Molecular characterization of the drug resistant tuberculosis epidemic in the Eastern Cape, South Africa

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DECLARATION

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

South Africa has a high burden of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB), with the Eastern Cape (EC) being one of the worst affected provinces in the country. This study provides the first in-depth analysis of the molecular epidemiology of drug-resistant TB in the EC.

A convenience sample of drug-sensitive and drug-resistant isolates was collected over one year by the National Health Laboratory Services in Port Elizabeth. These isolates were characterized by various molecular techniques. Our results were compared to data from three additional provinces, to contextualise the population structure of MDR-TB strains. Each province had a distinct population structure. The population structure of XDR-TB cases in the Western Cape was significantly influenced by strains originating from the EC. A high degree of clustering of drug resistance mutation patterns was detected in each setting, suggestive of transmission. Clustering was particularly pronounced in the EC, with 93% of pre-XDR and XDR-TB isolates belonging to the Atypical Beijing genotype. We showed that this genotype was programmatically selected through a weakened MDR-TB regimen that failed to recognise *inhA* defined ethionamide resistance. This weakened regimen has facilitated transmission and is the underlying cause of mortality. We propose that existing molecular assays which detect *inhA* mutations should be used to identify patients at risk of XDR-TB and to adjust treatment.

Through spoligotyping, restriction fragment length polymorphism typing and mutation analysis we demonstrated that the EC Atypical Beijing isolates evolved from a common progenitor, giving rise to two sub-groups, each with unique features, including mutations that confer resistance to up to 11 anti-TB drugs. This finding was supported by whole genome sequencing (WGS) and RNA sequencing demonstrating close relatedness and suggests the emergence and spread of totally drug-resistant TB in the EC.

We showed that isolates harbouring the *rrs* A1401G mutation displayed a decreased susceptibility to capreomycin, thereby questioning the utility of this drug in the treatment of XDR-TB when amikacin resistance was already noted. Importantly, strains harbouring the *rpoB*516 mutation were shown to be susceptible to rifabutin, despite low-level resistance to rifampicin (RIF). Therefore the use of rifabutin in the EC may improve therapeutic success and limit transmission of XDR-TB.

WGS was used to investigate molecular features that may confer a selective advantage to the EC Atypical Beijing genotype strains. These analyses revealed that all represented Atypical Beijing genotype strains – including those diagnosed as pan-susceptible – harboured a mutation in *ethA*,

conferring phenotypic ethionamide resistance. This surprising finding may explain the apparent increased ability of the Atypical Beijing genotype strains to develop higher drug-resistance in the context of an ethionamide-containing MDR-TB treatment regimen. It is unclear why some strains additionally acquire *inhA* promoter mutations. This requires further investigation.

A large number of genes were shown by RNAseq to be differentially regulated, however, their influence on the physiological properties of the bacillus remain to be determined.

Together these findings have challenged the use of standardised MDR-TB treatment without comprehensive DST. This view is now widely recognised but has not influenced the South African TB guidelines (2014) which promote treatment of RIF resistance without relevant knowledge of drug resistance. We propose that the effective treatment of highly resistant TB can only be achieved with the development of new drugs, new drug combinations and comprehensive rapid DST.

Opsomming

Suid-Afrika het ‘n hoë voorkoms van multi-middelweerstandige (MDR) en uiters middelweerstandige (XDR) tuberkulose (TB), veral in die Oos-Kaap. Hierdie studie bied die eerste in-diepte analise van die molekulêre epidemiologie van middelweerstandige TB in die Oos-Kaap.

‘n Gerieflikheidsteekproef wat oor een jaar geneem is en bestaan het uit middelsensitiewe sowel as middelweerstandige isolate is van die National Health Laboratory Services in Port Elizabeth ontvang. Hierdie isolate is deur verskeie molekulêre metodes gekarakteriseer. Ons resultate is vergelyk met data van drie addisionele provinsies om die populasiesamestelling van MDR-TB stamme in konteks te plaas. Elke provinsie het ‘n unieke populasiesamestelling getoon. Die populasiesamestelling van XDR-TB gevalle in die Wes-Kaap is beduidend deur stamme van die Oos-Kaap beïnvloed. ‘n Hoë mate van groepering van weerstandigheidspatrone is in elke provinsie gevind, wat dui op transmissie. Groepering was besonder duidelik in die Oos-Kaap, waar 93% van pre-XDR en XDR-TB isolate van die Atipiese Beijing genotipe was. Ons het getoon dat hierdie genotipe programmaties geselekteer is deur ‘n suboptimale MDR-TB behandelingsregime wat nie *inhA*-gedefiniëerde ethionamied weerstandigheid in ag neem nie. Hierdie ondoeltreffende behandelingsregime het transmissie gefasiliteer en is die onderliggende oorsaak van mortaliteit. Ons stel voor dat bestaande molekulêre toetse gebruik word wat *inhA* mutasies opspoor om XDR-TB risiko-pasiënte te identifiseer en hul behandeling dienooreenkomstig aan te pas.

Ons het gedemonstreer dat twee sub-groepe van Oos-Kaap Atipiese Beijing isolate ontwikkel het uit ‘n gemene voorsaat, elk met unieke eienskappe, insluitend mutasies wat weerstandigheid teen tot 11 middels veroorsaak. Hierdie bevinding word gerugsteun deur heelgenoom volgordebepaling en ribonukleïensuur volgordebepaling en dui op die ontluiking en verspreiding van algeheel middelweerstandige TB in die Oos-Kaap.

Ons het getoon dat isolate wat die *rrs* A1401G mutasie het, verminderde vatbaarheid vir capreomisien het, en dit bevraagteken die bruikbaarheid van hierdie middel in die behandeling van XDR-TB waar amikasienweerstandigheid teenwoordig is. Van belang is dat stamme wat die *rpoB*516 mutasie het, vatbaar is vir rifabutien, ten spyte van weerstandigheid teen rifampisien. Die gebruik van rifabutien kan dus die uitkomste van XDR-TB pasiënte in die Oos-Kaap verbeter, en ook transmissie beperk.

Heelgenoom volgordebepaling is gebruik om molekulêre eienskappe te ondersoek wat moontlik ‘n selektiewe voordeel kan bied aan die Oos-Kaapse Atipiese Beijing genotipe stamme. Ons het getoon dat alle verteenwoordigde Atipiese Beijing genotipe stamme – insluitend dié wat as algeheel

middelsensitief gediagnoseer is – ‘n *ethA* mutasie het wat ethionamied weerstandigheid veroorsaak. Dit mag die oënskynlike verhoogde vermoë van die Atipiese Beijing genotipe stamme om hoër weerstandigheid te ontwikkel verklaar.

Verder het ribonukleïensuur volgordebepaling getoon dat ‘n groot aantal gene verskillend gereguleer is. Hierdie verskille moet verder ondersoek word om die invloed daarvan op die fisiologiese eienskappe van die bacillus te verklaar.

Hierdie bevindinge betwis die gebruik van gestandardiseerde MDR-TB behandeling in die afwesigheid van omvattende middelsensitiwiteitstoetse. Hierdie siening word tans algemeen aanvaar, en tog het dit nie die Suid-Afrikaanse TB-riglyne (2014), wat behandeling van rifampisienweerstandigheid sonder die relevante kennis van middelweerstandigheid voorstaan, beïnvloed nie. Ons stel voor dat die effektiewe behandeling van hoogs weerstandige TB net bereik kan word deur die ontwikkeling van nuwe middels, nuwe kombinasies van middels en vinnige, omvattende middelsensitiwiteitstoetse.

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"Here, U is my lewe, U sorg vir my. Wat ek ontvang het, kom alles van U af. 'n Pragtige deel is vir my afgemeet, ja, wat ek ontvang het, is vir my mooi. Ek sal die Here loof wat my tot die regte insig gebring het." – Ps 16:5-7

Dedication

This is for

Dad, who inspired me to become a medical researcher

Louis, who encouraged me to live my dreams

The patients and doctors of the Eastern Cape, in whose lives I hope that this will make a difference.

Chapter 1 General introduction

Notes:

1. This thesis is presented as a collection of published and to-be-published works. Typesetting and references are in the format requested by the journal each paper was submitted to.
2. I published under the name of M. Tait and M. Klopper

Learn from yesterday, live for today, hope for tomorrow. The important thing is to not stop questioning. – Albert Einstein

Introduction

1.1 Background

1.1.1 TB in South Africa, in relation to the world

South Africa has one of the highest recorded incidences of tuberculosis (TB) world-wide; estimated at >1000 new TB cases per 100 000 population per year according to the World Health Organization (WHO) 2013 report (1). South Africa reports the highest number of confirmed MDR-TB (resistant to at least isoniazid and rifapicin) cases in the Afro-region of the WHO and ranks fourth among the world's high burden MDR-TB countries (1). In South Africa, 10% of all TB cases are believed to be MDR-TB of which one-tenth are XDR-TB (MDR with additional resistance to a fluoroquinolone and a second-line injectable drug) (1). The highest rates of MDR and XDR-TB were notified for the Western Cape, Eastern Cape and KwaZulu-Natal provinces (2). In a nationwide survey conducted by the National Institute for Communicable Diseases in 2008, 20.2% of all notified TB cases showed resistance to isoniazid and nearly half of these (9.6% of all cases) were MDR-TB (2). This represents a 3-fold increase since 2002, when it was shown that 3.1% of all TB cases (new and retreatment) had MDR-TB (2). These findings are supported by a report from the National Health Laboratory Service (NHLS) which showed a steady increase in the number of MDR-TB cases since 2004 (2), and a study in Khayelitsha which showed that MDR-TB was diagnosed in 4.4% of all TB cases in 2008 (3). These statistics appear significantly worse if we consider that only 8,200 of the projected 13,000 patients were diagnosed with MDR-TB or XDR-TB by the NHLS in 2008, suggesting a case detection rate of 63%. More worrying is the fact that no more than 50% of diagnosed cases were placed on MDR treatment in 2009 (Dr. Norbert Ndjeka, Director Drug-Resistant TB, TB and HIV, National Department of Health). Treatment success rates are below 50% for MDR-TB (4,5) with considerably poorer outcomes for XDR-TB (6,7). In South Africa today, the standard first-line regimen consists of a two-month initiation phase of isoniazid, rifampicin, pyrazinamide and ethambutol and four months continuation of isoniazid and rifampicin. MDR-TB is treated with six months of kanamycin, moxifloxacin, ethionamide, pyrazinamide and terizidone, followed by 18 months omitting kanamycin.

Research in the 1950's led to the dogma that drug-resistant strains were less fit than drug-susceptible strains, suggesting decreased ability to spread and cause disease (8). From this the notion was developed that if drug-susceptible TB was treated adequately, drug-resistant TB would disappear. However, there are many examples of the transmission of drug-resistant strains, including MDR-TB

and XDR-TB. This indicates that drug-resistant strains are sufficiently virulent to enable them to spread and cause disease (9–13). Molecular epidemiological studies have demonstrated that the drug-resistant TB epidemic in SA is endemic and driven by outbreaks (11,14–19). Today there is evidence that the success of drug-resistant strains may be attributable to compensatory mutations which allow it to overcome the loss of fitness associated with certain drug resistance-associated mutations (20,21). In addition, these strains are able to spread even more efficiently in a population co-infected with HIV (19).

1.1.2 The tuberculosis burden in the Eastern Cape

The Eastern Cape Province of South Africa is an area of special concern in terms of TB incidence, the high burden of drug-resistant TB (1) and HIV incidence (22). In 2006, two Eastern Cape districts (Amatole and Nelson Mandela Metropole) were identified as areas of particularly high TB incidence in the country, and as such were included in the TB Crisis Management Plan launched by the National Department of Health. This plan focused on social mobilization to ensure that TB is de-stigmatised, encouraging early treatment seeking and completion of treatment (23). However, at the time, diagnostic procedures relied on culture-based drug susceptibility testing (DST), and this was only done on request when a patient failed to respond to treatment. This lengthy process often leaves the infectious patient on standard and possibly ineffective treatment for several weeks before an accurate diagnosis has been made and appropriate treatment is initiated. During such diagnostic delay periods drug resistant TB (MDR-TB up to XDR-TB) will continue to spread in the community. Furthermore, inappropriate treatment may lead to the acquisition of additional resistance by chromosomal mutations in target genes.

Before commencement of this research project, only one small-scale study on the molecular features of the tuberculosis epidemic in the Eastern Cape has been done, showing an overrepresentation of the Atypical Beijing genotype among rifampicin resistant isolates (19).

1.1.3 Beijing Genotype

The *Mycobacterium tuberculosis* Beijing genotype was first described in 1995 and the name was coined based on the region from where the majority of these strains originated (24). This family of strains was initially identified by having similar IS*6110* banding patterns (24). IS*6110* is a transposon of which multiple copies may exist in a genome at various insertion sites, and is unique to members of the *M. tuberculosis* complex (25). Strains with this same IS*6110* banding pattern were shown to have identical spoligotype signatures, showing 9 specific spacer sequences (24). Beijing genotype strains have also been shown to have specific Regions of Difference (RDs, large deletions), compared to H37Rv (26).

The Beijing genotype is widespread in the world, including in South Africa (27,28) and is known to be highly transmissible and frequently associated with drug-resistance (27,29). The Beijing genotype can be divided into two distinct groups, namely Typical and Atypical (30). The Atypical Beijing genotype is defined by the absence of an IS*6110* insertion in the NTF region (*Rv0001 – Rv0002*), as opposed to Typical Beijing genotype that harbours an IS*6110* insertion on the right side of the NTF region, which can be detected by a PCR-based assay (31). Atypical and Typical Beijing genotype can also be distinguished by their IS*6110* restriction fragment length polymorphism (RFLP) pattern. RFLP additionally allows further sub-classification of Typical or Atypical Beijing genotypes, denoted by a strain family number. For example, strain family 29 (F29) is the predominant Typical Beijing genotype, whereas F31 is the most commonly seen Atypical Beijing genotype in the Eastern Cape. In addition, numerous other genetic changes have been shown to be characteristic of the Atypical and Typical Beijing genotypes, respectively (29). Phylogenetic analysis has positioned the Atypical Beijing genotype at the root of the tree which has led to the designation of “ancient” while the Typical Beijing genotype is positioned towards the termini leading to the designation “modern”.

1.1.4 World-wide distribution of Atypical Beijing strains

The Atypical Beijing is considered to be relatively rare in most settings worldwide, not frequently associated with drug-resistance (31), and less virulent than its typical counterpart (32). In a multi-site study, the Beijing genotype represented 6%, 53% and 71% of strains from the Netherlands,

Vietnam and Hong Kong, respectively. Among the Beijing genotype strains, the Atypical sub-lineage represented 21.2%, 25.4% and 13.6%, respectively, indicating that the distribution of both the Beijing genotype and its sub-lineages differ markedly in different settings across the world. In contrast to these relatively low prevalence settings, the Atypical sub-lineage represents approximately 79% of Beijing genotype strains in Japan (31); 50% among Aborigines in Taiwan (33); and 65% of drug-resistant Beijing genotype strains in the Eastern Cape, South Africa (17). However, in general, existing studies often do not specify which sub-lineage the observed Beijing strains belong to. Therefore, it is possible that the notion that Atypical Beijing strains are rare in most parts of the world, may be inaccurate.

1.1.5 Drug resistance associated with the Beijing genotype

In a small study from Brazil, Atypical Beijing genotype strains showed varying patterns of drug-resistance, ranging from fully drug-susceptible to polyresistant and MDR with additional streptomycin resistance (34). Similarly, in Japan, certain subdivisions of the Atypical Beijing genotype were significantly associated with drug resistance, whereas other subdivisions were underrepresented in the drug resistance groups (31). However, small sample sizes may have introduced bias in some of the subdivisions.

A study across the Netherlands, Vietnam and China (35) also showed that drug-resistance in Beijing subgroups varied by country. However, the published data only clearly indicate that drug-resistance in Beijing genotype strains was more prevalent in Vietnam than in the Netherlands, and that Atypical Beijing strains in these settings are more likely to be MDR or INH resistant and less likely to be streptomycin (STR) resistant than Typical Beijing strains (35). In the Eastern Cape, South Africa, Atypical Beijing genotype strains were significantly associated with pre-XDR and XDR, including STR resistance (18,19). The dominance of one particular strain type (Atypical Beijing) strengthens the view that drug-resistant strains may be equally fit to drug-susceptible strains or at a selective advantage in this setting (17,36).

Conversely, a study conducted in Beijing, China concluded that among isolates belonging to the Beijing strain family (92% of the total number of strains investigated) there was no statistical difference in resistance-causing mutations in *rpoB* or *katG* between strains of the Typical or Atypical sub-lineage (37).

These observations indicate that even within a sub-lineage of the Beijing genotype, considerable variation exists. It also shows that the features which define a strain as Atypical Beijing, is not responsible for the acquisition of resistance or fitness.

1.2 Hypotheses

We hypothesise that Atypical Beijing genotype strains historically acquired genomic mutations and initial drug-resistance, giving them a selective advantage to spread and acquire additional resistance in the absence of comprehensive drug susceptibility testing and adequate treatment.

1.3 Aims and thesis structure

The purpose of this study was to investigate the molecular characteristics which describe the phenotypic properties of the drug-resistant TB epidemic in the Eastern Cape Province and to contextualise the epidemic in relation to the TB strain population structure in South Africa. Chapter 2 is presented as three separate publications. The first publication (section 2.1 - 2.5) aimed to describe the molecular epidemiology of drug-resistant TB in four South African provinces. This study shows that the population structures of MDR-TB is distinct in these provinces and that the dominant strain type in the Eastern Cape Province has influenced the population structure of M(X)DR-TB in the Western Cape (17). The second publication (section 2.6 - 2.10) aimed to determine whether *inhA* promoter mutations are associated with XDR-TB in South Africa, and to assess the usefulness of the GenoType® MTBDR*plus* assay for the detection of mutations conferring INH resistance in order to guide treatment regimens for MDR-TB. It was shown that in three of these provinces the *inhA* promoter mutations predispose the strains to acquire mutations leading to the evolution of XDR-TB and that the GenoType® MTBDR*plus* assay can be used as a screening tool to identify patients at risk of XDR-TB (38). The third publication (section 2.11 - 2.15) aimed to elucidate whether and how standardized treatment impacted the strain population structure of drug-sensitive and drug-resistant *M. tuberculosis* in South Africa. This study demonstrated programmatic errors where the implementation of a standardised treatment regimen failed to recognise cross-resistance thereby

compromising treatment efficacy, culminating in the development and selection of highly resistant strains (36).

Chapter 3 aimed to describe the *Mycobacterium tuberculosis* strain population structure among MDR-TB and XDR-TB cases in the Eastern Cape Province of South Africa in order to determine whether the epidemic is driven by acquisition or transmission of resistance and to describe the extent of resistance within these strains (18). It showed that the population structure of different classes of drug-resistance (pan-susceptible, MDR-, pre-XDR- and XDR-TB) are distinct, and importantly, that Atypical Beijing genotype strains are vastly overrepresented among pre-XDR- and XDR-TB isolates. It was further shown that within the population of pre- and XDR-TB strains, two dominant clones exist, that are both resistant beyond the definition of XDR-TB, are endemic and are spreading. This was the first study to highlight TDR-TB in Africa and reported the highest number of these cases.

A concurrent study of the Eastern Cape isolates confirmed the association between the *rrs* A1401G mutation and capreomycin resistance. Chapter 4 (section 4.1-4.5) aimed to correlate the susceptibility levels of *M. tuberculosis* isolates against AMK and CAP with the molecular mechanisms that cause drug resistance and to determine whether the *rrs* and *tlyA* genes of isolates displaying resistance to both AMK and CAP contain specific mutations that mediate cross-resistance between the two drugs. The potential value of capreomycin in patients infected with strains with the mutation was explored by quantitative drug-susceptibility testing (39). This study led to a change in methods used to do routine capreomycin DST in the NHLS laboratory in Port Elizabeth. In addition, our study (Chapter 4, section 4.6-4.10) investigated the extent of cross-resistance between rifampicin and rifabutin in the largest clone of the drug resistant Atypical Beijing genotype (40). Our objectives were to correlate the MICs of RIF and RFB in a subset of M(X)DR *M. tuberculosis* isolates, to analyse the MIC data to establish whether cross-resistance occurs between RIF and its analogue RFB and to translate the gained knowledge into clinical practice for further assessment concerning RFBs potential to improve clinical outcome. This study confirmed that the rpoB516 mutation confers low level rifampicin resistance and retains susceptibility towards rifabutin. This finding has stimulated research towards determining whether rifabutin can be included in the regimen to treat patients with drug resistant strains harbouring the rpoB 516 mutation. Furthermore, this study has highlighted the importance of mycobacterial pharmacogenetics which will be an essential component of future molecular-based diagnostics.

In Chapter 5, we aimed to interrogate the genomes of highly resistant Atypical Beijing strains (resistant to up to 11 drugs) from the EC through high resolution next-generation sequencing. Through this analysis we aimed to infer evolutionary relationships, as well as to investigate genetic variation among individual isolates in order to find genetic traits that may be responsible for their increased transmissibility. We also sought to discover a novel mechanism of para-aminosalicylic acid (PAS) resistance, as six isolates were phenotypically resistant to PAS, but did not have mutations known to be associated with this phenotype. We demonstrated the phylogenetic relatedness of Atypical Beijing genotype strains, as well as the existence of two distinct clones, each with unique molecular characteristics. No genetic mechanism could be identified that may be related to PAS resistance in these isolates. However, preliminary evidence suggest underlying ethionamide resistance in all investigated Atypical Beijing genotype strains. This resistance would go undetected in most strains that are not also resistant to at least isoniazid and rifampicin. Furthermore, the ethionamide resistance undermines standard second-line therapy, leading to increased acquisition of drug-resistance.

The concept of totally drug-resistant TB, its definition and its management is discussed in Chapter 6. This review contextualises our findings with the current literature and thereby highlights the importance of comprehensive, rapid drug-susceptibility testing in order to prevent the acquisition of drug-resistance, as well as the spread of resistant TB.

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Chapter 2 Epidemiology of drug-resistant tuberculosis in the Eastern Cape in the South African context

This chapter consists of three peer-reviewed publications.

1. **Population structure of multi- and extensively drug-resistant *Mycobacterium tuberculosis* strains in South Africa.**

Chihota VN, Müller 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.

Published in Journal of Clinical Microbiology 2012 Mar;50(3):995-1002.

2. ***inhA* promoter mutations: a gateway to extensively drug-resistant tuberculosis in South Africa?**

Müller B, Streicher EM, Hoek KG, Tait M, Trollip A, Bosman ME, Coetzee GJ, Chabula-Nxiweni EM, Hoosain E, Gey van Pittius NC, Victor TC, van Helden PD, Warren RM.

Published in International Journal of Tuberculosis and Lung Disease 2011 Mar;15(3):344-51.

3. **Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa.**

Müller B, Chihota VN, Pillay M, Klopper M, Streicher EM, Coetzee G, Trollip A, Hayes C, Bosman ME, Gey van Pittius NC, Victor TC, Gagneux S, van Helden PD, Warren RM.

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My contribution to this work includes design and planning of the projects; database construction, management and analyses for the Eastern Cape isolates; all molecular laboratory analyses for the Eastern Cape isolates; and writing of the manuscripts.

There's a thrill in the process of digging, then piecing together the details like a puzzle. – Nancy Horain

The population structure of multi- and extensively drug-resistant tuberculosis in South Africa

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Abstract

Genotyping of multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains isolated from tuberculosis (TB) patients in four South African provinces (Western Cape, Eastern Cape, KwaZulu-Natal and Gauteng) revealed a distinct population structure of the MDR strains in all four regions despite the evidence of substantial human migration between these settings. In all analyzed provinces, a negative correlation between strain diversity and an increasing level of drug resistance (from MDR-TB to extensively drug-resistant TB, XDR-TB) was observed. Strains predominating in XDR-TB in the Western and Eastern Cape and KwaZulu-Natal provinces were strongly associated with harboring an *inhA* promoter mutation, potentially suggesting a role of these mutations in XDR-TB development in South Africa. Approximately

50% of XDR-TB cases detected in the Western Cape were due to strains probably originating from the Eastern Cape. This situation may illustrate how failure of efficient health care delivery in one setting can burden health clinics in other areas.

2.1 Introduction

Drug-resistant tuberculosis (TB) threatens TB control efforts throughout the world (1). In particular, multidrug-resistant (MDR) TB, defined by resistance to at least isoniazid (INH) and rifampicin (RMP), has profound effects on patient treatment outcomes, since these two most effective anti-TB drugs with the fewest side effects, must be replaced by less effective, more expensive and more toxic drugs (2). South Africa is among the countries with the worldwide highest numbers of MDR-TB cases (an estimated amount of 13,000 cases in 2008) (3). In a nationwide survey in 2008, 20.2% of all notified TB cases showed resistance to INH and nearly half of these (9.6% of all cases) were MDR (4). Six years earlier, in 2002, MDR-TB had been reported in a considerably lower proportion (3.1%) of TB cases, indicating a dramatic increase of MDR-TB, in recent years (4;5). Furthermore, a high rate of extensively drug-resistant (XDR) TB (defined by MDR-TB plus additional resistance to a fluoroquinolone and at least one second-line injectable drug) of 10.5% was estimated among MDR-TB cases tested for second-line drug resistance (4). MDR-TB is responsible for the majority of the financial burden of TB in South Africa with approximately 70% of the budget of the national TB control program allocated to the management of MDR-TB (6).

Various reports suggest that the drug-resistant TB epidemic in South Africa is primarily caused by the transmission of MDR strains and the amplification of resistance as a result of the use of inappropriate empiric drug regimens in the context of delayed drug-susceptibility testing (DST) (7-14). As suggested by mathematical models, future levels of M/XDR-TB will be largely determined by the efficiency of the transmission of MDR strains (15;16). Hence, the elaboration of strategies to contain the M/XDR-TB epidemic requires an understanding of the disease transmission dynamics. Our knowledge in this respect has increased considerably through the application of genotyping techniques such as spoligotyping (17) and IS*6110* restriction fragment length polymorphism (RFLP) analysis (18), enabling the detection of TB transmission chains and the study of the evolution of *M. tuberculosis* population structures (19). Worldwide, molecular genotyping of *M. tuberculosis* strains has led to the identification of strain families or lineages; some of which are associated with drug resistant-TB, including the Beijing

family of strains (10;11;20;21) the LAM genotype (22) and strains belonging to the IS*6110* low copy number clade (13). However, information on the genetic diversity of *M. tuberculosis* is primarily based on studies of drug-sensitive *M. tuberculosis* and specific data on the population structure of drug resistant strains is lacking (23).

This study aimed to decipher the population structure of M/XDR *M. tuberculosis* strains isolated from TB patients in four South African provinces. Our results help understanding the evolution and spread of M/XDR-TB in a high-incidence setting of drug-resistant TB and provide important baseline data for the assessment of future treatment changes and interventions.

2.2 Materials and Methods

2.2.1 Study Population

This study was approved by the Ethics Committees of the participating universities. Suspect drug-resistant isolates of TB patients attending public health facilities in South Africa are routinely subjected to DST. A random sample of diagnosed MDR isolates from the Western Cape, Eastern Cape, KwaZulu-Natal and Gauteng provinces was characterized by spoligotyping. Only one isolate per patient was included in the study. Isolates from patients visiting primary health care clinics in the Western Cape were collected from August 2000-December 2010. Isolates collected until end of 2007 only covered two of the four health districts of the Western Cape; however, from 2008 isolates were collected from all four health districts. Isolates from patients attending primary health care clinics in the Eastern Cape, referral hospitals in KwaZulu-Natal or referral hospitals in Gauteng, respectively, were collected from July 2008-November 2009, May 2005-April 2006 or March 2004-December 2007. Altogether, 2145, 503, 233 and 765 (a total of 3646) isolates from the Western Cape, Eastern Cape, KwaZulu-Natal and Gauteng, respectively, were included in the study, corresponding to approximately 15%, 21%, 17% and 27% of the MDR patients notified for the respective study period and province (Table 1) (24).

Table 1. Number of isolates analyzed by province and year.

