Fitness of multi- and extensively drug-resistant *Mycobacterium tuberculosis* clinical strains

By

Charissa Camille Naidoo

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Laboratory Medicine & Medical Sciences, University of KwaZulu-Natal

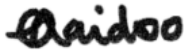
Supervisor: Prof. Manormoney Pillay

15 December 2015

DECLARATION

I, Charissa Camille Naidoo, declare that the work described in this thesis has not been submitted to UKZN or other tertiary institution for purposes of obtaining an academic qualification, whether by I or any other party. My contribution to the project was as follows:

i. Co-conceptualisation of research ideas
ii. Performance of all experimental work, data analysis and interpretation
iii. Writing up of individual manuscripts and thesis



15-Dec-2015

Charissa C. Naidoo — Date

For my parents, Michael and Evelyn Naidoo

ACKNOWLEDGEMENTS

- Dr. Manormoney Pillay, for her unwavering support and expertise during my postgraduate studies.

- Dr. Kogie Naidoo, for her mentorship during the CAPRISA Fellowship Programme.

- Professor William R. Jacobs, Prof. Michelle Larsen, Dr. Linda-Berney Meyer and the rest of Jacobs' Lab, Albert Einstein College of Medicine (AECOM), for hosting me from 1 December 2013 – 30 April 2014.

- Dr. Bing Chen (AECOM) for BSL3+ training

- My funders (2013-2015):
    - The College of Health Sciences
    - Medical Research Council (MRC) of South Africa
    - National Research Foundation (NRF) of South Africa
    - Columbia University-Southern African Fogarty AITRP
    - K-RITH - Connect Africa Scholarships
    - Department of Science and Technology – Women In Science

- Staff and students of Medical Microbiology & Infection Control, DDMRI.

- Prof. Balakrishna Pillay (Department of Microbiology, UKZN) for providing access to the Bio-Plex Instrument and Ms. Nontobeko Mvubu and Mrs. Dheshni Sewrathan (UKZN) for assistance.

- My parents, family and friends for their constant support and encouragement.

- Jesus, for always being faithful in every aspect of my life.

PRESENTATIONS

Naidoo CC, Pillay M.

Growth kinetics of multi- and extensively drug-resistant clinical strains of the F15/LAM4/KZN family of Mycobacterium tuberculosis

3rd TB Conference (ICC, Durban, South Africa), 12-15 June 2012 (Poster Presentation). *Winner of Discovery Health Award for Best Poster Presentation*

Naidoo CC, Pillay M.

Growth kinetics of multi- and extensively drug-resistant clinical strains of the F15/LAM4/KZN family

College of Health Sciences Research Symposium (UKZN, Durban, South Africa), 12 - 13 September 2012 (Poster Presentation)

Naidoo CC., Pillay M.

Fitness of MDR and XDR clinical strains of the F15/LAM4/KZN and Beijing families

K-RITH Scientific Symposium (The Hilton, Durban, South Africa), 10-11 October 2012 (Poster Presentation)

Naidoo CC, Pillay M.

The biological fitness of MDR & XDR clinical strains of the F15/LAM4/KZN and Beijing families

43rd Union World Conference on Lung Health (Kuala Lumpur, Malaysia), 13-17 November 2012 (Poster Presentation)

Naidoo CC, Pillay M

Amplified growth in co-culture with *Mycobacterium tuberculosis* strains suggests *in vitro trans*-complementation

18th Biennual Conference of the South African Society of Microbiology (Bela-Bela, South Africa), 23-27 November 2013 (Oral Presentation)

Naidoo CC, Pillay M

Fitness of MDR and XDR tuberculosis

CAPRISA (Durban, South Africa), 18 October 2013 (Oral presentation)

Naidoo CC, Pillay M.

Whole genome comparison of drug-resistant clinical strains from South Africa

5th Conference of the Union Asia Pacific Region (Sydney, Australia), 31 August – 2 September 2015 (Poster Presentation)

Naidoo CC. Pillay M, Naidoo K.

Mycobacterium tuberculosis isolates from HIV co-infected patients retain biological fitness at relapse

5th Conference of the Union Asia Pacific Region (Sydney, Australia), 31 August – 2 September 2015 (Poster Presentation)

PUBLICATIONS

Naidoo CC, Pillay M. Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. *Clin Microbiol Infect* 2014; 20: O361-369.

Naidoo CC, Pillay M. Fitness-compensatory mutations facilitate the spread of drug-resistant F15/LAM4/KZN and F28 *Mycobacterium tuberculosis* strains in KwaZulu-Natal, South Africa. *Submitted for publication*

Naidoo CC, Pillay M. Drug-resistant F15/LAM4/KZN *Mycobacterium tuberculosis* strains exhibit increased TNF-α and MIP-1β induction and high replication in THP-1 macrophages. *Manuscript in preparation*

Naidoo CC, Pillay M, Naidoo K. *Mycobacterium tuberculosis* isolates from HIV co-infected patients retain *in vitro* fitness at relapse. *Manuscript in preparation*

ETHICAL STATEMENT

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BE258/13).

TABLE OF CONTENTS

DECLARATION i

DEDICATION ii

ACKNOWLEDGEMENTS iii

PRESENTATIONS iv

PUBLICATIONS vi

ETHICAL STATEMENT vii

TABLE OF CONTENTS viii

LIST OF TABLES x

LIST OF FIGURES xii

LIST OF ABBREVIATIONS xiv

ABSTRACT xvi

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW 1

1.1 Introduction 2

1.2 Literature Review 5

1.2.1 *M. tuberculosis* strain diversity 5

1.2.1.1 Strain classification 5

1.2.1.2 Predominant genotypes in South Africa 6

1.2.2 Mechanisms of drug resistance 7

1.2.2.1 Isoniazid, rifampicin, ethambutol and pyrazinamide as first-line drugs 9

1.2.2.2 Fluoroquinolones and aminoglycosides as second-line drugs 10

1.2.2.3 Implications of drug resistance 11

1.2.3 Fitness costs, epistasis and compensatory mutations 11

1.2.3.1 Drug resistance-conferring mutations and fitness costs 12

1.2.3.2 Competitive costs of drug resistance 14

1.2.3.3 Virulence and immunogenicity 14

1.2.4 Hypothesis 16

1.2.5 Research Aim 16

1.2.6 Objectives 16

1.2.7 Scope of thesis 16

CHAPTER TWO: Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis* **17**

CHAPTER THREE: Whole-genome sequencing reveals fitness-compensatory mutations in F15/LAM4/KZN and F28 *Mycobacterium tuberculosis* genotypes **37**

CHAPTER FOUR: Drug-resistant F15/LAM4/KZN *Mycobacterium tuberculosis* strains exhibit increased TNF-α and MIP-1β induction and high replication in THP-1 macrophages **61**

CHAPTER FIVE: *Mycobacterium tuberculosis* isolates from HIV co-infected patients retain *in vitro* fitness at relapse **80**

CHAPTER SIX: SYNTHESIS AND FUTURE WORK **96**

APPENDIX **103**

REFERENCES **121**

LIST OF TABLES

Table 1.1	Anti-TB drugs and mechanisms of drug resistance	**8**
Table 2.1	Genotype and drug susceptibility of clinical strains used in the study	**20**
Table 2.2	Primer pairs and sequencing primer used for PCR amplification and DNA sequencing	**23**
Table 2.3	Distribution of nonsynonymous and synonymous mutations in selected clinical strains	**29**
Table 3.1	Genotype, drug susceptibility and sequencing statistics of clinical strains of *M. tuberculosis*	**43**
Table 3.2	Polymorphisms in genes associated with drug resistance	**47**
Table 3.3	Minimum inhibitory concentrations for isoniazid and ethambutol in clinical strains	**49**
Table 3.4	Polymorphisms in known or putative drug efflux pumps in clinical strains	**50**
Table 3.5	Polymorphisms in genes encoding *M. tuberculosis* virulence factors	**52**
Table 4.1	*M. tuberculosis* strain profiles	**64**
Table 5.1	Patient epidemiological data	**87**
Table 5.2	Drug resistance profiles of paired isolates	**87**
Table S2.1	Baseline (Day 0) CFU/ml readings corresponding to an OD_{600} of 0.015 in growth assays	**104**

Table S2.2 Relative fitness index of drug-resistant strains in comparison to drug-susceptible strains and H37Rv on day 24 **105**

Table S2.3 Significant *p*-values for the OD and CFU competition assays **106**

Table S3.1 Oligonucleotide primers used for SNP verification by Sanger Sequencing **115**

Table S3.2 Summary statistics clinical strains mapped to closely-related reference Genomes **116**

Table S3.3 Summary statistics for clinical strains mapped to H37Rv reference genome (4,411,532 bp) **117**

Table S3.4 Polymorphisms in re-sequenced V1435 relative to previously sequenced KZN 1435 **118**

Table S3.5 Polymorphisms in re-sequenced KZN605 relative to previously sequenced KZN 605 **119**

LIST OF FIGURES

Figure 1.1	Diverse strains contribute to drug resistance in KwaZulu-Natal	**6**
Figure 1.2	The influence of Darwinian fitness on the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR-) tuberculosis (TB)	**13**
Figure 2.1	Optical density (OD) measurements and colony forming units (CFU) counts for competitive KZN strain pairs	**26**
Figure 2.2	Mean competitive relative fitness for KZN, Beijing, F11 and F28 drug-resistant strains	**27**
Figure 3.1	Phylogenetic relationships of clinical strains	**42**
Figure 3.2	Venn diagrams depicting the distribution of non-synonymous single nucleotide polymorphisms in clinical strains	**45**
Figure 3.3	Functional categorisation of genes with non-synonymous SNPs (nSNPs) in different strain families	**51**
Figure 4.1	Uptake and intracellular growth indices of *M. tuberculosis* strains in THP-1 macrophages	**67**
Figure 4.2	Cytokines detected in the supernatants of *M. tuberculosis*-infected THP-1 macrophages at 4 hours	**69**
Figure 4.3	Cytokines detected in the supernatants of *M. tuberculosis*-infected THP-1 macrophages on day 5 only	**71**
Figure 4.4	Cytokines detected in the supernatants of *M. tuberculosis*-infected THP-1 macrophages on day 5	**72**

Figure 5.1 Optical density (OD_{600}) measurements for F15/LAM4/KZN (A), Beijing (B) and X3 (C) paired isolates **89**

Figure 5.2 Comparative growth of paired isolates as measured by colony forming units (CFU) counts **90**

Figure S2.1 Optical density measurements of clinical strains in independent cultures **107**

Figure S2.2 Absorbance measurements of clinical strains in independent cultures in the Alamar Blue assay **108**

Figure S2.3 Optical density measurements for susceptible strains paired with MDR (A) and XDR (B) F15/LAM4/KZN strains in competitive growth assays **109**

Figure S2.4 Optical density measurements for susceptible strains paired with Beijing MDR strains R35 (A) and R283 (B) in competitive growth assays **110**

Figure S2.5 Optical density measurements for F11 (A) and F28 (B) clinical strains in competitive growth assays **111**

Figure S2.6 Colony forming units counts for susceptible strains paired with MDR (A) and XDR (B) F15/LAM4/KZN strains in competitive growth assays **112**

Figure S2.7 Colony forming units counts for Beijing susceptible strains paired with MDR strains R35 (A) and R283 (B) in competitive growth assays **113**

Figure S2.8 Colony forming units counts for F11 (A) and F28 (B) clinical strains in competitive growth assays **114**

Figure S5.1 IS*6110*-RFLP images for patient isolates **120**

LIST OF ABBREVIATIONS

Abbreviation	Abbreviated term
ANOVA	Analysis of variance
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CFU	Colony forming units
CTAB	Cetyltrimethylammoniumbromide
dH_20	Distilled water
DMSO	Dimethyl suphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxydribonucleotide triphosphate
DS	Drug-susceptible
EMB	Ethambutol
ETH	Ethionamide
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HIV	Human Immunodeficiency Virus
IFN-γ	Interferon-gamma
IL	Interleukin
INH	Isoniazid
KAN	Kanamycin
KZN	KwaZulu-Natal
MCP-1	Monocyte chemotactic protein-1
MDR	Multidrug-resistant
MIC	Minimum inhibitory concentration
MIP-1β	Macrophage inflammatory protein -1β
mRNA	messenger RNA
MTBC	*Mycobacterium tuberculosis* complex
nSNP	Non-synonymous single nucleotide polymorphism
OADC	Oleic-acid-dextrose-albumin
OD_{600}	Optical density at 600nm
OFX	Ofloxacin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PE	Pro-Glu
PPE	Pro-Pro-Glu
PGRS	Polymorphic GC-rich repetitive sequences
RIF	Rifampicin
RNA	ribonucleic acid
RRDR	Rifampicin Resistance Determining Region
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SNP	single nucleotide polymorphism
sSNP	synonymous single nucleotide polymorphism
TB	Tuberculosis
TBE	Tris-borate-EDTA
TBDReaMDB	TB Drug Resistance Mutation Database
TE	Tris-EDTA
TNF-α	Tumor necrosis factor-alpha
WGS	Whole-genome sequencing
WHO	World Health Organisation
XDR	Extensively drug-resistant

ABSTRACT

The biological fitness of a pathogen is defined as its ability to reproduce, survive, cause disease and be transmitted. Drug-resistant *M. tuberculosis* isolates often exhibit reduced competitive ability against susceptible isolates in the absence of drug selection. However, compensatory mutations may, to some extent, offset fitness costs associated with resistance-conferring mutations. Most studies to date have focused on the fitness of isogenic laboratory strains or globally-relevant strains. Fewer studies have addressed the fitness of endemic strains which propagate in multidrug and extensively drug-resistant forms (MDR and XDR, respectively). In this study, the fitness of four genotypes which drive the transmission of tuberculosis (TB) in KwaZulu-Natal (KZN), South Africa was explored. This included F15/LAM4/KZN genotype strains, some of which were involved in the notorious XDR-TB outbreak in Tugela Ferry, KZN as well as Beijing, F11 and F28 genotype strains. Drug-resistant F15/LAM4/KZN and F28 genotype strains demonstrated increased *in vitro* fitness, whilst Beijing and F11 MDR strains had markedly reduced fitness. These findings correlated with whole-genome and Sanger sequencing data which revealed the presence of low/no-cost resistance-conferring mutations as well as intra- and intergenic compensatory mutations in drug-resistant F15/LAM4/KZN and F28 strains, respectively. In contrast, high cost *katG* mutations and no accompanying compensatory mutations were identified in Beijing and F11 MDR strains. During co-culture experiments, a novel observation was made whereby susceptible and resistant strains exhibited synergistic growth compared with axenic cultures i.e. *in vitro trans*-complementation. Drug-resistant F15/LAM4/KZN strains did not undergo fitness costs in THP-1 macrophages and produced increasing levels of TNF-α which may enhance tissue destruction and dissemination to other hosts. Although demonstrating similar intracellular fitness, susceptible and MDR Beijing, F11 and F28 strains induced heterogeneous cytokine responses. Thus, the lack of a direct relationship between bacillary burden and cytokine responses indicates that this diversity results from strain heterogeneity. Relapse isolates, including those from F15/LAM4/KZN and Beijing genotypes, may reactivate without any changes in biological fitness, thereby retaining the potential to re-transmit. Taken together, the enhanced fitness of drug-resistant F15/LAM4/KZN and F28 genotype strains is due to the presence of beneficial mutations, while the reduced fitness of MDR Beijing and F11 strains is associated with high cost mutations.

CHAPTER ONE

Introduction and Literature review

1.1 INTRODUCTION

The World Health Organization (WHO) reported approximately 9.6 million cases of tuberculosis (TB) globally in 2014 (WHO 2015). *Mycobacterium tuberculosis* is the etiologic agent of TB and is spread from person-to-person in the form of aerosolised droplets. Although one third of the human population is thought be infected with this pathogen, most individuals develop latent or asymptomatic TB (Lin and Flynn, 2010) and only 5-10% of infected individuals progress to active disease. Co-infection with the human immunodeficiency virus (HIV) and the rise in drug resistance pose significant threats to the control of TB (Raviglione and Smith, 2007; Chindelevitch et al. 2015), particularly in sub-Saharan Africa. In South Africa, nearly 80 % of the population are latently infected with TB and over 70 % of individuals with active disease are co-infected with HIV (National Strategic Plan 2012-2016).

A small population (2-5 %) of treated TB patients go on to develop recurrence, further complicating TB epidemics (Weis et al. 1994; Tuberculosis Trials Consortium, 2012). This may be due to reactivation of the initial infection (termed relapse) or reinfection with another *M. tuberculosis* strain (Sonnenberg et al. 2001; Verver et al. 2005). Using DNA fingerprinting, a recent study showed that relapse arose early after completion of anti-TB treatment while reinfection occurred after 12 months and constituted nearly 50 % of recurrent TB cases (Marx et al. 2014). Retreatment cases are often associated with increased costs which may prove problematic for developing countries with limited national TB budgets (Panjabi et al. 2007).

The diagnosis and treatment of TB remains a growing challenge, especially in patients who are infected with drug resistant strains of *M. tuberculosis*. Drug resistance can arise as a result of acquired resistance i.e. the selection of resistant *M. tuberculosis* populations during inadequate treatment regimens or by primary resistance i.e. infection with a resistant TB strain. In 2014, 4700 (3700-5900) new TB cases in South Africa were attributed to multidrug resistance (MDR) (WHO, 2015). Many studies have documented primary resistance as a major contributor to the increase of MDR-TB in South Africa (van Rie et al. 1999; Victor et al. 2007; Strauss et al. 2008; Johnson et al. 2010; Cox et al. 2010; Royce et al. 2013; Gandhi et al. 2014).

Extensively drug-resistant (XDR)-TB was first discovered in South Africa in 2006 (CDC, 2006). To put into perspective the enormity of XDR-TB in South Africa, incidence rates in this country are

said to be higher than total TB incidence rates in some low-incidence countries e.g. the United States (Lim et al. 2015). Treatment for MDR-TB can last up to 20 months, compared with 6 months for drug-susceptible TB (WHO 2015) and becomes progressively difficult in patients with XDR-TB and of recent, totally drug-resistant (TDR)-TB, as these regimens require the use of added drugs, posing considerable side-effects to patients (Banerjee et al. 2008; Dheda et al. 2014). If implemented properly, new drugs (i.e. bedaquiline) and treatment regimens may improve MDR- and XDR-TB outcomes, especially in South Africa which, together with the Russian Federation represent 75 % of MDR-TB patients currently being treated with bedaquiline (WHO, 2015).

Whereas most bacteria exploit mobile genetic elements for the propagation of drug resistance, *M. tuberculosis* acquires drug resistance through chromosomal mutations and gene rearrangements (Sandgren et al. 2009). Drug-resistant strains often demonstrate decreased competitive ability (or 'fitness costs') against susceptible strains in the absence of the drug (Gagneux et al. 2006). This 'fitness cost' is dependent on strain genetic background and specific drug resistance-encoding mutations which may be improved by compensatory mechanisms (Cohan et al. 1994; Schrag et al. 1997; Bottger et al. 1998; Reynolds 2000; Bjorkman et al. 2001; Maisnier-Patin and Andersson, 2004; Gagneux et al. 2006). Compensatory mutations play a significant role in the transmission of MDR and XDR-TB strains (Comas et al. 2012; de Vos et al. 2013; Casali et al. 2014; Cohen et al. 2015) and represent a key area of interest in TB research.

Much of the sequence diversity in *M. tuberculosis* is ascribed to inherent and acquired drug resistance (reviewed in Köser et al. 2012), with various public databases providing an informative platform for comparative analysis of resistance-encoding mutations in global *M. tuberculosis* strains. While whole-genome sequencing (WGS) efforts have substantially improved our understanding of compensatory mutations, transmission chains, estimation of mutation rates and phylogenetic classification, this method can be limited if not supported by additional data which exemplify clinical properties of *M. tuberculosis* strains (Takiff and Feo, 2015).

Most studies to date have focused on the fitness of isogenic laboratory strains or globally-relevant strains. Fewer studies have addressed the fitness of endemic strains which propagate in MDR and XDR forms i.e. the F15/LAM4/KZN genotype which was responsible for the XDR-TB outbreak in Tugela Ferry, KwaZulu-Natal in 2005 (Gandhi et al. 2006). This is the first study investigating the *in vitro* fitness of the F15/LAM4/KZN strains, as well as other relevant, genotype families including

Beijing, F11 and F28. These genotypes circulate within the KwaZulu-Natal Province– a region of South Africa with the second highest number of inhabitants (~10.3 million) of which 11.2% is infected with HIV (South African Statistics, 2015).

While the detection of low- or no- cost mutations and compensatory mutations among clinical *M. tuberculosis* strains has provided insight into drug resistance and fitness, this subject remains incompletely studied**.** Using clinically-relevant strains from KZN, South Africa, this study focused on the identification of resistance-conferring mutations and/or compensatory mutations and establishing their role in biological fitness. A better understanding on the fitness of MDR and XDR clinical strains is necessary for the improvement of strategies to interrupt their transmission.

1.2. LITERATURE REVIEW

1.2.1 *M. tuberculosis* strain diversity

Although the *M. tuberculosis* genome is more highly conserved than other pathogenic bacteria (Achtman, 2008), recent data supports that *M. tuberculosis* complex (MTBC) strains are more diverse than previously believed (Gagneux et al. 2007). The advent of genotyping allowed the identification of several polymorphisms in the *M. tuberculosis* genome, including single nucleotide polymorphisms (SNPs), large sequence polymorphisms (LSPs) and polymorphisms associated with repetitive sequences (Brown et al. 2010).

1.2.1.1 *Strain classification*

For a long time, IS*6110* restriction fragment length polymorphism (RFLP) was considered as the gold standard for genotyping of MTBC strains. One of the noteworthy features of this method was its ability to characterise successful strains, based on the quantity and geographical distribution of associated TB cases (van Soolingen, 2001; Mathema et al. 2006; Barnes and Donald, 2009). Although RFLP typing is still used today, newer genotyping methods utilising polymerase chain reaction (PCR) have taken precedence. These include spacer-oligo typing (Spoligotyping) and mycobacterial interspersed repetitive unit variable number tandem repeat (MIRU-VNTR) typing (Supply et al. 2000; Supply et al. 2001). Despite their usefulness in epidemiological studies, these standard genotyping techniques are variable in terms of their discriminative power and have a tendency for convergent evolution (Comas et al. 2009).

Given that horizontal gene transfer does not occur with MTBC and deleted regions within a particular strain cannot be regained, another method known as genomic deletion analysis was developed to classify strains on account of LSPs. Based on this analysis, MTBC was later categorised into seven major lineages (Hirsch et al. 2004; Mostowy et al. 2004; Tsolaki et al. 2004; Gagneux et al. 2006), with Lineages 2 (Beijing) and 4 (Euro-American) being the most geographically dispersed and virulent (Coscolla and Gagneux, 2014). Similar to other genotyping methods, LSPs fell short during phylogenetic classification of strains, with the introduction of whole-genome sequencing (WGS) providing a more robust measure of phylogenetic diversity. This next-generation sequencing technology currently exists as the new ‘gold standard’ for *M. tuberculosis* strain typing (Ford et al. 2012).

1.2.2.2 *Predominant genotypes in South Africa*

The overrepresentation of specific genotype families among cases of drug resistance and transmission is of paramount importance in relation to TB control regimens (Marais et al. 2006). In South Africa, the majority of MDR- and XDR-TB cases were reported in KwaZulu-Natal (KZN), Eastern Cape and Western Cape Provinces (NHLS, 2009). Upon closer inspection, a clear association was shown between specific genotypes and the appearance of XDR-TB in these provinces, probably as a result of selection by standard treatment regimens (Muller et al. 2013). Figure 1.1 depicts the distribution of drug resistance amongst diverse genotype families detected in KZN (Cohen et al. 2015), demonstrating a strong association of the F15/LAM4/KZN genotype with XDR-TB cases in the KZN Province. This is in agreement with multiple retrospective and/or prospective studies which report similar findings (Chihota et al. 2012; Muller et al. 2013; Gandhi et al. 2014).

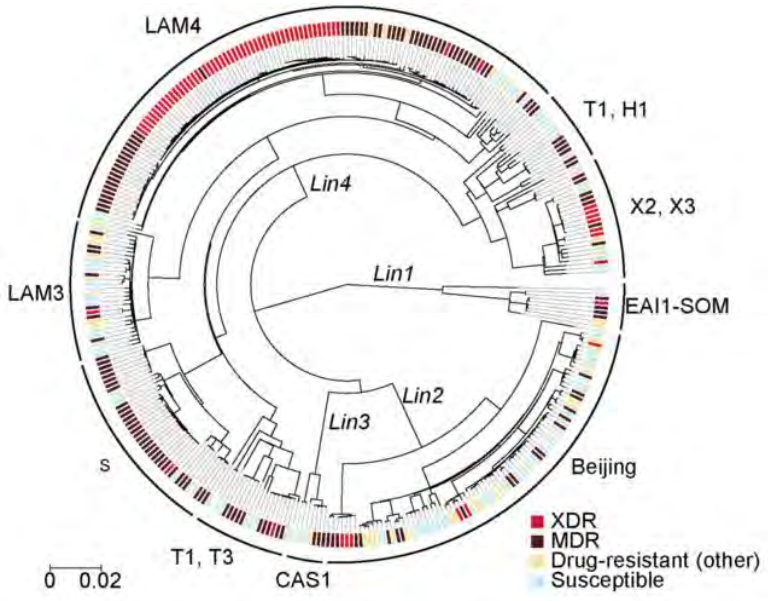


Figure 1.1 Diverse strains contribute to drug resistance in KwaZulu-Natal. Midpoint rooted maximum-likelihood phylogeny of 340 *M. tuberculosis* isolates. Four of the seven known *M. tuberculosis* lineages were identified: CAS (Lin1), Beijing (Lin2), EAI (Lin3), and Euro-American (Lin4). Digital spoligotyping identified 17 unique spoligotypes in the dataset; spoligotypes are shown on this figure if they are represented by three or more strains. Phenotypic XDR, MDR, poly- and monodrug resistance (labelled "Drug resistant other"), and pansusceptible strains are indicated by coloured tick marks at the tip of each leaf node (Cohen et al. 2015)

The F15/LAM4/KZN genotype was responsible for the Tugela Ferry XDR-TB outbreak, whereby 52 of 53 HIV- co-infected patients succumbed to death within a median time of 16 days (Gandhi et al. 2006). In a more recent examination, LAM4 strains (some of which represented Tugela Ferry clone members) were discovered to mutate at an astonishing rate of 0.61 SNPs per genome per year – a rate higher than any other reported for *M. tuberculosis* strains. Similar to a recent study by Gandhi et al. (2014), Figure 1.1 illustrates the association of the S genotype with MDR-TB (Cohen et al. 2015).

The Beijing genotype was first described by van Soolingen et al. (1995) and belongs to principle genetic group 1 on the basis of its *katG* 463Leu and *gyrA* 95Thr markers (Sreevatsan et al. 1997). The widespread distribution of Beijing strains (Bifani et al. 2002) can be accredited to its increased virulence in macrophages and animal models (Manca et al. 1999; Zhang et al. 1999; Manca et al. 2001; Li et al. 2002; Lopez et al. 2003; Tsenova et al. 2005). The LAM3 genotype on the other hand, is found distributed across 4 continents and 25 countries worldwide, and is a major contributor to TB in South Africa, particularly in the Western Cape Province (Victor et al. 2004). A C-T nucleotide substitution at codon 491 of the *rrs* gene found not to be associated with streptomycin resistance, is actually a useful phylogenetic marker for the F11 genotype (Victor et al. 2001).

1.2.2 Mechanisms of drug resistance

The rising epidemic of drug-resistant TB has compromised treatment strategies in various regions around the globe (WHO, 2015). Multidrug-resistant TB is caused by strains resistant to first-line anti-TB drugs, isoniazid (INH) and rifampicin (RIF) (WHO, 2015). Extensively drug-resistant TB is defined as resistance to any fluoroquinolone and injectable drug in addition to MDR-TB (WHO, 2015). The treatment of XDR-TB requires extended treatment times with expensive and less-effective second-line drugs, often causing adverse side effects in patients (Pietersen et al. 2014). Improved understanding of the mechanisms of drug resistance may help identify novel drug targets and enhanced methods of detecting drug resistance (Palomino and Martin, 2014). In the sections that follow, mechanisms of action and associated resistance to key drugs will be discussed. Table 1.1 provides a summary of the mechanisms of drug resistance in current and newly-discovered drugs with anti-TB activity (Müller et al. 2013).

Table 1.1 Anti-TB drugs and mechanisms of drug resistance (adapted from Müller et al. 2013)

Drug	Gene or gene region	Natural function of gene	Role in resistance formation when mutated
Isoniazid	*ahpC*	Alkyl hyperperoxide reductase	Compensatory mutations
	fabG	3-Oxacyl-thioester reductases	Unknown
	fadE24	Involved in fatty acid β-oxidation	Unknown
	inhA	Enoyl reductase	Alteration of drug target
	inhA promoter	Regulation of expression of *inhA*	Overexpression of drug target
	iniA	Efflux pump associated	Altered efflux pump activity
	katG	Catalase/peroxidase	Elimination of pro-drug conversion
Rifampicin	*rpoA*	α-Subunit of RNA polymerase	Compensatory mutations
	rpoB	β-Subunit of RNA polymerase	Alteration of drug target
	rpoC	β'-Subunit of RNA polymerase	Compensatory mutations
Pyrazinamide	*pncA*	Nicotinamidase	Elimination of pro-drug conversion
Streptomycin	*gidB*	7-Methylguanosine methyltransferase	Alteration of drug target
	rpsL	S12 ribosomal protein	Alteration of drug target
	rrs	16S rRNA	Alteration of drug target
Ethambutol	*embA*	Arabinosyl transferase	Alteration of drug target
	embB	Arabinosyl transferase	Alteration of drug target
	embC	Arabinosyl transferase	Alteration of drug target
	embR	Regulator of *embCAB* operon expression	Overexpression of drug target
	iniA	Efflux pump associated	Altered efflux pump activity
	rmlD	dDTP-4-dehydrorhamnose reductase	Unknown
Fluoroquinolones	*gyrA*	DNA gyrase	Alteration of drug target
	gyrB	DNA gyrase	Alteration of drug target
Kanamycin/amikacin	*rrs*	16S rRNA	Alteration of drug target
	rrs	16S rRNA	Compensatory mutations
Capreomycin/viomycin	*tlyA*	rRNA methlytransferase	Alteration of drug target
	rrs	16S rRNA	Alteration of drug target
Ethionamide	*inhA*	Enoyl reductase	Alteration of drug target
	inhA promoter	Regulation of expression of *inhA*	Overexpression of drug target
Para-amino salicylic acid	*thyA*	Thymidylate synthase A	Elimination of pro-drug conversion
PA-824 and OPC-67683	*Rv3547*	Hypothetical 16.4 kDa	Alteration of drug target
TMC207	*atpE*	ATP synthase	Alteration of drug target

1.2.2.1 *Isoniazid, rifampicin, ethambutol and pyrazinamide as first-line drugs*

Introduced in the early 1970s, RIF is still currently used as an effective first-line drug against TB. RIF exerts its activity against both replicating and non-replicating (slow metabolising) bacteria, by binding to the beta (β) subunit of RNA polymerase, thereby impeding the elongation of mRNA (Blanchard, 1996). Mutations conferring RIF-resistance primarily occur in an 81-bp region of the *rpoB* gene, also known as the rifampicin resistance-determining region (RRDR) (Ramaswamy et al. 1998), although mutations in other regions of *rpoB* have been described as well (Heep et al. 2001; Yuen et al. 2011). Single nucleotide polymorphisms in codons in 516, 526 and 531 are most frequently observed in RIF-resistant clinical isolates (Caws et al. 2006; Somoskovi et al. 2001). Nearly all RIF-resistant isolates have additional resistance to other drugs, particularly INH. Based on this finding, RIF is considered a representative marker for MDR-TB (Blanchard, 1996).

Isoniazid is a pro-drug that becomes activated by the action of catalase-peroxidase encoded by the *katG* gene in *M. tuberculosis* (Zhang et al. 1992). It acts to prevent mycolic acid synthesis in metabolically-active, replicating bacteria, via the NADH-dependent enoyl-acyl carrier protein reductase which is produced by *inhA* (Rawat et al. 2003). Whilst resistance to RIF occurs in *rpoB* alone, INH-resistance develops as a result of mutations in one or more genes including *katG*, *inhA*, *kasA*, *ahpC* and *ndh*. The S315T *katG* mutation is most frequently detected in clinical isolates and is also associated with a high level of INH resistance (MIC > 1 μg/ml) (Vilchèze et al. 2007; Fenner et al. 2012). Some *M. tuberculosis* isolates possess mutations in the *inhA* promoter region, which act to overexpress InhA or reduce its affinity for the INH-NAD adduct (Rozwarski et al. 1998). Moreover, *inhA* mutations have been implicated in resistance to ethionamide (ETH) - a second-line drug which shares structural similarities and a common target site to INH (Banerjee et al. 1994; Larsen et al. 2002).

Combinations of mutations in *inhA* regulatory and coding regions were recently shown to induce both high-level INH resistance and cross-resistance to ETH (Machado et al. 2013). While the involvement of genes and intergenic regions including *kasA*, *oxyR-ahpC* and *furA-katG* in INH resistance remains debateable, mutations in *ndh* have been shown to confer co-resistance to INH and ETH (Miesel et al. 1998; Vilcheze et al. 2005; Hazbón et al. 2006; Ando et al. 2011). A synonymous *mabA* mutation was recently shown to cause INH resistance via the overexpression of *inhA* in *M. tuberculosis* isolates (Ando et al. 2014).

Ethambutol is a bacteriostatic agent which hampers the biosynthesis of arabinogalactan in replicating bacteria (Takayama and Kilburn, 1989). The arabinosyl transferase enzyme is encoded by three *M. tuberculosis* genes (*embCAB*), which are arranged in an operon (Mikusová et al. 1995). Ethambutol resistance is commonly associated with the *embB* mutations, specifically those in codon 306 (Telenti et al. 1997; Sreevatsan et al. 1997), however some studies have reported its occurrence in ethambutol susceptible isolates as well (Ahmad et al. 2007). Although insufficient for causing high-level ethambutol resistance, *embB*306 mutations in combination with others i.e. those in *embC*, *Rv3806c* and *Rv3792* can increase EMB resistance, thereby resulting in a range of ethambutol MICs depending on the type and number of mutations (Safi et al. 2013).

Pyrazinamide acts against semi-dormant bacteria present in acidic environments i.e. TB lesions (Mitchison, 1985) by inducing cellular damage (Zhang and Mitchison, 2003). Similar to INH, PZA is a pro-drug which requires conversion into its active form – pyrazinoic acid (Konno et al. 1967; Scorpio and Zhang, 1996). This is facilitated by the pyrazinamidase/nicotinamidase enzyme encoded by the *pncA* gene (Konno et al. 1967; Scorpio and Zhang, 1996). PZA-resistant strains commonly harbour mutations in 561-bp or 82-bp regions of the *pncA* open reading frame or promoter region, respectively, however, mutations have been reported in other regions of the *pncA* gene as well (Scorpio et al. 1997; Juréen et al. 2008). Moreover, some PZA-resistant strains lack *pncA* mutations with some studies suggesting a role for the *rpsA* gene in PZA resistance, although data supporting this is limited (Alexander et al. 2012; Simons et al. 2013; Tan et al. 2014).

1.2.2.2 *Fluoroquinolones and aminoglycosides as second-line drugs*

Ofloxacin (OFX) and ciprofloxacin are presently utilised as second-line drugs against MDR-TB (WHO, 2014), whilst newer quinolones i.e. moxifloxacin and gatifloxacin are being tested for their efficacy as potential first-line drugs (Rustomjee et al. 2008; Palomino and Martin, 2013). Fluoroquinolones (FQ) inhibit topoisomerase II encoded by *gyrA* and *gyrB* genes in *M. tuberculosis* which promote supercoiling of DNA (Takiff et al. 1994; Aubry et al. 2004). Similar to *rpoB*, *gyrA* and *gyrB* contain a quinolone resistance-determining region (QRDR), with mutations at codons 90 and 94 being the most frequently identified (Cheng et al. 2004; Sun et al. 2008). Clinical isolates, irrespective of FQ susceptibility, all possess a naturally-occurring mutation at codon 95 (Musser, 1995). Efflux mechanisms have also been proposed as putative causes of FQ resistance in *M. tuberculosis* (Escribano et al. 2007).

Kanamycin (KAN) and amikacin are aminoglycosides used in the treatment of MDR-TB. By altering the level of 16S rRNA, these drugs act to inhibit protein synthesis (Alangaden et al. 1998). Mutations commonly conferring high-level KAN resistance are found at nucleotide positions 1400 and 1401 of the *rrs* gene, and occasionally position 1483 (Alangaden et al. 1998; Suzuki et al. 1998). Promoter mutations at position -10 and -35 in *eis* (encodes aminoglycoside acetyltransferase) have been found to confer low-level KAN resistance in 80 % of clinical isolates (Zaunbrecher et al. 2009; Campbell et al. 2011).

1.2.2.3 *Implications of drug resistance*

Drug resistance remains a complex process with some resistant strains harbouring uncharacterised mutations (Zhang et al. 2013). Implementation of new drugs in anti-TB treatment necessitates the responsibility of rapidly ascertaining resistance-conferring mutations to circumvent the evolution of resistant strains, thereby preserving the efficacy of new regimens (Trauner et al. 2014). Additional research is needed on the role of particular genetic mutations in the acquisition of MDR- and XDR-TB and its association with strain fitness. Efflux pumps are sometimes up-regulated in drug-resistant strains during drug selective pressure (Gupta et al. 2010), thus their role in drug resistance also warrants further research.

1.2.3 Fitness, epistasis and compensatory evolution

The concept of bacterial fitness has become an increasingly popular area of interest owing to its significant role in shaping the future of MDR and XDR-TB (Cohen and Murray, 2004; Blower et al. 2004). *M. tuberculosis* fitness is essentially dependent on the rates at which drug-susceptible and resistant populations compete, transmit between hosts and are eliminated from infected hosts, with the magnitude of these factors varying according to the degree and pattern of antibiotic usage (Andersson and Levin, 1999). Similar to other bacteria, *M. tuberculosis* strains carrying resistance-encoding mutations often undergo ‘fitness costs’ (Billington et al. 1999; Mariam et al. 2004; Gagneux et al. 2006). This cost is variable as it may depend on the type of mutation or genetic background of the strain (Gagneux et al. 2006). Mutations may differ in the level of costs imposed and there appears to be a strong selection for low- or no- cost mutations in clinical isolates (Bottger et al. 1998; Billington et al. 1999; Sander et al. 2002; Gagneux et al. 2006; Bottger and Springer, 2007).

Epistasis refers to the phenomenon whereby the phenotypic effect of one mutation differs depending on the presence of another mutation (Weinreich et al. 2005). This phenomenon may be positive or negative depending on whether the interactions between mutations are beneficial or deleterious to the organism. During positive epistasis, the evolution of drug resistance is enhanced as the collective cost of resistance-encoding mutations is lower than individual mutations which have additive fitness costs (Trindade et al. 2009). This is supported by the finding that specific combinations of mutations in *rpoB* (RIF resistance) and *gyrA* (OFX resistance) confer fitter status to MDR/XDR strains (Borrell et al. 2013). Another example of positive epistasis is the progressive development of ethambutol (EMB) resistance in *M. tuberculosis* where, in addition to *embB* M306V mutations, stepping-stone mutations in *Rv3806c* are acquired to augment EMB minimum inhibitory concentrations (MICs) (Safi et al. 2013). Negative epistasis on the other hand, limits the progression of drug resistance by exacerbating fitness costs (Trindade et al. 2009).

