiated.

2.7.2 Metabolic changes and efflux pumps activation

During infection, the *M. tuberculosis* bacilli reside in different microenvironmental conditions that include lung cavities or host macrophages that are characterized by nutrient starvation, oxidative stress, and acidic pH, all of which affect their metabolic statuses (Zhang et al., 2012). Such varied conditions constitute the basis for producing heterogeneous bacterial populations, including non-replicating persisters and growing bacteria with different capacities for persister formation (Zhang et al., 2012).

While persistence *in vitro* may be induced by a heterogeneous set of clues, such as hypoxia or starvation that allow *M. tuberculosis* to grow as a pellicle, a form of biofilm (Sambandan et al., 2013). Persisters may lead to a slower metabolism and transcription rate in cells without specific drug resistant mutation (Dhar and McKinney, 2010). In the presence of drugs, the average transcription rate decreases and EP activity increases (Motta et al., 2015). Moreover persistent bacteria with slow growth rate had decreased replication rate that reduced the expression of ribosomal proteins and FLQ target genes (*gyrA* and *gyrB*) (Walter et al., 2015).

Transcription studies on *M. tuberculosis* strains that lacked mutations in drug target genes had activated drug EPs (i.e DrrA) that may promote the persistence (Chatterjee et al., 2013). During treatment of XDR-TB patient, *iniBAC* operon coded by EPs was over-expressed on the fifth month of treatment and despite lobectomy procedure the infection still persisted (Eldholm et al., 2014).

Therefore, it is reasonable to assume that reduced cell growth and over-expression of EPs could lead to high levels of resistance (Motta et al., 2015). Thus EPs mediated resistance might be an important mechanism in persistent bacteria in patients on treatment (De Rossi et al., 2006).

2.8 Organization of drug efflux pumps in *Mycobacterium tuberculosis*

Drug EPs are transporter proteins involved in the natural removal of toxic substances from the interior of the cell to the exterior environment requires energy (Kumar and Schweizer, 2005; Gupta et al., 2010b; Da Silva et al., 2011). In terms of energy requirements and structural criteria EPs are divided into primary and secondary transporters.

Bacteria EPs, including *M. tuberculosis* have been classified into five superfamilies: ATP-binding cassette (ABC), major facilitator super-family (MFS), resistance nodulation-cell division (RND), small multidrug resistance (SMR) (**Figure 8**) (**Table 2**) and the multidrug and toxic compound extrusion (MATE) family (Gupta et al., 2006; Lynch, 2006).

The efflux pump proteins of MFS, SMR, RND and MATE families uses proton motive force (H^+ or Na^+) provided by trans-membrane electrochemical gradient of proton or sodium ion to drive the extrusion of drugs from the cell (Gupta et al., 2010b).

The primary ABC superfamily uses ATP as an energy source to pump drugs out of the cell cause multidrug efflux (Li and Nikaido, 2009; Gupta et al., 2010b). Secondary transporters (i.e MFS, RND, SMR, MATE) are the most dominant while ABC transporters are mainly substrate specific (Marquez, 2005).

In *M. tuberculosis* genome, genes encoding mainly for drug efflux transporters belong to ABC, MFS and RND families. Multidrug efflux systems are present on bacterial cell walls and limit the access of antimicrobial agents to their targets. Moreover, EPs appear to be one of the most widespread antibiotic resistance mechanisms among most microorganisms (De Rossi et al., 2002).

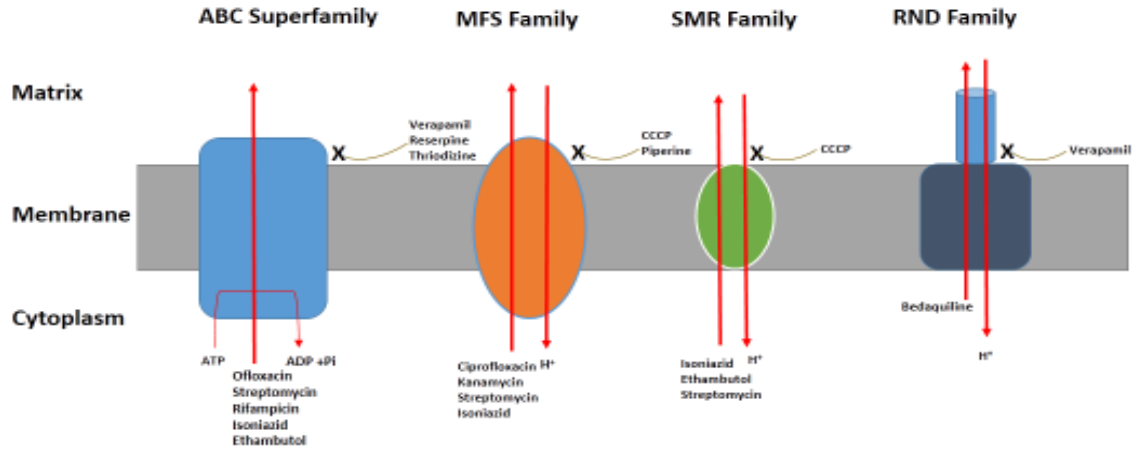

Figure 8: Efflux pump families in *Mycobacterium tuberculosis* that can be inhibited by anti-TB and various other compounds

(Adapted from Piddock., 2006)

Table 2: Efflux pump genes in *Mycobacterium tuberculosis* involved in drug resistance

Efflux Pump Family	Efflux pump Genes	Resistance to drugs	Inhibitors of Efflux pumps	Phenotypes	Reference(s)
ABC	Rv2936 (*ddrA*)			MDR-TB	(Chatterjee et al., 2013)
	Rv1687			MDR-TB	(Chatterjee et al., 2013)
	Rv1686			MDR-TB	(Chatterjee et al., 2013)
	Rv2937 (*ddrB*)	RIF INH, STR, EMB		MDR-TB	(Hao et al., 2011)
	Rv1456/57/58	FLQ		XDR-TB	(Pasca et al., 2004; De Rossi et al., 2006; Gupta et al., 2010a; Srivastava et al., 2010; Louw et al., 2011)
	Rv2686/87/88	OFX	CCCP, TDZ, VER		(Gupta et al., 2010a)
	Rv2477	OFX, STR			(Gupta et al., 2010a)
	Rv2938	STR			(Danilchanka et al., 2008)
	Rv0194	STR	RES		(Danilchanka et al., 2008; Li and Nikaido, 2009; Dinesh et al., 2013)
	Rv1217/18	OFX	VER, RES, CCCP		(Ramon-Garcia et al., 2007; Gupta et al., 2010a)
	Rv2209				

MFS	Rv1258c	STR, RIF, INH, FLQ	CCCP, Piperine, VER	MDR-TB	(Ainsa et al., 1998; De Rossi et al., 2002; Sharma et al., 2010; Louw et al., 2011; Ramon-Garcia et al., 2012)
	Rv1410c	CFZ	CCCP	MDR-TB	(Ramon-Garcia et al., 2009)
	Rv3728	INH, RIF		MDR-TB	(Jiang et al., 2008)
	Rv0783c				(Li and Nikaido, 2009; Dinesh et al., 2013)
	Rv2459	INH, EMB, STR			(Khanna et al., 2010)
	LfrA	FLQ			(da Silva et al., 2011)
	Rv2994	STR, FLQ, CIP			(De Rossi et al., 2002)
	Rv1634				(Gupta et al., 2010a; De Rossi et al., 2002))
					(De Rossi et al., 2002)
SMR	Rv3065 (*mmr*)	INH, EMB, STR			(Dutta et al., 2011; Rodrigues et al., 2012; Chatterjee et al., 2013; Dinesh et al., 2013; Andries et al., 2014)
RND	Rv0676c	BDQ	CCCP		(Andries et al., 2014)

ABC = ATP binding cassette; MFS = Major Facilitator Superfamily; RND = Resistance Nodulation Division; SMR = Small Multidrug Resistance, CIP = Ciprofloxacin; EMB = Ethambutol; INH = Isoniazid; KAN = Kanamycin; OFX = Ofloxacin; RIF = Rifampicin; STR = Streptomycin; BDQ = Bedaquiline; CFZ = Clofazimine; FLQ = Fluoroquinolone; VER = Verapamil; RES = Reserpine; TDZ = Thriodazine; CCCP = Carbon cyanide m-chlorophenylhydrazone; MDR = Multidrug-resistant; XDR = Extensively drug-resistant

2.9 Over-expression of efflux pumps causing drug resistance *in vitro*

The over-expression of EPs decreases the susceptibility of bacteria to specific drug or multiple drugs in clinical isolates (Ramon-Garcia et al., 2007; Gupta et al., 2010a). In many cases, EPs are part of an operon, with a regulatory gene controlling expression and increased expression is associated with resistance to the substrates (Webber, 2002). Over-expression or the increased activity of existing EPs in response to prolonged exposure to the sub-effective levels of anti-mycobacterial compounds, such as in the case of ineffective management of the TB patient, may render an organism increasingly resistant to one or more drugs employed in therapy (Viveiros et al., 2003). Moreover, treatment of M/XDR-TB can be more than 24 months and *M. tuberculosis* organism under toxic pressure may over-express EPs proteins leading to resistance. Thus, even in drug compliant patients, over-expression of EPs and slow metabolic processes in the case of persistent bacteria may be responsible for low level resistance to multiple drugs without the presence of drug resistant mutations with consequent treatment failure (Chatterjee et al., 2013).

The over-expression of EPs results in sub-therapeutic intracellular concentration of drugs and may cause subsequent treatment failure of two or more unrelated drugs (Viveiros et al., 2008). This was shown by simultaneous over-expression of Rv2459, Rv2728 and Rv3065 eps associated with drug-resistance to a combination of INH, EMB and STR drugs (da Silva et al., 2011). For instance, Rv1258c over-expression was shown to play a role in INH phenotypically resistant but genetically susceptible isolates (Adams et al., 2011). Transcriptome analysis of *M. bovis* revealed that disruption of the Rv1258c gene, encoding the tap protein, led to an extensive change in gene expression patterns during stationary phase, with no changes during exponential growth (Ramon-Garcia et al., 2012). It is possible that Rv1258c may play a role in the progression from low to high level resistance through elevated MICs levels (Wang et al., 2013). Most of the pumps studied, are Rv1218c and Rv3065 which belong to the ABC and SMR families respectively appear to play important roles in mediating the efflux of different antibiotics (Balganesh et al., 2012). Over-expression of ABC transporters of Rv1456c, Rv1457c and Rv1458c appeared in all the clinical isolates resistant to at least one of four first-line drugs, RIF, INH, STR and EMB (Hao et al., 2011).

Below we focus more specifically on overexpression of EPs that may lead to resistance to SLDs and therapeutic implications.

Fluoroquinolone efflux pump mediated resistance and inhibition

Over-expression of Rv1634 and Rv2686c-Rv2687c-Rv2688c EPs conferred resistance to FLQ by increasing MICs values by eight-fold when expressed in *M. smegmatis* (De Rossi et al., 2006; Louw et al., 2011). One of these putative pumps, Rv1634 decreases susceptibility to FLQs of the same class namely norfloxacin and ciprofloxacin (De Rossi et al., 2002). Several mycobacterial EPs associated with FLQ resistance have been described, including pumps of the MFS family (lfrA and Rv1258c) and ABC transporters (DrrAB, PstB and Rv2686c-2687c-2688c) (De Rossi et al., 2002). The FLQ resistant samples which lacked DNA *gyrase* mutations were found to have over-expressed EPs leading to increased MICs (Louw et al., 2011). Sparfloxacin is a more hydrophobic FLQ and strong interaction with MfpA protein coded by Rv3661 occurs, leading to intrinsic resistance (Montero et al., 2001). Using gene expression based analysis a tenfold increase was revealed in the Rv1258c transcript level in the presence of RIF and a sixfold increase in the presence of OFX drug (Siddiqi et al., 2004). Moreover, OFX stress altered the expression of two more EPs namely, Rv2477 and Rv2209 of ABC family (Gupta et al., 2010a). Apart from mutations within target genes, EPs are recognized as causes of resistance to FLQ group, however it remains to be seen if newer drugs of moxifloxacin and gatifloxacin are similarly affected. To counteract the effects of resistance by EPs, an EPI is used. Other FLQ drugs such as ciprofloxacin had MICs decreased in 30% of resistant strains that over-expressed EPs (Huang et al., 2013).

While the MIC levels of linezolid (a protein synthesis inhibitor) were slightly reduced in the presence of reserpine (Richter et al., 2007), compounds such as reserpine, verapamil (VER), carbonyl cyanide m-chlorophenylhydrazone (CCCP) and thioridazine (TDZ) have been shown to inhibit EPs overexpression of Rv2686c-Rv2687c-Rv2688c operon involved in drug resistance (De Rossi et al., 2006). The ABC transporters were inhibited by VER in the development of OFX resistance in *M. tuberculosis* isolates (Singh et al., 2011). Over-expression of Rv1634, used by the pathogen as a potential mechanism to resist drug activity is inhibited by TDZ (Dutta et al., 2011). Moreover FLQ resistant isolates without mutation in the DNA *gyrase* region which had their MICs reduced by reserpine, VER and CCCP indicating the expression of efflux mediated resistance (Sun et al., 2014).

2.10 Second-line injectables efflux pump mediated resistance and inhibition

The Rv1258c EP was shown to confer low-level resistance to aminoglycosides when expressed in *M. smegmatis* (Ainsa et al., 1998). For instance, an increase in *whiB7* (Rv3197) expression leads to upregulation of at least two different antibiotic resistance genes in the *whiB7* regulon namely *eis* and Rv1258c (Zaunbrecher et al., 2009) . Furthermore, a decrease in the MIC of KAN drug was observed in the knockout mutant of Rv3065 gene of the SMR family (Rodrigues et al., 2012). Transport proteins of the ABC and MFS families, i.e Rv2688, Rv2938, and Rv2994 have been observed to be over-expressed causing STR drug extrusion in *M. tuberculosis* (Gupta et al., 2006; Gupta et al., 2010a).

The aminoglycoside spectinomycin (a STR analog) was effluxed by Rv2333c EP when expressed in *M. bovis* (Ramon-Garcia et al., 2007). However, a new class of spectinomycin analog, spectinamide evaded Rv1258c over-expression through structural modification and restored MICs to susceptible levels by binding to 16S bacterial ribosomal subunit (Lee et al., 2014.). Over-expression of ABC transporter Rv0194 lead to an increased resistance of both *M. smegmatis* and *M. bovis* to multiple drugs including ampicillin, chloramphenicol, tetracyclin, vancomycin (VAC), erythromycin, novobiocin and STR (Danilchanka et al., 2008). The cyclic peptide, VAC was also effluxed by Rv1258c, Rv0849, Rv1218c and Rv3065 genes (Dinesh et al., 2013).

It has been noted that some isolates of *M tuberculosis* with a low-level resistance to aminoglycosides did not present with any mutations in the *rpsL*, *rrs*, and *gidB* gene sequences but show decreased MICs in the presence of an EPI (Spies et al., 2008). A knockout mutant of Rv1410c was studied and the levels of resistance to CFZ and VAC returned to the wild-type suggesting that it contributes to the intrinsic resistance to these drugs (Ramon-Garcia et al., 2009). Essentially, Rv1410c provides low level intrinsic resistance to a range of different antimicrobial compounds in *M. bovis* BCG (Zhang and Yew, 2009). The Rv1410c mutant was shown to have reduced resistance to RIF, AMK, MOX, linezolid, and rifabutin (Viale et al., 2014). Clearly, this demonstrates that Rv1410c of *M. tuberculosis* is involved in multiple resistance to many drugs.

The *p*-aminosalicyclic acid drug together with spectinomycin, was identified as specific Rv1258c substrates in *M. bovis* since a four-fold change in sensitivity was observed in resistant strains as compared to the wild-type in the presence of EPI (Ramon-Garcia et al., 2012). The spectinomycin drug is currently under developmental phase in the drug pipeline. More recently the MIC levels of recently endorsed BDQ drug were increased 4 fold together with overexpression of MmpL5 protein in resistant strain when compared to the control strain (Andries et al., 2014). Moreover, BDQ shown to inactivate EPs through the inhibition of ATP energy source (Lu et al., 2014). Cross-resistance in SLIDs is a common phenomenon and this could lead to poor patient treatment outcomes. Newer drugs such as BDQ that could inhibit EPs are crucial and should be considered in new treatment regimens.

MIC levels of aminoglycosides were reduced in the presence of CCCP, suggesting that a decrease in their extrusion was predominant (Ainsa et al., 1998). Since aminoglycosides are known to enter cells by an energy-dependent mechanism; CCCP can affect the levels of resistance to this group by decreasing both the uptake and extrusion through Rv1258c (Danilchanka et al., 2008). Furthermore, PIP, an EPI was shown to play a significant role in the inhibition of Rv1258c (Sharma et al., 2010). While VER reduces drug tolerance by inhibiting Rv1258c induced upon entry of the drug into intracellular matrix (Adams et al., 2011). On the contrary it was found that VER had an antagonistic effect when ribosome-targeting drugs such as STR, KAN, and CAP, as well as the cell wall agent cycloserine were used in macrophages (Adams et al., 2014). The Rv1258c EP is well characterized and may play a significant role in aminoglycoside resistance and the effects can be reversed by EPI.

2.11 Mutations within drug efflux pump genes

The introduction of WGS in *M. tuberculosis* genome analysis is revealing novel mutations within EPs. Earlier work has indicated that mutants causing an over-expression of EPs are a potential threat to overcoming drug resistance (Webber, 2002). For an EP to be involved as a mechanism of genetic drug resistance there should be a mutation that increases the expression of the pump and thereby reduces the drug concentration within the bacteria (Mayer and Takiff, 2014). Liu and Xie identified 20 known or putative EPs with non-synonymous mutations in MDR, pre-XDR and XDR-TB *M. tuberculosis* isolates but none in H37Rv strain (Liu and Xie, 2014). Non-synonymous mutations of M74T, R426H and I948V belonging to Rv0194, Rv0507 and Rv0676c respectively of the transporter families have been found in clinical isolates (Liu

and Xie, 2014). Detection of XDR-TB strains with non-synonymous mutations of P1098L, G198A and C213A within Rv0194, Rv1634, and Rv2688c EPs respectively in contrast to MDR-TB strains has been reported (Ilina et al., 2013). Resistant FLQ strains lacking DNA *gyrase* genetic changes showed various mutations in five EPs genes of Rv1217c, Rv0783c, Rv0849, Rv1877 and Rv2459 (Eilertson et al., 2012). Moreover, mutations were identified in transport proteins in 11% of samples that underwent WGS (Colangeli et al., 2014). Using WGS we detected number of mutations within Rv0987, Rv2039, Rv0402 in an OFX resistant isolate without *gyrA* mutation (Malinga et al., 2016).

Cross-resistance to STR and KAN due to over-expression of Rv1258c has been associated with a G133C mutation (Reeves et al., 2013). Rv3926 (*ddrA*) and Rv0849 had R262G and T403I mutations respectively in XDR-TB as compared to sensitive strains (Ioerger et al., 2009). Several EPs, including the ABC transporters of Rv0194 and Rv1463, were affected by a larger number of independent mutations in resistant strains relative to sensitive strains during WGS bioinformatic analysis (Farhat et al., 2013).

Genetic mutations in the form of insertions or deletions within Rv0678 caused MmpL5 protein over-expression (Milano et al., 2009). Other mutations within 5' untranslated region of *whiB7* that regulates Rv1258c, lead to KAN and STR cross-resistance in *M. tuberculosis* in the absence of *rrs* gene mutations (Reeves et al., 2013).

As previously stated a non-synonymous caused by single nucleotide polymorphism was observed in Rv0678 gene (A202G leading to S68G) causing an increased MIC in BDQ (Andries et al., 2014). Mutations within EPs mediating resistance over-expressed Rv1258c and treatment with inhibitors lead to reverse of resistance (Adams et al., 2011). Furthermore, drug candidate, SQ109 was inhibited by non-synonymous mutation Q40R of Rv0206c gene (MmpL) (Poce et al., 2013).

A summary of mutations in EPs is provided in **Table 3**.

Table 3: Mutations within efflux pumps gene causing drug-resistance in *Mycobacterium tuberculosis*

Efflux Pump Family	Efflux pump gene	Mutation	Drugs	Reference(s)
ABC	Rv0194	M74T, P1098L, A277V, V398M, G431R, L486M, F705I		(Liu and Xie, 2014), (Clark et al., 2013;Ilina et al., 2013;Farhat et al., 2013)
	Rv2688c	C213A		(Ilina et al., 2013)
	Rv1217c	V463C		(Eilertson, 2012)
	Rv1463	198E		(Farhat et al., 2013)
	Rv1704	93L	Fluoroquinolones	(Eilertson B 2012)
	Rv1272c	H613N		(Clark et al., 2013)
	Rv1273	G416V, C142K		(Clark et al., 2013)
	ddrA/B/C	H309N, G253A, G158A		(Clark et al., 2013)
RND	Rv0507 (MmpL2)	R426H		(Liu and Xie, 2014)
	Rv0676c (MmpL5)	I948V		(Liu and Xie, 2014)
	Rv0678 (regulator)	A202G/S68G C189A/S63R	Bedaquiline Clofazimine	(Andries et al., 2014)
	Rv0206c (MmpL3)	A644L, A677C	SQ 109 (Sequella)	(Ioerger et al., 2013)
MFS	Rv1634	G198A, T31GI47Y, G54C, V296A		(Clark et al., 2013;Ilina et al., 2013)
	Rv0849	V206G, T403C	Fluoroquinolones	(Ioerger et al., 2009)
	Rv1877	T5P	Fluoroquinolones	(Eilertson B 2012)
	Rv2549	L484T, A487P	Fluoroquinolones	(Eilertson B 2012)
	Rv2333c	69Y, I 202H, D68N, D21E		(Clark et al., 2013;Farhat et al., 2013)
	Rv1250	R278G, F549L		(Clark et al., 2013)
	Rv0783c	Q527H, G405D, R219G		(Clark et al., 2013)
	Rv2846c (EfpA)	N12K		(Clark et al., 2013)
	Rv1258c	G133C		(Reeves et al, 2013)

ABC = ATP binding cassette; MFS = Major facilitator superfamily; RND = Resistance nodulation-cell division

2.12 Concluding remarks

Drug resistance in *M. tuberculosis* is a complicated phenomenon involving both intrinsic and acquired mechanisms. Intrinsic mechanism in the form of EPs activation is regarded as the first step towards higher levels of resistance. Once drugs enter the organisms, over-expression and mutations in either EPs or target regions may lead to high level resistance. To counteract the effects of EPs activation, EPI in combination with drug regimens can be used in chemotherapy. Ideally, they will be combined with less effective SLDs to improve treatment outcomes. Clinically approved inhibitor, VER has therapeutic effects against most of the EPs that uses ATP as an energy source used in combination with BDQ it is highly effective since they both block ATP energy source. Moreover, SQ109 (Sequella) that blocks EPs currently undergoing phase II clinical trials is showing great potential. Indeed, EPs are becoming an attractive area of research in terms of drug development and diagnosis through mutations. Characterization of EPs in XDR-TB isolates will reveal new mechanisms of resistance not yet detected. This will improve our understanding of drug resistance and help us to develop new approaches in managing and eradicating the disease.

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Chapter 3: Performance evaluation of GenoType® MTBDRsl version 1 enhanced by DNA sequencing for detection of *Mycobacterium tuberculosis* second-line drug resistance in Southern Africa

3.1 Abstract

Background

Molecular detection of first and second-line drug-resistance in tuberculosis (TB) is important for early detection and treatment. Second-line drugs of ofloxacin (OFX), amikacin (AMK), kanamycin (KAN) and capreomycin (CAP) are crucial for treatment and prevention of extensively drug-resistant (XDR) TB. Molecular tests such as GenoType® MTBDR*sl* are important and should be evaluated in high burden regions of Africa. The study assessed the performance of GenoType® MTBDR*sl* version 1 (Hain Lifescience, GmbH, Nehren, Germany) against MGIT DST and used DNA sequencing (Sanger) to resolve discordance.

Methods

Six hundred and eighty-nine culture positive specimens were tested using GenoType® MTBDR*sl*. The diagnostic performance was compared to the gold standard MGIT DST method. Sanger DNA sequencing was performed on *gyrA*, *gyrB*, *rrs*, *eis*, *tlyA*, Rv1258c, Rv1634 and Rv0194. We further investigated the impact of new *rrs* gene mutations on minimum inhibitory concentrations (MIC) using microtiter plate (AlamarBlue®).

Results

A total of 632 (92.3%) of the 689 specimens included in the study had valid results for both tests. The sensitivity for detection of OFX, AMK, KAN, CAP, XDR-TB was 59.8%, 32.9%, 28.9%, 23.9% and 14.3% respectively. Sanger DNA sequencing enhanced OFX, AMK, KAN, CAP, or XDR-TB resistance detection with 72.2%, 47.1%, 62.2%, 46.7% and 53.6% respectively. Sanger sequencing significantly increased the diagnostic yield for KAN, CAP

and XDR-TB. The MIC of CAP on isolates with new G878A mutation ranged from 8 µg/ml to 64 µg/ml.