Year	Province											
	Western Cape			Eastern Cape			KwaZulu-Natal			Gauteng		
	Isolates	Notified	%	Isolates	Notified	%	Isolates	Notified	%	Isolates	Notified	%
2000	8	N/A	N/A	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A
2001	67	N/A	N/A	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A
2002	47	N/A	N/A	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A
2003	32	N/A	N/A	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A
2004	40	1085	3.7	0	N/A	N/A	0	N/A	N/A	152	537	34.0*
2005	87	1192	7.3	0	N/A	N/A	156	1024	22.9*	238	676	35.2
2006	106	1179	9.0	0	N/A	N/A	77	2200	10.5*	223	732	30.5
2007	158	1771	8.9	0	N/A	N/A	0	N/A	N/A	152	986	15.4
2008	426	2220	19.2	220	1501	29.3*	0	N/A	N/A	0	N/A	N/A
2009	566	2078	27.2	282	1858	16.6*	0	N/A	N/A	0	N/A	N/A
2010	592	N/A	N/A	0	N/A	N/A	0	N/A	N/A	0	N/A	N/A
Unknown	16	N/A	N/A	1	N/A	N/A	0	N/A	N/A	0	N/A	N/A
Total	2145	9525	14.5**	503	3359	20.5*	233	3346	16.5*	765	2931	26.9*

Isolates: Number of MDR isolates collected in the indicated year

Notified: Number of MDR-TB cases notified by the National Health Laboratory Service (NHLS), in the indicated year

%: Estimated proportion of isolates analyzed among MDR-TB cases detected during sampling months

N/A: No data available or not applicable

*Sampling only occurred during part of the indicated year. To estimate the proportion of notified MDR isolates analyzed, the number of notified cases during the sampling period was calculated pro rata temporis.

**The overall proportion of isolates analyzed only considered years for which NHLS data was available

2.2.2 Routine culture and drug susceptibility testing

Samples were decontaminated and subjected to bacterial culture using the BACTEC™ MGIT™ 960 Culture System for mycobacteria (27). Isolates from the Western Cape, Eastern Cape and Gauteng were subjected to routine DST by the National Health Laboratory Services (NHLS) using the indirect proportion method on Middlebrook 7H11 medium (25). In Gauteng, DST was done for INH, RMP, ethambutol (EMB) and streptomycin (SM) only; in the Western and Eastern Cape, DST was extended to ethionamide (ETH), kanamycin (KM), amikacin (AMK) and ofloxacin (OFX). However, in the Western Cape, testing for second line drugs only covered isolates collected in late 2006 onwards. In KwaZulu-Natal, during the study period, culture and DST was routinely done by the Regional Laboratory Service at Inkosi Albert Luthuli Central Hospital, using the agar proportion method on Middlebrook 7H10 agar plates containing INH, RMP, EMB, SM, KM and OFX (25).

2.2.3 Genotypic characterization

M. tuberculosis isolates from patients were subcultured on LJ medium and genotyped using spoligotyping (17). The spoligotype patterns were compared to the data deposited in the fourth international spoligotyping database (SpolDB4) (26), and grouped according to the different strain families as defined in SpolDB4. Sub-classification of Beijing genotypes into typical and atypical Beijing strains was performed on a random sample of Beijing genotypes from the Western Cape, Eastern Cape and Gauteng using a previously described PCR protocol or IS*6110* RFLP analysis (13;18). Beijing isolates from KwaZulu-Natal were not available for further sub-classification. A random sample of 13 typical and 19 atypical XDR Beijing isolates from the Western Cape and 65 atypical XDR Beijing isolates from the Eastern Cape were tested for mutations in the *inhA* promoter using previously described methods (27).

2.2.4 Definitions

MDR and XDR strains were classified according to WHO definitions (4). Pre-XDR-TB isolates were defined as MDR-TB isolates with additional resistance to either a fluoroquinolone (e.g. OFX) or a second-line injectable drug (capreomycin, KM or AMK) but not both (28). The MDR *sensu stricto* (*s.s.*) group excluded identified pre-XDR and XDR isolates.

2.2.5 Statistical analysis

Statistical analyses were done using STATA 10.0 (StataCorp LP, College Station, Texas, USA). The Pearson's chi-squared test was performed to test for a significant difference in the proportion of *M. tuberculosis* genotypes between two provinces or drug resistance groups. A p-value <0.05 was considered statistically significant.

2.3 Results

A sample of 3646 MDR isolates of *M. tuberculosis* collected between 2000 and 2010 from patients in four South African provinces, namely the Western Cape, Eastern Cape, KwaZulu-Natal and Gauteng, was genotyped by spoligotyping (17). A total of 220 spoligotype patterns were identified of which 122 could be grouped into 41 strain families according to the international spoligotyping database (SpolDB4) (26). The remaining isolates showed spoligotype patterns that did not match with any of the patterns in SpolDB4 (i.e., 206 isolates representing 98 different spoligotype patterns; 5.7% of all isolates; Table 2). The frequency distribution of isolates from all identified strain families stratified by origin and drug resistance group is presented in the Appendix.

Between the four South African provinces, Beijing strains were predominantly localized to the Western and Eastern Cape where 55% and 69% of all MDR isolates belonged to this genotype, respectively. Conversely, Beijing strains represented only 6% and 17% of all MDR isolates from KwaZulu-Natal and Gauteng, respectively (Table 2; Figure 1; $p<0.001$ for the pairwise comparison of the proportions between all provinces). Differentiation of a subset of the Beijing isolates into the sub-families of typical and atypical Beijing genotypes highlighted distinct population structures in the different provinces. In the Western Cape, 75% of all analyzed MDR isolates of the Beijing genotype belonged to the group of typical Beijing strains. In contrast, only 8% of the analyzed Beijing isolates from the Eastern Cape were members of the typical group and 92% belonged to the group of atypical Beijing strains (Table 3; Figure 1; $p<0.001$). In Gauteng, 83% of the tested Beijing isolates were members of the typical Beijing genotype (Table 3; Figure 1). Beijing isolates from KwaZulu-Natal were not available for further characterization.

Table 2. The frequency distribution of major MDR genotypes in four South African provinces stratified by drug resistance group.

Province	Drug resistance class	Strain families												Total	
		BEIJING		LAM4		S		T1		X1		Others			
		no.	%	no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
Western Cape	All MDR	1174	54.7	52	2.4	51	2.4	158	7.4	181	8.4	529	24.7	2145	100.0
	MDR *s.s.*	919	50.5	48	2.6	49	2.7	148	8.1	172	9.5	483	26.6	1819	100.0
	Pre-XDR	161	72.9	4	1.8	2	0.9	6	2.7	9	4.1	39	17.6	221	100.0
	XDR	94	89.5	0	0.0	0	0.0	4	3.8	0	0.0	7	6.7	105	100.0
Eastern Cape	All MDR	348	69.2	52	10.3	10	2.0	13	2.6	0	0.0	80	15.9	503	100.0
	MDR *s.s.*	144	50.5	46	16.1	9	3.2	13	4.6	0	0.0	73	25.6	285	100.0
	Pre-XDR	115	92.7	5	4.0	0	0.0	0	0.0	0	0.0	4	3.2	124	100.0
	XDR	89	94.7	1	1.1	1	1.1	0	0.0	0	0.0	3	3.2	94	100.0
KwaZulu-Natal	All MDR	15	6.4	61	26.2	71	30.5	30	12.9	0	0.0	56	24.0	233	100.0
	MDR *s.s.*	15	7.7	39	19.9	66	33.7	27	13.8	0	0.0	49	25.0	196	100.0
	Pre-XDR	0	0.0	4	33.3	3	25.0	1	8.3	0	0.0	4	33.3	12	100.0
	XDR	0	0.0	18	72.0	2	8.0	2	8.0	0	0.0	3	12.0	25	100.0
Gauteng	All MDR	128	16.7	128	16.7	56	7.3	109	14.2	0	0.0	344	45.0	765	100.0
All Provinces	All MDR	1665	45.7	293	8.0	188	5.2	310	8.5	181	5.0	1009	27.7	3646	100.0

MDR *s.s.*: MDR *sensu stricto*; MDR cases not including identified pre-XDR and XDR-TB cases

no.: Number of isolates belonging to a given strain family for a given drug resistance group and province

%: Proportion of isolates belonging to a given strain family for a given drug resistance group and province

LAM4 is also commonly referred to as F15/LAM4/KZN

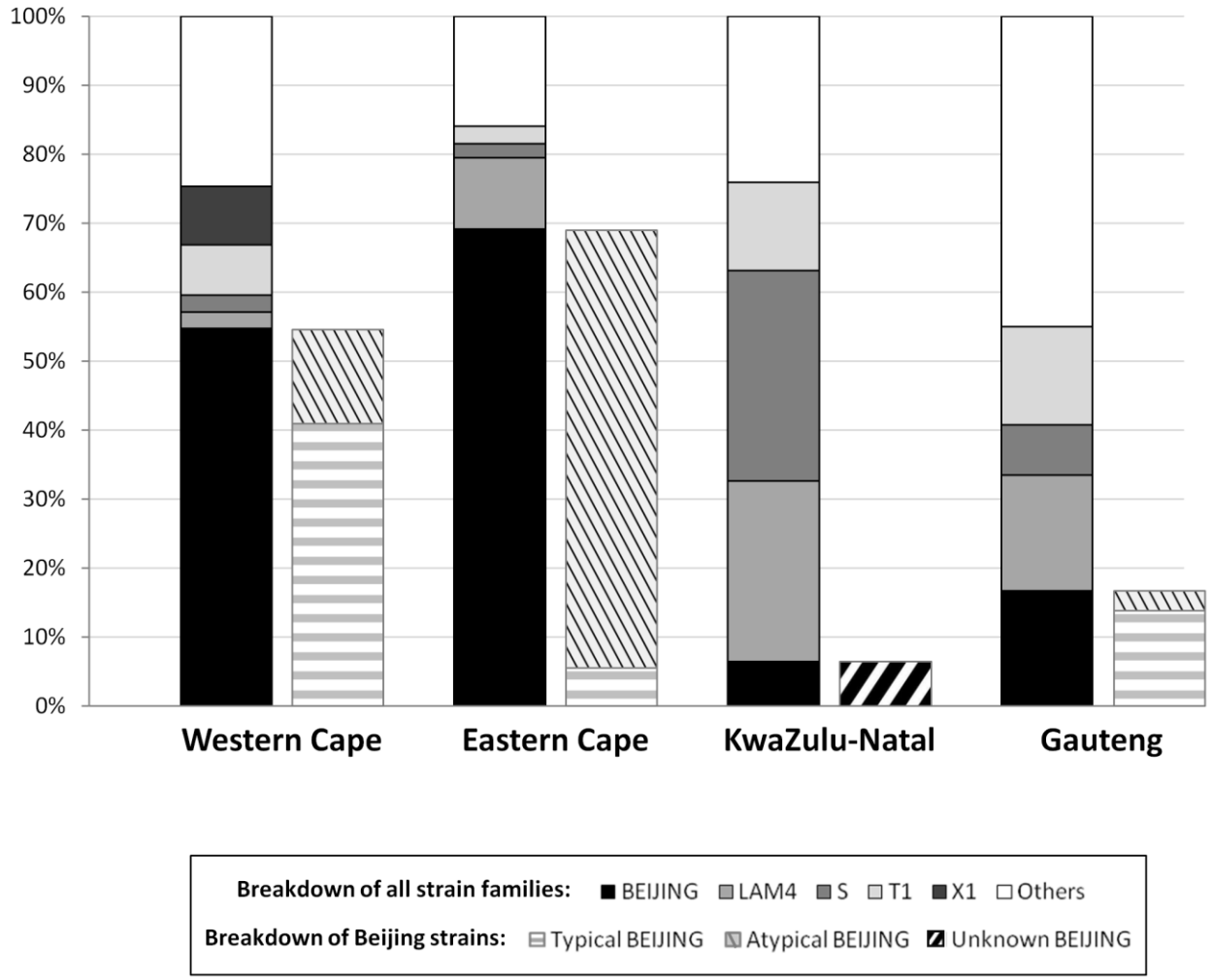

Figure 1: Frequency distribution of major MDR genotypes in four South African provinces. The proportion of isolates belonging to the Beijing, LAM4, S, T1, X1, or other genotypes is indicated in different shades of gray. The proportion of typical and atypical Beijing strains among the Beijing strains in Western Cape, Eastern Cape and Gauteng Provinces is shown in Table 2. Beijing strains from KwaZulu-Natal were not available for further characterization. LAM4 is also commonly referred to as F15/LAM4/KZN.

The concept of distinct population structures within the different provinces is further supported by our observation that the S and LAM4 (also referred to as F15/LAM4/KZN) families together constituted 57% of the isolates in KwaZulu-Natal but only 5%, 12% and 24% of the isolates in the Western Cape, Eastern Cape and Gauteng, respectively (Table 2; Figure 1; $p<0.001$ for the pairwise comparison of the proportions between all provinces). Interestingly, the X1 family of strains was only represented in isolates from the Western Cape (8% of all isolates) (Table 2; Figure 1). The strain population structure in Gauteng also differed from the other provinces. This was mostly suggested by the more equal frequency distribution of isolates from different strain families. While the two most frequently detected MDR strain families in the Western Cape, Eastern Cape and KwaZulu-Natal contributed 57% and more of all

isolates in these provinces, strains belonging to the two most prevalent genotypes in Gauteng only constituted 33% of the isolates in this province (Table 2; Figure 1). Of note, the EAI1_SOM and the H3 lineages were significantly more frequently isolated from MDR-TB patients in Gauteng (in 7% and 12% of the isolates, respectively) than in the other provinces (less than 2%; $p<0.001$), further illustrating the distinct population structure in Gauteng (Appendix).

Table 3. Proportion of typical and atypical Beijing strains.

Province	Drug resistance class	BEIJING					
		Typical		Atypical		All	
		no.	%	no.	%	no.	%
Western Cape	All MDR	168	75.0	56	25.0	224	100.0
	MDR *s.s.*	129	85.4	22	14.6	151	100.0
	Pre-XDR	25	67.6	12	32.4	37	100.0
	XDR	14	38.9	22	61.1	36	100.0
Eastern Cape	All MDR	16	7.9	186	92.1	202	100.0
	MDR *s.s.*	16	32.0	34	68.0	50	100.0
	Pre-XDR	0	0.0	85	100.0	85	100.0
	XDR	0	0.0	67	100.0	67	100.0
Gauteng	All MDR	67	82.7	14	17.3	81	100.0

MDR *s.s.*: MDR *sensu stricto*; MDR cases not including identified pre-XDR and XDR-TB cases

Stratification of the genotyping data for the Western Cape, Eastern Cape and KwaZulu-Natal by phenotypic drug resistance (no second-line DST results were available for Gauteng), revealed an increasingly marked predomination of one or two strain families from MDR *sensu stricto*- (MDR *s.s.*) to pre-XDR- and XDR-TB (Table 2; Figure 2). In the Western and Eastern Cape provinces, 90% or more of the XDR isolates belonged to the Beijing family of strains, representing a more than 75% increase compared to MDR *s.s.* ($p<0.001$ for both provinces; Table 2; Figure 2). Notably, the extrapolated overall proportion of atypical Beijing strains increased from 7% in the MDR *s.s.* group to 55% in the XDR group in the Western Cape, while the overall proportion of atypical Beijing strains increased from 34% in MDR *s.s.* to 95% in the XDR group in the Eastern Cape (Tables 1 and 2; Figure 2). Similarly, in KwaZulu-Natal,

the proportion of isolates belonging to the F15/LAM4/KZN family of strains increased from 20% in MDR *s.s.* cases to 72% in XDR-TB (Table 2).

A recent study revealed an association between drug-resistance mutations in the *inhA* promoter and XDR-TB in South Africa, however, without considering the influence of transmission (29). In a random, preliminary sample of XDR-TB isolates from the Western Cape, of 13 typical and 19 atypical Beijing isolates tested, 11 (85%) and 19 (100%) showed mutations in the *inhA* promoter, respectively. In the Eastern Cape, 62 (95%) of 65 tested XDR strains of the atypical Beijing family of strains showed a mutation in the *inhA* promoter. In a previous study by Ioerger et al., whole genome sequencing of 9 randomly chosen XDR isolates of the F15/LAM4/KZN family of strains from patients from different regions of KwaZulu-Natal revealed an *inhA* promoter mutation in all of these isolates (30). Thus, *inhA* promoter mutations are strongly linked to strains predominating in XDR-TB in at least three South African provinces.

2.4 Discussion

The present study represents a comprehensive description and comparison of the regional population structures of MDR *M. tuberculosis* strains in a high-burden country. Molecular characterization of MDR isolates from the Western and Eastern Cape, KwaZulu-Natal and Gauteng revealed distinct MDR-TB strain population structures in all four provinces and the geographical localization of specific strain families to different regions. In particular, typical and atypical Beijing strains are overrepresented in the neighboring coastal Western and Eastern Cape provinces and strains of the S and F15/LAM4/KZN family are present at significantly higher frequencies in KwaZulu-Natal (Table 2, Figure 1).

Distinct population structures between different regions may be explained by the lack of strain exchange between geographically separated populations, resulting in a 'disconnected' evolution and a progressive differentiation of these populations over time (31). Interestingly, the observed differences in the interprovincial MDR strain population structures parallel observations in the population structure of *M. bovis* in other parts of the world (31;32) but are unexpected as one might assume that internal migrations in South Africa could homogenize population structures between closely situated regions to a larger extent (33).

Nevertheless, our data provides evidence to suggest that limited strain exchange between different regions is occurring. This is especially indicated by the presence of most of the strain families in more than one province. Of all 3646 isolates analyzed within this study 3185 isolates (87%; representing 51 spoligotype patterns) exhibited a spoligotype pattern that was present in more than one province. Previous analyses of South Africa's internal migrations showed highest immigration rates for Gauteng (33); this is also reflected by a more equal frequency distribution of MDR-TB genotypes in this setting (Table 2; Figure 1). Residents from KwaZulu-Natal mostly immigrated to Gauteng and significantly less often to the Western or Eastern Cape provinces, perhaps explaining the more frequent isolation of MDR isolates belonging to the S and F15/LAM4/KZN family of strains in Gauteng and the very distinct population structure between KwaZulu-Natal and the Western and Eastern Cape provinces. Over 50% of the migrations to the Western Cape originated from the Eastern Cape (33), which possibly led to the introduction of the atypical Beijing strain genotype into the Western Cape. It could be speculated that the observed evidence for strain exchange between provinces is mostly based on recent human migrations while the generally different population structures between provinces could reflect earlier periods characterized by less migration (e.g. due to a restrictive migration policy during the apartheid era from 1948-1994) (33). Alternatively, the distinct population structures may be a result of multiple factors including geographically localized outbreaks or epidemics and distinct migration patterns in different settings.

An introduction of the atypical Beijing lineage from the Eastern Cape into the Western Cape and not vice versa is mostly supported by the much higher overall prevalence of this lineage in the Eastern Cape (Figure 1), a considerably higher diversity of IS*6110* RFLP patterns among the atypical Beijing strain population in the Eastern Cape compared to the Western Cape (data not shown) and a ten times higher number of human migrations from the Eastern to the Western Cape than vice versa (33). An introduction from other provinces not included in this study such as the neighboring Northern Cape (Figure 2) is less likely considering the human migratory behaviors in South Africa (33) and a significantly higher TB and MDR-TB incidence in the Eastern Cape compared to the Northern Cape (24), making spillover to the adjacent province more likely. Taken together, the current data suggests that the atypical Beijing lineage, accounting for more than 50% of all XDR-TB cases detected in the Western Cape, was introduced from the Eastern Cape.

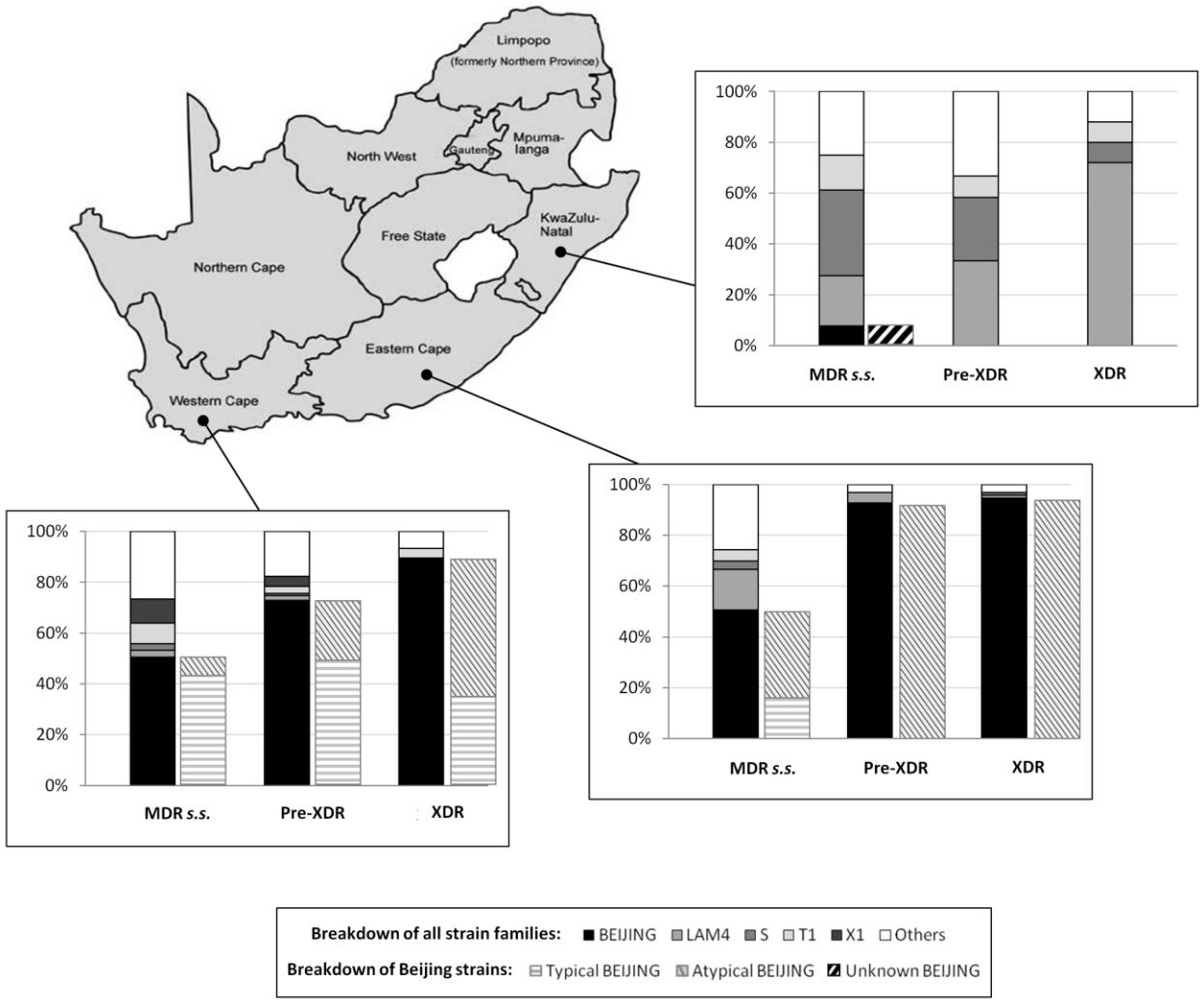

Figure 2: Frequency distribution of major MDR genotypes stratified by drug-resistance group for three South African provinces. The proportion of isolates belonging to the Beijing, LAM4, S, T1, X1 or other genotypes is indicated for MDR sensu stricto (MDR s.s.; not including pre-XDR and XDR isolates), pre-XDR and XDR cases (different shades of gray). The proportion of typical and atypical Beijing strains among the Beijing strains in the Western and Eastern Cape is extrapolated from Table 3. LAM4 is also commonly referred to as F15/LAM4/KZN.

XDR-TB cases caused by atypical Beijing strains in the Western Cape are for the most part unlikely to be a result of clonal transmission of XDR strains, as recent data revealed a variety of distinct second-line drug resistance mutations among IS*6110* RFLP clustered isolates (34). Thus, the majority of these XDR cases emerged through the amplification of resistance in primary MDR *s.s.* or pre-XDR-TB. The predominance of atypical Beijing strains in XDR-TB in the Western Cape, their significantly reduced contribution to MDR *s.s.* and pre-XDR-TB (Figure 2; Table 3) and the lack of evidence for the clonal transmission of XDR strains (34) suggests that a considerable amount of XDR-TB cases detected in the Western Cape could have developed from patients infected in the Eastern Cape who subsequently

migrated or returned to the Western Cape or who were seeking treatment outside their residential province. It seems conceivable that in particular patients with complicated disease residing in the economically more depressed Eastern Cape may decide to visit health clinics in the more affluent Western Cape where they may expect relatively superior quality of health care services (35). This situation could illustrate how relatively increased disease frequencies or shortage of access to efficient health care in a given region may burden health systems in adjacent areas. Similar scenarios are found in other regions of the world; e.g., MDR-TB clusters identified in Europe are associated with strains originating in eastern European countries (36). Equal access to functional health care services in closely situated regions is essential to prevent aggravation of circumstances in a given setting and the subsequent overload of health clinics in neighboring regions.

This study shows an intriguing, increasingly marked predomination of one single or two strain families from MDR *s.s.* to XDR-TB in all three provinces analyzed (Table 2; Figure 2). An association of certain strain families with XDR-TB could be attributed to their relatively more effective transmission as MDR strains, or, alternatively, may be explained by an enhanced intrinsic capacity to acquire resistance to second-line anti-TB drugs. We have recently demonstrated a significant association of *inhA* promoter mutations with XDR-TB in the Western and Eastern Cape provinces of South Africa (29). *InhA* promoter mutations confer low-level resistance to INH (used in first-line therapy for supposedly drug-sensitive TB) and high-level resistance to ETH (commonly used in second-line drug regimens for the treatment of MDR-TB) (37). We hypothesize that MDR strains, which have acquired such mutations at a previous instance (e.g. when exposed to INH during first-line therapy) would show an increased probability to gain resistance to other second-line drugs due to the treatment regimen having one less effective drug (29). Our preliminary data from the Western and Eastern Cape and a previous study in KwaZulu-Natal revealed that *inhA* promoter mutations are strongly linked to strains predominating in XDR-TB in at least three South African provinces. Similarly, in a study from Portugal, all of 26 XDR-TB isolates analyzed showed an *inhA* promoter mutation (38), suggesting that this association might be of importance in several parts of the world. These observations could be explained if, within the context of certain standardized second-line drug regimens, the presence of an *inhA* promoter mutation could increase the likelihood for a given strain to acquire additional resistance.

We previously recommended an adapted treatment regimen not including ETH for TB patients infected with strains harbouring an *inhA* promoter mutation (29). High-dose INH may be considered for treatment in such cases if no additional high-level resistance mutations (e.g. mutations in *katG* gene

codon 315) are present in infecting strains. Importantly, with the use of molecular probe assays such as the GenoType® MTBDR*plus* assay, now regularly used for genotypic DST in many settings, information about the presence of *inhA* promoter mutations in patient isolates is readily available to clinicians (29).

The present study has certain limitations. Most importantly, sample collection in the different provinces occurred during distinct time periods (Table 1), which could have caused artificial dissimilarities between the observed population structures. In the Western Cape Province, where isolates were collected over the longest period, an extensive increase in drug-resistant TB cases due to the Beijing family of strains could be observed with 32% and 63% of the isolates belonging to this family in 2001 and 2009, respectively. This is in accordance with previous reports from this region (10;39). Nevertheless, no considerable change was evident with respect to the different strain families comprising the most frequently observed MDR-TB isolates (data not shown), suggesting that temporal biases were unlikely to artificially create distinct strain population structure between the investigated regions. Secondly, it may not be able to exclude the possibility that the population bottlenecks occurring during the transitions from MDR *s.s.*- to pre-XDR- and XDR-TB could, in theory, account for the increasingly marked predomination of only one or two strain families from MDR *s.s.*- to XDR-TB. More research is needed to investigate the nature of this observation.

In conclusion, the data presented shows a distinct population structure and geographical localization of MDR strains of *M. tuberculosis* in four South African provinces. Evidence for provincial migration and in particular the introduction of XDR strains from the Eastern to the Western Cape highlights the need for uniformly implemented TB control programs. Only few strain families contribute to XDR-TB in South Africa, suggesting an enhanced ability of these strains to transmit as primary MDR strains or to acquire second-line resistance. In either case, inadequate diagnostic and treatment algorithms have probably led to the programmatic selection of these strains to become XDR strains. South African guidelines for the treatment of MDR-TB have recently been amended with terizidone replacing EMB for the treatment of MDR-TB and capreomycin, p-aminosalicylic acid and moxifloxacin, respectively, replacing KM (or AMK), PZA and OFX for the treatment of XDR-TB. It is questionable whether these changes will help curbing the emergence of XDR-TB or the predominance of the few transmitting MDR strains accounting for the majority of XDR-TB cases. This study provides important baseline data to understand and monitor future changes in the population structure of M/XDR strains of *M. tuberculosis* and to assess the impact of such interventions.