Compensatory mutations do not cause drug resistance but instead ameliorate costs associated with particular resistance-encoding mutations (Zur Wiesch et al. 2011). In *M. tuberculosis*, mutations in *ahpC* and *rpoA*/*rpoC* have been implicated in compensation of fitness defects associated with INH and RIF resistance, respectively (Sherman et al. 1996; Comas et al. 2012). Once a compensatory mutation has been acquired, positive epistasis between the resistance-encoding mutation and the compensatory mutation ensures that bacteria do not revert to wild-type status, even in the absence of drug selective pressure (Andersson and Hughes, 2010). In support of its epistatic involvement, studies have shown that the introduction of compensatory mutations into wild-type strains lacking corresponding resistance-encoding mutations, undergo fitness costs (Schrag et al. 1997; Trindade et al. 2009; Shcherbakov et al. 2010). The complex interplay between drug resistance-encoding mutations, compensatory mutations and strain genetic background warrants further research, particularly within the context of outbreak and other clinical strains.

1.2.3.1 *Drug resistance-conferring mutations and fitness costs*

The acquisition of resistance-encoding mutations in the absence of drugs is often associated with a biological fitness cost. Using laboratory-derived strains, several studies have provided significant insights into the fitness of bacteria harbouring drug resistance. Pym et al (2002) used isogenic strains to show that the S315T *katG* mutation is associated with the lowest fitness cost and is important for retaining virulence in INH-resistant mutants. Similarly, isogenic *rpoB* mutants were shown to undergo differential fitness costs depending on the type of resistance-encoding mutation,

with the S531L mutation being the most beneficial and the R529Q mutation posing the greatest cost (Gagneux et al. 2006). This study also demonstrated the importance of strain genetic background in the relative fitness of mutant strains. A landmark study later revealed the presence of compensatory mutations in RNA polymerase genes which improve overall fitness costs associated with RIF-resistance mutations (Comas et al. 2012). In subsequent studies, WGS detected *rpoA* and *rpoC* mutations in transmissible strains from various geographic locations, supporting the biological relevance of these fitness-compensatory mutations in *M. tuberculosis* isolates (de Vos et al. 2013; Casali et al. 2014; Cohen et al. 2015). Taken together, the most frequent resistance-conferring mutations in clinical isolates are believed to have low- or no- fitness cost. In the case of less frequently-occurring mutations, compensatory mutations assist in the amelioration of fitness costs. Figure 1.2 summarises the impact of fitness on the evolution of drug resistance (Müller et al. 2013).

Despite isogenic strains being practical and 'genetically controlled' (Brandis and Hughes, 2013) clinical strains are advantageous in that they 1) closely mimic the virulent nature of *M. tuberculosis* strains and 2) highlight the importance of genetic background in experimental fitness studies (Coscolla and Gagneux, 2011). In a pilot study by Davies et al. (2000), patient isolates which belonged to a single strain differed in fitness, with the drug-resistant isolate undergoing a physiological cost relative to the susceptible isolate. In contrast, a study utilising serial patient isolates of various lineages, demonstrated that the acquisition of drug resistance did not incur significant fitness costs – a finding related to low fitness costs associated with *katG*, *inhA* and *rpoB* mutations (Bhatter et al. 2013). Heterogeneity of drug resistance thus emphasizes the need for more work on the clinical relevance of fitness costs, epistasis and compensatory mutations.

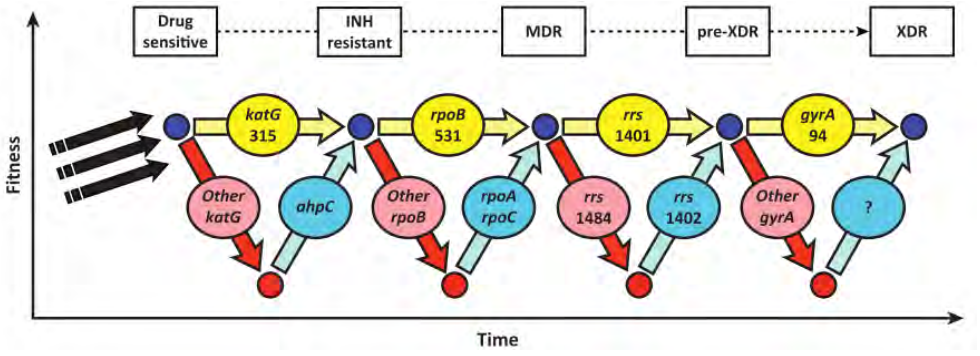


Figure 1.2 The influence of Darwinian fitness on the emergence of multidrug-resistant (MDR-) and extensively drug-resistant (XDR-) tuberculosis (TB). Higher and lower fitness levels of bacteria are

indicated by small blue or red circles, respectively. Yellow and red arrows and ovals indicate low-cost and high-cost resistance mutations, respectively. Blue arrows and ovals indicate compensatory mutations. Black arrows refer to baseline genomic characteristics of a particular strain genetic background. Abbreviation: INH, isoniazid (Müller et al. 2013)

1.2.3.2 *Competitive costs of drug resistance*

Simultaneous infections have been reported in individuals, in which two different strains co-exist in the same individual (van Rie et al. 2005). While this may not be a problem in low-incidence regions, TB patients from high-incidence settings e.g. South Africa, frequently present with multiple *M. tuberculosis* strains in the same sputum sample, albeit in different proportions (Warren et al. 2004). This phenomenon may be explained by two mechanisms related to the presence or absence of drugs, respectively: 1) during treatment with first-line drugs, susceptible populations are reduced, thereby permitting the growth of MDR populations and 2) following the subsequent removal of drug pressure (i.e. patient non-compliance or treatment completion), susceptible populations re-emerge and 'overgrow' due to differences in relative fitness (van Rie et al. 2005). Competitive *in vitro* assays comparing the fitness of resistant and susceptible isolates have revealed heterogeneity based on the mutations they harbour i.e. while some mutations may hamper growth and survival under oxidative stress; others may have negligible effects (Cohen and Murray, 2004).

1.2.3.3 *Virulence and immunogenicity*

Although fitness costs refer to reduced growth rates within a controlled environment, these costs can be extended to reduced transmissibility and decreased virulence of *M. tuberculosis* strains in model organisms (Andersson and Hughes, 2007). Given the centrality of macrophages in replication and survival of *M. tuberculosis*, extensive studies have interrogated *M. tuberculosis*-macrophage interactions as a means of understanding immunological, genetic and physiological determinants during *in vivo* infection (Schnappinger et al. 2003; Rengarajan et al. 2005; Giacomini et al. 2001). Several studies have used macrophage models to assess intracellular fitness of drug-resistant bacteria. Mariam et al. (2004) measured the independent and competitive fitness of 3 spontaneous RIF-resistant mutants in an U937 macrophage cell line, and showed decreased fitness compared with the parental strain. Similarly, Rey-Jurado et al. (2011) assessed the fitness *katG* S315T mutant and MDR isolates in J774.2 murine macrophages, and demonstrated reduced fitness relative to susceptible isolates. In an interesting study with respiratory and extrarespiratory strains, the latter was shown to have higher infectivity in human monocyte-derived macrophages (and mice),

highlighting the significance of infectivity as an indicator of extrapulmonary dissemination (de Viedma et al. 2005).

The immunogenicity of *M. tuberculosis* strains has been shown to regulate the outcome of infection in animal and human studies (Cooper and Khader, 2008; Kaufmann, 2002) with IFN-γ, TNF-α and IL-12 being essential factors in the control of infection (Flynn et al. 1993; Flynn et al, 1995; Cooper et al. 2011). Manca et al. 1999 compared the growth of clinical isolate CDC1551 to two laboratory strains, H37Rv and Erdman, and two clinical isolates, HN878 and HN60. CDC1551-infected mice survived longer than those infected with other strains. This was attributed to higher and quicker cytokine mRNA expression levels in response to CDC1551 and subsequent stabilisation of chronic infection with constant CFU counts. Thus, CDC1551 was found to be more immunogenic, rather than more virulent than other *M. tuberculosis* strains.

A later study by Barczak et al. (2005) looked at immune responses during dual infection with varying starting proportions of CDC1551 (hyperimmunogenic and hypovirulent) and W-Beijing isolate HN878 (hypoimmunogenic and hypervirulent). In all proportional comparisons, HN878 presented as the predominant strain indicating a growth advantage. Additionally, all groups containing HN878 progressed to death significantly faster than mice infected with CDC1551 alone. Interestingly, both strains had similar growth rates in liquid media, suggesting that the observations under *in vivo* condition were not due to differences in fitness. Similarly, increased *in vitro* fitness of drug-resistant F15/LAM4/KZN strains (Naidoo and Pillay, 2014) does not necessitate increased virulence as shown in a recent study (Smith et al. 2014). Their study showed decreasing virulence in increasingly drug-resistant strains (susceptible > MDR >XDR) however, it should be mentioned that all strains were virulent enough to kill SCID mice and exhibited high levels of necrosis relative to the Erdman strain (Smith et al. 2014). These findings were similar to earlier studies in guinea pigs models which demonstrated decreasing virulence in increasingly INH-resistant *M. tuberculosis* strains (Middlebrook and Cohn, 1953; Mitchison, 1954).

While it may not be clear how accurately *in vitro* findings correlate to *in vivo* conditions, laboratory studies can still reveal important information regarding resistance and fitness-compensatory mutations that may be conferred in the natural environment (Bjorkman and Andersson, 2000). Further research using clinical strains may improve our understanding of strain-specific differences in growth rates, virulence and transmission efficiencies.

1.2.4 Hypothesis

The enhanced fitness of specific *M. tuberculosis* genotypes is associated with the presence of beneficial drug resistance-conferring mutations and/or compensatory mutations.

1.2.5 Research aim

The overall aim of this study was to determine the biological fitness of F15/LAM4/KZN, Beijing, F11 and F28 clinical strains and correlate these findings with genotypic data.

1.2.6 Objectives

i. To determine the laboratory fitness of clinical *M. tuberculosis* strains using *in vitro* growth, viability and competition assays.
ii. To correlate mutations in common drug resistance-conferring genes i.e. *katG, inhA, ahpC, rpoA, rpoB, rpoC, gyrA* and *rrs* with fitness.
iii. To elucidate the mutational profiles of selected clinical strains by WGS and correlate with biological fitness.
iv. To determine the virulence of clinical strains, as surrogates of fitness, by measuring uptake, intracellular replication and cytokine responses in THP-1 macrophages
v. To ascertain whether relapse isolates undergo fitness costs relative to initial isolates, following passage in HIV-positive individuals with TB recurrence after successful treatment.

1.2.7 Scope of thesis

This thesis comprises six chapters and is presented in a "thesis by manuscript" format. The first chapter includes the introduction and literature review. In Chapter two, the fitness of clinical strains is elucidated using various *in vitro* assays and correlated with mutations in common drug resistance-encoding genes. Using a WGS approach, the mutational profiles of selected clinical strains are examined in Chapter three, with particular focus on drug resistance and fitness-compensatory mutations. Chapter four addresses the virulence properties of clinical strains in a THP-1 macrophage model, in terms of uptake efficiencies, growth indices and multi-cytokine responses. In Chapter five, the fitness of paired isolates is explored as a putative contributor to relapse in HIV-positive TB patients. Chapter six includes overall conclusions and recommendations for future research.

CHAPTER TWO

Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*

Naidoo CC, [1] Pillay M. [1]

[1]Medical Microbiology and Infection Control, University of KwaZulu-Natal, Durban, South Africa

Original article published in: Clinical Microbiology and Infection 2014; 20: O361-369

ABSTRACT

The role of fitness in transmission of drug-resistant strains has been explored in previous studies; but has not been established for F15/LAM4/KZN strains, which were responsible for the extensively drug-resistant tuberculosis (XDR-TB) outbreak in Tugela Ferry, South Africa. The biological fitness of 15 clinical strains representing the F15/LAM4/KZN, Beijing, F11 and F28 families was determined by growth, viability and competition assays and correlated with DNA sequencing of eight genes associated with drug resistance and putative compensatory mechanisms. Similar growth rates were observed among susceptible, multidrug-resistant (MDR) and XDR strains of the KZN and F28 genotypes. In contrast, Beijing and F11 MDR strains demonstrated significantly reduced fitness. Resistant strains exhibited heterogeneous fitness profiles in competition with different susceptible strains, suggesting strain dependence. In addition, co-culture growth rates were consistently higher than independent growth rates in 13/14 competition pairs. All 14 drug-resistant strains retained viability, at a low CFU/mL, when paired with susceptible strains. The persistence of such resistant strains could consequently support the acquisition of additional drug-resistance-conferring mutations and/or the evolution of compensatory mechanisms. Frequently occurring mutations were detected in KZN and F28 resistant strains whereas, the Beijing MDR strain harboured a less common *katG* mutation and the F11 MDR strain had no *katG* mutation. Contrary to drug-resistant Beijing and F11 strains, the successful transmission of KZN strains, particularly during the outbreak, may be attributed to the presence of drug-resistance-conferring mutations associated with little or no associated fitness costs. Amplified growth in co-culture may be suggestive of *in vivo trans*-complementation.

INTRODUCTION

Multidrug-resistant tuberculosis (MDR-TB) represented 3.7 % of new cases and 20 % of previously treated cases worldwide. An alarming 9 % of the average fraction of all these cases were extensively drug-resistant (XDR) -TB [1]. South Africa, which is ranked number 1 of 22 high TB-burdened countries globally, reported 1.8 % of new TB cases and 6.7 % of previously treated TB cases as MDR-TB [1]. Of these, 10 % were XDR-TB, which is among the highest globally [2].

Mathematical models have predicted the successful spread of drug-resistant *Mycobacterium tuberculosis* strains to be dependent on strain fitness [3]. The fitness of a pathogen is dependent on the rates at which drug-susceptible and drug-resistant cell populations compete in an infected organism and the environment, are transmitted between organisms, and are eliminated from infected

organisms [4]. The magnitude of these factors is variable depending on the degree and pattern of antibiotic usage [4]. Drug-resistant strains often demonstrate decreased competitive ability against susceptible strains in the absence of the drug [5]. This fitness 'cost' is dependent on strain genetic background and specific drug-resistance-conferring mutations, which can be improved by compensatory evolution [6].

The F15/LAM4/KZN genotype, first detected in the province of KwaZulu-Natal in the mid-1990s, was largely associated with drug resistance [7]. The emergence of the MDR forms of this genotype during the same period coincided with increasing HIV prevalence rates and limited access to antiretroviral treatment [7]. Second-line resistance began to emerge between 1997 and 2000, followed by the isolation of the first XDR strain in 2001, preceding the outbreak of the XDR-F15/LAM4/KZN strains in Tugela Ferry in 2005 [8].

Between 2004 and 2005, the Beijing genotype accounted for 11 % of MDR strains; the F11 genotype for 7 % of all MDR and 10 % of pre-XDR and XDR strains; and the F28 genotype represented the highest proportion (49 %) among the MDR, 30 % of pre-XDR and 9 % of the XDR strains in KwaZulu-Natal (M. Pillay, personal communication).

Both MDR and XDR strains of F15/LAM4/KZN, F11 and F28 are transmitted regardless of fitness costs believed to be associated with drug-resistance-conferring mutations [9]. In this study, we explore for the first time, the fitness of the F15/LAM4/KZN, F11 and F28 families, compared with the Beijing family, which has been previously extensively characterized [10]. Although it is not clear how accurately in vitro findings correlate to in vivo conditions, laboratory data can elucidate important information regarding resistance, fitness and compensatory mutations that may be conferred in the natural environment [11]. The overrepresentation of specific genotype families among cases of drug resistance and the subsequent successful transmission of these strains are of paramount importance in relation to TB control regimens [12].

METHODS AND MATERIALS

M. tuberculosis isolates

The genotypes and the respective drug-resistance profiles of the 15 clinical isolates included in this study are listed in Table 2.1. Genotypes of the isolates were determined by spoligotyping in

previous studies as per the manufacturer's instructions (Isogen Bioscience B.V., Hyderabad, AP, India). Drug susceptibility was determined using the 1% agar proportion method [13].

Table 2.1 Genotype and drug susceptibility of clinical strains used in the study

Strain	Missing spoligo spacers	ST numbers	Genotype family	Drug resistance profile	Type
V9124	21-24, 33-36, 40	60	F15/LAM4/KZN	None	Susceptible
V4207	21-24, 33-36, 40	60	F15/LAM4/KZN	None	Susceptible
V1435	21-24, 33-36, 40	60	F15/LAM4/KZN	IRS	MDR
V2475	21-24, 33-36, 40	60	F15/LAM4/KZN	IR	MDR
KZN605	21-24, 33-36, 40	60	F15/LAM4/KZN	IRSEOKCN	XDR
X162	21-24, 33-36, 40	60	F15/LAM4/KZN	IRSEOKCN	XDR
B910	1-34	1	Beijing	None	Susceptible
B1528	1-34	1	Beijing	None	Susceptible
R35	1-34	1	Beijing	IRS	MDR
R283	1-34	1	Beijing	IR	MDR
R490	9-11, 21-24, 33-36	33	F11	None	Susceptible
R271	9-11, 21-24, 33-36	33	F11	IR	MDR
R104	9, 10, 33-36	34	F28	None	Susceptible
R443	9, 10, 33-36	34	F28	IRON	MDR
R262	9, 10, 33-36	34	F28	IRSEOKCN	XDR

I: Isoniazid; R: Rifampicin; S: Streptomycin; E: Ethambutol; O: Ofloxacin; K: Kanamycin; C: Capreomycin; N: Niacinamide, MDR: multidrug-resistant; XDR: extensively drug-resistant

Preparation of *M. tuberculosis* inoculum

Frozen stocks were prepared by inoculating 100 µL of each strain into Middlebrook 7H9 broth (20 mL supplemented with 10 % oleic albumin dextrose catalase (OADC), 0.05 % Tween-80 and 0.5 % glycerol). After aerobic incubation at 37 °C, with agitation, aliquots of 1000 µL were frozen once an optical density at 600 nm (OD_{600}) of 0.9–1.2 was reached. For the growth and competition assays, frozen stocks were inoculated into 100 mL of Middlebrook 7H9 broth in triplicate, incubated with agitation at 37°C until an OD_{600} of 1.6–1.8 was reached. After centrifugation at 1780 **g** at 4 °C for 10 min, the pellet was washed twice with PBS containing 0.05 % Tween-80 and re-

suspended in 1 mL of Middlebrook 7H9 broth. Homogeneous suspensions were obtained by vigorous pipetting.

Fitness of axenic cultures

Re-suspended cultures were back-diluted, in triplicate, in 25 mL of Middlebrook 7H9 broth, to an OD_{600} of 0.015 using the following formula: total OD units X (amount to back-dilute) = (0.015) X (25 mL). The CFU/mL corresponding to OD_{600} 0.015 ranged from 3.65E + 05 to 3.10E + 06 for all strains (Table S2.1). Cultures were incubated aerobically with shaking at 37°C for 24 days.

Growth and metabolic activity were measured at 4-day intervals for 24 days. In addition, OD_{600} measurements were taken at 0, 24, 48 and 70 h during the lag phase. To ensure reproducibility and accuracy, all measurements were recorded in triplicate for each of the three biological replicates. The CFUs were quantified in triplicate on Middlebrook 7H11 agar (containing 10 % OADC, 0.05 % Tween-80 and 0.5 % glycerol) after performing a ten-fold dilution series with PBS (containing 0.05 % Tween-80). For each strain family, the resistant strains were compared with the susceptible strains by a comparison of fitness (W) [14].

$$W = ln\ (R_{final} \div R_{initial}) / ln\ (S_{final} \div S_{initial})$$

Where *ln* represents the natural logarithm and R and S represent the CFU/ml of the resistant and susceptible strains, respectively. The fitness of the drug susceptible strain represented 1. Therefore, if $W < 1$, then the resistant strain encountered a fitness cost, and if $W > 1$, then it was regarded as fit.

Alamar Blue assay

Alamar Blue (Life Technologies, Fairland, Roosevelt Park, South Africa) (10 µl) was added to 100 µl of culture in triplicate a clear 96-well microtitre plate. Broth and Alamar blue alone served as negative controls and H37Rv and the reagent represented the positive control. After aerobic incubation overnight at 37 °C, a colour change from blue to pink was indicative of metabolically active cells. The absorbance was measured at 600 nm in the GloMax system (Promega Corp. Madison, WI. USA), using Alamar Blue and broth as a blank.

Pairwise Competition

Cultures were back-diluted in duplicate into 30 ml of broth. Thereafter, 10 ml each of a drug-

susceptible and resistant culture were transferred into a 30 ml inkwell bottle, resulting in a total of 20 ml each of mixed and single cultures. OD_{600} and CFU counts were performed, in triplicate, at 6-day intervals for 30 days on the aerobically agitated cultures at 37 °C.

Counts of CFU were quantified in triplicate on Middlebrook 7H10 agar (containing 10 % OADC, 0.05 % Tween-80 and 0.5 % glycerol) with and without antibiotics. Plates containing isoniazid (1 mg/L) and rifampicin (1 mg/L) were used to test MDR isolates [13]. In addition, to these antibiotics, XDR isolates were inoculated onto agar plates containing kanamycin (5 mg/L) and ofloxacin (2 mg/L) [15].

After 21 - 25 days of aerobic incubation at 37 °C, the number of drug-resistant colonies (R) was counted on the antibiotic plates. The number of drug-susceptible colonies (S) was enumerated as the difference between the number of colonies on the non-antibiotic and antibiotic plates. These colony counts were then used to quantify the CFU/ml used to calculate the mean competitive relative fitness index (*W*) for each drug-resistant strain.

PCR amplification

A total of three susceptible, four MDR, and three XDR clinical isolates was examined for drug resistance-conferring and compensatory mutations by DNA sequencing. DNA was isolated as previously described [15]. The primers listed in Table 2.2 were used to amplify the following target genes: *katG*, *inhA*, *ahpC, rpoA*, *rpoB*, *rpoC*, *gyrA* and *rrs*. PCR was performed in a 25-µl mixture consisting of 0.25 mM dNTPs, each primer (0.25 µM), 0.5 U of *EconoTaq* Polymerase (Lucigen), 1 x PCR buffer and 5 µl of template DNA. H37Rv was used as the positive control and nuclease-free water as the negative control. The thermal cycling conditions included denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 20 s, 55°C for 1 min and 72°C for 30 s and a final extension at 72 °C for 5 min. PCR products were visualized in 1.5 % agarose gels containing ethidium bromide using GeneSnap (SYNGENE).

Table 2.2 Primer pairs and sequencing primer used for PCR amplification and DNA sequencing

Locus	Nucleotide sequence (5' – 3')	Product size (base pairs)	Reference
katGF	GCAGATGGGGCTGATCTACG		Zenteno-Cuevas *et al*. (2009)
katGR	AACTCGTCGGCCAATTCCTC	580	Zenteno-Cuevas *et al*. (2009)
inhAF	TGCAATTTATCCCAGCGAAG		Primer 3 (v 0.4.0)
inhAR	ACCGAAATGCAGGTAGTGCT	886	Primer 3 (v 0.4.0)
ahpCF	GCGATGCCGATAAATATGGT		Primer 3 (v 0.4.0)
ahpCR	ATACCTGCGGATTTCGTGTC	877	Primer 3 (v 0.4.0)
rpoAF	GAGTACTACTCAAAGTAAACCC		Comas *et al*. (2012)
rpoAR	CTTCTGATGTGAAGACGACC	1239	Comas *et al*. (2012)
rpoA	AACAACTCGACCAGCGTCTT	Sequencing primer	Comas *et al*. (2012)
rpoBF	TGGTCCGCTTGCACGAGGGTC		van der Zanden *et al*. (2003)
rpoBR	CTCAGGGGTTTCGATCGGGCA	437	van der Zanden *et al*. (2003)
rpoCF	TCCGACTTGAACGACCTGTA		Comas *et al*. (2012)
rpoCR	GTTGCGCTCGTCGTGGTTCA	1298	Comas *et al*. (2012)
gyrAF	CAGCTACATCGACTATGCGA		Takiff *et al*. (1994)
gyrAR	GGGCTCGGTGTACCTCAT	320	Takiff *et al*. (1994)
rrs1F	AAGGGCTGCGATGCCGCGAG		Primer 3 (v 0.4.0)
rrs1R	AAGTCCGAGTGTTGCCTCAGG	433	Primer 3 (v 0.4.0)
rrs2F	AGGTGTGGGTTTCCTTCCTT		Primer 3 (v 0.4.0)
rrs2R	CGTTGCTGATCTGCGATTAC	540	Primer 3 (v 0.4.0)
rrs3F	CTGAGATACGGCCCAGACTC		Primer 3 (v 0.4.0)
rrs3R	TAAGGTTCTTCGCGTTGCATC	656	Primer 3 (v 0.4.0)
rrs4F	GTGGCCGTTTGTTTTGTCAG		Primer 3 (v 0.4.0)
rrs4R	TGCAATATTCCCCACTGC	437	Primer 3 (v 0.4.0)

F: forward primer; R: reverse primer

DNA Sequencing

Purification and sequencing of the PCR products were performed by Inqaba Biotec using the PCR forward primers, with the exception of the *rpoA* gene for which a sequencing primer was synthesised (Table 2.2). Termination reactions were performed using BigDye® Terminator v3.1 (ABI, Life Technologies). The labelled fragments were purified using the Zymo Research Sequencing Clean-up Kit and subsequently analysed in a 3500XL Genetic Analyzer (ABI, Life Technologies). Multiple sequence alignment was performed using BioEdit software. Due to the re-

annotation of the H37Rv genome [16], the new numbering system of the *rrs* gene was used in this study.

Statistical Analysis

All statistical analysis was performed using SPSS software version 19.0 (IBM). One-way Repeated Measures ANOVA was used for multiple comparisons of the Mean ± SD obtained in the lag phase, OD and Alamar Blue assays. One-way ANOVA was used to compare mean relative competitive fitness.

RESULTS

OD assay

No significant differences were found among any of the resistant strains compared with susceptible ones during the lag phase of growth (data not shown). Relatively similar growth was observed among susceptible, MDR and XDR-F15/LAM4/KZN strains (Figure S2.1). However, both Beijing MDR strains (R35 and R283) presented with a lower OD than the susceptible strains (B910 and B1528). This was significantly different between R35 and B910 ($p=0.016$), R283 and B910 ($p=0.022$) and R35 and R283 ($p=0.046$). Similarly, susceptible (S) strains F11-R490 and F28-R104 demonstrated increased fitness over MDR strains F11-R271 ($p=0.009$) and F28-R443 ($p=0.16$), respectively.

CFU assay

In comparison to H37Rv, fitness of three of the four F15/LAM4/KZN drug-resistant strains was reduced (Table S2.2). X162 (XDR) displayed increased fitness compared with both susceptible and reference strains, whereas KZN605 (XDR) was the least fit of the drug-resistant strains in all three comparisons. V1435 (MDR) was only less fit than H37Rv. V2475 (MDR) and KZN605 were significantly less fit than X162 relative to V4207 (S) ($p=0.017$ and $p=0.012$, respectively) and H37Rv ($p=0.012$ and $p=0.008$, respectively).

Beijing-R35 (MDR) was less fit than Beijing-B1528 (S) and H37Rv but not B910 (S). Beijing-R283 (MDR), however, was fitter than all three susceptible strains. No significant differences were found between any of these comparisons.

F11-R271 (MDR) presented with fitness costs compared with F11-R490 and reference strain H37Rv. F28-R443 (MDR) was marginally less fit than R104 (S), but not less fit than H37Rv, whereas F28-R262 (XDR) was less fit than both.

Alamar Blue assay

The Alamar Blue assay (Figure S2.2) showed similar metabolic activity for the F15/LAM4/KZN strains, with significant differences in viability only between V4207 (S) and V2475 (MDR) ($p=0.022$). Among the Beijing family, the viability of MDR strain R283 was significantly reduced compared with both susceptible strains, B910 ($p=0.026$) and B1528 ($p=0.030$). R35 (MDR) showed significantly lower fitness than B910 ($p=0.021$) and B1528 ($p=0.021$). Similar to the OD assay, the F11 strain R271 (MDR) displayed significantly reduced viability compared with R490 (S) ($p=0.004$). Among the F28 family, although R104 (S) grew at a consistently higher rate than R443 (MDR), it did not reach significance. R262 (XDR) displayed increased fitness after day 12, with the exception of day 20, which suggests a pipetting error.

Competitive growth

In pairwise competition experiments, similar trends were observed in the OD and CFU assays (Figure 2.1), whereby the susceptible strain exhibited significantly higher growth (Table S2.3; Figures S2.3-2.8) than its resistant counterpart throughout the experimental period. The exception was the KZN-X162 (XDR), whose growth had surpassed that of both the susceptible strains by day 30 of the OD assay, but this was not statistically significant. The growth of this strain was almost identical to KZN-V9124 (S) until day18 (Figure 2.1D) but lower than that of KZN-V4207 (S) in the CFU assay.

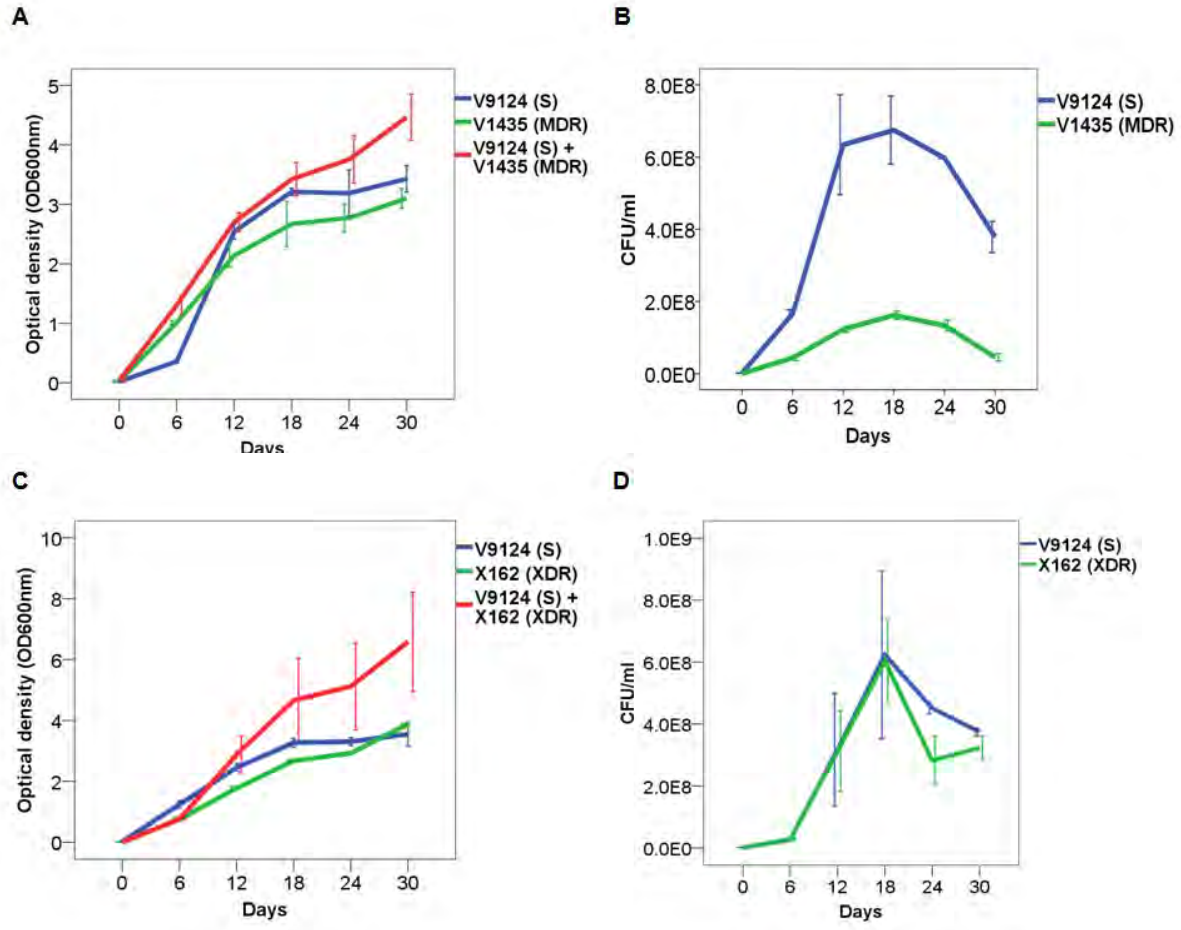


Figure 2.1 Optical density (OD) measurements and colony forming units (CFU) counts for competitive KZN strain pairs. (A) OD values and (B) CFU/ml for V9124 (S) + V1435 (MDR). (C) OD values and (D) CFU/ml for V9124 (S) + X162 (XDR). Data is represented as the mean ± SD of duplicate experiments. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant.

Optical density values for co-culture were consistently higher than those of independent cultures (Figure 2.1A, 2.1C Figures S2.3 –S2.5) with the exception of the Beijing pair, B1528 (S) + R283 (MDR). The growth of the former was higher than in co-culture from day 12, but this was not significant.

All the KZN MDR, three of the four Beijing MDR and the F11 MDR pairs experienced fitness costs in competition with susceptible strains (Figure 2.2). In contrast, both KZN XDR strains were fit. The F28 XDR strain exhibited a minimal competitive cost paired with R104 (S). Higher fitness indices were observed for all KZN MDR and XDR strains when paired with V9124 (S) compared with V4207 (S), and for Beijing-resistant strains when paired with B1528 (S) compared with B910 (S). This difference was statistically significant between the KZN605 (XDR) pairs (p=0.017). Furthermore, R283 (MDR) (paired with B1528) was the fittest strain among the four genotype families, whereas the F11 strain R271 (MDR) was the least fit.

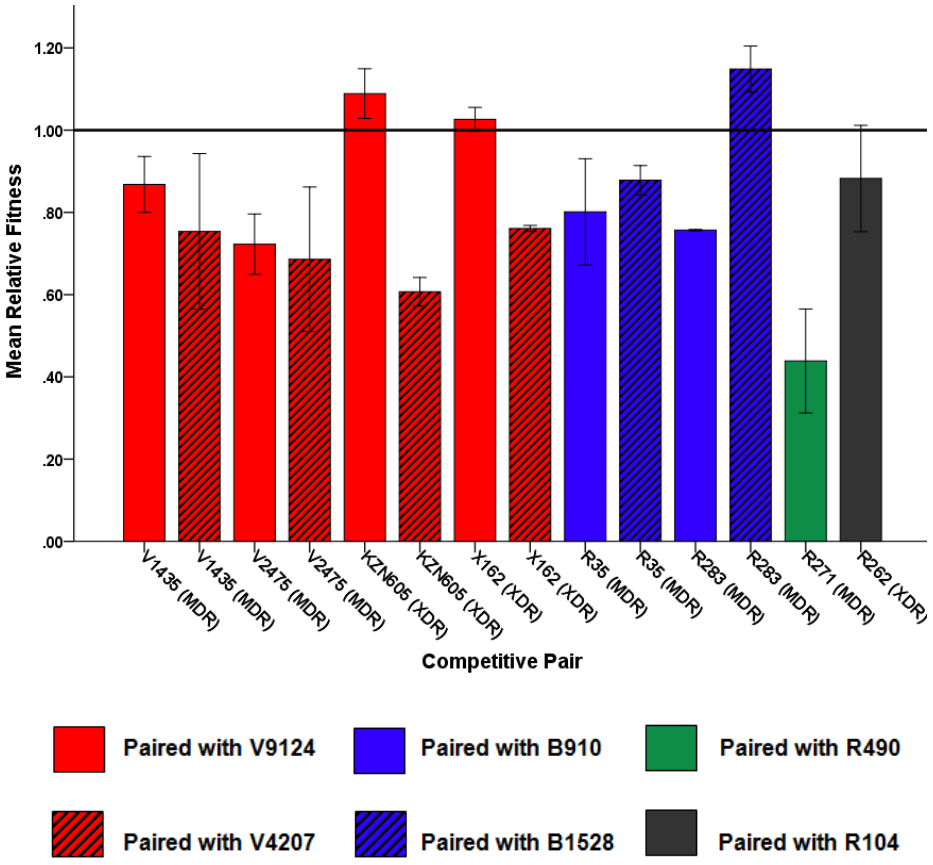


Figure 2.2 Mean competitive relative fitness for KZN, Beijing, F11 and F28 drug-resistant strains. The fitness index of the susceptible strain is taken as 1 (solid black line). Fitness indices below 1 indicate fitness costs and fitness indices above 1 indicate no loss of fitness. The data above is represented as the mean ± SD of duplicate experiments. MDR: multidrug-resistant; XDR: extensively drug-resistant.

Molecular characterisation

Eight genes associated with drug resistance were sequenced in eleven clinical strains and H37Rv on day 12 of the growth assays. Multiple sequence alignment with H37Rv in Tuberculist showed no differences between both reference strains. Sequencing detected four different mutations in *rpoB*, two in *gyrA* and *katG*, one in *rpoC a*nd *rrs*, and none in *rpoA*, *inhA* or *ahpC* (Table 2.3). Identical mutations were present in both KZN and the F28 XDR strains: double mutations Asp516Gly/Leu533Pro in *rpoB* and Ala90Val/ Ser95Thr in *gyrA*, and single mutations Ser315Thr in *katG*, A1402G in *rrs* and Ala542Ala in *rpoC*. Different *rpoB* mutations were harboured in the three MDR strains belonging to the different families. However, Ser531Leu in *rpoB* was common to both MDR F11 and Pre-XDR F28. All drug-resistant KZN and F28 harboured Ser315Thr, whereas Beijing harboured Ser315Asn in *katG*. Ser95Thr in *gyrA* was present in all sensitive strains, in addition to Ala542Ala in *rpoC* KZN.

Table 2.3 Distribution of nonsynonymous and synonymous mutations in selected clinical strains

Strain	Genotype family	Resistance profile	*rpoA*	*rpoB*	*rpoC*	*katG*	*inhA*	*ahpC*	*gyrA*	*rrs*
V9124	F15/LAM4/KZN	None	-	-	Ala542Ala	-	-	-	Ser95Thr	-
V2475	F15/LAM4/KZN	IR	-	Asp516Thr	Ala542Ala	Ser315Thr	-	-	Ser95Thr	-
KZN605	F15/LAM4/KZN	IRSEOKCN	-	Asp516Gly Leu533Pro	Ala542Ala	Ser315Thr	-	-	Ser95Thr Ala90Val	A1402G
X162	F15/LAM4/KZN	IRSEOKCN	-	Asp516Gly Leu533Pro	Ala542Ala	Ser315Thr	-	-	Ser95Thr Ala90Val	A1402G
B910	Beijing	None	-	-	-	-	-	-	Ser95Thr	-
R35	Beijing	IRS	-	Leu533Pro	-	Ser315Asn	-	-	Ser95Thr	-
R490	F11	None	-	-	Ala542Ala	-	-	-	Ser95Thr	C492T
R271	F11	IR	-	Ser531Leu	Ala542Ala	-	-	-	Ser95Thr	C492T
R104	F28	None	-	-	-	-	-	-	Ser95Thr	-
R443	Beijing	IRSEK	-	Ser531Leu	-	Ser315Thr	-	-	Ser95Thr	-
R262	Beijing	IRSEOKCN	-	Asp516Gly Leu533Pro	Ala542Ala	Ser315Thr	-	-	Ser95Thr Ala90Val	A1402G
H37Rv	Laboratory	None	-	-	-	-	-	-		-

I: Isoniazid; R: Rifampicin; S: Streptomycin; E: Ethambutol; O: Ofloxacin; K: Kanamycin; C: Capreomycin; N: Niacinamide

DISCUSSION

From an epidemiological perspective, the principal dynamic that impacts the spread of drug-resistant strains is the fitness cost associated with the resistance mutations they harbour [17]. In our study, MDR and XDR strains representing the F15/LAM4/KZN and F28 genotypes demonstrated enhanced fitness despite the burden of carrying drug-resistance-conferring mutations. In contrast, Beijing and F11 MDR strains exhibited significantly reduced fitness.