Conclusion

A combination of GenoType® MTBDR*sl* version 1 with DNA sequencing significantly improved detection of KAN, CAP and XDR-TB through detection of various mutations. Incoporation of *rrs* and *eis* mutations in new molecular assays such as GenoType® MTBDR*sl* version 2 might improve the rapid detection of resistance to second-line drugs.

3.2 Introduction

Sub-Saharan Africa has approximately 1.3 million new cases of tuberculosis (TB) and ranks among the highest in the world (Aubry et al., 2014). Treatment of TB is long and requires use of first line drugs rifampicin (RIF) and isoniazid (INH). Failure of first line treatment requires use of ineffective second-line antibiotics that are costly and can lead to more adverse side effects (Campbell et al., 2011). These drugs include several fluoroquinolone (FLQ) compounds such as ofloxacin (OFX) and the injectable drugs amikacin (AMK), capreomycin (CAP), and kanamycin (KAN). Timely detection of resistance to second-line drugs is of key importance to optimize treatment and to direct infection control measures to block transmission of multidrug resistant (MDR)-TB (Hu et al., 2013). As the number of drug-resistant cases increases each year, the need to supplement the current time-consuming, growth-based resistance detection methods with more rapid molecular drug susceptibility tests becomes more pressing (Reeves et al., 2013). Generally, detection of drug-resistant *M. tuberculosis* is performed by culture and drug susceptibility testing (DST) in liquid or on solid media (Engstrom et al., 2012). This often has a long turn-around time, requires high biosafety standards, and depends on highly trained staff.

Molecular assays are easy to perform, require less infrastructure and provide patients with timely results. The GenoType® MTBDR*sl* line probe assay (LPA) was introduced in 2010 for rapid detection of FLQ, AMK, KAN and CAP. Most of the studies were done in low burden countries and reported high sensitivity and specificity (Hillemann et al., 2009; Ajbani et al., 2012). However, larger studies in settings with a high TB burden and circulating drug-resistant TB are required to demonstrate the feasibility of the use of the assay for the routine detection of XDR-TB strains. Using LPAs may offer the further advantage of identifying specific

mutations conferring resistance which may allow the tailoring of optimized individual treatment regimens for patients infected with XDR-TB patients where effective treatment options are severely restricted (Kiet et al., 2010). To overcome delays in reporting a diagnosis, LPA tests such as GenoType® MTBDR*plus* have been endorsed by the World Health Organization (WHO) for rapid detection of MDR-TB. GenoType® MTBDR*sl* version 1 was not recommended by WHO experts for replacing phenotypic DST (Theron et al., 2014). However, it was recommended for the identification of patients harbouring potentially high-level resistant codon-94 mutants and thereby allowing for the timely adjustment of treatment options (Rigouts et al., 2016). However, larger studies in settings with a high TB burden and drug resistance are required to demonstrate the feasibility of the use of the assay for the routine detection of XDR-TB strains (Ignatyeva et al., 2012). The sensitivity and predictive value of LPA depends on the inclusion of all mutations conferring resistance, and on the analysis of the frequency at which these mutations are found in TB strains in various geographical settings (Ajbani et al., 2012; Barnard et al., 2012). However, conventional phenotypic culture-basesd DST should be used in the follow-up evaluation of patients with a negative result especially in settings with a high pre-test probability for resistance to FLQ (WHO, 2016a). More comparative diagnostic accuracy data are needed from strains from different geographic regions (for example, Eastern Europe), where resistance-causing mutations that fall outside of the genes targeted by GenoType® MTBDR*sl* are less common than in drug-resistant strains from South Africa (Theron et al., 2014). More research should include genetic sequencing as a reference standard that targets all known resistance-determining mutations and not just those detectable using GenoType® MTBDR*sl* (Theron et al., 2014). In cases where mutations are not found in phenotypically resistant isolates other drug-resistant mechanisms, such as efflux pumps (EPs), have been suggested to play a role (Huang et al., 2011; Engstrom et al., 2012). Therefore, the study of mutations in EP genes might help to better understand the different mechanisms of resistance and could improve the performance of molecular diagnosis (Hu et al., 2013). Detection of XDR-TB strains with non-synonymous mutations within Rv0194 and Rv1634 EPs in contrast to MDR-TB strains has been reported (Ilina et al., 2013). Furthermore, Rv0194 and Rv1634 have been implicated in aminoglycosides and FLQ resistance respectively (Danilchanka et al., 2008;Louw et al., 2011). Moreover, cross-resistance among second-line injectable drugs due to over-expression of Rv1258c has been associated with a G133C mutation (Reeves et al., 2013).

In this study, we evaluated the GenoType® MTBDR*sl* indirectly on culture isolates and sequenced target genes and EPs on discordant cases.

3.3 Materials and Methods

3.3.1 Bacterial cultures and specimen origin

Six hundred and eighty-nine culture isolates received by the South African Medical Research Council Supranational Reference Laboratory from regional reference laboratories within the Southern African development community during the period 2008-2012 were analysed. The isolates were confirmed to be 333 MDR-TB (48.3%), 16 RIF monoresistant (2.3%), 64 INH monoresistant (9.3%), or 86 susceptible (12.5%). Isolates with no first-line susceptibility data available, numbered 189 (27.4%). One contaminated specimen was reported.

3.3.2 Drug susceptibility testing

Second-line drug susceptibility testing (DST) was done on OFX, KAN, CAP and AMK. Drug susceptibility testing was performed on BACTEC MGIT 960 in accordance with WHO critical concentration citeria: 2µg/ml for OFX, 1 µg/ml for AMK, 5µg/ml for KAN and 2.5µg/ml for CAP. The laboratory participated in the annual proficiency programme for DST coordinated by the Mycobacteriology Unit at the institute of Tropical Medicine, Antwerp, Belgium during the study period.

For quality assurance, DST performed by technicians from our laboratory had 95% concordance with the the WHO's external quality control assurance programme (Mativandlela et al., 2013). Upon detection of XDR-TB, DST for FLQ and second-line injectable drugs (SLIDs) was repeated twice.

3.3.3 GenoType® MTBDR*sl* assay

Extraction of DNA was performed by the boiling method. Briefly, 1000 µl of a mycobacterium growth indicator tube (MGIT) culture was transferred to a 1.5ml Eppendorf tube and centrifuged at 8000 rpm and the supernatant discarded. The remaining pellet was suspended in 1X TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA) and boiling the suspension

for 5 min. The extracted DNA was analyzed and stored at -20°C for further processing. DNA amplification was performed as recommended by the manufacturer. For amplification, 35 µl of a primer-nucleotide mixture (provided with the kit), amplification buffer containing 2.5 mM $MgCl_2$, 1.25 U Hot Start *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 5 µl of the preparation of mycobacterial DNA in a final volume of 50 µl were used. The amplification protocol consisted of 15 min of denaturing at 95°C; 10 cycles comprising 30 seconds at 95°C and 120 seconds at 58°C; an additional 20 cycles comprising 25 seconds at 95°C, 40 seconds at 53°C, and 40 seconds at 70°C; and a final extension at 70°C for 8 min.

3.3.4 Polymerase chain amplification and DNA sequencing

Crude DNA was isolated from MGIT cultures by a boiling method. Briefly, 1000 µl of culture was transferred to a 1.5ml Eppendorf tube and centrifuged at 8000 rpm and the supernatant discarded. The pellet was re-suspended in 100µl of deionised water, heat killed (20 minutes), sonicated (15 minutes) and supernatant transferred to a new tube and stored at -20°C until further processing. GenoType® MTBDR*sl* was run on all specimens, as previously explained, in accordance with manufacturer instructions (Hain Lifescience, Germany) (Hillemann et al., 2009).

Discordant isolates between GenoType® MTBDR*sl* and BACTEC MGIT 960 were amplified into 10 genes by PCR using primers of *gyrA*, *gyrB*; *rrs* (500, 900, and 1400), *eis*, *tlyA*, Rv0194, Rv1258c, Rv1634 synthesized by integrated DNA technologies (**Table 4**). The 25µl of the cocktail reaction was made up of 11.5µl of Hot Start mix (Kapa Biosystems, Cape Town, South Africa), 1µl each of sense and antisense primer, 7.5 µl distilled H_2O (dH_2O), and 2µl of DNA.

The amplification protocol was performed at 95°C (15 minutes), followed by 30 cycles of 95°C (30 seconds), 60°C (30 seconds), 72°C (30 seconds), with a final step at 72°C for 5 minutes. The PCR products were purified using purification kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol, to remove unincorporated primers and nucleotides. Direct sequencing of the genes was performed at Central Analytical Facility, Stellenbosch, South Africa. These sequences were subjected to multiple sequence alignment using BioEdit software version 7.2 (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Table 4: Primer list used in the study

Primer	Sequence	Target size	Reference
gyrA	Forward: 5'ccctgcgttcgattgcaac3' Reverse: 5'cttcggtgtacctcatcgcc3'	423bp	(Campbell et al.,2011)
gyrB	Forward:5'gagttggtgcggcgtaagagc3' Reverse: 5'cggccatcaagcacgatcttg3'	115bp	(Brossier et al., 2010)
***rrs* 500**	Forward:5'gatgacggccttcgggttgt3' Reverse: 5'tctagtctgcccgtatcgcc3'	123bp	(Honore and Cole, 1994)
***rrs* 900**	Forward:5'gtagtccacgccgtaaacgg3' Reverse: 5'aggccacaagggaacgccta3'	222bp	(Honore and Cole, 1994)
***rrs* 1400**	Forward: 5'gtccgagtgttgcctcagg3' Reverse: 5'gtcaactcggaggaaggtgg3'	516bp	(Campbell et al., 2011)
eis	Forward:5'gcgtaacgtcacggcgaaattc3' Reverse: 5'gtcagctcatgcaaggtg3'	567bp	(Campbell et al., 2011)
tlyA	Forward:5'atgtcggataggccagctg3' Reverse:5'acttttttctacgcgcgccgtgc3'	555bp	(Campbell et al., 2011)
Rv0194	Forward: 5'gcgacctacttgctgatgta3' Reverse: 5'cgctggaactccagtgataa3'	700bp	This study
Rv1258c	Forward:5'cggcattcctgatcctgtt3' Reverse: 5'cgtgtggtcggtgaagtatt3'	700bp	This study
Rv1634	Forward:5'tatctgggcacctccatagt3' Reverse: 5'accgacatggtcaggtaaatc3'	700bp	This study

3.3.5 Spoligotyping

The PCR products were amplified using primers purchased from manufacturer (Ocimum Biosciences, India) and the procedure was performed as previously described (Kamerbeek et al., 1997). After amplification, hybridization was performed on denatured DNA using a 43-spacer membrane. The direct repeat region was amplified by PCR with primers derived from the direct repeat (DR) spacer sequences. The amplified PCR product was hybridized to a set of 43 immobilized oligonucleotides on the membrane. The products were detected by chemiluminescence (Amersham Biosciences) and by exposure to X-ray film (HyperfilmECL, Amersham). The spoligotypes were reported by using a binary code as previously described (Cowan et al., 2002).

3.3.6 Minimum inhibitory concentrations of second-line drugs

To determine the minimum inhibitory concentration (between 64.0 and 0.125µg/ml) levels of OFX, AMK, KAN and CAP resistant isolates of *M. tuberculosis* strains, based on conventional DST with 16 and six XDR-TB and pre-XDR-TB, respectively, a microplate AlamarBlue® assay (MABA) was performed as described elsewhere (Collins and Franzblau, 1997). Briefly, the cultures were grown to mid-log phase on 7H9 (BD Biosciences, San Jose, CA, USA) with OADC. Once an OD of 0.6 was reached, 100 µl of the culture was added to a

solution of 98µl of 7H9 OADC and 2µl of CAP, AMK and KAN drug (Sigma Aldrich). AlamarBlue® reagent (Thermo Fischer, US) and 10% v/v Tween 80 of 25 µl each were added to the wells of the microplate and further incubated for 24 hours. After one day of incubation, resistance was detected by change of a blue to pink colour. The MIC was recorded as the well without colour change at the lowest concentration. The H37Rv (ATCC 27294) was used as negative control and was susceptible to all drugs tested. Two technical and two biological replicates were grown separately and tested on different days.

3.3.7 Statistical analysis

Data generated was entered on MS Access and Epi Info. Statistical analysis performed on STATA 10 and Graphpad Prism (version 7.0; GraphPad Soſtware, USA, www.graphpad.com). Sensitivity, specificity, positive predictive values, negative predictive values were calculated. Standards for reporting diagnostic accuracy studies (STARD) reporting was used. *P*-values less than 0.05 were considered significant.

3.4 Results

3.4.1 Performance of MTBDR*sl* and DNA sequencing

GenoType® MTBDR*sl* LPA was conducted on 689 isolates. Of these, valid results were obtained for 636 (92.3%). In the other 52 isolatess, one isolate was contaminated, 45 showed invalid LPA results for OFX or SLIDs, and 7 isolates missed results for the *rrs* region. Of 636 isolates with valid results, 97 were phenotypically to OFX while 546 were susceptible. For injectable drugs, 70 and 556, 82 and 554, 92 and 544, were AMK, KAN and CAP resistant and susceptible respectively. The performance characteristics of GenoType® MTBDR*sl* against MGIT DST are summarised in (**Table 5**). GenoType® MTBDR*sl* assay detected *gyrA* mutations in 65 (67%) isolates, three (3.1%) had missing wild type probes and 29 (29.9%) had no mutations detected. Ten (14.7%) of the 68 isolates were phenotypically susceptible to OFX drug. The GenoType® MTBDR*sl* assay also detected A1401G mutation in 22 (47%) and one (10%) of the cases with AMK/KAN/CAP and AMK/KAN resistance, respectively. The sensitivity of the LPA (likelihood for detection of resistance) against MGIT DST ranged from moderate to low. For OFX, AMK, KAN, CAP and XDR-TB (OFX plus any of the injectables) the values were 59.8%, 32.9%, 28.0% and 23.9%, respectively. Specificity (accuracy of the result) values were generally very high for OFX, AMK, KAN, and XDR-TB (98.4%, 100%,

100% and 100%, respectively. A total of 102/689 (14.8%) discordant results were analysed further by DNA sequencing. Employing Genotype MTBDR*sl* and DNA sequencing in combination increased the sensitivity for detection of the second-line drugs defining XDR-TB (**Tables 6 and 7**). The detection efficiency (sensitivity and specificity) of the two technologies in combination for resistance to OFX, AMK, KAN, CAP or XDR-TB, respectively, was as follows: 72.2% and 99.8%; 47.1% and 97.0 %; 62.2% and 98.0%; 46.7% and 98.3%; and 53.6% and 100%. Compared to GenoType MTBDR*sl*, DNA sequencing significantly improved the sensitivity for detection of resistance to KAN (28.0% vs 62.2%; *P*<0.05), CAP (23.9% vs 46.7%; *P*<0.05) and XDR-TB (14.3% vs 53.6%; *P*<0.05).

Table 5: Performance characteristics of GenoType® MTBDR*sl* against culture DST for XDR-TB defining second-line drug resistance

GenoType® MTBDR*sl*	Culture DST (95%CI)									
	OFX		AMK		KAN		CAP		XDR-TB	
	R	S	R	S	R	S	R	S	R	S
R	58	10	23	0	23	0	22	1	8	0
S	39	529	47	566	59	554	70	543	48	580
Total	97	539	70	566	82	554	92	544	56	580
Sensitivity	59.8% (49.3-69.0)		32.9% (22.1-45.1)		28.0% (18.7-39.1)		23.9% (15.6-33.9)		14.3% (6.4-26.2)	
Specificity	98.4% (96.5-99.1)		100% (-)		100% (-)		99.8% (98.8-100)		100% (-)	

CI = Confidence Interval; OFX = Ofloxacin; AMK = Amikacin; KAN = Kanamycin; CAP = Capreomycin; XDR-TB = Extensively drug resistant tuberculosis

Table 6: Performance characteristics of GenoType® MTBDR*sl* and DNA sequencing combined against culture DST for XDR-TB defining second-line drug resistance

GenoType® MTBDR*sl*	Culture DST (95%CI)									
	OFX		AMK		KAN		CAP		XDR-TB	
	R	S	R	S	R	S	R	S	R	S
R	72	10	33	17	51	11	43	9	30	0
S	25	529	37	549	31	543	49	535	26	580
Total	97	539	70	566	82	544	92	544	56	580
Sensitivity	74.2% (64.3-82.6)		47.1% (35.1-59.4)		62.2% (50.8-72.7)		46.7% (36.3-57.4)		53.6% (39.7-67.0)	
Specificity	98.4% (96.5-99.1)		97% (95.1-98.2)		98.0% (96.4-99.0)		98.3% (96.8-99.2)		100% (-)	

CI = Confidence Interval; OFX = Ofloxacin; AMK = Amikacin; KAN = Kanamycin; CAP = Capreomycin; XDR-TB = Extensively drug resistant tuberculosis

Table 7: Direct comparison of the performance parameters of GenoType® MTBDR*sl* and DNA sequencing results in detection of XDR-TB defining second-line drug resistance

Parameter	OFX (95% CI)		AMK (95% CI)		KAN (95% CI)		CAP (95% CI)		XDR-TB (95% CI)	
	MTBDR*sl*	*gyrA/B*	MTBDR*sl*	*rrs*	MTBDR*sl*	*eis/rrs*	MTBDR*sl*	*rrs/tlyA*	MTBDR*sl*	Sequencing
Sensitivity	59.8% (49.3-69.6%)	74.2% (64.3-82.6%)	32.9% (22.1-45.1%)	47.1% (35.1-59.4%)	28.0% (18.7-39.1%)	62.2% (50.8-72.7%)	23.9% (15.6-33.9%)	46.7% (36.3-57.4%)	14.3% (6.4-26.2%)	53.6% (39.7-67.0%)
Specificity	98.4% (96.8-99.2%)	99.8% (98.5-99.9%)	100.0% (-)	97.0% (95.1-98.2%)	100.0% (-)	98.0% (96.4-99.0%)	99.8% (98.8-100.0%)	98.3% (96.8-99.2%)	100% (-)	100% (-)
PPV	85.3% (74.6-92.7%)	98.6% (90.6-99.7%)	100% (-)	66.0% (51.2-78.8%)	100% (-)	82.3% (70.5-90.8%)	95.7% (78.1-99.9%)	82.7% (69.7-91.8%)	100% (-)	100% (-)
NPV	93.1% (90.7-95.0%)	95.2% (93.4-97.0%)	92.3% (89.8-94.3%)	93.7% (91.3-95.5%)	90.4% (87.7-92.5%)	92.3% (91.6-96.2%)	88.6% (85.7-90.9%)	91.6% (89.0-93.7%)	92.4% (89.9-94.3%)	95.7% (93.7-97.1%)

CI = Confidence Interval; OFX = Ofloxacin; AMK = Amikacin; KAN = Kanamycin; CAP = Capreomycin; XDR-TB = Extensively drug resistant tuberculosis

3.4.2 Fluoroquinolone resistance

DNA sequencing revealed 14 mutations within *gyrA*, *gyrB*, Rv1634, genes. For OFX phenotypic resistance, 68 and 14 mutations (*n*=82) were detected by GenoType® MTBDR*sl* and DNA sequencing respectively.

Most of the mutations were D94G (43.6%) and A90V (21.8%) by GenoType® MTBDR*sl*, while sequencing revealed mutations within *gyrB* and Rv1634 regions (**Table 8**). DNA sequencing revealed eight mutations in *gyrA* regions which were missed by GenoType® MTBDR*sl*.

It is possible that mixed populations of FLQ resistant/sensitive strains were present in the set of isolates, and not for the other TB drugs. The E21Q mutation appeared in all of the samples, however is not associated with resistance. The S95T appeared in 31 (79.5%), classifying the samples into geno-group 2 and remaining 8 into geno-group 3. Of 27 samples sequenced for *gyrB*, 3 had mutations while the rest were wild type. Sequencing resolved 14/36 (38.9%) of OFX discordant samples.

Table 8: Summary of *gyrA/B* and Rv1634 mutations

Gene	Mutation detected	MTBDR*sl*	DNA Sequencing	Total
gyrA	A90V	17	2	19
	D94A	5		5
	D94A/G	2		2
	D94G	28	2	30
	D94G/A90V/D94N/Y	1		1
	D94H	4		4
	D94N/Y	3	3	6
	no WT3	3		3
	S91P	1		1
	S91P/D94G	1		1
	G88C	0	1	1
	H70R	0	1	1
	no WT3	3	0	3
gyrB	I545H	0	1	1
	D500E	0	1	1
	D554E	0	1	1
Rv1634	A26V	0	1	1
	G198R	0	1	1
Total		68	14	82

3.4.3 Second-line injectable resistance

The GenoType® MTBDR*sl* assay detected A1401G mutation in 23 cases of both AMK and KAN in 22 cases of CAP resistance. Sequencing of *rrs* (500,900 and 1400) was performed in AMK/KAN/CAP resistant isolates.

Most of the mutations were detected in the *rrs* 900 region as compared to *rrs* 500 and 1400 regions. For KAN resistance, additional *eis* sequencing revealed 13 mutations, with the majority being *eis* C14T mutation. Both *rrs* and *eis* regions were used to determine the overall KAN resistance. An additional *tlyA* region was sequenced and combined with *rrs* region to determine the overall CAP resistance. Rv0194 and Rv1258c EPs were sequenced in all these cases and their mutations appeared in SLID cross resistant cases. The mutations are summarized in **Table 9**.

Table 9: Summary of mutations of *rrs* (500, 900, 1400), *eis*, *tlyA*, Rv1258c and Rv0194 involved in amikacin, kanamycin and capreomycin

Gene	MTBDR*sl*	DNA Sequencing	Total
rrs 1400	23		26
A1401G		1	
T1238A		1	
A1219T		1	
*rrs*900	0		22
G878A		21	
G985T		1	
*rrs*500	0		6
A514C		2	
C517T		3	
eis	0		13
C12T		2	
C14T		8	
G37T		3	
tlyA	0		2
T257G		2	
Rv0194	0		2
R83G		1	
G170V		1	
Rv1258	0		1
Y177H		1	
Total	23	49	72

3.4.4 Genotypic diversity of the samples

A total of 102 discordant samples were spoligotyped to reveal *M. tuberculosis* families. Of these, 42 were XDR-TB, 9 were OFX mono-resistant, 7 were resistant to all injectables, 10 were resistant to two injectables, and 34 were resistant to only one injectable. Spoligotyping revealed genotypic diversity within this population.

The families detected were S (31), X3 (29), T1 (10), Beijing (8), X2 (1), X1 (1), EIA 1 SOM (1), Beijing (8), H3 (5) LAM 3 (2), LAM 11-ZWE (2), LAM 4 (1), Orphan (11). New or Orphan families were found namely T (1), T2 (1), S (1), and LAM (3) variants. Lineage 4 (EA, H, L, X, T, S) was the most dominant with 87 samples versus 15 for lineage 2 (Beijing, EIA). The A1401G mutation was widely distributed among all genotypes.

The new mutation of G878A was found in 16/31 of the EuroAmerican X3 genotype compared to 5/88 in the rest of the genotypes (95% confidence interval: 5.98-58.94; $P<0.0001$).

3.4.5 Genotypic mutations and minimum inhibitory concentrations

We selected 22 isolates displaying genotypic/phenotypic discordance regarding resistance to SLDs. The isolates had newer or novel mutations within *gyrA*, *rrs*, *eis* and EP (Rv1258c) genes. A total of eight *eis* (including two with *gyrA* mutations), 11 *rrs* and one each for *gyrA*, Rv1258c, and *tlyA* genes.

Five isolates displayed moderate to high MICs (8-64 µg/ml) to CAP drugs belonging to EuroAmerican X3 genotype. Three isolates had their MIC level at 32µg/ml, two were of S genotype. One isolate belonging to Beijing genotype had a moderate MIC of 8 µg/ml. The AMK/KAN cross-resistant isolates with C14T mutations showed MIC levels of 4-16 µg/ml for both drugs.

The OFX MIC were also different *gyrA* mutations. However, five isolates had their MIC at 32µg/ml and thus were independent of genotype (**Table 10**).