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TABLE A1 Strain families stratified by province and drug resistance group

	Result by province and drug resistance group[a]																									
	Western Cape								Eastern Cape								KwaZulu-Natal								Gauteng, all MDR		Total	
	MDR *sensu stricto*		Pre-XDR		XDR		All MDR		MDR *sensu stricto*		Pre-XDR		XDR		All MDR		MDR *sensu stricto*		Pre-XDR		XDR		All MDR					
Strain family	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Beijing	919	50.5	161	72.9	94	89.5	1,174	54.7	144	50.5	115	92.7	89	94.7	348	69.2	15	7.7	0	0.0	0	0.0	15	6.4	128	16.7	1,665	45.7
CAS1_DELHI	6	0.3	0	0.0	0	0.0	6	0.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	0.3	8	0.2
CAS1_KILI	2	0.1	0	0.0	0	0.0	2	0.1	0	0.0	0	0.0	0	0.0	0	0.0	5	2.6	1	8.3	0	0.0	6	2.6	10	1.3	18	0.5
EAI1_SOM	4	0.2	0	0.0	0	0.0	4	0.2	1	0.4	0	0.0	0	0.0	1	0.2	2	1.0	0	0.0	0	0.0	2	0.9	52	6.8	59	1.6
EAI5	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.5	0	0.0	0	0.0	1	0.4	1	0.1	2	0.1
EAI5 or EAI3	2	0.1	0	0.0	0	0.0	2	0.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	0.1
H1	7	0.4	0	0.0	0	0.0	7	0.3	1	0.4	0	0.0	0	0.0	1	0.2	0	0.0	0	0.0	0	0.0	0	0.0	9	1.2	17	0.5
H3	14	0.8	3	1.4	0	0.0	17	0.8	6	2.1	2	1.6	0	0.0	8	1.6	0	0.0	0	0.0	0	0.0	0	0.0	90	11.8	115	3.2
H3-T3	4	0.2	0	0.0	0	0.0	4	0.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.1	5	0.1
H4	1	0.1	0	0.0	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.0
LAM1	6	0.3	0	0.0	0	0.0	6	0.3	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	3	0.4	9	0.2
LAM1-LAM4	1	0.1	0	0.0	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.0
LAM10_CAM-S	1	0.1	0	0.0	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.0
LAM11_ZWE	2	0.1	0	0.0	0	0.0	2	0.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	6	0.8	8	0.2
LAM2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	0.3	2	0.1
LAM3	124	6.8	2	0.9	2	1.9	128	6.0	24	8.4	0	0.0	0	0.0	24	4.8	10	5.1	1	8.3	2	8.0	13	5.6	28	3.7	193	5.3
LAM3 and S/convergent	1	0.1	0	0.0	0	0.0	1	0.0	1	0.4	0	0.0	0	0.0	1	0.2	0	0.0	0	0.0	0	0.0	0	0.0	4	0.5	6	0.2
LAM4	48	2.6	4	1.8	0	0.0	52	2.4	46	16.1	5	4.0	1	1.1	52	10.3	39	19.9	4	33.3	18	72.0	61	26.2	128	16.7	293	8.0
LAM5	1	0.1	0	0.0	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.0
LAM9	15	0.8	2	0.9	0	0.0	17	0.8	1	0.4	0	0.0	1	1.1	2	0.4	0	0.0	0	0.0	0	0.0	0	0.0	14	1.8	33	0.9
MANU1	1	0.1	0	0.0	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.0
MANU2	9	0.5	1	0.5	0	0.0	10	0.5	0	0.0	0	0.0	2	2.1	2	0.4	2	1.0	0	0.0	0	0.0	2	0.9	1	0.1	15	0.4
S	49	2.7	2	0.9	0	0.0	51	2.4	9	3.2	0	0.0	1	1.1	10	2.0	66	33.7	3	25.0	2	8.0	71	30.5	56	7.3	188	5.2
T1	148	8.1	6	2.7	4	3.8	158	7.4	13	4.6	0	0.0	0	0.0	13	2.6	27	13.8	1	8.3	2	8.0	30	12.9	109	14.2	310	8.5
T1_RUS2	1	0.1	0	0.0	0	0.0	1	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.0
T2	4	0.2	1	0.5	0	0.0	5	0.2	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	0.3	7	0.2
T2-S	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.5	0	0.0	0	0.0	1	0.4	0	0.0	1	0.0
T2-T3	2	0.1	0	0.0	0	0.0	2	0.1	2	0.7	0	0.0	0	0.0	2	0.4	0	0.0	0	0.0	0	0.0	0	0.0	4	0.5	8	0.2
T3	16	0.9	0	0.0	0	0.0	16	0.7	1	0.4	0	0.0	0	0.0	1	0.2	7	3.6	1	8.3	0	0.0	8	3.4	13	1.7	38	1.0
T4_CEU1	9	0.5	1	0.5	0	0.0	10	0.5	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	10	0.3
T5	22	1.2	8	3.6	1	1.0	31	1.4	4	1.4	0	0.0	0	0.0	4	0.8	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	35	1.0
T5_RUS1	2	0.1	0	0.0	0	0.0	2	0.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	2	0.1
U	24	1.3	5	2.3	1	1.0	30	1.4	2	0.7	1	0.8	0	0.0	3	0.6	0	0.0	0	0.0	0	0.0	0	0.0	3	0.4	36	1.0
U (likely H)	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	8	1.0	8	0.2
U (likely H3)	3	0.2	0	0.0	0	0.0	3	0.1	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	3	0.1
X1	172	9.5	9	4.1	0	0.0	181	8.4	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	181	5.0
X1-LAM9	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	0	0.0	1	0.5	0	0.0	1	4.0	2	0.9	0	0.0	2	0.1
X2	29	1.6	3	1.4	0	0.0	32	1.5	2	0.7	0	0.0	0	0.0	2	0.4	2	1.0	0	0.0	0	0.0	2	0.9	8	1.0	44	1.2
X3	49	2.7	4	1.8	1	1.0	54	2.5	5	1.8	0	0.0	0	0.0	5	1.0	14	7.1	1	8.3	0	0.0	15	6.4	37	4.8	111	3.0
Not included in SpolDB4	121	6.7	9	4.1	2	1.9	132	6.2	23	8.1	1	0.8	0	0.0	24	4.8	4	2.0	0	0.0	0	0.0	4	1.7	46	6.0	206	5.7
Total	1,819	100.0	221	100.0	105	100.0	2,145	100.0	285	100.0	124	100.0	94	100.0	503	100.0	196	100.0	12	100.0	25	100.0	233	100.0	765	100.0	3,646	100.0

[a] MDR *sensu stricto*, MDR cases not including identified pre-XDR and XDR-TB cases; No., number of isolates belonging to a given strain family for a given drug resistance group and province; %, proportion of isolates belonging to a given strain family for a given drug resistance group and province. LAM4 is also commonly referred to as F15/LAM4/KZN.

Are *inhA* promoter mutations in *Mycobacterium tuberculosis* a gateway to the emergence of extensively drug resistant tuberculosis in South Africa?

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Abstract

SETTING: Western Cape and Eastern Cape Provinces in South Africa.

OBJECTIVE: To assess a potential association between the evolution of extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* and mutations in *katG* or the *inhA* promoter.

DESIGN: Analysis of a population sample of drug resistant strains of *M. tuberculosis*.

RESULTS: In the Western Cape and Eastern Cape Provinces, respectively, the percentage of strains exhibiting *inhA* promoter mutations increased significantly from 47.8% and 70.7% in multidrug resistant (MDR) strains to 85.5% and 91.9% in XDR strains. Data from the Western Cape revealed that significantly more XDR strains showed mutations in the *inhA* promoter than in *katG* (85.5% vs. 60.9%; $p<0.0001$) although the respective proportions were equal for INH resistant non-MDR strains (~30%).

CONCLUSIONS: *InhA* promoter mutations are strongly associated with XDR tuberculosis in South Africa. This is most probably due to the dual resistance to ethionamide and (low-dose) isoniazid

conferred by *inhA* promoter mutations. The use of molecular probe assays such as the GenoType® MTBDR*plus* assay, which allow the detection of *inhA* promoter mutations, could enable adjustment of the treatment regimens depending on the pharmacogenetic properties of the mutations detected.

2.6 Introduction

Multidrug resistant tuberculosis (MDR-TB), defined by the resistance to at least isoniazid (INH) and rifampicin (RIF), threatens TB control programs in many parts of the world (1). Amplification of drug resistance in MDR-TB can lead to virtually untreatable extensively drug resistant tuberculosis (XDR-TB), defined as MDR-TB with additional resistance to a fluoroquinolone and one of the injectable second line drugs kanamycin (KAN), amikacin (AMI) or capreomycin (CAP) (2-5). Cases of drug resistant TB emerge through ineffective treatment, patient non-adherence to therapy or the transmission of drug resistant strains of *M. tuberculosis* (2-4).

In South Africa, all new cases of TB are treated with a combination of four anti-TB drugs [INH, RIF, pyrazinamide (PZA) and ethambutol (EMB)] in the absence of drug susceptibility testing (DST) (6;7). Streptomycin is added to this regimen for retreatment cases while DST is being performed. If resistance to INH and RIF is detected the treatment regimen is adjusted to include second line anti-TB drugs. Since 2002 the treatment of MDR-TB has been largely standardized and includes a fluoroquinolone [mostly ofloxacin (OFX)], an aminoglycoside (AMI or KAN), PZA, EMB or cycloserine, and ethionamide (ETH) (6;8). This regimen assumes that resistance to EMB and PZA is rare. However, there is mounting microbiological evidence to suggest a strong association between MDR-TB and EMB and PZA resistance (6;9;10). Furthermore, several molecular epidemiological studies have suggested that MDR-TB is largely transmitted (11-13). This questions the validity of the current treatment guidelines (6) and calls for the development of rapid diagnostics for DST (14).

On a molecular level, drug resistance in *M. tuberculosis* develops through spontaneous mutations in target genes followed by the natural selection of these resistant bacteria upon exposure to anti-TB drugs (3;4). Various drug resistance causing mutations in *M. tuberculosis* have been characterized, to date (3;15;16). It is well documented that mutations in the *inhA* promoter confer low-level resistance to INH and cross-resistance to ETH, while mutations in *katG* at codon 315 exclusively confer high-level resistance to INH (16-19). Similarly, mutations in the *rpoB* gene account for >95% of RIF resistance (20). Thus, molecular assays identifying specific drug resistance causing mutations may be used to accelerate DST, which is considered critical to prevent amplification and

transmission of drug resistance (14). In this respect, the WHO approved GenoType® MTBDR*plus* assay has been implemented in several settings to assist culture based DST; it detects most of the mutations conferring RIF resistance as well as the principal mutations in the *katG* gene and *inhA* promoter which confer INH resistance (14).

Considering the dual resistance to INH and ETH conferred by the *inhA* promoter mutation and the use of these drugs for first and second line treatment, respectively, this study aimed to determine whether *M. tuberculosis* strains harbouring mutations in the *inhA* promoter are associated with XDR-TB, in South Africa. Moreover, we assessed the usefulness of the GenoType® MTBDR*plus* assay for the detection of mutations conferring INH resistance in order to guide treatment regimens for MDR-TB.

2.7 Materials and Methods

2.7.1 Data from the Western Cape Province

Between 2001 and 2007, drug resistant isolates from patients visiting health facilities in two of the four health districts in the Western Cape Province were collected. From 2008, drug resistant isolates from patients visiting health facilities in all of the health districts were collected. Routine DST was done by the National Health Laboratory Services (NHLS) and included testing for INH, RIF and EMB resistance. In 2007, routine DST was revised to include testing for OFX, AMI and ETH resistance. Mutation analyses of *katG* and the *inhA* promoter was done on a convenience sample of INH resistant non-MDR (INH mono-resistance and poly-resistant) and MDR strains. The same mutation analysis was done on the complete set of XDR-TB strains. Mutations were identified by dot-blot techniques or partial gene sequencing as explained elsewhere (21). For the analysis of the frequency of mutations in *katG* and the *inhA* promoter of INH resistant strains (Table 1), only sequencing data was considered. For the analysis of mutation patterns in different drug resistance classes (Tables 2 and 3, Figures 1 and 2), all available data including results from dot-blot analyses was considered. Only one *M. tuberculosis* isolate per patient was integrated in our analysis.

2.7.2 Data from the Eastern Cape Province

During the period from July 2008 to November 2009, all MDR-TB isolates cultured from patients visiting heath facilities in the Eastern Cape Province were collected. Routine DST was done by the

NHLS of the Nelson Mandela Bay Municipality, Eastern Cape. Mutations in the *inhA* promoter were identified by DNA sequencing (21).

2.7.3 Statistical analyses

Associations between distinct drug resistance groups and the occurrence of mutations in *katG* or the *inhA* promoter have been tested by the Pearson's chi-squared test. Difference in the proportions of XDR strains with mutations in *katG* or the *inhA* promoter have been tested by the Mc Nemar's test. Analyses have been performed in Stata/IC v10.1

2.8 Results

2.8.1 Western Cape Province

Table 1 lists the mutations identified by sequencing of the *katG* gene and the *inhA* promoter. *KatG* mutations at codon 315 and *inhA* promoter mutations at nucleotide position -15 constituted the majority (34.7% and 55.9%, respectively) of the observed sequence changes (Table 1). Of all identified mutations, the MTBDR*plus* assay could potentially detect alterations in *katG* codon 315 and in the *inhA* promoter at nucleotide positions -8, -15 and -17, either specifically through a mutation probe or through the absence of a hybridization signal for one of the wild-type probes. Consequently, in theory, the MTBDR*plus* assay might detect up to 96.3% of the mutation events here identified by sequencing (Table 1).

To investigate any potential associations between sequence changes in *katG* or the *inhA* promoter and XDR-TB, we calculated the proportion of strains with mutations in these genetic regions for different drug resistance classes (INH resistant non-MDR strains, MDR strains and XDR strains; Table 2, Figure 1). The percentage of strains with an *inhA* promoter mutation increased significantly from 30.1% in INH resistant non-MDR strains to 58.5% in MDR strains and 85.5% in XDR strains (overall 2.8-fold increase, $X2=74.6$, $p<0.001$; Table 2, Figure 1). Although the percentage of strains with a *katG* mutation also increased, the rate of increase was lower (2.1-fold, $X2=28.7$, $p<0.001$) and fully attributable to strains that also harboured an *inhA* promoter mutation (Table 2, Figure 1). Significantly more XDR strains showed mutations in the *inhA* promoter than in *katG* (85.5% vs. 60.9%, $p<0.0001$) although the respective proportions were equal for INH resistant non-MDR strains (~30%; Table 2, Figure 1).

Table 1 Frequency of mutations identified by sequencing in *katG* and the *inhA* promoter of INH resistant strains of *M. tuberculosis* in the Western Cape Province

Gene	Position	Mutation	Present	Absent	Sum	% Present	% Mutations	% Cumulative mutations
inhA promoter	-15	point mutation	224	157	381	58.8%	55.9%	55.9%
inhA promoter	-17	point mutation	21	360	381	5.5%	5.2%	61.1%
inhA promoter	-8	point mutation	2	379	381	0.5%	0.5%	61.6%
katG	315	point mutation	139	277	416	33.4%	34.7%	96.3%
katG	387	point mutation	2	414	416	0.5%	0.5%	96.8%
katG	77	point mutation	1	415	416	0.2%	0.2%	97.0%
katG	299	point mutation	1	415	416	0.2%	0.2%	97.3%
katG	302	point mutation	1	415	416	0.2%	0.2%	97.5%
katG	314	insertion	1	415	416	0.2%	0.2%	97.8%
katG	320	point mutation	1	415	416	0.2%	0.2%	98.0%
katG	408	point mutation	1	415	416	0.2%	0.2%	98.3%
katG	483	point mutation	1	415	416	0.2%	0.2%	98.5%
katG	119	deletion	3	130	133	2.3%	0.7%	99.3%
katG	76	point mutation	1	132	133	0.8%	0.2%	99.5%
katG	137	point mutation	1	132	133	0.8%	0.2%	99.8%
katG	198	point mutation	1	132	133	0.8%	0.2%	100.0%
Total			401					

Position: Amino-acid positions are indicated for transcribed regions (*katG*) and base-pair positions are indicated for untranscribed regions (*inhA* promoter)
Present: Among the INH resistant strains tested, number of strains showing a point mutation for the indicated gene at the indicated position
Absent: Among the INH resistant strains tested, number of strains not showing a mutation for the indicated gene at the indicated position
Sum: Number of INH resistant strains tested for a specific mutation for the indicated gene at the indicated position
% Present: Among the INH resistant strains tested, percentage of strains showing a mutation for the indicated gene at the indicated position
% Mutations: Number of times a specific mutation has been detected divided by the total number of detected mutation events
Total: Total number of detected mutation events

Importantly, 41.7% of all INH resistant non-MDR strains did not exhibit any mutations in katG or the inhA promoter (Table 2, Figure 1). However, this was significantly less frequently observed in MDR- and not seen in XDR-TB cases. Also of note, a proportion of almost 40% of the MDR and XDR strains only showed a mutation in the inhA promoter without any detected mutation in katG (Table 2, Figure 1).

Table 2 Patterns of point mutations in the *inhA* promoter and *katG* by drug resistance class in strains of *M. tuberculosis* in the Western Cape Province

Mutation	non-MDR INH^R			MDR			XDR		
	N	%	CI	N	%	CI	N	%	CI
inhA promoter*katG*	61	29.6%	23.5%-36.4%	100	39.5%	33.5%-45.8%	27	39.1%	27.6%-51.6%
inhA promoter∩*katG*	1	0.5%	0.0%-2.7%	48	19.0%	14.3%-24.4%	32	46.4%	34.3%-58.8%
*katG**inhA* promoter	58	28.2%	22.1%-34.8%	73	28.9%	23.4%-34.9%	10	14.5%	7.2%-25.0%
only others	86	41.7%	34.9%-48.8%	32	12.6%	8.8%-17.4%	0	0.0%	0.0%-5.2%*
inhA promoter	62	30.1%	23.9%-36.9%	148	58.5%	52.2%-64.6%	59	85.5%	75.0%-92.8%
katG	59	28.6%	22.6%-35.3%	121	47.8%	41.5%-54.2%	42	60.9%	48.4%-72.4%
Total	206	100.0%	N/A	253	100.0%	N/A	69	100.0%	N/A

MDR group includes XDR group
inhA promoter*katG*: Strains with mutations detected in the *inhA* promoter but not in *katG*
inhA promoter∩*katG*: Strains with mutations detected in the *inhA* promoter and in *katG*
*katG**inhA* promoter: Strains with mutations detected in *katG* but not in the *inhA* promoter
only others: Strains with no mutations detected in *katG* or the *inhA* promoter
inhA promoter: Strains with mutations detected in the *inhA* promoter
katG: Strains with mutations detected in *katG*
Total: Total number of strains tested
N: Number of strains of the same drug resistance group with the respective drug mutation pattern
%: Percentage of strains of the same drug resistance group with the respective drug mutation pattern
CI: 95% confidence intervals
* One-sided, 97.5% confidence interval

2.8.2 Eastern Cape Province

We analyzed an extensive set of MDR-TB strains isolated from TB patients in the Eastern Cape Province in order to determine whether the association between the *inhA* promoter mutation and XDR-TB is restricted to the Western Cape Province. Table 3 and Figure 2 show all mutations identified in the *inhA* promoter and their mutation frequencies in MDR and XDR strains.

In this setting, the proportion of isolates with an *inhA* promoter mutation also increased significantly during amplification of resistance from MDR-TB (70.7%) to XDR-TB (91.9%; 1.3-fold increase; X2=13.9, p<0.001; Table 3, Figure 2). In contrast to the Western Cape Province, the *inhA* promoter mutation at nucleotide position -17 was the predominant mutation found in this region.

Table 3 Frequency of point mutations in MDR and XDR strains of *M. tuberculosis* in the Eastern Cape Province

Gene	Position	MDR								XDR		
		Present	Absent	Sum	% Present	% Mutation	N	%	CI	N	%	CI
inhA	-17	134	129	263	51.0%	72.0%	134	51.0%	44.7%-57.1%	62	83.8%	73.4%-91.3%
inhA	-15	30	233	263	11.4%	16.1%	30	11.4%	7.8%-15.9%	4	5.4%	1.5%-13.3%
inhA	-8	22	241	263	8.4%	11.8%	22	8.4%	5.3%-12.4%	2	2.7%	0.3%-9.4%
only others		N/A	N/A	N/A	N/A	N/A	77	29.3%	23.8%-35.2%	6	8.1%	3.0%-16.8%
inhA	all	N/A	N/A	N/A	N/A	N/A	186	70.7%	64.8%-76.2%	68	91.9%	83.2%-97.0%
Total		186					263	100.0%		74	100.0%	

MDR group includes XDR group

Position: Nucleotide positions are indicated

Present: Among the MDR strains tested, number of strains showing a point mutation for the indicated gene at the indicated position

Absent: Among the MDR strains tested, number of strains not showing a mutation for the indicated gene at the indicated position

Sum: Number of MDR strains tested for a specific point mutation for the indicated gene at the indicated position

% Present: Among the MDR strains tested, percentage of strains with a point mutation detected for the indicated gene at the indicated position

% Mutations: Number of times a specific mutation has been detected divided by the total number of detected mutation events

N: Number of strains of the same drug resistance group with the respective drug mutation pattern

%: Percentage of strains of the same drug resistance group with the respective drug mutation pattern

CI: 95% confidence intervals

Total: Total number of detected mutation events or total number of MDR/XDR cases

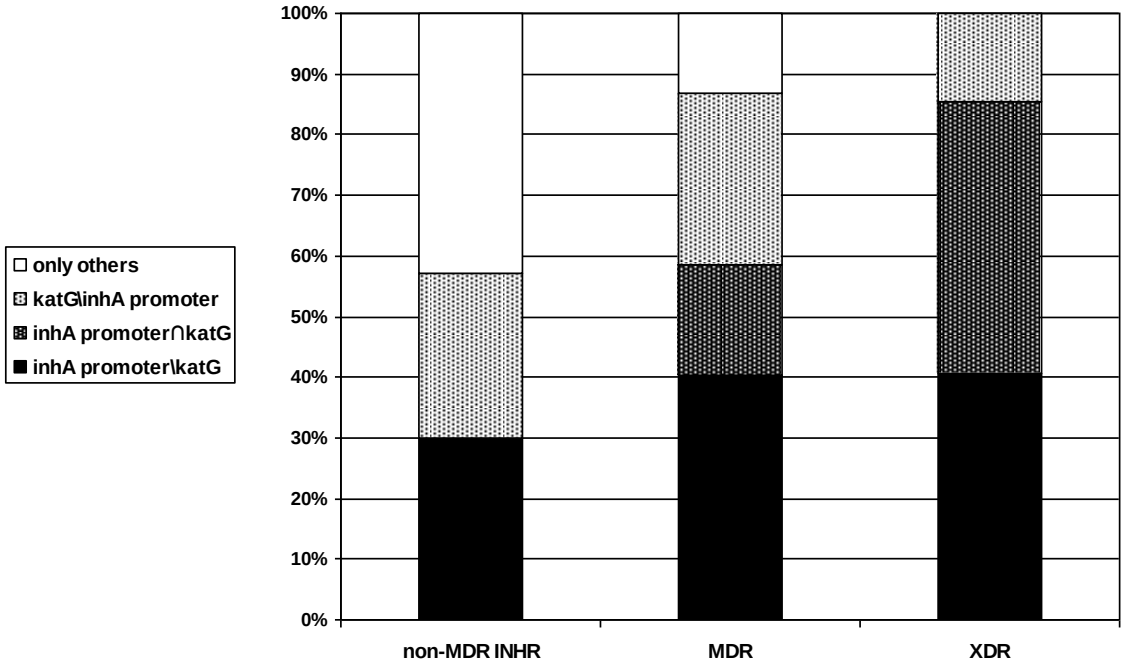

Figure 1: Patterns of point mutations in the *inhA* promoter and *katG* by drug resistance class in strains of *M. tuberculosis* in the Western Cape Province. MDR group includes XDR group; only others = isolates with no mutations detected in *katG* or the *inhA* promoter; *katGinhA* promoter = isolates with mutations detected in *katG* but not in the *inhA* promoter; *inhA* promoter∩*katG* = isolates with mutations detected in the *inhA* promoter and in *katG*; *inhA* promoter*katG* = isolates with mutations detected in the *inhA* promoter but not in *katG.***

2.9 Discussion

Our results show a significant association between *inhA* promoter mutations and the emergence of XDR-TB from MDR-TB in two different settings in South Africa (Table 2, Figure 1). This suggests that MDR strains harbouring an *inhA* promoter mutation have a selective advantage to become XDR strains, within the current treatment regimen. Two distinct processes may explain the observed accumulation of XDR-TB strains with an *inhA* promoter mutation. First, a significant proportion of XDR-TB strains initially might have been susceptible to ETH; however, continuous exposure to this drug could have led to the acquisition of *inhA* promoter mutations and ETH resistance (3;4;15-19). Second, *inhA* promoter mutations may have evolved during exposure to INH during first line treatment. MDR strains evolving from such strains also would have demonstrated cross resistance to ETH. If ETH resistance remained undetected there was an increased probability for these strains to acquire resistance to second line drugs due to the treatment regimen having one less effective drug.

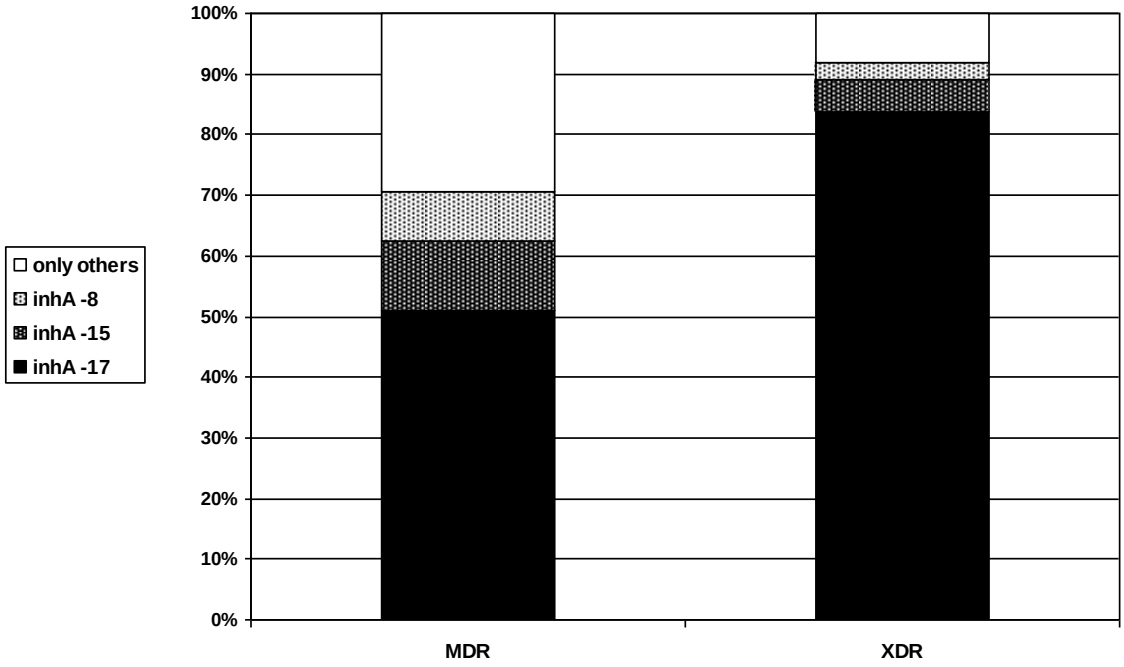

Figure 2: Frequency of point mutations in MDR and XDR strains of *M. tuberculosis* in the Eastern Cape Province. MDR group includes XDR group; only others = isolates with no mutations detected in the *inhA* promoter; inhA -8/-15/-17 = isolates with mutations detected in the *inhA* promoter at nucleotide position -8 or -15 or -17, respectively

The strong association between the presence of *inhA* promoter mutations and XDR-TB may also apply to other regions in South Africa and other parts of the world. In a recent study in Portugal, 98.0% of 50 tested MDR strains showed resistance to ETH and 91.4% of 58 tested MDR strains showed an *inhA* promoter mutation (22). A retrospective study on XDR-TB isolates from four provinces of South Africa showed that 30 of the 41 strains tested (73.2%), were resistant to ETH (23). These findings may again be explained by the latter scenario described above.