All the F15/LAM4/KZN, Beijing and F28 resistant strains had a fitness index above 0.7 in the CFU growth and competition assays, with the exception of KZN605 and V2475, which had fitness indices of 0.61 and 0.65, respectively, in competition with V4207. Hypothetically, co-infection with V4207 may impede the successful spread of these two resistant strains to other hosts, unless selected for by antibiotic pressure. The mean fitness of 0.44 ± 0.13 of F11-R271 (MDR) in pairwise competition is hardly surprising, given its reduced growth in axenic cultures (Figures S2.1 and S2.2). Therefore, this strain would not be expected to spread successfully in the population.

Pairwise competition revealed three generalized patterns of growth. First, there was an 'overgrowth' of susceptible strains in the CFU results obtained, most likely attributable to differences in fitness between co-infecting susceptible and resistant strains [10, 18]. However, all 14 drug-resistant strains retained viability, at a low CFU/mL, when paired with susceptible strains. The persistence of such resistant strains could consequently support the acquisition of additional drug resistance-conferring mutations and/or the evolution of compensatory mechanisms.

Second, fitness profiles of the F15/LAM4/KZN and Beijing resistant strains during competition were heterogeneous, i.e. all the former strains had higher fitness paired with KZN-V9124 (S) compared with KZN-V4207 (S), with significant differences between both KZN605 (XDR) pairs ($p=0.022$). Similarly, the Beijing MDR strains had higher fitness with B910 (S) compared to B1528 (S). This is most likely due to fitness differences between susceptible strains. Together, the results are suggestive of strain dependence. Lastly, growth in co-culture was amplified compared with single cultures in 13 of 14 competition pairs. To the best of our knowledge, this phenomenon in co-culture noted after 2 weeks of growth, has not been reported in *M. tuberculosis* before. We hypothesise that one strain may be promoting or suppressing the growth of the other by means of a putative growth factor, or other signalling molecules. In contrast, Bhatter et al. [14] recently showed mutual inhibition of growth of susceptible and resistant strains from the MANU genotype during a

competition assay. Hence, differences in competitive behaviour may be a reflection of biological differences between strain genotypes.

The high frequency of Ser315Thr *katG* mutations in clinical isolates in our study illustrate its importance in conserving catalase/peroxidase activity, virulence and reduced conversion of isoniazid into active form, while maintaining mycobacterial fitness [19]. The Beijing MDR strain, however, harboured a different mutation (Ser315Asn) in *katG.* This mutation, although associated with a small reduction (4%) in virulence, presented with significantly reduced catalase–peroxidase activity compared with four other *katG* mutants, including the Ser315Thr mutant, in a study by Unissa et al. [20]. The presence of the Ser315Asn mutation could consequently explain the reduced fitness of Beijing-R35 (MDR) in all the assays.

All seven resistant strains harboured mutations in the sequenced 81-bp hot-spot region of *rpoB* that accounts for >95% of rifampicin resistance in clinical *M. tuberculosis* strains [21]. The XDR strains harboured a common mutation (Asp516Gly) that was distinguishable from the MDR F15/LAM4/KZN strain (Asp516Tyr). This supports the findings of others that propose independent acquisition of *rpoB* mutations for MDR and XDR F15/LAM4/KZN strains [22]. In addition to Asp516Gly, the XDR strains possessed a second *rpoB* mutation at codon 533, identical to the mutation present in the Beijing MDR strain.

The F11 and F28 MDR strains possessed the most frequently occurring rifampicin resistance-conferring mutation, Ser531Leu associated with minimal or no fitness loss in clinical isolates [21, 23]. Taken together with the Ser315Thr mutation in *katG*, the increased fitness of F28-R443 (MDR) in the OD, Alamar Blue and CFU assays can be attributed to its predisposition to 'beneficial' fitness mutations in *rpoB* and *katG*. Despite the presence of Ser531Leu in F11-R271 (MDR), it grew poorly in comparison to the susceptible strain in the growth and competition assays. The reduced fitness of this strain may be attributed to an unidentified *katG* mutation that it may harbour, which may be slowing down its growth despite the beneficial *rpoB* mutation it possesses.

No mutations were detected in the *inhA* sequenced region in any of the isolates. Mutations have been reported in the *inhA* promoter region for KZN-V2475 (MDR) and another F15/LAM4/KZN XDR strain, KZN-R506 [22]. However, we had not sequenced this region because of financial constraints. No putative compensatory mutations were detected in the *ahpC*, *rpoA* or *rpoC* genes for

any clinical strain. However, there could be other mechanisms of compensation for which whole genome sequencing could provide more insight. The presence of synonymous mutation Ala542Ala in *rpoC* for F15/LAM4/KZN strains, F11 strains and F28-R262 (XDR) is in accordance with a recent study that demonstrated this mutation in Lineage 4 strains only [24]. A natural polymorphism, thought to be unassociated with fluoroquinolone susceptibility [25], was observed at codon 95 of *gyrA* in all 11 clinical strains. In addition, both resistant and susceptible F11 strains possessed the C492T polymorphism in *rrs*, which is useful as a phylogenetic marker for such strains [26].

Identical mutations were detected in *katG* (Ser315Thr), *rpoB* (Asp516Gly, Leu533Pro), *rpoC* (Ala542Ala), *gyrA* (Ala90Val, Ser95Thr) and *rrs* (A1402G) for all three XDR strains. This is supported by whole genome sequencing of *M. tuberculosis* XDR strains that revealed only a few differences among them [22]. These mutations that confer resistance to first- and second-line drugs appear to be favourable to the XDR organisms, as displayed by their unimpeded growth in the growth and competition assays of this study. A recent comparison of the XDR strains from the Tugela Ferry outbreak to a panel of phylogenetically distinct drug-resistant and susceptible strains of *M. tuberculosis* showed very little substantiation for compensatory mechanisms [27]. This has significant implications for the pathogenicity and transmissibility of these strains, which carry mutations in several essential genes. The possibility arises that these strains may be more suited to compromised populations, including HIV-infected individuals, in which the organism's minimal fitness costs are negligible.

There are a few limitations of this study. First, only co-cultures were plated out and we were therefore unable to correlate these findings with axenic susceptible and resistant CFU counts in the competition assays. Second, only a limited number of genes and gene regions could be sequenced in this study. Therefore, other significant mutations correlating with observed fitness phenotypes may have gone undetected.

In conclusion, paired growth of competing resistant and susceptible strains illustrated a slight 'additive' effect in comparison to independent growth. This *in vitro* finding may be postulated to represent an *in vivo* occurrence with implications in high-incidence TB settings, including South Africa, that are burdened with a wide spectrum of strain diversity and drug resistance. Furthermore, strains with differing drug-resistance phenotypes [28] and strain genotypes [29] have been found to

co-exist in hosts in South Africa. Our findings demonstrate that the successful spread of F15/LAM4/KZN strains during the Tugela Ferry XDR-TB outbreak in 2005 may be attributed to the presence of drug resistance-conferring mutations associated with little or no fitness cost. This conclusion is in agreement with previous observations that drug-resistant F15/LAM4/KZN strains are highly transmissible [7] and highlights the importance of TB surveillance systems, which may prevent future epidemics caused by fit resistant strains.

ACKNOWLEDGEMENTS

The authors are grateful for the financial support provided by the National Research Foundation (Innovation Master's Scholarship, grant no. 74567) and the College of Health Sciences, University of KwaZulu-Natal. This work has been presented at the 3rd TB conference (Durban) and 43rd Union World Conference on Lung Health (Kuala Lumpur) in 2012. We thank Rupal R Shah for kindly providing us with the growth and competition assay protocol. We are indebted to Dr Adrie Steyn, KwaZulu-Natal Research Institute for TB and HIV, for his insightful contribution.

REFERENCES

1. World Health Organization. Global tuberculosis report 2012. WHO/HTM/TB/2012.6. Geneva, Switzerland: WHO, 2012.
2. World Health Organization. Multidrug and extensively drug-resistant TB (M/XDR-TB): 2010 global report on surveillance and response. WHO/HTM/TB/2010.3. Geneva, Switzerland: WHO, 2010.
3. Cohen T, Murray M. Modelling epidemics of multidrug-resistant *M. tuberculosis* of heterogeneous fitness. Nat Med 2004; 10: 1117-1121.
4. Andersson DI, Levin BR. The biological cost of antibiotic resistance. Curr Opin in Microbiol.1999; 2: 489-493.
5. Gagneux S, Long C D, Small P M, Van T, Schoolnik G K, Bohannan B J. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science. 2006; 312: 1944-1946.
6. Maisnier-Patin S, Andersson DI, Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res Microbiol. 2004; 155: 360-369.
7. Pillay M, Sturm AW. Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. Clin Infec Dis. 2007; 45: 1409-1414.

8. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. The Lancet. 2006; 368:1-6.
9. Chihota VN, Muller B, Mlambo CK, Pillay M, Tait M, Streicher EM, Marais E, van der Spuy GD, Hanekom M, Coetzee G, Trollip A, Hayes C, Bosman ME, Gey van Pittius NC, Victor TC, van Helden PD, Warren RM. Population structure of multi- and extensively drug-resistant *Mycobacterium tuberculosis* strains in South Africa. J Clin Microbiol. 2012; 50:995-1002.
10. Toungoussova OS, Caugant DA, Sandven P, Mariandyshev AO, Bjune G. Impact of drug resistance on fitness of *Mycobacterium tuberculosis* strains of the W-Beijing genotype. FEMS Immunol Med Microbiol 2004; 42: 281-290.
11. Bjorkman J, Andersson DI. The cost of antibiotic resistance from a bacterial perspective. Drug Resistance Updates. 2000; 3: 237-245.
12. Marais BJ, Victor TC, Hesseling AC, Barnard M, Jordaan A, Brittle W, Reuter H, Beyers N, van Helden PD, Warren RM, Schaaf HS. Beijing and Haarlem genotypes are overrepresented among children with drug-resistant tuberculosis in Western Cape Province of South Africa. J Clin Microbiol. 2006; 44: 3539-3543.
13. NCCLS. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard. NCCLS document M24-A [ISBN 1-56238-500-3]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2003.
14. Bhatter P, Chatterjee A, D'souza D, Tolani M, Mistry N. Estimating fitness by competition assays between drug susceptible and resistant *Mycobacterium tuberculosis* of predominant lineages in Mumbai, India. PLoS One. 2012; 7: 1-8.
15. Van Soolingen D, de Haas PE, Kremer K. Restriction Fragment Length Polymorphism (RFLP) typing of mycobacteria. Methods Mol Med. 2001; 54:165-203.
16. Camus JC, Pryor MJ, Medique C, Cole ST. Re-annotation of the genome sequence of *Mycobacterium tuberculosis* H37Rv. Microbiol. 2002; 148: 2967-2973.
17. Andersson DI. The biological cost of mutational antibiotic resistance: any practical conclusions? Curr Opin Microbiol. 2006; 9:461-465.
18. Mariam DH, Mengistu Y, Hoffner SE, Andersson DI. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 2004; 48: 1289-1294.

19. Pym AS, Saint-Joanis B, Cole ST. Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implications for transmission in humans. Infec & Immunity. 2002; 70: 4955-4960.
20. Unissa AN, Narayanan S, Selvakumar N. Virulence in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from South India. Int J Mol Clin Microbiol. 2011; 1: 87-96.
21. Zenteno-Cuevas R, Zenteno JC, Cuellar A, Cuevas B, Sampieri CL, Riviera JE, Parissi. Mutations in *rpoB* and *katG* genes in *Mycobacterium isolates* from Southeast Mexico. Mem Inst Oswalso Cruz. 2009; 104: 468-472.
22. Loerger TR, Koo S, No E, Chen X, Larsen M, Jacobs WR, Pillay M, Sturm AW, Sacchettini. Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. PLoS One. 2009; 4: 1-9.
23. Billington OJ, McHugh TD, Gillespie SH. Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1999; 43: 1866-1869.
24. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, Galagan J, Niemann S, Gagneux S. Whole genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identified compensatory mutations in RNA polymerase. Nature Genetics. 2012; 44: 106-110.
25. Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiwirth B, Cole ST, Jacobs WR, Telenti A. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother. 1994; 38:773-780.
26. Victor TC, van Rie A, Jordaan AM, Richardson M, van der Spuy GD, Beyers N, van Helden PD, Warren R. Sequence polymorphism in the *rrs* gene of *Mycobacterium tuberculosis* is deeply rooted within an evolutionary clade and is not associated with streptomycin resistance. J Clin Microbiol. 2001; 39: 4184-4186.
27. Motiwala AS, Dai Y, Jones-Lopez EC, Hwang S, Lee JS, Cho SN, Via LE, Barry III CE, Alland D. Mutations in extensively drug-resistant *Mycobacterium tuberculosis* that do not code for known drug-resistance mechanisms. J Infect Dis. 2010; 201: 881-888.
28. van Rie A, Victor TC, Richardson M, Johnson R, van der Spuy GD, Murray EJ, Beyers N, Gey van Pittius NC, van Helden PD, Warren RM. Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. Am J Respir Crit Care Med. 2005; 172: 636-642.

29. Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, Gey van Pittius NC, van Helden PD. Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Respir Crit Care Med. 2004; 169: 610-614.

CHAPTER THREE

Fitness-compensatory mutations facilitate the spread of drug-resistant F15/LAM4/KZN and F28 *Mycobacterium tuberculosis* strains in KwaZulu-Natal, South Africa

Naidoo CC, [1] Pillay M. [1]

[1]Medical Microbiology and Infection Control, University of KwaZulu-Natal, Durban, South Africa

Submitted for publication

ABSTRACT

While the acquisition of drug resistance is often accompanied by fitness costs, *Mycobacterium tuberculosis* has developed mechanisms to overcome these costs in the form of compensatory mutations. In an attempt to dissect strain-specific differences in biological fitness, ten *M. tuberculosis* genomes, representing F15/LAM4/KZN, Beijing, F11 and F28 genotypes were sequenced on the Illumina MiSeq platform. Drug-susceptible F15/LAM4/KZN strains differed by 43 SNPs, demonstrating that heterogeneity exists even among closely-related strains. We found unique, non-synonymous single nucleotide polymorphisms (SNPs) in the *sigA* and *grcC1* genes of MDR and XDR F15/LAM4/KZN strains, respectively. The F28 MDR strain harboured a novel *ubiA* mutation in combination with its *embB* M306I mutation, which may be related to ethambutol resistance. In addition, it possessed a low-frequency *rpoC* mutation, suggesting that this strain was in the process of developing compensation. In contrast, no compensatory mutations were identified in Beijing and F11 MDR strains, corroborating its low *in vitro* fitness. Clinical strains also harboured unique SNPs in a number of important genes associated with virulence, highlighting the need for future studies which examine the correlation of genetic variations with phenotypic diversity. In summary, whole-genome sequencing revealed the presence of fitness-compensatory mutations in F15/LAM4/KZN and F28 genotypes which predominate in MDR and XDR forms in KwaZulu-Natal, South Africa.

INTRODUCTION

Since the discovery of the first anti-tuberculosis (TB) drug about 70 years ago, *M. tuberculosis* has progressively evolved from monodrug resistant to multidrug resistant (MDR), extensively drug resistant (XDR) and recently, totally drug-resistant (TDR) forms, in various parts of the world (WHO, 2014). Anti-TB drugs, which are generally designed to target essential, highly conserved genes, may be impeded by chromosomal mutations which alter the target site (Sandgren et al. 2009). The acquisition of drug resistance is often associated with a fitness 'cost' as mutations may affect the normal function of target genes, thereby reducing the growth rate of *M. tuberculosis* (Andersson and Levin, 1999; Billington et al. 1999; Gillespie 2002). Moreover, strains carrying identical resistance-encoding mutations may differ in their ability to spread from person-to-person, demonstrating the significance of strain genetic background in the context of fitness (Gagneux et al. 2006; Fenner et al. 2012). Compensatory mutations however, may help restore the fitness of drug-resistant strains (Sherman et al. 1996; de Vos et al. 2013).

The World Health Organisation (WHO) estimated that 1 out of every 100 people in South Africa develops active TB disease every year (WHO, 2014). As a result of poor TB control programmes and treatment strategies, *M. tuberculosis* strains have been found to disseminate in MDR (isoniazid and rifampicin resistance) and XDR (MDR in addition to resistance to any fluoroquinolone and at least one injectable drug) forms in South Africa (Pillay and Sturm 2007; Victor et al. 2007; Cox et al. 2010; WHO, 2014; Cohen et al. 2015). Moreover, the appearance of XDR-TB is strongly associated with specific strain genotypes (Muller et al. 2013; Cohen et al. 2015), including the F15/LAM4/KZN (KZN) genotype which caused the largest XDR-TB outbreak, in Tugela Ferry, KwaZulu-Natal (Gandhi et al. 2006). Our recent study demonstrated increased fitness for KZN strains in laboratory culture, contrasting with Beijing and LAM3 MDR strains which were characterized by impeded growth and metabolic profiles (Naidoo and Pillay 2014). Competitive fitness assays also showed consistently lower fitness indices for most drug-resistant strains, when compared with susceptible competitor strains (Naidoo and Pillay 2014). In support of this, preliminary sequencing of selected resistance-conferring genes revealed the presence of low or no fitness cost mutations in KZN and F28 strains and high fitness cost mutations in Beijing and F11 strains.

Despite the recent advances in understanding the biological cost of drug resistance, this subject remains incompletely studied among clinical strains. This is in part due to the difficulty in assessing the effects of strain-specific variation on TB pathogenesis. The introduction of next-generation sequencing technology may help decipher the functional and clinical consequences of *M. tuberculosis* genetic diversity, displayed in the form of single nucleotide polymorphisms (SNPs), small insertions and deletions (indels) and gene rearrangements (Coscolla and Gagneux 2014). In this study, we correlated whole-genome sequencing (WGS) data with previous experimental fitness data of ten clinical strains from South Africa. Comparative genomic analysis revealed the existence of significant sequence diversity among clinical strains and helped uncover novel mutations - some of which may aid in the amelioration of fitness costs.

METHODS

Clinical strains

Ten *M. tuberculosis* strains were selected for WGS analysis. These included four susceptible, four MDR and two XDR strains for which drug susceptibility testing was performed previously (Naidoo and Pillay 2014). Spoligotyping and IS*6110*-RFLP typing were performed previously (Naidoo and Pillay 2014), classifying strains into four families, namely: F15/LAM4/KZN (n = 4), Beijing (n = 2), F11/LAM3 (n =2) and F28/S (n = 2). Mycobacteria were grown in Middlebrook 7H9 broth (Difco) containing 10% oleic albumin dextrose catalase (OADC) (BD Biosciences), 0.5% glycerol (Sigma) and 0.05% Tween-80 (Sigma) at 37 °C, until an optical density (OD_{600}) of 1.

Whole genome sequencing

Genomic DNA was isolated using the CTAB-lysozyme method (Larsen et al. 2007) and purified using the Genomic DNA Clean & Concentrate Kit™ (Zymo Research, Pretoria, South Africa). Whole-genome sequencing was performed using the Illumina MiSeq Sequencer (Illumina) at Inqaba Biotec (Pretoria, South Africa). An Illumina-provided tagmentation kit (Nextera) was used for library preparation. Paired-end massively parallel sequencing (2 x 300 bp) was carried out with a MiSeq v3 sequencing kit.

Read mapping

Raw sequence reads were trimmed to remove adaptor sequences and low quality sequences using the CLC Genomics Workbench (version 7.5.1; QIAGEN, Aarihus, Denmark). The '*Map Reads to Reference'* function was used to assemble paired reads from all strains to the *M. tuberculosis* H37Rv reference genome (NCBI accession # N000962) using the following parameters: similarity fraction = 0.8, length fraction = 0.5, mismatch cost of 2, deletion cost of 3 and insertion cost of 3. Similarly, KZN strains were mapped against KZN-4207 (NCBI accession # CP001662), a previously sequenced susceptible strain. In addition, re-sequenced MDR and XDR- KZN strains, V1435 and KZN605, were mapped against previous versions by the Broad Institute (www.broad.mit.edu/annotation/genome/mycobacterium_tuberculosis_spp) (NCBI accession # CP001658 and # NC_018078, respectively). Beijing strains were mapped against HN878 (NCBI accession # NZ_CM001043) and F11 strains were mapped against the F11 reference genome (NCBI accession # NC_009565). Since no reference genome was available for F28, this sequence was mapped against H37Rv only. All statistical information was derived from the resulting table

generated by the software. The '*k-mer based tree construction*' function in the CLC Genomics Workbench was used for phylogram construction. Regions with low coverage were filled from the reference sequence (H37Rv) and quality scores were used for conflict resolution.

SNP analysis and confirmation

Drug resistance-conferring mutations were characterised using publicly available databases including TBDreaMDB and the Broad Institute Tuberculosis Drug Resistance Mutation Database, together with recently published reports. Variants were called on the basis of high quality (Phred score of Q30) and more than 70% of the reads reflecting the SNP. SNPs with less than ten reads mapping to the reference were excluded from analysis. This included repetitive sequence regions which usually map poorly due to high GC content. Verification of a subset of SNPs was accomplished by PCR using Q5 High-Fidelity DNA Polymerase (NEB) and primers listed in Table S3.1. Sanger sequencing was performed on the ABI 3500XL Genetic Analyzer using forward primers. Bioedit software and chromatograms were used to analyse sequences for the presence of wild-type or mutant peaks. Non-synonymous SNPs (nSNPs) were categorised into functional groups according to Tuberculist (Lew et al. 2011). A selection of genes encoding virulence factors was inspected for nSNPs, across the four strain families (Smith, 2003). We used the '*InDels and Structural Variants Tool*' on paired reads mapped to H37Rv with the following parameters: *P*-value threshold = 0.0001, maximum number of mismatches = 2 and minimum number of reads = 2.

Minimum inhibitory concentration (MIC) testing of isoniazid and ethambutol

Strains were cultured on Middlebrook 7H11 agar (Difco) and adjusted to a McFarland no. 1 turbidity standard in phosphate buffered saline with Tween-80. Serially-diluted suspensions were inoculated onto Middlebrook 7H10 agar containing 10% OADC, 0.5% glycerol and drug concentrations ranging from 0.0625 to 16 mg/L for isoniazid (INH) and 0.4687 to 120 mg/L for ethambutol (EMB). Strains were classified as having low-level or high-level resistance in the presence of 0.2 mg/L or 1 mg/L of INH, respectively. Two critical breakpoints of 5 and 7.5 mg/L were tested for EMB.

Ethical approval

Ethical approval was obtained from the Biomedical Research Ethics Committee, University of KwaZulu-Natal, South Africa (BE258/15).

RESULTS

Whole genome sequencing

Mapping reports detailing summary statistics of read distribution are described in Table S3.2-S3.3. Sequencing reads were deposited into the NCBI database under the accession number PRJNA305082. Sequencing depth in strains ranged from 20 to 60 X with average read length of 230 bp (Table 3.1). Phylogenetic relationships among clinical strains are shown in Figure 3.1.

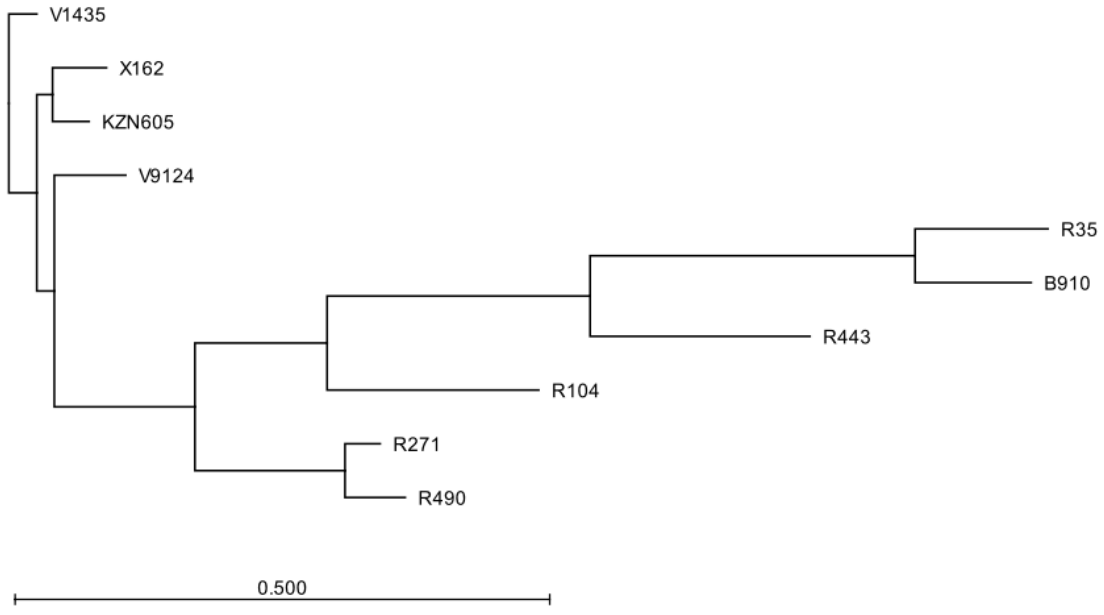


Figure 3.1 Phylogenetic relationships of clinical strains. The tree was constructed by the neighbour-joining method using the CLC Genomics Workbench.

Table 3.1 Genotype, drug susceptibility and sequencing statistics of clinical strains of *M. tuberculosis*

Strain	Missing spacers [a]	Genotype	Drug resistance [a]	Type [b]	Read count	Average read length (bp)	Average depth of coverage
V9124	21-24, 33-36, 40	F15/LAM4/KZN	-	DS	925,818	265	55.6 X
V1435	21-24, 33-36, 40	F15/LAM4/KZN	IR	MDR	475,657	233	25.1 X
KZN605	21-24, 33-36, 40	F15/LAM4/KZN	IREOKCN	XDR	505,153	226	25.8 X
X162	21-24, 33-36, 40	F15/LAM4/KZN	IREOKCN	XDR	532,585	235	28.4 X
B910	1-34	Beijing	-	DS	458,138	231	24.0 X
R35	1-34	Beijing	IRS	MDR	1,021,876	244	56.4 X
R490	9-11, 21-24, 33-36	F11/LAM3	-	DS	719,873	189	30.8 X
R271	9-11, 21-24, 33-36	F11/LAM3	IR	MDR	1,362,298	193	59.6 X
R104	9-10, 33-36	F28/S	-	DS	380,439	229	19.7 X
R443	9-10, 33-34	F28/S	IRSEK	MDR	389,783	253	20.7X

[a] I: isoniazid, R: rifampicin, E: ethambutol, O: ofloxacin, K: kanamycin, C: capreomycin, N: niacinamide, S: streptomycin.

[b] DS: drug-susceptible, MDR: multidrug-resistant, XDR: extensively drug-resistant

Mapping of F15/LAM4/KZN strains to KZN-4207

Sequence reads from KZN strains mapped uniquely to 4,394,985 bases (99 %) of the KZN-4207 reference genome (Table S3.1). The susceptible strain, KZN-V9124 had 43 nSNPs relative to KZN-4207, of which 37 were unique and 6 were shared with the drug-resistant strains (Figure 3.2a)). Seven nSNPs were common to the MDR (KZN-V1435) and XDR strains (KZN-605 and KZN-X162) of which 4 had no definitive link to drug resistance. Drug-resistant strains had previously reported mutations in resistance-conferring genes (Ioerger et al. 2009). Mapping of the XDR strains to KZN-4207 revealed 3 unique mutations in KZN-X162. Of these, one mutation (L129M) identified in the *grcC1* gene (Rv0562) encoding polyprenyl-diphosphate synthase, was not detected in previously-sequenced KZN genomes. Fifteen frameshift mutations were identified in V1435 (MDR) with respect to the Broad's sequence (Table S3.4), as well as a nSNP in *sigA,* albeit with low read frequency (<70%). Sanger sequencing of *sigA* revealed the presence of two peaks at the target nucleotide site, confirming it was not a false positive. Fewer differences were evident between KZN605 and the previously-sequenced genome. These included 5 frameshift mutations (4 in conserved hypothetical genes and 1 in a gene encoding a RifB protein) and 2 nSNPs in conserved hypothetical genes (Table S3.5).

Mapping of Beijing strains to HN878

A total of 120 and 135 nSNPs were detected in susceptible and MDR Beijing strains respectively, of which 65 were shared (Figure 3.2b). The majority of shared mutations (46 %) occurred within hypothetical proteins, underscoring the need for functional assignment to yet uncharacterised genes. Unique nSNPs in the susceptible strain included those in *moaA1* and *rpfB*, whilst the MDR strain harboured nSNPs in *sigK* and double mutations in *murD*.

Mapping of F11 strains to F11 reference

A comparison of the susceptible and MDR F11 strains to the F11 reference genome revealed 54 and 52 nSNPs, respectively (Figure. 3.2c). A total of 30 and 28 nSNPs were uniquely found in the susceptible and MDR strain, respectively. Of particular interest, was a Val104Leu mutation detected in the *hrp1* gene (Rv2626c) of the susceptible strain. This gene is a member of the dormancy regulon and is strongly up-regulated under hypoxic conditions (Sharpe et al. 2008). The MDR strain harboured a mutation in *vapC38* (Rv2494) which encodes a toxin previously shown to have increased expression during nutrient starvation, with plausible importance in the establishment of latent infection (Albrethsen et al. 2013).

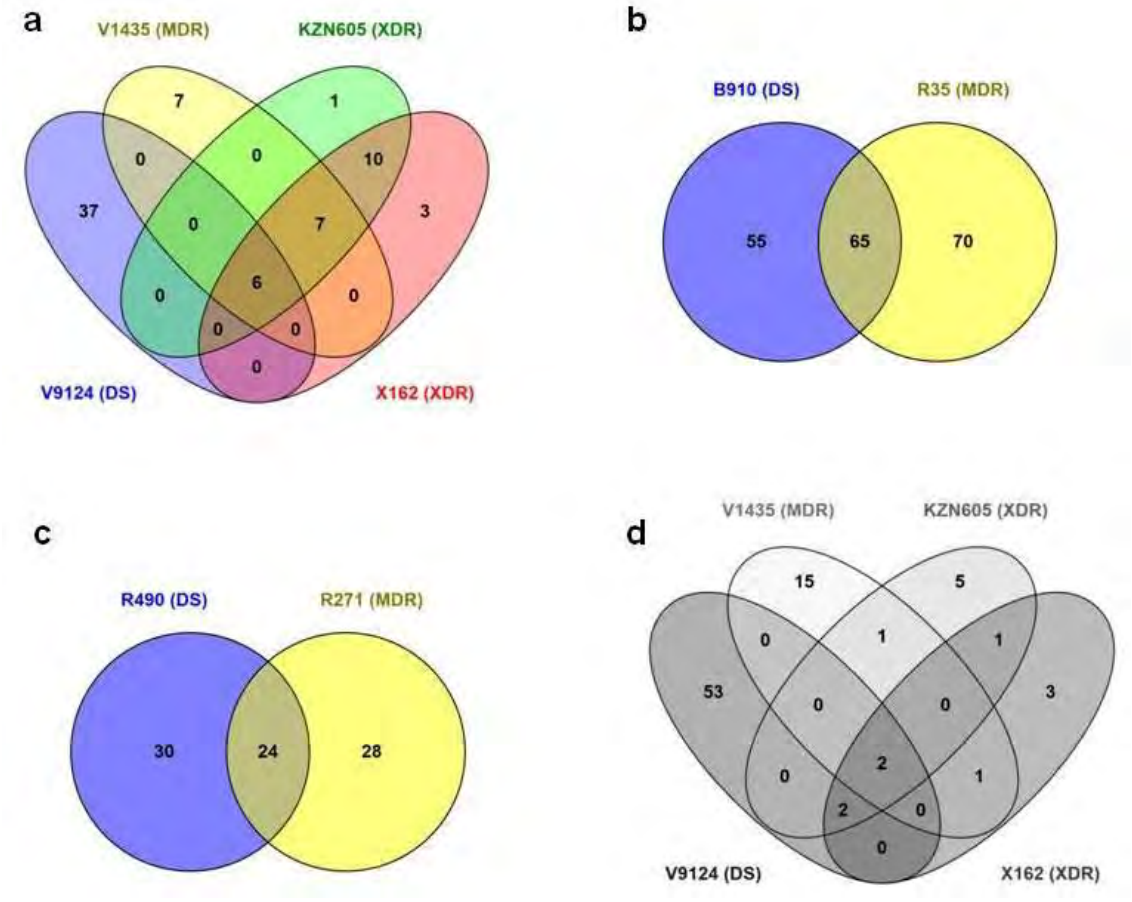


Figure 3.2 Venn diagrams depicting the distribution of non-synonymous single nucleotide polymorphisms in clinical strains. (a) F15/LAM4/KZN strains relative to KZN-4207, (b) Beijing strains relative to HN878, (c) F11 strains relative to F11 reference genome and (d) large indels (100 – 500 bp) in F15/LAM4/KZN strains relative to H37Rv reference genome. DS: drug-susceptible, MDR: multidrug-resistant, XDR: extensively drug-resistant

Drug resistance and efflux pumps

Reads from the ten strains were uniquely mapped to an average of 4,341,256 bases (98.4 %) of the H37Rv reference genome (Table S3.3). A total of 50 mutations spanning 25 genes associated with drug resistance, were identified (Table 3.2). For INH resistance, we analysed *katG*, *ndh*, *accD6*, *kasA*, *Rv1592c* and *mabA*. Drug-resistant KZN and F28 strains had the canonical *katG* Ser315Thr mutation however different INH MICs (Table 3.3). Although the Beijing-MDR strain had a different substitution at codon 315, it maintained a comparably high INH MIC. In contrast, the F11

MDR strain possessed a rare *katG* mutation corresponding to the lowest INH MIC detected in this study. Mutations detected in *ndh*, *accD6*, *kasA* and *Rv1592c* did not correlate well with INH resistance as these were present in susceptible strains (Table 3.2). A recently characterised, *mabA*^{g609a} silent mutation (Ando et al. 2014) was detected in our Beijing MDR strain. Drug-resistant KZN strains harboured a mutation (T1673432A) -8 upstream of *mabA*.

Rifampicin-resistant strains exhibited mutations across five codons in *rpoB*. Interestingly, KZN-V1435 had a secondary *rpoB* mutation which was reflected by 61% of the reads. Sanger sequencing indicated the presence of double nucleotide peaks (G and A) at position 1460, confirming it was a true variant (data not shown). Both susceptible and MDR Beijing strains harboured a sSNP in *rpoB*. The MDR-F28 strain possessed an *rpoC* compensatory mutation previously associated with the Ser450Leu *rpoB* mutation (de Vos et al. 2013), however only 42% of the reads reflected this SNP. Re-sequencing of *rpoC* revealed the presence of two peaks at nucleotide position 1448 (data not shown), confirming that it was a true variant.

M. tuberculosis possesses three Emb homologs of which *embA* and *embB* which are co-transcribed; *embC* on the other hand is co-transcribed with *dprE1*, Rv3791 (*dprE2*) and Rv3792 (*aftA*) (Goude et al. 2008). Four *embB* mutations were observed in KZN, F11 and F28 resistant strains corresponding to different levels of EMB resistance (Table 3.3). The susceptibility of strains V9124, B910, R490 and R104 to EMB was confirmed by MICs equivalent to 1.875 mg/L. Despite having an *embB* mutation, the F11 MDR strain presented with a lower EMB MIC than the Beijing MDR strain without an *embB* mutation.

For fluoroquinolone resistance, one of five *gyrA* mutations correlated with ofloxacin resistance (A90V), whilst others were common to both susceptible and resistant strains. All SNPs identified in our examination of *gyrB*, *thyA*, *ddlA* and *gidB* were present in both susceptible and -resistant strains, thus ruling out their association with drug resistance.

Table 3.2 Polymorphisms in genes associated with drug resistance

Drugs	Gene	Name	Nucleotide change	AA change	Genotype	Strain (s)
FQ	*Rv0005*	*gyrB*	281C→T	P94L	F11	R490, R271
	Rv0006	*gyrA*	61G→C	E21Q	All	All
			269C→T	A90V	F15/LAM4/KZN	KZN605, X162
			284G→C	S95T	All	All
			739G→A	G247S	F15/LAM4/KZN	V9124, V1435, KZN605, X162
			2003G→A	G668D	All	All
ETH-INH	*Rv0486*	*mshA*	560C→T	A187V	Beijing	B910, R35
	Rv3199c	*nudC*	716C→G	P239R	Beijing	B910, R35
THZ	*Rv0644c*	*mmaA2*	639A→C	E213D	Beijing	B910, R35
RIF	*Rv0667*	*rpoB*	1303G→T	D435T	F15/LAM4/KZN	V1435
			1304A→G	D435G	F15/LAM4/KZN	KZN605, X162
			1349C→T	S450L	F11, F28	R271, R443
			1355T→C	L452P	F15/LAM4/KZN; Beijing	KZN605, X162; R35
			[a]1460A→G	N487S	F15/LAM4/KZN	V1435
			3225T→C	-	Beijing	B910, R35
			3317T→C	I1106T	F15/LAM4/KZN	KZN605, X162
	Rv0668	*rpoC*	[a]1448T→G	V483G	F28	R443
			1626C→G	-	F15/LAM4/KZN; F11	V9124, V1435, KZN605, X162; R490, R271
INH	*Rv1483*	*mabA*	609G→A	-	Beijing	R35
	Rv1592c		963A→G	E321E	Beijing	B910, R35
			964A→G	I322V	Beijing	B910, R35
	Rv1854c	*ndh*	53T→C	V18A	F28	R104
	Rv2245	*kasA*	805G→A	G269S	F15/LAM4/KZN	V9124, V1435, KZN605, X162
	Rv2247	*accD6*	686A→G	D229G	Beijing	B910, R35
	Rv2764c	*thyA*	604A→G	T202A	F15/LAM4/KZN; F11	V9124, V1435, KZN605, X162; R490, R271
	Rv1908c	*katG*	944G→C	S315T	F15/LAM4/KZN; F28	V1435, KZN605, X162; R443
			944G→T	S315N	Beijing	R35

			700G→A	G234R	F11	R271
			1388G→T	R463L	Beijing	B910, R35
CS	*Rv1704c*	*cycA*	278G→T	R93L	All	All
	Rv2981c	*ddlA*	1093A→G	T365A	F15/LAM4/KZN; F11; F28	V9124, V1435, KZN605, X162; R490, R271; R104, R443
PZA	*Rv2043c*	*pncA*	395G→C	G132A	F15/LAM4/KZN	V1435
			456_457insC	T153fs	F15/LAM4/KZN	KZN605, X162
EMB	*Rv3792*	*aftA*	513G→A	-	Beijing	R35
			1032C→T	-	F28	R104
			1343C→G	P448R	F28	R104
	Rv3793	*embC*	2781C→T	-	All	All
	Rv3794	*embA*	228C→T	-	Beijing	B910, R35
	Rv3795	*embB*	916A→G	M306V	F15/LAM4/KZN	V1435, KZN605, X162
			918G→C	M306I	F28	R443
			1217G→A	G406D	F11	R271
			[a]1489C→A	G406K	F28	R443
	Rv3806c	*ubiA*	563T→C	V188A	F15/LAM4/KZN	KZN605, X162
			112G→T	A38S	F28	R443
STR	*Rv3919c*	*gidB*	47T→G	L16R	F15/LAM4/KZN; F11	V9124, V1435, KZN605, X162; R490, R271
			583T→C	Y195H	Beijing	B910
			276A→C	E92N	Beijing	B910, R35
			615A→G	-	Beijing	B910, R35
KAN	*Rvnr01*	*rrs*	1400A→G	-	F15/LAM4/KZN	KZN605, X162
	Rvnr02	*rrl*	2398G→A	-	F15/LAM4/KZN	KZN605, X162

Drug resistance groups: FQ, fluoroquinolones; ETH-INH, ethionamide-isoniazid; THZ, thiacetazone; RIF, rifampicin; INH, isoniazid; CS, cycloserine; PZA, pyrazinamide; EMB, ethambutol; STR, streptomycin; KAN, kanamycin. [a] denotes low-frequency (< 70%) variants confirmed by PCR and Sanger sequencing.