Table 10: Pattern of mutations and minimum inhibitory concentrations of second-line drugs with different genotypes

Strain	DST R profile	Gene	Mutations	OFX MIC	KAN MIC	AMK MIC	CAP MIC	Genotype
Second-line injectable drugs with ofloxacin								
361	AMK/KAN/CAP/OFX	*eis*	C12T	4	8	8	16	X3
429	AMK/KAN/CAP/OFX	*rrs*	G878A	2	4	8	8	Beijing
102	AMK/KAN/CAP/OFX	*eis*	C14T	0.5	16	16	64	S
S358	AMK/KAN/CAP/OFX	Rv1258c	Y177H	64	16	16	32	H
SS2238	AMK/KAN/CAP/OFX	*gyrA*	D94G	2	4	2	8	LAM
671	KAN/OFX	*eis*	G37T	8	4	ND	ND	T1
SS908	AMK/KAN/OFX	*eis, gyrA*	C14T, A90V	4	8	16	ND	X3
SS1989	AMK/KAN/OFX	*eis, gyrA*	C14T, S91P	16	16	4	ND	X3
Second-line injectable drugs								
121	AMK/KAN	*eis*	C14T	ND	8	8	ND	X3
151	AMK/KAN	*eis*	C14T	ND	4	4	ND	X3
1799	KAN	*ers*	G985T	ND	4	ND	ND	S
887	KAN	*eis*	G37T	ND	8	ND	ND	S
211	KAN	*rrs*	A1219T	ND	16	ND	ND	T1
B107	KAN	*rrs*	C517T	ND	4	ND	ND	T1
591	CAP	*rrs*	G878A	ND	ND	ND	32	S
569	CAP	*rrs*	G878A	ND	ND	ND	32	S
49	CAP	*rrs*	G878A	ND	ND	ND	32	X3
94	CAP	*rrs*	G878A	ND	ND	ND	8	X3
212	CAP	*rrs*	G878A	ND	ND	ND	32	X3
1040	CAP	*rrs*	G878A	ND	ND	ND	32	X3
33	CAP	*rrs*	G878A	ND	ND	ND	64	S
S85	CAP	*tlyA*	T257G	ND	ND	ND	8	S
H37Rv	Sensitive	none	WT	ND	2	1	1	

OFX = Ofloxacin; AMK = Amikacin; KAN = Kanamycin; CAP = Capreomycin; MIC = Minimum inhibitory concentration; R= Resistant

3.5 Discussion

The performance of GenoType® MTBDR*sl* in our study was enhanced by DNA sequencing. DNA sequencing is a robust method but cannot replace GenoType® MTBDR*sl* in low resource settings. The low sensitivity of 58% for OFX was similar to other studies (Lacoma et al., 2012; Kontsevaya et al., 2013) as with the SLIDs (Ignatyeva et al., 2012; Said et al., 2012). This is lower than the pooled sensitivity of 85.6% for FLQ group (WHO, 2016a). We found a moderate sensitivity for OFX (74.2%) but good sensitivity for KAN (62%) but poor sensitivity for CAP (46.7%), and AMK (32.9%) detection as compared to conventional method of culture DST after sequencing. DNA sequencing is more accurate in detecting second-line drug resistance while line probe assay for second-line DST still posed with some limitations (Huang et al, 2011). The detection of *gyrA* mutations in FLQ susceptible isolates could be due to over-the-counter availability and widespread short-course use as broad spectrum antibiotics for the treatment of respiratory infections, allowing resistant populations to emerge but not be fully selected (Kiet et al, 2010). Others also found moderate sensitivity for OFX (81%) but poor sensitivity for AMK (41.7%), CAP (57%), KAN (29%) and XDR-TB (41%) detection as compared to conventional methods including cultures plus DST (Ignatyeva et al., 2012). However, a large cohort of 516 patients, found high sensitivity of the GenoType® MTBDR*sl* in detecting OFX (90.7%), AMK (100%), and XDR (92.3%) and high specificity for all categories (≥98%) (Barnard et al., 2012). The low sensitivity of GenoType® MTBDR*sl* in detecting XDR-TB at 14% was also seen in an earlier Russian study (Kontsevaya et al., 2013). However, detection of XDR-TB increased with sequencing at 56% with high specificity of 100%. Currently the new version of GenoType® MTBDR*sl* with high sensitivity and specificity is recommended as the initial test for second-line rapid DST (WHO, 2016a).

The sensitivity of the test for OFX was higher than for SLIDs overall, as the overall sensitivity in respect of the latter group of drugs was affected by a low sensitivity to AMK and CAP (47.1% and 46.7% respectively). Specificity for all SLIDs was excellent, ranging from 97% to 100% for AMK/CAP. The values of PPV and NPV were lower for injectables when combined with sequencing. Other studies also showed lower PPV and NPV in SLIDs than for FLQs (Ilina et al., 2013; Kontsevaya et al., 2013). When GenoType® MTBDR*sl* was tested directly on decontaminated specimens the overall sensitivity and specificity for AMK was 100%, where the sensitivity was in the range of 70–100% and specificity was 100% (Ajbani et al., 2012). The summarized sensitivity of GenoType® MTBDR*sl* was 87.4%, 82.6%, 82%, 44.4%, for

FLQs, AMK, CAP and KAN, respectively (Feng et al., 2013). A systematic review found that, when used indirectly on culture isolates, GenoType® MTBDR*sl* had higher pooled sensitivity for detection of FLQ resistance (83.1%) than for detection of SLIDs resistance (76.9%) and XDR-TB (70.9%) (Theron et al., 2014). The sensitivity of detection of XDR-TB was 13.6%, increasing to 42.9% if KAN was excluded (Kontsevaya et al., 2013).

A high diversity of mutations causing OFX and SLIDs were detected in the study. Similar to other studies the most prevalent mutation was the D94G (GAC-GGC) exchange indicated by the omission of the WT3 band together with the appearance of the MUT3C band, followed by the A90V (GCG-GTG) exchange in the same codon and the D94A (GAC-GCC) mutation (Hillemann et al., 2009; Huang et al., 2011). Five and four cases of A90V and D94G mutation were susceptible to OFX drug. It is reported that D94A and D94N, occur in less than 1% of susceptible isolates, leaving open the possibility these were likely phenotypic DST errors (Avalos et al., 2015). Sequencing of *gyrB* region revealed five mutations of D500E, D554E, K490R and I545H. Mutations in the *gyrB* gene were also associated with FLQ resistance but at a much lower frequency (Avalos et al., 2015). Most significantly we detected two mutations within Rv1634, namely A26V and G198R in FLQ resistant isolates. Similarly, the G198R mutations in Rv1634 was detected in an XDR-TB strain from Russia (Ilina et al., 2013). About 15–18% of FLQ resistant strains with no identified mutation may possess an alternate mechanism of resistance (Louw et al., 2011). It seems unlikely that molecular diagnostics based on *gyrA* mutations alone will have global sensitivities exceeding 95%, and may suffer from geographic variability (Avalos et al., 2015). More markers are needed to achieve higher sensitivities.

From our results, the most common mutation, *rrs* A1401G, came from 23 isolates that showed cross-resistance to AMK, KAN and CAP. The mutations outside *rrs* that were not detected by GenoType® MTBDR*sl* contributed to decreased sensitivity. Other studies reported that *eis* gene promoter alone or combined with *rrs* accounted for 28.8% or 86.5% respectively, of KAN resistance (Jin et al., 2013). The inclusion of probes for the detection of mutations at the level of the *eis* promoter region in the new version of the assay, GenoType® MTBDR*sl* version 2.0, leads to a significant increase in test sensitivity for the detection of KAN resistance compared to the original version (Tagliani et al., 2015). Our results confirmed that sequencing of the *eis* region significantly increased the sensitivity of KAN resistance from 28.0% to 62.2% ($P<0.05$). An increase in the test sensitivity, negatively affected its specificity, which decreased from

99.5% in MTBDR*sl* version 1 to 91.1% (Tagliani et al., 2015). Our KAN specificity was also slightly reduced from 100% to 98.0%.

Molecular detection CAP is often low due to the sole use of *rrs* A1401G in GenoType® MTBDR*sl*. Resistance to CAP is associated with *rrs* A1401G mutation, while the *tlyA* mutations are involved in mono-resistance (Engstrom et al., 2011). However, not all CAP resistant strains contain A1401G mutation and has moderate sensitivity (van Ingen et al., 2010) (Huang et al., 2009; Georghiou et al., 2012). Recently 20 strains phenotypically resistant to SLIDs were detected as fully wild-type by the GenoType® MTBDR*sl* version 2.0 assay and 65% of the majority were resistant to CAP (Tagliani et al., 2015). In our study the combination of GenoType® MTBDR*sl* and significantly improved the detection of CAP resistance (23.9% vs 46.7%, P <0.05). Moreover, the detection of G878A mutation in the *rrs* gene may improve our efforts in in molecular detection of CAP resistance.

Cross-resistance among AMK, KAN and CAP is common and due to A1401G mutation (Maus et al., 2005;Jugheli et al., 2009). However, 20% of cross resistant isolates lack the mutation (Georghiou et al., 2012). It has been shown that mutations within *M. tuberculosis* transporter proteins lead to cross-resistance due to EP mechanisms (Jugheli et al., 2009; Engstrom et al., 2011). We detected one and two mutations in Rv1258c and Rv0194 respectively in cross resistant cases. EPs have the potential to detect cross-resistance since they extrude broad spectrum drugs (Lu et al., 2014). Moreover, future molecular diagnostics will need to include more mutations in more genes in order to accurately and sensitively detect resistance and cross-resistance to AMK, KAN and CAP for clinical decision-making purposes (Georghiou et al., 2012).

The *eis* C14T mutation causes very high levels of KAN resistance (MICs 16 to 32 µg/ml) (Gikalo et al., 2012) and this was observed in our study. The G878A mutation with MIC range of 8-64µg/ml may be a novel mutation for CAP resistance and an intrinsic resistant marker for X3 genotype (Malinga et al., 2016). Most recently, Reeves et al. also mentioned that a CAP MIC of 8 µg/ml with no mutations within *rrs* gene could be due to an unidentified mechanism (Reeves et al., 2015). In our study, isolates with the mutation A90V had MICs at or above the critical concentrations of 4.0 µg/ml for OFX, also seen in other studies (Sirgel et al., 2012; Kambli et al., 2015).

In September 2013, a World Health Organization (WHO) Guideline Development Group reviewed the evidence (11 published and 7 unpublished studies) and recommended that SL-LPA not be used as a replacement test for phenotypic drug susceptibility testing (DST) and noted, in addition, that the assay did not allow for detection of specific resistance to individual drugs within the SLID or fluoroquinolone groups (WHO, 2013).

The study had several limitations due to its retrospective nature, however. There were missing data of culture isolates submitted from regional reference laboratories. The chances of mixed strains (resistant and susceptible genetic profiles) was also possible and this could have caused low performance of GenoType® MTBDR*sl* assays.

The inclusion of probes for the detection of mutations at the level of the *eis* promoter region in the new version of the assay, GenoType® MTBDR*sl* version 2.0, leads to a significant increase in test sensitivity for the detection of KAN resistance compared to version 1 (Tagliani et al., 2015). The proportion of false-negative results is concerning and means that the test will likely only be usable in clinical practice as a "rule-in" test for drug resistance, with further DST being required in patients who have a susceptible GenoType® MTBDR*sl* result (Theron et al., 2014). Most recently, WHO endorsed the GenoType® MTBDR*sl* version 2 as the most reliable way to "rule out" resistance to SLIDs. It is also a critical prerequisite for identifying MDR-TB patients who are eligible for the newly recommended shorter regimen. Avoiding placing patients who have resistance to second-line drugs on this regimen lessen the development of XDR-TB (WHO, 2016b).

3.6 Conclusion

In conclusion, the GenoType® MTBDR*sl* version 1 assay is a useful assay for detecting resistance to second-line drugs and combined with sequencing it has the potential to have high sensitivity. Addition of more molecular probes as in the case of new GenoType® MTBDR*sl* version 2 has proved to be worthwhile, especially with the inclusion of *eis* promoter region. Therefore, we hypothesise better performance characteristic for KAN and OFX detection in GenoType MTBDR*sl* version 2. Particularly, *rrs* G878A mutation for CAP and other SLIDs may achieve greater performance of molecular assays and thus replacing culture based methods in the future.

3.7 References

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Chapter 4: Draft genome sequences of two extensively drug-resistant strains of *Mycobacterium tuberculosis*

4.1 Abstract

Background

Whole genome sequencing enables novel insights into mycobacterial genomes with unprecedented depth. The increased incidence of extensively drug-resistant tuberculosis (XDR-TB) is worrisome. Understanding drug resistance at a genomic level level will allow us to detect novel mutations affecting important functions in *Mycobacterium tuberculosis*.

Methods

Genomic DNA was extracted from two XDR-TB strains andthe whole genome of *Mycobacterium tuberculosis* sequenced on the Illumina HiSeq platform at the Broad Institute (Cambridge, MA, USA). Reads from two strains were assembled and annotated to the H37Rv genome. Functional effects of amino acid mutations were predicted using the protein variation effect analyser (PROVEAN) tool.

Results

Whole genome sequencing had a coverage of 119 and 57 respectively for RSA 114 and RSA 187. We detected a total 797 and 734 nonsynonymous changes relative to H37Rv. The non-synonymous changes were detected in drug target, efflux pumps, lipid metabolism and type VII secretion pathway genes. Three efflux pump genes of Rv0987, Rv2039c and Rv0402c were predicted to have functional effects at protein level.

Conclusion

Our study demonstrated high number of non-synonymous changes in key metabolic processes of *M. tuberculosis*. Gene mutations with predicted functional changes at a protein level have the potential to cause drug resistance through efflux pumps. Further experimental studies are needed to elucidate their roles to different drugs.

4.2 Introduction

The whole genome sequencing (WGS) of *Mycobacterium tuberculosis* (*M. tuberculosis*) H37Rv strain was first published by Stewart Cole and colleagues in 1998 (Cole et al., 1998). New information on the biology, metabolism, and evolution lead to a deeper understanding of this infectious pathogen including large protein families related to fatty acid and polyketide biosynthesis, regulation, drug efflux pump (EP) transporters, and the proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) proteins were identified (Ioerger et al., 2010).

Comparative WGS studies have the potential to identify biologically significant mutations in drug-resistant *M. tuberculosis* strains by providing an unbiased scan of the total genomic changes that accompany resistance acquisition (Motiwala et al., 2010). Application of WGS offers the power to track the evolutionary mechanisms that promote development and transmission of drug resistance in pathogen populations with unparalleled resolution (Casali et al., 2014). Few groups have reported WGS of extensively drug-resistant (XDR) tuberculosis (TB) strains (Koser et al., 2013; Kulandai et al., 2014). Such investigations have a potential to reveal powerful pathways that make XDR-TB hyper-resistant. Discordance between genotypic and phenotypic methods hampers rapid detection of XDR-TB. Mutations outside the drug target genes could account for the rest of the phenotypic resistance. Since drug efflux transporters can be overexpressed by the presence of mutations, this may be linked to their ability to cause XDR-TB. Non-synonymous mutations in ABC transporters of Rv0194 (M74T and P1098L), Rv1218c (Q243P), Rv1458c (T133A) and Rv2688 (P156T) have been found in XDR-TB strains (Kuan et al., 2015). Novel bioinformatics tools can be used to positively predict functional effect of amino acid substitutions and indels (Choi et al., 2012). A transmembrane protein, Rv2994 that gained a stop codon was predicted to be involved in drug efflux system (Coker et al., 2016). We used a combination of WGS and bioinformatics tools to detect and predict respectively novel mutations that may be involved in drug efflux. We initially selected eight XDR-TB isolates for WGS using Illumina HiSeq 2000 platform. Six isolates failed the sequencing runs and two were successful. The latter were used to gain a better understanding of the genome-wide characteristics of XDR-TB isolates.

4.3 Materials and methods

4.3.1 Strains and drug susceptibility testing

We describe the draft genome sequence of two XDR-TB clinical isolates of *M. tuberculosis* belonging to EuroAmerican S clade. The clinical isolates (RSA184 and RSA114) were isolated from patients from Swaziland and RSA114 was obtained from a 25-year-old man. Drug susceptibility testing (BACTEC MGIT) according to WHO critical concentration citeria: 2µg/ml for OFX, 1 µg/ml for AMK, 5µg/ml for KAN and 2.5µg/ml for CAP. Spoligotyping was performed as per standard protocols (Kamerbeek et al., 1997). Drug sensitivity testing of RSA184 isolate revealed resistance to streptomycin (STR), rifampicin (RIF), isoniazid (INH), ethambutol (EMB), pyrazinamide (PZA), ofloxacin (OFX), kanamycin (KAN), capreomycin (CAP), amikacin (AMK) and ethionamide (ETH). The RSA114 was resistant to INH, RIF, EMB, STR, PZA, OFX, CAP and ETH. The isolates had similar resistance profiles.

4.3.2 Genomic DNA extraction from mycobacteria

For DNA extraction the *N*-cetyl-*N*,*N*,*N*,-trimethyl-ammonium bromide (CTAB) method was used. A volume of 1.5 ml of liquid culture was centrifuged (12000 rpm, 5 min) and the pellet re-suspended in 400 µl of TE (Tris, EDTA). This was heated for 10 min at 85ºC to inactivate the bacteria and 50 µl of lysozyme 10 mg/ml were added and incubated for minimum of 1 hour (but normally overnight) at 37ºC. Subsequently, 50 µl of the proteinase K (10mg/ml) (Qiagen) and 100µl of SDS mix was added to each sample, and the suspension incubated for 30 min at 65ºC in a waterbath. After this, 100 µl of NaCl 5M and 100 µl of CTAB/NaCl pre-warmed at 65ºC were added and samples incubated for a further 10 min at 65ºC. After incubation, the mixture was added to 1000 µl of chloroform:isoamyl alcohol in a ratio of 24:1. Samples were mixed by invertion for 10 sec before centrifugation (13000 rpm, 5 min). The aqueous phase was transferred to a fresh tube containing 560 µl of cold isopropanol, and samples were precipitated at -20ºC for at least 30 min. Nucleic acids were collected by centrifugation (13000 rpm, 10 min) and the pellets washed with 1 ml of cold EtOH 70%. After centrifugation (13000, 5 min) the pellet was vacuum dried, dissolved in 40 µl of double distilled water and treated with 1 µl of RNAse 1 mg/ml for 15 min. DNA was stored at -20ºC.

4.3.3 Genome sequencing, analysis and annotation

Illumina sequencing libraries were prepared as previously described (Walker et al., 2014) and sequenced using the Illumina HiSeq platform at the Broad Institute (Cambridge, USA). Reads from RSA114 and RSA184 were assembled into draft genomes using ALLPATHS with Pilon-correction as previously described (Kurtz et al, 2004). Genes were annotated by aligning each assembly to H37Rv genome (CP003248.2) using Nucmer (Kurtz et al, 2004). For those genes not cleanly mapping to H37Rv, the protein-coding genes were predicted with Prodigal (Hyatt et al., 2010). The tRNAs were identified by tRNAscan-SE (Lowe , 1997) and rRNA genes were predicted using RNAmmer (Lagesen , 2007). Genome statistics are listed in **Table 11**. We also confirmed the experimental spoligotype predictions using a previously described computational spoligotyping approach (Cohen et al., 2015). Sequence reads were aligned to the *M. tuberculosis* H37Rv reference genome using Burrons-Wheeler Aligner (BWA) (Li et al., 2010) and Pilon was used to identify variants. The neutral and deleterious characteristics of the amino acid variants was described using protein variation effect analyser (PROVEAN) prediction software (Choi et al., 2012).

4.4 Results

4.4.1 Genomic data

We detected a total of 797 and 734 non-synonymous changes relative to H37Rv for RSA114 and RSA184, respectively. The RSA 114 and 184 genomes were sequenced at coverage of 119x and 57x respectively. The assembly of RSA114 yielded a length of 4416700 nucleotides in 45 contigs which were placed in 29 scaffolds the sequences were 4.4 Mbp for both samples. Compared to RSA184, lesser assembly size with a length of 43899272 nucleotides with higher contigs at 68 which were placed in 6 scaffolds. The average GC content for both isolates was at 65%. A total of 680 and 604 non-synonymous changes were recorded for both RSA114 and RSA184 in respect of H37Rv. SNPs distribution in the two XDR-TB strains shows sharing of 502 polymorphisms (**Figure 9**). Specific genes were identified and functionally annotated using UniProt (www.uniprot.org). Functional categories were retrieved from the Tuberculist Clusters of Orthologous Groups (COG) database (http://tuberculist.epfl.ch/). We describe each genetic change within drug target genes, EPs, lipid metabolism and secretion systems.

Table 11: Comparison of genomic data for two extensively drug resistant TB isolates

Details	RSA 114	RSA 184
Assembly Info		
Coverage	119x	57x
Assembly size	4416700nt	4389272nt
Scaffold count	29	67
Contig count	45	68
Protein coding gene count	4020	4019
rRNA	3	3
tRNA	45	45
Pseudogene	20	21
Structural overview		
Genic	3993485 nt, 90.07% cov, 65.85% GC	3971780nt, 90.14% cov, 65.83%GC
Intergenic	438702nt, 9,93% cov, 63.02%GC	432917nt, 9.86% cov, 65.89% GC
Protein coding	3991907nt, 90.03 % cov, 65.88% GC	3970202 nt,90.10% cov, 65.89% GC
Non-synomous changes	797	734

nt = nucleotide; cov = coverage; GC = Guanine + Cytosine

Figure 9: Venn diagram of single nucleotide polymorphism in two XDR-TB isolates, RSA184 and RSA114

4.4.2 Drug resistance markers

For the two XDR-TB strains the patterns of mutations conferring resistance were determined according to TB dream (Sandgren et al., 2009). Our analysis revealed known genetic markers in *gyrA, rpoB, katG, rpsL* associated with the resistant phenotype. Compensatory mutation of P1040S at *rpoC* region was detected in RSA114 (**Table 12**). There was a lack of *gyrA* mutation in RSA114 strain and other mechanisms could be responsible for resistance. The strains had different *rpoB* gene mutations of ile491P and S450L. The S450L is a common mutation that corresponding to S531L in diagnostic assays, while ile491Phe (I521P) is a rare mutation. The *ndh* mutation of V18A was detected in both strains and may cause cross-resistance between ethionamide and isoniazid. Interestingly, RSA114, which lacked known resistance-conferring *gyrA* mutations, had 14, 7, and 4 non-synonymous changes in genes encoding EPs, phthiocerol dimycocerosates (PDIM) and type VII secretion (T7S) systems, respectively.

Table 12: Drug target genes

Rv number	Gene	Drug	Function	RSA 114	RSA 184
Rv0006	*gyrA*	OFX	Information pathway	Ile245Thr	Asp94Gly
Rv0006	*gyrA*		Information pathway	Glu21Gln	Gln613Glu
Rv0006	*gyrA*		Information pathway	Gly668Asp	Asp94Gly
Rv0710	*rspL*	STR	Information pathway	K43L	K43L
Rv1854c	*ndh*	ETH/INH		V18A	V18A
Rv1908c	*katG*	INH	Virulance, detoxification and adaptation	Ser315Thr	Ser315Thr
Rv0067	*rpoB*	RIF	Information pathway	Ser450Leu	Ile491Phe
Rv0068	*rpoC*		Information pathway	P1040S	WT
MTB000019	*rrs*	AMK/KAN/CAP		A1401G	A1401G

OFX = Ofloxacin; STR = Streptomycin; ETH = Ethionamide; INH = Isoniazid; RIF = Rifampicin; AMK = Amikacin; KAN = Kanamycin; CAP = Capreomycin

4.4.3 Efflux pumps

We detected EP gene mutations within two families namely the ATP binding cassette (ABC) and the resistance nodulating-cell division (RND). Both strains had amino acid changes in eight

similar and four dissimilar genes. Of interest, RSA184 had two amino acid changes, M74T and A134L, in ABC Rv0194. The RSA114 had amino acid changes within three genes (Rv0402c, Rv0507, Rv0676) belonging to the RND family. No OFX resistant mutations were detected in the *gyrA* gene of the RSA114 strain. We checked for mutations within genes coding for EP transporters (**Table 13**). We found dissimilar mutations in EP genes Rv2688 of P156T and G141A.