Our population study has some limitations. Mutation analyses of *katG* and the *inhA* promoter of isolates from the Western Cape Province was done on a convenience sample of INH resistant non-MDR and MDR strains. However, the majority of the XDR strains from this setting have been analyzed and no bias is evident for this group of strains. We know that the Beijing lineage of strains was overrepresented in our sample of the INH resistant non-MDR and MDR strains. In the Western Cape Province, drug resistant Beijing strains are strongly associated with *inhA* promoter mutations [(11), unpublished results]. Therefore, our analyses overestimated the proportion of strains with an *inhA* promoter mutation in INH resistant non-MDR and in MDR strains. Moreover, XDR strains were overrepresented in our sample of MDR strains of the Western Cape Province. Therefore, the increase in the proportion of strains with an *inhA* promoter mutation from non-MDR INH resistant

strains to XDR strains is likely to be even more pronounced and our analyses have been very conservative with regards to this observation.

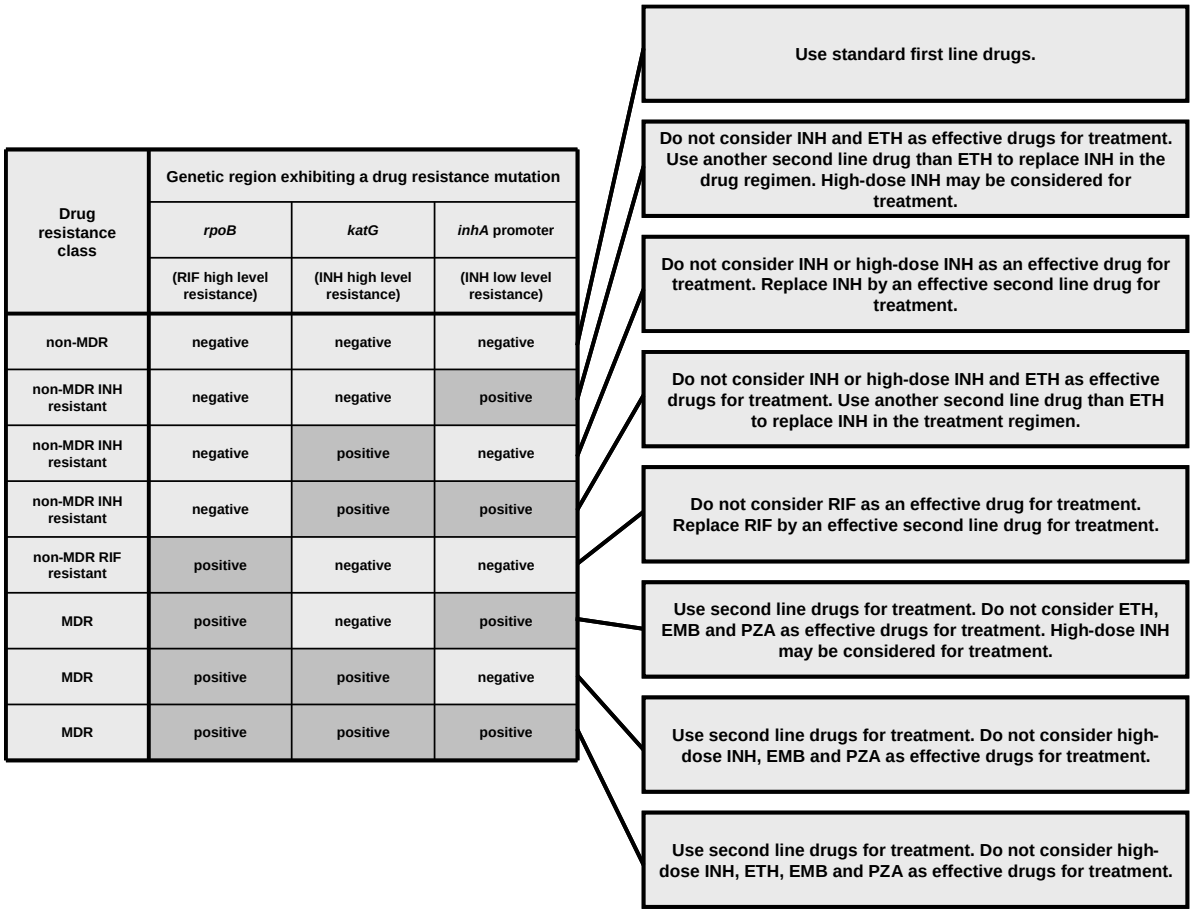

Figure 3: Proposed guidelines for the individualized treatment of TB patients in South Africa contingent on MTBDR*plus* DST results

Also, our results may have been influenced by the considerable contribution of transmission of drug resistant strains to MDR-TB, in South Africa (11-13). Therefore, the percentage composition of distinct drug resistance mutations in different drug resistance groups may fluctuate if significant changes in the transmission rates of strains occur that are associated with specific mutations.

Our study suggests that in principle, the MTBDR*plus* assay could detect 96.3% of all mutation events identified by sequencing of the *katG* gene and the *inhA* promoter of an extensive sample of INH resistant strains of *M. tuberculosis* (Table 1). However, our results clearly showed that mutations in genes other than *katG* or the *inhA* promoter accounted for more than 40% and 12% of all cases of

INH resistant non-MDR and MDR cases, respectively. This illustrates that culture based phenotypic DST should not be replaced but only assisted by molecular drug resistance typing techniques in order to achieve the highest possible sensitivity for the detection of drug resistant isolates. Failing this will inevitably lead to the selection and spread of INH resistant strains with properties that remain undetected by the applied molecular identification methods.

The MTBDR*plus* assay has been implemented for routine DST in several countries including some provinces in South Africa. With the introduction of the assay to assist culture based DST, information on the presence of the most important drug resistance mutations in isolates of *M. tuberculosis* from TB patients becomes readily available to clinicians. This may allow adjustment of the treatment regimens depending on the pharmacogenetic properties of the mutations detected (24).

For TB patients infected with strains exhibiting an *inhA* promoter mutation, ETH must not be considered an effective drug of the treatment regimen. However, because the *inhA* promoter mutation only confers low-level resistance to INH, high-dose or standard dose INH may be included in the treatment regimen of such cases if the infecting strains do not also exhibit any additional high-level INH resistance mutations (24). In many settings, the most frequently observed high-level INH resistance mutations are found in codon 315 of *katG* (3), which are detectable by the MTBDR*plus* assay (14). The information that high-dose INH may be included in treatment regimens is particularly valuable in XDR-TB cases, for which only few active drugs remain available. In this respect, our observation that approximately 40% of the MDR and XDR strains from the Western Cape Province exhibit a mutation in the *inhA* promoter but not in *katG*, is of special importance.

In Figure 3, we propose guidelines for the individualized treatment of TB patients based on the drug resistance mutation patterns detected by the MTBDR*plus* assay. These recommendations also consider our previous observations of a high percentage of EMB and PZA resistance in MDR strains in South Africa (6).

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Programmatically selected multidrug-resistant strains drive the emergence of extensively drug-resistant tuberculosis in South Africa

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Abstract

BACKGROUND: South Africa shows one of the highest global burdens of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB). Since 2002, MDR-TB in South Africa has been treated by a standardized combination therapy, which until 2010 included ofloxacin, kanamycin, ethionamide, ethambutol and pyrazinamide. Since 2010, ethambutol has been replaced by cycloserine or terizidone. The effect of standardized treatment on the acquisition of XDR-TB is not currently known.

METHODS: We genetically characterized a random sample of 4,667 patient isolates of drug-sensitive, MDR and XDR-TB cases collected from three South African provinces, namely, the Western Cape, Eastern Cape and KwaZulu-Natal. Drug resistance patterns of a subset of isolates were analyzed for the presence of commonly observed resistance mutations.

RESULTS: Our analyses revealed a strong association between distinct strain genotypes and the emergence of XDR-TB in three neighbouring provinces of South Africa. Strains predominant in XDR-TB increased in proportion by more than 20-fold from drug-sensitive to XDR-TB and accounted for up to 95% of the XDR-TB cases. A high degree of clustering for drug resistance mutation patterns was detected. For example, the largest cluster of XDR-TB associated strains in the Eastern Cape, affecting more than 40% of all MDR patients in this province, harboured identical mutations concurrently conferring resistance to isoniazid, rifampicin, pyrazinamide, ethambutol, streptomycin, ethionamide, kanamycin, amikacin and capreomycin.

CONCLUSIONS: XDR-TB associated genotypes in South Africa probably were programmatically selected as a result of the standard treatment regimen being ineffective in preventing their transmission. Our findings call for an immediate adaptation of standard treatment regimens for M/XDR-TB in South Africa.

2.11 Introduction

The emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) threatens disease control efforts throughout the world (1–3). Drug-resistant TB may be acquired if bacteria harbouring spontaneously emerging drug resistance mutations (Table 1) are positively selected due to e.g. inadequate treatment regimens, poor drug quality or patient non-compliance (2,4–6). Alternatively, drug-resistant TB may also occur through the transmission of already resistant strains; termed primary resistance. High rates of primary resistance reflect poor transmission control essentially due to delays in drug susceptibility testing and initiation of appropriate treatment (2,5).

Globally, in 2011, there were an estimated 310,000 incident cases of MDR-TB among cases reported to have tuberculosis of which 9% were XDR-TB (3,4). Increasing incidence rates for MDR-TB were recorded in several settings with South Africa being among the most severely affected countries (1,7,8). In South Africa, 10% of all TB cases are believed to be MDR-TB of which again one-tenth are XDR-TB (1,7,8). Highest rates of MDR and XDR-TB were notified for the Western Cape, Eastern Cape and KwaZulu-Natal provinces (9) with treatment success rates below 50% for MDR-TB and considerably poorer outcomes for XDR-TB (10,11). There is convincing evidence that MDR-TB in South Africa is caused mostly by the transmission of MDR strains, as suggested by well-documented clonal outbreaks and elevated rates of primary resistance (in some places as high as 80%) among MDR-TB cases (12–17). Similarly, transmission of MDR strains is likely to be a main driver of MDR-TB in many other high-burden countries (2,5,18).

New TB patients in South Africa are treated according to WHO guidelines with isoniazid (H), rifampicin (R), ethambutol (E) and pyrazinamide (Z) (19). Since 2002, MDR-TB treatment is also standardized and until 2010 included a fluoroquinolone (FQ; mostly ofloxacine (Ofx)), kanamycin (Km), ethionamide (Eto), E and Z (20). This regimen neglected high proportions of E and Z resistance among MDR-TB cases and cross-resistance to Eto if infecting strains previously acquired an *inhA* promoter mutation (Table 1) (21,22). An only marginally improved MDR-TB regimen was implemented in 2010, which replaced E with cycloserine or terizidone (Cs/Trd) (20). Standardized chemotherapy for MDR-TB is necessary in resource-limited settings where drug susceptibility testing (DST) cannot be performed regularly (19). The design of standardized regimens however, requires the prior determination of the spectrum of resistances present in the community (19). Culture-based resistance surveys not incorporating strain genotyping data do not enable examining whether detected resistances are transmitted jointly (by the same strain) or independently (by different strains). The absence of this knowledge has important implications for the design of standardized treatment regimens.

Previous studies in South Africa observed an association of specific genotypes of *M. tuberculosis* with XDR-TB (23). Specifically, the R220 genotype, a subgroup of the typical Beijing family of strains, the R86 genotype, a subgroup of "atypical" Beijing strains and the F15/LAM4/KZN genotype, a subgroup of the LAM4 family, were identified as commonly transmitted drug-resistant strains in the Western Cape, Eastern Cape and KwaZulu-Natal, respectively (15,16,23–26). In order to elucidate whether and how standardized treatment impacted the strain population structure of drug-sensitive and drug-resistant *M. tuberculosis* in South Africa, we characterized in detail an extensive collection

of clinical TB isolates from these provinces and analyzed resistance patterns of XDR-TB associated strains.

2.12 Materials and Methods

2.12.1 Ethics statement

This study was approved by the Ethics Committees of Stellenbosch University and the University of KwaZulu-Natal. The Stellenbosch Health Research Ethics Committee approved a waiver of consent for the retrospective genotypic analysis of routinely collected *M. tuberculosis* isolates after patient identifiers were removed. The University of KwaZulu-Natal Ethic Committee approved the prospective collection and genotyping of *M. tuberculosis* isolates after obtaining written consent.

2.12.2 Study population, routine culture and drug susceptibility testing

A comprehensive sample of clinical drug-resistant TB isolates collected during different time periods from the whole area of the Western Cape, Eastern Cape and KwaZulu-Natal province were analysed (Figure 1). Only one isolate per patient was included in the study. Subsets of this sample collection were used previously to describe the population structure of MDR *M. tuberculosis* strains in these provinces (23) and drug resistance mutations of strains of the Eastern Cape Province (27). These isolates characterised formerly were further complemented with a comparable, random sample of diagnosed drug-sensitive and mono-/poly-resistant isolates in order to analyse a larger spectrum of resistance patterns and a wider geographical area compared to previous studies. Routine culture and DST was performed at the National Health Laboratory Service (NHLS) in the respective provinces as described previously (23). The location of healthcare facilities attended by the TB patients was recorded to analyse the geographical distribution of *M. tuberculosis* genotypes identified.

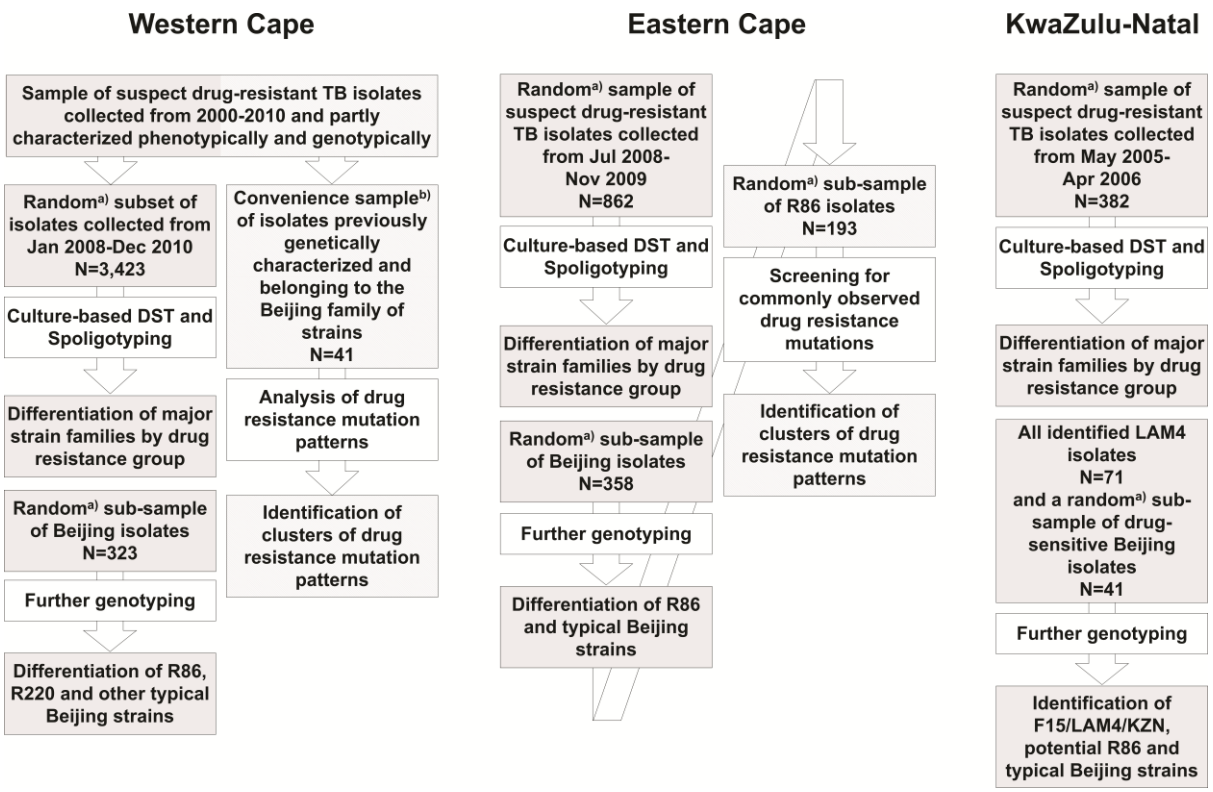

Figure 1: Selection of study population. Grey boxes indicate sample sets used to analyse the strain population structures in the three South African provinces. Boxes with striped pattern indicate sample sets used to characterise drug resistance mutation patterns among XDR-TB associated genotypes. a) Computer-based random sampling was applied. b) Review of an extensive collection of data generated within multiple previous studies.

2.12.3 Definition of drug resistance groups

M. tuberculosis isolates were classified into different drug resistance groups based on routine DST (23). Drug-sensitive isolates were susceptible to all drugs tested (at least H and R). Mono-/Poly-resistant isolates were resistant to one or multiple first-line anti-TB drugs but were not MDR. MDR and XDR isolates were classified according to WHO definitions (19). Pre-XDR-TB isolates were defined as MDR-TB isolates with additional resistance to either a FQ or a second-line injectable drug (Km, amikacin (Am) or capreomycin (Cm)) but not both. The MDR *sensu stricto* (*s.s.*) group excluded identified pre-XDR and XDR isolates from MDR isolates.

2.12.4 Genotypic characterisation

Initial genotyping of random samples of *M. tuberculosis* isolates was done by spoligotyping according to the protocol described by Kamerbeek et al (28) and the isolates were grouped into recognised strain families by comparison to previously reported spoligotype patterns (29,30). A randomly selected subset of Beijing isolates from all drug resistance groups from the Western and Eastern Cape and a subset of only drug-sensitive Beijing isolates from KwaZulu-Natal were further differentiated into typical and "atypical" Beijing isolates by PCR (Figure 1) (14). Computer-based random sampling was applied to randomly select isolates. Based on similar IS*6110* RFLP patterns and whole genome sequencing data it was previously established that "atypical" Beijing strains in the Western and Eastern Cape represent one single genotype herein referred to as R86 (14,25,31). Typical Beijing isolates from the Western Cape were distinguished into R220 and non-R220 isolates by PCR (Figure 1) (32). LAM4 isolates from KwaZulu-Natal were differentiated into F15/LAM4/KZN and other LAM4 isolates by IS*6110* RFLP analysis (Figure 1) (16). A random subsample of identified MDR R86 isolates from the Eastern Cape was tested for the presence of drug resistance mutations in the *inhA* promoter and the genes *katG*, *rpoB*, *pncA*, *embB*, *rrs* and *gyrA* by PCR amplification of genetic regions commonly observed to harbour resistance mutations and subsequent sequencing of these PCR products (Table 1, Figure 1) (33–37). Similarly, data from an extensive collection of drug-resistant isolates from the Western Cape was reviewed for records on Beijing isolates tested for the presence of resistance mutations in the same genetic regions (Table 1, Figure 1). However, no data on streptomycin resistance mutations in *rrs* were available (Table 1). Isolates with identical drug resistance mutation patterns were grouped by *pncA* mutations, which are highly diverse and may allow identifying genetically related groups of strains (27).

Table 1. Drug resistance-associated genetic regions analyzed

Genetic region	Region covered*	No. of base-pairs	Resistance
katG gene	2154968...2155387	420	H
inhA promoter	1673261...1673506	246	H, Eto
rpoB gene	760822...761258	437	R
embB gene	4247302...4247561	260	E
pncA gene	2288652...2289266	615	Z
rrs gene (around nucleotide position 513)	1472283...1472852	570	S
rrs gene (around nucleotide position 1401)	1473184...1473373	190	Km, Am, Cm
gyrA gene	7355...7698	344	Many FQs, e.g. Ofx

*Genetic region covered by PCR with respect to nucleotide positions in H37Rv
H: Isoniazid; Eto: Ethionamide; R: Rifampicin; E: Ethambutol; Z: Pyrazinamide; S: Streptomycin; Km: Kanamycin; Am: Amikacin; Cm: Capreomycin; FQ: Fluoroquinolone; Ofx: Ofloxacin

2.13 Results

Molecular characterisation of a random sample of 4,667 clinical TB isolates collected from the whole area of the Western Cape, Eastern Cape and KwaZulu-Natal provinces of South Africa revealed an increasing predomination of a single genotype of strains from drug-sensitive to XDR-TB, in each of the three provinces (Figure 2). In the Eastern Cape and KwaZulu-Natal, the proportion of isolates belonging to the R86 and the F15/LAM4/KZN genotype, respectively, underwent a 27- and 44-fold increase from drug-susceptible to XDR-TB and accounted for 95% and 72% of all XDR-TB cases (Figure 2, Table S1). In the Western Cape, the percentage of R86 isolates also increased significantly from drug-sensitive to XDR-TB cases. However, a previous study indicated that R86 isolates detected in the Western Cape, may to a large extent represent TB patients from the economically depressed Eastern Cape seeking treatment in the more affluent Western Cape (23). Thus, if R86 isolates are disregarded, the R220 genotype most strongly contributes to drug-resistant TB in the Western Cape, in line with previous results (15). Noteworthy, R220 isolates expand significantly in proportion (24-fold) from drug-sensitive to mono-/poly-resistant TB (Figure 2, Table S1).

Genotypes predominant in XDR-TB were infrequently detected among drug-sensitive TB cases (Figure 2). In all three provinces investigated, R220, R86 and F15/LAM4/KZN strains accounted for less than 5% of the drug-sensitive TB cases, making them considerably less abundant than the typical Beijing, LAM3 and T1 genotypes, which each represented between 10% and 41% of all drug-sensitive isolates (Table S1). Interestingly, while the strain population structure among MDR-TB isolates was fundamentally different between the three provinces (23), it appeared to be similar for drug-sensitive isolates (Figure 2).

Drug resistance patterns of XDR-TB associated genotypes were analysed by assessing the presence of commonly observed resistance mutations in the *inhA* promoter and the genes *katG*, *rpoB*, *pncA*, *embB*, *rrs* and *gyrA* (Table 1). A random sample of 193 MDR isolates of the R86 genotype from the Eastern Cape and 41 conveniently selected MDR isolates from the Western Cape representing a variety of different Beijing genotypes (R86, R220 and other typical Beijing strains) were selected (Figures 1, 3 and 4). Apart from H and R resistance mutations, various additional resistance-conferring mutations were detected. Moreover, mutation patterns were highly clustered (Figures 3 and 4). Most strikingly, 69% of the R86 isolates from the Eastern Cape analyzed (133/193 MDR isolates analyzed) harboured as many as seven identical resistance mutations in the *inhA* promoter

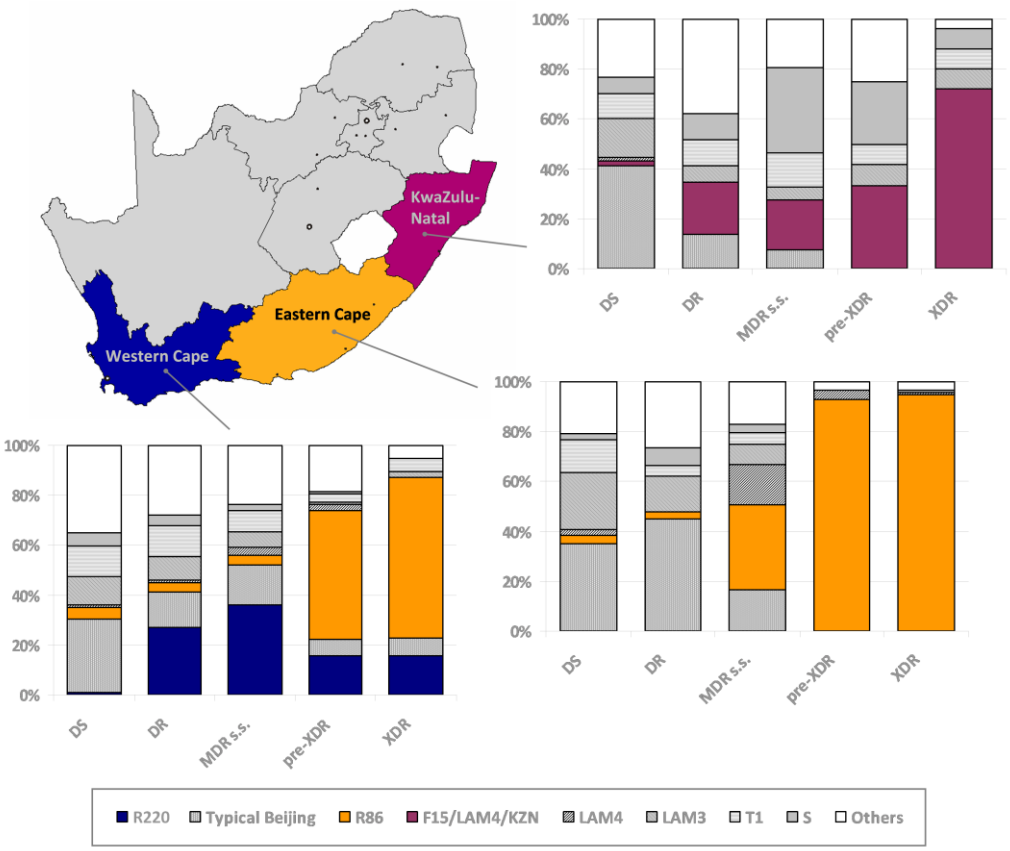

Figure 2: Strain population structure of drug-sensitive (DS), mono-/poly-resistant (DR), sensu stricto multidrug-resistant (MDR *s.s.*; excluding identified pre-XDR and XDR isolates), pre-extensively drug-resistant (pre-XDR) and extensively drug resistant (XDR) isolates in three provinces of South Africa. The R220, R86 and F15/LAM4/KZN genotypes, respectively, represent a subgroup of the typical Beijing, "atypical" Beijing and LAM4 family (14–16,22–24). Based on similar IS*6110* RFLP patterns and whole genome sequencing data it was previously shown that "atypical" Beijing strains in the Western and Eastern Cape, unlike in other parts of the world, represent one single genotype herein referred to as R86 (23,25,27). The specific presence of R220 and F15/LAM4/KZN genotypes was only assessed in the Western Cape and KwaZulu-Natal, respectively, where these genotypes were known to be frequent among XDR-TB cases (22).

and the genes *katG*, *rpoB*, *pncA*, *embB* and *rrs* suggesting that this cluster represents a commonly transmitted pre-XDR strain resistant to at least H, R, Z, E, S, Eto, Km, amikacin (Am) and capreomycin (Cm) (Table 1, Figure 3) (23,27). XDR-TB cases that have emerged from infection with this strain showed a variety of different *gyrA* mutations, suggesting that FQ resistance was acquired subsequently and perhaps due to the mismanagement of primary pre-XDR-TB. Nevertheless, a sub-group of 44 isolates showed for example an identical *gyrA* D94G mutation, potentially indicating community spread of XDR strains (Figure 3).