Table 3.3 Minimum inhibitory concentrations for isoniazid and ethambutol in clinical strains

Strain	Genotype	Isoniazid MIC (μg/ml)	Ethambutol MIC (μg/ml)
V9124	F15/LAM4/KZN	Susceptible	1.875
V1435	F15/LAM4/KZN	>16	ND
KZN605	F15/LAM4/KZN	>16	30
X162	F15/LAM4/KZN	>16	30
B910	Beijing	Susceptible	1.875
R35	Beijing	>16	7.5
R490	F11/LAM3	Susceptible	1.875
R271	F11/LAM3	1	5
R104	F28/S	Susceptible	1.875
R443	F28/S	8	30

Isoniazid (INH) and ethambutol (EMB) MICs were determined by the agar proportion method. Triplicate and duplicate experiments were performed for INH and EMB, respectively. The critical breakpoint for INH was 1 μg/ml and for EMB was 5 and 7.5 μg/ml. ND: not determined

Previous studies have shown associations between drug efflux pumps and pathogenicity/virulence (Piddock 2006; Bina et al. 2009). Of the 36 SNPs occurring in 20 genes encoding drug efflux pumps, 31 (86 %) were present in susceptible strains (Table 3.4), suggesting that drug resistance is not always acquired by genetic modification of efflux pumps. Mutations in *drrA* and *ctpB* (G23S) - a gene believed to encode a putative copper transporter (Knapp et al. 2015), were uniquely present in the drug-resistant KZN strains.

Table 3.4 Polymorphisms in known or putative drug efflux pumps in clinical strains

Gene	Name	Nucleotide change	Amino acid change	Genotype	Strain (s)
Rv0050	*ponA1*	1891_1892 ins	P631del ins PS	F11, F28	R490, R271, R104, R443
		1891C→T	P631S	F15/LAM4/KZN	V9124, V1435, X162
		184_1885 ins	P628_P629 ins P	Beijing	B910
		1891_1892 ins	P631del ins PPS	Beijing	B910
		1891_1892 ins	P631del ins PPPS	Beijing	R35
Rv0103c	*ctpB*	65T→C	L22S	All	All
		67G→A	G23S	F15/LAM4/KZN	V1435, KZN605, X162
		749C→T	A250V	Beijing	B910
Rv0107c	*ctpI*	4711_4712 ins T	S1571fs	All	All
		3409G→T	V1137L	Beijing	B910
Rv0174	*mce1F*	1109T→C	L370P	All	All except R104
Rv0194		221T→C	Met74T	All	All
		3293C→T	P1098L	Beijing	B910, R35
Rv0425c	*ctpH*	2065A→G	M689V	All	All
		197A→C	N66T	F11	R490, R271
Rv0507	*mmpL2*	1277G→A	R426H	All	All
Rv0545c	*pitA*	145C→T	P49S	All	All
Rv0589	*mce2A*	152T→C	F51S	All	All
Rv0676c	*mmpL5*	2842A→G	I948V	All	All
		2381C→T	T794I	Beijing	B910, R35
		2299G→A	D767N	Beijing	B910, R35
Rv1183	*mmpL10*	1222A→G	T408A	Beijing	B910, R35
Rv1258c		580_581 insC	E194fs	Beijing	B910, R35
Rv1634		592G→C	G198R	Beijing	B910, R35
Rv1730c		1483_1485 del	F495del	Beijing	B910, R35
		1305G→C	D435E	Beijing	B910, R35
		1326_1332 del	D442fs	Beijing	R35
		1313_1324 del	T438_D442 del ins N	Beijing	R35
Rv2687c		658C→T	R220W	F15/LAM4/KZN	V9124, V1435, KZN605, X162
Rv2688c		466C→A	P156T	All	All
Rv2936	*drrA*	784A→G	R262G	F15/LAM4/KZN	V1435, KZN605, X162
Rv3331	*sugI*	116C→T	P39L	F11	R490, R271
		1268C→T	P423L	All	All
Rv3522	*itp4*	471G→C	W157C	Beijing	B910
		971C→T	T324I	All	All
Rv3833		313G→A	V105I	All	All

Del, deletion; ins, insertion; fs, frameshift

Functional categorisation of SNPs

Genes harbouring nSNPs relative to H37Rv were functionally categorised according to Tuberculist. The vast majority of nSNPs, regardless of strain family, were in genes encoding cell and cell wall processes followed by intermediary metabolism and respiration and conserved hypotheticals (Figure 3.3). Closer examination of 13 genes encoding known virulence factors revealed clustering of strain genotypes with mutations in specific genes i.e. *mmaA4* for KZN strains; *glcB*, *fadD26*, *fadD28, plcA, plcC, mbtB* and *lipF* for Beijing strains; *narG* and *mmpL7* for F11 strains; and *plcB* for F28 strains (Table 3.5). Whilst Beijing, F11 and F28 harboured distinct mutations in *mas* (Rv2940c), the KZN group was unique in that it lacked mutations in this gene.

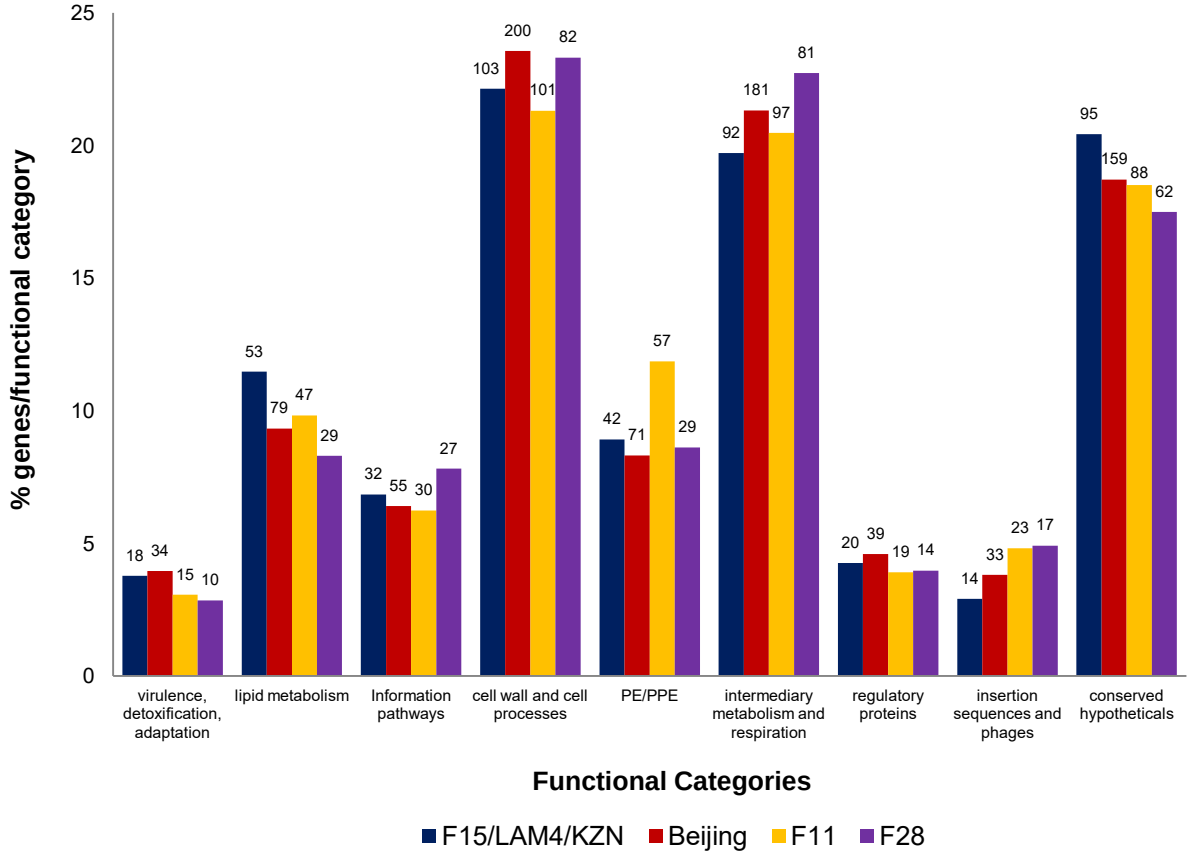


Figure 3.3 Functional categorisation of genes with non-synonymous SNPs (nSNPs) in different strain families. Number of nSNPs in each functional group is depicted above bars

Table 3.5 Polymorphisms in genes encoding *M. tuberculosis* virulence factors

Gene	Name	Nucleotide change	Amino acid change	Strain (s)	Strain (s)
Rv0642c	*mmaA4*	494A→G	N165S	F15/LAM4/KZN	V9124, V1435, KZN605, X162
Rv1161	*narG*	2999G→A	R1000H	F11	R490, R271
Rv1837c	*glcB*	310G→A	G104S	Beijing	B910, R35
Rv1860	*apa*	406T→C	F136L	All	All
Rv2349c	*plcC*	1081G→T	G361C	Beijing	B910, R35
Rv2350c	*plcB*	1406T→C	L469S	F28	R104
Rv2351c	*plcA*	1336A→G	T466A	Beijing	B910, R35
Rv2383c	*mbtB*	2020G→C	V647L	Beijing	B910, R35
Rv2930	*fadD26*	338C→A	S113Y	Beijing	B910
Rv2940c	*mas*	1057G→A, 1193C→G	A353T, P398R	F28	R104
		3044A→C	D1015A	F11	R490
		6013A→C	T2005P	Beijing	B910, R35
Rv2941	*fadD28*	1306A→G	T436A	Beijing	B910, R35
Rv2942	*mmpL7*	1651G→A	A551T	F11	R490
Rv3487c	*lipF*	697C→T	R233C	Beijing	B910, R35

Indels

Indels represent a significant source of phenotypic variability and may enhance the pathogenesis of infectious agents (Liu et al. 2014). Small (<100 bp) and large (100 – 500 bp) insertions and deletions were analysed in strains relative to H37Rv. In total, 562 small indels and 207 large indels were identified in all strains, of which 96 were in PE-PPE-PGRS regions - a significant source of antigenic variability (Sampson 2011). Within the KZN group, 2 indels i.e. a large deletion (321 bp) and insertion (124 bp), were shared amongst all 4 strains in Rv0145 and Rv2112c, respectively (Fig. 3.2d). Susceptible strains, KZN-V9124 and F28-R104 harboured the highest (57) and lowest number (8) of large indels, respectively. A higher degree of overlap was observed within the Beijing group, with 15 shared indels spanning PPE-PE-PGRS regions, hypothetical genes and transmembrane proteins.

DISCUSSION

In our previous study, we showed that specific genotypes i.e. F15/LAM4/KZN and F28 were better suited to drug-resistant forms than Beijing and F11 strains, in terms of their *in vitro* fitness (Naidoo and Pillay, 2014). In the current study, we addressed the role of resistance-conferring mutations and/or compensatory mutations in the fitness of these clinical strains. Mapping of the KZN

susceptible strain (V9124) to the reference genome (KZN-4207) revealed a surprising number (43) of nucleotide variations. This genetic dissimilarity between both susceptible strains most likely accounts for our previous observations, whereby resistant strains exhibited differential growth when paired with V9124 or KZN-V4207 in competitive growth assays (Naidoo and Pillay, 2014).

The KZN-MDR strain, V1435, contained a novel, low-frequency *sigA* mutation that was absent in the KZN 1435 genome previously sequenced by the Broad Institute. Moreover, this mutation was undetected in other strains sequenced in this study. Since variants with at least 30% of the Illumina reads reflecting a SNP can exist as sub-populations within the *M. tuberculosis* genome (Black et al. 2015), we re-sequenced the *sigA* gene. Double peaks were observed in the Sanger chromatogram, confirming it as a true variant. An earlier study showed attenuation of an *M. bovis* strain carrying a *sigA* mutation (Collins, 1995) while a later study showed that the overexpression of the *sigA* gene enhanced intracellular growth in macrophages and mouse lungs (Wu et al. 2004). V1435 demonstrated comparable growth to V9124, KZN605 and X162 in laboratory culture (Naidoo and Pillay, 2014) however the virulence of this strain remains to be determined. Mapping of the XDR strain, KZN605 to the previous version revealed fewer genetic differences that were predominantly observed in hypothetical genes, thus their significance remains elusive. Overall, we speculated that these changes were due to adaptation during laboratory culture (although passages were kept to a minimum) or could reflect differences in read depth.

Comparative assessment of the KZN XDR strains revealed the presence of some formerly reported, as well as a unique mutation in the *grcC1* gene of X162 - a mutation that was unreported in any of the previously-sequenced KZN-XDR strains (Ioerger et al. 2009). Intriguingly, this mutation was one of 3 *grcC1* mutations detected in an outbreak strain which infected 69 patients in Bern, Switzerland (Stucki et al. 2015). Orthologs of this essential gene (in *M. tuberculosis*) are highly conserved across the mycobacteria family, advocating the potential significance of this gene in isoprenoid metabolism (Mann et al. 2012). Mutations that were shared, yet uniquely present in the KZN XDR strains, were found in Rv2000 (L275P) and Rv3471c (D64E), as previously reported (Ioerger et al. 2009). Overexpression of Rv2000 in a recent study resulted in no change in susceptibility to EMB, kanamycin, rifampicin and streptomycin (He et al.2015). Thus, their role in drug resistance or epistatic interactions remains to be determined. In addition to the canonical M306V *embB* mutation, both XDR strains harboured an accessory mutation in Rv3806c *(ubiA*), which functions to increase EMB resistance without imposing fitness deficits (Safi et al. 2013).

Because mutations in the rifampicin-resistance determining region (RRDR) are associated with fitness costs, the presence of intragenic mutations in highly transmissible strains is worthy of closer inspection. Drug-resistant KZN strains in this study harboured previously-reported secondary (N568S) and tertiary (I1187T) mutations outside the RRDR (Ioerger et al. 2009) which represent putative compensatory mutations (Cohen et al. 2015). Further research with isogenic mutants may provide more insight regarding the level of compensation offered by such mutations in the evolution of fit drug-resistant strains.

HN878 was selected as a reference for Beijing strains on the basis of its pan-susceptibility and widespread inclusion in comparative studies (Manca et al. 2001). Both Beijing strains had *gyrA* Ser95Thr and *katG* R463L mutations, verifying that they belonged to principle genetic group 1 (Sreevatsan et al. 1997). The *rpoB* sSNP present in both Beijing strains was shown to be a phylogenetic marker rather than a predictor of rifampicin resistance (Comas et al. 2012). Synonymous SNPs (sSNPs) are believed to have little effect on bacterial phenotype or fitness, however some sSNPs have been shown to play an important role in the generation of alternative transcriptional start sites (Coscolla and Gagneux 2014). In addition to the *katG* alteration, the Beijing MDR strain had a synonymous G609A mutation in the *mabA* gene. Commonly found in INH-resistant clinical isolates, this synonymous mutation results in the amplification of transcriptional levels of *inhA*, and may represent an alternative mechanism of INH resistance in *M. tuberculosis* (Ando et al. 2014). Similarly, synonymous mutations in Rv3792 are believed to increase EMB MICs by upregulating *embC*, explaining the increased EMB MIC of the Beijing MDR strain relative to susceptible strains. Given that Ser315Asn *katG* mutants exhibit nearly half the catalase-peroxidase activity of Ser315Thr mutants (Unissa et al. 2012) and that no compensatory mutations related to INH and rifampicin resistance were identified, the reduced *in vitro* fitness of the Beijing MDR strain is hardly surprising. While the Beijing genotype is highly transmissible in its susceptible form, it does not disseminate equally well in drug-resistant forms (notably XDR) in South Africa, and is most likely due to high fitness costs associated with drug resistance (van de Spuy et al. 2009; Ioerger et al. 2010). This is supported by the diversity of resistance-encoding mutations among XDR Beijing strains from South Africa (Ioerger et al. 2010), Russia (Casali et al. 2014) and Japan (Iwamoto et al. 2008), suggesting that mutations are independently acquired rather than clonally spread (Ioerger et al. 2009).

Clinical strains which harbour identical resistance-conferring mutations may demonstrate variable MICs (Kim et al. 2003). This was the case for KZN and F28 drug-resistant strains which had different INH MICs despite sharing the same *katG* S315T mutation. Whilst the S315T mutation confers little or no fitness cost, other *katG* mutants may be compensated by *ahpC* mutations which ameliorate catalase-peroxidase activity (Sherman et al. 1996). Likewise, the fitness of rifampicin-resistant strains harbouring *rpoB* mutations may be restored by *rpoC* compensatory mutations (de Vos et al. 2013). Even though both F11 and F28 MDR strains possessed a S531L mutation, only the F28 strain had an *rpoC* mutation, explaining its high fitness (Naidoo and Pillay, 2014). The finding that only 42 % of the reads reflected the *rpoC* SNP indicates that the genome was in the process of acquiring compensation in response to a changing environment (Black et al. 2014). Furthermore, 36% of the mapped reads from the F28 MDR strain reflected a newly-identified nSNP (A38S) in *ubiA*. The significance of this SNP in EMB resistance and compensation requires further study. We surmise that the absence of compensatory *ahpC* and *rpoC* mutations to complement the rare *katG* and S351L *rpoB* mutations in the F11 MDR strain may explain its marked reduction in fitness, as evidenced by previous growth and metabolic assays (Naidoo and Pillay 2014). Although the F11 MDR strain had a G406D *embB* mutation, this strain was susceptible to EMB at the critical breakpoint of 5 mg/L, substantiating that this mutation confers a low level of resistance.

We acknowledge that the whole genome sequencing of a greater number of strains would provide an improved framework for the detection of strain-specific SNPs associated with drug resistance and physiological fitness. However, due to financial constraints, we limited the number of strains sequenced to those used in our previous biological fitness and competitive assays. A selection of low-frequency alleles was verified by Sanger sequencing, all of which proved to be true variants. This supports a recent study by Black et al. (2015) which demonstrated that significant SNPs may be missed at higher frequency cut-offs. The presence of subpopulations within the *M. tuberculosis* genome raises the question of how genetic heterogeneity shapes treatment outcomes, especially in highly endemic regions e.g. South Africa, where selection of drug resistance is significantly influenced by TB control programmes (Chihota et al. 2012; Muller et al. 2013).

Whole-genome sequencing provides an ideal platform for comparative analysis of dominant strains to better understand drug resistance and fitness-compensatory mutations. Whilst there are certain limitations to experimental models, we and others (Gagneux et al. 2006; Spies et al. 2012) have shown that laboratory studies are crucial in understanding the effects of resistance-encoding

mutations on replicative fitness. Further studies evaluating the fitness of highly circulating strains may provide added insight on their epidemiological success and possibly reveal new ways to combat TB. Clinical strains possess considerable genetic diversity which may influence physiological fitness i.e. growth characteristics, virulence and transmissibility. The results of this study corroborate our previous work which showed that 1) specific genetic backgrounds are better suited to coping with drug resistance i.e. F15/LAM4/KZN and F28 strains and 2) resistance-conferring mutations with the lowest fitness costs are favoured in highly transmissible strains. Whole-genome sequencing also revealed the presence of novel SNPs in drug-resistant F15/LAM4/KZN strains, some of which may serve as fitness-compensatory mutations.

Conflict of interest: The authors declare that they have no conflict of interest.

REFERENCES

1. Albrethsen J, Agner J, Piersma SR, Hojrup P, Pham TV, Weldingh K, Jimenez CR, Andersen P, Rosenkrands I (2013) Proteomic profiling of *Mycobacterium tuberculosis* identifies nutrient-starvation-responsive toxin-antitoxin systems. Mol Cell Proteom 12; 1180–1191.
2. Andersson DI, Levin BR (1999) The biological cost of antibiotic resistance. Curr Opin Microbiol 2: 489-493.
3. Ando H, Miyoshi-Akiyama T, Watanabe S, Kirikae T (2014) A silent mutation in *mabA* confers isoniazid resistance on *Mycobacterium tuberculosis*. Mol Microbiol 91: 538-47.
4. Billington OJ, McHugh TD, Gillespie SH (1999) Physiological cost of rifampicin resistance induced in vitro in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 43:1866-1869.
5. Bina XR, Philippart JA, Bina JE (2009) Effect of the efflux inhibitors 1-(1-naphthylmethyl)-piperazine and pheny-arginine-beta-naphthylamide on antimicrobial susceptibility and virulence factor production in *Vibrio cholera*. J Antimicrob Chemoth 63:103-108.
6. Black PA, de Vos M, Louw GE, van der Merwe RG, Dippenaar A, Streicher EM, Abdallah AM, Sampson SL, Victor TC, Dolby T, Simpson JA, van Helden PD, Warren RM, Pain A (2015) Whole genome sequencing reveals genomic heterogeneity and antibiotic purification in *Mycobacterium tuberculosis* isolates. BMC Genomics 16: 857.
7. Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, Corander J, Bryant J, Parkhill J, Nejentsev S, Hortsmann RD, Brown T, Drobniewski F (2014) Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet 46: 279-286.

8. Cohen KA, Abeel T, McGuire AM, Desjardins CA, Munsamy V, Shea TP, Walker BJ, Bantubani N, Almeida DV, Alvarado L, Chapman SB, Mvelase NR, Duffy EY, Fitzgerald MG, Govender P, Gujja S, Hamilton S, Howarth C, Larimer JD, Maharaj K, Pearson MD, Priest ME, Zeng Q, Padayatchi N, Grosset J, Young SK, Wortman J, Mlisana KP, O'Donnell MR, Birren BW, Bishai WR, Pym AS, Earl AM (2015) Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole Genome Sequencing and Dating Analysis of *Mycobacterium tuberculosis* Isolates from KwaZulu-Natal. PLoS Medicine 12: 1-22.
9. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M, Galagan J, Niemann S, Gagneux S (2012) Whole genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identified compensatory mutations in RNA polymerase. Nat Genet 44: 106–110.
10. Coscolla M, Gagneux S (2014) Consequences of genomic diversity in *Mycobacterium tuberculosis*. Semin Immunol 26:431-444.
11. Cox HS, McDermid C, Azevedo V, Muller O, Coetzee D, Simpson J, Barnard M, Coetzee G, van Cutsem G, Goemaere E (2010) Epidemic levels of drug resistant tuberculosis (MDR and XDR-TB) in a high HIV prevalence setting in Khayelitsha, South Africa. PLoS One. 5: e13901.
12. de Vos M, Müller B, Borrell S, Black PA, van Helden PD, Warren RM, Gagneux S, Victor TC (2013) Putative compensatory mutations in the *rpoC* gene of rifampin resistant *Mycobacterium tuberculosis* are associated with ongoing transmission. Antimicrob Agents Chemother 57:827-832.
13. Fenner L, Egger M, Bodmer T, Altpeter E, Zwahlen M, Jaton K, Pfyffer GE, Borrell S, Dubuis O, Bruderer T, Siegrist HH, Furrer H, Calmy A, Fehr J, Stalder JM, Ninet B, Böttger EC, Gagneux S; Swiss HIV Cohort Study and the Swiss Molecular Epidemiology of Tuberculosis Study Group (2012) Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother 56: 3047-3053.
14. Gagneux S, Burgo MV, DeRiemer K, Encisco A, Munoz S, Hopewell PC, Small PM, Pym AS (2006) Impact of bacterial genetics on the transmission of isoniazid-resistant *Mycobacterium tuberculosis*. PLoS Pathol 2:e61.
15. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. Lancet 368: 1–6.
16. Gillespie SH (2002) Evolution of Drug Resistance in *Mycobacterium tuberculosis*: Clinical and Molecular Perspective. Antimicrob Agents Chemother 46: 267-274.

17. Goude R, Amin AG, Chatterjee D, Parish T (2008) The critical role of *embC* in *Mycobacterium tuberculosis*. J Bacteriol 190:4335-4341.
18. He L, Wang X, Cui P, Juin J, Chen J, Zhang W, Zhang Y (2015) *ubiA* (Rv3806c) encoding DPPR synthase involved in cell wall synthesis is associated with ethambutol resistance in *Mycobacterium tuberculosis*. Tuberculosis 95: 149-154.
19. Ioerger TR, Koo S, No E, Chen X, Larsen MH, Jacobs Jr. WR, Pillay M, Sturm A, Sacchettini JC (2009) Genome analysis of multi- and extensively-drug-resistant tuberculosis from KwaZulu-Natal, South Africa. PLoS One 4:1-9.
20. Ioerger TR, Feng Y, Chen X, Dobos KM, Victor TC, Streicher EM, Warren RM, Gey van Pittius NC, van Helden PD, Sacchettini JC (2010) The non-clonality of drug resistance in Beijing genotype isolates of *Mycobacterium tuberculosis* from the Western Cape of South Africa. BMC Genomics 11: 670.
21. Iwamoto T, Yoshida S, Suzuki K, Wada T (2008) Population structure analysis of the *Mycobacterium tuberculosis* Beijing Family indicates an association between certain sublineages and multidrug-resistance. Antimicrob Agents Chemother 52: 3805-3809.
22. Kim SY, Park YJ, Kim WI, Lee SH, Ludgerus Chang C, Kang SJ, Kang CS (2003). Molecular analysis of isoniazid resistance in *Mycobacterium tuberculosis* isolates recovered from South Korea. Diagn Microbiol Infect Dis 47: 497-502.
23. Knapp GS, Lyubetskaya A, Peterson MW, Gomes ALC, Ma Z, Galagan JE, McDonough KA (2015) Role of intragenic binding of Camp responsive protein (CRP) in regulation of the succinate dehydrogenase genes *Rv0249c-Rv0247c* in TB complex mycobacteria. Nucleic Acids Res 43:5377-5393.
24. Larsen MH, Biermann K, Tandberg S, Hsu T, Jacobs Jr. WR (August 2007) Genetic Manipulation of *Mycobacterium tuberculosis*. Curr Protoc Microbiol 10A.2.1-10a.2.21
25. Lew JM, Kapopoulou A, Jones LM, Cole ST (2011) TubercuList—10 years after. Tuberc Edinb Scotl 91: 1–7.
26. Liu F, Hu Y, Wang Q, Li HM, Gao GF, Liu CH, Zhu (2014) Comparative genomic analysis of *Mycobacterium tuberculosis* clinical isolates. BMC Genomics 15:469.
27. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, Barry CE, Freedman VH, Kaplan G (2001) Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. Proc Natl Acad Sci USA 98:5752-5757.

28. Mann FM, Xu M, Davenport EK, Peters RJ (2012) Functional characterisation and evolution of the isotuberclosinol operon in *Mycobacterium tuberculosis* and related mycobacteria. Front Microbiol 3: 368.
29. Muller B, Chihota VN, Pillay M, Klopper M, Streicher EM, Coetzee G, Trollip, Hayes C, Bosman ME, van Pittius G, Victor TC, Gagneux S, van Helden PD, Warren RM (2013) Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa. PLoS One 8:1-9.
30. Naidoo CC, Pillay M (2014) Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. Clin Microbiol Infect 20:O361-369.
31. Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venny/index.html
32. Piddock LJ (2006) Multidrug-resistance efflux pumps? Not just for resistance. Nat Rev Microbiol 4: 629-636
33. Pillay M, Sturm AW (2007) Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. Clin Infect Dis 45: 1409–1414.
34. Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, McNeil M, Peterson SN, Chatterjee D, Fleischmann R, Alland D (2013) Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes. Nat Genet 45:1190-1197.
35. Sampson SL (2011) Mycobacterial PE/PPE proteins at the host-pathogen interface. Clin Dev Immunol 1-11.
36. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB (2009) Tuberculosis drug resistance mutation database. PLoS Medicine 6:0132-0136.
37. Sharpe ML, Gao C, Kendall SL, Baker EN, Lott JS (2008) The structure and unusual protein chemistry of hypoxic response protein 1, a latency antigen and highly expressed member of the DosR regulon in *Mycobacterium tuberculosis*. J Mol Biol 383:822-836.
38. Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE 3rd, Stover CK (1996) Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. Science 272:1641-1643.
39. Smith I (2003) *Mycobacterium tuberculosis* pathogenesis and molecular determinants of virulence. Clin Microbiol Rev 16:463-496.

40. Spies FS, von Groll A, Ribeiro AW, Ramos DF, Ribeiro MO, Costa ERD, Martin A, Palomino JC, Rossetti ML, Zaha A, da Silva PEA (2013) Biological cost in *Mycobacterium tuberculosis* with mutations in the *rpsL*, *rrs*, *rpoB* and *katG* genes. Tuberculosis 94: 150-154.
41. Sreevatsan S, Pan X, Stockbauer KE, Connell KD, Kreiswirth BN, Whittam TS, Musser JM (1997) Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionary recent global dissemination. Proc Natl Acad Sci USA 94: 9869-9874.
42. Stucki D, Ballif M, Bodmer T, Coscolla M, Maurer A, Droz S, Butz C, Borrell S, Längle C, Feldmann J, Furrer H, Mordasini C, Helbling P, Rieder HL, Egger M, Gagneux S, Fenner L (2015) Strain-specific single nucleotide polymorphism typing combined with targeted whole-genome sequencing. J Infect Dis 211: 1306-1316.
43. Unissa AN, Narayanan S, Selvakumar N (2011) Virulence in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from South India. Int J Mol Clin Microbiol 1: 87–96.
44. van der Spuy GD, Kremer K, Ndabambi SL, Beyers N, Dunbar R, Marais BJ, van Helden PD, Warren RM (2009) Changing *Mycobacterium tuberculosis* population highlights clade-specific pathogenic characteristics. Tuberculosis 89: 120-125.
45. Victor TC, Streicher EM, Kewley C, Jordaan AM, van der Spuy GD, Bosman M, Louw H, Murray M, Yound D, van Helden PD, Warren RM (2007) Spread of an emerging *Mycobacterium tuberculosis* drug-resistant strain in the Western Cape of South Africa. Int J Tuberc Lung Dis 11: 195–201.
46. WHO (2014) Global Tuberculosis Report. http://www.who.int/tb/publications/global_report/en/
47. Wu S, Howard ST, Lakey DL, Kipnis A, Samten B, Safi H, Gruppo V, Wizel B, Shams H, Basaraba RJ, Orme IM, Barnes PF (2004) The principal sigma factor *sigA* mediates enhanced growth of *Mycobacterium tuberculosis* in vivo. Mol Microbiol. 51: 1551-1562.

CHAPTER FOUR

Drug-resistant F15/LAM4/KZN *Mycobacterium tuberculosis* strains exhibit increased TNF-α and MIP-1β induction and high replication in THP-1 macrophages

Naidoo CC, [1] Pillay M. [1]

[1]Medical Microbiology and Infection Control, University of KwaZulu-Natal, Durban, South Africa

Manuscript in preparation

ABSTRACT

The F15/LAM4/KZN (KZN) genotype is frequently associated with multidrug resistance (MDR) and extensive drug-resistance (XDR) in KwaZulu-Natal, South Africa. Ten clinical strains, representing KZN and other predominant genotypes, and H37Rv were used to infect THP-1 macrophages. Uptake and intracellular growth indices were measured at 4 hours and on day 5, respectively. In addition, 17 cytokines were examined at both intervals in *M. tuberculosis*-infected macrophage supernatants, using a commercial multiplex cytokine assay. There were no significant differences for uptake by macrophages. The MDR KZN strain presented with the highest growth index (1.64 ± 0.05), followed closely by one of two XDR KZN strains (1.59 ± 0.02), while the other (X162) had comparable replication to the susceptible KZN strain. High levels of IL-1β and MIP-1β elicited by X162 early in infection may have limited its replication. H37Rv had the lowest growth index (1.36 ± 0.03). XDR KZN strains elicited higher levels of TNF-α compared with other strains ($p<0.05$) and uninfected macrophages at 4 hours. These strains may exploit the secretion of TNF-α for tissue destruction and ultimately, its transmission to other hosts. Increasing levels of MIP-1β production corresponded with increasing degrees of drug resistance in KZN strains, while no clear trends were observed between remaining cytokines and drug resistance in this genotype. Although exhibiting similar replication, susceptible and MDR strains within Beijing, F11 and F28 genotypes produced heterogeneous cytokine responses. The lack of a direct relationship between bacillary burden and cytokine responses indicate that this diversity results from strain heterogeneity.

INTRODUCTION

Tuberculosis (TB) and the human immunodeficiency virus (HIV) are the primary contributors to mortality resulting from infectious diseases, with strategies to combat these diseases often hampered by drug resistance (WHO, 2014). Upon inhalation, *M. tuberculosis* bacilli are delivered to the alveolar spaces of the lung where they encounter macrophages. During infection with *M. tuberculosis*, these professional phagocytes engulf and process infectious bacilli, and present them to other immune cells (Hocking *et al*., 1979). Some bacilli however, are able to survive and replicate regardless of the harmful nitric oxide (NO) and reactive nitrogen intermediates (RNI) present within phagosomes and may even prevent phagosome-lysosome fusion (reviewed in Ehrt and Schnappinger, 2009). The ability of mycobacteria to resist RNI is believed to be dependent on the strain, dose and time (O' Brien et al. 1994; Rhoades and Orme, 1997; Long et al. 1999; Long et al. 2005). Alveolar macrophages play an integral role in connecting innate and adaptive immunity within hosts (Flynn and Chan, 2003). One of the key determinants in the progression of TB

infection is the production of cytokines/chemokines, which orchestrate the containment of infectious organisms and granuloma formation (Fuller et al. 2003; Mihret and Abebe, 2013). Both interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) have been described as the forefront cytokines in the antimycobacterial Th1 pathway (Lin and Flynn, 2010).

Since macrophages are critical for intracellular replication and survival of *M. tuberculosis*, extensive research has focused on establishing immunological, genetic and physiological properties of *M. tuberculosis*-macrophage interactions (Schnappinger et al., 2003; Rengarajan et al., 2005; Giacomini et al., 2001). Studies employing cell culture and mouse models have demonstrated that strains of different *M. tuberculosis* genotypes display variability in immune responses (Hoal-van Helden et al. 2001; Lopez et al. 2003; Chacón-Salinas et al. 2005; Sarkar et al. 2012; Chakraborty et al. 2013). Moreover, virulent strains tend to exhibit increased growth and survival in macrophages (Silver et al. 1998; Zhang et al. 1998; Park et al. 2006; Rajavelu and Das, 2007). Data for Beijing strains are conflicting, with some studies reporting low proinflammatory responses and growth rates compared to other genotype strains in macrophages (Tanveer et al. 2009; Chakraborty et al. 2013), and others reporting high production of TNF-α in human macrophages (Chacón-Salinas et al. 2005). Beijing strains have also to be shown to cause higher mortality rates in BALB/c mice compared to H37Rv (Lopez et al. 2003). Survival times of SCID mice infected with F15/LAM4/KZN strains varied, with susceptible strains causing rapid mortality compared with strains carrying multidrug-resistance (MDR) and extensive drug-resistance (XDR) (Smith et al. 2014).

Drug-resistant strains sometimes undergo fitness costs, depending on the resistance-encoding mutations they harbour and their genetic background (Gagneux et al. 2006). Fitness costs refer to decreased growth rates, virulence or transmission efficiencies that are measureable in specific environments (Andersson and Hughes, 2008). The *in vitro* growth rates of four clinically-relevant genotypes that predominate in KwaZulu-Natal, South Africa were previously characterised (Naidoo and Pillay, 2014) however their virulence properties remained to be determined. Increased fitness was observed for drug-resistant F15/LAM4/KZN and F28 genotype strains, contrasting with Beijing and F11 MDR strains which had incurred fitness costs (Naidoo and Pillay, 2014). In the current study, as surrogates of fitness, we assessed uptake, intracellular growth and cytokine responses of the same clinical strains with different genotypes and levels of drug resistance, in THP-1 macrophages. The 17-plex kit was selected on the basis of its inclusion of several proinflammatory

(IFN-γ and TNF-α) and anti-inflammatory (e.g. IL-10) cytokines associated with *M. tuberculosis*-infected macrophages.

METHODS

M. tuberculosis strains

The H37Rv laboratory reference strain and ten clinical strains comprising drug-susceptible, MDR and XDR types were included in this study (Table 4.1). Strains were cultured in Middlebrook 7H9 broth (Difco) supplemented with 10 % oleic acid albumin dextrose (OADC) enrichment (Becton Dickinson), 0.5 % glycerol and 0.05 % Tween-80 until an optical density (OD_{600}) of 0.6-1.

Table 4.1 *M. tuberculosis* strain profiles

Strain	Genotype	Type [a]	Drug resistance [b]
V9124	F15/LAM4/KZN	DS	-
V1435	F15/LAM4/KZN	MDR	IR
KZN605	F15/LAM4/KZN	XDR	IRSEOKCN
X162	F15/LAM4/KZN	XDR	IRSEOKCN
B910	Beijing	DS	-
R35	Beijing	MDR	IRS
R490	F11/LAM3	DS	-
R271	F11/LAM3	MDR	IR
R104	F28/S	DS	-
R443	F28/S	MDR	IRSEK
H37Rv	Laboratory reference	DS	-

[a] DS: drug-susceptible; MDR: multidrug-resistant, XDR: extensively drug-resistant

[b] I, isoniazid; R, rifampicin; E, ethambutol; O, ofloxacin; K, kanamycin; C, capreomycin; N, niacinamide

Cell culture

The THP-1 human derived macrophage cell line (ATCC TIB-202) was propagated in RPMI-1640 (Lonza) containing 10% foetal bovine serum (Biowest) (RPMI-C) at 37 °C (with 5% CO_2). Cells were enumerated by trypan blue exclusion prior to reconstitution with phorbol 12-myristate 13-acetate (50 ng/ml) for overnight differentiation into macrophages. Cells were seeded at 2 X 10^5 cells/ml in 24-well cell culture plates (Porvair) for intracellular growth assays, or in 25 cm^2 flasks (Porvair) for cytokine analysis.

Macrophage infection

Bacterial cultures were pelleted by centrifugation at 2000 x g for 10 minutes and resuspended in 1 mL of RPMI-C. Bacteria were passaged 10 times through a 21-gauge needle (attached to a 1-ml syringe) and diluted to 2 X 10^5 bacteria per ml in RPMI-C. Following overnight incubation, monolayers were washed twice with warm PBS to remove non-adherent cells. Infection media was freshly prepared immediately before use. Macrophages were infected, in triplicate wells, at a multiplicity of infection (MOI) of 1:1. After 4 hours of incubation at 37 °C (5 % CO_2), monolayers were washed three times with warm PBS to remove extracellular bacteria and fresh media was added to day 5 wells. For colony forming unit (CFU) enumeration at 4 hours and 5 days post-infection, macrophages were lysed for 20 minutes with 0.1 % Triton X-100. Serially-diluted lysates were plated, in triplicate, onto Middlebrook 7H10 medium (Difco) containing 10 % OADC and 0.5% glycerol and incubated for 3 weeks at 37 °C. Three independent macrophage experiments were performed for each strain. To confirm the MOI during each experiment, bacterial inocula was plated onto Middlebrook 7H10 medium and incubated for 3 weeks.

Cytokine determination

For cytokine analysis, macrophages were infected as described above in 25 cm^2 flasks (10 ml) at an MOI of 1:1. Cell-free culture supernatants were collected after 4 hours and 5 days of infection by filtering through 0.22 µm units. Supernatants were reconstituted with 0.5% bovine serum albumin (BSA) prior to storage at -70 °C. The Bio-Plex Pro ™ Human Cytokine 17-plex assay (Bio-Rad) was performed according to the manufacturer's instructions. Briefly, a standard curve was generated by serially-diluting cytokine standards in RPMI-C (with 0.5% BSA) to ensure comparability to samples. Each strain was tested in triplicate wells from one representative experiment. A 50 µl volume of samples, uninfected controls (day 0 or day 5), blank (medium only) and cytokine standards was added to a 96-well plate containing 50 µl of coupled beads, and incubated with gentle

shaking (at room temperature) for 60 minutes. Detection antibodies and streptavidin-PE were added to the plate with incubation and washing steps preceding each addition. After the last wash step, 125 µl of assay buffer was added to each well and the plate was read on the Bio-Plex array reader. Cytokine data analysis was performed using the Bio-Plex Manager software, version 6.1.