Table 13: Efflux pump gene mutations

RSA114			**RSA184**		
Rv number	Family	Mutations	Rv number	Family	Mutations
ABC transporters (Similar genes)					
Rv0194	ABC	Met74Thr	Rv0194	ABC	Met74Thr
Rv1739	ABC	Gln68His	Rv1739c	ABC	Arg134Leu
Rv2688c	ABC	Pro156Thr	Rv2688c	ABC	Pro156Thr
Rv0930	ABC	Met1	Rv0930	ABC	Met1
Rv0987	ABC	Glu854Ala	Rv0987	ABC	Leu415Phe
Rv2398c	ABC	Gly141Ala	Rv2398c	ABC	Gly141Ala
Rv3041c	ABC	Arg140Cys	Rv3041c	ABC	Gln328Lys
ABC transporters (Different genes)					
Rv1683	ABC	Arg409_Gly410in	Rv2836c	ABC	Leu21Met
Rv1668c	ABC	Pro348fs	Rv0986	ABC	Leu390Pro
Rv1683	ABC	Arg409_Gly410ins	Rv1522c	RND	Ser694Arg
Rv2039c	ABC	Val131Phe	Rv2333c	ABC	Ile19Thr
RND transporters (Similar genes)					
Rv0676c	RND (MmpL5)	Ile948Val	Rv0676c	RND (mmpL5)	Ile948Val
Rv0402c	RND (MmpL1)	Gly272Arg	Rv0402c	RND (mmpL1)	Arg659del Gly272Arg
RND transporters (Different genes)					
Rv0507	RND (MmpL2)	Arg426His			

ABC = ATP binding cassette; RND = Resistance nodulating-cell division

4.4.4 Lipid metabolism

We detected amino acid changes within genes coding for lipid moieties of *ppsA, ppsC, ppsD, ppsE, masD, pks12, pks 6* and *pks 15* genes. Some genetic mutations were similar among the

isolates while some were unique. For instance, *ppsA, ppsC, ppsE, mas, pks15* and *pks6* were common among RSA114 and RSA184. The RSA114 had additional changes in *ppsD* and *pks6* while RSA 184 had changes within *pks12*. Both of the polyketide synthetase (*pks)* genes code for multifunctional enzymes which contains a B-ketoacyl synthase and acyltransferase activities. The genes *ppsA-ppsE* (Rv2931-Rv2935) and *mas* encode a type I modular polyketide synthase responsible for biosynthesis of the PDIM with PpsA-PpsC sequentially loading ketide units onto long-chain fatty acids and PpsE subsequently extending the phthiocerol further by adding a 4-methyl branch and malonyl- or methylmalonyl-CoA, respectively (Bisson et al., 2012). Changes within these genes may lead to altered cell wall structure through disruption of PDIM synthesis which may affect drug permeability (**Table 14**).

Table 14: Lipid moieties genetic changes

RSA114			**RSA184**		
Rv number	**Gene**	**Mutation**	**Rv number**	**Gene**	**Mutation**
Similar genes					
Rv2931	*ppsA*	Asp624Glu,Ala803Thr, Gln808Lys,Gln808Arg Asn809Asp,Arg877His His955Pro	Rv2931	*ppsA*	Asp624Glu,Ala803Thr,Gln808Lys,Gln808Arg Asn809Asp,Arg877His His955Pro
Rv2933	*ppsC*	Thr906_Pro907ins	Rv2933	*ppsC*	His895Pro
Rv2935	*ppsE*	Gln690Pro	Rv2935	*ppsE*	Gln690Pro
Rv2940c	*mas*	Pro398Arg Ala353Thr	Rv2940c	*mas*	Ala353Thr
Rv2947c	*pks15*	Val333Ala	Rv2947c	*pks15*	Ala1115Val Val333Ala
Rv0050	*ponA1*	Pro629_Ser630ins	Rv0050	*ponA1*	Pro629_Ser630ins
Rv0035	*fadD34*	Leu106_Leu107ins	Rv0035	*fadD34*	Leu106_Leu107ins
Different genes					
Rv2934	*ppsD*	Val295Met	Rv2048c	*pks12*	Pro3649Ala, Ala3564Thr His2147Gln
Rv0405	*pks6*	Arg1402Pro			

4.4.5 Type VII secretion systems

The T7S systems are large gene clusters that encode the dedicated ATP-dependent secretory apparatus required to export specific members of the 6-kDa early secreted antigenic target

(ESAT-6) protein family, together with their corresponding effector proteins (Stinear et al., 2008). They are divided into five genes namely ESAT-6 secretion (ESX) 1-5. We identified changes within ESX 1 genes namely EspH, EspB, EspL and EspK. Mutations were found mostly in EspH and EspB genes (**Table 15**).

Table 15: Mutational changes within secretion pathways

RSA114			**RSA184**		
Rv number	**Gene**	**Mutation**	**Rv number**	**Gene**	**Mutation**
Rv3876	EspL	Lys93Asn	Rv3867	EspH	Lys93Asn
Rv3881c	EspB	Asn16Ser	Rv3881c	EspB	Asn16Ser
Rv3876	EspL	Pro183Leu Arg254Gln	Rv3876	EspL	Pro183Leu Arg254Gln
Rv3885c		Gly253fs	Rv3879c	EspK	Val269fs

4.4.6 Mutations within functional groups

We further characterised the protein functions of mutations for sample RSA114 and RSA184 into eight groups namely virulence detoxification and adaptation (VDA), PE-PPE, insertion sequences and phages (ISP), intermediary metabolism and respiration (IMR), lipid metabolism (LM), regulatory proteins (RP), cell wall and cell processes (CP) and information pathway (IP).

The mutations in each sample were evenly matched except for groups within PE-PPE, VDA, ISP and CP and IP. The strains possessed very few mutations within the RP group and appeared to be under less evolutionary pressure. In all samples, the PE-PPE group possessed a high number (>200) of nsSNPs under high evolutionary pressure. This was confirmed by a study indicating that PE-PPE genes play important roles in virulence and invasion of immune system through antigen variation (Vordermeier et al., 2012). In *M. tuberculosis*, the 167 genes encoding for PE-PPE proteins cover about 10% of the coding capacity (Cole et al., 1998).

Three groups of isolates were distinguished, those with high numbers (>100) of nsSNPs, namely PE-PPE, CP and IMR; an intermediary group with a moderate number (>40 but ≤100) of nsSNPs, namely ISP and LM; and groups with a low number (≤40) of nsSNPs, namely VDA, IP and RP. It is noteworthy that sample RSA184 had very few nsSNPs in ISP group when compared to RSA114 (43 vs 70 nsSNPs). Furthermore, RSA 114 had lower nsSNPs in the

VDA group compared to RSA 184 (14 vs 28 nsSNPs). Similar nsSNPs between the two isolates were found in IMR, CP and IP groups (**Figure 10**).

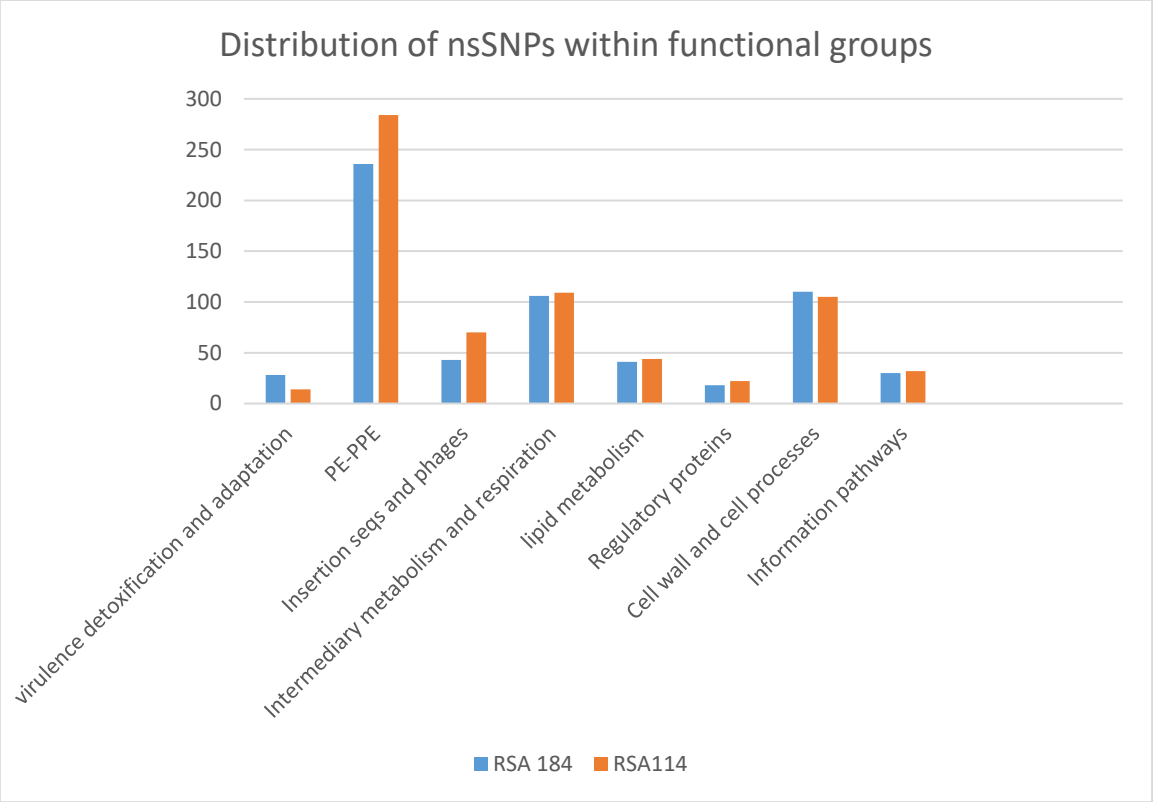

Figure 10: The frequency of amino acid changes within functional groups

4.4.7 Functional effect of amino acid changes within efflux pump genes

We determined the causal effect of amino acid changes within EP genes that could have functional importance. A PROVEAN tool was used, which provides a generalized approach to predicting the functional effects of protein sequence variations, including single or multiple amino acid changes and in-frame insertions and deletions (Choi et al., 2012). The effect of the amino acid change can either be neutral or deleterious.

In the two isolates, we identified mutations within Rv0987, Rv2039c and Rv0402c, encoding EPs having deleterious effects (**Table 16**).

Table 16: A summary of efflux pump gene mutations with neutral and deleterious effects

RSA 114			RSA 184		
Efflux pump gene	**Amino acid**	**PROVEAN**	**Efflux Pump gene**	**Amino acid**	**PROVEAN**
Rv0194	Met74Thr	Neutral	Rv0194	Met74Thr	Neutral
Rv2688c	Pro156Thr	Neutral	Rv2688c	Pro156Thr	Neutral
Rv0987	Leu415Phe	Deleterious	Rv0987	Leu415Phe	Deleterious
Rv2398c	Gly141Ala	Neutral	Rv2398c	Gly141Ala	Neutral
Rv2836c	Leu21Met	Neutral	Rv2836c	Leu21Met	Neutral
Rv1522c	Ser694Arg	Neutral	Rv1522c	Ser694Arg	Neutral
Rv2039c	Val131Phe	Deleterious	Rv2039c	Val131Phe	Deleterious
Rv2333c	Ile19Thr	Neutral	Rv2333c	Ile19Thr	Neutral
Rv0676c	Ile948Val	Neutral	Rv0676c	Ile948Val	Neutral
Rv0402c	Gly272Arg	Deleterious	Rv0402c	Arg659del	Deleterious

4.5 Discussion

The draft genomes of the two XDR-TB strains reported here provided novel insights into hyper-resistant phenotypes. The comparative analysis of the two strains belonging to the EuroAmerican S genotype revealed high diversity of mutations in most genes. Drug target genes detected are within *rpoB*, *katG*, *embB*, *gyrA*, *rpsL* and *rrs* genes. Also, a compensatory mutation of P1040S within *rpoC* was detected in one strain with a *rpoB* Ser450Leu mutation. This latter mutation was previously reported to evolve with putative compensatory mutation and may have low fitness cost (Cohen et al., 2015). The *rpoB* I491F detected in one strain is also a compensatory mutation, but may have no role in RIF resistance (Cohen et al., 2015).

Mutations within EPs are emerging as potential markers of resistance. Detection of XDR-TB strains with non-synonymous mutations of P1098L and C213A within Rv0194 and Rv2688c, respectively, in contrast to MDR-TB strains, has been reported previously (Liu et al., 2014). Similar non-synonymous mutations in ABC transporters Rv0194 (M74T and P1098L) were found in an XDR-TB case in Malaysia (Kuan et al., 2015). Mutations within Rv0987, Rv2039c and Rv0402c, encoding EPs, are predicted by PROVEAN to impact efflux activity (Choi et al., 2012). In addition, the enrichment of the pathway involved in ABC transporters approached

significance, suggesting a probably significant role of drug efflux in XDR-TB isolates (Yu et al., 2015).

Apart from ABC transporters, MmpL genes belonging to the RND family of membrane proteins might be involved in drug-resistance. Non-synonymous mutations of M74T, R426H and I948V belonging to Rv0194, Rv0507 (MmpL2) and Rv0676c (MmpL5) of the transporter families have been found in clinical isolates (Liu et al, 2014;Yu et al, 2015). The two XDR-TB strains RSA114 and RSA184 had mutations at MmpL1, MmpL2, MmpL5 genes of the RND family, which are responsible for transport of lipids such as phthiocerol dimycolates (PDIMs) and phenolic glycolipids (PGLs) to the external surface of the mycobacteria (Bailo et al., 2015). The MmpL proteins which translocate relatively large lipophilic PGLs need energy to carry the substrate specific to these proteins (Grzegorzewicz et al., 2012). In our analysis, there were genetic changes within ABC and RND transporters, suggesting that both transporters may be involved in lipid transport.

The *ppsA* and *ppsE*, *mas*, *fadD28* are also involved in PDIM/PGL synthesis, reinforcing their role in mycocerosate synthesis (Yu et al., 2012). The MmpL1 gene is colocated in the genome with *pks* genes, suggesting a role in transport of the products of the proteins encoded by these *pks* genes (Domenech et al., 2005). The co-localization of MmpL with *pks* and *fadD* in the *M. tuberculosis* genome proposes a function for these proteins in the transport of complex lipids (Domenech et al., 2005). The *pks* genes encoding the polyketide synthases are involved in the lipopolysaccharide and complex lipids biosynthesis (Kuan et al., 2015). Further investigation of the functional relationships between these families of proteins will help to determine whether *fadD*, *pks* and *mmpL* genes identified here have compensatory roles in drug-resistance. Indeed, it is likely that some drug resistance associated genes identified here, in addition to others identified in earlier reports, may have compensatory roles (Zhang et al., 2013).

We detected similar mutations within *fad34* and *mas* genes in the two XDR-TB strains. Both *fadD32* and Rv1739c (ABC transporters), are involved in cell wall biosynthesis and the appearance of mutations could indicate increased evolutionary pressures (Farhat et al., 2013). The mutations within *mas* genes are common and have been detected in two strains of H37RvLP and H37RvJO which encode mycocerosic acid synthase and are also involved in PDIM biosynthesis (Ioerger et al., 2010). Interestingly, genes involved in mycolic acid synthesis were prominently overexpressed in XDR-TB strains suggesting that the production

of mycolic acid probably increased in these strains (Yu et al., 2015). T7S systems are not only required for the export of ESAT-6 type proteins, but also for the export of enzymes involved in the synthesis of mycolic acids, PDIMs and PGLs e.g. *pks13, kasB, kasA, mmaA4, pks5, mas, pks15/1, ppsD, and ppsE*. (Mendum et al., 2015). A high density of SNPs was also found in the ESX-1 locus (RD1 region), which includes a T7S system (Liu et al., 2014). One can assume, therefore, that erroneous activity of these enzymes leads to disorders in cell wall structure and in its permeability for anti-TB agents (Ilina et al., 2013).

Although many of the newly identified drug resistance–associated genes and IGRs are poorly characterized, some, such as representatives of the *fadD*, *pks* and MmpL families (*fadD14, fadD30, pks2, pks8, pks15, pks17* and MmpL1), are of notable interest (Zhang et al., 2013). Interestingly, we identified *ppsA* as a target of independent mutation in strains resistant to RIF which had nonsynonymous mutations in *rpoB*; in addition to other drugs, raising the possibility that rifampicin resistance–causing *rpoB* mutations may lead to alterations in cell wall metabolism, possibly because of altered transcription (Zhang et al., 2013). There seems to be a connection between the lipid forming genes, EPs and secretions system. Thus, such systems may exhibit compensatory roles in combination with drug resistant genes that makes the *M. tuberculosis* organism highly tolerant to drug treatment. Moreover, drug-resistance is caused by a series of systems working together, thus the appearance of mutations in drug target genes is accompanied by alterations or modifications in other systems.

Our study is hampered by the small number of isolates used for the WGS analysis. There was also a lack of genetic diversity as the isolates only belonged to EuroAmerican S clade. Further prediction models were used on EP mutations and our results should be interpreted with caution.

4.6 Conclusion

In this study, we provided draft genomes for two XDR-TB strains belonging to EuroAmerican S genotype. We detected both known and unknown genetic mutations within drug targets, EPs, lipid metabolism and secretion pathways which may impact on resistance. This study further increases our knowledge of the *M. tuberculosis* drug resistance mechanism. The amino acid variants detected in this study might play important roles in diagnostic and drug development processes.

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Chapter 5: RNA sequencing and inhibition of efflux pump genes involved in second-line drug resistance in *Mycobacterium tuberculosis*[1]

5.1 Abstract

Background: The emergence of drug resistance in extensively drug resistant tuberculosis (XDR-TB) is a major threat in the control and ultimate elimination of the disease. Our understanding of drug resistant mechanisms is mostly limited to target mutations. Other mechanisms are outside drug target regions such as efflux could contribute to sustained resistance. We used RNA sequencing and pathways analysis provide novel insights into drug resistance. Further testing with efflux pump inhibitors (EPIs) was done on second-line drugs to determine effect on minimum inhibitory concentration (MIC).

Methods and Results: We used 11 XDR-TB, six MDR-TB and two susceptible strains to determined unique RNA genes belonging to efflux pumps, lipid metabolism in the XDR-TB subgroup. Further analysis on four XDR-TB stains showed significant (P<0.05) of differentially expressed efflux pump (EP) genes compared to H37Rv. The Rv2686/87/88 operon was significantly expressed in strains with *gyrA* mutations. Comparison of RNA sequencing and MIC results provided insights into the role of efflux pump inhibitors on overexpressed genes. EPIs of piperine and verapamil had different effects on each drug

[1] *PLEASE NOTE: This chapter has been prepared in the form of a manuscript for publication in the journal BioMed Central Medicine; therefore, the applicable editorial style has been adopted in preparing the narrative and list of references below. However, to preserve consistency in the Table of Contents, numbering of headings, tables and figures follows the overall style and format used for the rest of this thesis.*

tested. The protein-protein interaction network revealed novel pathways of EP genes with type VII secretion pathways.

Conclusions: The study identified unique genes that are overexpressed in the XDR-TB strains. The different genes/proteins/pathways identified in the study could provide novel insights into drug resistance. Knowledge into pathways of overexpressed genes in drug resistance could impact positively on further research and development.

Keywords: *M. tuberculosis*, second-line drug-resistance, efflux pump genes, minimum inhibitory concentration

5.2 Background

Drug resistance in *Mycobacterium tuberculosis (M. tuberculosis)*, as in any other bacterium, is an outcome of multiple mechanisms operating simultaneously [1]. Drug target mutations are the main causes of resistance, however efflux pumps (EPs) appear to be involved in the process [2]. EPs are membrane proteins that export substrates from bacterial and eukaryotic cells [3]. These pumps efflux multiple substrates, including antibiotics, from within the cell to the outer environment [4]. Efflux-mediated drug resistance in *M. tuberculosis* could be due to one or more EPs working alone or in coordination [1].

Recent evidence suggests that EPs could be an important mechanism in drug-resistance within patients [5]. RNA sequencing of isolates grown in the absence of drug challenge revealed that the efflux-associated *iniBAC* operon was up-regulated over time [5]. Efflux genes including the *Tap* protein coded for by Rv1258c, bacA (Rv1819c), and mmr (Rv3065) were induced 3-fold during days 7–14 in patients on treatment [6]. Up regulation of drug EPs, ABC transporters, transmembrane proteins and stress response transcriptional factors (whiB) was observed in the MDR isolates [7].

Few studies utilized RNA sequencing of *M. tuberculosis* isolates to investigate the novel events that lead to drug resistance such as EPs and tolerance [5, 8]. Genetic

expression of EP genes has always been studied under drug stress [9, 10]. However, few researchers have studied efflux gene expression without inducing drug stress [5, 11]. The differences in expression levels between drug-resistant and susceptible controls could suggest *M. tuberculosis* EP genes may be a new method to diagnose resistant TB [12]. Few studies have reported EPs expression on isolates resistant to second-line drugs [9, 13]. The expression levels of 11 genes (*efpA*, Rv0849, Rv1250, P55 (Rv1410c), Rv1634, Rv2994, stp, Rv2459, *pstB*, *drrA* and *drrB*) were significantly higher (P<0.05) in nine MDR isolates than in 10 pan-sensitive isolates [14]. RNA sequencing to identify upregulation of efflux genes that contribute to persister formation established that cells with upregulated efflux genes showed elevated persistence and can cause bacterial drug tolerance [15].

The role of EPs in promoting drug tolerance opens up a potentially powerful approach for shortening TB treatment. The use of efflux pump inhibitors (EPIs) would target not only bacterial growth, but also drug tolerance [16]. The possible ways of blocking the effects of efflux of an antimicrobial compound by the use of EPI compounds have been described elsewhere [13]. Verapamil (VER) and piperine (PIP) are common EPIs and were shown to inhibit Rv1258c overexpressed in a drug resistant strain [17, 18]. Reduced MICs of ofloxacin (OFX) were observed in the presence of VER suggesting the importance of EPs in OFX resistance in *M. tuberculosis* [1]. Thus, using EPI can be an alternative strategy to improving the efficacy of less potent second-line drugs.

The functional associations of *M. tuberculosis* efflux pump proteins on the STRING database (http://string.embl.de/) could provide novel insights. The STRING database predicts protein-protein interactions based upon physical and functional associations, such as available high-throughput data, co-expression, genomic context, and text mining of available literature using a medium to confidence value to define protein-protein interactions [19]. The aims of our study were (i) to determine transcriptomic differences between XDR, MDR and susceptible groups; (ii) to quantify the level of EP gene expressions and compare these to efflux pump inhibition in the

presence of EPIs; and (iii) to determine protein-protein interactions of overexpressed EP genes.

5.3 Methods

5.3.2 Strains phenotype and genotype

A total of 18 clinical strains made up of 11 XDR-TB, five MDR-TB and two susceptible. Among the XDR-TB isolates, four clinical strains were further characterized by molecular and phenotypic methods. Four sublineages (belonging to EuroAmerican lineage S, X and LAM genotype). The isolates were phenotypically resistant to OFX and injectables drugs and were selected based on level of resistance to second-line drugs. GenoType® MTBDR*sl* (Hain Lifescience GmbH, Nehren, Germany) was performed to screen for common mutations in *gyrA* and *rrs* mutations were screened as per the manufacturer's instructions. The discordant isolates detected by GenoType® MTBDR*sl* were further analysed by DNA sequencing for *rrs* and *eis* genes. Spoligotyping of the isolates was performed as explained elsewhere to reveal the genotypes according to manufacturer instructions [20].

5.3.3 Strain growth

The strains were grown on Lowenstein-Jensen (LJ) solid medium for 3-4 weeks. Colonies were scraped from the LJ slants and incubated in 50ml Middlebrook 7H9 (BD Biosciences, San Jose, CA, USA) medium (Becton Dickinson, Sparks, MD 21152, USA) supplemented with 0.2% (v/v) glycerol 10%, oleic albumin-dextrose-catalase (OADC) in a 250 duran bottle. The duran was placed on a magnetic stirrer for two weeks in a 37 °C until it reached an OD of 0.6-0.8 nm. The cultures were inspected for contamination by Ziehl-Neelsen gram staining and cultured on blood agar plates. Once the culture was clear of contamination, a 50% glycerol stock was prepared and stored at -80 °C.

5.3.4 Minimum inhibitory concentrations

To determine the MIC levels of OFX, AMK, KAN, CAP resistant cases of *M. tuberculosis* strains based on conventional DST, a microplate AlamarBlue® assay (MABA) was performed as described previously [17]. Briefly, the cultures were grown on to mid-log phase on 7H9 (BD Biosciences, San Jose, CA, USA) supplemented with OADC. Once an OD of 0.6 was reached, 100 µl of the culture was added to a solution of 98 µl of 7H9 OADC and 2 µl of AMK, KAN or CAP. The AMK, KAN and CAP (Sigma Aldrich) drug concentrations ranged from 0.125 to 64 µg/ml. ForEPIs, 2 µl of 50 µM of VER and PIP were added to each drug. AlamarBlue® reagent (Thermo Fischer, US) and 10% v/v Tween 80 of 25 µl each were added to the wells of the microplate and further incubated for 24 hours. After one day of incubation, resistance was detected by change of a blue to pink colour. The MIC was recorded as the well without colour change at the lowest concentration. The H37Rv was used as positive control and was susceptible to all drugs tested. Two technical and biological replicates were grown separately and tested on different days.