A second cluster representing 17% of the R86 isolates from the Eastern Cape (32/193 MDR isolates analyzed), was characterized by identical mutations in *katG*, *rpoB*, *pncA*, *embB* and *rrs* conferring resistance to H, R, Z, E and S (Table 1; Figure 3) (27). Presumably, a sub-branch of this strain subsequently acquired resistance to Eto through an *inhA* promoter mutation (38), to Km, Am and Cm

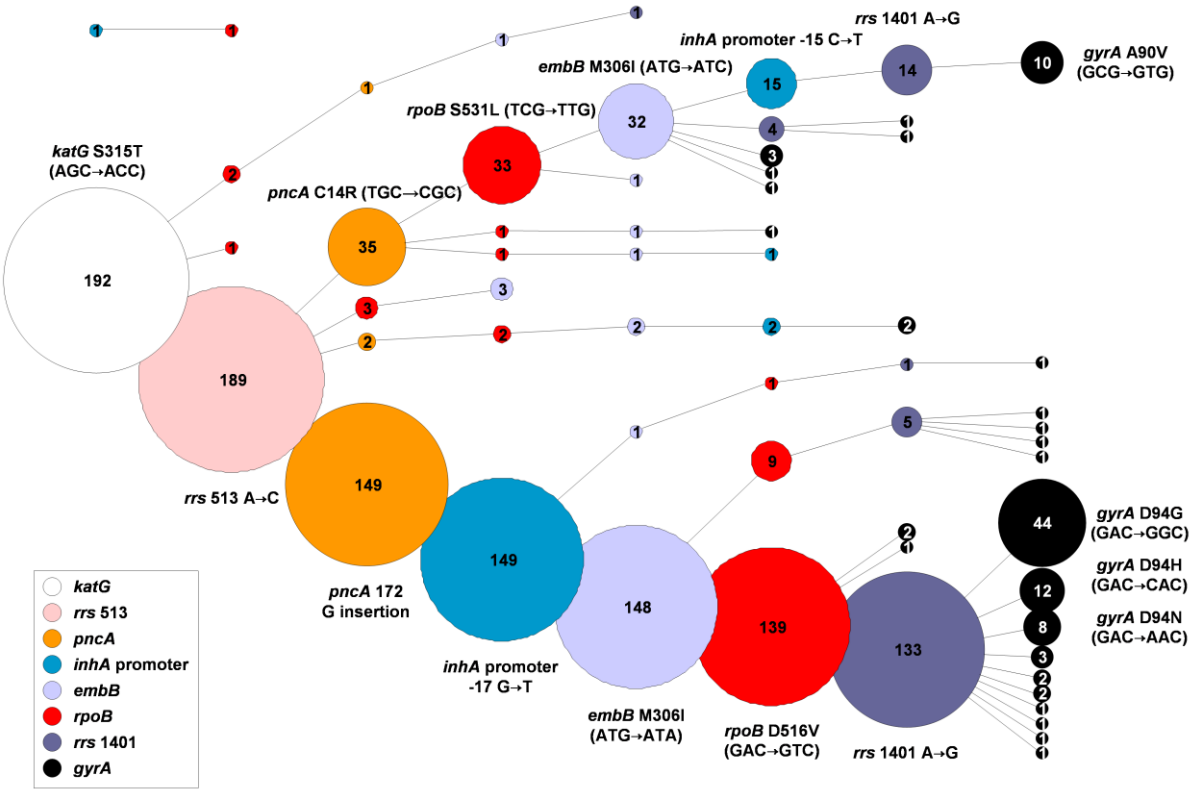

Figure 3: Drug resistance mutation pattern in a random selection of 193 MDR R86 isolates from the Eastern Cape. Different colours indicate different drug resistance associated genes. The area of the circles is proportional to the number of isolates (indicated in the centre of each circle) harbouring an identical drug resistance mutation for the respective resistance gene as well as all circles connected to the left. Principal branches of the tree were defined by resistance mutations in *pncA*. Other first-line drug resistance mutations were connected by logical deduction to maximize clustering and were followed by second-line resistance mutations. However, the order of acquisition of resistance mutations may remain debatable in some cases.

through an additional mutation in *rrs* (39,40) and finally to FQs due to the acquisition of a *gyrA* A90V resistance mutation (Figure 3) (6,41).

Analysis of the drug resistance mutation patterns of a convenience sample of 41 MDR Beijing isolates from the Western Cape, revealed that the two major R86 clusters detected in the Eastern Cape were also present in this province, albeit at a different relative frequency (Figures 3 and 4). For the remaining R220 and other typical Beijing isolates analyzed, clustered mutation patterns for at least *pncA* and *embB* were found in 8 out of 16 cases (Figure 4), indicating a widespread combined presence of Z and E resistance among these strains, in the Western Cape.

The clusters of strains defined above by genotype and drug resistance mutation patterns (Figures 3 and 4) were geographically widespread within the Eastern and Western Cape (Table 2), indicating historical spread. In the Eastern Cape, the two predominant clusters among MDR isolates of the R86

genotype were detected in four and three different municipal districts, respectively. In the Western Cape, despite the small sample size, isolates of four out of five clusters as defined by distinct *pncA* mutations were identified in more than one district (Table 2).

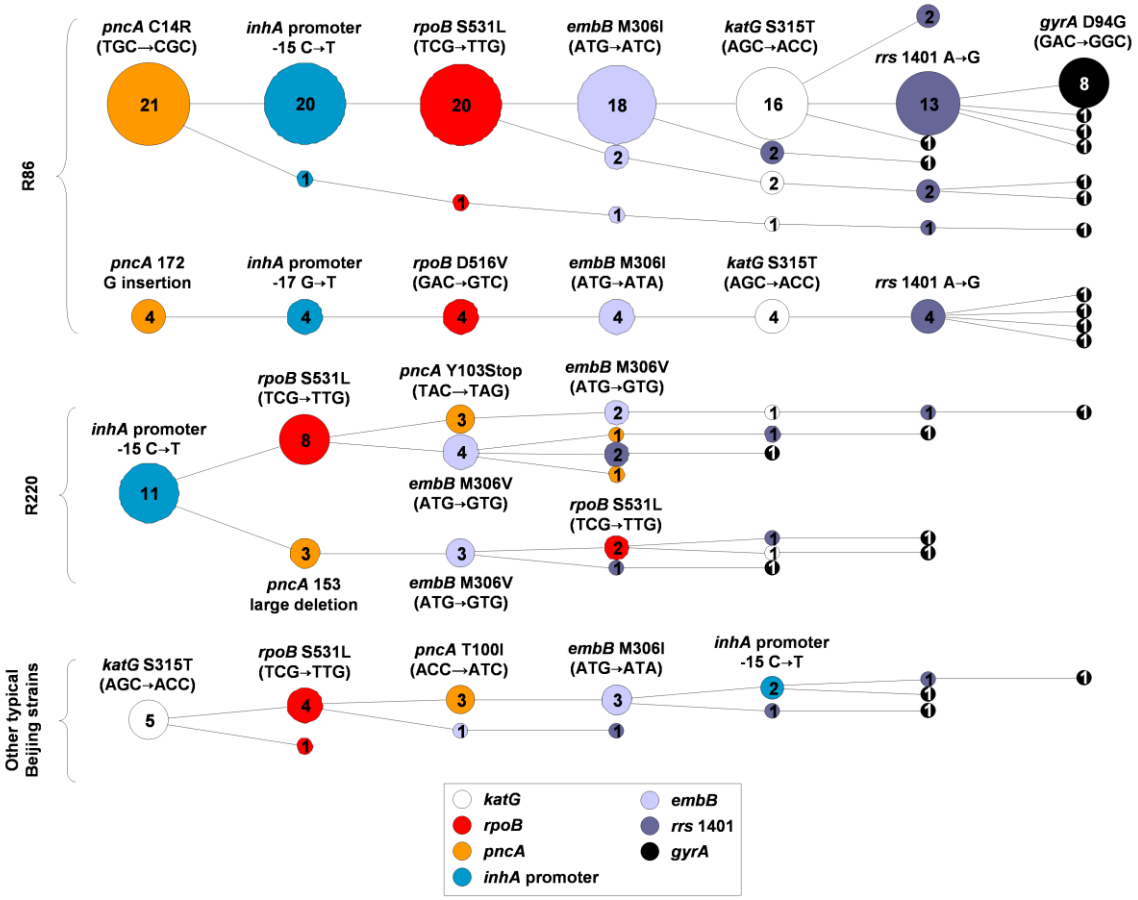

Figure 4: Drug resistance mutation pattern in a convenience sample of 41 MDR Beijing isolates from the Western Cape. No data was available for the streptomycin resistance determining region in *rrs* (Table 1). For more information see figure legend of Figure 3.

2.14 Discussion

The present data shows a strong association between distinct strain genotypes and the emergence of XDR-TB in three neighbouring provinces of South Africa (23). XDR-TB associated genotypes were infrequently found among drug-sensitive TB cases, of which typical Beijing, LAM3 and T1 were the most prevalent genotypes in all three provinces (Figure 2). This observation is counterintuitive, if it was supposed that the proportion of genotypes causing XDR-TB was a result of random fluctuations. Under such conditions it would be plausible to assume that genotypes predominant among drug-

sensitive TB cases would have been more likely to become overrepresented among XDR-TB cases (Table 2, Figure 2). Instead, the association of the R220, R86 and F15/LAM4/KZN genotypes with XDR-TB suggests an increased ability of these strains to acquire multiple drug-resistance mutations or to transmit as drug-resistant strains. However, the relatively distant phylogenetic relationship of these XDR-TB associated strain genotypes (42,43) argues against the possibility of genetic background accounting for this observation.

Table 2. Geographical distribution of selected clusters of isolates

Province	Genotype	Drug resistance mutation pattern	Municipal District	$N_{Isolate}$	%
EC	R86	*katG* S315T/*rrs* 513 A→C/*pncA* C14R/*rpoB* S531L/*embB* M306I	Amathole	19	59.4
			Nelson Mandela Bay	12	37.5
			OR Tambo	1	3.1
EC	R86	*katG* S315T/*rrs* 513 A→C/*pncA* C14R/*rpoB* S531L/*embB* M306I/*inhA* promoter -15 C→T/*rrs* 1401 A→G/*gyrA* A90V	Amathole	7	70.0
			Nelson Mandela Bay	2	20.0
			OR Tambo	1	10.0
EC	R86	*katG* S315T/*rrs* 513 A→C/*pncA* 172 G insertion/*inhA* promoter -17 G→T/*embB* M306I/*rpoB* D516V/*rrs* 1401 A→G	Amathole	30	22.6
			Cacadu	12	9.0
			Chris Hani	1	0.8
			Nelson Mandela Bay	90	67.7
EC	R86	*katG* S315T/*rrs* 513 A→C/*pncA* 172 G insertion/*inhA* promoter -17 G→T/*embB* M306I/*rpoB* D516V/*rrs* 1401 A→G/*gyrA* D94G	Amathole	9	20.5
			Cacadu	3	6.8
			Chris Hani	1	2.3
			Nelson Mandela Bay	31	70.5
WC	R86	*pncA* C14R/*inhA* promoter -15 C→T/*rpoB* S531L/*embB* M306I/*katG* S315T/*rrs* 1401 A→G	Cape Town	12	92.3
			Eden	1	7.7
WC	R86	*pncA* 172 G insertion/*inhA* promoter -17 G→T/*rpoB* D516V/*embB* M306I/*katG* S315T/*rrs* 1401 A→G	Cape Town	3	75.0
			Eden	1	25.0
WC	R220	*inhA* promoter -15 C→T/*rpoB* S531L/*pncA* Y103Stop	Cape Town	2	66.7
			Overberg	1	33.3
WC	R220	*inhA* promoter -15 C→T/*pncA* 153 large deletion/*embB* M306V	Cape Town	2	66.7
			Cape Winelands	1	33.3
WC	Other typical Beijing	*katG* S315T/*rpoB* S531L/*pncA* T100I/*embB* M306I	Cape Town	3	100.0

EC: Eastern Cape Province
WC: Western Cape Province
$N_{isolate}$: Number of isolates of a cluster detected in the municipal district indicated
%: Proportion of isolates of a cluster detected in the municipal district indicated

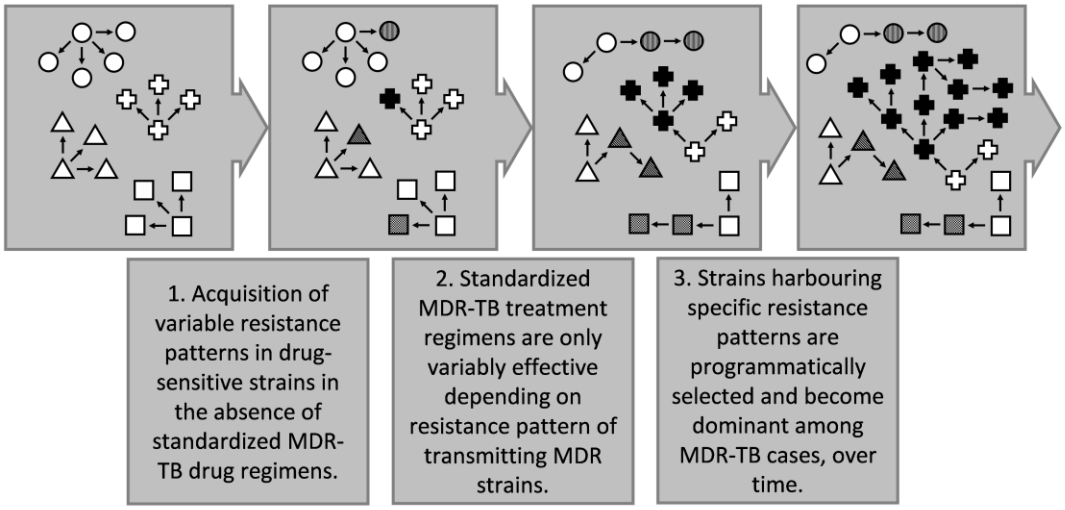

Figure 5: Model for the evolution of XDR-TB associated strain families.

Drug resistance mutation patterns of isolates of XDR-TB associated genotypes in the Eastern and Western Cape provinces were highly clustered (Figure 3). Unfortunately, isolates of the XDR-TB associated F15/LAM4/KZN genotype in KwaZulu-Natal were not further characterised within this study and therefore the relationship between genotype and clustering could not be evaluated. However, in line with our observations for the Western and Eastern Cape, a previous whole genome sequence analysis of nine XDR F15/LAM4/KZN isolates from patients of different settings in KwaZulu-Natal revealed nearly identical genome sequences including matching drug resistance mutations (26). Together, this data suggests that in South Africa, XDR-TB emerges mainly due to ongoing transmission of specific MDR *s.s.* or pre-XDR genotypes that are sub-optimally treated by programmatic treatment regimens, or partly, directly through the transmission of XDR strains of these genotypes (11,25,27).

It is likely however, that our analyses convey a relative overestimate of the proportion of transmission of primary pre-XDR and XDR strains as the Km/Am/Cm resistance mutation (*rrs* 1401 A→G) and the FQ resistance mutations (*gyrA* D94G and the *gyrA* A90V) detected among the largest clusters of isolates, belong to the most frequently observed resistance mutations for these drugs (6,44). Indeed, for the Km/Am/Cm resistance mutations observed in *rrs*, only a very low diversity was observed (Figures 3 and 4) (39,40). Thus, it is likely that these mutations have been acquired

independently multiple times among clustered isolates and clustering may not (or to a lesser extent) represent the clonal spread of pre-XDR and XDR strains.

Even if FQ and Km/Am/Cm resistance mutations in *gyrA* and *rrs* are disregarded, 72% (139/193) and 8% (15/193) of the MDR R86 isolates from the Eastern Cape belonged to one of two major clusters of isolates harbouring identical resistance mutations to at least H, R, Z, E, S and Eto (Figure 3). Similarly, altogether 63% (26/41) of the MDR Beijing isolates from the Western Cape tested belonged to one of altogether five clusters of isolates with identical resistance mutations to at least H, R, Z, E, and Eto (Figure 4). Given this data and the frequency distribution of different genotypes among MDR-TB cases (Table 2), we can estimate that at least 48% and 28% of all MDR-TB cases in the Eastern and Western Cape, respectively, were caused by a strain resistant to at least H, R, Z, E, S and Eto at the time of infection. Considering published whole genome sequences of XDR F15/LAM4/KZN isolates (26) and if FQ and Km/Am/Cm resistance mutations are disregarded, this genotype also shows primary resistance to at least H, R, E, Z, S and Eto and accounts for 26% of all MDR-TB cases in KwaZulu-Natal (Table 2). Importantly, since only specific XDR-TB associated genotypes were analyzed, the proportion of MDR-TB cases with resistances to additional anti-TB drugs than H and R may be even higher.

Given the standard MDR-TB drug regimens in South Africa (currently consisting of Ofx, Km, Eto, Trd/Cs and Z) and if excluding *rrs* and *gyrA* mutations, TB patients infected with these strains are exposed to three effective drugs only (Ofx, Km and Cs/Trd); this is less than the four effective drugs recommended by the WHO (45). If many of these transmitting strains in fact also harboured a primary *rrs* 1401 A→G mutation, the treatment regimen would consist of two effective drugs only. Under these conditions, even the standardized XDR-TB treatment regimen in South Africa, currently consisting of moxifloxacin, Cm, Eto, para-aminosalicylic acid and Cs/Trd would be inappropriate to treat infected patients (20). Noteworthy, the previous MDR-TB regimen endorsed until 2010, which used E instead of Cs/Trd, resulted in an even higher chance of resistance development as it consisted of only two or one effective drug, respectively. This clearly demonstrates the inadequacy of current treatment regimens in South Africa to prevent spread of XDR-TB associated strains and calls for an immediate adaptation of MDR treatment algorithms. Moreover, our findings highlight the urgent need for rapid first- and second-line DST for all TB cases at treatment onset.

A likely scenario for the evolution of XDR-TB associated strains in South Africa is depicted in Figure 5. It could be speculated that the use of non-standardized drug regimens before 2002 facilitated the emergence and transmission of strains with different resistance patterns. Possibly, the

implementation of standardized MDR-TB treatment subsequently promoted the spread of strains harbouring resistances against which the regimen was less effective. These strains could have emerged originally as early as in the 1950's when TB treatment was not well controlled and mostly included H, S and para-aminosalicylic acid only (46). This is supported by the very widespread presence of identical H and S resistance mutations in isolates from the Eastern Cape, indicating that these mutations were acquired at an initial stage (Figure 3). However, importantly, improved TB control and standardized MDR-TB treatment probably curbed the emergence of new resistant strains and transmission of strains harbouring unfavourable resistance patterns. Thus, the programmatic use of an only variably effective MDR-TB treatment regimen could explain the predomination of only a few strain families among XDR-TB cases. Although an impact of strain genetic background on the propensity to develop MDR/XDR-TB has been suggested (47), according to this model, the acquisition of advantageous resistance patterns would have occurred by chance and independent of strain genetic background, explaining the association of different, distantly related genotypes with XDR-TB in different provinces. Associations of a few specific genotypes with MDR and XDR-TB were observed in several countries throughout the world (48–51), suggesting similar mechanisms for the emergence of XDR-TB.

This work highlights the value of molecular epidemiological tools to perform drug resistance surveys and to decipher how individual resistances may be linked and transmitted. Moreover, this data will help designing more effective and urgently needed MDR-TB treatment regimens for South Africa. Failure to do so will rapidly enhance spread and amplification of resistance among XDR-TB associated strains.

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Table S1. Strain population structure in the Western Cape, Eastern Cape and KwaZulu-Natal Provinces of South Africa.

Province	Family	Subfamily	Drug resistance group																		
			DS			DR			MDR *s.s.*			pre-XDR			XDR			MDR			All
			N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N	%	95%CI	N
Western Cape	BEIJING	All	34	35,1	25.6-45.4	782	44,9	42.5-47.3	743	56,0	53.3-58.7	133	73,9	66.8-80.1	67	87,0	77.4-93.6	943	59,5	57.1-62.0	1759
	BEIJING	*R220* [a]	*1*	*3,3*	*0.1-17.2*	*54*	*60,7*	*49.7-70.9*	*63*	*64,3*	*54.0-73.7*	*12*	*21,4*	*11.6-34.4*	*9*	*18,0*	*8.6-31.4*	*84*	*41,2*	*34.4-48.3*	*139*
	BEIJING	*Typical (excl. R220)* [a]	*25*	*83,3*	*65.3-94.4*	*28*	*31,5*	*22.0-42.2*	*28*	*28,6*	*19.9-38.6*	*5*	*8,9*	*3.0-19.6*	*4*	*8,0*	*2.2-19.2*	*37*	*18,1*	*13.1-24.1*	*90*
	BEIJING	*R86* [a]	*4*	*13,3*	*3.8-30.7*	*7*	*7,9*	*3.2-15.5*	*7*	*7,1*	*2.9-14.2*	*39*	*69,6*	*55.9-81.2*	*37*	*74,0*	*59.7-85.4*	*83*	*40,7*	*33.9-47.8*	*94*
	BEIJING	*R220* [b]	*1*	*1,2*	*N/A*	*474*	*27,2*	*N/A*	*478*	*36,0*	*N/A*	*29*	*15,8*	*N/A*	*12*	*15,7*	*N/A*	*518*	*32,7*	*N/A*	*994*
	BEIJING	*Typical (excl. R220)* [b]	*28*	*29,2*	*N/A*	*246*	*14,1*	*N/A*	*212*	*16,0*	*N/A*	*12*	*6,6*	*N/A*	*5*	*7,0*	*N/A*	*230*	*14,5*	*N/A*	*504*
	BEIJING	*R86* [b]	*5*	*4,7*	*N/A*	*62*	*3,5*	*N/A*	*53*	*4,0*	*N/A*	*93*	*51,5*	*N/A*	*50*	*64,4*	*N/A*	*195*	*12,3*	*N/A*	*261*
	LAM4	All	1	1,0	0.0-5.6	19	1,1	0.7-1.7	42	3,2	2.3-4.3	4	2,2	0.6-5.6	0	0,0	0.0-4.7	46	2,9	2.1-3.9	66
	LAM4	*F15/LAM4/KZN* [c]	*0*	*0,0*	*0.0-3.7*	*6*	*0,3*	*0.1-0-7*	*37*	*2,8*	*2.0-3.8*	*3*	*1,7*	*0.3-4.8*	*0*	*0,0*	*0.0-4.7*	*40*	*2,5*	*1.8-3.4*	*46*
	LAM4	*Non-F15/LAM4/KZN* [c]	*1*	*1,0*	*0.0-5.6*	*13*	*0,7*	*0.4-1.3*	*5*	*0,4*	*0.1-0.9*	*1*	*0,6*	*0.0-3.1*	*0*	*0,0*	*0.0-4.7*	*6*	*0,4*	*0.1-0.8*	*20*
	LAM3	All	11	11,3	5.8-19.4	164	9,4	8.1-10.9	85	6,4	5.1-7.9	2	1,1	0.1-4.0	2	2,6	0.3-9.1	89	5,6	4.5-6.9	264
	T1	All	12	12,4	6.6-20.6	218	12,5	11.0-14.2	112	8,4	7.0-10.1	6	3,3	1.2-7.1	4	5,2	1.4-12.8	122	7,7	6.4-9.1	352
	S	All	5	5,2	1.7-11.6	73	4,2	3.3-5.2	31	2,3	1.6-3.3	2	1,1	0.1-4.0	0	0,0	0.0-4.7	33	2,1	1.4-2.9	111
	Others	All	34	35,1	25.6-45.4	486	27,9	25.8-30.1	314	23,7	21.4-26.0	33	18,3	13.0-24.8	4	5,2	1.4-12.8	351	22,2	20.1-24.3	871
	Total	All	97	100,0	N/A	1742	100,0	N/A	1327	100,0	N/A	180	100,0	N/A	77	100,0	N/A	1584	100,0	N/A	3423
Eastern Cape	BEIJING	All	111	38,5	32.9-44.4	34	47,9	35.9-60.1	144	50,5	44.6-56.5	115	92,7	86.7-96.6	89	94,7	88.0-98.3	348	69,2	64.9-73.2	493
	BEIJING	*Typical* [a]	*101*	*91,0*	*84.1-95.6*	*29*	*93,5*	*78.6-99.2*	*17*	*32,7*	*20.3-47.1*	*0*	*0,0*	*0.0-3.9*	*0*	*0,0*	*0.0-5.1*	*17*	*7,9*	*4.7-12.3*	*147*
	BEIJING	*R86* [a]	*10*	*9,0*	*4.4-15.9*	*2*	*6,5*	*0.8-21.4*	*35*	*67,3*	*52.9-79.7*	*93*	*100,0*	*96.1-100.0*	*71*	*100,0*	*94.9-100.0*	*199*	*92,1*	*87.7-95.3*	*211*
	BEIJING	*Typical* [b]	*101*	*35,1*	*N/A*	*32*	*44,8*	*N/A*	*47*	*16,5*	*N/A*	*0*	*0,0*	*N/A*	*0*	*0,0*	*N/A*	*47*	*9,4*	*N/A*	*180*
	BEIJING	*R86* [b]	*10*	*3,5*	*N/A*	*2*	*3,1*	*N/A*	*97*	*34,0*	*N/A*	*115*	*92,7*	*N/A*	*89*	*94,7*	*N/A*	*301*	*59,8*	*N/A*	*313*
	LAM4	All	6	2,1	0.8-4.5	0	0,0	0.0-5.1	46	16,1	12.1-20.9	5	4,0	1.3-9.2	1	1,1	0.0-5.8	52	10,3	7.8-13.3	58
	LAM4	*F15/LAM4/KZN* [c]	*2*	*0,7*	*0.1-2.5*	*0*	*0,0*	*0.0-5.1*	*45*	*15,8*	*11.8-20.6*	*5*	*4,0*	*1.3-9.2*	*1*	*1,1*	*0.0-5.8*	*51*	*10,1*	*7.6-13.1*	*53*
	LAM4	*Non-F15/LAM4/KZN* [c]	*4*	*1,4*	*0.4-3.5*	*0*	*0,0*	*0.0-5.1*	*1*	*0,4*	*0.0-1.9*	*0*	*0,0*	*0.0-2.9*	*0*	*0,0*	*0.0-3.8*	*1*	*0,2*	*0.0-1.1*	*5*
	LAM3	All	66	22,9	18.2-28.2	10	14,1	7.0-24.4	24	8,4	5.5-12.3	0	0,0	0.0-2.9	0	0,0	0.0-3.8	24	4,8	3.1-7.0	100
	T1	All	38	13,2	9.5-17.7	3	4,2	0.9-11.9	13	4,6	2.5-7.7	0	0,0	0.0-2.9	0	0,0	0.0-3.8	13	2,6	1.4-4.4	54
	S	All	7	2,4	1.0-4.9	5	7,0	2.3-15.7	9	3,2	1.5-5.9	0	0,0	0.0-2.9	1	1,1	0.0-5.8	10	2,0	1.0-3.6	22
	Others	All	60	20,8	16.3-26.0	19	26,8	16.9-38.6	49	17,2	13.0-22.1	4	3,2	0.9-8.1	3	3,2	0.7-9.0	56	11,1	8.5-14.2	135
	Total	All	288	100,0	N/A	71	100,0	N/A	285	100,0	N/A	124	100,0	N/A	94	100,0	N/A	503	100,0	N/A	862
KwaZulu-Natal	BEIJING	All	50	41,3	32.4-50.6	4	13,8	3.9-31.7	15	7,7	4.4-12.4	0	0,0	0.0-26.5	0	0,0	0.0-13.7	15	6,5	3.7-10.4	69
	BEIJING	*Typical* [a]	*41*	*100,0*	*91.4-100.0*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*41*
	BEIJING	*R86* [a]	*0*	*0,0*	*0.0-8.6*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*N/D*	*N/A*	*N/A*	*0*
	BEIJING	*Typical* [b]	*50*	*41,3*	*N/A*	*4*	*13,8*	*N/A*	*15*	*7,7*	*N/A*	*0*	*0,0*	*N/A*	*0*	*0,0*	*N/A*	*15*	*6,5*	*N/A*	*69*
	BEIJING	*R86* [b]	*0*	*0,0*	*N/A*	*0*	*0,0*	*N/A*	*0*	*0,0*	*N/A*	*0*	*0,0*	*N/A*	*0*	*0,0*	*N/A*	*0*	*0,0*	*N/A*	*0*
	LAM4	All	4	3,3	0.9-8.2	6	20,7	8.0-39.7	39	20,0	14.6-26.3	4	33,3	9.9-65.1	18	72,0	50.6-87.9	61	26,3	20.7-32.5	71
	LAM4	*F15/LAM4/KZN*	*2*	*1,7*	*0.2-5.8*	*6*	*20,7*	*8.0-39.7*	*39*	*20,0*	*14.6-26.3*	*4*	*33,3*	*9.9-65.1*	*18*	*72,0*	*50.6-87.9*	*61*	*26,3*	*20.7-32.5*	*69*
	LAM4	*Non-F15/LAM4/KZN*	*2*	*1,7*	*0.2-5.8*	*0*	*0,0*	*0.0-11.9*	*0*	*0,0*	*0.0-1.9*	*0*	*0,0*	*0.0-26.5*	*0*	*0,0*	*0.0-13.7*	*0*	*0,0*	*0.0-1.6*	*2*
	LAM3	All	19	15,7	9.7-23.4	2	6,9	0.8-22.8	10	5,1	2.5-9.2	1	8,3	0.2-38.5	2	8,0	1.0-26.0	13	5,6	3.0-9.4	34
	T1	All	12	9,9	5.2-16.7	3	10,3	2.2-27.4	27	13,8	9.3-19.5	1	8,3	0.2-38.5	2	8,0	1.0-26.0	30	12,9	8.9-17.9	45
	S	All	8	6,6	2.9-12.6	3	10,3	2.2-27.4	66	33,8	27.2-41.0	3	25,0	5.5-57.2	2	8,0	1.0-26.0	71	30,6	24.7-37.0	82
	Others	All	28	23,1	16.0-31.7	11	37,9	20.7-57.7	38	19,5	14.2-25.8	3	25,0	5.5-57.2	1	4,0	0.1-20.4	42	18,1	13.4-23.7	81
	Total	All	121	100,0	N/A	29	100,0	N/A	195	100,0	N/A	12	100,0	N/A	25	100,0	N/A	232	100,0	N/A	382
All	Total	All	506	N/A	N/A	1842	N/A	N/A	1807	N/A	N/A	316	N/A	N/A	196	N/A	N/A	2319	N/A	N/A	4667

DS: Drug-sensitive; DR: Non-multidrug-resistant drug-resistant isolates (also commonly referred to as mono- and poly-resistant isolates);
MDR *s.s.* : Multidrug-resistant *sensu stricto* (excluding all identified pre-extensively drug-resistant and extensively drug-resistant isolates);
pre-XDR: Pre-extensively drug-resistant isolates; XDR: Extensively drug-resistant isolates; MDR: Multidrug-resistant (including MDR *s.s.* , pre-XDR and XDR-TB cases).
N: Number of isolates; %: Proportion of isolates; 95% CI: 95% confidence interval N/A: Not applicable; N/D: No data;

[a]A subset of Beijing strains was further categorized. The number of isolates identified as members of the typical or atypical Beijing subfamily is indicated. Proportions in this case relate to the number of Beijing isolates characterized.