Statistical analysis

Intracellular uptake and growth indices were analysed by one-way ANOVA with Fisher's LSD test, using SPSS v.22.0 (IBM Statistics). Cytokine responses were analysed by one-way ANOVA with Tukey's post-hoc test. A *p* value less than 0.05 was statistically significant.

Ethical approval

This study was approved by the Biomedical Review Ethics Committee at the University of KwaZulu-Natal, Durban, South Africa (BE258/13).

RESULTS

Uptake by macrophages

Four KZN (one susceptible, one MDR and two XDRs) and two strains (one susceptible and one MDR) of Beijing, F11 and F28 genotypes were used to infect THP-1 macrophages. Mycobacterial uptake was measured by dividing the number of CFU/ml at 4 hours by the number of CFU/ml in the starting inoculum (Figure 4.1A). No statistically significant differences were observed at 4 hours however, some general observations were made. Beijing and F11 strains had similar uptake at 4 hours post-infection, with the MDR strains being taken up two to three times more efficiently by macrophages, than susceptible strains and H37Rv. The reverse was observed for KZN and F28 genotypes, with higher uptake levels for susceptible strains.

Intracellular growth

Intracellular growth was calculated as the CFU/ml on day 5 divided by the CFU/ml at 4 hours. The KZN MDR strain, V1435 had the highest growth index of 1.64 ± 0.05, followed closely by the XDR strain, KZN605 (1.59 ± 0.02) (Figure 4.1B). Despite high initial uptake, the susceptible KZN strain, V9124 had a lower growth index than V1435 (p=0.009) and the XDR strain, KZN605 (p=0.031), but equivalent growth to the other XDR strain, X162. Susceptible strains within Beijing, F11 and F28 genotypes had similar growth indices to MDR strains. H37Rv had the lowest growth

index (1.36 ± 0.03); this was significantly lower than V1435 (p=0.004), KZN605 (p=0.016) and Beijing strains, B910 (p=0.039) and R35 (p=0.031).

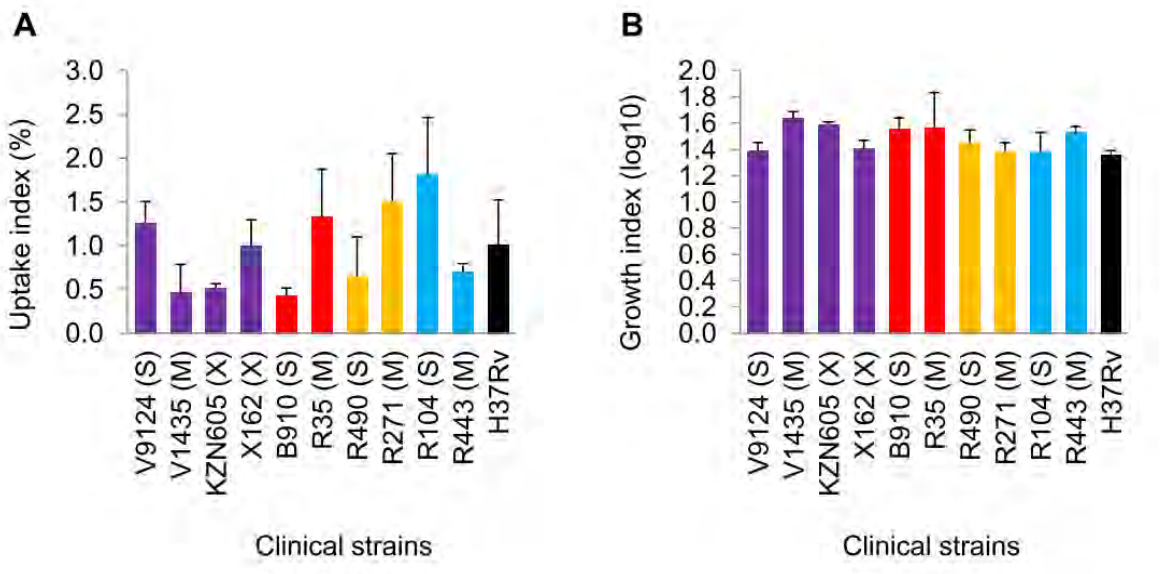


Figure 4.1 Uptake and intracellular growth indices of *M. tuberculosis* strains in THP-1 macrophages. (A) Strain uptake was calculated as the CFU/ml at 4 hours divided by the CFU/ml in the starting inoculum, expressed at a percentage (mean ± SE). (B) Intracellular growth indices were calculated as the log CFU/ml on day 5 divided by the log CFU/ml at 4 hours (mean ± SD). Data represents three independent experiments. Purple bars: F15/LAM4/KZN strains, red bars: Beijing strains, yellow bars: F11 strains, blue bars: F28 strains. S: drug-susceptible, M: multidrug-resistant, X: extensively drug-resistant

Cytokine responses at 4 hours

Of 17 cytokines examined, interleukin (IL)-5, IL-7 and IL-13 were undetected. Uninfected THP-1 macrophages produced relatively high levels of cytokine production. This is in accordance with a study by Aldo et al. (2013) which demonstrated that uninfected macrophages produce high levels of proinflammatory cytokines in response to PMA-differentiation. The following cytokines were elicited at 4 hours: TNF-α, IL-1β, IL-2, IL-8, IL-17, granulocyte-colony stimulating factor (G-CSF), monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein -1β (MIP-1β) (Figure 4.2). Overall, the most highly expressed cytokines were IL-1β, IL-8, MCP-1 and MIP-1β. With the exception of the KZN605 and R35, similar cytokines responses were detected amongst all strains for IL-2, IL-8 and G-CSF at 4 hours.

Within the KZN genotype, increasing levels of MIP-1β were observed for KZN strains, with X162 exhibiting the most, followed by KZN605 and V1435 with intermediate levels, and V9124 with the least. This was significant between the XDR strains and the susceptible strain ($p<0.001$), X162 vs. the MDR strain ($p<0.001$) and KZN605 ($p=0.012$). X162 also had higher expression of IL-1β compared to V9124 and KZN605 at 4 hours ($p<0.001$). The XDR strains, KZN605 and X162, were the strongest inducers of TNF-α at 4 hours (vs. R490: $p=0.007$ and $p=0.012$, respectively, vs. H37Rv: $p=0.004$ and $p=0.007$, respectively and vs. all other strains: $p<0.001$). KZN strains had similar responses for remaining cytokines at 4 hours.

The Beijing susceptible strain (B910) had higher secretion of IL-1β ($p=0.006$) than the MDR strain (R35), while similar responses were observed for other cytokines. Compared to its MDR counterpart (R271), the susceptible F11 strain (R490) had higher production of IL-1β ($p<0.001$), MIP-1 ($p=0.013$) and TNF-α ($p=0.006$) at 4 hours. F28 strains did not differ in their cytokine responses at 4 hours. Interestingly, H37Rv and R104 had comparable cytokine responses with the exception of TNF-α at 4 hours ($p=0.004$).

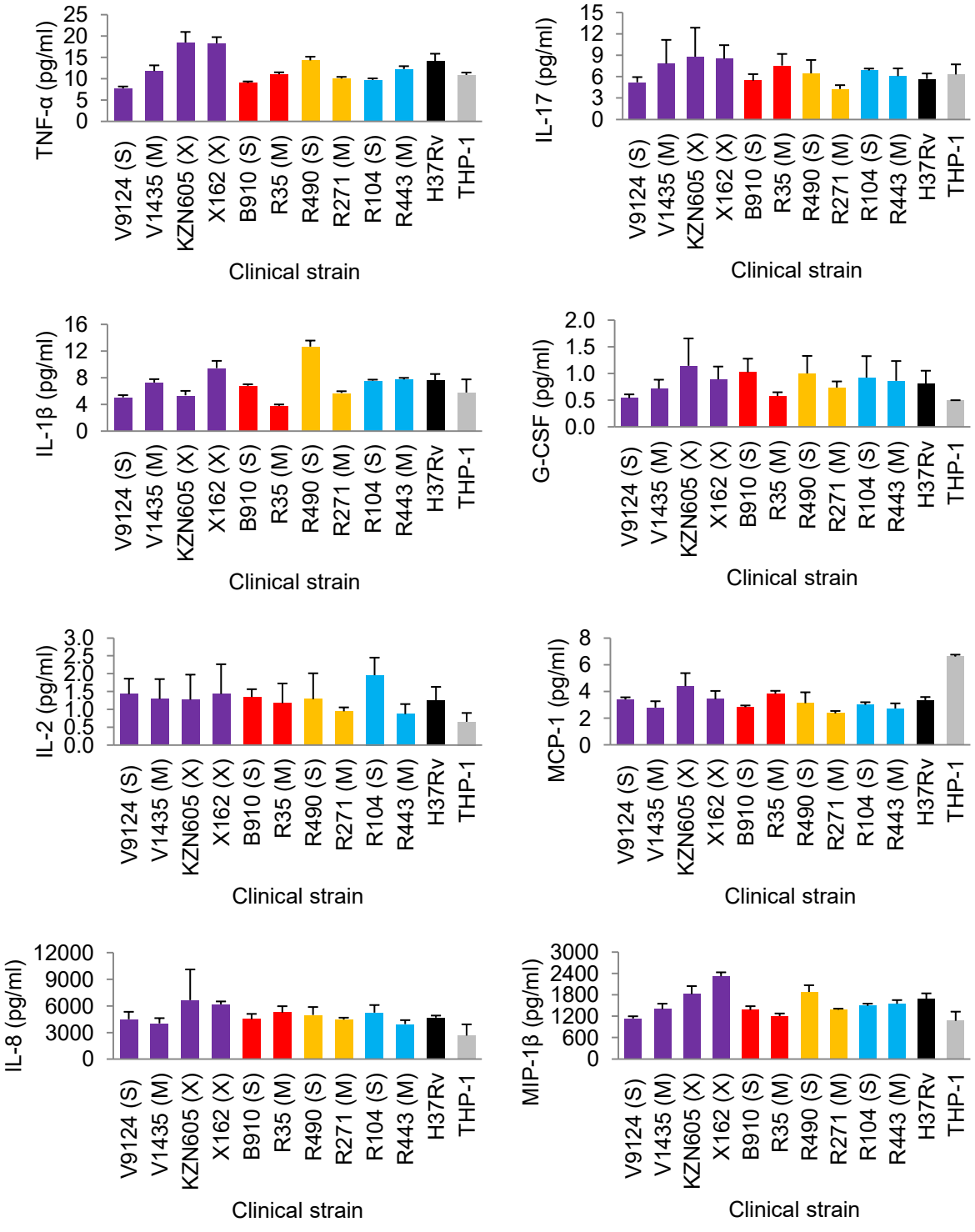


Figure 4.2 Cytokines detected in the supernatants of *M. tuberculosis*-infected THP-1 macrophages at 4 hours. Purple bars: F15/LAM4/KZN strains, red bars: Beijing strains, yellow bars: F11 strains, blue bars: F28 strains. Data represents the mean ± SD. S: drug-susceptible, M: multidrug-resistant, X: extensively drug-resistant

Cytokine responses at 5 days

Cytokines IL-4, IL-6, IL-10, IL-12 (p70), IFN-γ and granulocyte macrophage-colony stimulating factor (GM-CSF) were secreted on day 5 only (Figure 4.3) while others i.e. TNF-α, IL-1β, IL-2, IL-8, IL-17, G-CSF and MCP-1 (Figure 4.4) were secreted at both time points. Similar cytokines responses were detected for IL-10, IL-12 (p70) and IL-17, with the exception of KZN605 and R35, which produced significantly higher amounts of cytokines than other strains, particularly MCP-1 at 5 days ($p<0.001$).

For IL-1β, V9124 and KZN605 had higher production than X162 and V1435 ($p<0.001$). The Beijing MDR strain, R35 had significantly higher secretion of ten cytokines including IL-1β, IL-2, IL-4, IL-6, IL-10, G-CSF, GM-CSF, MCP-1, TNF-α and IFN-γ than the susceptible Beijing strain on day 5 ($p<0.05$). More significant differences were observed between the F28 strains on day 5, with the susceptible strain (R104) eliciting significantly higher levels of IL-1β ($p<0.001$), IL-6 ($p=0.001$) and GM-CSF ($p=0.039$) than the MDR strain (R443). In contrast, R443 did not induce significantly higher levels of any cytokines compared to R104. Notably, H37Rv had the highest observed IL-17 response on day 5, however this was only significant with R443 ($p=0.025$).

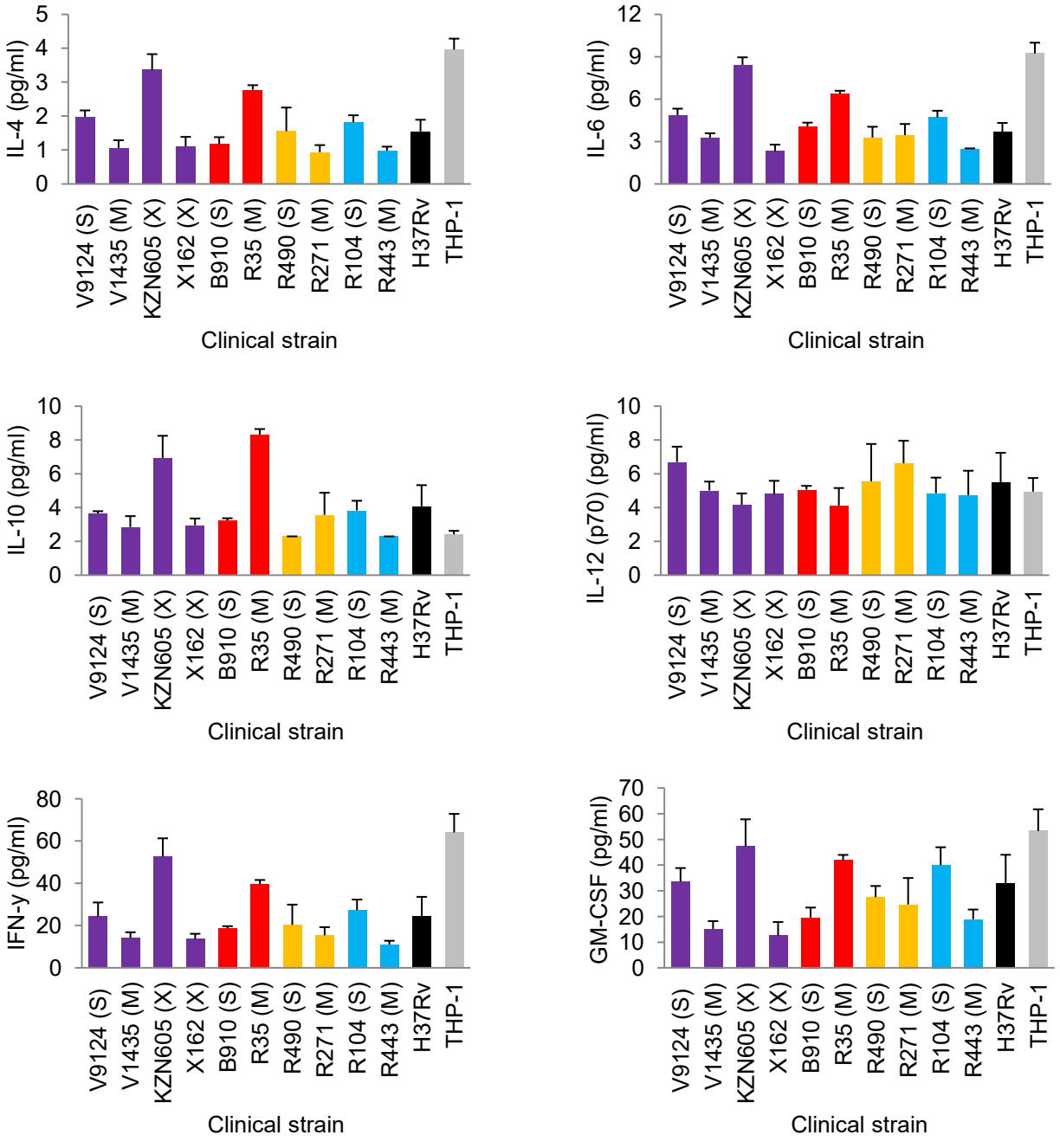


Figure 4.3 Cytokines detected in the supernatants of *M. tuberculosis*-infected THP-1 macrophages on day 5 only. Purple bars: F15/LAM4/KZN strains, red bars: Beijing strains, yellow bars: F11 strains, blue bars: F28 strains. Data represents the mean ± SD. S: drug-susceptible, M: multidrug-resistant, X: extensively drug-resistant

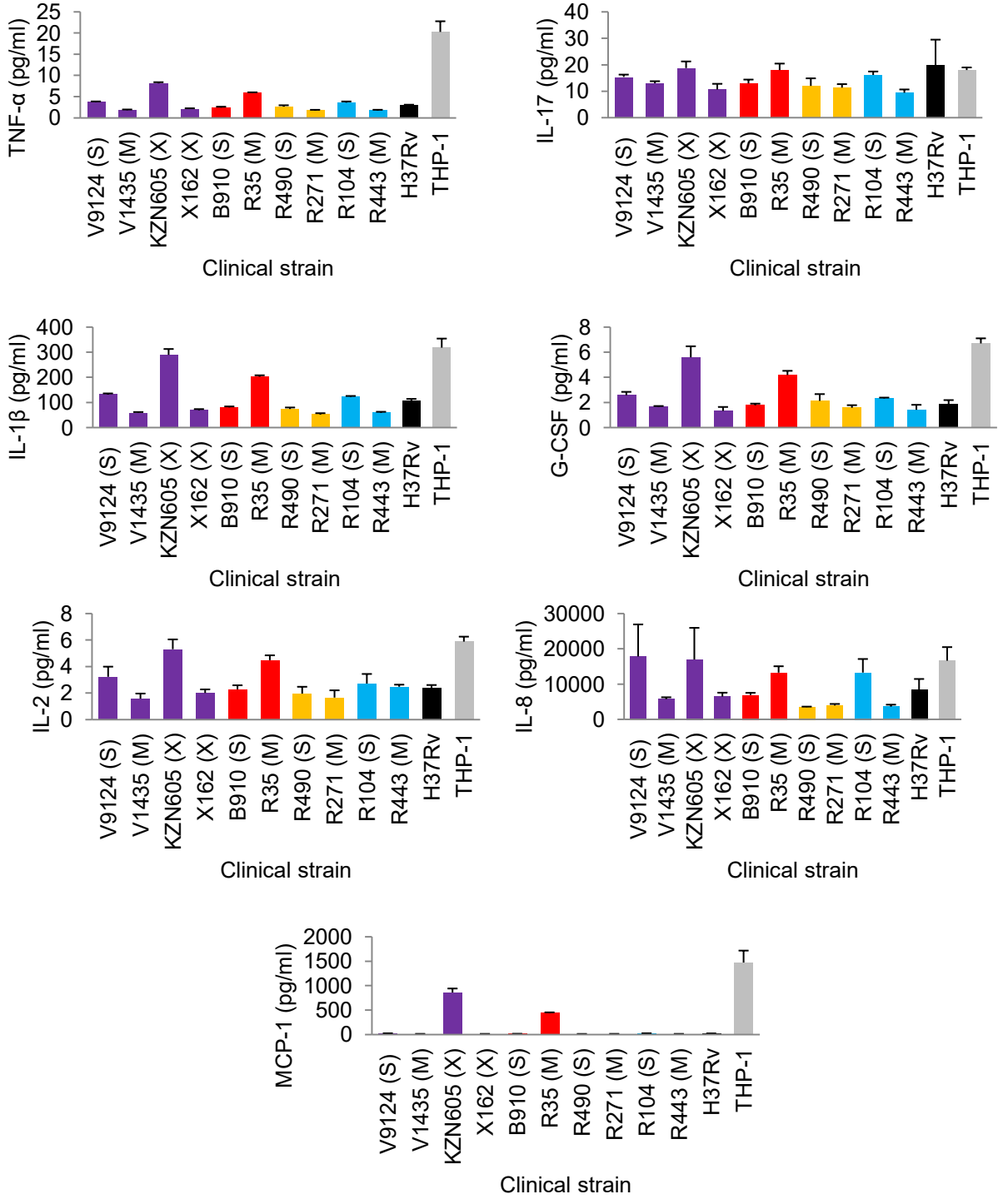


Figure 4.4 Cytokines detected in the supernatants of *M. tuberculosis*-infected THP-1 macrophages on day 5**.** Purple bars: F15/LAM4/KZN strains, red bars: Beijing strains, yellow bars: F11 strains, blue bars: F28 strains. Data represents the mean ± SD. S: drug-susceptible, M: multidrug-resistant, X: extensively drug-resistant

DISCUSSION

Strain-specific variation has been increasingly demonstrated to play a significant role in virulence and immunogenicity in recent years. Using diverse clinical strains from South Africa, we addressed the effect of genotype and drug resistance on mycobacterial fitness, in the form of intracellular replication and cytokine induction at two different time points in macrophages. Similar to recent studies (Sarkar et al. 2012; Chakraborty et al, 2013), we found no significant differences in the uptake efficiencies of clinical strains however our findings differed for Beijing (and two KZN) strains which had significantly higher intracellular growth than H37Rv.

The majority of cytokines were low or undetected at 4 hours, however interesting patterns of expression were observed for IL-1β, MIP-1β and TNF-α. While increasing levels of MIP-1β and TNF-α corresponded with increasing degrees of drug resistance in KZN strains (i.e. XDR > MDR > susceptible), the reverse was observed for F11 strains, whereby the susceptible strain induced significantly higher production. Likewise, susceptible Beijing and F11 strains which exhibited higher IL-1β production than MDR strains contrasted with the KZN genotype, which did not show any correlation between IL-1β production and drug resistance.

Recent work demonstrated increased necrosis/cytotoxicity in A549 epithelial cells following infection with XDR KZN strains however the role of TNF-α was not explored (Smith et al. 2014; Ashiru and Sturm, 2015). TNF-α has been described as a 'double-edged sword': it not only controls *M. tuberculosis* infection but also causes extensive tissue destruction (reviewed in Mootoo et al. 2009). In this study, XDR strains were the highest inducers of TNF-α at 4 hours post-infection. Increased production of TNF-α has been reported previously by highly virulent isolates in human monocytes and macrophages (Valway et al. 1998; Engele et al. 2002. We speculate that the XDR KZN strains may exploit the secretion of TNF-α for cell necrosis, thereby increasing their chances of being released into surrounding tissues (Bocchino et al. 2005; Vandenabeele et al. 2010).

While cytokines may be secreted by either T helper cells 1 (Th1) or 2 (Th2), the cell-mediated immune response to TB is primarily driven by Th1 secretion of cytokines, including IFN-γ. This cytokine is responsible for the induction of antimicrobial activity by macrophages (Condos et al. 2003; Wallis 2005; Park et al. 2007), whereby IFN-γ inhibits IL-4 production but promotes the secretion of IL-12 (p70) and together, these cytokines direct microbial recognition by innate effector cells (Rivero-Lezcano, 2008). Previous studies show that TB patients with predominantly

Th1 cytokines (i.e. IFN-γ), experience milder forms of pulmonary disease, whereas patients with high levels of Th2 cytokines (i.e. IL-4) have more severe forms of disease (Dlugovitzky et al. 1997; Dlugovitzky et al. 1999). Moreover, MCP-1 is able to activate the promoter of IL-4, thereby enhancing the Th2 response (Karpus et al. 1997).

In our current study, IFN-γ, IL-4 and IL-12 (p70) were only detected on day 5, with IFN-γ having the highest response and IL-4 having the lowest response. No significant differences were observed in the induction of IL-12 (p70), however nine of 11 strains produced three to six-fold more IL-12 (p70) than IL-4. The remaining strains, KZN605 and R35, had similar IL-4 levels (3.36 and 2.77 pg/ml, respectively) to IL-12 (p70) (4.16 and 4.12 pg/ml, respectively). Moreover, while other strains appeared to suppress IL-4 and MCP-1 production on day 5, KZN605 and R35 produced significantly higher amounts than these strains ($p<0.01$ except IL-4: R35 vs. R104 where $p=0.047$). Increasing levels of these cytokines may lead to higher morbidity/mortality in individuals infected with these mycobacterial strains, as elevated IL-4 expression is often associated with tissue destruction and apoptosis (Seah et al. 2001). This is also supported by the fact that KZN605 is a member of the historical XDR- F15/LAM4/KZN clone associated with rapid disease progression and time to death of 52 of 53 HIV-infected patients in Tugela Ferry in 2005 (Gandhi et al. 2006).

Beijing isolates are believed to induce low levels of pro-inflammatory cytokines such as IL-6, IL-10 and TNF-α relative to H37Rv and clinical isolates *in vitro* (Reed et al. 2004; Wang et al. 2010). In the present study, this was observed for the susceptible strain only, whereas the MDR strain, R35 had induced significantly higher production of these and most other cytokines (including IFN-γ, $p=0.013$) compared to H37Rv and clinical strains. Interestingly, R35 possesses a non-synonymous mutation in a gene encoding a mammalian cell entry family protein, *mce1C* (*Rv0171*) (Naidoo and Pillay, 2015, submitted) which could be associated with its virulence in THP-1 macrophages, however, this needs clarification by further research.

Despite commencing experiments with similar MOIs, strains within, and among, genotypes displayed differential replication and cytokine responses i.e. Beijing strains exhibited similar replication but differed in cytokine induction, with the MDR strain being highly immunogenic. F11 strains replicated at similar rates but differed in some of their cytokines responses, with the susceptible strain eliciting significantly higher levels of IL-1β, MIP-1 and TNF-α following phagocytosis at 4 hours. Similarly, F28 strains which had comparable replication, differed in their

secretion of cytokines with the susceptible strain producing significantly more IL-1β, IL-6 and GM-CSF than the MDR strain on day 5. Thus, there appears to be no direct relationship between intracellular bacillary burden and cytokine responses in macrophages, but is rather a reflection of strain heterogeneity.

The increased intracellular replication of two drug-resistant KZN strains (including the outbreak strain KZN605) confirms that little or no fitness costs accompany their infection of THP-1 macrophages. Inconsistent findings in the other XDR strain may be explained by the high levels of IL-1β and MIP-1β produced during the first 4 hours of infection which could have limited its ability to replicate thereafter, although not to a level below that of the susceptible KZN strain. Indeed, MIP-1β being one of the main beta-chemokines produced during *M. tuberculosis* infection has been shown to suppress mycobacterial growth (Saukkonen et al. 2002). MDR strains from Beijing and F11 genotypes which had comparable growth indices to susceptible strains in macrophages, demonstrated significant fitness costs in laboratory medium (Naidoo and Pillay, 2014), affirming that *in vitro* fitness does not always correlate with *in vivo* fitness. Only two time points were measured in this study, thus the long-term growth kinetics of these strains in other models of infection remains to be determined.

Extensive tissue destruction due to increased TNF-α production may have contributed to the high mortality rates caused during the Tugela Ferry XDR-TB outbreak (Gandhi et al. 2006). While KZN strains displayed diverse phenotypes associated with drug resistance, no clear associations could be made for MDR Beijing, F11 and F28 genotype strains and their cytokine responses. Thus, this study provides further evidence that clinical strains exhibit heterogeneous virulence properties, and necessitates additional research to determine how these differences influence TB infection, disease progression and outcome in human hosts.

ACKNOWLEDGEMENTS

We thank Prof. Thumbi Ndung'u (K-RITH) for kindly providing the THP-1 monocytic cell line; Prof. Balakrishna Pillay (UKZN) for providing access to the Bio-Plex Instrument and Ms. Nontobeko Mvubu (UKZN) for assistance; the National Research Foundation of South Africa (CCN: grant number 88959, MP: grant number 90508 and Incentive Funds for Rated Researchers) and College of Health Sciences (UKZN) (Doctoral scholarship and project running costs). Both

authors were responsible for conceptualisation, analysis and interpretation of data. CCN performed experiments.

REFERENCES

1. WHO. Global Tuberculosis Report 2014. WHO/HTM/TB/2014.08, Geneva, Switzerland, 2014
2. Hocking WG, Golde DW. The pulmonary-alveolar macrophage. N Engl J Med. 1979; 301:639.
3. Ehrt S, Schnappinger D. Mycobacterial survival strategies in the phagosome: defense against host stress. Cell Microbiol. 2009; 11: 1170-1178.
4. O' Brien L, Carmichael J, Lowrie DB, Andrew PW. Strains of *Mycobacterium tuberculosis* differ in susceptibility to reactive nitrogen intermediates in vitro. Infect Immun. 1994; 62: 5187–5190
5. Rhoades ER, Orme IM. Susceptibility of a panel of virulent strains of *Mycobacterium tuberculosis* to reactive nitrogen intermediates. Infect Immun. 1997; 65: 1189–1195.
6. Long R, Light B, Talbot JA. Mycobacteriocidal action of exogenous nitric oxide. Antimicrob Agents Chemother. 1999; 43: 403–405.
7. Long R, Jones R, Talbot J, Mayers I, Barrie J, Hoskinson M, Light B. Inhaled nitric oxide treatment of patients with pulmonary tuberculosis evidenced by positive sputum smears. Antimicrob Agents Chemother. 2005; 49: 1209–1212.
8. Flynn JL, Chan J. Immune evasion by *Mycobacterium tuberculosis*: living with the enemy. Curr Opin Immunol. 2003; 15: 450-455.
9. Fuller CL, Flynn JL, Reinhart TA. In situ study of abundant expression of proinflammatory chemokines and cytokines in pulmonary granulomas that develop in cynomolgus macaques experimentally infected with *Mycobacterium tuberculosis*. Infect Immun. 2003; 71: 7023-7034
10. Mihret A, Abebe M. Cytokines and chemokines as biomarkers of tuberculosis. J Mycobac Dis. 2013; 3: 1000128.
11. Lin PG, Flynn JL. Understanding latent tuberculosis: a moving target. Journal of Immunology. 2010; 185: 15–22.
12. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, Schoolnik GK. Transcriptional adaptation of *Mycobacterium tuberculosis* within macrophages: insights into the phagosomal environment. J Exp Med. 2003; 198: 693-704.

13. Rengarajan J, Bloom BR, Rubin EJ. Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages. Proc Natl Acad Sci USA. 2005; 102: 8327–8332.
14. Giacomini E, Iona E, Ferroni L, Miettinen M, Fattorini L, Orefici G, Julkunen I, Coccia EM. Infection of human macrophages and dendritic cells with *Mycobacterium tuberculosis* induces a differential cytokine gene expression that modulates T cell response. J Immunol. 2001; 166: 7033-7041.
15. Hoal-van Helden EG, Stanton LA, Warren R, Richardson M, van Helden PD. Diversity of in vitro cytokine responses by human macrophages to infection by *Mycobacterium tuberculosis* strains. Cell Biol Int 2001; 25: 83–90.
16. Lopez B, Aguilar D, Orozco H, Burger M, Espitia C, et al. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol 2003; 133: 30–37.
17. Chacón-Salinas R, Serafín Lopez J, Ramos-Payán R, Méndez-Aragón P, Hernández-Pando R, et al. Differential pattern of cytokine expression by macrophages infected in vitro with different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol. 2005; 140: 443–449.
18. Sarkar R, Lenders L, Wilkinson KA, Wilkinson RJ, Nicol MP. Modern lineages of *Mycobacterium tuberculosis* exhibit lineage-specific patterns of growth and cytokine induction in human monocyte-derived macrophages. PLoS One. 2012; 7: e43170.
19. Chakraborty P, Kulkarni S, Rajan R, Sainis K. Drug resistant clinical isolates of *Mycobacterium tuberculosis* from different genotypes exhibit differential host responses in THP-1 cells. PLoS ONE. 2013; 8: e62966.
20. Rajavelu P, Das SD. A correlation between phagocytosis and apoptosis in THP-1 cells infected with prevalent strains of *Mycobacterium tuberculosis*. Microbiol Immunol. 2007; 51: 201.
21. Park JS, Tamayo MH, Gonzalez-Juarrero M, Orme IM, Ordway DJ. Virulent clinical isolates of *Mycobacterium tuberculosis* grow rapidly and induce cellular necrosis but minimal apoptosis in murine macrophages. J Leukoc Biol. 2006; 79: 80–86.
22. Silver RF, Li Q, Ellner JJ. Expression of virulence of *Mycobacterium tuberculosis* within human monocytes: virulence correlates with intracellular growth and induction of tumor necrosis factor alpha but not with evasion of lymphocyte-dependent monocyte effector functions. Infect Immun. 1998; 66: 1190–1199.
23. Zhang M, Gong J, Lin Y, Barnes PF. Growth of virulent and avirulent *Mycobacterium tuberculosis* strains in human macrophages. Infect Immun. 1998; 66: 794–799.

24. Tanveer M, HasanZ, Kanji A,Hussain R, Hasan R. Reduced TNF-alpha and IFN-gamma responses to Central Asian strain 1 and Beijing isolates of *Mycobacterium tuberculosis* in comparison with H37Rv strain. Trans R Soc Trop Med Hyg 2009; 103: 581–587.
25. Smith KLJ, Saini D, Bardarov S, Larsen M, Frothingham R, Gandhi NR, Jacobs Jr. WR, Sturm AW, Lee S. Reduced virulence of an extensively drug-resistant outbreak strain of *Mycobacterium tuberculosis* in a murine model. PLoS ONE. 2014; 9: e94953
26. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJM. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science. 2006; 312: 1944:1946.
27. Andersson DI, Hughes D, Baquero F, Nombela C, Cassell GH, Gutiérrez-Fuentes JA. Effects of antibiotic resistance on bacterial fitness, virulence, and transmission. Evolutionary biology of bacterial and fungal pathogens. 2008; 307-318.
28. Naidoo CC, Pillay M. Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. *Clin Microbiol Infect*. 2014; 20:O361-369.
29. Ashiru OT, Sturm AW. Cytotoxicity induction in A549 alveolar epithelial cells by *Mycobacterium tuberculosis* isolates cultured in the presence and absence of oxygen. J Basic and Applied Sciences. 2015; 11: 118-124
30. Mootoo A, Stylianou E, Arias MA, Reljic R. TNF-α in tuberculosis: a cytokine with a split personality. Inflammation & allergy – Drug Targets. 2009; 8: 53-62.
31. Valway SE, Sanchez MPC, Shinnick TF, Orme I, Agerton T, Hoy D, Scott Jone J, Westmoreland H, Onorato IM. An outbreak involving extensive transmission of a virulent strain of *Mycobacterium tuberculosis*. 1998; 338: 633-639.
32. Engele M, Stöbel E, Castiglione K, Schwerdtner N, Wagner M, Bölcskei P, Röllinghoff M, Stenger S. Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent *Mycobacterium tuberculosis*. J Immunol. 2002; 168: 1328-1337.
33. Bocchino M, Galati D, Sanduzzi A, Colizzi V, Brunetti E, Mancino G. Role of mycobacteria-induced monocyte/macrophage apoptosis in the pathogenesis of human tuberculosis. Int J Tub Lung Dis. 2005; 9: 375–383
34. Vandenabeele P, Galluzzi L, Berghe TV, Kroemer G. Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat Rev Mol Cell Biol. 2010; 11: 700-714.
35. Condos R, Raju B, Canova A, Zhao BY, Weiden M, Rom WN, Pine R. Recombinant gamma interferon stimulates signal transduction and gene expression in alveolar macrophages in vitro and in tuberculosis patients. Infect Immun 2003; 71: 2058—64.

36. Wallis RS. Reconsidering adjuvant immunotherapy for tuberculosis. Clin Infect Dis. 2005; 41: 201-208
37. Park S, Cho S, Lee I, Jeon D, Hong S, Smego Jr RA, Cho S. Subcutaneously administered interferon-gamma for the treatment of multidrug-resistant pulmonary tuberculosis. Int J Infect Dis. 2007; 11: 434-440.
38. Rivero-Lezcano OM. Cytokines as immunomodulators in tuberculosis therapy. Recent Pat Antiinfect Drug Discov. 2008; 3: 168-176.
39. Dlugovitzky D, Torres-Morales A, Rateni L, et al. Circulating profile of Th1 and Th2 cytokines in tuberculosis patients with different degrees of pulmonary involvement. FEMS Immunology and Medical Microbiology. 1997; 18: 203–207.
40. Dlugovitzky D, Bay ML, Rateni L, et al. In vitro synthesis of interferon-γ, interleukin-4, transforming growth factor β and interleukin-1β by peripheral blood mononuclear cells from tuberculosis patients: Relationship with the severity of pulmonary involvement. Scandinavian Journal of Immunology, 1999; 49: 210–217.
41. Karpus WJ, Lukacs NW, Kennedy KJ, Smith WS, Hurst SD, Barrett TA. Differential CC chemokine-induced enhancement of T helper cell cytokine production. J Immunol. 1997; 158: 4129-4136.
42. Seah GT, Rook GAW. IL-4 influences apoptosis of mycobacterium-reactive lymphocytes in the presence of TNF-α. Journal of Immunology. 2001; 167:1230–1237.
43. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, Zeller K, Andrews J, Friedland G. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. The Lancet. 2006; 368:1-6.
44. Reed M, et al. A glycolipid of hypervirulent tuberculosis strains that inhibits the innate immune response. Nature. 2004; 431: 84 – 87.
45. Wang C, et al. Innate immune response to *Mycobacterium tuberculosis* Beijing and other genotypes. PLoS One. 2010: 25:e13594.
46. Saukkonen JJ, Bazydlo B, Thomas M, Strieter RM, Keane J, Kornfield. β-Chemokines are induced by *Mycobacterium tuberculosis* and inhibit its growth. Infect Immun. 2002; 70: 1684-1693.

CHAPTER FIVE

Mycobacterium tuberculosis isolates from HIV co-infected patients retain *in vitro* fitness at relapse

Naidoo CC, [1] Pillay M, [1] Naidoo K. [2]

[1] Medical Microbiology and Infection Control, University of KwaZulu-Natal, Durban, South Africa

[2] Centre for the AIDS Programme of Research in South Africa (CAPRISA) and the MRC-CAPRISA HIV-TB Pathogenesis and Treatment Research Unit, Doris Duke Medical Research Institute, Private bag X7, Congella, 4013, Durban, South Africa

Manuscript in preparation

ABSTRACT

Tuberculosis retreatment cases are often associated with low cure rates and increased risk for the development of drug-resistant TB. In this study, three HIV-positive patients who had successfully completed anti-tuberculosis treatment later developed relapse, despite improved overall health status. The *in vitro* fitness of paired *M. tuberculosis* isolates was investigated as a putative contributor to relapse. DNA fingerprint patterns of paired isolates were verified by IS*6110*-restriction fragment length polymorphism typing and spoligotyping. Drug susceptibility profiles, *in vitro* growth and drug resistance-encoding mutations were compared between initial and relapse isolates. Each patient harboured isolates with identical fingerprint patterns, drug susceptibility profiles, minimum inhibitory concentrations and mutations at initial infection and relapse. Growth rates were indistinguishable for initial and relapse isolates on day 12, as measured by colony counts. Although some differences were evident on day 20, these did not achieve statistical significance. Taken together, these findings supports the assertion that isolates recovered at the time of relapse most likely represents primary-infecting isolates. Importantly, this preliminary study shows that *M. tuberculosis* isolates may reactivate without significant changes in *in vitro* fitness, despite prolonged exposure to anti-tuberculosis drugs and host immunity.