5.3.5 RNA preparation and sequencing

The 1 ml stock cultures were thawed on ice and inoculated in a 50 ml 7H9 (BD Biosciences, San Jose, CA, USA) supplemented with OADC (BD, sparks). After incubation 3-4 week's incubation, the 50 ml culture was measured on spectrophotometer for an OD of 0.6-1 nm. Thereafter poured in a 50 ml falcon tube (Scientific group, Randburg, South Africa)) and centrifuged for 3000 rpm for 15 minutes. The supernatant was discarded and 1ml of TRIzol (Life Technologies, Gaithersburg, MD) added. RNA was isolated using the crude method and purified using RNeasy kit (Qiagen). Following extraction, the RNA samples were depleted of ribosomal RNA, and subsequently used for sequencing library preparation for whole transcriptome analysis using TruSeq RNA sample preparation v2 kit (Illumina Inc. San Diego, CA). The size of the selected libraries was quantified by

PicoGreen assay (Life Technologies, Carlsbad, CA) and visualized with an Agilent Bioanalyzer using a DNA 1000 kit (Agilent Technologies, Waldbronn, Germany). This was followed by a cluster generation step in an Illumina cBOT TM instrument following the manufacturer's protocol using TruSeqTM SR Cluster Kits v3 (Illumina Inc.; San Diego, CA). Finally, six indexed libraries were loaded into each flowcell lane and sequencing (75 bp single end) was performed on an Illumina HiSeqH2000 instrument (Illumina, Inc.) as per the manufacturer's protocol. Multiplexed single-read runs were carried out with a total of 57 cycles per run (including 7 cycles for the index se quences). Illumina HCS 2.0.12/RTA 1.17.21.3/SAV 1.8.20 Software was used for real-time image processing and base calling. Library preparation and sequencing were performed by the University of Texas Southwestern Genomics and Microarray Core Centre.

5.3.6 RNA sequencing data analysis

The CLC Genomic workbench (v8) was used for alignment of sequencing reads. The datawas analyzed for the quality control of the reads, and alignment of reads was made to *M. tuberculosis* reference genome NC_000962.The data was normalized and statistical test was performed to find the significantly differentially expressed genes (DEG) with statistically significant *P*-value (≤0.05) with Bonferroni post-test correction. We defined DEG as ≥1.0-fold change with a significant *P*-value ≤ 0.05. Analyses were performed using CLC Genomics workbench 8.0.

5.3.7 Protein-protein interaction network

We analysed EP genes that are connected to each other using STRING ver.10. The network is made up of nodes that are connected to each gene or protein to determine their level of association.

5.4 Results

5.4.1 Transcriptomic comparison of XDR to MDR and to susceptible TB strains

To identify genes overexpressed in XDR strains, we performed transcriptomic comparison in relation to MDR strains and susceptible strains. Pairwise comparisons of reads per kb per million mapped reads (RPKM) between the three groups were performed in order to identify overexpression categories: (i) Genes upregulated in XDR relative to MDR and susceptible TB strains; and (ii) Genes unique to both XDR and MDR TB strains, relative to the susceptible group. Using a 1.5-fold overexpression, we identified 1257 (30.7%) genes unique to XDR-TB and 577 (14.1%) genes unique to MDR-TB strains (**Figure 11**). A further 1887 (46.1%) genes were common among all strains (**Table 17**). Among the genes unique to XDR-TB, genes involved in efflux, lipid metabolism and non-coding RNA were identified with a 1.5-fold overexpression (**Table 18**).

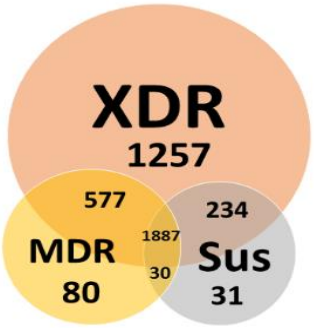

Figure 11: Comparison of unique and shared gene overexpression categories from three sample sets of extensively drug resistant (XDR), multidrug resistant (MDR) and susceptible (Sus) *M. tuberculosis* strains

Table 17: Number of differentially genes expressed per group of strains

Group	Frequency (*n*)	Percent (%)	Cumulative percent (%)
XDR	1257	30.7	30.7
MDR	80	2.0	32.7
Susceptible	31	0.8	33.4
XDR and MDR	577	14.1	47.5
XDR and susceptible	234	5.7	53.2
MDR and susceptible	30	0.7	53.9
XDR, MDR and susceptible	1887	46.1	100

MDR=multidrug-resistant; XDR=extensively drug-resistant

Table 18: List of genes that are unique to the XDR tuberculosis group

Function	Functional category	Gene
Drug efflux	Conserved hypothetical	Rv0607
	Cell wall and cell processes	Rv1410c
	Cell wall and cell processes	Rv3197
	Cell wall and cell processes	Rv1877
	Cell wall and cell processes	Rv2459
	Cell wall and cell processes	Rv2944
Lipid metabolism	Cell wall and cell processes	lprQ
	Regulatory proteins	PhoP
	Cell wall and cell processes	PstB
	Cell wall and cell processes	rpfA
	Cell wall and cell processes	rpfD
	Cell wall and cell processes	Rv2994
	Cell wall and cell processes	Rv3876
Non-coding RNA	Conserved hypothetical	Rv1989
	Intermediary metabolism and respiration	Rv1990
	Regulatory proteins	Rv2027c
	Virulence, detoxification and adaptation	Rv3134c

5.4.2 Phenotypic and genotypic strain characteristics

Four XDR-TB strains used in the study had mutations in either *gyrA* or *eis* genes or both (**Table 19**). All strains belonged to lineage 4 and two were assigned to Euro-American S spoligotype. The MIC readings ranged from 2-16 µg/ml, depending on the drug.

Table 19: Phenotypic and genotypic characteristics of strains

Strain	Mutation	CAP (µg/ml)	AMK (µg/ml)	KAN (µg/ml)	OFX (µg/ml)	Spoligotype
SS908	A90V, C14T	ND	2	16	4	Euro-American X
SS1989	S91P, C14T	ND	4	16	16	Euro-American S
2238	D94G	8	2	4	2	Latin American Mediterrian
361	C12T	ND	4	4	4	Euro-American S

CAP = Capreomycin; AMK = Amikacin; KAN = Kanamycin; OFX = Ofloxacin; ND = Not done

5.4.3 Minimum inhibitory concentrations of second-line drugs in the presence of efflux pump inhibitors

The MICs of the antibiotics AMK, KAN, CAP and OFX, individually or in the presence of EPI of PIP and VER, are presented in **Table 20** for each strain enrolled in the study.

All the drugs tested in H37Rv, except for OFX were reduced in the presence of an EPI compound. Two of the strains (SS908 and SS1989) with high level KAN resistance (16 µg/ ml) were not affected EPIs and had *eis* C14T mutations. The AMK resistance was affected by PIP EPI compound. Only one OFX resistant strain (2238) with D94G mutation had its MIC reduced in the presence of PIP compound. Furthermore, one CAP resistant strain had its MIC reduced.

Table 20: MICs of second-line drugs with efflux pump inhibitors

Drug	Strain number and corresponding drug MIC (µg/ ml)				
	H37Rv	**SS908**	**SS1989**	**2238**	**361**
AMK	0.5	2	4	2	4
AMK+PIP	**0.25**	**1**	**2**	2	4
AMK+VER	**0.25**	2	4	2	4
KAN	2	16	16	4	4
KAN+PIP	2	16	16	4	4
KAN+VER	**0.25**	16	16	4	4
OFX	0.5	4	16	2	4
OFX+PIP	0.5	4	16	**0.5**	4
OFX+VER	0.5	4	16	2	4
CAP	4	ND	ND	8	ND
CAP+PIP	**≤0.25**	ND	ND	8	ND
CAP+VER	**≤0.25**	ND	ND	**4**	ND
PIP	50	>100	>100	50	>100
VER	>100	>100	>100	50	>100

AMK=Amikacin; CAP=Capreomycin; KAN=Kanamycin; OFX=Ofloxacin; PIP=Piperine; VER=Verapamil; ND=Not done. The values in bold highlight the reversal changes observed with the addition of efflux pump inhibitors.

5.4.4 RNA expression levels of efflux pump genes

The expression levels of EP genes in the four strains have been observed relative to H37Rv. In total, there were 14 genes that were differentially expressed among the strains. Three of the strains (SS1989, SS908 and 2238) had their genes upregulated (as opposed to strain SS361) and significantly expressed (P<0.05) above 1.5-fold changes. The operon Rv2686/87/88 was consistently overexpressed in these three samples (P<0.01), and Rv1686c and Rv1456c were significantly upregulated in all samples. Rv0194 and Rv1258c were also upregulated in all samples, while Rv1634 was upregulated in two samples and downregulated in the other two. When comparing the RNA levels of each EP gene for each strain with that of H37Rv, only three strains showed increased expression of EP genes. One

strain (SS361) showed a five-fold increased expression of Rv1634 EP. Both 361 and SS1989 had a one and three-fold increase respectively of Rv1258c EP. The Rv0194 gene had a one-fold increase when expressed in SS908 strain. The 2238 strain had low level expression in all genes analysed. The fold changes are summarized in **Figure 12** below.

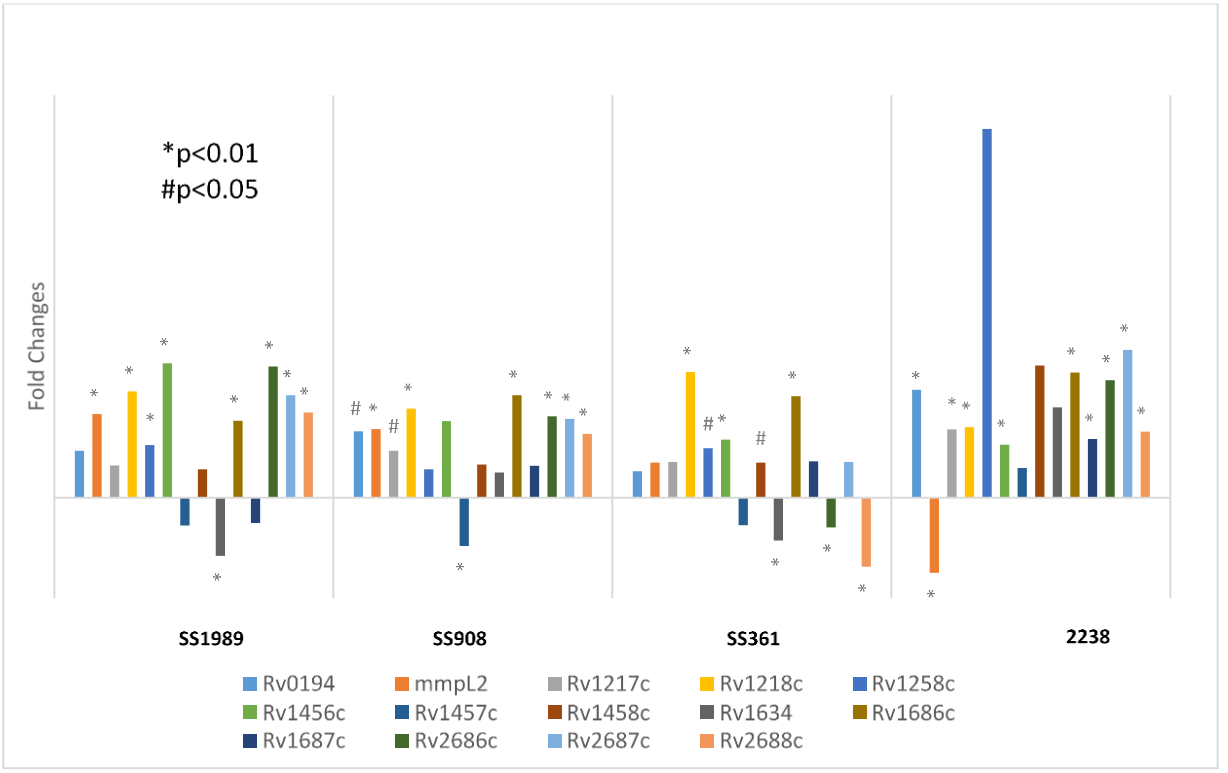

Figure 12: Efflux pump genes upregulated and downregulated in four samples with different phenotype

5.4.5 Protein–protein interactions network

A protein protein network that summarizes all known or predicted protein interactions in an organism is shown in **Figure 13**.

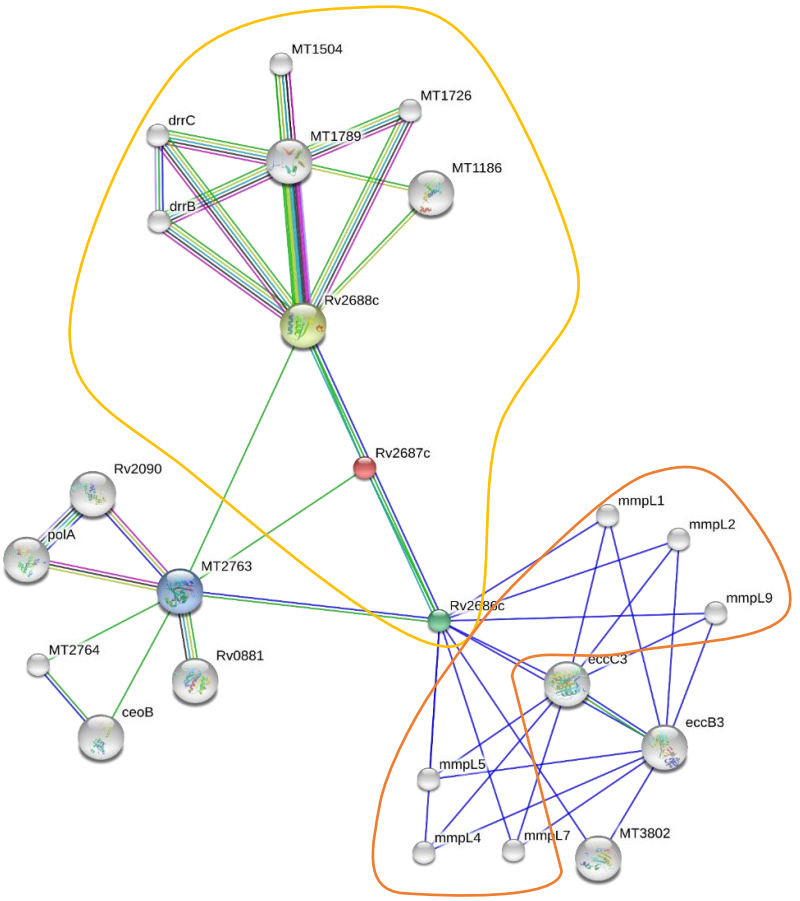

Figure 13: The protein-protein network of genes belonging to different efflux pump families

ATP-binding cassette (ABC) and resistance nodulation-cell division (RND) circled in yellow and orange respectively. The connections are computed based on multiple evidence, gene fusion, gene neighbourhood, co-occurance, experiments, text mining, database and gene-co-expression. Thicker lines represent stronger associations.

The STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins) is dedicated to functional associations between proteins, on a global scale [21].The number of associations between genes for are indicated confidence ranges with different scores. (low confidence: scores <0.4; medium:0.4 to 0.7; high: >0.7). Two distinct groups of ABC and RND were indicated on the in the STRING map. The Rv2686c ABC transporter was shown to form a link between the two groups. Protein interaction was high (0.7) between Rv2686 with MmpL4 (Rv0450) and eccB3

(Rv0507) genes. The eccB3 is part of type VII secretion (T7S) that is involved in iron uptake together with MmpL genes. The link between the three secretion systems of ABC, RND and T7S was medium to high (0.4-0.7).

5.5 Discussion

Our study employed transcription and functional studies for assessing EP activity in XDR-TB isolates. Most of the genes unique to XDR-TB belonged to drug efflux, lipid metabolism and non-coding RNA. For instance, Rv1410c and genes in the Lpr family function together to position mycobacterial lipids in the cell wall [22]. Furthermore, Rv1410c could serve as a candidate to the vaccine design against drug-resistant *M tuberculosis* due to its TB immunotherapy [23]. The rpfA and rpfD of resuscitation promoting factors over-expressed in XDR-TB strains are crucial for both cell wall hydrolysis and reactivation [24]. They also stimulate regrowth of inert and otherwise non culturable bacteria, and enable reactivation of chronic TB in mice [25]. Such genes could allow the XDR-TB phenotype to persist for months in the lungs.

In the present study, non-coding RNA molecules were found and these could play a role in the transition to dormancy [41]. The differences between XDR and MDR-TB isolates suggest that quantifying the expression levels of *M. tuberculosis* EP genes may be a new method to diagnose resistant TB and provide a state into the physiology of the bacteria [26].

Our study, as with other studies, confirms that EPIs have a potential use in TB treatment [9, 10]. The reduction of MIC levels in the background of resistant mutations could be a significant milestone in the fight against drug resistant bacteria. Three isolates had their MICs reduced in the presence of *gyrA* and *eis* mutations. Most interestingly the isolate with the D94G mutation had its mutation reduced by PIP. In our study, PIP was the most effective EPI as compared to VER. As in other studies, isolates with OFX resistance did not have MIC reduction in the presence of either PIP or VER [13]. For these isolates, efflux activity seems to have

little contribution for OFX resistance [27]. Moreover, other studies have shown that VER did not contribute to MIC reduction of moxifloxacin in clinical isolates [28].

The MIC levels of AMK were reduced by two-fold in two isolates without the presence of *rrs* mutations. However, the isolates had *eis* C14T mutation which is implicated in KAN mono resistance with an MIC level of 16 µg/ml [29]. Thus, EP genes might be implicated in AMK in cross-resistant cases with KAN drug which was reported in other studies [30, 31]. Concerning the CAP resistant strain, the resistance was reduced by one-fold in the presence of VER. This indicates an involvement of an ABC transporter in CAP resistance. Moreover, a cyclic peptide, vancomycin (VAC), was shown to be extruded by an overexpressed ABC transporter [32].

The operon Rv2686/Rv2687c/Rv2688c was significantly overexpressed in samples with *gyrA* mutations. An eightfold increase in the FLQ MIC was observed in clones overexpressing the entire Rv2686/Rv2687/Rv2688c operon [33]. Only one sample had reduced FLQ MIC in the presence of PIP. Other ABC transporters such as Rv1686c and Rv1218c were significantly expressed in the study, but not linked to resistance [34] [1]. VER was not effective with most second-line drugs. However, for CAP, a good synergistic effect has been reported with rifampicin [9, 35]. RNA was isolated from bacteria grown in the absence of antibiotics, indicating that stable transcriptional changes had taken place. Our findings suggest that drug efflux could be an important mechanism effectuating drug resistance also within patients [5].

The Rv1634, Rv1258c and Rv0194 EP gene expressions were differently expressed in all samples. The Rv1634 gene was expressed by 3-fold in an OFX isolate that was inhibited PIP compound. Most recently the Rv1634 showed the highest expression level in drug resistant strain as compared to drug susceptible strain [12]. It also decreases susceptibility to other FLQ drugs of the same class, such as norfloxacin and ciprofloxacin [36]. Both drugs are hydrophilic and thus suggest that OFX could also be effluxed by Rv1634. Louw et al. also reported that Rv1634 overexpression in RIF resistant strains that lead to resistance to OFX [9].

Early detection and inhibition of Rv1634 upregulation is important to prevent resistance to OFX.

The Rv1258c gene was shown to confer low-level resistance to aminoglycosides when expressed in *M. smegmatis* [37]. This protein has been previously shown to be over expressed in response to OFX [9, 36, 38]. Only one strain (2238) showed a high expression of Rv1258c (14 fold). The Rv1258c has a PIP binding pocket at position Arg141 and this occurs through hydrogen binding [17]. Most interestingly two isolates with low level Rv1258c expression had their AMK MIC reduced in the presence of PIP. The isolates were also cross-resistant to KAN and had *eis* C14T mutation. Moreover, both Rv1258c and *eis* genes are upregulated by *whiB7* (Rv3197A) which might contribute to cross-resistance of aminoglycosides [39]. Thus, Rv1258c over-expression together with *eis* mutation could be important in diagnosis of AMK/KAN cross-resistance.

The Rv0194 was expressed in all study samples. Efflux activity of Rv0194 has been shown to cause multiple drug resistance to various compounds [32]. Among them, VAC, which is similar to anti tuberculosis drug CAP, is extruded by Rv0194. One isolate which was resistant to CAP, had its MIC reduced in the presence of VER and displayed high level (4 fold) Rv0194 expression. The Rv0194 could be an additional mechanism responsible for CAP resistance.

The identification of EPs specific pathway activation may provide insights into potential novel targets that may be useful for the design of drugs and diagnostics. Using protein-protein interactions we mapped a connection between ABC, RND and T7S pathways (EccB3 and EccC3). The T7Spathways of EccB3 gene is a component of the ESX-3T7S, one of five paralogous clusters (ESX-1 through ESX-5) in the *M. tuberculosis* genome [40]. The component of the T7S membrane complex namely EccC has the same homology to a component of other established specialized protein secretion systems [43]. The analysis provides an invaluable insight to understanding the origin of EP mechanism that may be involved in transport of compounds (including drugs) from the cytoplasm to the

extracellular matrix. Targeting such novel pathways not inhibited by existing drugs may reduce the chance of cross-resistance with current drugs [41].

In conclusion, we examined the RNA expression levels of EP genes in strains without drug inducement and determined the effect of EPIs in XDR-TB strains. Similarly, to others we did not notice a straight correlation between the EP gene expression and the reduction of the antibiotic resistance levels by the EPIs [13]. The reduction of AMK MIC by PIP in cross-resistant cases is an important finding and this could be due to its affinity with Rv1258c. Moreover, EPs may play a role in resistance together with drug target mutations. The study highlights the importance of EP genes in diagnosis and treatment of XDR-TB. The novel pathway detected in our study could highlight the multiple processes involved in drug resistance.

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Chapter 6: The role of efflux pumps Rv0194, Rv1258c and Rv1634 on antimicrobial susceptibilities and virulence in *Mycobacterium tuberculosis*[2]

6.1 Abstract

Mycobacterium tuberculosis (M. tuberculosis) uses efflux pumps (EPs) to extrude toxic substances from microrganisms. We amplified and sequenced EPs genes Rv0194, Rv1258c and Rv1634, all of which have the potential to cause drug resistance to various second-line anti-tuberculosis compounds. Bionformatic analysis revealed transmembrane helices in all genes that are characteristic of EPs. The *M. tuberculosis* Rv0194, Rv1258c and Rv1634 genes were cloned and conferred low-level resistance to amikacin (AMK), kanamycin (KAN), capreomycin (CAP) and ofloxacin (OFX). In the presence of the EPI, piperine, the minimum inhibitory concentrations (MICs) were reduced for the Rv1258c and Rv1634 clones in the presence of kanamycin (KAN) and ofloxacin (OFX). We found that biofilm formation by the *M. tuberculosis* of Rv1258c and Rv0194 were distinct from that by Rv1634 *M. tuberculosis*. EPs genes of Rv0194 and Rv1258c contribute to biofilm formation as they formed a pellicle under hypoxia stress. Our study highlights the relationship between biofilms and EPs which could be the basis of multidrug resistance in *M. tuberculosis.*

[2] *PLEASE NOTE: This chapter has been prepared in the form of a manuscript for publication in the Journal of Bacteriology; therefore, the applicable editorial style has been adopted in preparing the narrative and list of references below. However, to preserve consistency in the Table of Contents, numbering of headings, tables and figures follows the overall style and format used for the rest of this thesis.*

IMPORTANCE: EPs genes of Rv1258c, Rv1634 and Rv0194 are part of the cell envelope transport system of *M. tuberculosis*. They remove toxic substances, including drugs from the interior of the cell to the outside environment. In this work, we report the formation of biofilms by Rv1258c and Rv0194 EP clones. The EP genes over-expressed in biofilms could indirectly cause drug-resistance. EPI, piperine binds Rv1258c binding pocket and reduce its activity.

6.2 Introduction

Drug resistance in *Mycobacterium tuberculosis* (*M. tuberculosis)*, as in any other bacterium, is an outcome of multiple mechanisms operating simultaneously. Through upregulation and down regulation of genes involved in the same cellular function, including transcription, drug efflux and protein synthesis, compensation for drug resistance mechanisms occurs (1, 2, 3, 4).

Acquisition of genetic mutations is the first step towards drug resistance; however, it is also possible that bacterial strains are phenotypically resistant or tolerant to antibiotics while they do not harbour any mutations. In *M. tuberculosis*, such organisms possess the ability to survive despite exposure to antibiotics and are referred to as “persisters” (5). Persisters are genetically identical to phenotypically susceptible bacteria and appear to be non-replicating and in a metabolically altered state (6). Persistent bacteria arise within a subset of infected macrophages during treatment and cause induction of efflux pumps (EPs), thereby also favouring the accumulation of mutations (7-9). *In vitro*, persistence may be induced by a heterogeneous set of environmental conditions, such as hypoxia or starvation that allow *M. tuberculosis* to grow as a pellicle, a form of biofilm (10). Similar to biofilms, macrophages create a physical barrier between bacteria and the immune system (11). Furthermore, treatment of drug-resistant TB is long and the survival of persisters in macrophages can occur. Finally, it is thought that biofilms harbour specialized persisters, in the form of cells that are phenotypically drug resistant (10).