[b]Extrapolated number for typical and atypical Beijing isolates. The sum of the extrapolated number of isolates corresponds to the total number of Beijing isolates.

[c]Differentiation into F15/LAM4/KZN and non-F15/LAM4/KZN based on spoligotype pattern, only (for isolates from KwaZulu-Natal, IS*6110* RFLP analysis was used in addition)

Chapter 3 Emergence and spread of extensively and totally drug-resistant tuberculosis in the Eastern Cape

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What has been is what will be, and what has been done is what will be done and there is nothing new under the sun. – Ecclesiastes 1:9

Emergence and spread of Extensively and Totally Drug Resistant Tuberculosis in South Africa

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Abstract

Factors driving the increase in drug-resistant tuberculosis (TB) in the Eastern Cape, South Africa are not understood. A convenience sample of 309 drug-susceptible and 342 multidrug-resistant (MDR)-TB isolates, collected between July 2008 and July 2009, were characterised by spoligotyping, DNA fingerprinting, insertion site mapping and targeted DNA sequencing. Analysis of the molecular-based data showed diverse genetic backgrounds among drug-sensitive and MDR sensu stricto isolates, as opposed to restricted genetic backgrounds among pre-extensively drug-resistant (pre-XDR)-TB and XDR-TB isolates. Second-line drug resistance was associated with the atypical Beijing genotype ($p<0.001$). DNA fingerprinting and sequencing demonstrated that the pre-XDR and XDR atypical Beijing isolates had evolved from a common progenitor with 85% and 92%, respectively,

being clustered, indicating transmission. Ninety-five percent of the atypical XDR Beijing isolates had mutations known to confer resistance to 10 anti-TB drugs, while additional resistance to para-aminosalicylic acid suggests emergence of totally drug-resistant TB.

3.1 Introduction

The emergence of drug-resistant tuberculosis (TB) is of major concern to TB control in South Africa. A country-wide survey in 2002 revealed that 1.8% of all new TB cases and 6.7% of previously treated TB cases had multidrug-resistant (MDR)-TB (resistant to at least isoniazid and rifampicin) (1). This translates to an estimated annual case load of 13 000 MDR-TB cases, placing South Africa fourth among the world's high-burden MDR-TB countries (1). However, this may be an under-estimation as two recent studies (2, 3) suggested that the proportion of MDR-TB cases may be significantly higher than the WHO estimate (3). Alarmingly, only 4143 of the 9070 patients (46%) diagnosed with MDR-TB in 2009 were started on treatment, possibly due to resource constraints, creating a situation where control was bound to fail (4). This is supported by the diagnosis of 594 extensively drug resistant (XDR)-TB cases (MDR plus additional resistance to a fluoroquinolone and any second-line injectable drug) in that year (4). The cure rate of patients with drug-resistant TB is less than 50% for MDR-TB cases (5), while culture conversion was observed in only 19% of XDR-TB cases during the follow-up period, irrespective of HIV status (6).

The majority of MDR-TB and XDR-TB cases in South Africa are detected in KwaZulu-Natal, the Western Cape and the Eastern Cape provinces (4). Statistics from the Eastern Cape showed the largest increase in the number of MDR-TB cases, rising from 836 cases in 2006 to 1858 cases in 2009 (2.2 fold increase) (4). The reason for this dramatic increase in MDR-TB cases remains to be determined.

Molecular epidemiological data from the neighbouring Western Cape province have demonstrated that MDR-TB is primarily transmitted (7, 8), accounting for nearly 80% of reported MDR-TB cases (2). To date, only one molecular epidemiological study has been reported for the Eastern Cape (9), showing that 50% of rifampicin-resistant TB isolates (including MDR-TB cases) belonged to the Beijing genotype with "atypical" Beijing strains being significantly overrepresented. These strains

harboured rare mutations in the *inhA* promoter (G-17A) and *rpoB* gene (GAC516GTC), which have previously been associated with a high fitness cost (10). The authors demonstrated that the spread of these strains was facilitated by HIV co-infection, thereby raising concern for the spread of drug-resistant strains in vulnerable populations (9).

A recent epidemiological study conducted in the Eastern Cape estimated that 75.6% of XDR-TB cases with complete data were due to on-going transmission (11). Treatment outcomes were dismal with 58% of cases dying within one year, while culture conversion was observed in only 8.4% of cases after 143 days of treatment (11), raising concern that these patients are infected with an untreatable form of TB. This situation is similar to the Tugella Ferry outbreak in KwaZulu-Natal (12), which emphasised the need for improved basic control measures, including rapid diagnostics and infection control (13).

This study aimed to describe the *Mycobacterium tuberculosis* strain population structure among MDR-TB and XDR-TB cases in the Eastern Cape Province of South Africa in order to determine whether the epidemic is driven by acquisition or transmission of resistance and to describe the extent of resistance within these strains. It is envisaged that these findings will inform TB control to implement measures to curb either emergence or spread of drug-resistance.

3.2 Materials & Methods

3.2.1 Study population

Sputum specimens were collected from high risk TB suspects (previously treated cases and close contacts of known drug resistant cases) in accordance with the National TB Control program. Specimens that were collected at health care facilities in the Eastern Cape were submitted to the National Health Laboratory Service (NHLS) in Port Elizabeth for TB drug susceptibility testing (DST). From July 2008 to July 2009 a convenience sample of sputum cultures shown to be either fully drug-susceptible or resistant to at least isoniazid and rifampicin (MDR-TB) by the NHLS were submitted to Stellenbosch University for subsequent genotyping. Only limited demographic and clinical data was available for each patient: unique identifier (assigned by the NHLS), sputum date, clinic/hospital of

origin and routine DST pattern. The unique identifier was used to identify the first available isolate from 309 drug-susceptible and 342 MDR-TB cases included in the study. This study was approved by the ethics committee of Stellenbosch University, Faculty of Health Sciences (N09/11/296).

3.2.2 Drug susceptibility testing

Sputum samples were processed by the NHLS for routine TB diagnosis by smear microscopy and culture. Each sputum specimen was decontaminated using the standard NALC-NaOH method and cultured in MGIT960 medium until a positive growth index was observed. The presence of *M. tuberculosis* was confirmed by spoligotyping (see below). DST was done by the indirect proportion method using the BACTEC MGIT 960 system (BD Bioscience, Sparks, MD) according to the manufacturer's instructions. Initially, resistance against isoniazid and rifampicin was tested, followed by testing for streptomycin and ethambutol resistance if the isolate was resistant to either isoniazid or rifampicin. Second-line drug susceptibility testing was done on 7H10 medium containing 2µg/ml ofloxacin, 4µg/ml amikacin or 5µg/ml ethionamide. DST for para-aminosalicylic acid was done at Stellenbosch University in MGIT 960 medium containing 4.0µg/ml, 8µg/ml and 16µg/ml para-aminosalicylic acid (14).

3.2.3 Molecular-based analysis

Crude DNA was prepared by boiling a 200µl aliquot of a positive MGIT culture and this was used as template for subsequent PCR analysis (15). Each isolate was spoligotyped using the internationally standardized method (16) and grouped into genotypes according to previously described spoligotype signatures (17). Beijing genotype strains were sub-classified as either "typical" or "atypical" according to the presence or absence of an IS*6110* insertion in the NTF region (18),(19). The atypical Beijing genotype strains were further classified by using the internationally standardized IS*6110* DNA fingerprinting method (20). In atypical Beijing strains that were drug-sensitive according to DST, sensitivity to isoniazid and rifampicin was confirmed by sequencing of the katG and rpoB genes. In MDR atypical Beijing strains, mutations conferring resistance to isoniazid, rifampicin,

ethambutol, pyrazinamide, ofloxacin, streptomycin, amikacin, kanamycin and capreomycin were identified by sequencing of the *inhA* promoter, *katG*, *rpoB*, *embB*, *pncA*, *gyrA*, and *rrs* genes, respectively (21, 22). Isolates were grouped as either MDR-TB *sensu stricto* (*s.s.*, MDR strains

Table 1: Spoligotype classification of drug-sensitive and MDR-TB isolates

Spoligotype family	ST* Number	Culture-based DST				Molecular-based DST		
		Sensitive (%)	MDR*ss* † (%)	Pre-XDR‡ (%)	XDR§ (%)	MDR*ss* (%)	Pre-XDR (%)	XDR (%)
Atypical Beijing¶	1	11 (3.6)	41 (27.0)	98 (92.5)	78 (92.9)	29 (22.5)	85 (87.6)	103 (95.4)
Typical Beijing¶	1	108 (35.0)	19 (12.5)	0 (0)	0 (0)	18 (14.0)	1 (1.0)	0 (0)
H	36; 47; 50; 62	7 (2.3)	2 (1.3)	1 (0.9)	0 (0)	2 (1.6)	1 (1.0)	0 (0)
LAM3	33; 130; 211	66 (21.4)	12 (7.9)	2 (1.9)	0 (0)	12 (9.3)	0 (0)	0 (0)
LAM4	60; 811	6 (1.9)	32 (21.1)	4 (3.8)	2 (1.9)	29 (22.5)	3 (3.1)	2 (1.9)
LAM (other)	4; 20; 42; 398	7 (2.3)	1 (0.7)	0 (0)	1 (1.2)	1 (0.8)	0 (0)	1 (0.9)
MANU2	1247	0 (0)	0 (0)	0 (0)	2 (2.4)	1 (0.8)	0 (0)	1 (0.9)
S	34; 71	8 (2.6)	8 (5.3)	0 (0)	1 (1.2)	8 (6.2)	0 (0)	1 (0.9)
T	44; 53; 73; 254; 926; 1240	51 (16.5)	18 (11.8)	0 (0)	0 (0)	13 (10.1)	5 (5.2)	0 (0)
U	443; 519; 790	1 (0.3)	2 (1.3)	0 (0)	0 (0)	2 (1.6)	0 (0)	0 (0)
X	18; 92; 119; 1751	6 (1.9)	3 (2.0)	0 (0)	0 (0)	3 (2.3)	0 (0)	0 (0)
CAS	21; 26; 1092	4 (1.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Orphan	Not assigned	34 (11.0)	14 (9.2)	1 (0.9)	0 (0)	11 (8.5)	2 (2.1)	0 (0)
Total		309	152	106	84	129	97	108
Total MDR				342			334#	

* ST - Shared Type *(17)*; † MDR*s.s.* – Multidrug-resistant *sensu strictu*; ‡ Pre-XDR – Pre-extensively drug-resistant; § XDR – extensively drug-resistant; ¶ For Beijing isolates a distinction was made between typical and atypical based on the presence or absence of an IS*6110* insertion in the NTF region (*18*),(*19*); # Molecular-based DST total differs from culture-based DST total, because some results were not available.

excluding identified pre-XDR (MDR plus additional resistance to either a fluoroquinolone or any second-line injectable anti-TB drug) (23) and XDR strains), pre-XDR-TB or XDR-TB according to high confidence mutations. This method of grouping was selected given that routine drug susceptibility testing was not done for all of the anti-TB drugs on all of the isolates. Furthermore, a poor correlation was observed between high confidence mutations and routine second-line DST (see Results). Isolates were considered to belong to the same cluster (implying on-going transmission) if identical mutations were observed in all of the genes sequenced.

3.3 Results

A convenience sample of 309 drug-sensitive and 342 MDR-TB isolates from patients from the Eastern Cape Province was collected during the study period. These were submitted to Stellenbosch University for molecular-based analysis. Analysis of the population structure of these isolates by spoligotyping identified 52 and 29 different spoligotype patterns among drug-sensitive and MDR-TB strains, respectively. Among drug-sensitive and MDR isolates, 22/52 and 14/29 spoligotype patterns were previously recorded in the SpolDB4 database. These represented 275/309 (89.0%) of drug-sensitive and 327/342 (95.6%) of MDR isolates. It is noteworthy that 84% of MDR isolates constituted only 3 different spoligotypes (Table 1), namely Beijing, LAM3 and LAM4. This is an indication of transmission of these strains.

Table 1 shows the breakdown of spoligotypes against the degree of drug resistance, where drug resistance is expressed as either culture-based or molecular-based DST. In this study, we used molecular-based DST to define the extent of drug-resistance in routinely diagnosed MDR-TB isolates. The *katG* or *rpoB* regions of 8 isolates could not be amplified and could therefore not be classified according to molecular based DST. Accordingly, 119 (38.5%) drug-sensitive and 236 (69%) of the MDR-TB patients were infected with a Beijing genotype strain. Sub-classification of Beijing genotype strains showed that 11/119 (9.2%) drug-sensitive and 217/236 (91.9%) MDR strains belonged to the "atypical" subgroup of the Beijing genotype as indicated by the absence of an IS*6110* element in the NFT region.

Analysis of mutations conferring resistance to first and second-line anti-TB drugs allowed grouping of the MDR isolates: 136 MDR *s.s.*, 98 pre-XDR and 108 XDR. Using these groupings, isolates with a

higher degree of resistance, were more likely to have an atypical Beijing genotype (drug sensitive: 11/309 (3.6%, 95% CI: 1.8%-6.3%); MDR *s.s.*: 29/136 (21.3%, 95% CI: 14.8%-29.2%) vs pre-XDR: 85/98 (86.7%, 95% CI: 78.4%-92.7%) vs XDR: 103/108 (95.4%, 95% CI: 89.5%-98.5%)).

DNA sequencing data for the first available isolate from each patient infected with an MDR atypical Beijing strain (n = 217) and IS*6110* fingerprinting for a subset of these isolates (n = 110) was analysed to establish whether the over-abundance of the atypical Beijing genotype among patients with pre-XDR-TB and XDR-TB strains reflected on-going transmission. IS*6110* DNA fingerprinting showed that all of these patients were infected with closely related atypical Beijing strains with only minor differences in the banding patterns (Figure 1 and 2), thereby suggesting clonal dissemination.

Table 2 shows that 216/217 (99.5%) of the MDR atypical Beijing genotype strains harboured an identical *katG* (AGC315ACC) mutation, while 209/217 (94.9%) had a distinctive *rrs* (A513C) gene mutation. This suggests that these mutations were acquired prior to dissemination. Subsequently, resistance to rifampicin, ethambutol, pyrazinamide, amikacin and ofloxacin was acquired in various combinations. Of the 29 atypical Beijing MDR *s.s*. isolates, 22 (75.9%) were grouped into 4 clusters according to mutations (mutation pattern, MP) in the *inhA* promoter and *katG*, *rpoB*, *embB*, *pncA*, *rrs* and *gyrA* genes (cluster size ranged from 3 to 12 cases; Table 2: MP2, MP17, MP32, MP34) while 7 had unique mutation patterns (Table 2: MP23, MP25, MP30, MP31, MP41, MP44, MP48). Similarly, the 85 atypical pre-XDR Beijing isolates showed 11 different mutation patterns, of which 81 (95.3%) were clustered into 7 clusters (cluster size ranged from 2 to 62 cases; Table 2: MP3, MP5, MP18, MP26, MP28, MP35, MP38). The genotype of the largest pre-XDR-TB cluster was characterised by an *inhA* promoter mutation at position -17 and the *katG* AGC315ACC, *rpoB* GAC516GTC, *embB* ATG306ATA, *rrs* A513C and *rrs* A1401G nucleotide substitutions as well as an insertion in the *pncA* gene at position 172G. This mutation pattern was characteristic of 79% (81/103) of the atypical Beijing XDR-TB isolates and for ease of reference will be called MP5 (see Table 2). By contrast, only 3 of the 29 atypical Beijing MDR *s.s.* isolates showed the same mutation pattern for these genes excluding the *rrs*A1401G mutation (MP2). Ten different atypical XDR Beijing mutation patterns emerged from the MP5 progenitor by mutation in the *gyrA* gene. Of these, 6 mutation patterns showed clustering (cluster size ranged from 2 to 46 cases, MP6-11) and 4 had unique mutations conferring ofloxacin resistance (MP12-16). The presence of clustering among both the pre-XDR and XDR genotypes suggests transmission following acquisition of additional resistance. Of the remaining 22 atypical XDR Beijing isolates, 12 distinct resistance mutation patterns were observed, of which 11 isolates were clustered (MP27) and 11 had unique genotypes (MP19-22, MP24, MP29, MP39-40, MP42-43, MP47).

Table 2: Geographical distribution of atypical Beijing genotype isolates and their mutation patterns

Number of isolates harbouring a defined mutation in genes known to confer drug resistance

katG	*rrs*	*inhA* promoter	*embB*	*pncA*	*rpoB*	*rrs*	*gyrA*	MP†	DR‡	District municipalities where patients were diagnosed
315ACC	513CAC	G-17A	306ATA	WT	516GTC	A1401G	WT	MP1	Pre-XDR	OT
216	209	162	161	1	1	1	1			
				Ins172G	516GTC	WT	WT	MP2	MDR¶	AM, NMB
				159	149	6	3			
							94GGC	MP3	Pre-XDR	NMB,CC
							2			
							94GCC	MP4	Pre-XDR	NMB
							1			
						A1401G	WT*	MP5	Pre-XDR	AM, NMB, CC, OT§§
						143	62			
							94GGC	MP6	XDR	AM, NMB, CC††, CH‡‡
							47			
							94AAC	MP7	XDR	AM, NMB
							10			
							94CAC	MP8	XDR	AM, NMB
							12			
							94GCC	MP9	XDR	NMB
							2			
							94TAC	MP10	XDR	AM, NMB
							4			
							90GTG	MP11	XDR	AM
							2			
							Unique mutations	MP12-16	XDR§	AM#, NMB**
							5			
					516TCC	WT	WT	MP17	MDR	AM, CH
					10	4	4			
						A1401G	WT*	MP18	Pre-XDR	AM, OT
						6	2			
							Unique mutations	MP19-22	XDR	NMB
							4			
				14CGC	516TCC	WT	WT	MP23	MDR	AM
				1	1	1	1			
			306ATC	Ins172G	516TCC	A1401G	94GGC	MP24	XDR	NMB
			1	1	1	1	1			

*WT – Wild Type; †MP – mutation pattern; ‡DR – Drug-resistance; §XDR – extensively drug-resistant tuberculosis; ¶MDR – mutidrug-resistant tuberculosis; # AM – Amathole; **NMB - Nelson Mandela Bay; ††CC – Cacadu; ‡‡CH - Chris Hani; §§OT - OR Tambo Continued…

Table 2 continued: Geographical distribution of atypical Beijing genotype isolates and their mutation patterns

Number of isolates harbouring a defined mutation in genes known to confer drug resistance

katG	*rrs*	*inhA* promoter	*embB*	*pncA*	*rpoB*	*rrs*	*gyrA*	MP†	DR‡	District municipalities where patients were diagnosed
		-15	306ATC	14CGC	531TTG	WT	WT	MP25	MDR¶	AM
		20	17	17	17	1	1	MP26	Pre-XDR	AM
						A1401G	WT*	MP27	XDR§	AM#, NMB**, OT††, AN‡‡
						16	5	MP28	Pre-XDR	NMB
							90GTG	MP29	XDR	NMB
							11	MP30	MDR	NMB
			306GTG	31AGC	531TTG	WT	90GTG	MP31	MDR	NMB
			3	2	3	2	3	MP32	MDR	NMB
				WT		A1401G		MP33	Pre-XDR	CH§§
				1		1		MP34	MDR	AM, NMB, OT, AN
		WT	WT	14GCG	531TTG	WT	WT	MP35	Pre-XDR	NMB
		27	1	1	1	1	1	MP36-37	Pre-XDR	AM,NMB
			306ATA	14CGC	531TTG	WT	WT	MP38	Pre-XDR	AM, NMB
			1	1	1	1	1	MP39-40	XDR	AM, NMB
			306ATC	WT	526TAC	WT	WT	MP41	MDR	NMB
			25	3	3	3	3	MP42	XDR	NMB
				14CGC	516GTC	WT	88TGC	MP43	XDR	CC
				22	1	1	1	MP44	MDR	NMB
					531TTG	WT	WT	MP45	pre-XDR	CC¶¶
					21	17	12	MP46	Pre-XDR	AM
							91CCG	MP47	XDR	AM
							3	MP48	MDR	NMB
							Unique mutations			
							2			
						A1401G	WT			
						4	2			
							Unique mutations			
							2			
	WT	WT	WT	WT	516TAC	WT	WT			
	7	6	4	4	2	1	1			
						A1401G	94GCC			
						1	1			
					MIX	MIX	94GCC			
					1	1	1			
					531TTG	WT	WT			
					1	1	1			
			306ATA	34TAG	516TAC	WT	MIX			
			2	2	2	1	1			
						A1401G	WT			
						1	1			
		-15	WT	WT	531TTG	A1401G	94GCC			
		1	1	1	1	1	1			
WT	WT	-15	WT	NR	531TTG	WT	WT			
1	1	1	1		1	1	1			

*WT – Wild Type; †MP – mutation pattern; ‡DR – Drug-resistance; §XDR – extensively drug-resistant tuberculosis; ¶MDR – mutidrug-resistant tuberculosis; # AM – Amathole; **NMB - Nelson Mandela Bay; ††CC – Cacadu; ‡‡CH - Chris Hani; §§OT - OR Tambo

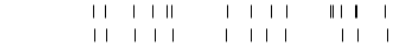
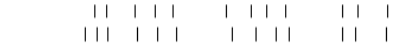
IS*6110* DNA fingerprint pattern	n	Mutation pattern	Cluster	Clinic	District Municipality
	1	MP6	12050	Empilweni	NMB
	1	MP8	12050	Mdantsane	AM
	1	MP6	12051	New Brighton	NMB
	1	MP6	12051	Chatty	NMB
	1	MP9	12052	Mabandla	NMB
	1	MP10	12053	Motherwell	NMB
	1	MP8	12054	Soweto	NMB
	1	MP6	12068	Chatty	NMB
	3	MP8	12055	Jose Pearson	NMB
	1	MP8	12055	Silvertown	NMB
	2	MP8	12055	Schauder	NMB
	1	MP8	12055	Chatty	NMB
	3	MP8	12055	Kwazakhele	NMB
	1	MP8	12055	Fort Grey	AM
	1	MP8	12055	Ezibeleni	AM
	1	MP8	12055	Port Alfred	CC
	1	MP5	12055	St Albans Prison	NMB
	1	MP5	12055	Govan Mbeki	NMB
	1	MP6	12055	Masakhane	NMB
	1	MP6	12055	Tshangana	NMB
	1	MP7	12055	Mdantsane	AM
	1	MP17	12055	Empilweni	NMB
	1	MP5	12029	Empilweni	NMB
	1	MP8	12020	Jose Pearson	NMB
	1	MP47	12021	Nkqubela	AM
	1	MP43	12014	Themba	NMB
	1	MP2	86	Frankfort	AM
	1	MP5	86	Jose Pearson	NMB
	1	MP5	86	Schauder	NMB
	1	MP6	86	Wells Estate	NMB
	1	MP8	86	Motherwell	NMB
	1	MP8	86	Kwazakhele	NMB
	1	MP8	86	Zwide	NMB
	1	MP8	86	Walmer	NMB
	1	MP8	86	Kwamagxaki	NMB
	1	MP8	86	Ndevana	AM
	1	MP8	86	Mdantsane	AM
	1	MP8	86	Glenmore	AM
	1	MP8	86	Nkwenkwezi	CC
	1	MP10	86	Openshaw	AM
	1	MP19	86	Kwazakhele	NMB
	1	MP24	86	Jose Pearson	NMB
	1	MP25	86	Zanempilo, Bisho	NMB
	1	MP25	86	Nu	AM
	1	MP25	86	Nkqubela	AM
	1	MP32	86	Mdantsane	AM
	1	MP8	11973	Lunga Kobese	NMB
	1	MP8	12040	Empilweni	NMB
	1	MP8	12011	Walmer	NMB
	1	MP6	12018	Fort Grey	AM
	1	MP29	12017	Motherwell	NMB
	1	MP5	11846	Frankfort	AM
	1	MP8	11846	Motherwell	NMB
	1	MP8	11846	Wells Estate	NMB
	1	MP8	11846	Jama	AM
	1	MP18	11846	Rosedale	NMB
	1	MP8	12009	Motherwell	NMB
	1	MP8	12043	Nompumelelo	AM
	1	MP8	12043	Lizo Ngcana	NMB
	1	MP8	12043	Motherwell	NMB
	1	MP25	12043	Gompo	AM
	1	MP25	12043	Mt Frere, Trnsk	OT
	1	MP10	12022	Walmer	NMB
	1	MP8	12022	Fort Grey	AM
	1	MP8	12023	Fort Grey	AM
	2	MP10	12028	Walmer	NMB
	1	MP4	11846	Port Elizabeth	NMB
	1	MP1	11845	Ginsberg	AM
	1	MP8	11819	Jose Pearson	NMB
	1	MP8	11838	Kwazakhele	NMB

Figure 1: IS*6110* DNA fingerprint patterns of a subset (63/85) of atypical Beijing pre-XDR-TB isolates and their geographical origin. MP, Mutation pattern; NMB, Nelson Mandela Bay; AM, Amathole; CC, Cacadu; OT, Oliver Tambo

IS*6110* DNA fingerprint pattern	n	Mutation pattern	Cluster	Clinic	District Municipality
	1	MP11	11846	Empilweni	NMB
	1	MP11	11822	Uitenhage	NMB
	1	MP11	11822	Chatty	NMB
	1	MP11	11848	Winterberg	AM
	1	MP11	12041	Nkqubela	AM
	1	MP11	12057	Alicedale	CC
	1	MP11	11252	Dimbaza	AM
	1	MP11	12030	Booyens Park	NMB
	1	MP11	12055	Helenvale	NMB
	1	MP11	12055	West End	NMB
	2	MP11	12055	Zwide	NMB
	1	MP11	12055	Kwamagxaki	NMB
	1	MP11	12055	Tanduxolo	NMB
	1	MP11	12055	Kirkwood	CC
	1	MP11	12055	Temba	CC
	1	MP11	12055	Marselle	CC
	1	MP11	12055	Canzibe	OT
	1	MP11	12055	Nolita	OT
	1	MP11	12065	Temba	CC
	1	MP11	12061	Isolomzi	NMB
	1	MP11	86	Tanduxolo	NMB
	2	MP11	86	Booyens Park	NMB
	1	MP11	86	New Brighton	NMB
	1	MP11	86	Uitkyk	NMB
	2	MP11	86	Walmer	NMB
	2	MP11	86	Empilweni	NMB
	1	MP11	86	Motherwell	NMB
	1	MP11	86	Govan Mbeki	NMB
	1	MP11	86	Soweto, PE	NMB
	1	MP11	86	Nkqubela	AM
	1	MP11	86	Sweetwaters	AM
	1	MP11	86	Bezville	AM
	1	MP11	86	Temba	CC
	1	MP11	86	Korsten, PE	CC
	1	MP11	12060	Motherwell	NMB
	1	MP11	12024	Zwide	NMB
	1	MP11	12063	Max Madlingozi	NMB
	1	MP11	11846	Tshabo	AM
	1	MP11	11846	Mabandla	NMB
	1	MP11	11840	Max Madlingozi	NMB
	1	MP11	11840	Walmer	NMB

Figure 2: IS*6110* DNA fingerprint patterns of a subset (81/103) of atypical Beijing XDR-TB isolates and their geographical origin. MP, Mutation pattern; NMB, Nelson Mandela Bay; AM, Amathole; CC, Cacadu; OT, Oliver Tambo

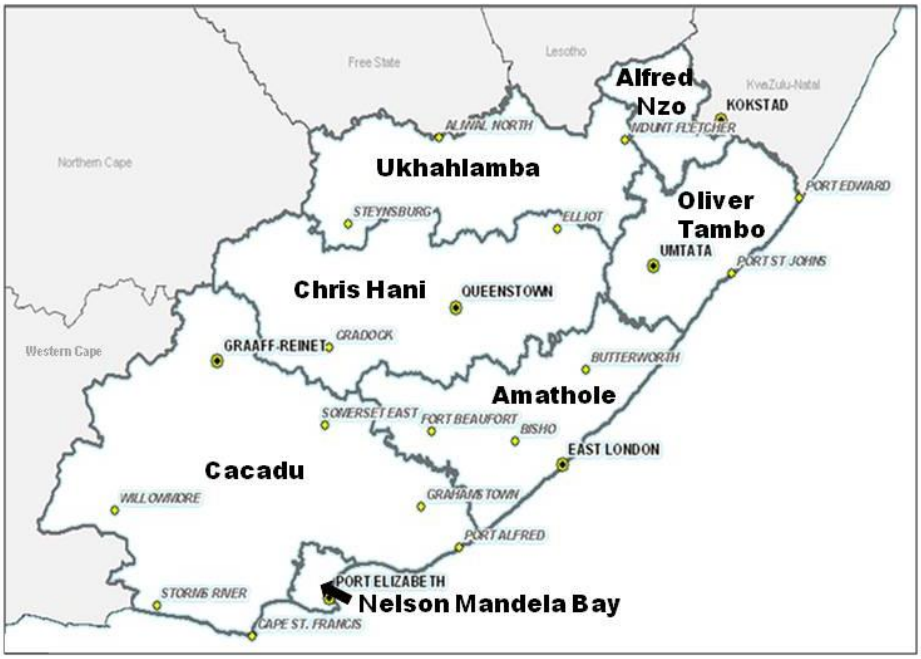


Figure 3: Map showing district municipalities in the Eastern Cape.