INTRODUCTION

The World Health Organization estimated that 13 % of the 9 million individuals with tuberculosis (TB) in 2013 were co-infected with the human immunodeficiency virus (HIV) (WHO, 2014). HIV facilitates the progression to active TB disease (Daley et al. 1996) causing high rates of mortality (Sileshi et al. 2013), and is also a notable risk factor for TB recurrence (Sonnenberg et al. 2001). *Mycobacterium tuberculosis* isolates causing recurrence can be classified into two groups: the diagnosis of TB with an isolate having a restriction fragment length polymorphism (RFLP) pattern similar or different to that from a previous TB episode defined as relapse or reinfection, respectively. Recent estimates suggest that 51 % of notified retreatment cases in South Africa were due to relapse (WHO, 2014).

Weakened immune responses and low CD4 counts, characteristic of HIV infection, are significant triggers of reactivation disease (Lawn et al. 2005). Other predictors of recurrent TB disease include, but are not limited to, poor patient adherence to anti-TB treatment, residual cavitation, TB treatment lasting less than 37 weeks (reviewed comprehensively in Panjabi et al. 2007) and reinfection with a different strain of *M. tuberculosis* (Chaisson et al. 2010). Primary drug resistance has also been

described as a strong risk factor contributing to TB relapse and treatment failure (Quy et al. 2003). *M. tuberculosis* acquires drug resistance as a result of mutations within the bacterial genome which are thought to impose a 'fitness cost', usually occurring in the form of reduced growth *in vitro* or *in vivo* (Schrag et al. 1997). However, the role of *M. tuberculosis* fitness in the context of recurrence remains elusive.

Several studies show that subpopulations of bacteria may evade complete eradication by the immune system and establish latency (Gupta et al. 2012). Tuberculous granulomas which primarily aid in controlling infection ironically permit survival of latent TB bacilli within infected, inactivated cells (Tufariello et al. 2003). The transition of such bacteria into an active, virulent form is termed reactivation or relapse (Biketov et al. 2007). A number of mycobacterial factors may induce reactivation including resuscitation promoting factors which are thought to play an important role in forcing mycobacteria out of a state of dormancy or latency (Biketov et al. 2007). The DosS/DosT-DosR regulon has been shown to mediate the transition of *M. tuberculosis* from aerobic to anaerobic respiration and may also facilitate the resumption of replication following re-exposure to oxygen (Leistikow et al. 2010).

Relapse often occurs 6 to 12 months after treatment completion. In the present study, three patients developed relapse 15 to 48 months after successful TB-treatment completion. Patients were HIV-positive and were receiving highly active antiretroviral therapy (HAART). Here we compare the fitness and drug susceptibility patterns of initial and relapse isolates (with identical DNA fingerprint patterns), as putative contributors to relapse.

METHODS

Patients

This is a subset of a prospective, cohort study investigating the incidence of TB recurrence in adult TB/HIV co-infected patients stable on HAART, and who had previously completed therapy for pulmonary TB. This study was conducted from June 2005 to August 2013 at the CAPRISA eThekwini clinic in KwaZulu-Natal Province, South Africa. All patients received standardized TB therapy as per the South African National TB Control Program Guidelines (2005). New TB cases were treated with a fixed-drug combination of rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and pyrazinamide (PZA) dosed according to weight, for a 2-month intensive phase. The 4-

month continuation phase included a fixed-drug combination of INH and RIF. Retreatment cases received a 60-day intensive phase (including streptomycin), followed by a 100-day continuation phase. All patients were initiated on a once-daily antiretroviral therapy regimen; didanosine, lamivudine and efavirenz, dosed according to weight. Patients were followed up for 5 years, at 3-monthly intervals. TB symptoms screening and clinical evaluation were conducted 3-monthly; chest x-rays and laboratory evaluations were conducted at baseline and 6-monthly. Patients were deemed cured based on the following criteria: completion of TB treatment based on protocol-defined uninterrupted TB treatment, with a clinical and radiologic response to TB treatment without two adequate sputum samples obtained at or beyond 2 months, including one at or beyond 5 months of treatment (Dr. K Naidoo, personal communication). Among 489 patients with a previous TB cure/TB treatment completion, 68 (14 %) patients developed recurrent TB. The diagnosis of recurrent TB was made microbiologically via induced sputum collected at baseline, every 3 months or on suspicion of TB. Sputum smear microscopy and culture were performed on all sputum specimens. Routine drug susceptibility testing which became available only after 2008 was conducted on cultures. This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BF 051/09).

Clinical isolates

Fifteen pairs of initial and recurrent isolates were previously genotyped by IS*6110*-RFLP typing. Of these, three sets of paired isolates (Table 5.1) were selected on the basis of matching RFLP patterns. Isolates were grown on Middlebrook 7H11 medium (Difco) supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (Becton Dickinson) and 0.5% glycerol for 2-3 weeks at 37 °C. Mycobacterial harvests were heat-inactivated for 30 minutes at 80 °C. Genomic DNA was isolated by the cetyltrimethylammonium bromide (CTAB) method with some modifications (van Soolingen et al. 2001). RFLP typing was carried out using the method described previously (van Embden et al. 1993). Restriction fragment length polymorphism patterns were visualized using enhanced chemiluminescence (Amersham Pharmacia, Buckinghamshire, England). For each pair, we verified that initial and relapse isolates shared identical RFLP profiles. Spoligotyping was performed as previously described (Kamerbeek et al. 1997) using a commercially available kit (Ocimum Biosolutions, Hyderbad, India).

Drug susceptibility testing

Drug susceptibility testing was performed using the 1% agar proportion method on Middlebrook 7H10 medium (Difco) supplemented with 10% OADC, 0.5% glycerol and appropriate drugs (Sigma) as previously described (NCCLS, 2003). The following concentrations were used: 1.0 mg/L INH and RIF, 7.5 mg/L EMB, 2 mg/L ofloxacin (OFX) and streptomycin, and 5 mg/L kanamycin and ethionamide (ETH). After 3 weeks of incubation at 37 °C, the number of colony forming units (CFU) on drug-containing medium was compared with the number on drug-free medium. Isolates with growth on the drug-containing medium of > 1% of that on drug-free medium were classified as drug-resistant.

Minimum inhibitory concentrations

Minimum inhibitory concentrations (MIC) were determined in a 96-well plate format using Alamar Blue (Invitrogen) as previously described (Franzblau et al. 1998). The following drug concentration ranges were tested: for INH and OFX, 0.125 – 32 mg/L; RIF, 0.06 – 16 mg/L; EMB, 0.5 – 128 mg/L and ETH, 0.003 – 8 mg/L. Wells containing inoculum only (drug-free) served as controls. The MIC was defined as the lowest drug concentration at which no colour change from blue to pink occurred. MICs were carried out in duplicate experiments per clinical isolate to confirm reproducibility.

Molecular resistance

The following genes were examined in paired isolates: 580 bp of the *katG* gene, 437 bp of the *rpoB* gene, 863 bp of the *embB* gene, 670 bp of the *pncA* gene, 320 bp of the *gyrA* gene, 1278 bp and 342 bp of the *ethA* gene. Template DNA was amplified using Q5 Hot Start High-Fidelity DNA Polymerase (NEB, Scientific Group, SA) and primer sequences from previously reported studies (Takiff et al. 1994; van der Zanden et al. 2003; Zenteno-Cuevas et al. 2009; Brossier et al. 2011; Alexander et al. 2012; Bakula et al. 2013). Polymerase chain reactions (PCR) were performed in a 25-µl reaction mixture consisting of 1X Q5 Reaction Buffer, 0.4 µM forward and reverse primers, 1X Q5 High GC Enhancer, 0.2 mM dNTPs, 0.02 U/µl Q5 DNA polymerase and 50 ng of template DNA. Nuclease-free water was used as a negative control and the *M. tuberculosis* laboratory strain H37Rv was used as a positive control. Thermal cycling conditions included an initial denaturation step at 98 °C for 1.5 min, followed by 35 cycles as follows: denaturation at 98 °C for 10s, annealing at 58-70°C (depending on primer set) for 10s and extension at 72°C for 2 min, and a final extension step of 2 min at 72 °C. Amplifications were confirmed by gel electrophoresis in 1% agarose gels.

Amplified products were outsourced for purification and subsequent sequencing on the 3500XL Genetic Analyzer platform (ABI, Life Technologies). Mutations were identified by aligning sequences to the *M. tuberculosis* H37Rv reference (GenBank accession number NC_000962) using BioEdit software (Ibis Biosciences, Carlsbad, CA).

Fitness assay

Isolates were grown from an optical density at 600 nm (OD_{600}) of 0.015 in 25 ml of Middlebrook 7H9 medium (Difco) containing 10% OADC, 0.5% glycerol and 0.05% Tween-80 (Sigma) as previously described (Naidoo and Pillay, 2014). Serially-diluted culture was plated onto triplicate Middlebrook 7H11 agar plates and incubated for 21 days at 37 °C. Three independent experiments were performed for each isolate pair. OD_{600} and CFU counts were recorded on days 0 (baseline), 12 (log-phase) and 20 (late-log phase).

Statistical analysis

Differences in mean relative fitness indices between initial and relapse isolates on days 12 and 20 were calculated by one-way analysis of variance (ANOVA) (IBM SPSS Statistics version 22.0, Armonk, NY). A p-value < 0.05 was considered as statistically significant.

RESULTS

Patient demographics

Paired isolates from one male and two female patients were analysed. Viral loads and CD4 counts were improved at the time of relapse, albeit only a marginal increase in CD4 counts was observed for patient three (Table 5.1). Viral load information was missing at the time of initial TB infection for patient two however undetectable viral loads were recorded at the time of relapse. Patients one and two showed increased body mass indices (BMI) at the time of relapse, whereas the BMI of patient three was slightly reduced. Patients one and two both had a previous history of TB disease for which they received eight and six months of anti-TB treatment, respectively. These episodes occurred prior to enrolling into this study. Both patients presented with their third episode of TB 34 and 48 months respectively, after successful treatment completion for their second episode of TB while in the study. Thus, paired isolates from episodes two and three were analysed for patients one and two. Patient three had no past history of TB, received six months of standardised anti-TB treatment upon infection and presented with TB recurrence 15 months later.

Fingerprinting analysis

Among 15 paired isolates obtained during our prospective cohort study, we identified 3 sets of paired isolates by RFLP typing, with identical DNA fingerprints (Figure S5.1), representing F15/LAM4/KZN (KZN), Beijing and X3 genotypes. Spoligotyping confirmed the paired isolates were identical.

Table 5.1 Patient epidemiological data

Patient	Episode	Isolate	Missing spacers	Genotype	Age	Gender	CD4 count (cells/mm^3)	Viral load (cpm)	BMI
1	2007	74684	21-24, 33-36, 39-40	F15/LAM4/KZN	28	Male	100	73300	18.5
	2011	11370	21-24, 33-36, 39-40	F15/LAM4/KZN	31		565	342	20.4
2	2006	066069	1-34	Beijing	37	Female	233	Unknown	21.6
	2010	102570	1-34	Beijing	42		911	Undetectable	24.5
3	2005	73078	4-12, 18, 33-36	X3	30	Female	203	83900	26.5
	2007	05129	4-12, 18, 33-36	X3	31		277	<400	25.8

Table 5.2 Drug resistance profiles of paired isolates

Pair	Isolate ($_{episode}$)	Drug resistance[a]	MICs (µg/mL)[b]					Mutations					
			I	R	Et	E	O	*katG*	*rpoB*	*ethA*	*embB*	*gyrA*	*pncA*
1	74684 ($_{initial}$)	IREtEO	> 32	16	> 8	> 128	2	S315T	L452P	-	M306V	S95T	+c in A152
	11370 ($_{relapse}$)	IREtEO	> 32	16	> 8	> 128	2	S315T	L452P	-	M306V	S95T	+c in A152
2	066069 ($_{initial}$)	none	-	-	-	-	-	-	-	-	-	S95T	-
	102570 ($_{relapse}$)	none	-	-	-	-	-	-	-	-	-	S95T	-
3	05129 ($_{initial}$)	IEtO	> 32	-	> 8	-	> 8	S315T	-	-	-	S95T	-
	73078 ($_{relapse}$)	IEtO	> 32	-	> 8	-	> 8	S315T	-	-	-	S95T	-

[a] I: isoniazid, R: rifampicin, Et: Ethionamide, E: ethambutol, O: ofloxacin.

[b] MICs were determined in duplicate microplate-based Alamar Blue assays.

Phenotypic drug resistance

Drug susceptibility patterns and MICs were identical for initial and relapse isolates (Table 5.2). Patient one isolates were phenotypically resistant to INH, RIF, ETH, EMB and OFX. Patient two isolates were susceptible to all drugs tested in this study. Patient 3 bore poly-resistant isolates (resistance to a combination of two or more drugs that do not constitute MDR) with phenotypic resistance to INH, ETH and OFX only.

Mutations associated with drug resistance

Isoniazid resistance in KZN and X3 paired isolates was associated with the S315T *katG* mutation (Table 5.2). No mutations were identified in the *ethA* gene, indicating the contribution of other genes to ETH resistance. KZN isolates which were phenotypically resistant to RIF and EMB, bore corresponding L452P and M306V mutations in *rpoB* and *embB*, respectively. Although susceptibility to PZA was not tested, the *pncA* gene was sequenced to identify potential mutations associated with PZA resistance. We identified an insertion (+c) at codon 152 of *pncA* in both KZN isolates. No ofloxacin resistance-conferring mutations were detected in *gyrA* in KZN and X3 isolates. As expected, Beijing paired isolates did not harbour any drug resistance-conferring mutations.

Fitness assays

F15/LAM4/KZN and X3 relapse isolates had significantly higher OD_{600} values than initial isolates on day 12 but similar OD_{600} measurements on day 20 (Figure 5.1). Beijing paired isolates had similar OD_{600} readings on days 12 and 20. Colony forming units counts which are more reliable than OD_{600} for quantifying viable microorganisms, showed nearly identical growth rates for initial and relapse isolates on day 12 (Figure 5.2). On day 20, more visible differences were evident between initial and relapse isolates of the KZN and X3 genotypes however, these were not statistically significant. Interestingly, Beijing isolates grew poorly in laboratory culture, compared to KZN and X3 isolate pairs. Statistically significant differences were observed on day 20 between the KZN relapse isolate and Beijing initial ($p = 0.006$) and relapse ($p = 0.007$) isolates.

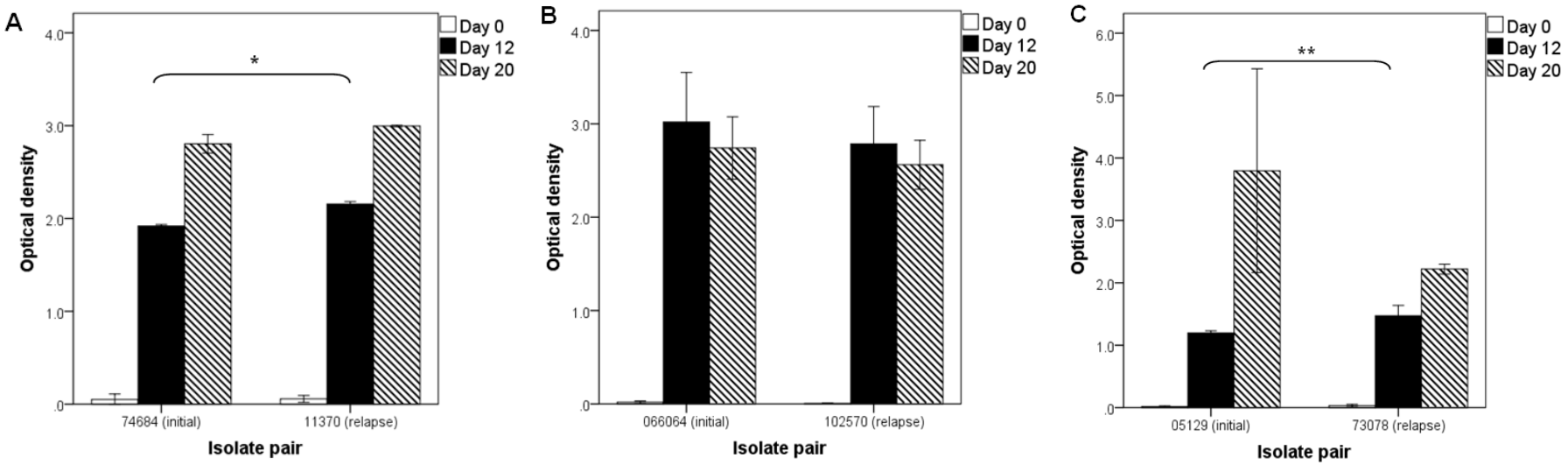


Figure 5.1 Optical density (OD_{600}) measurements for F15/LAM4/KZN (A), Beijing (B) and X3 (C) paired isolates. Data represents the mean ± SD of three independent experiments. * $P < 0.001$, ** $P = 0.049$.

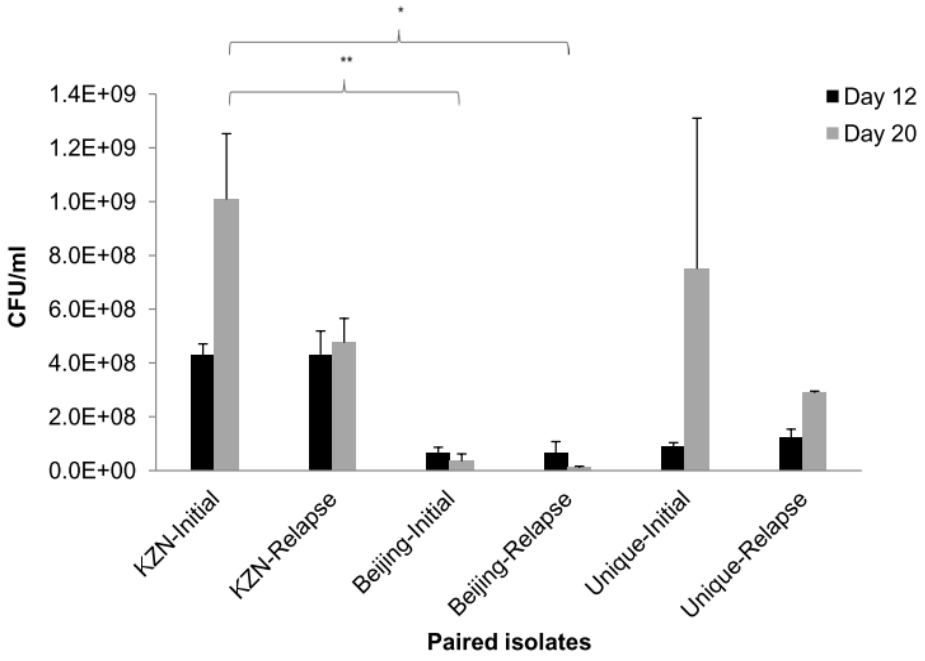


Figure 5.2 Comparative growth of paired isolates as measured by colony forming units (CFU) counts. Data represents the mean ± SD of three independent experiments, analysed by one-way analysis of variance (ANOVA). * $P = 0.007$, ** $P = 0.006$.

DISCUSSION

Patients in this study had developed TB relapse despite their improved health status. This is in accordance with other studies which suggest that under favourable conditions i.e. improved CD4 counts, the risk of TB is still high and that HIV infection may impose further immune impairments unrelated to CD4 counts (Lawn et al. 2005). This prompted us to investigate the biological fitness of paired isolates as a contributor to relapse in these patients.

Identical drug susceptibility profiles, MICs and resistance-conferring mutations were obtained for paired isolates which had matching RFLP and spoligotyping patterns at baseline infection and relapse. Taken together, this finding supports the assertion that isolates recovered at the time of relapse most likely represents primary-infecting isolates, which under permissive *in vivo* conditions were capable of reactivating from a latent state (Gupta et al. 2012). Growth rates, as measured by CFU, were not statistically different for initial and relapse isolates, demonstrating that clinical isolates do not necessarily undergo fitness costs during reactivation.

Generally, persistent bacilli are harder to eradicate if they exist in necrotic lung regions, are in a low metabolic state or exhibit reduced susceptibility to anti-TB drugs (Boshoff and Barry, 2005). The most probable explanation for relapse in these patients could be that a subpopulation of infecting mycobacteria shifted into a non-replicating (dormant) state, thereby rendering them phenotypically resistant to anti-TB drugs (Gengenbacher and Kaufmann, 2012). As a result of standardised sputum inspection methods, these bacilli perhaps went undetected and patients were deemed cured. Subsequent to termination of anti-TB treatment, some bacilli may have recovered, with unchanged fitness, and replicated to high enough bacterial loads causing relapse. Indeed, mutations identified in *katG* and *rpoB* in the KZN isolates are thought to confer high-level drug resistance whilst sustaining mycobacterial fitness (Naidoo and Pillay, 2014). This process may have also allowed for the acquisition of compensatory mutations in already drug-resistant isolates which could outcompete less fit and less resistant mycobacteria (Andersson and Hughes, 2010), however further examination is required. We did not detect any mutations in *gyrA*, thus OFX resistance in the KZN and X3 isolates may be attributable to mutations outside of the sequenced quinolone resistance-determining region. Intriguingly, the KZN paired isolates demonstrated increased *in vitro* fitness compared with Beijing and X3 pairs, corroborating previous studies which show specific genetic backgrounds may cope better with fitness costs associated with drug resistance (Naidoo and Pillay, 2014; Gagneux et al. 2006).

We cannot conclusively rule out reinfection with the same strain, especially in endemic settings that promote the circulation of specific predominant *M. tuberculosis* strains (Shen et al. 2006). Whole-genome sequencing of *M. tuberculosis* isolates from sputum specimens could distinguish highly related but genetically distinct strains, affording resolution that is not possible with other fingerprinting methods (Bryant et al. 2013). Moreover, the inclusion of additional assays measuring fitness would strengthen the findings of this study. Nevertheless, these preliminary findings indicate that *M. tuberculosis* isolates may retain *in vitro* fitness at the point of relapse, and warrant further inspection. This could have significant implications for future TB control programs as retreatment cases are often associated with lower cure rates (Murray et al. 1999) and increased risk for the development of drug-resistant TB (Costello et al. 1980). Molecular epidemiological tools which accurately distinguish between exogenous reinfection and TB relapse, together with the development of new, effective anti-TB agents may aid in improved surveillance and management of TB, particularly in settings with high incidence rates of TB and HIV. Until improved therapeutic

regimens become implemented, lengthier courses of TB therapy may be necessary for reducing the number of retreatment cases.

HIV-associated TB is the commonest clinical manifestation of TB in sub-Saharan Africa, with ongoing vulnerability to TB previously demonstrated in HIV-infected patients. Population studies have further suggested that wide-scale ART access will reduce TB incidence, however this study demonstrates that the individual risk of relapse after successful TB treatment completion, in stable patients on HAART, still exists.

ACKNOWLEDGMENTS

This work was supported by the Howard Hughes Medical Institute (grant number 55007065) as well as the Centers for Disease Control and Prevention (CDC) (Cooperative Agreement number UY2G/PS001350-02). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of either the Howard Hughes Medical Institute or the CDC. Patient care was supported by the KwaZulu-Natal Department of Health and the U.S. President's Emergency Plan for AIDS Relief (PEPFAR). The research infrastructure to conduct this trial, including the data management, laboratory and pharmacy cores were established through the US National Institutes for Health's Comprehensive International Program of Research on AIDS grant (CIPRA, grant number AI51794). KN was supported by the Columbia University-South Africa Fogarty AIDS International Training and Research Program (AITRP, grant number D43 TW000231). CCN was supported by the Medical Research Council of South Africa. The funding sources listed here did not have any role in the analysis or preparation of the data in this manuscript, nor was any payment received by these or other funding sources for this manuscript. Preliminary findings were presented at the 5th Conference of the Union Asia Pacific Region (2015). CCN MP KN conceived and designed the experiments, CCN MP performed the experiments and analysed the data, KN contributed reagents and materials, CCN MP KN wrote the paper.

Conflict of interest: none declared.

REFERENCES

1. WHO. Global Tuberculosis Report 2014. WHO/HTM/TB/2014.08, Geneva, Switzerland, 2014.

2. Daley CL, Small PM, Schecter GF et al. An outbreak of tuberculosis with accelerated progression among persons infected with the human immunodeficiency virus: an analysis using restriction fragment length polymorphisms. N Engl J Med. 1996; 326: 231-235.
3. Sileshi B, Deyessa N, Girna B, Melese M, Suarez P. Predictors of mortality among TB-HIV co-infected patients being treated for tuberculosis in Northwest Ethiopia: a retrospective study. BMC Infect Dis. 2013; 13: 297-307.
4. Sonnenberg P, Murray J, Glynn JR, Shearer S, Kambashi B, Godfrey-Faussett P. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. Lancet. 2001; 358: 1687-1693.
5. Lawn SD, Bekker LG, Wood R. How effectively does HAART restore immune responses to *Mycobacterium tuberculosis*? Implications for tuberculosis control. AIDS 2005; 19: 1113-1124.
6. Panjabi R, Comstock GW, Golub JE. Recurrent tuberculosis and its risk factors: adequately treated patients are still at high risk. Int J Tuberc Lung Dis. 2007; 11: 828-837.
7. Chaisson RE, Churchyard GJ. Recurrent Tuberculosis – Relapse, Re-infection and HIV. J Infect Dis. 2010; 201: 653-355.
8. Quy HT, Lan NT, Borgdoff MW et al. Drug resistance among failure and relapse cases of tuberculosis: is the standard re-treatment regimen adequate? Int J Tuberc Lung Dis. 2003; 7: 631-636.
9. Schrag SJ, Perrot V, Levin BR. Adaptation to the fitness costs of antibiotic resistance in *Eschericia coli*. Proc R Soc Lond B. 1997; 264: 1287-1291.
10. Gupta A, Kaul A, Tsolaki AG, Kishore U, Bhakta S. *Mycobacterium tuberculosis*: immune evasion, latency and reactivation. Immunobiol. 2012; 217: 363-374.
11. Tufariello JM, Chan J, Flynn JL. Latent tuberculosis: mechanisms of host and bacillus that contribute to persistent infection. Lancet Infect Dis. 2003; 3: 578-590.
12. Biketov S, Popotov V, Ganina E, Downing K, Kana BD, Kaprelyants A. The role of resuscitation promoting factors in pathogenesis and reactivation of *Mycobacterium tuberculosis* during intra-peritoneal infection in mice. BMC Infect Dis. 2007; 7: 146-153.
13. Leistikow RL, Morton RA, Bartek IL, Frimpong I, Wagner K, Voskuil MI. The *Mycobacterium tuberculosis* DosR regulon assists in metabolic homeostasis and enables rapid recovery from nonrespiring dormancy. J Bacteriol. 2010; 192: 1662-1670.
14. The South African National Tuberculosis Control Programme Guidelines, 2005.
15. Van Soolingen D, de Haas PE, Kremer K. Restriction fragment length polymorphism (RFLP) typing of mycobacteria. Methods Mol Med. 2001; 54: 165–203.

16. van Embden JD, Cave MD, Crawford JT et al. Strain identification of *M. tuberculosis* by DNA fingerprinting: recommendation for a standardized methodology. J Clin Microbiol. 1993; 31: 406-409.
17. Kamerbeek J, Schouls L, Kolk A et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. J Clin Microbiol. 1997; 35: 907-914.
18. NCCLS. Susceptibility Testing of Mycobacteria, Nocardiae, and Other Aerobic Actinomycetes; Approved Standard. NCCLS, Pennsylvania, USA, 2003.
19. Franzblau SG, Witzig RS, McLaughlin JS et al. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate alamar blue assay. J Clin Microbiol. 1998; 36: 362-366.
20. Zenteno-Cuevas R, Zenteno JC, Cuellar A et al. Mutations in *rpoB* and *katG* genes in Mycobacterium isolates from Southeast Mexico. Mem Inst Oswalso Cruz. 2009; 104: 468-472.
21. Van der Zanden AGM, Te Koppele-Vije EM, Vijaya BN, van Soolingen D, Schouls LM. Use of DNA extracts from Ziehl-Neelsen-stained slides for molecular detection of rifampicin resistance and spoligotyping of *Mycobacterium tuberculosis*. J. Clin Microbiol. 2003; 41 : 1101-1108.
22. Bakuła Z, Napiórkowska A, Bielecki J, Augustynowicz-Kopeć E, Zwolska Z, Jagielsji T. Mutations in the *embB* gene and their association with ethambutol resistance in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from Poland. Biomed Res Int. 2013; in press. http://dx.doi.org/10.1155/2013/167954
23. Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: a role for *pncA* but not *rpsA*. J Clin Microbiol. 2012; 50: 3726-3728.
24. Takiff HE, Salazar L, Guerrero C et al. Cloning and nucleotide sequence of *Mycobacterium tuberculosis gyrA* and *gyrB* genes and detection of quinolone resistance mutations. Antimicrob Agents Chemother. 1994; 38: 773–780.
25. Brossier F, Veziris N, Truffot-Pernot C, Jarlier V, Sougakoff W. Molecular investigation of resistance to the antituberculous drug ethionamide in multidrug-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2011; 55: 355-360.
26. Naidoo CC, Pillay M. Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. Clin Microbiol Infect. 2013; 20: O361-369.

27. Boshoff HI, Barry CE 3rd. Tuberculosis - metabolism and respiration in the absence of growth. Nat Rev Microbiol. 2005; 3: 70-80.
28. Gengenbacher M, Kaufmann SH. *Mycobacterium tuberculosis*: success through dormancy. FEMS Microbiol Rev. 2012; 36: 514-532.
29. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol. 2010; 8: 260–271.
30. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJM. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science. 2006; 312: 1944-1946.
31. Shen G, Xue Z, Shen X et al. Recurrent tuberculosis and exogenous reinfection, Shangai, China. Emerg Infect Dis. 2006; 12: 1776-1778.
32. Bryant JM, Harris SR, Parkhill J et al. Whole-genome sequencing to establish relapse or reinfection with *Mycobacterium tuberculosis*: a retrospective observational study. Lancet Respir Med. 2013; 10: 786-792.
33. Murray J, P. Sonnenberg P, Shearer SC, Godfrey-Faussett P. Human immunodeficiency virus and the outcome of treatment for new and recurrent pulmonary tuberculosis in African patients. Am J Respir Crit Care Med. 1999; 159: 733-740.
34. Costello HD, Caras GJ, Snider DEJ. Drug resistance among previously treated tuberculosis patients: a brief report. Am Rev Respir Dis. 1980; 121: 313-316.

CHAPTER SIX

Synthesis

SYNTHESIS AND FUTURE WORK

MDR-TB cases comprised a small proportion (~ 5%) of total TB cases in 2014 (WHO, 2015). However, given that biological fitness is a primary determinant in the transmission of *M. tuberculosis*, and that low- or no- cost mutations are frequently selected for in clinical isolates, the potential for MDR and even XDR-TB to expand is an imperative concern. While the Beijing genotype is associated with MDR and XDR-TB globally and in South Africa, non-Beijing strains propel the transmission of drug resistance in the KZN Province (Muller et al. 2013; Gandhi et al. 2014; Cohen et al. 2015). To test the hypothesis that the enhanced fitness of specific genotypes is associated with beneficial resistance-conferring mutations and/or compensatory mutations, four clinically-relevant genotypes were selected for evaluation of fitness. These included susceptible, MDR and/or XDR strains from F15/LAM4/KZN, Beijing, F11 and F28 genotypes.

The first objective of this research was divided into two parts: (1) to determine the laboratory fitness of these strains using a number of growth, viability and competition assays, and (2) to correlate phenotypic findings with mutations in selected genes associated with drug resistance. Susceptible and resistant F15/LAM4/KZN and F28 strains had shown similar growth kinetics as measured in axenic experiments, whilst Beijing and F11 MDR strains exhibited significantly reduced fitness relative to susceptible strains. Sequencing of common genes associated with INH, RIF, OFX and KAN resistance revealed the presence of low- or no-cost mutations in F15/LAM4/KZN and F28 resistant strains, whilst the Beijing MDR strain harboured a less frequently-occurring *katG* mutation associated with a high fitness cost. The absence of a mutation in the sequenced region of *katG* indicated that a less frequently-occurring mutation with high costs were present in the F11 MDR strain.

During competitive growth assays, apparent differences were revealed even amongst genetically similar strains. This highlights the importance of carefully selecting comparator strains to avoid bias when assessing laboratory fitness. The use of standardised reference strains alone would bias results i.e. a commonly-used reference strain, H37Rv, was isolated over a century ago and its serial passage in laboratory culture may have compromised its use as a comparator strain in fitness studies.

An interesting phenomenon was observed in co-culture experiments with susceptible and resistant strains, where growth was amplified relative to axenic cultures. This phenomenon was applicable to all four genotypes and was not reported previously in *M. tuberculosis*. Similar to *M. tuberculosis*,

Staphylococcus aureus strains which acquire drug resistance-conferring mutations often undergo fitness costs. A study by Hammer et al. (2014) showed that co-culture of two distinct *S. aureus* mutants led to the improvement of fitness to that of wild-type levels. This synergism was attributed to the exchange of metabolites between mutants which not only enhanced growth but also increased the production of virulence factors and pathogenic effects (Hammer et al. 2014). Likewise, some sort of metabolite exchange may take place between susceptible and resistant *M. tuberculosis* strains during co-culture. Attempts to elucidate the mechanism behind this synergistic growth phenomenon were difficult without a defined genetic target, thus these findings in *M. tuberculosis* remain solely observational. Nevertheless, the implications of this phenomenon in TB patients are far-reaching as many studies have reported the existence of multiple-strain infections in South Africa (Richardson et al. 2002; Warren et al. 2004; van Rie et al. 2005; Cohen et al. 2011). The findings by Hammer et al. (2014) were extended to the interspecies level, whereby members of the human microbiota enhanced the fitness of *S. aureus* mutants (Hammer et al. 2014). Thus, further studies examining the influence of interspecies interactions on the growth of *M. tuberculosis* may provide significant insight into host pathogenesis.

The second objective expanded on the first, by addressing the role of genome-wide mutations in drug resistance, compensation and fitness of selected clinical strains. WGS analysis provided an understanding of some of the phenotypes that were observed in the first study. Firstly, resistant KZN strains demonstrated heterogeneous fitness when paired with different susceptible competitors (V9124 and V4207), indicating that susceptible strains differed in fitness. Mapping of V9124 to V4207 revealed a total of 43 SNPs, accounting for their differences in fitness. Secondly, WGS helped uncover mutations that were missed during preliminary amplicon sequencing i.e. the rare *katG* mutation associated with high-level INH resistance (1 µg/ml) in the F11 MDR strain and intragenic *rpoB* mutations associated with compensation in MDR and XDR KZN strains. This highlights the disadvantage of solely targeting 'hot-spot' genetic regions, as other significant mutations related to drug resistance could be overlooked.

Of the 10 sequenced strains, two (V1435 and KZN605) were previously sequenced by the Broad Institute. These strains were re-sequenced to ensure comparability to other strains cultured under the same conditions. Using WGS, a variety of drug resistance-encoding mutations were indentified, including a novel *ubiA* mutation in the F28 MDR strain. Stepping-stone mutations related to EMB resistance are of recent interest (Safi et al. 2013), thus the significance of this mutation warrants

further research. Some isolates carry resistance without defined gene mutations, suggesting that other mechanisms (e.g. efflux pumps) are at play (Choudhuri et al. 2002; Ramon-Garcia et al. 2009; Balganesh et al. 2010). Although resistance was explained by mutations in well-characterised genes for all resistant strains included in this study, efflux pumps were explored as an alternate source of resistance. With the exception of KZN genotype strains which harboured mutations specific to resistant phenotypes, a large proportion of mutations in genes encoding efflux pumps were common to both susceptible and resistant strains. This underscores the need for further studies which demonstrate clear relationships between drug resistance and spontaneous mutations related to efflux pumps.

Whereas some groups perform WGS on single colonies which may not be a true representation of the genomic diversity within a sample, others which sequence a complete *M. tuberculosis* population may minimize the amount of diversity present in a sample (Black et al. 2015). However, this is dependent on read depth, a feature which can be improved so as to not miss any heterogeneity at target alleles (Black et al. 2015). In this study, a selection of low-frequency alleles in *rpoB*, *rpoC* and *sigA* genes were verified by Sanger sequencing, of which all proved to be true variants. These mutations highlight the versatility of *M. tuberculosis* organisms which are able to lose or gain mutations in response to changing selective pressures (Black et al. 2015). It also raises the question of how genetic heterogeneity shapes treatment outcomes, especially in high TB-burden regions such as South Africa, where antibiotic programmatic selection exists.

Whole-genome sequencing also afforded valuable insights into the distribution of nSNPs in specific genes encoding virulence factors. Beijing, F11 and F28 genotypes clustered together with (different) nSNPs in the *mas* gene. The F15/LAM4/KZN genotype was set apart from other genotypes with nSNPs in the *mmaA4* gene only. Although spontaneous mutations in the *mmaA4* have been implicated in thiacetazone resistance (Alahari et al. 2009), this mutation was common to both susceptible and resistant KZN strains. Using a transposon library of mutants in macrophages, Dao et al. (2008) detected that the inactivation of the *mmaA4* gene resulted in increased induction of IL-12p40 and attenuation of the mutant in macrophages and mice. Thus, further studies elucidating the role of *mmaA4* mutations may reveal interesting data related to IL-12-mediated host responses and virulence (Dao et al. 2008). Taken together, WGS provided significant insights into the genetic diversity among *M. tuberculosis* strains, on the basis of both drug resistance and genetic background. As WGS by itself is not capable of dissecting strain-specific differences in

transmissibility or virulence, future studies should aim to integrate clinical data with genomic sequence data to improve our understanding on the success of *M. tuberculosis*. Moreover, declining costs of WGS means that this technology could be implemented on a wider basis, therefore necessitating the development of user-friendly software and accessible genomic databases (Takiff and Feo, 2015).

Because *in vitro* assays do not fully mimic *in vivo* conditions, the fitness of *M. tuberculosis* strains was examined in a human monocytic-like cell line (THP-1) as part of the third objective. The use of macrophages derived from transformed monocytic cell lines are suitable representations of early stages of infection with *M. tuberculosis* (Theus et al. 2005). Uptake efficiencies, intracellular replication and cytokine induction were assessed at two time points. Increased or equal intracellular replication was demonstrated for resistant KZN strains relative to the susceptible KZN strain in macrophages. Previous studies assessing the virulence of KZN strains showed increased invasion and necrosis of A549 cells by XDR strains (Ashiru et al. 2010; Ashiru and Sturm, 2015). Increased levels of TNF-α were produced by XDR strains relative to other strains. These strains may exploit the secretion of TNF-α for tissue destruction and facilitate its transmission to other hosts. During the experimental phase of this thesis, another research group assessed the virulence of F15/LAM4/KZN genotype strains in a murine model and found an indirect relationship between drug resistance and virulence, with XDR strains demonstrating the lowest virulence (Smith et al. 2014). While all but one strain tested in Smith et al. (2014) differed from those included in this thesis, it is plausible that macrophage findings do not embody that of mouse models, and necessitates further research.

Beijing and F11 MDR strains did not undergo intracellular replication costs relative to susceptible strains, which was surprising given their reduced *in vitro* fitness. However, these strains differed in their cytokine responses, an observation probably associated with differences in drug susceptibilities rather than bacillary burden. As with *in vitro* assays, the F28 MDR strain displayed comparable fitness to the susceptible strain in THP-1 macrophages. While these strains elicited similar levels of most cytokines, the susceptible strain produced significantly higher levels of IL-1β, IL-6 and GM-CSF compared with the MDR strain. An interesting observation was that the susceptible F28 strain had very similar replication and cytokine responses to H37Rv, with only one exception i.e. TNF-α at 4 hours.