The growth of bacteria within the pellicle biofilm that confers drug tolerance is still unclear (10). The bacteria represent a phenotypically resistant, ‘‘drug-tolerant’’ population; and their minimum inhibitory concentrations (MICs) remain unchanged (12). Increased expression of EPs may contribute to drug tolerance in bacteria (13, 14). Adams and colleagues proposed that EPs induced by macrophages lead to drug-tolerance, an important barrier *in*

vivo to shorten TB treatment (7). A biofilm matrix provides a physical barrier to environmental stresses and allows for the emergence of a drug-tolerant phenotype (15).

Cell wall lipids contribute to mycobacterial biofilm formation and have immunomodulatory properties that are essential to the infectious strategy of pathogenic mycobacteria (15). However, it has remained largely unknown how the drug-tolerant biofilm of *M. tuberculosis* relates to its pathogenicity (16). Biofilms are bacterial aggregates that grow at phase interfaces (air/liquid in the case of the *M. tuberculosis* pellicle), elaborate a matrix of some type, and gain resistance to killing by stressors such as antibiotics (17). Bacteria within biofilm communities are typically associated with a complex architecture of extracellular material that contains secreted molecules such as polysaccharides, lipids, proteins and DNA (15). Biofilm in bacteria can significantly enhance their tolerance to antibiotics (18). There is a relationship between EPs and biofilm secretion, which has come to the forefront only recently (19). Control of this relationship is critical for successful therapy of multidrug resistant bacterial infections, which have become rather commonplace (20). Because biofilm formation involves secretion of compounds, efflux systems of the cells are considered to be involved in this process (20).

Some EP genes (e.g. Rv0202c) have been shown to contribute to the biogenesis of the mycobacterial cell wall and biofilm formation (15). An ABC transporter, Rv1819 might also be involved in transport of one of the long-chain fatty acids (21). Both Rv0194 and Rv1819c are classified under multidrug transporters that extrude drugs to the external environment (22). We hypothesize that the mycobacterial Rv0194, Rv1258c and Rv1634 might also be involved in the transport of one of the long-chain fatty acids that lead to biofilm formation, thus causing resistance (21), and conducted the study reported here to further elucidate this thesis. The clones were tested for growth when exposed to ofloxacin (OFX), kanamycin (KAN), amikacin (AMK) and capreomycin (CAP) in the presence or absence of the efflux pump inhibitors (EPIs) of verapamil (VER) and piperine (PIP). We included H37Rv wild type strain as a control.

6.3 Materials and methods

6.3.1 DNA amplification and bioinformatics features of Rv1258c, Rv1634 and Rv0194 genes

The genomic regions on Rv1634, Rv1258c and Rv0194 were PCR amplified using primers in **Table 21**. Briefly DNA extracted from *M. tuberculosis* H37Rv used as template DNA was extracted using a boiling method (23). Amplification of the PCR products was done according to the following protocol: 25 µl of the cocktail reaction was made up of 11.5 µl of Hot Start mix (Kapa Biosystems, Cape Town, South Africa), 1 µl each of sense and antisense primer, 7.5 µl distilled H_2O (dH_2O), and 2 µl of DNA. The PCR products were run on agarose gel electrophoresis (700bp). The products were confirmed by DNA sequencing (Central Analytical Facility, Stellenbosch, South Africa). Bioinformatic analysis was performed on BioEdit and the amino acid sequences were blasted on TMHMM server version 2.0. (http://www.cbs.dtu.dk).

Table 21: Primer list for amplification of Rv1258c, Rv1634 and Rv0194

Primer	Direction	Sequence	Reference
Rv1258c	Forward	TTCCCGAAATACTTCACCGAC	This study
	Reverse	CAACACGGCATACCCCAG	
Rv1634	Forward	TGCGGTGTTTGTGGTAGTC	This study
	Reverse	CCGACATGGTCAGGTAAATCC	
Rv0194	Forward	TGGTGAAGTGGTTTTCGACG	This study
	Reverse	CGGAATTCGCAGGTTGATG	

6.3.2 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used, are listed in **Table 22**. The PCR products were ligated to pDrive and cloning was performed in *Escherichia coli* DH5α (Zymo Research, US). The clones were selected on Luria-Bertani (LB) medium at 37°C. The shuttle vector pOLYG was cut with *BamHI* and *HindIII* to produce pSODIT (24) (**Figures 14 and 15**). The PCR clones were also cut with the same enzymes and ligated to pOLYG. The *M. tuberculosis* H37Rv strain were grown in Middlebrook 7H9 (BD Biosciences, San Jose, CA, USA) medium, 0.2%

glycerol supplemented with albumin, dextrose and catalase (OADC) (Becton Dickson and company). Hygromycin and kanamycin (KAN) were added where necessary to grow the plasmids.

Table 22: Bacterial strains and plasmids

Bacterial strains	**Reference**
E. coli DH5α	Zymo
M. tuberculosis	ATCC
Plasmids	**Reference**
PDrive	Qiagen
pOLYG-1258-700	This study
pOLYG-1634-700	This study
pOLYG-0194-700	This study

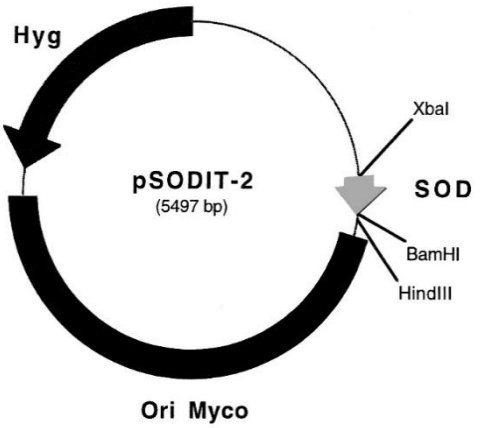


Figure 14: Plasmid map of pOLYG with *BamHI* and *HindIII* restriction sites

A map of the expression vector with mycobacterial origin of replication and hygromycin resistant marker (24)

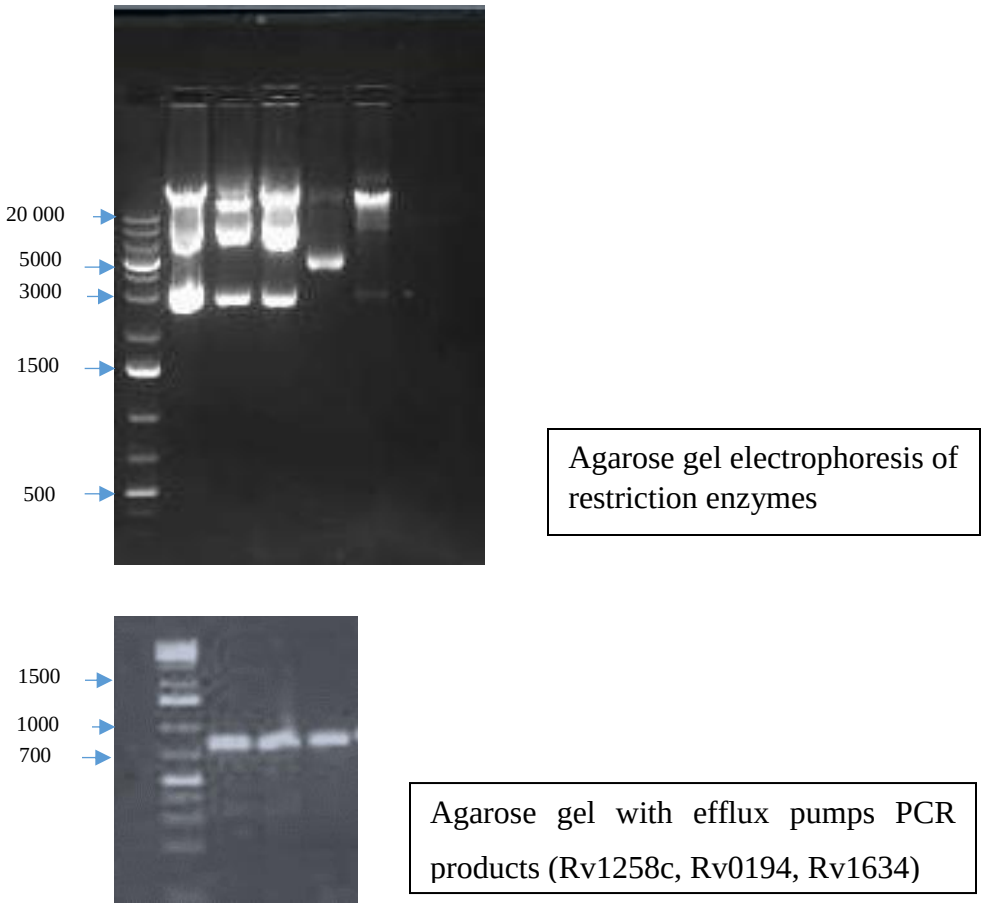


Figure 15: Agarose gels depicting vectors with/without clones and amplified PCR products

Top: Line 1-3 represent the cloned plasmid cut with restriction enzymes, line 4 is the vector and line 5 is the *E. coli* genome; Bottom: Efflux pump genes (Rv1258c, Rv0194 and Rv1634) PCR products

6.3.3 Antibiotics and inhibitors

OFX, AMK, KAN, CAP and EPIs of PIP and VER were obtained from Sigma, USA. Hygromycin 50 µg/ml was obtained from Roche, Germany. Piperine was dissolved in dimethyl sulphoxide (DMSO, Sigma, USA), and the rest of the drugs were dissolved in distilled water (D/w; Span Diagnostics, India). All drugs and inhibitors were filter sterilized through 0.22 µm filters (Millipore, USA).

6.3.4 Construction of Rv1258c, Rv0194 and Rv1634

The DNA fragments of three genes (approx. 700bp) were ligated into pDrive cloning vector to generate p1258c, p0194, p1634 plasmids (**Figure 14**). The plasmids were cut with either *BamHI* or *HindIII* restriction enzymes as per manufacturer instructions (Thermo Scientific). The plasmids were ligated to pOLYG and cut with *BamHI* or *HindIII* to produce pOLYG1258/0194/1634. A volume of 400 µl electrocompetent cells mixed with plasmid DNA, including controls, were transferred to an electroporation cuvette (0.2 cm gap-BioRad). Electroporation was performed with a Gene Pulser (Bio-Rad Laboratories Inc., Richmond, Calif.) at 2.5 kV, 25 µF, and 1,000 V. The cells were revived by adding 800 µl 7H9 (BD Biosciences, San Jose, CA, USA), supplemented with OADC and glycerol.

6.3.5 Colony morphology analysis

M. tuberculosis cells transformed with pOLYG1258/0194/1634 were grown on Middlebrook 7H9 (BD Biosciences, San Jose, CA, USA) agar to analyse the colony morphology. The plates were incubated for 24 h at 37°C under humidified conditions**.**

6.3.6 Microplate AlamarBlue® assay

To determine the minimum inhibitory concentration (MIC) levels of *M. tuberculosis* strains resistant to OFX, AMK, KAN and CAP, based on conventional DST, a microplate AlamarBlue® assay (MABA) was performed as described elsewhere (25). Briefly, the cultures were grown to mid-log phase on 7H9 with OADC. Once an OD of 0.6 was reached, 100 µl of the culture was added to a solution of 98 µl of 7H9 withOADC and 2 µl of AMK, KAN or CAP. The AMK, KAN and CAP (Sigma Aldrich) drug concentrations ranged from 0.125 ug/ml to 64 ug/ml. ForEPIs, 2 µl of 50 µM of VER and PIP were added to each drug, with concentrations ranging from 64 ug/ml to 0.125 to µg/ml. A 25 µl quantity of AlamarBlue® reagent (Thermo Fischer, US) and 10% v/v Tween 80 each were added to the wells of the microplate and incubated for 24 hours. After one day of incubation, resistance was detected by change of a blue to pink colour. The MIC was recorded as the well without colour change at the nearest (lowest) concentration. The H37Rv was used as negative control and was

susceptible to all drugs tested. Two technical and two biological replicates were grown separately and tested on different days.

6.3.8 Biofilm formation

The four clones, namely Rv1258c, Rv0194, Rv1634 and wild type, were cultured for biofilm growth in 28-well polystyrene plates using published protocols (26). Cells were grown on 7H9 medium complemented with 50 µg hygromycin until they reached an absorbance of 0.6 at 600 nm. These were diluted 1:100 in Sautons medium without a detergent and 2 ml added in duplicates. The plates were wrapped in parafilm and incubated for 5 weeks without shaking. Plates were assessed weekly for 5 weeks.

6.4 Results

6.4.1 Bioinformatics characterization

Bioinformatic analysis of Rv1634 with TMHMM server version 2 revealed 14 transmembrane helices of the 450 amino acid full length gene. The partial gene expression of Rv1634 clone with 250 amino acids had eight transmembrane helices. The sequence alignment of Rv1634 with A, B and C motifs as observed, are mainly found in the MFS superfamily (**Figure 16**). The Rv1258c full length gene had 12 transmembrane helices with approximately 400 amino acids. The 250 amino acid partial clone was predicted to have six transmembrane helices. The multiple sequence alignment of Rv1258c had three motifs of A, B and C. A PIP binding site at position G130 amino acid was previously reported by Sharma et al (27) (**Figure 17**). The Rv0194 efflux gene of ABC family has predicted nine transmembrane helices. The 250 amino acid clone had two transmembrane helices. The multiple sequence alignment had 5 loops between the transmemebrane helices. Two longer loops (loops 2 and 4) were present within the membrane spanning the domain of the Rv0194 gene (**Figure 18**).

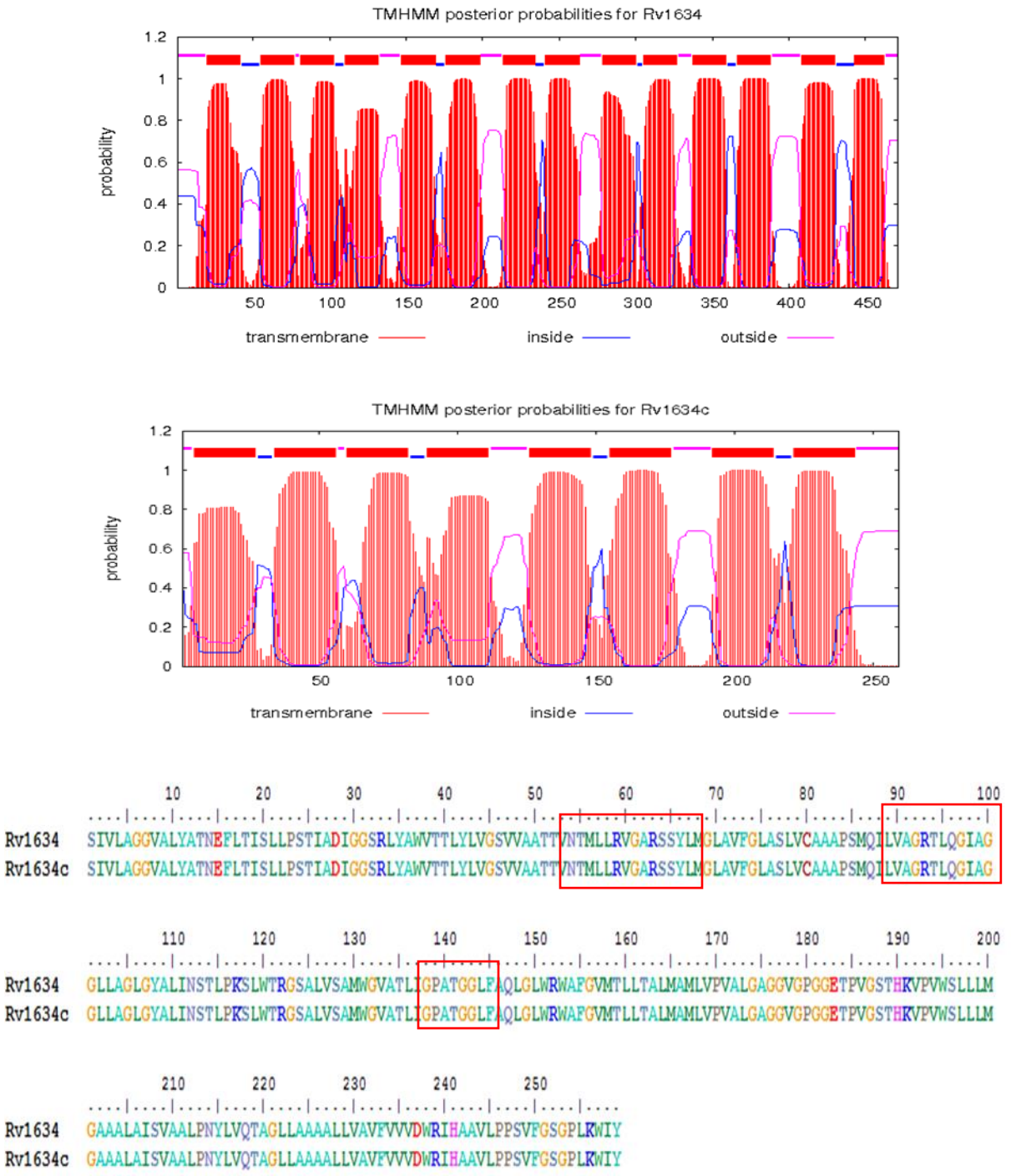
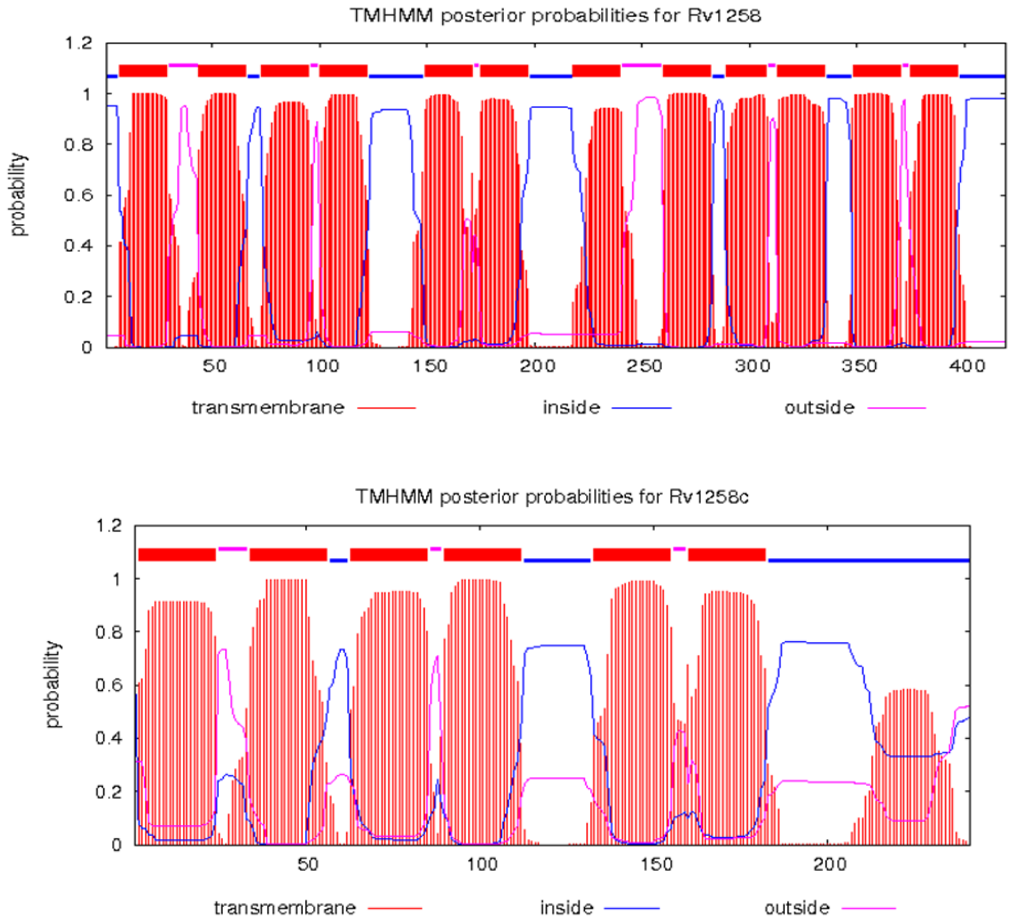


Figure 16: Schematic diagram depicting structural and genomic features of Rv1634

Top: 450 amino acid of full length gene with 14 transmembrane helixes; Middle: 250 amino acid clone with eight transmembrane; Bottom: Amino acid alignment with three motifs

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                 20        30        40        50        60        70        80        90       100       110
                 |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
Rv1258           ILFATLMAAAGDGVSIVAFPWLVLQREGSAGQASIVASATMLPLLFATLVAGTAVDYFGRRRVSMVADALSGAAVAGVPLVAWGYGGDAVNVLVLAVLAA
Rv1258c          VLFATLMAAAGDGVSIVAFPWLVLQREGSAGQASIVASATMLPLLFATLVAGTAVDYFGRRRVSMVADALSGAAVAGVPLVAWGYGGDAVNVLVLAVLAA

                120       130       140       150       160       170       180       190       200       210
                 |....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|.
Rv1258           LAAAFGPAGMTARDSMLPEAAARAGWSLDRINGAYEAILNLAFIVGPAIGGLMIATVGGITTMWITATAFGLSILAIAALQLEGAGKPHHTSRPQGLVSG
Rv1258c          LAAAFGPAGMTARDSMLPEAAARAGWSLDRINGAYEAILNLAFIVGPAIGGLMIATVGGITTMWITATAFGLSILAIAALQLEGAGKPHHTSRPQGLVSG