Spatial analysis of the patient origins showed that pre-XDR and XDR isolates with an atypical Beijing genotype were present in 5 of 8 district municipalities (Figure 3, Table 2). The largest atypical pre-XDR Beijing genotype cluster (MP5) was identified in 4 adjacent district municipalities (Table 2), while the largest XDR-TB cluster (MP6) was identified in 3 of these districts as well as an additional district, suggesting historic spread of these genotypes.

Table 3: Correlation of culture-based and molecular-based Drug-susceptibility testing among atypical Beijing isolates

Drug/gene	CB-DST* R† MB-DST‡ R	CB-DST S§ MB-DST S	CB-DST R MB-DST S	CB-DST S MB-DST R	Total	Correlation
INH¶/*katG*	*217*	9	1	0	227	99.6%
RIF#/*rpoB*	219	9	0	0	228	100%
STR**/*rrs*500	191	6	2	16	215	91.60%
EMB††/*embB*	56	5	2	152	215	28.40%
ETH‡‡/*inhA* promoter	76	25	5	86	192	52.60%
OFL§§/*gyrA*	78	93	0	29	200	85.50%
AMK¶¶/*rrs*1400	167	32	7	9	215	92.60%
CAP##/*rrs*1400	21	38	1	155	215	27.40%

* CB-DST – culture-based drug susceptibility testing; †R – resistant; ‡MB-DST – molecular-based DST; §S – sensitive; ¶INH – Isoniazid; #RIF – Rifampicin; **STR – Streptomycin; ††EMB – Ethambutol; ‡‡ETH – Ethionamide; §§OFL – Ofloxacin; ¶¶AMK – Amikacin; ##CAP – Capreomycin

The presence of mutations in target genes known to confer resistance with high confidence suggested 95.1% (98/103) of the atypical XDR Beijing isolates were resistant to at least ten anti-TB drugs: isoniazid, rifampicin, ethambutol, pyrazinamide, streptomycin, amikacin, kanamycin, capreomycin, ethionamide and ofloxacin. The extent of drug resistance in these isolates was under-estimated by routine DST (Table 3). The correlation between molecular-based drug-resistance and routine culture-based DST was 99.6% for isoniazid, 100% for rifampicin, 28% for ethambutol, 92% for streptomycin, 93% for amikacin, 27% for capreomycin, 52% for ethionamide and 86% for ofloxacin (Table 3). Routine DST for pyrazinamide, kanamycin, cycloserine and para-aminosalicylic acid was not determined. DST for para-aminosalicylic acid was done at Stellenbosch University on 45 isolates of which 9 showed resistance at a level of > 4.0µg/ml.

3.4 Discussion

Review of routine DST results highlights the severity of the drug-resistant TB epidemic in South Africa (4), and thereby emphasises the urgent need to curb the rising incidence of drug resistance in the country. This can only be achieved by the implementation of appropriate intervention strategies which are based on knowledge of the mechanisms fuelling this epidemic. Recently, molecular epidemiological techniques have been used in combination with classical epidemiological data to enhance our understanding of the TB epidemic in different settings. Those studies have quantified the relative proportion of acquisition versus transmission, and have described the population structure of *M. tuberculosis* over time (7, 9, 21, 23, 24). Using these approaches we show that patients with MDR-TB in the Eastern Cape could be divided into two distinct groups: patients infected with MDR-TB *s.s.* showed diverse genetic backgrounds, while patients infected with pre-XDR-TB and XDR-TB showed restricted genetic backgrounds.

The finding that the pre-XDR-TB and XDR-TB strains are genetically distinct when compared to the MDR-TB *s.s.* strains is counterintuitive as we would expect all MDR-TB strains to have had an equal chance of acquiring resistance to second-line anti-TB drugs. The absence of second-line resistance among a large number of different MDR-TB genotypes suggests that under the current MDR-TB treatment regimen acquisition of additional resistance in MDR *s.s.* strains is reduced. Conversely, analysis of the DNA sequencing data showed a significant association between the atypical Beijing genotype and mutations conferring second-line resistance. This demonstrates that this genotype has acquired resistance to the level of pre-XDR-TB, which in turn has spread and thereafter has acquired additional resistance to the level of XDR-TB followed again by transmission. An alternative

explanation would be that the atypical Beijing genotype acquires resistance conferring mutations more readily than other genotypes. However, it is highly unlikely that the convergent evolution of seven different mutations would occur within a single genotype.

Spatial analysis of the pre-XDR-TB cases infected with this clone shows a wide distribution suggesting that this genotype has been in circulation for an extended period. This was further supported by the analysis of the evolutionary order in which resistance was acquired (Table 2) which showed that the ancestral clone first acquired resistance to isoniazid and streptomycin. This could be explained by the treatment regimen used in the early 1960s, which was based on the combination of isoniazid and streptomycin (26). A similar conclusion was drawn from whole genome sequence data, which predicted that mutations conferring resistance to isoniazid and streptomycin were deeply rooted in the atypical Beijing genotype (27).

Given the extent of resistance in pre-XDR-TB strains and the extremely limited treatment options available, it is inevitable that the emergence of ofloxacin resistance would follow. This was supported by our molecular-based analysis of the XDR-TB isolates which demonstrates that resistance to a fluoroquinolone had been acquired independently on a number of different occasions (several different *gyrA* mutations were observed) followed by amplification through transmission (clustering of XDR phenotypes was observed). However, the true extent of acquisition may be higher than predicted given that the XDR-TB isolates were cultured from patients who resided in different district municipalities and contact was unlikely due to the long distances.

We suggest that the absence of routine second-line drug susceptibility testing and treatment of MDR-TB with an inadequate standardized regimen according to the 2002 guidelines (6 months intensive phase: kanamycin, ethionamide, pyrazinamide, ofloxacin, and cycloserine or ethambutol; 12–18 months continuation phase: ethionamide, ofloxacin and cycloserine or ethambutol) (28) may have led to the inappropriate treatment of undiagnosed pre-XDR-TB cases. This would have prolonged the period of infectiousness leading to transmission to close contacts as well as increasing the risk of amplification of resistance (28,29). This has been recently addressed with the implementation of a revised treatment regimen (28) as well as routine second-line DST which is now done on all isolates shown to be resistant to rifampicin. However, these tests are culture-based, exacerbating diagnostic delay and possible transmission. This can be partially resolved with the implementation of a genetic-based second-line drug susceptibility test (29). However, the extent of resistance associated with the atypical Beijing genotype makes treatment options extremely difficult

as these isolates are resistant to all first-line anti-TB drugs (isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin) and many of the second-line drugs (amikacin, kanamycin, ofloxacin, ethionamide capreomycin). A limited number of isolates were also resistant to para-aminosalicylic acid. This suggests that the atypical Beijing genotype clone is evolving towards total drug resistance (TDR, defined as in vitro resistance to all first-line drugs, as well as aminoglycosides, cyclic polypeptides, fluoroquinolones, thioamides, serine analogues and salicylic acid derivatives (30)).

Our molecular-based results are in accordance with a recent study from the Eastern Cape, which documented extremely poor treatment outcomes for XDR-TB cases (11). The authors showed high mortality (58.4%) and low culture conversion rates (8.4%) over a follow-up period of 143 days. They concluded that only 1.7 drugs per patient was regarded as "effective", based on DST results and/or previous treatment records. Given that this study was conducted concurrently with ours, it is highly likely that a large proportion of their patients were also infected with XDR-TB strains with an atypical Beijing genotype. Thus the poor treatment outcome may be related to the extent of drug-resistance, however, we cannot exclude the possibility that the atypical Beijing genotype contributes to morbidity and mortality. A further concern is the observation that this clone is now spreading to other provinces in South Africa, possibly due to migration. In the Western Cape, an estimated 55% of XDR-TB cases harbour isolates with the atypical Beijing genotype (32).

We acknowledge that this study has a number of limitations. Firstly clinical data was not available for this study and therefore it was not possible to establish the impact of drug resistance on treatment outcome. However, we do not believe that the strains reported by Kvasnovsky et al (11) differ from those analysed in this study as both studies were conducted concurrently. Secondly, our analysis of a convenience sample may have led to an over estimation of the proportion of pre-XDR-TB and XDR-TB cases in the Eastern Cape Province. Thirdly, our use of mutational data to categorise patients as MDR *s.s.*, pre-XDR and XDR is not the accepted standard. However, genetic DST has been endorsed by the WHO for first-line anti-TB drugs and there is mounting evidence that high confidence mutations accurately predict second-line drug resistance (33).

The diagnostic dilemma facing TB control managers in the Eastern Cape is how to rapidly identify cases at risk of harbouring the atypical Beijing genotype to prioritize DST, ensure isolation and administer appropriate treatment. Previous studies have shown a strong association between *inhA* promoter mutations and preXDR-TB and XDR-TB (34). Given that the Genotype® MTBDR*plus* test (35) has been implemented as the diagnostic standard in most NHLS laboratories in South Africa we

propose that this test could be used as a rapid screening tool to identify patients harbouring drug resistant atypical Beijing strains (34). It is therefore essential that TB control managers make use of this information in an attempt to contain the spread of this virtually untreatable form of TB.

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Chapter 4 The value of selected drugs in the treatment of XDR-TB patients in the Eastern Cape

The work in this chapter has been published as two peer-reviewed publications.

1. **Mutations in the *rrs* A1401G gene and phenotypic resistance to amikacin and capreomycin in *Mycobacterium tuberculosis*.**

Sirgel FA*, Tait M*, Warren RM, Streicher EM, Böttger EC, van Helden PD, Gey van Pittius NC, Coetzee G, Hoosain EY, Chabula-Nxiweni M, Hayes C, Victor TC, Trollip A.

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2. **The rationale for using rifabutin in the treatment of MDR and XDR tuberculosis outbreaks.**

Sirgel FA, Warren RM, Böttger EC, Klopper M, Victor TC, van Helden PD.

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My contribution to these works include design and planning of the projects, molecular characterization of the Eastern Cape isolates and writing and editing of the manuscripts.

It's the little details that are vital. Little things make big things happen. – John Wooden

Mutations in the *rrs* A1401G gene and cross-resistance between Amikacin and Capreomycin in clinical isolates of *Mycobacterium tuberculosis.*

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Abstract

The availability of antituberculosis drugs to treat extensively drug-resistant tuberculosis is limited. The aminoglycosides amikacin and kanamycin, and the cyclic polypeptide capreomycin are important injectable drugs in the treatment of multidrug-resistant tuberculosis. Capreomycin is recommended as a substitute for amikacin or kanamycin if resistance to either of them is suspected. However, cross-resistance between amikacin/kanamycin and capreomycin in *Mycobacterium tuberculosis* was observed in clinical isolates and laboratory-generated mutants that contain single nucleotide polymorphisms. In this study, the genetic mechanisms that confer phenotypic levels of resistance to amikacin and capreomycin in 50 clinical isolates of *M. tuberculosis* were investigated. The isolates were cultured from patients resident in the Eastern Cape Province of South Africa and then subjected

to DNA sequencing of the *rrs*- (1400-1500 region) and the *tlyA*- (entire region) genes. The phenotypic resistance of each isolate was quantified in MGIT 960 and compared to the sequenced data. All isolates with a nucleotide substitution at position 1401 (A→G) in the *rrs* gene showed high-level resistance (>20 µg/ml) to amikacin. A 100% correlation was also found between the presence of the *rrs*(A1401G) mutation and decreased phenotypic susceptibility to capreomycin at MICs of 10 to 15 µg/ml. No other mutations in either the *rrs* or *tlyA* genes were detected. Complete (100%) cross-resistance between amikacin and capreomycin in *M. tuberculosis* isolates with an A-to-G change at position 1401 of the *rrs* gene was therefore observed in this study. Our findings provide important information that could be used to guide the management of drug resistant tuberculosis.

4.1 Introduction

Therapeutic options in the treatment of pulmonary tuberculosis (TB) are limited and this is a major concern in view of the increasing incidence of drug resistant *M. tuberculosis*. Drug susceptible *M. tuberculosis* infections are treated with a combination of first-line drugs: isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB). Therapy with all 4 drugs is administered for an initial intensive phase of 2 months followed by a 4 month continuation phase with only INH and RIF (16, 17). Lengthy dosing regimens with multiple drugs increase the risk of non-adherence and this is often the reason for treatment failure. Patients may thus acquire drug resistance during therapy or it may arise in previously treated individuals due to relapse of the initial infection (4, 7). Primary drug resistance may also occur as a result of drug resistant strains that are transmitted to treatment-naïve patients. First-line drugs, especially INH and RIF, are the most effective and best tolerated amongst the antituberculosis drugs. Resistance to at least INH and RIF indicates that multidrug-resistant TB (MDR) has emerged (7). Effective treatment of MDR-TB requires prolonged administration of expensive and less potent second-line drugs that are often poorly tolerated (4). MDR-TB patients are thus at increased risk of acquiring additional resistance, eventually giving rise to extensively drug resistant TB (XDR-TB). XDR-TB has emerged independently worldwide and is defined as MDR-TB that has acquired additional resistance to any fluoroquinolone such as ofloxacin (OFX) and at least 1 of the 3 injectable second-line drugs: amikacin (AMK) kanamycin (KAN) or capreomycin (CAP) (4, 16, 17). XDR-TB is extremely difficult to cure because of limited and expensive treatment options. Delays in the detection and therapy of infected individuals are further complications as M(X)DR strains are expected to spread readily in vulnerable communities (5). The transmission of XDR-TB especially amongst individuals co-infected with the human immunodeficiency virus could easily develop into

XDR-TB epidemics with devastating public health consequences (5). The standard MDR-TB treatment regimen used in South Africa is based on the recommended guidelines of the World Health Organization (WHO) and includes the following drugs: KAN or AMK (injectable); OFX; terizidone; ethionamide (ETH) and PZA (16, 17). AMK and KAN are aminoglycosides with a high level of cross-resistance between them, while CAP is a structurally unrelated cyclic polypeptide antibiotic (3). However, cross resistance in *M. tuberculosis* between AMK/KAN and CAP has been observed in both clinical isolates and laboratory-generated mutants that contain single nucleotide polymorphisms (SNPs) in the *rrs* gene between nucleotides 1400 and 1500 (9, 13, 15). AMK/KAN and CAP primarily affect protein synthesis in *M. tuberculosis* and resistance to these drugs is associated with changes in the 16S rRNA (*rrs*) (2, 8, 9, 12). Mutations in the 1400 region of the *rrs* gene and in particular those at positions 1401, 1402 and 1484 are somehow linked with cross-resistance between CAP, AMK and KAN (1, 2, 9, 13, 15). Resistance to CAP has also been associated with mutations in the *tlyA* gene, although such mutants are less often seen as those with an A-to-G substitution at position 1401 in the *rrs* gene (9, 12, 13). The *tlyA* gene encodes a putative rRNA methyltransferase and mutations that inactivate this gene in *M. tuberculosis* results in CAP and viomycin resistance (8, 12).

Between June 2008 and November 2009, 310 M(X)DR *M. tuberculosis* clinical isolates, cultured by the National Health Laboratory Services (NHLS) in the Eastern Cape of South-Africa were sent to Stellenbosch University for genotyping. This cohort of strains was subjected to DNA sequencing of the *rrs* and *tlyA* genes to establish the mutational mechanisms responsible for AMK and CAP resistance. A subset of these strains was then selected for quantitative drug susceptibility testing (QDST) against AMK and CAP. The objectives of the study were (i) to correlate the susceptibility levels of *M. tuberculosis* isolates against AMK and CAP with the molecular mechanisms that cause drug resistance; (ii) to determine whether the *rrs* and *tlyA* genes of isolates displaying resistance to both AMK and CAP contain specific mutations that mediate cross-resistance between the two drugs; and (iii) to consider possible applications of our findings in further studies, particularly in regions with a high incidence of M(X)DR-TB.

4.2 Methods

4.2.1 Clinical isolates

The M(X)DR clinical isolates of *M. tuberculosis* used in this study were obtained from the NHLS in Port Elizabeth (Eastern Cape Province, South Africa) where they were collected during the period June 2008 to November 2009. A total of 310 isolates were initially cultured and subjected to routine DST of first- and second- line drugs by the NHLS laboratory. The BD BACTEC MGIT 960 SIRE system (BD Bioscience, Sparks, MD) was used for DST of first-line drugs at critical concentrations (CCs) of 1.0 µg/ml for SM; 0.1 µg/ml for INH; 1.0 µg/ml for RIF; and 5.0 µg/ml for EMB (17). DST of the second-line drugs was determined according to the 7H11-agar proportional method at CCs of 10.0 µg/ml for AMK; 2.5 µg/ml for ETH; 2.0 µg/ml for OFX and 10 µg/ml for CAP (17). Subsequently, the isolates were sent to Stellenbosch University where they were subjected to DNA sequencing of the *rrs* (1400-1500 region) and the *tlyA* genes as previously described (10). A subset of 50 M(X)DR isolates was randomly selected from the above group with each isolate representing a separate patient. The cohort included strains that were: susceptible to both AMK and CAP; resistant to AMK; and resistant to both drugs (see Table 1). The test isolates were sub-divided according to their drug susceptibility patterns into three groups: MDR; pre-XDR and XDR (15). Twenty strains were MDR (19 of these showed additional resistance to 1 or more of the alternative antituberculosis drugs, excluding the fluoroquinolones and the injectable drugs); 15 pre-XDR (MDR with additional resistance to either a fluoroquinolone or an injectable); and 15 XDR (MDR with additional resistance to both a fluoroquinolone and an injectable). Among the test strains 20 were susceptible to both AMK and CAP; 17 were resistant to both AMK and CAP; while 13 were resistant to AMK, but susceptible to CAP when tested on Middlebrook 7H11-agar medium (see Table 1). Each of the 50 isolates was re-tested by QDST to quantify the level of AMK and CAP resistance. The automated BACTEC MGIT 960 instrument equipped with the TBeXiST application and EpiCentre^{TM}V5.69A software (BD Bioscience, Erebodegem, Belgium) was used for QDST as previously described (14). AMK was tested at 1.0 µg/ml, 4.0 µg/ml and 20.0 µg/ml, and CAP at 2.5 µg/ml, 5.0 µg/ml, 10.0 µg/ml, 15.0 µg/ml, and 25.0 µg/ml. *M. tuberculosis* strain H37Rv (ATCC 27294) was included as a control and subjected to all the relevant drug concentrations. The MICs were determined with the MGIT 960 according to the proportion method that defines resistance as the ability of 1% of the strains (inoculum) to grow at a particular drug concentration. Strains with MICs of ≥ 1.0 µg/ml for AMK and ≥ 2.5 µg/ml for CAP were considered as resistant based on the CCs suggested by the WHO (17).

4.2.2 Antimicrobial agents used for QDST

AMK and CAP were obtained from Sigma Aldrich South Africa. Stock solutions of AMK at 1680 µg/ml and CAP at 2100 µg/ml were respectively prepared in distilled water. The drugs were then sterilised by filtration through a Millex-GV syringe-driven filter with a membrane pore size of 0.22 µm. Aliquots of stock solutions were stored at -80°C in screw-cap polypropylene cryovials up to 6 months. Stock solutions were further diluted in sterile distilled water as required. AMK was diluted 1:5 and 1:20 for a concentration range of 1680 µg/ml, 336 µg/ml and 84 µg/ml. From each of the latter dilutions, 100 µl was respectively added into 8.3 ml MGIT medium for a final drug concentration range of 20.0 µg/ml, 4.0 µg/ml and 1.0 µg/ml. From the CAP stock solution, four additional dilutions: 1:1.67 µg/ml, 1:2.5 µg/ml, 1:5 µg/ml and 1:10 µg/ml were prepared to provide a concentration range of 2100 µg/ml, 1260, µg/ml, 840 µg/ml, 420 µg/ml and 210 µg/ml. From each of these 100 µl was transferred into 8.3 ml MGIT tubes to obtain a final drug concentration range of 25.0 µg/ml, 15.0 µg/ml, 10.0 µg/ml, 5.0 µg/ml and 2.5 µg/ml.

4.3 Results

4.3.1 Molecular detection of mutations

During a period of 18 months, 310 M(X)DR-TB isolates were received from NHLS in Port Elizabeth. DNA sequencing of the *rrs* gene detected the A1401G mutation in 181 of the isolates (58%). Amongst the 50 selected test isolates, 35/35 (100%) of the AMK/CAP resistant strains as determined by QDST, were identified with the *rrs*(A1401G) SNP. The remaining 15 strains lacked the latter mutation and were susceptible to both AMK and CAP (see Table 1). Alternative mutations that are linked to AMK/KAN or CAP resistance in either the *rrs* or *tlyA* genes were not observed in any of the 50 test strains.

4.3.2 QDST of clinical isolates against AMK and CAP

The control strain H37Rv (ATCC 27294) was susceptible to AMK and CAP in both routine DST and QDST, as expected. According to routine DST, 20 of the selected 50 M(X)DR strains tested susceptible to both AMK and CAP on Middlebrook 7H11-medium at CCs of 4.0 µg/ml and 10.0 µg/ml, respectively

Table 1: Mutations and susceptibility profiles of amikacin and capreomycin in clinical isolates of *M. tuberculosis*

Resistance classification	Routine DST profile		QDST MGIT 960[b]			
No. of clinical isolates	(7H11-agar)[a]		MIC in µg/ml[c]		Gene mutations[d]	
	AMK	CAP	AMK	CAP	*rrs* gene	*tlyA* gene
XDR						
6	R	S	>20 (R)	10 (R)	A1401G	None
7	R	R	>20 (R)	10 (R)	A1401G	None
2	R	R	>20 (R)	15 (R)	A1401G	None
Pre-XDR					A1401G	None
6	R	S	>20 (R)	10 (R)	A1401G	None
1	R	S	>20 (R)	15 (R)	A1401G	None
7	R	R	>20 (R)	10 (R)	A1401G	None
1	R	R	>20 (R)	15 (R)	A1401G	None
MDR						
5	S	S	>20 (R)	10 (R)	A1401G	None
15	S	S	<1.0 (S)	≤2.5 (S)	None	None
Control						
H37Rv (ATCC 27294)	S	S	<1.0 (S)	≤2.5 (S)	Not Done	Not Done

[a] Critical concentrations on 7H11 agar: AMK, 4.0mg/L; CAP, 10mg/L.
[b] QDST, quantitative drug susceptibility testing.
[c] Critical concentrations in MGIT 960 medium: AMK, 1.0mg/L; CAP, 2.5mg/L.
[d] The entire *tlyA* gene and region 1400-1500 of the *rrs* gene were sequenced.
DST, drug susceptibility testing; QDST, quanititative DST; MIC, minimum inhibitory concentration; AMK, amikacin; CAP, capreomycin; S, susceptible; R, resistant.

(see Table 1). However, in this study and by QDST in MGIT 960 at CCs of 1.0 µg/ml for AMK and 2.5 µg/ml for CAP, 5 of the 20 strains were found to be resistant to both drugs while the other 15 were truly susceptible. The remaining 30 strains also tested resistant to both AMK and CAP by QDST. Routine DST on the other hand, only found 17 of these 30 strains resistant to both AMK and CAP, while 13 showed resistance to AMK only (Table 1). The agreement between routine DST and QDST results regarding the detection of both AMK and CAP susceptibility was 15 out of 20 isolates (75%); AMK resistance was 30 out of 35 strains (85.7 %); and both AMK and CAP resistance was 17 out of 35 strains (48.60%). However, the QDST findings correlated with molecular data which indicated an A-to-G nucleotide change at position 1401 of the *rrs* gene in all 35 isolates that were resistant to both AMK and CAP. Our results therefore showed a 100% correlation between the presence of the A1401G mutation and cross-resistance between AMK and CAP.

4.4 Discussion

The aminoglycosides AMK or KAN and the cyclic polypeptide CAP together with fluoroquinolones play an important role in the treatment of MDR-TB (7, 17). Evidence that CAP is bactericidal against non-replicating *M. tuberculosis* has renewed interest in this drug despite its limitations due to renal and auditory toxicities (6, 7). It has also been demonstrated that the risk of treatment failure and mortality increases when CAP resistance emerges among MDR-TB cases (11). High levels of cross-resistance between the closely related aminoglycosides AMK and KAN have previously been reported (2, 9). CAP has therefore been recommended as the preferred choice to replace AMK or KAN if resistance to either of them is suspected as cross-resistance across drug classes may only in part be expected (7, 17). The introduction of CAP therapy may be based on the treatment history of the patient when DST results are still pending. However, a relationship between AMK/KAN and CAP resistance due to the presence of either an A1401G (referred to as A1400G in ref 2), C1402T or a G1484T *rrs* mutation in *M. tuberculosis* strains (13) may compromise the use of CAP in M(X)DR treatment. In this study, the occurrence of cross-resistance between AMK and CAP in clinical isolates obtained from the Eastern Cape Province of South Africa was examined. Phenotypic and genotypic resistance to AMK and CAP were respectively determined by QDST and compared to DNA sequencing of the *rrs* (1400-1500 region) and *tlyA* genes. QDST in MGIT 960 showed high-level AMK resistance (>20 µg/ml) in 35 of the 50 M(X)DR isolates while a lower level of CAP resistance was observed in the same isolates (MICs of 10 to 15 µg/ml). A nucleotide substitution at position 1401(A→G) in the *rrs* gene was present in all of the 35 AMK-CAP resistant strains. No mutations were detected in the 1400-1500 region of the *rrs* gene in any of the 15 AMK-CAP susceptible isolates, while all 50 isolates had wild-type *tlyA* genes. These results agree with previous findings that a mutation at *rrs* position 1401 not only confers high-level AMK/KAN resistance in *M. tuberculosis*, but also mediates CAP resistance. In contrast, mutation C1402T is associated with CAP-resistance (also viomycin), low-level KAN resistance and no resistance to AMK, whereas mutation G1484T is linked to high-level AMK and CAP resistance (also KAN and viomycin) in *M. tuberculosis* (13)). The absence of mutations C1402T and G1484T in the *rrs* gene further supports the QDST findings. Mutations in the *tlyA* genes that are associated with CAP resistance (but no resistance to AMK or KAN) were also not detected (8, 12, 13). Discrepancies between routine DST and QDST results were probably due to the different techniques that were used. QDST was done in MGIT 960 as opposed to Middlbebrook 7H11-agar that was used for routine DST. A 100% correlation between the QDST results and the mutational data regarding AMK-and CAP-susceptibilities were obtained and these findings were considered to be more accurate than

conventional DST on 7H11-agar plates. The agreement between routine DST and mutational findings of AMK and CAP resistance was 86% and 49%, respectively.