To understand fitness in the context of TB recurrence, the last aim assessed the drug resistance patterns and growth kinetics of paired isolates using microbiological and molecular techniques. In the light of improved health status of HIV patients at the time of TB relapse, the *in vitro* fitness of *M. tuberculosis* isolates was investigated as a putative contributor to relapse. Isolates were collected from three HIV-positive patients who had successfully completed anti-TB treatment but later developed relapse. Matching fingerprint patterns of paired isolates were re-confirmed by IS*6110*-RFLP typing and spoligotyping. Isolates did not develop additional drug resistance and MICs remained unchanged at the point of relapse. Furthermore, initial and relapse isolates had indistinguishable growth kinetics supporting that *M. tuberculosis* can reactivate without significant changes in fitness, despite prolonged exposure to anti-TB drugs and host immunity. To the best of our knowledge, this has not been shown before in relapse isolates. Interestingly, pre-XDR KZN isolates had higher fitness (as measured by CFU) than pan-susceptible Beijing paired isolates, verifying that the KZN genotype does not undergo significant fitness costs in resistant forms. Recent WGS analysis of recurrent isolates revealed few (if any) SNP differences between initial and relapse isolates whereas strains causing reinfection differed from initial isolates by at least 1306 SNPS (Guerra-Assuncao et al. 2015). While their findings emphasise the usefulness of WGS for strain differentiation, it also highlights the preservation of genomic diversity in relapse isolates which would translate into retained potential for transmission to other hosts.

LIMITATIONS

Given the diversity of circulating genotypes in the KZN Province and other regions of South Africa, it is acknowledged that the inclusion of additional strains and genotypes (i.e. CAS, EAI1-SOM, X and T) would strengthen the findings of the thesis. Furthermore, due to financial constraints, WGS was only possible for a few strains within each genotype. One limitation of using the Bio-Plex Pro ™ Human Cytokine 17-plex assay (Bio-Rad) was the overall low sensitivity for some analytes. Given the exorbitant cost of this kit, validation by a more sensitive method i.e. enzyme-linked immunosorbent assay (ELISA) would have proven too costly. Additional time points may have provided more insight into the replication and cytokine induction of clinical strains in macrophages. While interesting observations were made in the study of relapse isolates, additional time intervals and fitness assays would have supported these findings.

CONCLUSIONS

This study showed that the enhanced fitness of drug-resistant KZN and F28 genotype strains is due to the presence of beneficial resistance-conferring mutations and compensatory mutations, while the reduced fitness of MDR Beijing and F11 strains is associated with high cost *katG* mutations and the absence of compensatory mutations. In support of this, the predominance of KZN and S (or F28) strains among MDR and/or XDR cases have been well-documented, whereas Beijing and LAM3 (or F11) strains frequents among susceptible TB-cases in KZN (Pillay and Sturm, 2007; Andrews et al. 2008; Pillay and Sturm, 2010; Gandhi et al. 2014; Cohen et al. 2015). Moreover, the existence of XDR KZN predecessors prior to the HIV epidemic in South Africa indicates that the selection of drug resistance can take place even in low-prevalence HIV settings (Cohen et al. 2015). Taken together, this highlights the importance of TB surveillance systems which may prevent future epidemics caused by fit resistant strains.

APPENDIX

Table S2.1 Baseline (Day 0) CFU/ml readings corresponding to an OD_{600nm} of 0.015 in growth assays

Genotype family	Strain	Drug resistance profile	CFU/ml
F15/LAM4/KZN	V9124	S	2.50E+06
	V4207	S	3.10E+06
	V1435	MDR	1.90E+06
	V2475	MDR	2.05E+06
	KZN605	XDR	1.50E+06
	X162	XDR	1.00E+06
Beijing	B910	S	2.70E+06
	B1528	S	2.05E+06
	R 35	MDR	2.10E+06
	R 283	MDR	1.70E+06
F11	R 490	S	3.75E+05
	R 271	MDR	3.65E+05
F28	R 104	S	7.00E+05
	R 443	MDR	8.00E+05
	R 262	XDR	1.55E+06

S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

Table S2.2 Relative fitness index of drug-resistant strains in comparison to drug-susceptible strains and H37Rv on day 24

Genotype family	Strain	Drug resistance profile	Relative Fitness Index	Susceptible competitor
F15/LAM4/KZN	V1435	MDR	1.05 ± 0.23	V9124
			1.14 ± 0.15	V4207
			0.86 ± 0.10	H37Rv
	V2475	MDR	0.93 ± 0.13	V9124
			1.02 ± 0.08	V4207
			0.78 ± 0.06	H37Rv
	KZN605	XDR	0.92 ± 0.17	V9124
			1.00 ± 0.09	V4207
			0.76 ± 0.04	H37Rv
	X162	XDR	1.25 ± 0.15	V9124
			1.36 ± 0.05	V4207
			1.04 ± 0.06	H37Rv
Beijing	R 35	MDR	1.01 ± 0.04	B910
			0.99 ± 0.02	B1528
			0.94 ± 0.04	H37Rv
	R 283	MDR	1.08 ± 0.01	B910
			1.05 ± 0.04	B1528
			1.01 ± 0.06	H37Rv
F11	R 271	MDR	0.99 ± 0.06	R 490
			0.83 ± 0.04	H37Rv
F28	R 443	MDR	0.98 ± 0.02	R 104
			1.01 ± 0.07	H37Rv
	R 262	XDR	0.91 ± 0.04	R 104
			0.93 ± 0.04	H37Rv

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

Table S2.3 Significant *p*-values for the OD and CFU competition assays

Genotype family	Pairwise competition	OD assay *p*-value	CFU assay *p*-value
F15/LAM4/KZN	V9124 (S) > V1435 (MDR)	0.035	0.034
	V1435 < V4207 (S) + V1435	0.038	
	V9124 > V2475 (MDR)	0.022	
	V4207 > V2475	0.013	
	V9124 > KZN605 (XDR)	0.027	
	V4207 > KZN605	0.035	0.044
	X162 (XDR) < V4207 + X162	0.045	
Beijing	B910 (S) > R35 (MDR)	0.048	0.01
	R35 < B910 + R35	0.016	
	B1528 (S) > R35	0.044	
	B910 > R283 (MDR)		0.027
	R283 < B910 + R283	0.016	
	B1528 > R283	0.043	
F11	R490 (S) > R271 (MDR)		0.034
	R271 < R490 + R271	0.026	

A *p*-value less than 0.05 was considered significant. > represents more fit, < represents less fit. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

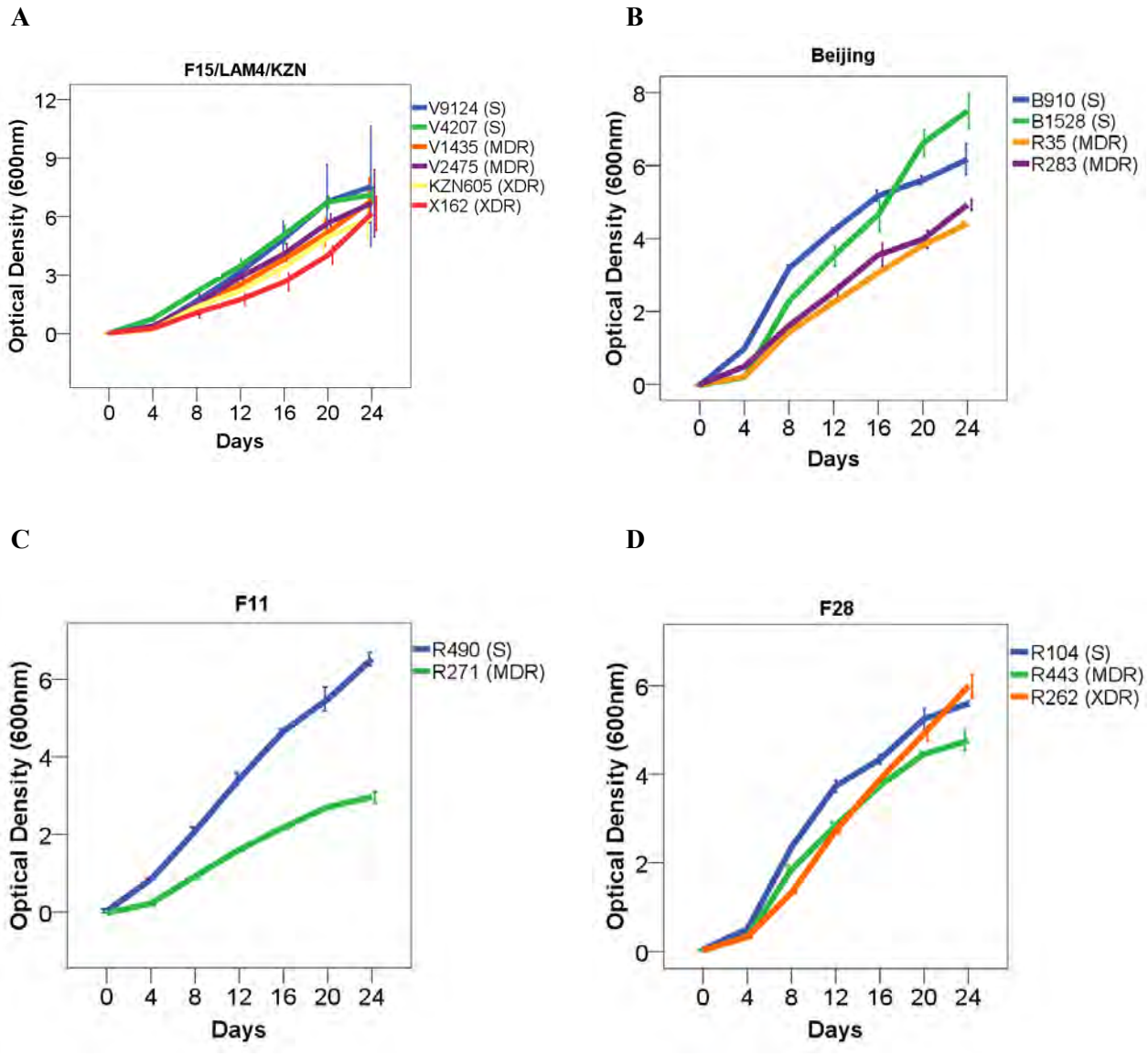


Figure S2.1 Optical density measurements of clinical strains in independent cultures. Growth rates were determined for the F15/LAM4/KZN (A), Beijing (B), F11 (C) and F28 (D) genotype families. Data represents the mean ± standard deviation of triplicate cultures per strain. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

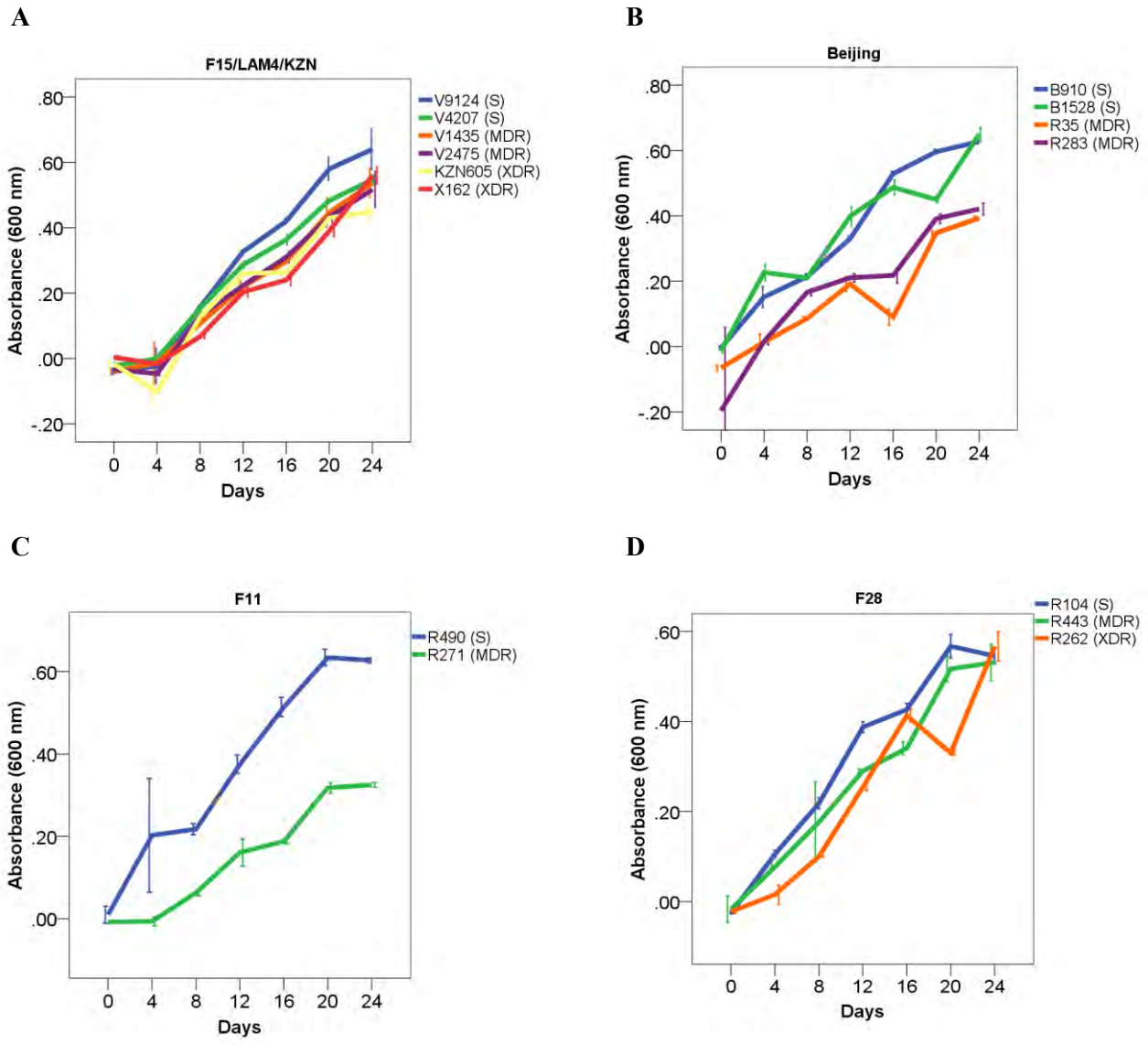


Figure S2.2 Absorbance measurements of clinical strains in independent cultures in the Alamar Blue assay. Metabolic rates were determined for the F15/LAM4/KZN (A), Beijing (B), F11 (C) and F28 (D) genotype families. Data represents the mean ± standard deviation of triplicate cultures per strain. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

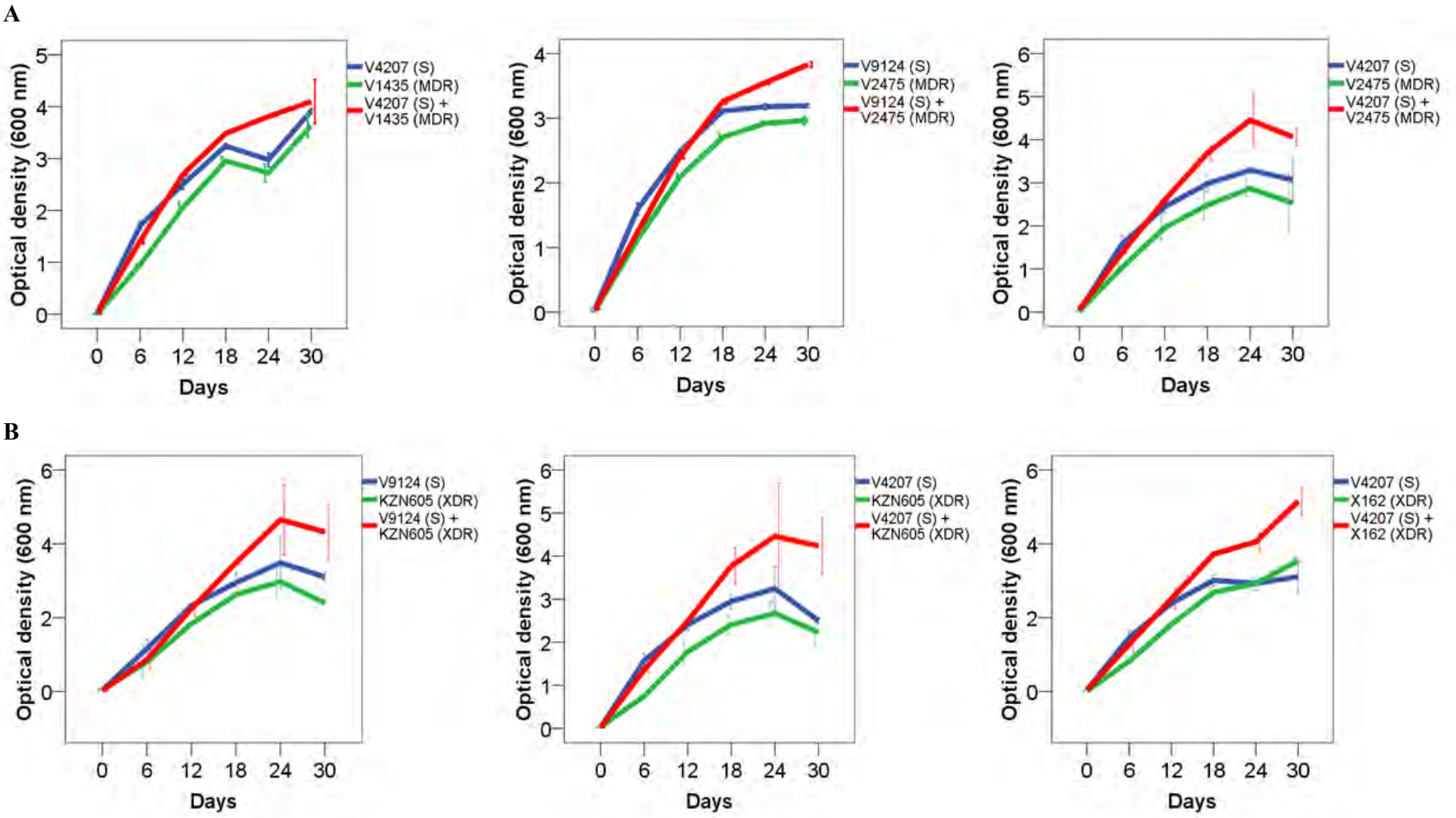


Figure S2.3 Optical density measurements for susceptible strains paired with MDR (A) and XDR (B) F15/LAM4/KZN strains in competitive growth assays. Data represents the mean ± standard deviation of duplicate experiments. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

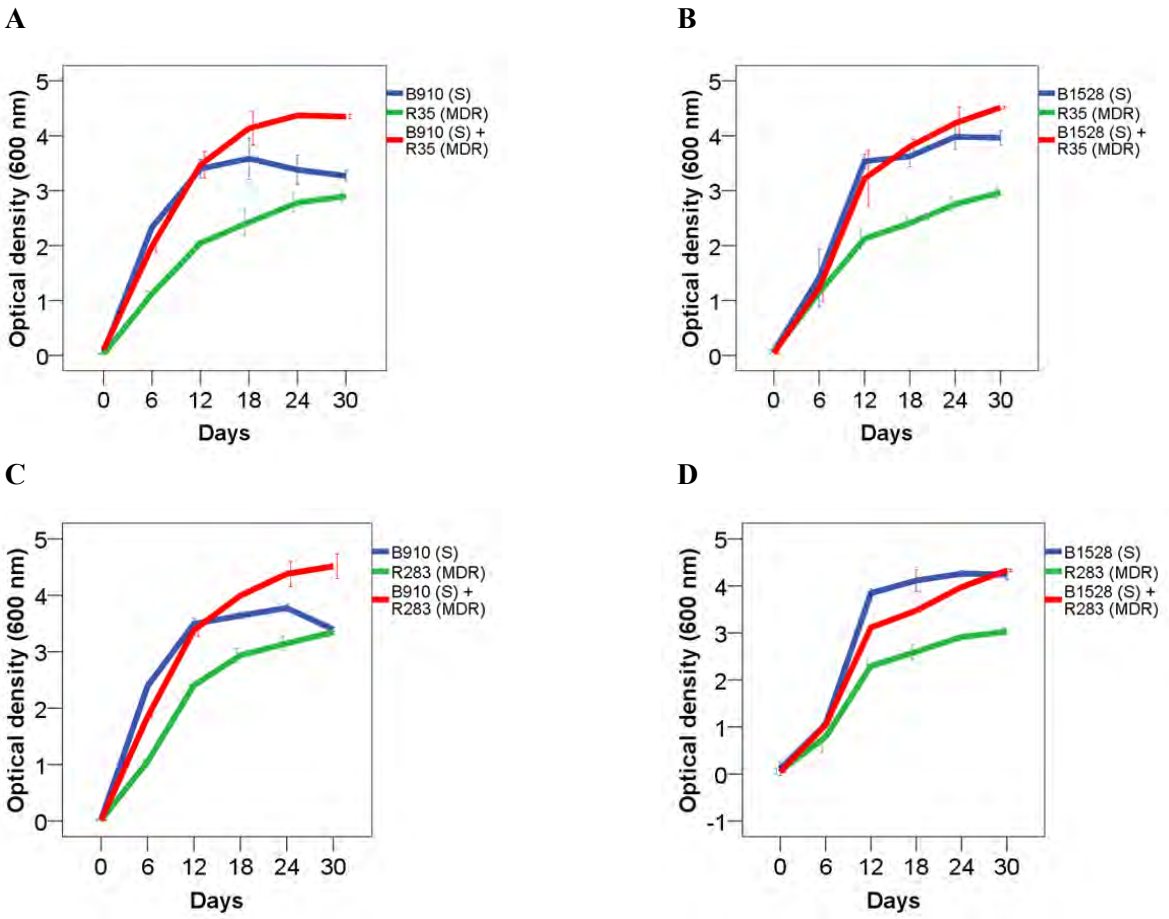


Figure S2.4 Optical density measurements for susceptible strains paired with Beijing MDR strains R35 (A) and R283 (B) in competitive growth assays. Data represents the mean ± standard deviation of duplicate experiments. S: susceptible; MDR: multidrug-resistant.

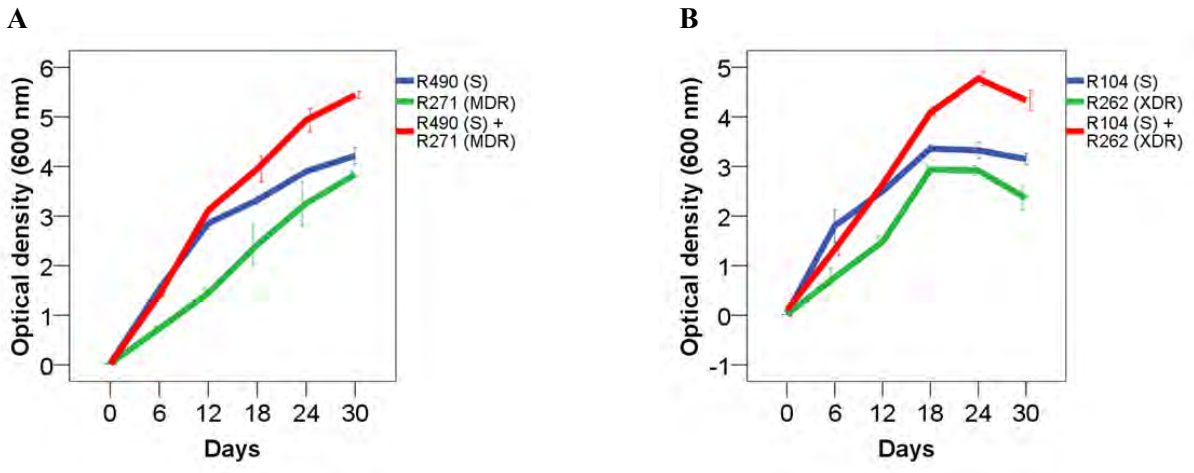


Figure S2.5 Optical density measurements for F11 (A) and F28 (B) clinical strains in competitive growth assays. Data represents the mean ± standard deviation of duplicate experiments. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

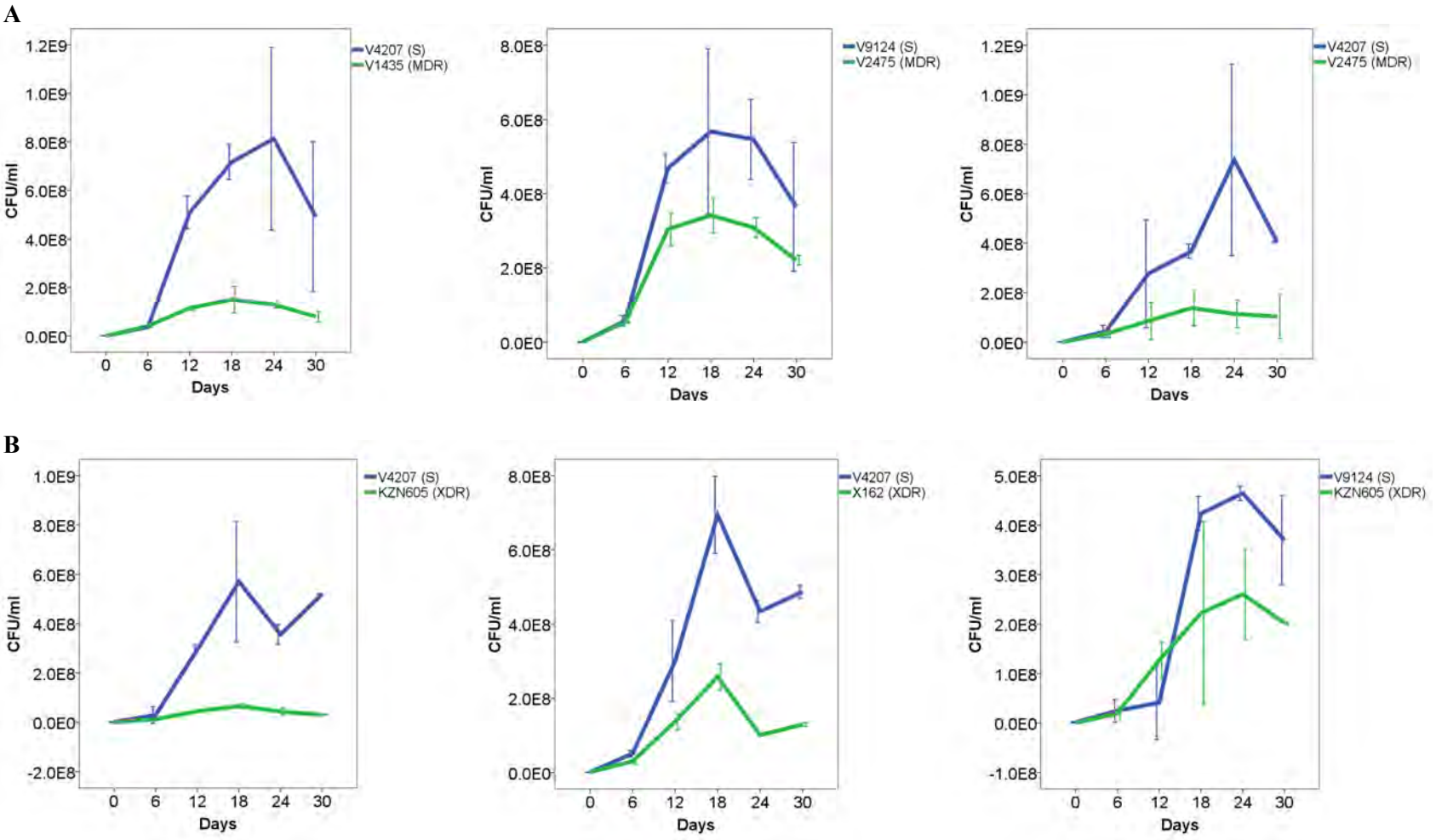
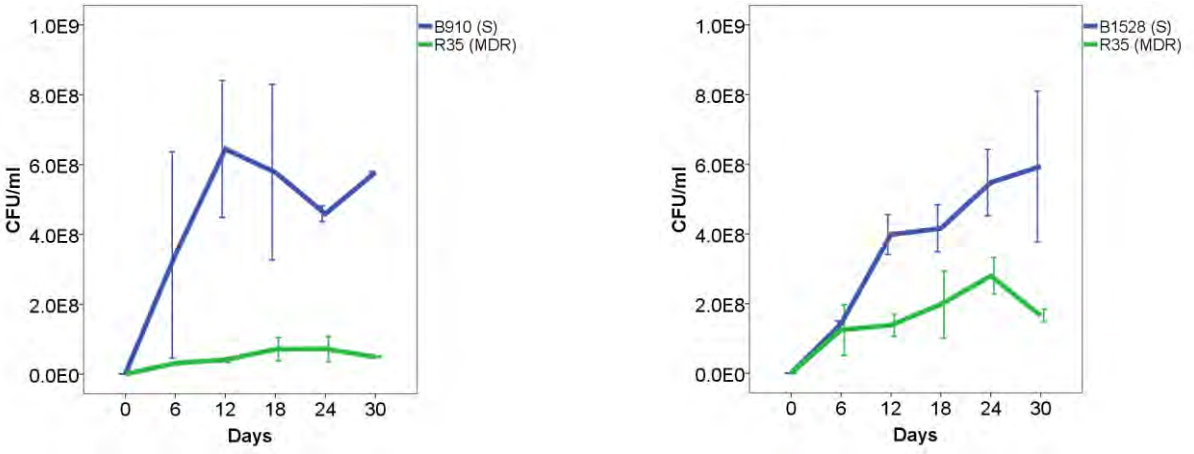


Figure S2.6 Colony forming units counts for susceptible strains paired with MDR (A) and XDR (B) F15/LAM4/KZN strains in competitive growth assays. Data represents the mean ± standard deviation of duplicate experiments. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

A



B

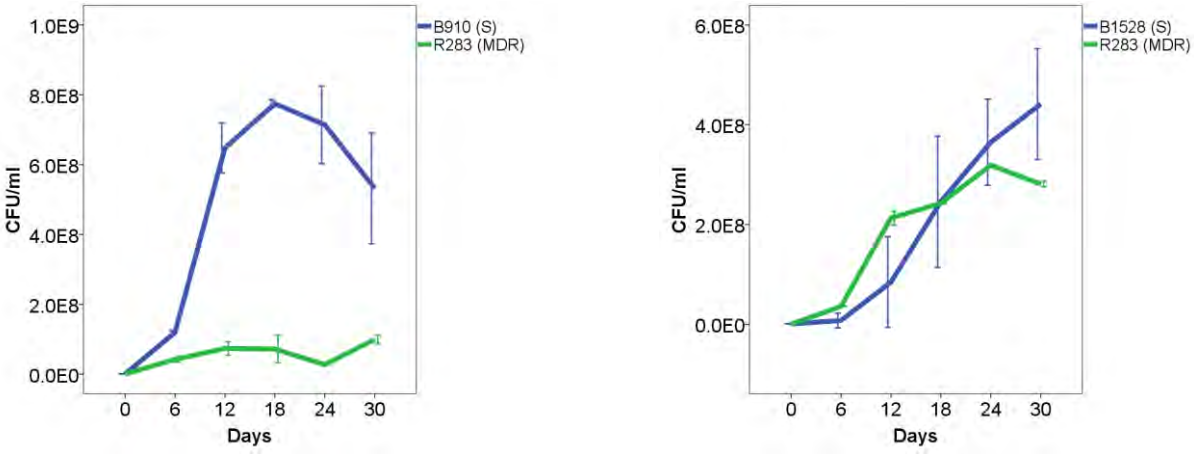


Figure S2.7 Colony forming units counts for Beijing susceptible strains paired with MDR strains R35 (A) and R283 (B) in competitive growth assays. Data represents the mean ± standard deviation of duplicate experiments. S: susceptible; MDR: multidrug-resistant

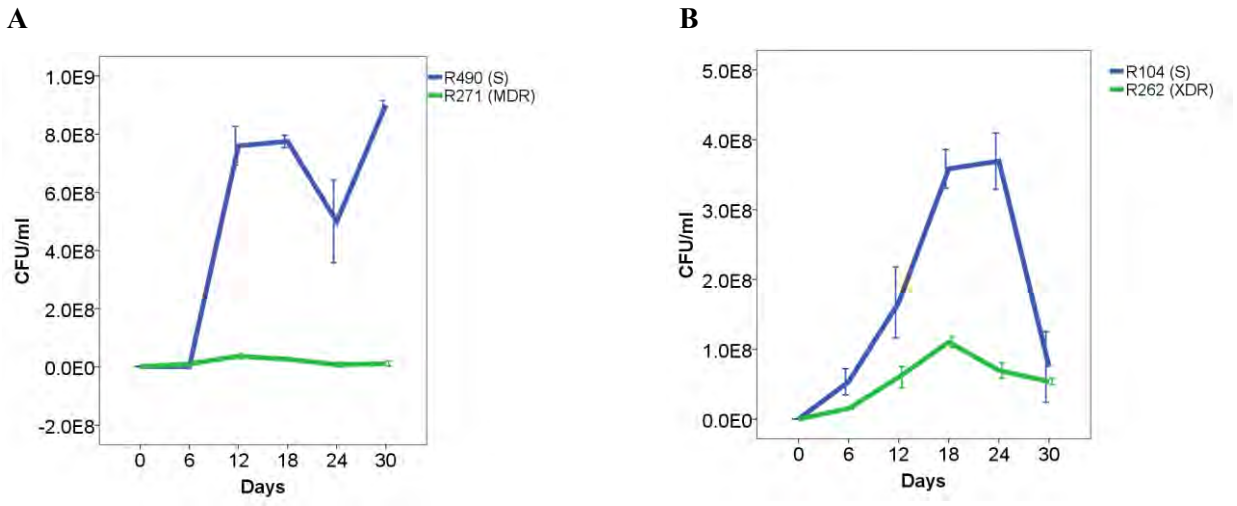


Figure S2.8 Colony forming units counts for F11 (A) and F28 (B) clinical strains in competitive growth assays. Data represents the mean ± standard deviation of duplicate experiments. S: susceptible; MDR: multidrug-resistant; XDR: extensively drug-resistant

Table S3.1 Oligonucleotide primers used for SNP verification by Sanger Sequencing

Oligo	5' – 3' sequence	Product size (bp)	Annealing temperature (°C)
katG_L	gccggtcaagaagaagtacg	591	67
katG_R	ctcttcgtcagctcccactc		
rpoB_L[1]	ctgatccaaaaccagatccg	440	64
rpoB_R[1]	tacacgatctcgtcgctaac		
embB_L[2]	cgacgccgtggtgatattcg	863	70
embB_R[2]	ccacgctgggaattcgcttg		
pncA_L[3]	ggcgtcatggaccctatatc	670	64
pncA_R[3]	caacagttcatcccggttc		
rpoC_L	agaacatcaagagcgccaag	223	66
rpoC_R	atctggtcaccgtcgaagtc		
sigA_L	gcagatacgacgcactgaaa	947	66
sigA_R	ggctagctcgacctcttcct		
rpoB_L	agctgagccaattcatggac	239	65
rpoB_R	cgtttcgatgaacccgaac		

[1]Ao W, Aldous S, Woodruf E, Hicke B, Rea L, Kreiswirth B, Jenison R. Rapid detection of *rpoB* gene mutations conferring rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol*. 2012; 50: 2433-2440. [2]Bakuła Z, Napiórkowska A, Bielecki J *et al*. Mutations in the *embB* gene and their association with ethambutol resistance in multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from Poland. *Biomed Res Int* 2013; *in press*. http://dx.doi.org/10.1155/2013/167954. [3]Alexander DC, Ma JH, Guthrie JL *et al*. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: a role for *pncA* but not *rpsA*. *J Clin Microbiol* 2012; 50:3726-3728.

Table S3.2 Summary statistics clinical strains mapped to closely-related reference genomes

	V9124 [a]	V1435 [a]	KZN605 [a]	X162 [a]	B910 [b]	R35 [b]	R490 [c]	R271 [c]
Mapped Reads	1,830,877	937,477	999,617	1,055,628	899,721	2,018,291	1,406,634	2,681,225
% of total reads	98.88	98.55	98.94	99.10	98.19	98.75	97.70	98.41
Average read length	265.15	232.95	225.41	235.45	230.76	243.35	188.51	193.25
Unmapped Reads	20,759	13,837	10,689	9,542	16,555	25,461	33,112	43,371
% of total reads	1.12	1.45	1.06	0.90	1.81	1.25	2.30	1.59
Average read length	285.21	268.05	278.23	282.43	261.55	274.64	198.23	207.32

[a] Mapped to the KZN-V4207 reference genome (4,394,985 bp); [b] mapped to the HN878 reference genome (4,404,672 bp); [c] mapped to the F11 reference genome (4,424,435 bp).

Table S3.3 Summary statistics for clinical strains mapped to H37Rv reference genome (4,411,532 bp)

	V9124	V1435	KZN605	X162	B910	R35	R490	R271	R104	R443
Mapped Reads	1,827,320	935,638	997,781	1,053,750	901,327	2,018,291	1,401,910	2,672,172	749,682	765,817
% of total reads	98.69	98.35	98.76	98.93	98.37	98.75	97.37	98.08	98.53	98.24
Average read length	265.15	232.93	225.40	235.44	230.88	243.39	188.50	193.23	228.92	234.04
Unmapped Reads	24,316	15,676	12,525	11,420	14,949	25,461	37,836	52,424	11,196	13,749
% of total reads	1.31	1.65	1.24	1.07	1.63	1.25	2.63	1.92	1.47	1.76
Average read length	282.44	265.20	270.97	275.33	258.15	271.56	197.38	205.96	253.78	263.66

Table S3.4 Polymorphisms in re-sequenced V1435 relative to previously sequenced KZN 1435

Gene	Name	Function	Coding region change	Amino acid change
TBMG_00302		predicted protein	1701_1702 ins G	P568fs
TBMG_00540	*menA*	1,4-dihydroxy-2-naphthoate octaprenyltransferase	701_702 ins C	A234fs
TBMG_00782		conserved hypothetical protein	547 del C	H183fs
TBMG_00937		transferase	1139_1140 ins C	T380fs
TBMG_01038	*ppsD*	phenolpthiocerol synthesis type-I polyketide synthase	3823 del A	I1275fs
TBMG_01735	*kasA*	3-oxoacyl-[acyl-carrier protein] synthase 1	1001_1002 ins C	A334fs
TBMG_02215	*malQ*	4-alpha-glucanotransferase	1342 del C	P448fs
TBMG_02275	*folP2*	dihydropteroate synthase 2	632 del C	P211fs
TBMG_04073		conserved hypothetical protein	809_810 ins G	A270fs
TBMG_04086	*trcS*	two component system sensor histidine kinase	873_874 ins C	P292fs
TBMG_03038		conserved hypothetical protein	26delG	R9fs
TBMG_03046		monooxygenase	65_66 ins C	R22fs
TBMG_04096		monooxygenase	1173 del C	P391fs
TBMG_03128		conserved hypothetical protein	1378_1379 ins C	A460fs
TBMG_03778		conserved hypothetical protein	468 del G	G156fs

Del, deletion; ins, insertion; fs, frameshift mutation

Table S3.5 Polymorphisms in re-sequenced KZN605 relative to previously sequenced KZN 605

Gene	Function	Nucleotide change	Amino acid change
TBXG_000209	Conserved hypothetical protein	62delG	G21fs
TBXG_001385	Conserved hypothetical protein	863delT	F288fs
TBXG_001918	rifB protein	425delA	E1419fs
TBXG_002105	Conserved hypothetical protein	293delG	R98fs
TBXG_002696	Conserved hypothetical protein	552delA	K184fs
TBXG_003461	Conserved hypothetical protein	799C→T	A267T
TBXG_003898	Conserved hypothetical protein	445→G	N149H

Del, deletion; fs, frameshift mutation

A **B**

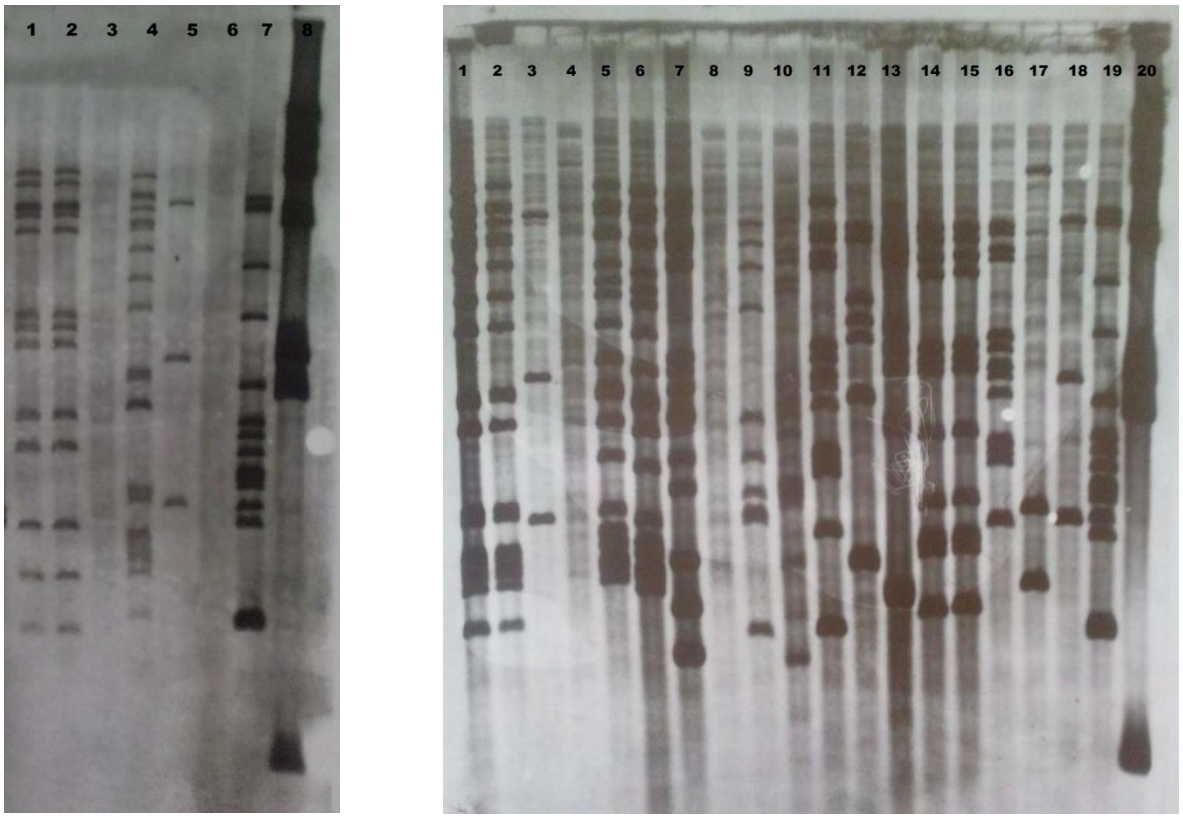


Figure S5.1 IS*6110*-RFLP images for patient isolates. (A) Lanes 1-2: F15/LAM4/KZN isolates 74684 and 11370, respectively; lane 7: H37Rv control; lane 8: lambda DNA cut with *Hin*DIII. (B) Lanes 1-2: Beijing isolates 066069 and 102570, respectively; lane 3 and 18: X3 isolates 05129 and 73078, respectively; lane 19: H37Rv control; lane 20: lambda DNA cut with *Hin*DIII.