                220       230       240       250
                 |....|....|....|....|....|....|....|..
Rv1258           IAEGLRFVWNLRVLRTLGMIDLTVTALYLPMESVLFPKYFT
Rv1258c          IAEGLRFVWNLRVLRTLGMIDLTVTALYLPMESVLFPKYFT
```

Figure 17: Schematic diagram depicting structural and genomic features of Rv1258c

Top: Full gene showing 400 amino acids with 12 transmembrane helices; Middle: A 200 amino acid clone showing seven transmembrane helices; Bottom: Amino acid alignment with three motifs and a piperine binding site (27)

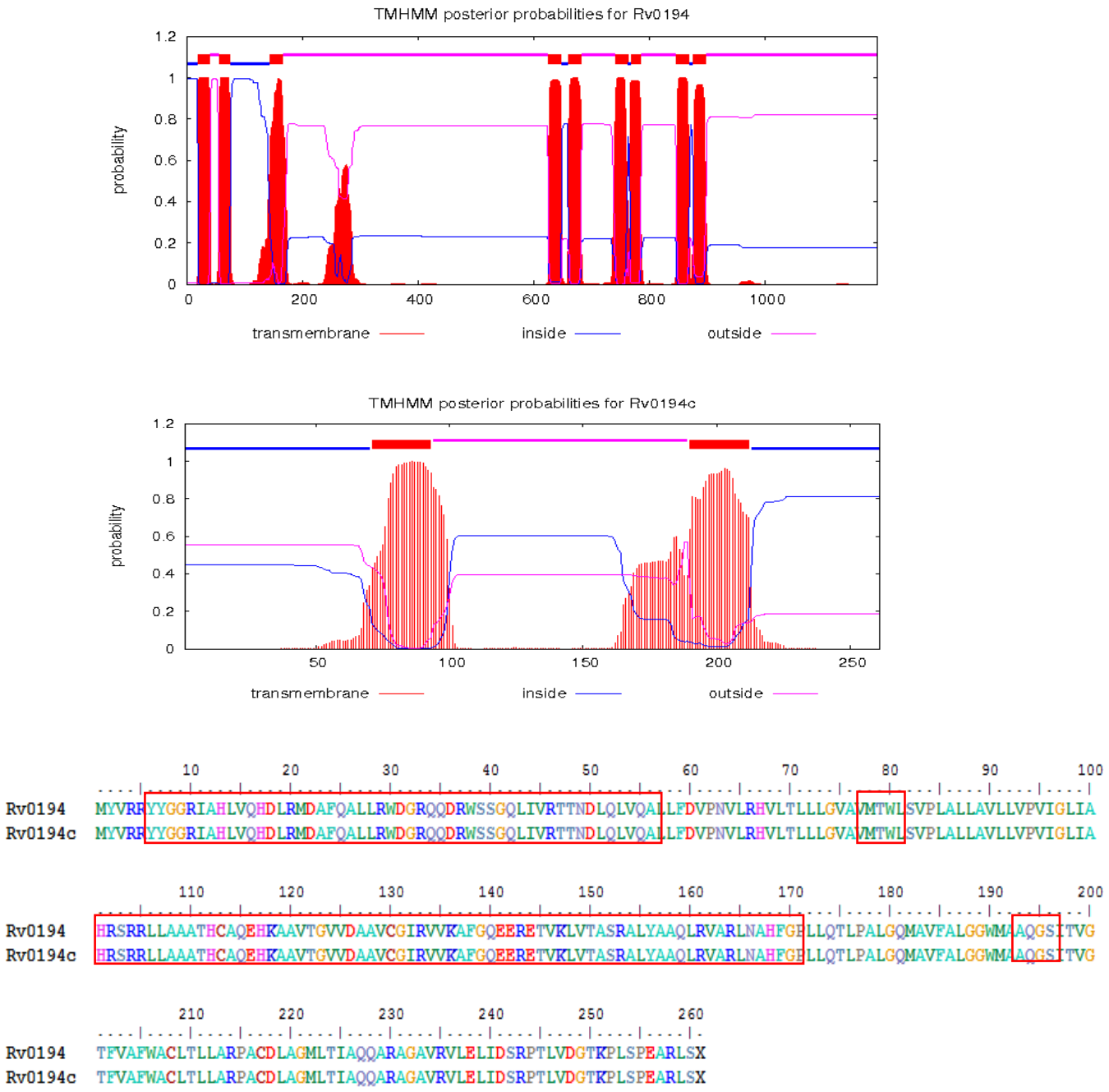


Figure 18: Schematic diagram depicting structural and genomic features of Rv0194

Top: full length Rv0194 with nine transmembrane helices; Middle: Rv0194 clone with two transmembrane helices; Bottom: An amino acid alignment with four loops highlighted

6.4.2 Cloning and construction of Rv1258c, Rv1634, Rv0194

The amplification of Rv1258c, Rv1634 and Rv0194 was amplified by primers as shown in **Table 21**. The amplified products were cloned into pDrive vector (Qiagen). Restriction digestion of the with *BamHI* and *HindIII* was performed and the product clonedinto pOLYG. The resulting plasmids were designated pOLYG-1258-700, pOLYG-1634-700, pOLYG-0194-700 (**Table 22**). The plasmids were electrophorated into H37Rv and changes under different conditions was recorded.

6.4.3 Antimicrobial susceptibility testing

The antimicrobial testing of clones was performed on agar plates and microtiterplates. All clones gave susceptible results both on solid and liquid culture media. In combination with the EPI, PIP, the MICs of OFX and KAN were reduced. More specifically, this was associated with the EP clones, Rv1258c and Rv1634. None of the Rv0194 clones had their MICs reduced in the presence of EPIs (**Table 23**).

Table 23: Comparative minimum inhibitory concentrations for *Mycobacterium tuberculosis* isolates carrying different efflux pump genes

Drug	H37Rv	p1258c	p1634	p0194
AMK	0.5	0.5	0.5	0.5
AMK+PIP	0.5	0.5	0.5	0.5
AMK+VER	0.5	0.5	0.5	0.5
CAP	1	1	1	1
CAP+PIP	1	1	1	1
CAP+VER	1	1	1	1
KAN	2	2	2	2
KAN+PIP	2	**1**	**1**	2
KAN+VER	2	2	2	2
OFX	0.5	0.5	0.5	0.5
OFX+PIP	0.5	0.5	**0.25**	0.5
OFX+VER	0.5	0.5	0.5	0.5

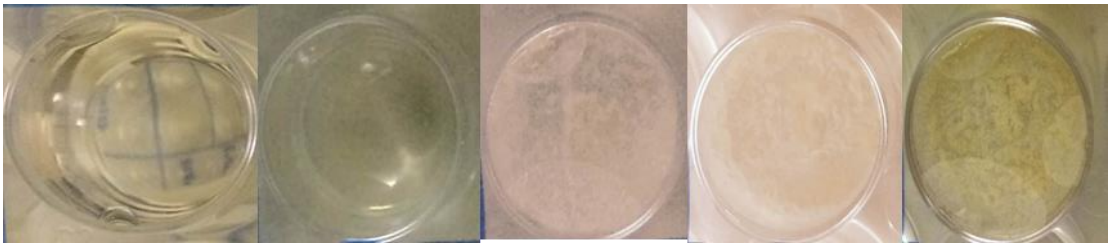
AMK=Amikacin; CAP=Capreomycin; KAN=Kanamycin; OFX=Ofloxacin; PIP=Piperine; VER=Verapamil. The values in bold highlight the reversal changes observed with the addition of efflux pump inhibitors.

6.4.4 Biofilm formation

Biofilm formation and morphological changes were inspected for five weeks. The H37Rv control strain pellicle appeared by week 3, and in clones Rv0194 and Rv1258c, the pellicle formed by week 4. Both Rv0194 and Rv1258c formed closed biofilm, but Rv1634 showed loose biofilm (**Figures 19 and 20**).

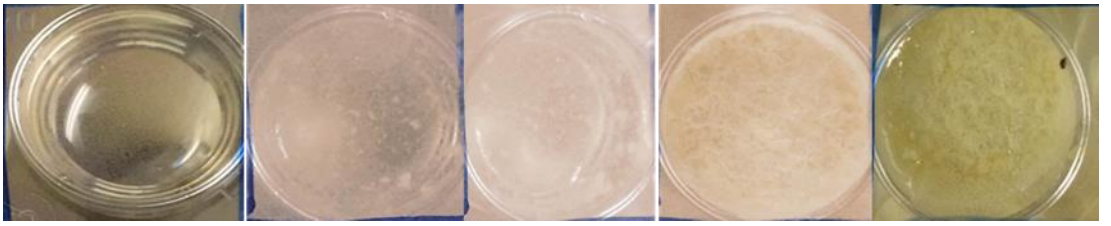
Rv0194

Week 1 Week 2 Week 3 Week 4 Week 5



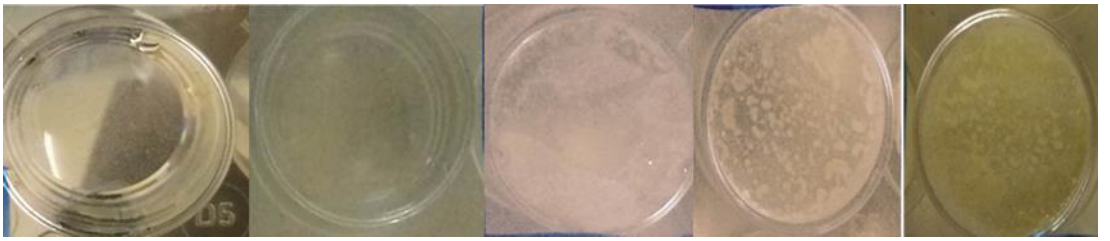
Rv1258c

Week 1 Week 2 Week 3 Week 4 Week 5



Rv1634

Week 1 Week 2 Week 3 Week 4 Week 5



H37Rv

Week 1 Week 2 Week 3 Week 4 Week 5

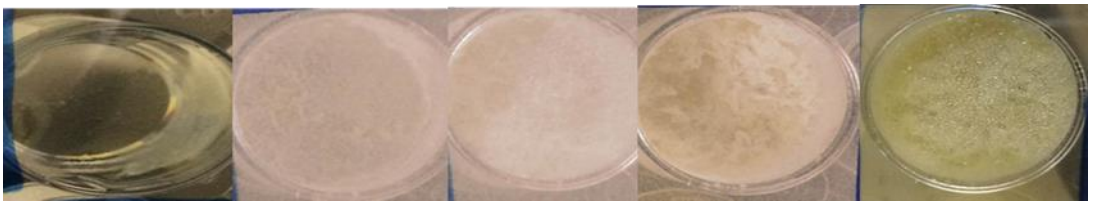
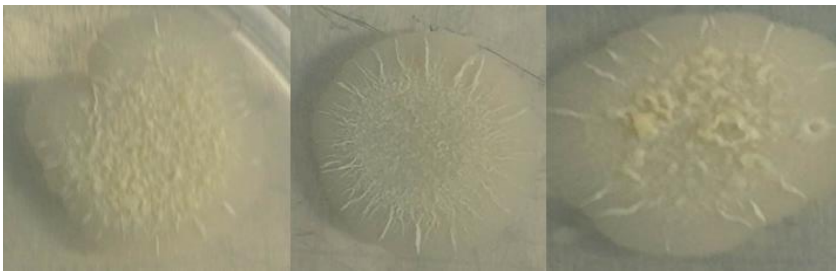


Figure 19: Biofilm formation in Rv0194, Rv1258c, and Rv1634 clones, and H37Rv, over 5 weeks



a) Rv0194 b) Rv1634 c) Rv1258c

Figure 20: a) Growth morphology of Rv0194 clone with rough phenotype b) Smooth phenotype of Rv1634 clone c) Rv1258c clone with rough phenotype

6.5 Discussion

Most chronic and persistent bacterial infections are associated with biofilm growth, a strategy that has probably accelerated the emergence and rapid spread of MDR-TB bacteria (28). Drug EP activity has also previously been shown to be associated with biofilm development and drug resistance (29). In terms of energy requirement, efflux activity uses adenosine triphosphate and proton motive force to extrude compounds to the external environment. In this study, we characterized three EP genes of Rv0194, Rv1258c and Rv1634 involved in resistance to second-line drugs.

The Rv0194 belonging to ABC family consists of two nucleotide binding domains and two membrane binding domains fused together, which are normally detected in eukaryotes (22). The enhanced gene fusion allows export of lipids which could contribute to increased multidrug resistance (30). The Rv1258c and Rv1634 belonging to MFS are arranged in 12 to 14 transmembrane-spanning domains with conserved amino acid motifs. Conservation of such motifs among proteins responsible for the transport of a wide variety of structurally disparate compounds implies that they play some vital structural or functional role rather than directly interacting with their substrate(s) (31).

Most EP genes have been cloned into appropriate plasmids that facilitates their replication in *M. smegmatis* and caused resistance to several drugs (24, 31, 32). Few genes were expressed in *M. tuberculosis* with varying degree of resistance. Similar to findings from other studies, there was no increase in MIC levels of drugs (33). We did not detect an increase in resistance of clones to OFX, KAN, AMK or CAP. However, when the Rv1634 and Rv1258c clones were grown under exposure to the EPI PIP, a single dilution in MIC was detected in OFX and KAN drugs. It was previously observed that plasmids containing Rv1634 and Rv1258c clones conferred resistance to OFX and aminoglycosides in *M. smegmatis*, demonstrating that a novel FLQ resistant determinant is present in *M. tuberculosis* (31). Moreover, the involvement of Arg141 in the Rv1258c gene could contribute to the inhibitory activity of PIP and may facilitate the inhibition of the EP related resistance mechanism in *M. tuberculosis* (27).

As with previous studies, we found no evidence of increased resistance to CAP when Rv1258c was over-expressed (34). Furthermore, Rv1258c EP has been shown to cause drug-tolerance to the hydrophobic antibiotics (i.e rifampicin) (12). Most of the drugs studied here were hydrophilic. Resistance factors of *M. smegmatis* expressing Rv0194 were low and the same as those observed for heterologous expression of drug EPs of *M. tuberculosis* (30).

Because biofilm formation involves secretion of compounds, efflux systems of the cells might be involved in this process (20). Bacteria within biofilm communities are typically associated with a complex architecture of extracellular material that contains secreted molecules such as polysaccharides, lipids, proteins and DNA (15). The Rv0194 gene was previously predicted to contribute to the transportation of lipids which may indirectly cause resistance in mycobacteria (30). In our study, we prove that Rv0194 and Rv1258c contributes to biofilm formation as they formed a pellicle under hypoxia stress.

Similarly, Rv0202c gene (MmpL11) of resistance nodulating-cell division (RND) contributed to biofilm formation when expressed in *M. smegmatis* (15). Notably, export of lipids by Rv0194 cannot be ruled out as an additional explanation for the increased drug resistance of the Rv0194 expressing strain (30). However, we did not detect any increase in MIC readings in any of the clones studied and thus could not detect any drug tolerance. By virtue of their anti-EP activities, it seems that EPIs have great potential as antibiofilm drugs (35). Confinement-induced drug tolerance in *M. tuberculosis* may contribute to persistence in

TB patients, where drugs that are rapidly potent *in vitro* require prolonged administration to achieve comparable effects (36).

In conclusion, our study demonstrates the importance of EPs in biofilm formation. Future studies are needed to properly understand the role of EPs in lipid secretion which may inversely cause drug resistance. The usage of EPI in persistent bacteria should be further investigated as it may inhibit biofilm formation through EPs.

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Chapter 7: Concluding remarks

7.1 Summary

A better understanding of efflux pump (EP) mechanisms will assist in combating drug resistance. In this study, we explored the impact of EP gene mechanisms on second-line drugs used in the treatment of multidrug resistant (MDR) and extensively drug resistant (XDR) tuberculosis (TB). We focused on the impact of EP genes on drug resistance to second-line drugs of ofloxacin (OFX) and injectable drugs. The findings in this study aims to show the importance of EP genes in drug-resistant strains with or without target gene mutations.

EP gene mechanisms when combined with drug target mutations can make strains difficult to treat. Drug target mutations are well known and can be detected by current diagnostic assays. Rapid diagnostic assays offer relief but confirmatory drug susceptibility testing (DST) are needed to put patients on effective regimens. The discordance between phenotypic tests and molecular assays often occurs and could be due to other mechanisms. Other drug target and EP genes could be responsible for the discordance. Our study characterized discordant strains using genomic and transcriptomic methods. Inhibitors of EP gene activity were used to reverse resistance in the presence and absence of drugs. Such information will contribute in understanding the complexity of second-line drug resistance, whereby multiple mechanisms within the bacteria are activated.

Theme 1: **Enhancing molecular assays for detection of resistance to second-line and future drugs**

The GenoType® MTBDR*sl* (version 1) was compared with culture DST for rapid detection of second-line phenotypic resistance of OFX, amikacin (AMK), kanamycin (KAN) and capreomycin (CAP) drugs. We detected a significant increase in the

diagnostic yield by 62.2%, 46.7%, 53.6% for KAN, CAP and XDR-TB respectively against culture when we combined GenoType MTBDR*sl* and Sanger sequencing. Most importantly, we detected the *rrs* G878A mutation that could be a novel CAP resistant marker. The marker has been previously detected in our region (Daum et al, 2012) and its inclusion in future molecular assays would be beneficial. Our study detected new genetic markers that could be used in the design of future assays. Of importance is the impact of genetic mutations on minimum inhibitory concentrations (MICs) to each drug. This observation could be used by clinicians to tailor the best treatment for patients. Currently, the advanced version of GenoType® MTBDR*sl* (version 2) has been recommended by World Health Organization for detecting of second-line drug resistance within 24-48 hours.

This recommendation is an advancement in rapid diagnosis of XDR-TB. The assay has been modified with *eis* and *gyrB* genetic markers for enhanced detection of KAN and fluoroquinolones respectively. The use of KAN drug differs from patient to patient and due to toxicity may lead to hearing loss or side effects. The other available injectable drug, CAP part of the conventional MDR-TB regimens can be used in certain patients. The common *rrs* A1401G mutation is detected by GenoType® MTBDR*sl* often can be confused with cross-resistance to KAN and AMK drugs. Thus, we propose the inclusion of G878A mutation for rapid detection of CAP resistance.

Future drugs of Bedaquiline (BDQ), delamanid and SQ109 (Sequella) are currently on clinical trials and lack standardized *in vitro* DST assays. Genotypic markers could be used for resistance monitoring on patients receiving this drugs. The EP, MmpL5 belonging to the resistance nodulating-cell division (RND) family upregulation leads to clofazimine (CFZ) and BDQ cross-resistance due to mutations within Rv0678 regulatory gene (Milano et al., 2009). Compassionate use of BDQ prescribed to XDR-TB and pre-XDR-TB patients together with CFZ as part of combination therapy (Somoskovi et al., 2014). The non-synonymous S68G within Rv0678 regulatory gene caused an increased in MIC levels of BDQ drug (Andries et al., 2014). Another drug SQ109 was inhibited by non-synonymous mutation Q40R of MmpL4 gene (Rv0206c)

(Poce et al., 2013). Delamanid resistance is due to mutations within *fbiA* and *fgd1* that are coincided with phenotypic resistance (Bloemberg et al., 2015). The introduction of these drugs into new regimens will require standardized rapid DST assays. Of interest is the new GeneXpert XDR-TB assay that will detect isoniazid and second-line drugs anticipated for release in 2017 in the diagnostic market.

Theme 2: **The diagnostic utility of RNA based tests for detection of tuberculosis resistance and treatment monitoring towards personalized medications**

Transcriptomic changes were detected in 11 XDR-TB, six MDR-TB and two susceptible strains. Unique RNA signatures belonging to EP and lipid metabolism genes in the XDR-TB subgroup were detected. The detected genes could be used as novel markers for rapid detection of XDR-TB. Significant changes of differentially expressed EP genes compared to H37Rv were also detected. The Rv2686/87/88c operon belonging of ABC family was significantly expressed in XDR-TB strains with *gyrA* mutations. Our study used RNA sequencing to detect novel genetic expressions within XDR-TB strains. Some overexpressed genes are also implicated in bacterial persistence. Such observation could have prognostic implications and detect patients that do not respond to therapy timeously. Genetic markers unique to XDR-TB strains could be used to develop new diagnostic assays.

Monitoring bacterial physiology during human TB treatment is feasible through *M. tuberculosis* RNA expression profiling (Walter et al., 2015). Transcriptomic changes occur upon exposure of bacteria with drugs and EP genes are usually over-expressed. A set of EP genes could be used to determine resistance that may not be detected by genomic tests. EP gene operons were up-regulated over time in patients without drug induction and thus indicate important mechanism of resistance (Eldholm et al., 2014). Our study also detected upregulated operons that have been shown to cause EP mediated resistance.

Transcriptional responses during the first 14 days of standard anti-tuberculous therapy indicated that persister-type bacilli are the dominant population in human sputum

(Honeyborne et al., 2016). Persisters could lead to drug tolerance through over-expression of EP genes thus cause prolonged treatment. Through biofilms formation occurring under hypoxia, persisters could be induced to express EP proteins. We cloned and expressed three EPs for biofilm formation under hypoxic conditions. The Rv1258c EP gene causes drug tolerance which can be reversed by EPI.

Positron emission tomography integrated with computer tomography technique provides a unique opportunity to non-invasively image the whole body for diagnosing, staging and assessing therapy response in many infectious and inflammatory diseases (Ankrah et al., 2016). This new technique will allow identification of persistent bacteria that can be targeted with effective drugs.

Theme 3: **Combining efflux pump inhibitors with second-line drugs to enhance tuberculosis therapy**

EPIs of piperine (PIP) and verapamil (VER) were used in combination we second-line drugs. We did not observe a significant MIC change of XDR-TB strains in the presence ofEPIs. Only one strain with *gyrA* mutation had an MIC reduction in the presence of an EPI. The presence of drug target mutations could have hampered the inhibitory effects of PIP and VER compounds. Therefore, clones of Rv0194, Rv1258c and Rv1634 EP genes were used to evaluate the effect of PIP and VER compounds. We observed a reduction in OFX and KAN MICs by PIP with Rv1258c and Rv1634 clones. Further analysis revealed a PIP binding marker in Rv1258c gene. This information is important in optimizing EP inhibitory therapies.

Novel approaches that combine EPIs with antibiotics to reduce TB treatment are needed. An approved EPI (i.e. VER) used to treat high blood pressure, can be combined with other TB drugs shorten treatment (Gupta et al., 2013). The successful use of VER as an adjunct drug for TB therapy in a mouse model provided a scientific framework for human studies of this treatment-shortening combination.

Newly approved drugs such as BDQ can also be combined with VER to increase its efficacy. Both *in vivo* and *in vitro* studies demonstrated the efficacy of VER and BDQ (Gupta et al., 2014;Gupta et al., 2015). Although BDQ is an effective drug it may cause cardiac arrhythmias but when combined with VER it can lessen the side effects (Srikrishna et al., 2015). This combination therapy could beneficial to patients on shorter MDR-TB regimen. In our study the best combination was of AMK and PIP drugs. A combination of RIF and PIP was also shown to be effective both *in vivo* and *in vitro* (Sharma et al., 2010; Sharma et al., 2014). The Rv1258c is well characterized and has a binding affinity for PIP compound. This was observed in our study and opens the possibility of combination therapies with EPIs. Novel compounds such as spectinamides that blocks Rv1258c have shown impressive activity against M/XDR-TB strains (Madhura et al., 2016). The spectinamides form part of the drug discovery pipeline, with more data on its potential use likely to be available soon.

EPIs can also be used as part of pathogen-directed adjunct therapies to improve treatment outcomes. This will supplement antibiotic therapy and shorten the course of treatment (Li et al., 2016). Thioridazine (TDZ), a neuroleptic used for the therapy of psychosis and has shown to treat XDR-TB patients (Amaral et al., 2010). It has indirect inhibition of EP genes and an increase of cell-envelope permeability and this can contribute to a more rapid accumulation of antibiotic compounds in the bacterial cell (de Keijzer et al., 2016).

Inhibition of EPs is a promising area of development that can optimize the efficacy of current drugs. The ability of EPIsto act in the background of target mutations is a great milestone in treatment of drug resistance. Newer drugs that are accelerated through the pipeline could be combined with EPIs to improve their efficacy. The drug development pipeline has impressive compounds that are designed through EP mechanisms (i.e SQ109 and spectinamides).

The EPIs can also be used as compassionate treatment for XDR-TB. Due to long treatment times for XDR-TB, drug tolerance can develop and with modern medical

imaging mrthods, it should be possible to detect unresponsive lesion with high resolution. The prospect of introducing personalized medication in treatment of XDR-TB is gaining momentum. To conclude, EP gene activation and inhibition is an important new dirsction that might enable optimal management of, and ultimately eradication of TB bacilli.

7.2 Future Research

The increasing burden of TB and its drug resistant forms is a challenge to ending the epidemic by 2035. Alternative tools to combat drug resistance are needed especially in high TB disease burden. A better understanding of the prevalence of EPs genes in different genotypes of various geographic settings is needed. Drug EPs present in the bacterial cell wall offers an attractive option for combating the disease. The Rv1258c, Rv1634 and Rv0194 genes cause low level drug resistance that when combined with target mutations could make *Mycobacterium tuberculosis* hyper-resistant. Combining conventional drugs with EPIs could be a way to treat patients effectively.

Genetic expression of RNA molecules is unlocking powerful biomarkers used by pathogen in the disease process. The biomarkers can be optimized for diagnosis and prognosis of the disease. Especially the detection of persistent bacteria in sputum that can lead to drug tolerance and impact on patient treatment outcomes. The detection of novel connections between EP genes and type VII secretion (T7S) pathways is important and should be further explored. Importantly because T7S system and EPtransporters form part of the cell membrane. The discovery of non-coding RNA offers new class for drug targets for development. Future studies to optimize biomarker based RNA tests should be prioritized.

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Appendix A: Detailed methods

A1 Spoligotyping

Amplification of the spacers was accomplished by using the primers direct repeat forward (a) and direct repeat reverse (b), which enable amplification of the whole direct repeat (DR) region using s 5µl of DNA. The PCR products were labelled with biotin, because primer DRa is biotinylated. A total of 50 µl of the reaction mixture was used for the PCR. The reaction mixture composed of 25 µl KapaTaq ready mix (5mM Tris-HCl, 5mM KCl, 0.7mM $MgCl_2$, pH 9.0, deoxynucleoside triphosphates (dNTPs), 2µl of 200mM of each of the primers DRa and DRb and 19 µl of nuclease free water and 2µl of the sample DNA. The primers that were used are DRa (forward) with sequence: 5'-GGTTTTGGGTCTGACGAC-3', 59 biotinylated and DRb (reverse) (5'-CCGAGAGGGGACGGAAAC-3'). The numbers of spacer oligonucleotides 1 to 43 correspond to the spacer numbers. The PCR was performed on a Vereti thermocycler (Thermo fischer, USA). The PCR amplification consisted of an initial denaturation step at 96ºC for 3 minutes, followed by 30 cycles of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 30 seconds. Hybridization of the biotin-labelled PCR products to the immobilized spacer-oligonucleotides that represent spacers of known sequence was the next step in spoligotyping. The presence of spacers was visualized on film as black squares after incubation with streptavidin-peroxidase and ECL-detection (Amersham Buster GmbH and Co. KG. Braunschweig, Germany). The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus.

Briefly, a nylon membrane (Isogen Biosciences. BV. Marseen. The Netherlands) was activated by using 16% (wt/vol) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma). The oligonucleotides were applied to the membrane in parallel by using a mini-blotter system (MN45; Immunetics, Cambridge, Mass, United Kingdom). After a short incubation, the membrane was inactivated by using 100 mMNaOH and washed in 2X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH2PO4, and 1 mM EDTA (pH 7.7) (Gibco BRL Life Technologies, Inc.) supplemented with 0.1% sodium dodecyl sulphate (SDS; Sigma, Germany).

For hybridization, 20µl of the amplified PCR product was diluted in 150ml of 2X SSPE/0.1% SDS. The PCR product was then heat denatured for 10 minutes at 99°C and immediately snap cooled on ice (4°C). The membrane was washed at 55ºC in pre-warmed 2XSSPE/0.1%SDS for 5 minutes. The membrane was then placed on a support cushion in a mini-blotter in such a way that the channels of the mini-blotter apparatus were perpendicular to the rows of oligonucleotides deposited previously. Residual fluid was then removed from the mini-blotter by aspiration using a vacuum suction system or a Venturi pump. The parallel channels were carefully filled with diluted denatured PCR product, avoiding air bubbles. The PCR product was left to hybridize for 60 minutes at 55ºC on a horizontal surface. The samples were removed from the mini-blotter by aspiration.

After hybridization, the membrane was removed from the mini-blotter into a washing chamber and washed twice in 250ml of 2X SSPE/0.5% SDS for 10 min at 55°C. The membrane was incubated in 1:4,000 diluted streptavidin-peroxidase conjugate (Roche diagnostics. Indianapolis, USA) (40ml 2XSSPE/0.5%SDS warmed to 42ºC with 10µl of 500U/ml) for 45 to 60 min at 42ºC. Thereafter, membrane was washed twice, for 10 min each time, in 250ml of 2X SSPE/0.5% SDS at 42ºC. The membrane was rinsed with 250 ml of 2X SSPE for 5 min at room temperature. The membrane was incubated in 20ml (10ml solution 1 +10ml solution 2) ECL detection liquid (Amersham Buster GmbH and Co. KG. Braunschweig, Germany) with gentle shaking for 90 seconds.

A2 RNA extraction

The 1 ml stock culture was thawed on ice and inoculated in a 50 ml 7H9 (BD Biosciences, San Jose, CA, USA)7H9 (BD Biosciences, San Jose, CA, USA) supplemented with OADC (BD, sparks). After incubation 3-4 week's incubation, the 50 ml culture was measured on spectrophotometer for and OD of 0.6-1 nm. Thereafter poured in a 50 ml falcon tube (Scientific group) and centrifuged for 3000 rpm for 15 minutes. The supernatant was discarded and 1ml of TRIzol (Life Technologies, Gaithersburg, MD). The suspension was transferred to 2ml screw capped tube containing silica beads (IEPSA Medical diagnostics, South Africa) and ribolyzed (reciprocal shaker, Hybaid) at 6 W for 20 seconds. Thereafter the tube was cooled on ice for 2 minutes between pulses. The ribolysis was repeated for 3 cycles. The ribolysed samples were centrifuged at 13000rpm for 1min and the TRIZOL solution and cellular debris above the beads was transferred to a 2ml tube containing 300µl chloroform/isoamyl alcohol (24:1) (SigmaAldrich, St. Louis, Germany). The tube was inverted several times and then centrifuged at 13000rpm for 5 min. The top aqueous layer was transferred to a new 1.5ml tube and the crude RNA was precipitated with the addition of an equal volume of isopropanol (Merck, Darmstadt, Germany). The samples were then incubated at -20 °C overnight. The crude RNA was collected by centrifugation at 12000 x g, 30 min at 4 °C and the pellet was washed with 70% ethanol (Merck, Darmstadt, Germany). The crude RNA was collected by centrifugation at 12000 x g, for 10 min at 4°C, the 70% ethanol was aspirated and the RNA pellet was air-dried and dissolved in 89µl RNase-free water.

A3 RNA purification

RNA purification was done using RNeasy Mini kit (Qaigen) as per manufacturer's instructions. Briefly the RNA sample was adjusted to 100µl distilled water. A volume of 250µl absolute alcohol was added to 100µl RNA solution and mixed. The sample was transferred to the spin column with collection tube and centrifuged for 8000xg for 15 seconds. On-column digestion was performed using RNase-free DNase set (Qiagen) by adding 10µl DNase stock solution to 70µl buffer. The mix of 80µl was

added directly to the RNeasy easy column membrane and placed on a benchtop at room temperature for 15 minutes. After incubation, 350µl buffer RW1 was added to the column and centrifuged at ≥ 8000xg for 15 seconds. The flow-through was discarded, 500µl of RPE buffer was added and the column centrifuged for 2 minutes at ≥8000 xg to wash membrane. After the centrifugation 30 µl RNase free water directly to the column was placed in a 1.5 ml tube (supplied) and centrifuged for 1 min at ≥8000xg to elute the RNA. The RNA solution was stored at -80ºC until processing.

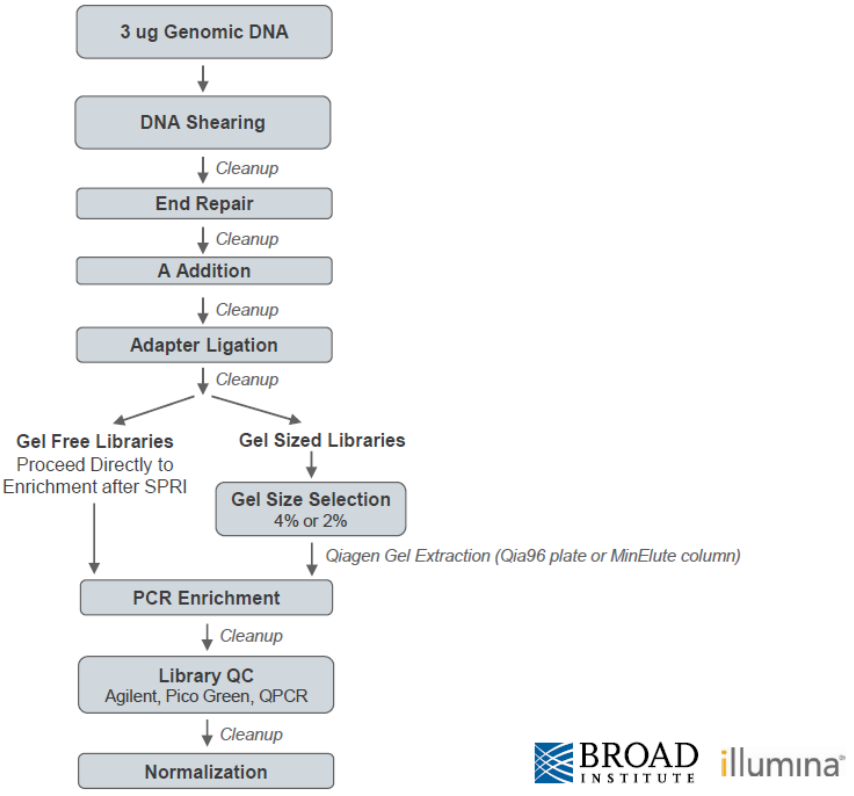


Figure A1: Sample preparatory workflow at Broad Institute

Appendix B: Data listings

B1 Genomic data: RSA114 and RSA184

Mycobacterium tuberculosis TB_RSA114.1

Organism Information

Description	None
Web Publication Date	2014-07-15
Genbank BioProject	228108
Taxonomy ID	1422030

Assembly Information

Assembly Size	4416700 nt
Sequencing Technology	Illumina
Coverage	119x
Scaffold count	29
Contig count	45

Annotation Information

Protein-coding Gene Count	4040

Structural Overview

	Length	Cov	GC
Genic	3993485 nt	90.07%	65.88%
Intergenic	438702 nt	9.93%	63.02%
Protein-coding	3991907 nt	90.03%	65.88%
5' UTR	329 nt	0.01%	61.70%
3' UTR	1249 nt	0.03%	61.97%

Length Distribution

	Min	Median	Mean	N50	Max
Gene	30 nt	840 nt	988.49 nt	1230 nt	11151 nt
mRNA	30 nt	840 nt	988.49 nt	1230 nt	11151 nt
CDS	30 nt	840 nt	988.10 nt	1230 nt	11151 nt
5' UTR	64 nt	85 nt	109.67 nt	180 nt	180 nt
3' UTR	268 nt	432 nt	416.33 nt	432 nt	549 nt
Intergenic Distance	1 nt	82 nt	142.67 nt	271 nt	5606 nt

Mycobacterium tuberculosis TB_RSA184.1

Organism Information

Description	None
Web Publication Date	2014-07-15
Genbank BioProject	228179
Taxonomy ID	1422100

Assembly Information

Assembly Size	4389272 nt
Sequencing Technology	Illumina
Coverage	57x
Scaffold count	67
Contig count	68

Annotation Information

Protein-coding Gene Count	4019

Structural Overview

	Length	Cov	GC
Genic	3971780 nt	90.14%	65.89%
Intergenic	432917 nt	9.86%	62.83%
Protein-coding	3970202 nt	90.10%	65.89%
5' UTR	329 nt	0.01%	61.70%
3' UTR	1249 nt	0.03%	61.97%

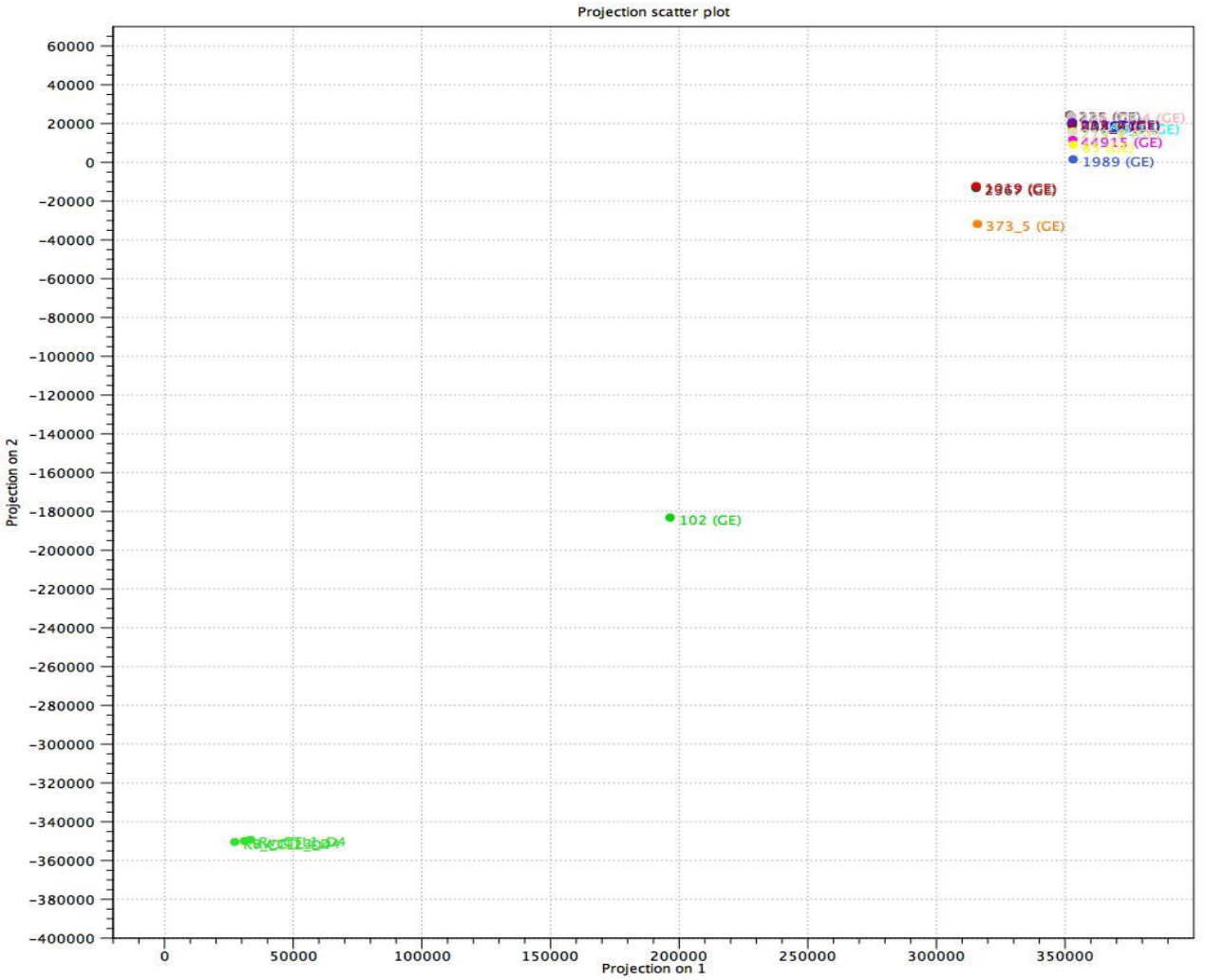
Length Distribution

	Min	Median	Mean	N50	Max
Gene	40 nt	843 nt	988.25 nt	1230 nt	11151 nt
mRNA	40 nt	843 nt	988.25 nt	1230 nt	11151 nt
CDS	40 nt	843 nt	987.86 nt	1230 nt	11151 nt
5' UTR	64 nt	85 nt	109.67 nt	180 nt	180 nt
3' UTR	268 nt	432 nt	416.33 nt	432 nt	549 nt
Intergenic Distance	1 nt	81 nt	140.65 nt	266 nt	5556 nt

B2 RNA quantity and quality

No.	Sample ID	RNA Reading	Unit	A260	A280	260/280	µL Volumes	Total ug	Sample	Profile
1	78	30.2	ng/µl	0.754	0.411	1.84	18	**0.5436**	RNA	MDR
2	85	175.1	ng/µl	4.378	2.162	2.02	18	**3.1518**	RNA	MDR
3	102	141.1	ng/µl	3.528	1.996	1.77	18	**2.5398**	RNA	XDR
4	213-1	117.3	ng/µl	2.932	1.53	1.92	18	**2.1114**	RNA	MDR
5	217-6	80.1	ng/µl	2.002	1.245	1.61	18	**1.4418**	RNA	XDR
6	225	198	ng/µl	4.949	2.586	1.91	18	**3.564**	RNA	XDR
7	226/11-4	50.5	ng/µl	1.264	0.693	1.82	18	**0.909**	RNA	XDR
8	231	60.6	ng/ul	1.516	0.806	1.88	18	**1.098**	RNA	MDR
8	358-8	76.4	ng/µl	1.911	1	1.91	18	**1.3752**	RNA	XDR
9	361	183.9	ng/µl	4.598	2.294	2	18	**3.3102**	RNA	XDR
10	373-5	10.4	ng/µl	0.261	0.23	1.14	18	**0.1872**	RNA	XDR
11	908-9	33.4	ng/µl	0.835	0.479	1.74	18	**0.6012**	RNA	XDR
12	1989	7.5	ng/µl	0.187	0.128	1.46	18	**0.135**	RNA	XDR
12	1019	29.1	ng/µl	0.728	0.447	1.63	18	**0.5238**	RNA	XDR
14	2238-11	151.2	ng/µl	3.779	1.933	1.95	18	**2.7216**	RNA	XDR
15	2367	50.5	ng/µl	1.262	1.152	1.1	18	**0.909**	RNA	MDR
16	44209/3	4.1	ng/µl	0.262	1.152	1.1	18	**0.909**	RNA	Susceptible
17	44915	136.2	ng/µl	3.405	1.755	1.94	18	**2.4516**	RNA	Susceptible

B3 Projection scatter plot of RNAsequenced samples



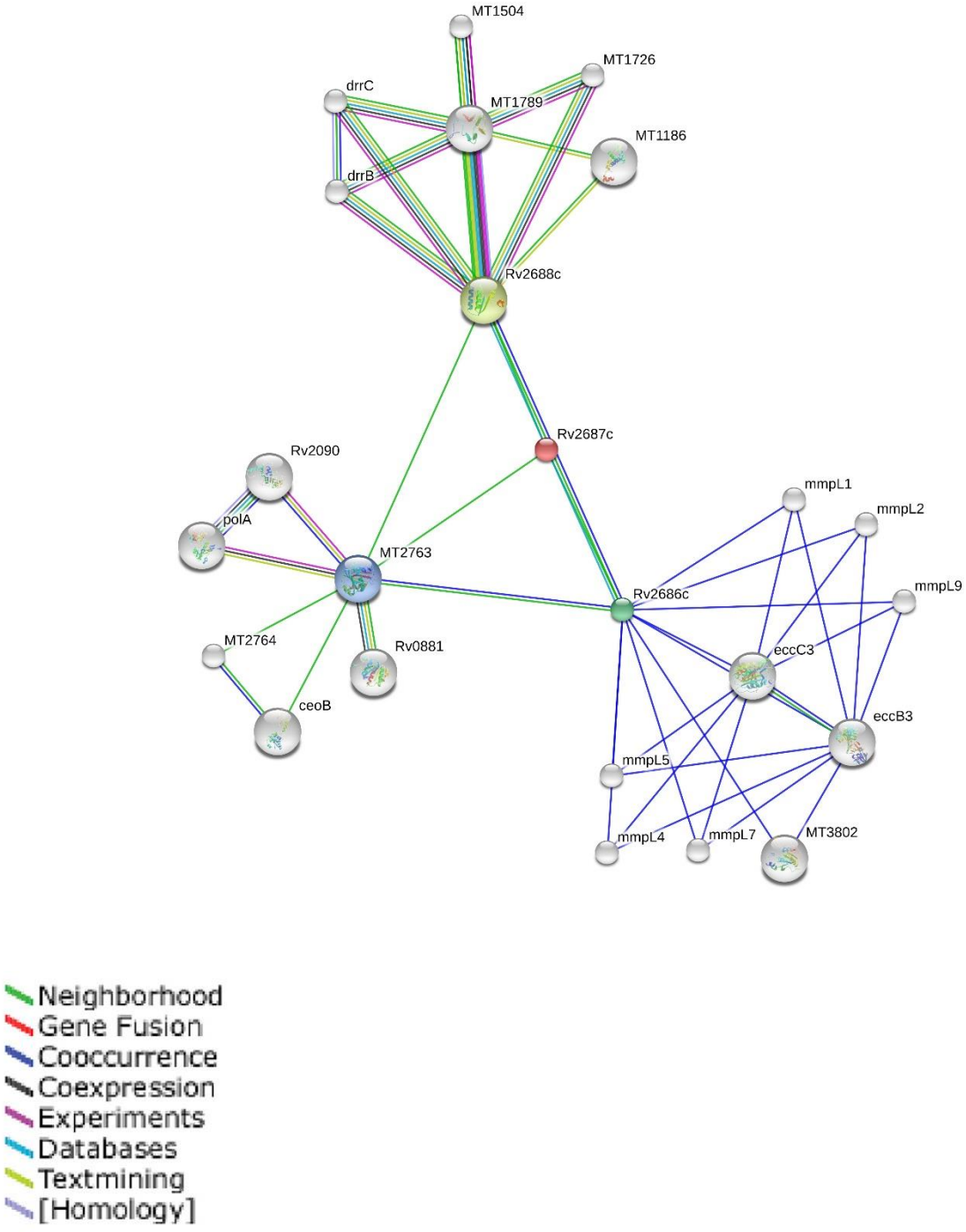
B4 Differentially expressed genes of drug efflux

Feature ID	Baggerley's test: LM_1989 vs Control normalized values - Weighted proportions fold change	Baggerley's test: LM_1989 vs Control normalized values - P-value	Baggerley's test: LM_1989 vs Control normalized values - Bonferroni	Baggerley's test: LM_2238 vs Control normalized values - Weighted proportions fold change	Baggerley's test: LM_2238 vs Control normalized values - P-value	Baggerley's test: LM_2238 vs Control normalized values - Bonferroni	Baggerley's test: LM_361 vs Control normalized values - Weighted proportions fold change	Baggerley's test: LM_361 vs Control normalized values - P-value	Baggerley's test: LM_361 vs Control normalized values - Bonferroni	Baggerley's test: LM_908-9 vs Control normalized values - Weighted proportions fold change	Baggerley's test: LM_908-9 vs Control normalized values - P-value	Baggerley's test: LM_908-9 vs Control normalized values - Bonferroni	Family
Rv0194	1.858	0.203223	1	4.293022	0.000776	1	1.058879	0.912767	1	2.640687	0.036745	1	ABC
mmpL2	3.326562	9.8E-13	4.01E-09	-2.99259	6.72E-06	0.02754	1.394509	0.083082	1	2.732583	7.12E-09	2.92E-05	RND
Rv1217c	1.279589	0.449271	1	2.706775	0.000767	1	1.422035	0.273604	1	1.857674	0.04643	1	ABC
Rv1218c	4.228787	3.39E-07	0.00139	2.810458	0.000571	1	5.002018	5.05E-09	2.07E-05	3.542137	1.32E-05	0.054073	ABC
Rv1258c	2.096391	0.014846	1	14.67594	0	0	1.972642	0.026528	1	1.133059	0.703525	1	MFS
Rv1456c	5.343018	1.27E-13	5.22E-10	2.102748	0.004085	1	2.315317	0.001021	1	3.043624	6.19E-06	0.025345	ABC
Rv1457c	-1.10791	0.634568	1	1.180712	0.425581	1	-1.09179	0.683202	1	-1.92085	0.004886	1	ABC
Rv1458c	1.130547	0.482915	1	5.248059	0	0	1.39019	0.053277	1	1.324767	0.100995	1	ABC
Rv1634	-2.31915	1.08E-05	0.044251	3.595124	0	0	-1.71663	0.00317	1	1.006696	0.968786	1	MFS
Rv1686c	3.064066	0.002034	1	4.974514	2E-06	0.008209	4.043103	6.13E-05	0.251098	4.079359	5.37E-05	0.220066	ABC
Rv1687c	-1.01326	0.963486	1	2.339011	0.000994	1	1.439777	0.18437	1	1.273277	0.38619	1	ABC
Rv2209	-2.24651	2.69E-07	0.001101	-1.42682	0.016121	1	-2.85304	1.32E-10	5.41E-07	-2.71019	7.31E-10	2.99E-06	ABC
Rv2686c	5.224613	3.85E-10	1.58E-06	4.675591	9.63E-09	3.94E-05	-1.18646	0.612703	1	3.239205	3.5E-05	0.143172	ABC
Rv2687c	4.070488	5.15E-06	0.021112	5.881589	1.06E-09	4.33E-06	1.418439	0.324653	1	3.132351	0.000359	1	ABC
Rv2688c	3.388027	7.55E-09	3.09E-05	2.622554	1.06E-05	0.043299	-2.75527	0.000366	1	2.542024	2.19E-05	0.089642	ABC

ABC=ATP-binding cassette; MFS=Major facilitator superfamily; RND=Resistance nodulation-cell division

B5 Protein-protein network of genes and interactions

Network of genes



Protein-protein interaction with scores

Node	Node 2	Neighbor-hood	Gene fusion	Phylogenetic co-occurance	Homology	Co-expression	Experi-ments	Data-base	Text-mining
Rv2688c	Rv2686c	0.899	0	0.778	0	0	0.491	0	0.987
eccC3	eccB3	0.859	0	0.781	0	0	0	0	0.967
drrC	drrB	0.859	0	0.781	0	0	0	0	0.894
Rv2688c	Rv1457c	0.705	0	0	0	0.093	0.248	0.339	0.203
Rc2688c	Rv1686c	0.705	0	0	0	0.093	0.248	0.339	0.212
Rv2688c	Rv2686c	0.859	0	0	0	0	0	0	0
Rv2686c	eccC3	0	0	0.694	0	0	0	0	0
Rv2686c	MmpL2	0	0	0.686	0	0	0	0	0
Rv2686c	MmpL4	0	0	0.727	0	0	0	0	0
eccB3	MmpL2	0	0	0.745	0	0	0	0	0
Rv2686c	eccB3	0	0	0.733	0	0	0	0	0

Low confidence: <0.4; Medium confidence: 0.4 to 0.7; High confidence: >0.7

Appendix C: Ethics certificate

UNIVERSITEIT VAN PRETORIA

UNIVERSITY OF PRETORIA

YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

18/11/2015

Lesibana Malinga

Department of Internal Medicine
Division of Infectious Diseases
University of Pretoria

Dear Lesibana Malinga

RE.: 206/2012 - Letter dated 16 November 2015

NUMBER	206/2012
TITLE OF THE PROTOCOL	Characterization of efflux pumps genes involved in second-line drug resistance of tuberculosis
PRINCIPAL INVESTIGATOR	Student Name & Surname: Lesibana Malinga Dept: Internal Medicine, Division of Infectious Diseases; University of Pretoria. Cell: 0723593170 E-Mail: lesibana.malinga@mrc.ac.za

We hereby acknowledge receipt of the following document:

- Extension for study until June 2016.

This will be processed in due course and filed.

With regards

Dr R Sommers

Dr Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria
•Tel:012-3541330 Fax:012-3541367 / 0866515924

•Web: //www.healthethics-up.co.za •H W Snyman Bld (South) Level 2-34

Appendix D: Publications based on thesis

Copies of the following publications are attached:

1. Malinga, L.A., Abeel, T., Desjardins, C., Dlamini, T., Cassell, G., Chapman, S., Birren, B., Earl, A., and Van der Walt, M. (2016) Draft genome sequence of two extensively drug-resistant strains of *Mycobacterium tuberculosis* belonging to the Euro-American S lineage. *Genome Announcement* 4(2), e01771-15
2. Malinga, L.A., Brand, J., Olorunju, S., Stoltz, A., and Van der Walt, M. (2016) Molecular analysis of genetic mutations among cross-resistant second-line injectable drugs reveals a new resistant mutation in *Mycobacterium tuberculosis. Diagnostic Microbiology and Infectious Disease* 81(4), 433-7.