REFERENCES

1. Achtman M. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu Rev Microbiol. 2008; 62: 53-70.
2. Ahmad S, Jaber AA, Mokaddas E. Frequency of *embB* codon 306 mutations in ethambutol-susceptible and -resistant clinical *Mycobacterium tuberculosis* isolates in Kuwait. Tuberculosis (Edinb.) 2007; 87: 123–129.
3. Alangaden, GJ, Kreiswirth, BN, Aouad A, Khetarpal M, Igno FR, Moghazeh SL et al. Mechanism of resistance to amikacin and kanamycin in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 1998, 42, 1295–1297.
4. Alexander DC, Ma JH, Guthrie JL, Blair J, Chedore P, Jamieson FB. Gene sequencing for routine verification of pyrazinamide resistance in *Mycobacterium tuberculosis*: A role for *pncA* but not *rpsA*. J. Clin. Microbiol. 2012; 50: 3726–3728.
5. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? Nat Rev Microbiol. 2010; 8: 260–271.
6. Andersson DI, Hughes D. Effects of Antibiotic Resistance on Bacterial Fitness, Virulence, and Transmission. In Evolutionary Biology of Bacterial and Fungal Pathogens; Baquero, F., Nombela, C., Cassell, G.H., Gutiérrez-Fuentes, J.A., Eds.; ASM Press: Washington, DC, USA, 2007; pp. 307–318.
7. Ando H, Kitao T, Miyoshi-Akiyama T, Kato S, Mori T, Kirikae T. Downregulation of *katG* expression is associated with isoniazid resistance in *Mycobacterium tuberculosis*. Mol. Microbiol. 2011, 79, 1615–1628.
8. Ando H, Miyoshi-Akiyama T, Watanabe S, Kirikae T. A silent mutation in *mabA* confers isoniazid resistance on *Mycobacterium tuberculosis*. Mol Microbiol 2014; 91: 538-47.
9. Andrews JR, Gandhi NR, Moodley P, Shah NS, Bohlken L, Moll AP et al. Exogenous reinfection as a cause of multidrug-resistant and extensively drug-resistant tuberculosis in rural South Africa. J Infect Dis. 2008; 198:1582-9
10. Ashiru OT, Pillay M, Sturm AW. Adhesion to and invasion of pulmonary epithelial cells by the F15/LAM4/KZN and Beijing strains of *Mycobacterium tuberculosis*. J Med Microbiol. 2010; 59: 528-33.
11. Ashiru OT, Sturm AW. Cytotoxicity induction in A549 alveolar epithelial cells by *Mycobacterium tuberculosis* isolates cultured in the presence and absence of oxygen. J Basic and Applied Sciences. 2015; 11: 118-124

12. Aubry A, Pan XS, Fisher LM, Jarlier V, Cambau, E. *Mycobacterium tuberculosis* DNA gyrase: Interaction with quinolones and correlation with antimycobacterial drug activity. Antimicrob. Agents Chemother. 2004, 48, 1281–1288.
13. Balganesh M, Dinesh N, Sharma S, Kuruppath S, Nair AV, Sharma U. Efflux Pumps of *Mycobacterium tuberculosis* Play a Significant Role in Antituberculosis Activity of Potential Drug Candidates. Antimicrob. Agents Chemother. 2012; 56: 2643-2651.
14. Banerjee A, Dubnau E, Quemard A, Balasubramanian V, Um KS, Wilson T et al. *InhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. Science 1994; 263: 227–230.
15. Banerjee R, Schecter GF, Flood J, Porco TC. Extensively drug-resistant tuberculosis: new strains, new challenges. Expert Rev Anti Infect Ther. 2008; 6: 713-724.
16. Barnes PF, Cave M Donald. Molecular epidemiology of tuberculosis. N Engl J Med 2009; 349: 1149-1156.
17. Bhatter P, Mistry N. Fitness of acquired drug-resistant *Mycobacterium tuberculosis* isolates from DOTS compliant patients. Tuberculosis. 2013; 93: 418-424
18. Bifani PJ, Mathema B, Kurepina NE, Kreiswirth BN. Global dissemination of the *Mycobacterium tuberculosis* W-Beijing family strains. Trends Microbiol. 2002; 10: 45-52.
19. Billington OJ, McHugh TD, Gillespie SH. Physiological cost of rifampin resistance induced in vitro in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 1999; 43: 1866-1869.
20. Bjorkholm B, Sjolund M, Falk PG, Berg OG, Engstrand L, Andersson DI. Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. Proc. Natl. Acad. Sci. USA. 2001; 98:14607-14612.
21. Bjorkman J, Andersson DI. The cost of antibiotic resistance from a bacterial perspective. Drug Resistance Updates. 2000; 3: 237-245.
22. Bjorkman J, Andersson DI. The cost of antibiotic resistance from a bacterial perspective. Drug Resistance Updates. 2000; 3: 237-245.
23. Black PA, de Vos M, Louw GE, van der Merwe RG, Dippenaar A, Streicher EM et al. Whole genome sequencing reveals genomic heterogeneity and antibiotic purification in *Mycobacterium tuberculosis* isolates. BMC Genomics 2015; 16: 857.
24. Blanchard JS. Molecular mechanisms of drug resistance in *Mycobacterium tuberculosis*. Annu. Rev. Biochem. 1996; 65: 215–239.

25. Blower SM, Chou T. Modeling the emergence of the 'hot zones': tuberculosis and the amplification dynamics of drug resistance. Nat Med. 2004; 10: 1111-1116.
26. Borrell S, Teo Y, Giardina F, Streicher EM, Klopper M, Feldmann J et al. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. Evolution, Medicine, and Public Health. 2013; 1:65–74
27. Bottger EC, Springer B, Pletschette M, Sander P. Fitness of antibiotic-resistant microorganisms and compensatory mutations. Nature Medicine. 1998; 4: 1343-1344.
28. Bottger EC, Springer B. Tuberculosis: drug resistance, fitness, and strategies for global control. Eur J Pediatr. 2007; 167: 141–148.
29. Brandis G, Hughes D. Genetic characterization of compensatory evolution in strains carrying *rpoB* Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates. J Antimicrob Chemother. 2013; 68: 2493-7.
30. Brown T, Nikolayevskyy V, Velji P, Drobniewski F. Associations between *Mycobacterium tuberculosis* strains and phenotypes. Emerg Infect Dis. 2010; 16: 272-280.
31. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM et al. Molecular detection of mutations associated with first-and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 2011; 55: 2032–2041.
32. Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I et al. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet 2014; 46: 279-286.
33. Caws M, Duy PM, Tho DQ, Lan NT, Hoa DV, Farrar J. Mutations prevalent among rifampin and isoniazid-resistant *Mycobacterium tuberculosis* isolates from a hospital in Vietnam. J. Clin. Microbiol. 2006; 44: 2333–2337.
34. Centers for Disease Control and Prevention. 2006. Emergence of *Mycobacterium tuberculosis* with extensive resistance to second-line drugs worldwide, 2000 –2004. MMWR Morb Mortal Wkly Rep 55:301–305.
35. Cheng AF, Yew WW, Chan EW, Chin ML, Hui MM, Chan RC. Multiplex PCR amplimer conformation analysis for rapid detection of *gyrA* mutations in fluoroquinolone-resistant *Mycobacterium tuberculosis* clinical isolates. Antimicrob. Agents Chemother. 2004; 48: 596–601.

36. Chihota VN, Müller B, Mlambo CK, Pillay M, Tait M, Streicher EM et al. The population structure of multi- and extensively drug-resistant tuberculosis in South Africa. J Clin Microbiol. 2012; 50: 995-1002.
37. Chindelevitch L, Menziers NA, Pretorius C, Stover J, Salomon JA, Cohen T. Evaluating the potential impact of enhancing HIV treatment and tuberculosis control programmes on the burden of tuberculosis. J R Soc Interface. 2015; 12: 1-14.
38. Choudhuri BS, Bhakta S, Barik R, Basu J, Kundu M, Chakrabarti P. Overexpression and functional characterization of an ABC (ATP-binding cassette) transporter encoded by the genes *drrA* and *drrB* of *Mycobacterium tuberculosis*. Biochem J. 2002. 367:279-85
39. Cohan FM, King EC, Zawadzki P. Amelioration of the deleterious pleiotropic effects of an adaptive mutation in *Bacillus subtilis*. Evolution. 1994; 48: 81-95.
40. Cohen KA, Abeel T, McGuire AM et al. Evolution of Extensively Drug-Resistant Tuberculosis over Four Decades: Whole Genome Sequencing and Dating Analysis of *Mycobacterium tuberculosis* Isolates from KwaZulu-Natal. PLoS Medicine. 2015; 12: 1-22.
41. Cohen T, Murray M. Modeling epidemics of multidrug-resistant *M. tuberculosis* of heterogenous fitness. Nat Med. 2004; 10: 1117 – 1121.
42. Cohen T, Wilson D, Wallengren K, Samuel EY, Murray M. Mixed-Strain Mycobacterium tuberculosis Infections among Patients Dying in a Hospital in KwaZulu-Natal, South Africa. Journal of Clinical Microbiology. 2011; 49:385–8.
43. Collins EM, Kawakami RP, De Lisle GW, Pascopella L, Bloom BR, Jacobs Jr, WR. Mutation of the principal σ factor causes loss of virulence in a strain of the *Mycobacterium tuberculosis* complex. Proc Natl Acad Sci USA. 1995; 92: 8036-8040.
44. Comas I, Borrell S, Roetzer A, Rose G, Malla B, Kato-Maeda M et al,. Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. Nat. Genet. 2012; 44: 106–110.
45. Comas I, Gagneux S. A role for systems epidemiology in tuberculosis research. Trends Microbiol. 2011; 19: 492–500.
46. Comas I, Homolka S, Niemann S, Gagneux S. Genotyping of genetically monomorphic bacteria: DNA sequencing in *Mycobacterium tuberculosis* highlights the limitations of current methodologies. PLoS ONE 2009; 4:e7815.
47. Cooper AM, Khader SA. The role of cytokines in the initiation, expansion, and control of cellular immunity to tuberculosis. Immunol Rev 2008; 226: 191-204.

48. Cooper AM, Mayer-Barber KD, Sher A. Role of innate cytokines in mycobacterial infection. Mucosal Immunol 2011; 4: 252-260.
49. Coscolla M, Gagneux S. Consequences of genomic diversity in *Mycobacterium tuberculosis.* Seminars in Immunology. 2014; 26: 431-444.
50. Cox HS, McDermid C, Azevedo V, Muller O, Coetzee D, et al. Epidemic levels of drug resistant tuberculosis (MDR and XDR-TB) in a high HIV prevalence setting in Khayelitsha, South Africa. PLoS One 2010; 5: e13901.
51. Dao DN, Sweeney K, Hsu T, Gurcha SS, Nascimento IP, Roshevsky D, et al. Mycolic Acid Modification by the *mmaA4* Gene of *M. tuberculosis* Modulates IL-12 Production. PLoS Pathog. 2008; 4: e1000081
52. de Vos M, Müller B, Borrell S, Black PA, van Helden PD, Warren RM, et al. Putative compensatory mutations in the *rpoC* gene of rifampin resistant *Mycobacterium tuberculosis* are associated with ongoing transmission. Antimicrob Agents Chemother 2013; 57:827-832.
53. Dheda K, Gumbo T, Gandhi NR et al. Global control of tuberculosis: from extensively drug-resistant to untreatable tuberculosis. Lancet Respir Med. 2014; 14: 1-18
54. Escribano I, Rodríguez JC, Llorca B, García-Pachon E, Ruiz M, Royo G. Importance of the efflux pump systems in the resistance of *Mycobacterium tuberculosis* to fluoroquinolones and linezolid. Chemotherapy 2007; 53: 397–401.
55. Fenner L, Egger M, Bodmer T, Altpeter E, Zwahlen M, Jaton K, et al. Effect of mutation and genetic background on drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2012; 56: 3047–3053.
56. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, et al. An essential role for interferon gamma in resistance to *Mycobacterium tuberculosis* infection. J Exp Med 1993; 178: 2249-2254.
57. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, et al. Tumor necrosis factor-alpha is required in the protective immune response against *Mycobacterium tuberculosis* in mice. Immunity 1995; 2: 561-572.
58. Ford C, Yusim K, Ioerger T, Feng S, Chase, Greene M, et al. *Mycobacterium tuberculosis* – heterogeneity revealed through whole genome sequencing. Tuberculosis. 2012; 92: 194-201.
59. Gagneux S, DeRiemer K, Van T, Kato-Maeda M, de Jong BC, Narayanan S, et al. Variable host–pathogen compatibility in *Mycobacterium tuberculosis*. Proc Natl Acad Sci USA 2006; 103:2869–73

60. Gagneux S, Long CD, Small PM, Van T, Schoolnik GK, Bohannan BJ. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science. 2006; 312: 1944-1946.
61. Gagneux S, Small PM. Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. Lancet Infect Dis. 2007; 7: 328-337.
62. Gandhi NR, Brust JCM, Moodley P, Weissman D, Heo M, Ning Y, et al. Minimal Diversity of Drug-Resistant *Mycobacterium tuberculosis* Strains, South Africa. 2014; 20: 426-433
63. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, Lalloo U, et al. Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. The Lancet. 2006; 368: 1575-1580.
64. Garcia de Viedma D, Lorenzo G, Cardona PJ, Rodriguez NA, Gordillo S, Serrano MJ, et al. Association between the infectivity of *Mycobacterium tuberculosis* strains and their efficiency for extrarespiratory infection. J Infect Dis. 2005; 192: 2059-65.
65. Guerra-Assunção JA, Crampin AC, Houben RMGJ et al. Large-scale whole genome sequencing of *M. tuberculosis* provides insights into transmission in a high prevalence area. eLife. 2015; 4: e05166.
66. Gupta AK, Katoch VM, Chauhan DS, Sharma R, Singh M, Venkatesan K, et al. Microarray analysis of efflux pump genes in multidrug-resistant *Mycobacterium tuberculosis* during stress induced by common anti-tuberculous drugs. Microb Drug Resist. 2010; 16: 21–28.
67. Hammer ND, Cassat JE, Noto MJ, Lojek LJ, Chadha AD, Schmitz JE et al. Inter- and intraspecies metabolite exchange promotes virulence of antibiotic-resistant *Staphylococcus aureus*. Cell Host Microbe. 2014; 16:531-7
68. Hazbón MH, Brimacombe M, Bobadilla del Valle M, Cavatore M, Guerrero MI, Varma-Basil M, et al. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 2006; 50: 2640–2649.
69. Heep M, Brandstatter B, Rieger U et al. Frequency of *rpoB* mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. J Clin Microbiol 2001; 39: 107–110.
70. Hirsh AE, Tsolaki AG, DeRiemer K, Feldman MW, Small PM. Stable association between strains of *Mycobacterium tuberculosis* and their human host populations. Proc Natl Acad Sci USA 2004; 101: 4871–6.

71. Johnson R, Warren RM, van der Spuy GD, Gey Van Pittius NC, Theron D, et al. Drug-resistant tuberculosis epidemic in the Western Cape driven by a virulent Beijing genotype strain. Int J Tuberc Lung Dis 2010; 14: 119–121.
72. Juréen P, Werngren J, Toro JC, Hoffner S. Pyrazinamide resistance and *pncA* gene mutations in *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 2008; 52: 1852–1854.
73. Kaufmann SH. Protection against tuberculosis: cytokines, T cells, and macrophages. Ann Rheum Dis. 2002; 6: ii54-58.
74. Konno K, Feldmann FM, McDermott W. Pyrazinamide susceptibility and amidase activity of tubercle bacilli. Am. Rev. Respir. Dis. 1967; 95: 461–469.
75. Larsen MH, Vilchèze C, Kremer L, Besra GS, Parsons L, Salfinger M, et al. Overexpression of *inhA*, but not *kasA*, confers resistance to isoniazid and ethionamide in *Mycobacterium smegmatis*, *M. bovis* BCG and *M. tuberculosis*. Mol. Microbiol. 2002; 46: 453–466.
76. Li Q, Whalen CC, Albert JM, Larkin R, Zukowski L, Cave MD, Silver RF: Differences in rate and variability of intracellular growth of a panel of *Mycobacterium tuberculosis* clinical isolates within a human monocyte model. Infect Immun 2002; 70:6489-6493.
77. Lim JR, Gandhi NR, Mthiyane T, Mlisana K, Moodley J, Jaglal P, et al. Incidence and Geographic Distribution of Extensively Drug-Resistant Tuberculosis in KwaZulu-Natal Province, South Africa. PLoS One. 2015; 10: e0132076.
78. Lin PG, Flynn JL. Understanding latent tuberculosis: a moving target. Journal of Immunology. 2010; 185: 15–22.
79. Lopez B, Aguilar D, Orozco H, Burger M, Espitia C, Ritacco V, et al. A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. Clin Exp Immunol. 2003; 133: 30-37.
80. Machado D, Perdigão J, Ramos J, Couto I, Portugal I, Ritter C et al. High-level resistance to isoniazid and ethionamide in multidrug-resistant *Mycobacterium tuberculosis* of the Lisboa family is associated with *inhA* double mutations. J Antimicrob Chemother. 2013; 68: 1728–1732.
81. Maisnier-Patin S, Andersson DI, Adaptation to the deleterious effects of antimicrobial drug resistance mutations by compensatory evolution. Res Microbiol. 2004; 155: 360-369
82. Manca C, Tsenova L, Barry CE, Bergtold A, Freeman S, Haslett PA, et al. *Mycobacterium tuberculosis* CDC1551 induces a more vigorous host response *in vivo* and *in vitro*, but is not more virulent than other clinical isolates. J Immunol. 1999; 162: 6740-6746.

83. Manca C, Tsenova L, Bergtold A, Freeman S, Tovey M, Musser JM, et al. Virulence of a *Mycobacterium tuberculosis* clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha/beta. Proc Natl Acad Sci USA 2001; 98: 5752-5757.

84. Marais BJ, Victor TC, Hesseling AC, Barnard M, Jordaan A, Brittle W, Reuter H et al. Beijing and Haarlem genotypes are overrepresented among children with drug-resistant tuberculosis in Western Cape Province of South Africa. J Clin Microbiol. 2006; 44: 3539-3543.

85. Mariam DH, Mengistu Y, Hoffner SE, Andersson DI. Effect of *rpoB* mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2004; 48: 1289-1294.

86. Marx FM, Dunbar R, Enarson DA, Williams BG, Warren RM, van der Spuy GD, et al. The temporal dynamics of relapse and reinfection tuberculosis after successful treatment: a retrospective cohort study. Clin Infect Dis. 2014; 58: 1676-1683

87. Mathema B, Kurepina NE, Bifani PJ, Kreiswirth BN. Molecular epidemiology of tuberculosis: current insights. Clin Microbiol Rev 2006; 19: 658–685.

88. Middlebrook G, Cohn ML. Some observations on the pathogenicity of isoniazid-resistant variants of tubercle bacilli. Science 1953; 118: 297–9.

89. Miesel L, Weisbrod TR, Marcinkeviciene JA, Bittman R, Jacobs WR Jr. NADH dehydrogenase defects confer isoniazid resistance and conditional lethality in *Mycobacterium smegmatis*. J. Bacteriol. 1998; 180: 2459–2467.

90. Mikusová K, Slayden RA, Besra GS, Brennan PJ. Biogenesis of the mycobacterial cell wall and the site of action of ethambutol. Antimicrob. Agents Chemother. 1995; 39: 2484–2489.

91. Mitchison DA. The action of antituberculosis drugs in short-course chemotherapy. Tubercle. 1985; 66: 219–225.

92. Mitchison DA. Tubercle bacilli resistant to isoniazid; virulence and response to treatment with isoniazid in guinea-pigs. Br Med J 1954; 1: 128–30.

93. Mostowy S, Cousins D, Behr MA. Genomic interrogation of the dassie bacillus reveals it as a unique RD1 mutant within the *Mycobacterium tuberculosis* complex. J Bacteriol 2004; 186: 104–109.

94. Mostowy S, Onipede A, Gagneux S, Niemann S, Kremer K, Desmond EP, et al. Genomic analysis distinguishes *Mycobacterium africanum*. J Clin Microbiol 2004; 42: 3594–3599.

95. Müller B, Borrell S, Rose G, Gagneux S. The heterogeneous evolution of multidrug-resistant *Mycobacterium tuberculosis*. Trends Genet. 2013; 29:160-169.
96. Müller B, Chihota VN, Pillay M, Klopper M, Streicher EM, Coetzee G, et al. Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa. PLoS One 2013; 8:1-9.
97. Musser, J.M. Antimicrobial agent resistance in mycobacteria: Molecular genetic insights. Clin. Microbiol. Rev. 199; 8: 496–514.
98. Naidoo CC, Pillay M. Increased *in vitro* fitness of multi- and extensively drug-resistant F15/LAM4/KZN strains of *Mycobacterium tuberculosis*. Clin Microbiol Infect. 2014; 20:O361-369.
99. National Health Laboratory Services. National Institute for Communicable Diseases annual report 2009 [cited 2015/11/30]. http://www.nicd.ac.za/assets/files/Annual_report_2009.pdf
100. National Strategic Plan for HIV, STIs and TB (2012 -2016). Date accessed: 07 December 2015. url: www.sahivsoc.org/upload/documents/National_Strategic_Plan_2012.pdf
101. Palomino JC, Martin A. Drug resistance mechanisms in *Mycobacterium tuberculosis*. Antibiotics. 2014; 3: 317-340.
102. Palomino JC, Martin A. Tuberculosis clinical trial update and the current anti-tuberculosis drug portfolio. Curr Med Chem. 2013; 20: 3785–3796.
103. Panjabi R, Comstock GW, Golub JE. Recurrent tuberculosis and its risk factors: adequately treated patients are still at high risk. Int J Tuberc Lung Dis. 2007; 11: 828-837.
104. Pietersen E, Ignatius E, Streicher EM, Mastrapa B, Padanilam X, Pooran A, et al. Long-term outcomes of patients with extensively drug-resistant tuberculosis in South Africa: a cohort study. Lancet. 2014; 383: 1230-1239.
105. Pillay M, Sturm AW. Evolution of the extensively drug-resistant F15/LAM4/KZN strain of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa. Clin Infec Dis. 2007; 45: 1409-1414.
106. Pillay M, Sturm AW. Nosocomial transmission of the F15/LAM4/KZN genotype of *Mycobacterium tuberculosis* in patients on tuberculosis treatment. Int J Tuberc Lung Dis. 2010; 14: 223-30.
107. Ramaswamy S, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. Tuber Lung Dis. 1998; 79: 3–29.

108. Ramón-García S, Martín C, Thompson CJ, Aínsa JA. Role of the *Mycobacterium tuberculosis* P55 efflux pump in intrinsic drug resistance, oxidative stress responses, and growth. Antimicrob Agents Chemother. 2009; 53: 3675-82
109. Raviglione MC, Smith IM. XDR tuberculosis – implications for global public health. N. Engl. J. Med. 2007; 356: 656–659
110. Rawat R, Whitty A, Tonge PJ. The isoniazid-NAD adduct is a slow, tight-binding inhibitor of *InhA*, the *Mycobacterium tuberculosis* enoyl reductase: Adduct affinity and drug resistance. Proc. Natl. Acad. Sci. USA 2003; 100: 13881–13886.
111. Rengarajan J, Bloom BR, Rubin EJ. Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages. Proc Natl Acad Sci USA. 2005; 102: 8327-32
112. Reynolds MG. Compensatory evolution in rifampicin-resistant *Escherichia coli*. Genetics. 2000; 156: 1471-1481.
113. Richardson M, Carroll NM, Engelke E, van der Spuy GD, Salker F, Munch Z, et al. Multiple *Mycobacterium tuberculosis* strains in early cultures from patients in a high-incidence community setting. Journal of Clinical Microbiology. 2002; 40: 2750–4.
114. Royce S, Falzon D, van Weezenbeek C, Dara M, Hyder K, Hopewell P, et al. Multidrug resistance in new tuberculosis patients: burden and implications. Int J Tuberc Lung Dis. 2013; 17: 511–3.
115. Rozwarski DA, Grant GA, Barton DH, Jacobs WR Jr., Sacchettini JC. Modification of the NADH of the isoniazid target (*InhA*) from *Mycobacterium tuberculosis*. Science 1998; 279: 98–102.
116. Rustomjee R, Lienhardt C, Kanyok T, Davies GR, Levin J, Mthiyane T, et al. A Phase II study of the sterilising activities of ofloxacin, gatifloxacin and moxifloxacin in pulmonary tuberculosis. Int. J. Tuberc. Lung Dis. 2008; 12: 128–138.
117. Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, et al. Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes. Nat Genet 2013; 45: 1190-1197.
118. Safi H, Lingaraju S, Amin A, Kim S, Jones M, Holmes M, McNeil M, Peterson SN, Chatterjee D, Fleischmann R, et al. Evolution of high-level ethambutol-resistant tuberculosis through interacting mutations in decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes. Nat. Genet. 2013; 45: 1190–1197.

119. Sander P, Springer B, Prammananan T, Sturmfels A, Kappler M, Pletschette M, et al. Fitness cost of chromosomal drug resistance-conferring mutations. Antimicrob Agents Chemother. 2002; 46: 1204–1211.
120. Sandgren A, Strong M, Muthukrishnan P, Weiner BK, Church GM, Murray MB. Tuberculosis drug resistance mutation database. PLoS Med. 2009; 6: e1000002.
121. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, et al. Transcriptional Adaptation of *Mycobacterium tuberculosis* within Macrophages: Insights into the Phagosomal Environment. J Exp Med. 2003; 198:693-704.
122. Schrag SJ, Perrot V, Levin BR. Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*. Proc R Soc Lond B Biol Sci. 1997; 264: 1287-1291.
123. Scorpio A, Lindholm-Levy P, Heifets L, Gilman R, Siddiqi S, Cynamon M, Zhang Y. Characterization of *pncA* mutations in pyrazinamide-resistant *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. 1997; 41: 540–543.
124. Scorpio A, Zhang Y. Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. Nat. Med. 1996; 2: 662–667.
125. Shcherbakov D, Akbergenov R, Matt T, Sander P, Andersson DI, Bottger EC. Directed mutagenesis of *Mycobacterium smegmatis* 16S rRNA to reconstruct the in vivo evolution of aminglycoside resistance in *Mycobacterium tuberculosis*. Mol Microbiol. 2010; 77: 830 – 840.
126. Sherman DR, Mdluli K, Hickey MJ, Arain TM, Morris SL, Barry CE. 3rd, et al. Compensatory *ahpC* gene expression in isoniazid-resistant *Mycobacterium tuberculosis*. Science. 1996, 272, 1641–1643.
127. Simons SO, Mulder A, van Ingen J, Boeree MJ, van Soolingen D. Role of *rpsA* gene sequencing in diagnosis of pyrazinamide resistance. J. Clin. Microbiol. 2013; 51: 382.
128. Smith KLJ, Saini D, Bardarov S, Larsen M, Frothingham R, Gandhi NR, et al. Reduced virulence of an extensively drug-resistant outbreak strain of *Mycobacterium tuberculosis* in a murine model. PLoS ONE. 2014; 9: e94953
129. Somoskovi A, Parsons LM, Salfinger M. The molecular basis of resistance to isoniazid, rifampin, and pyrazinamide in *Mycobacterium tuberculosis*. Respir Res 2001; 2: 164–168.
130. Sonnenberg P, Murray J, Glynn JR, Shearer S, Kambashi B, Godfrey-Faussett P. HIV-1 and recurrence, relapse, and reinfection of tuberculosis after cure: a cohort study in South African mineworkers. Lancet. 2001; 358: 1687–93.

131. South African Statistics. Mid-year population estimates. 2015 Date accessed: 07 December 2015. url: https://www.statssa.gov.za/publications/P0302/P03022015.pdf.
132. Sreevatsan S, Pan X, Stockbauer KE, Connell ND, Kreiswirth BN, Whittam TS, et al. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc Natl Acad Sci USA 1997; 94: 9869-9874.
133. Sreevatsan S, Stockbauer KE, Pan X, Kreiswirth BN, Moghazeh SL, Jacobs WR Jr, Telenti A, Musser JM. Ethambutol resistance in *Mycobacterium tuberculosis*: Critical role of *embB* mutations. Antimicrob. Agents Chemother. 1997; 41: 1677–1681.
134. Strauss OJ, Warren RM, Jordaan A, Streicher EM, Hanekom M, Falmer AA, et al. Spread of a low-fitness drug-resistant *Mycobacterium tuberculosis* strain in a setting of high human immunodeficiency virus prevalence. J Clin Microbiol 2008; 46: 1514–1516.
135. Sun Z, Zhang J, Zhang X, Wang S, Zhang Y, Li C. Comparison of *gyrA* gene mutations between laboratory-selected ofloxacin-resistant *Mycobacterium tuberculosis* strains and clinical isolates. Int. J. Antimicrob. Agents 2008; 31: 115–121.
136. Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D, Locht C. Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. J Clin Microbiol 2001; 39: 3563–3571.
137. Supply P, Mazars E, Lesjean S, Vincent V, Gicguel B, Locht C. Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. Mol Microbiol 2000; 36: 762–771.
138. Suzuki Y, Katsukawa C, Tamaru A, Abe C, Makino M, Mizuguchi Y, Taniguchi H. Detection of kanamycin-resistant *Mycobacterium tuberculosis* by identifying mutations in the 16S rRNA gene. J Clin Microbiol 1998; 36; 1220–1225.
139. Takayama K, Kilburn JO. Inhibition of synthesis of arabinogalactan by ethambutol in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. 1989; 33: 1493–1499.
140. Takiff HE, Feo O. Clinical value of whole-genome sequencing of *Mycobacterium tuberculosis*. Lancet Infect Dis. 2015; 15:1077-90
141. Takiff HE, Salazar L, Guerrero C, Philipp W, Huang WM, Kreiswirth B, et al. Cloning and nucleotide sequence of *Mycobacterium tuberculosis* gyrA and gyrB genes and detection of quinolone resistance mutations. Antimicrob. Agents Chemother. 1994; 38: 773–780

142. Tan Y, Hu Z, Zhang T, Cai X, Kuang H, Liu Y, Chen J, Yang F, Zhang K, Tan S et al. Role of *pncA* and *rpsA* gene sequencing in detection of pyrazinamide resistance in *Mycobacterium tuberculosis* isolates from southern China. J. Clin. Microbiol. 2014; 52: 291–297.
143. Telenti A, Philipp WJ, Sreevatsan S, Bernasconi C, Stockbauer KE, Wieles B, Musser JM, Jacobs WR Jr. The *emb* operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. Nat. Med. 1997; 3: 567–570.
144. Theus SA, Cave MD, Eisenach KD. Intracellular macrophage growth rates and cytokine profiles of *Mycobacterium tuberculosis* strains with different transmission dynamics. J Infect Dis. 2005; 191:453-60.
145. Trauner A, Borrell S, Reither K, Gagneux S. Evolution of drug resistance in tuberculosis: recent progress and implications for diagnosis and therapy. Drugs. 2014; 74: 1063-1072.
146. Trindade S, Sousa A, Xavier KB, Dionisio F, Ferreira MG, Gordo I. Positive epistasis drives the acquisition of multidrug resistance. PLoS Genet. 2009; 5: e1000578.
147. Tsenova L, Ellison E, Harbacheuski R, Moreira AL, Kurepina N, Reed MB, et al. Virulence of selected *Mycobacterium tuberculosis* clinical isolates in the rabbit model of meningitis is dependent on phenolic glycolipid produced by the bacilli. J Infect Dis 2005; 192: 98-106.
148. Tsolaki AG, Hirsh AE, DeRiemer K, Enciso JA, Wong MZ, Hannan M, et al. Functional and evolutionary genomics of *Mycobacterium tuberculosis*: insights from genomic deletions in 100 strains. Proc Natl Acad Sci USA 2004; 101: 4865–70.
149. Tuberculosis Trials Consortium. Once-weekly rifapentine and isoniazid versus twice-weekly rifampin and isoniazid in the continuation phase of therapy for drug-susceptible pulmonary tuberculosis: a prospective, randomized clinical trial among HIV-negative persons. Lancet. 2002; 360: 528–34.
150. van Rie A, Victor TC, Richardson M, Johnson R, van der Spuy GD, Murray EJ, et al. Reinfection and mixed infection cause changing *Mycobacterium tuberculosis* drug-resistance patterns. Am J Respir Crit Care Med. 2005; 172: 636-642.
151. van Rie A, Warren RM, Beyers N, Gie RP, Classen CN, Richardson M, et al. Transmission of a multidrug-resistant *Mycobacterium tuberculosis* strain resembling ‘‘strain W’’ among noninstitutionalized, human immunodeficiency virusseronegative patients. J Infect Dis 1999; 180: 1608–1615.
152. van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of east Asia. J Clin Microbiol 1995; 33: 3234-3238.

153. van Soolingen D. Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements. J Int Med 2001; 249: 1–26.
154. Verver S, Warren RM, Beyers N, Richardson M, van der Spuy GD, Borgdorff MW, et al. Rate of reinfection tuberculosis after successful treatment is higher than rate of new tuberculosis. Am J Respir Crit Care Med. 2005; 171: 1430–5.
155. Victor TC, de Haas PEW, Jordaan AM, van der Spuy GD, Richardson M, van Soolingen D, et al. Molecular characteristics and global spread of *Mycobacterium tuberculosis* with a Western Cape F11 genotype. J Clin Microbiol. 2004; 42: 769-772.
156. Victor TC, Streicher EM, Kewley C, Jordaan AM, van der Spuy GD, Bosman M, et al. Spread of an emerging *Mycobacterium tuberculosis* drug-resistant strain in the western Cape of South Africa. Int J Tuberc Lung Dis. 2007; 11: 195–201.
157. Victor TC, van Rie A, Jordaan AM, Richardson M, van der Spuy GD, Beyers N, et al. Sequence Polymorphism in the *rrs* Gene of *Mycobacterium tuberculosis* Is Deeply Rooted within an Evolutionary Clade and Is Not Associated with Streptomycin Resistance. J Clin Microbiol. 2001; 39: 4184-4186.
158. Vilchèze C, Jacobs WR Jr. The mechanism of isoniazid killing: Clarity through the scope of genetics. Annu. Rev. Microbiol. 2007; 61: 35–50.
159. Vilchèze C, Weisbrod TR, Chen B, Kremer L, Hazbón MH, Wang F, et al. Altered NADH/NAD+ ratio mediates coresistance to isoniazid and ethionamide in mycobacteria. Antimicrob. Agents Chemother. 2005; 49: 708–720.
160. Warren RM, Victor TC, Streicher EM, Richardson M, Beyers N, Gey van Pittius NC, et al. Patients with active tuberculosis often have different strains in the same sputum specimen. Am J Respir Crit Care Med. 2004; 169: 610-614.
161. Weinrech DM, Watsson RA, Chao L. Perspective: sign epistasis and genetic constraint on evolutionary trajectories. Evolution. 2005; 8: 260-271.
162. Weis SE, Slocum PC, Blais FX, King B, Nunn M, Matney GB, et al. The effect of directly observed therapy on the rates of drug resistance and relapse in tuberculosis. N Engl J Med. 1994; 330: 1179–84.
163. WHO Global Tuberculosis Report 2015 http://www.who.int/tb/publications/global_report/en/
164. Yuen KY, Yam WC. Mutations outside the rifampicin resistance-determining region associated with rifampicin resistance in *Mycobacterium tuberculosis*. J Antimicrob Chemother. 2011; 66: 730–733.

165. Zaunbrecher MA, Sikes RD Jr., Metchock B, Shinnick TM, Posey JE. Overexpression of the chromosomally encoded aminoglycoside acetyltransferase eis confers kanamycin resistance in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA 2009; 106: 20004–20009.
166. Zhang H, Li D, Zhao L, Fleming J, Lin N, Wang T, et al. Genome sequencing of 161 *Mycobacterium tuberculosis* isolates from China identifies genes and intergenic regions associated with drug resistance. Nat Genet. 2013; 45: 1255–1260.
167. Zhang M, Gong J, Yang Z, Samten B, Cave MD, Barnes PF: Enhanced capacity of a widespread strain of *Mycobacterium tuberculosis* to grow in human macrophages. J Infect Dis 1999; 179: 1213-1217
168. Zhang Y, Heym B, Allen B, Young D, Cole S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. Nature 1992; 358: 591–593.
169. Zhang Y, Mitchison D. The curious characteristics of pyrazinamide: A review. Int. J. Tuberc. Lung Dis. 2003; 7: 6–21.
170. Zur Wiesch PA, Kouyos R, Engelstadter J, Regoes RR, Bonhoeffer S. Population biological properties of drug-resistance evolution in infectious diseases. Lancet Infect Dis. 2011; 11: 236-247.