The presence of high level AMK resistance as opposed to a relatively low level of CAP resistance among the test strains reflect a history of AMK/KAN usage prior to CAP treatment (15). It is likely that decreased susceptibility to CAP occurred even before it was included in the treatment regimen. The pre-selection of low-level CAP resistance as a result of AMK/KAN therapy may imply that the drug has no further clinical relevance in the treatment of XDR patients. The possibility of administering CAP at increased dosages to eliminate low-level CAP-resistant mutants is not an option because of toxicity considerations (7). DST is however, based on a single critical concentration which does not necessarily reflect clinical resistance (14). Peak serum concentration levels of 20–47 µg/ml for CAP are achieved between 1 and 2 hours after a single daily dosage of 15-20 mg/kg by intramuscular injection (3, 7). In this study the MICs (10 to 15 µg/ml) of the CAP resistant isolates were above the CC of 2.5 µg/ml, but substantially below the achievable peak serum levels. Protein binding of CAP is relatively low at 20% and the concentration of free drug at the point of infection may be sufficient for an adequate therapeutic effect when patients are infected with low-level CAP resistant mutants (3). Therapeutic options for M(X)DR-TB are severely restricted and the omission of CAP based on the current DST criteria needs to be considered with caution.

This study supports existing data that the A1401G mutation in the *rrs* gene mediates cross-resistance between AMK and CAP and that it could be regarded as a genetic marker to demonstrate resistance to both drugs (2, 9). Rapid and accurate detection of AMK/KAN and CAP resistance is important to guide treatment regimens and to improve infection control measures that may limit the spread of M(X)DR-TB. Our findings have significant implications on the management of XDR-TB in the Eastern Cape as nearly 60% of MDR cases in the region harbour the *rrs* A1401G mutation. Therefore, we propose that clinical studies be carried out to establish a clear correlation between the current critical concentration, quantitative susceptibilities, mutational data, pharmacokinetics and clinical outcome as a basis for a realistic DST interpretation.

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The Rationale for using Rifabutin in the Treatment of MDR and XDR Tuberculosis Outbreaks

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Abstract

Genetically related *Mycobacterium tuberculosis* strains with alterations at codon 516 in the *rpoB* gene were observed amongst a substantial number of patients with drug resistant tuberculosis in the Eastern Cape Province (ECP) of South Africa. Mutations at codon 516 are usually associated with lower level rifampicin (RIF) resistance, while susceptibility to rifabutin (RFB) remains intact. This study was conducted to assess the rationale for using RFB as a substitution for RIF in the treatment of MDR and XDR tuberculosis outbreaks. Minimum inhibitory concentrations (MICs) of 34 drug resistant clinical isolates of *M. tuberculosis* were determined by MGIT 960 and correlated with *rpoB* mutations. RFB MICs ranged from 0.125 to 0.25 µg/ml in the 34 test isolates thereby confirming phenotypic susceptibility as per critical concentration (CC) of 0.5 µg/ml. The corresponding RIF MICs ranged between 5 and 15 µg/ml, which is well above the CC of 1.0 µg/ml. Molecular-based drug susceptibility testing provides important pharmacogenetic insight by demonstrating a direct correlation between defined *rpoB* mutation and the level of RFB susceptibility. We suggest that isolates with marginally reduced susceptibility as compared to the epidemiological cut-off for wild-type strains (0.064 µg/ml), but lower than the current CC (≤0.5 µg/ml), are categorised as intermediate. Two breakpoints (0.064 µg/ml and 0.5 µg/ml) are recommended to distinguish

between susceptible, intermediate and RFB resistant strains. This concept may assist clinicians and policy makers to make objective therapeutic decisions, especially in situations where therapeutic options are limited. The use of RFB in the ECP may improve therapeutic success and consequently minimise the risk of ongoing transmission of drug resistant *M. tuberculosis* strains.

4.6 Introduction

Mutations within an 81-bp fragment of the *rpoB* gene that encodes the ß subunit of DNA-dependent RNA polymerase are responsible for RIF resistance in *M. tuberculosis* [1–4]. This domain is found between *rpoB* codons 507 and 533 and is referred to as the Rifampicin Resistance Determining Region (RRDR). More than 95% of RIF-resistant isolates have been shown to possess mutations within the RRDR of the *rpoB* gene [1–7]. Mutations in the RRDR at codons 531, 526 and 513 are generally associated with high-level RIF-resistance [2–4]. In contrast, amino acid substitutions resulting from specific changes at codons 511, 514, 515, 516, 518, 521, 522 and 533 are correlated with lower levels of RIF-resistance [2,8,9]. Mutations in the *rpoB* gene that confer high-level RIF-resistance (MICs, ≥32 µg/ml) in *M. tuberculosis* have been associated with cross-resistance to RFB (MIC, ≥4.0 µg/ml) (2,8,9). Conversely, isolates that exhibit lower levels of RIF-resistance MICs (≤16 µg/ml) were found to remain phenotypically susceptible to RFB based on a CC of 0.5 µg/ml [2,8,9]. Single nucleotide polymorphisms (SNPs) outside the RRDR near the beginning of the *rpoB* gene have also been described to be associated with RIF resistance [5]. This region has recently been suggested for inclusion as an additional target for the detection of cross-resistance between RIF and RFB [5]. High-level RIF-resistance is almost always encountered in clinical practice, while mutants with lower levels of resistance are less frequently reported [10]. The clinical impact of low-level RIF-resistance in multidrug-resistant (MDR) tuberculosis (TB) and extensive drug-resistant (XDR) TB, collectively referred to as M(X)DR-TB, is less well-studied and understood.

Epidemiological data based on molecular methods demonstrated that large numbers of M(X)DR patients in the Eastern Cape Province (ECP) of South Africa are infected with similar *M. tuberculosis* isolates of the atypical Beijing genotype [11]. These isolates had comparable sequence alterations in the *inhA* promoter, *katG*, *rpoB*, *pncA*, *embB* and *rrs* (500 and 1400 regions) genes which mediate isoniazid (INH), RIF, pyrazinamide, ethambutol (EMB), streptomycin and amikacin/kanamycin resistance, respectively (11). These similarities suggest genotypic clustering of circulating strains

which are likely responsible for wide-spread transmission of M(X)DR-TB in the ECP. Of particular interest is the high proportion (77%) of the atypical Beijing isolates harbouring SNPs at codon 516 in the *rpoB* gene (11), which is expected not to mediate RFB-resistance [2,9,12]. Based on these observations, it was decided to investigate the possibility of using RFB as a substitute for RIF to treat these M(X)DR TB-infected patients. Hence, our objectives: (i) to correlate the MICs of RIF and RFB in a subset of M(X)DR *M. tuberculosis* isolates (ii) to analyse the MIC data to establish whether cross-resistance occurs between RIF and its analogue RFB and, (iii) to translate the gained knowledge into clinical practice for further assessment concerning RFBs potential to improve clinical outcome.

4.7 Materials and Methods

4.7.1 Clinical Isolates

Amongst a collection of 342 M(X)DR *M. tuberculosis* clinical isolates obtained from patients resident in the ECP, South Africa, 217 (63%) were previously characterised as members of the atypical Beijing lineage [11]. Within this group, 77% (168/217) harboured a mutation at codon 516 in the *rpoB* gene and 151/168 (90%) of these had an Asp516Val (GAC→GTC) alteration. A convenience sample of 34/217 isolates (Table 1) with SNPs in the *rpoB* gene at codon 516 was selected for this study. The isolates had known sequence alterations in the *inhA* promoter, *katG* (315), *rpoB* (516), *pncA* (Ins172) and *embB* (306) genes [11]. In addition, high confidence drug-resistance conferring mutations were observed in the *rrs* (500 and 1400 regions) and *gyrA* genes. H37Rv (ATCC 27294) and 27 *M. tuberculosis* clinical isolates were included for quality control purposes. Two of the isolates were genotypically and phenotypically resistant and 25 susceptible to RIF (Table 1).

4.7.2 MIC determinations

MICs for all selected isolates were determined for RIF and RFB by quantitative drug susceptibility testing (QDST) in BACTEC MGIT 960 eXtended individual Susceptibility Testing (TB eXiST) for EpiCenter TM 92 V5.75A, (BD Bioscience, Erembodegem, Belgium) as previously described (13). The drugs were purchased from Sigma-Aldrich, South Africa. RIF and RFB were dissolved in dimethyl sulfoxide and then diluted in sterile distilled water. Stock solutions of each drug were prepared at

concentrations that were at least 84 times higher than the highest test concentration used. The stock solutions were filter sterilized and small aliquots were then stored at -80°C. The MICs for RIF were determined at 0.5 µg/ml,1.0 µg/ml, 10.0 µg/ml, 15.0 µg/ml and 20.0 µg/ml and for RFB at 0.03 µg/ml, 0.06 µg/ml, 0.125 µg/ml, 0.25 µg/ml, 0.5 µg/ml and 1.0 µg/ml. CCs of 1.0 µg/ml and 0.5 µg/ml were used to determine the susceptibilities of the strains against RIF and RFB, respectively [14]. The relative resistance (RR) of the isolates against the drugs was measured by: Mutant MICs/Wild-type MICs.

Table 1: MICs and relative resistance of rifampicin an rifabutin in *M. tuberculosis*

Genotype	*rpoB* Mutants (n)	Rifampicin MIC µg/ml	[b]RR	Rifabutin MIC µg/ml	RR
Atypical Beijing	D516T (1)	5.0	10	0.125	2
	D516S (4)	5.0-15	10-30	0.125-0.25	2-4
	D516V (29)	10-15	20-30	0.125-0.25	2-4
Undetermined	[a]Wild-type (26)	≤0.5	-	≤0.06	-
Typical Beijing	S531L (1)	>10	>20	>1.0	>16
Atypical Beijing	Q510P (1)	>10	>20	>1.0	>16

[a] Twenty-five clinical isolates with unknown genotype plus one H37Rv strain were included as controls.
[b] RR indicates relative resistance: Mutant MIC/Wild-type MIC.

4.8 Results

The MICs for RIF and RFB as determined in this study are summarised in Table 1. The selected 34 isolates possessed either an Asp516Tyr (n = 1), Asp516Ser (n = 4) or an Asp516Val (n = 29) *rpoB* mutation. The RIF MICs for these isolates were 5 µg/ml (n =2) and 10 – 15 µg/ml (n = 32), which clearly distinguished them from H37Rv and the 25 wild-type strains which displayed MICs of ≤0.5 µg/ml. The level of RIF resistance were thus 5- to 15-fold above the CC, but much lower than those generally displayed by strains harbouring mutations at codons 526 and 531 (MICs, ≥50 to ≥250 µg/ml) [7,10]. However, the corresponding MICs for RFB ranged between 0.125 and 0.25 µg/ml, which was 2- to 4- fold below its CC of 0.5 µg/ml [14]. The MICs for the 26 wild-type strains ranged from ≤0.03 to≤0.06 µg/ml for RFB. Two mutant control strains with SNPs at codons Ser531Leu and Gln510Pro in the *rpoB* gene had MICs of >10 µg/ml and >1.0 µg/ml for RIF and RFB, respectively. These susceptibility levels were well within the resistance ranges of the respective drugs.

4.9 Discussion

The 34 clinical isolates were phenotypically susceptible to RFB as per CC, despite of their resistance to RIF and the presence of SNPs at codon 516 in the *rpoB* gene (Table 1). However, a shift in the RFB MICs, from ≤0.03–0.06 µg/ml for wild-type strains to 0.125–0.25 µg/ml for the mutant isolates was observed. The corresponding MIC shift for RIF was from ≤ 0.5 µg/ml to 5.0–15.0 µg/ml. Based on these findings, the relative resistance of the drugs (Table 1) shows that RFB was less affected by the mutations at codon 516 in the *rpoB* gene as compared to RIF [2,8,9]. The decreased susceptibility to RFB may not predict clinical resistance, but indicate that mutations at codon 516 in the RRDR are associated with incomplete cross-resistance between RIF and RFB. More recently, an epidemiological cut-off (ECOFF) concentration of 0.064 µg/ml was proposed for RFB based on the Middlebrook 7H10 dilution method [15]. T he ECOFF is defined as the highest concentration within the MIC distribution of wild-type strains (i.e. isolates lacking resistance mechanisms) [15]. A breakpoint for RFB, based on clinical evidence has not yet been established. According to the CC (0.5 µg/ml) endorsed by the World Health Organization [14], our results suggest that a substantial proportion M(X)DR TB patients in the ECP may benefit from a treatment regimen that substitute RIF for RFB. This strategy is feasible only if the strains that remain susceptible to RFB are readily detectable. Molecular assays are therefore useful to assist culture-based drug susceptibility testing (DST) in identifying isolates with specific mutations that are associated with RIF-resistance, while they remain susceptible to RFB. The GenoType® MTBD*plus* assay (Hain LifeScience GmbH, Nehren, Germany) is designed to detect most of the mutations that confer RIF- and INH- resistance and has been suggested to be an important tool to define RFB susceptibility [16]. However, molecular assays with enhanced discriminating capacity are needed for identifying mutations that confer low-level or incomplete cross-resistance to analogue drugs. This information is crucial, particularly for the rifamycins, INH, the fluoroquinolones and the injectable drugs as the definition of MDR- and XDR- TB is based on these compounds (17). Furthermore, mutations outside the RRDR, in the beginning of the *rpoB* gene, have also been associated with resistance to both RIF and RFB, while others confer resistance only to RIF [5]. Molecular assays that exclude this region and only target the RRDR in the *rpoB* gene may give a genotypic susceptible result which does not match the resistance phenotype [5].

Previous studies reported on borderline RIF-resistance, missed by standard DST [10,18]. The particular isolates possessed *rpoB* mutations that were associated with low-level RIF-resistance as confirmed by their MICs [10,18]. In another study, isolates from an outbreak of MDR-TB were identified with low-level RIF-resistance [9]. All the isolates harboured an Asp516Tyr mutation in the

rpoB gene and displayed MICs of 0.5–2 µg/ml for RIF and 0.2–0.5 µg/ml for RFB [9]. These studies suggest that low-level RIF-resistance can be overcome by the use of higher RIF doses or alternatively, by replacing RIF with RFB [9,10]. A retrospective study in South Korea showed an 85.7% (12/14) treatment success in patients with RFB-susceptible MDR-TB infections who received RFB as an additional drug [19]. In our study, the MIC distribution for RFB was above the ECOFF, but below the standard CC. Using of the ECOFF as clinical breakpoint for RFB as recently suggested [15] may be misleading as strains with decreased susceptibility that remain treatable may be overlooked. *M. tuberculosis* strains with moderate decreases in susceptibility to RFB should rather be classified as intermediate. Clinical isolates in this category can be distinguished from those that are clearly susceptible or resistant by introducing a second breakpoint and/or by the use of a molecular assay. Our results and existing data [9,10,14,15,18] suggest an intermediate classification that encompasses MICs above the ECOFF (0.6 µg/ml), but below or equal to the current CC (0.5 µg/ml). The peak serum concentration of RFB at a single dosage of 300 mg ranges from 0.4 µg/ml to 0.6 µg/ml [20]. Increased RFB dosages may not be an option to increase these levels due to possible toxicity issues. However, the pharmacokinetics of RFB are in part misleading as its blood levels do not reflect the concentrations that are attained in infected cells and tissues where the drug tends to accumulate [20]. Furthermore, a twofold reduction in the MICs of both RFB and EMB has previously been indicated owing to synergy between these two drugs when used together [20].

Our study reinforces the notion that the heterogeneous MIC levels observed in drug resistant *M. tuberculosis* strains may have important therapeutic implications [21–24]. Of particular relevance is the presence of mutations that confer low-level drug-resistance as it offers possibilities for more effective treatment of drug resistant TB [21–24]. Knowledge of incomplete cross-resistance between the rifamycins and the identification of isolates with intermediate RFB susceptibility should assist clinicians to make objective therapeutic decisions regarding its potential use in M(X)DR-TB treatment-regimens. Designing a treatment regimen for M(X)DR-TB is challenging and the substitution of one or two drugs in a failing regimen must be done with caution to avoid the development of further resistance. Relevant clinical studies are thus imperative to establish appropriate RFB-based regimens that warrant favourable clinical outcome.

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Competing Interest

The authors have declared that no competing interest exist.

4.10 References

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Chapter 5 In-depth analysis of the epidemiologically dominant TB strain in the Eastern Cape

Scrutinizing the Evolution of TDR-TB in the Eastern Cape Province of South Africa by Next Generation Sequencing

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This work will be presented for publication to BioMed Central

My contribution to this work included design and planning of the project, initial genotyping (spoligotyping, PCR-typing, Sanger sequencing and RFLP), culturing of selected isolates, DNA- and RNA extraction and purification and bioinformatics analyses of whole genome sequencing and RNAseq data.

The more I learn, the more I learn how little I know – Socrates

Scrutinizing the Evolution of Beyond XDR-TB in the Eastern Cape Province of South Africa by Next Generation Sequencing

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Abstract

The drug-resistant TB epidemic in the Eastern Cape is out of control. We have previously shown programmatic selection of the Atypical Beijing genotype to be the dominant strain type among pre-XDR-TB, and XDR-TB in the region. Inappropriate treatment of these cases continues amplify resistance resulting in untreatable TB which is resistant to up to 11 anti-TB drugs. Contrary to dogma these highly resistant strains have retained their ability to transmit and cause disease. However, the evolutionary mechanisms underlying the retention of virulence remains unknown. In this study, we employed next generation sequencing to determine the evolutionary history of the Atypical Beijing genotype strains from the region, and to investigate molecular features that may predispose these strains to become increasingly drug-resistant, and to efficiently spread. Phylogenetic analysis confirmed the evolution and sequential acquisition of resistance markers. These strains initially evolved resistance to isoniazid and streptomycin in response to historic inadequate treatment regimens before acquiring resistance to EMB, PZA, RIF and second-line injectable drugs in two distinct branches of the Atypical Beijing genotype, each harbouring unique features. Furthermore, we show that all representative Atypical Bejing strains – including those diagnosed as pan-susceptible – harbour a mutation in *ethA*, which has been associated with ethionamide (ETH) resistance. This result may explain the apparent increased ability of the Atypical

Beijing strains to evolve higher drug-resistance. RNA sequencing confirmed the distinct nature of the two groups of drug-resistant isolates, and revealed the massive up-regulation of the *InhA* operon in isolates with an *inhA* promoter mutation, suggesting a mechanism of resistance. Furthermore, the toxin-antitoxin pair *vapBC* was affected by a DNA mutation that resulted in altered RNA expression. The effect of this change on the cell needs to be investigated further.

5.1 Introduction

There is growing acknowledgement of the problem of tuberculosis (TB) with drug resistance beyond the four drugs that define extensively drug-resistant (XDR) TB, unofficially called totally drug-resistant TB (TDR-TB). Evidence suggest that TDR-TB may be the result of acquisition of resistance (1–3) or it may be due to primary resistance (4) or a combination thereof (5). A recent report documented the widespread transmission of a highly resistant *Mycobacterium tuberculosis* strain with an Atypical Beijing genotype in the Eastern Cape (EC) Province of South Africa (4). The Beijing genotype has previously been associated with increased ability to spread and with multidrug-resistance (MDR) (6,7). However, the Atypical subgroup – distinguished from Typical Beijing primarily through the absence of an IS*6110* in the NTF region – is not frequently observed world-wide, with the exception of countries in the Far East (8–10). Atypical Beijing strains are usually also seen at low frequency in South African settings (11). However, in the EC, Atypical Beijing strains are overrepresented among pre-extensively drug-resistant (pre-XDR) and XDR (93%) isolates, as well as multidrug-resistant (MDR, 48%) TB isolates. In contrast, it is seen at relatively low frequency (4%) among drug susceptible isolates) (4). Furthermore, recent figures suggest an increasing incidence of Atypical Beijing strains in the Western Cape Province (WC), in particular among XDR-TB patients (11), suggesting an influence through migration from the EC, with additional local transmission (Streicher, personal communication). It is therefore important to investigate the molecular mechanisms driving the epidemic in order to understand the reasons for this strain's increased transmissibility.

A range of molecular techniques are available to assess transmission, as well as other specific features of the genome. These include spoligotyping (12), Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeat (MIRU-VNTR) typing (13) and IS*6110* DNA fingerprinting (14). However, none of these methods provide a complete catalogue of genomic variation as they target a limited set of repeat sequences. Furthermore, results from the different methods may yield

discrepant results, as evolution of these features are independent of each other (15). Similarly, targeted Sanger sequencing only reveals a small portion of the genome, and is therefore only suitable to evaluate specific areas of the genome. A more thorough understanding of transmission and evolution can be facilitated through the application of whole genome sequencing (WGS). This technology has the potential to identify variants that may contribute to a given phenotype.

In this study, we aimed to interrogate the genomes of highly resistant Atypical Beijing strains (resistant to up to 11 drugs) from the EC through high resolution next-generation sequencing. Through this analysis we aimed to infer evolutionary relationships, as well as to investigate genetic variation among individual isolates in order to find genetic traits that may be responsible for their increased transmissibility. We also sought to discover a novel mechanism of PAS resistance, as six isolates were phenotypically resistant to PAS, but did not have mutations known to be associated with this phenotype.

5.2 Materials and methods

5.2.1 Strain selection

Twenty-nine Atypical Beijing genotype isolates originating from health care facilities from across the EC and processed by the National Health Laboratory Services in Nelson Mandela Metropole were selected. This selection was based on resistance profiles obtained through phenotypic drug susceptibility testing (DST) as well as targeted DNA sequencing. The selection included 11 pan-susceptible, 4 MDR-TB, 4 pre-XDR-TB and 10 XDR-TB isolates (including 6 TDR-TB isolates - resistant to 11 of the available first-and second line drugs). Twenty-seven of these isolates were shown by RFLP to belong to strain family 31, a sub group of the Atypical Beijing genotype. The remaining two isolates were both drug-sensitive, one belonging to a different Atypical Beijing sub-group, family 27, and the other could not be assigned to a known strain family.

A group of unrelated strains (n = 93, 42 genotypes), originating from the Western Cape (including Atypical Beijing strains) and Kwa-Zulu Natal, as well as published genomes and *M. bovis* strains originating from the Kruger National Park, Western Cape and Kwa-Zulu Natal were included in

phylogenetic analysis. These strains were selected to represent a variety of strains seen in South Africa, to improve the robustness of the phylogenetic analysis, and to determine the uniqueness of certain variants within the Atypical Beijing genotype.

Ethical approval has been obtained from the Ethics Committee of Stellenbosch University.

5.2.2 DNA sequencing

Each of the selected strains was cultured under biosafety level 3 conditions on 7H10 media, and grown to confluence. The bacteria were heat-killed prior to standardised phenol/chloroform DNA extraction (16).

Purified DNA from each isolate was used to prepare paired-end genomic libraries using TruSeq DNA Sample Preparation Kits V2 (Illumina Inc, San Diego, CA, USA). Each library was sequenced on an Illumina HiSeq2000 to a pre-calculated theoretical depth of coverage of 150x.

5.2.3 DNA sequence analysis

Next-generation sequencing data obtained from the Illumina HiSeq2000 platform was analysed using a pipeline that was developed by our research group (Van der Merwe, manuscript in preparation). Briefly, reads are assessed with FastQC (17), followed by trimming with Trimmomatic (18). The latter assesses each read individually with a sliding-window approach and trims accordingly. The average quality of the bases considered needs to be above 20 and a read may not be shorter than 36 bases. The processed reads are subsequently used as input in three different open source alignment tools, namely Burrows-Wheeler Aligner (BWA) (19), Novoalign (20), and SMALT (21), using *M. tuberculosis* H37Rv (GenBank NC000962.2) as a reference genome (19,21). Trimmed reads were used as input in each alignment tool with standard parameters. The alignment files were assessed with Qualimap (22) to determine the number of reads that mapped to the reference genome and the depth of coverage. A summary of these statistics are given in Table 2.

Considering indels only in isolates from the EC, a total of 5208 indels (excluding changes in PE/PPE genes and highly repetitive regions) were called in 29 isolates by Unified Genotyper. Of the 5208 indels, 3018 were called with high confidence (i.e. by all three alignment algorithms, namely, BWA, Novoalign and SMALT). Indels occurring in only 1-6 isolates were regarded as unlikely to confer any significant change in terms of evolution of drug resistance and were disregarded, given that no clear pattern in terms of drug resistance could be discerned (i.e. there was a mixture of drug-resistant and -susceptible isolates in each group of isolates with the same indel). Similarly, indels present in all isolates (n=117 indels) or in a proportion of isolates that represent both drug-sensitive and drug-resistant strains were disregarded, based on the observation that such events were not unique to drug-resistant isolates and thereby could not explain a fitness advantage.

The Genome Analysis Tool Kit (GATK) (23) was used as recommended in the user documentation to call single nucleotide polymorphisms (SNPs) in the alignment files from the different mapping algorithms used.

Only variants (SNPs) identified by GATK in all of the alignments that overlap in both position and base identity were written to a high-confidence SNP file with the use of an in-house script, for each isolate analysed. SNPs were filtered for PE/PPE family genes, repeat regions and SNPs with a quality value below 200. These high-confidence variants were used in further analyses.

5.2.4 Phylogeny

A concatenated sequence containing high-confidence variable sites (coding and non-coding SNPs) for each isolate was prepared. These sequences were written to a single multi-FASTA file and were used as input in Modelgenerator (24) to determine the optimal substitution model that fits the data structure. The general time reversal (GTR) model with gamma (G) distribution scored the lowest in the hierarchical likelihood ratio test (Bayesian information criterion (BIC)), and thus described the substitution pattern occurring in the dataset most accurately. The GTR+G model of substitution was subsequently applied to construct a maximum likelihood or minimum evolution phylogeny of the isolates included in this analysis with MEGA 6 (25), RaxML (26) and PhyML (27) with 1000 bootstrap pseudo-replicates. In a separate phylogeny, known drug-resistance causing SNPs (Table 1) were

removed from the SNP string before tree construction in an attempt to improve the resolution of the tree by reducing homoplasy. In further selected analyses, TT147 was omitted due to inferior sequencing quality (Table 2) which may contribute noise to the phylogeny. The robustness of the different trees and branches were assessed by comparing the topologies of trees generated by different methods.

Table 1: Known drug-resistance causing single nucleotide polymorphisms (SNPs) that were excluded from relevant phylogenies

Gene name	Rv number	Drug resistance	Codon	Genomic position*
gyrA	Rv0006	FQ	90	7570
gyrA	Rv0006	FQ	91	7572
gyrA	Rv0006	FQ	94	7581
gyrA	Rv0006	FQ	94	7582
rpoB	Rv0667	RIF	516	761110
rpoB	Rv0667	RIF	531	761155
rrs	MTB000019	SM	pos 513	1472359
rrs	MTB000019	AM/KAN/CAP	pos 1401	1473246
inhA promoter	pre-Rv1483	INH,ETH	pos -17	1673423
inhA promoter	pre-Rv1483	INH,ETH	pos -15	1673425
katG	Rv1908c	INH	315	2155168
pncA	Rv2043c	PZA	14	2289202
embB	Rv3795	EMB	306	4247431

*Refers to genomic position relative to H37Rv (GenBank NC000962.2)

5.2.5 RNA sequencing

The selected strains were grown in supplemented 7H9 media to OD 0.6-0.7 in biosafety level 3 conditions, prior to RNA extraction with the FastRNA Pro™ Blue Kit (MP Biomedicals). All extractions were done in biological triplicate. Total RNA extraction was followed by DNase (Promega, Madison, WI) treatment in technical duplicate and the purified RNA was stored at -80°C. The selected samples were prepared for shipment at ambient temperature by preservation in RNAstable (Biomatrica) tubes.

The quantity and quality of RNA was assessed using a Nanodrop (ND-1000, Labtech) and the Agilent 2100 Bioanalyzer (Agilent; Palo Alto, CA, USA) per manufacturer's recommendation.

A subset of the purified RNA samples were selected for RNA sequencing, including 3 biological replicates of 2 drug-susceptible (TT372, TT700), 3 MDR-TB (TT50, TT248, TT321, TT648), 3 Pre-XDR-TB (TT57, TT145, TT160), 4 XDR-TB (TT48, TT99, TT154, TT219) and 6 TDR-TB (TT46, TT61, TT62, TT98, TT135, TT147) isolates.

Ribosomal RNA depletion was done using the RiboMinus-based rRNA depletion kit (Invitrogen). For mRNA enrichment, Invitrogen's RiboMinus™ Prokaryotic kit was used according to manufacturer's instructions.

Construction of double-stranded cDNA libraries was done using Illumina TruSeq™ RNA sample preparation kit V2 (Low-Throughput protocol) according to manufacturer's protocol. Briefly, first strand cDNA was synthesized from enriched and fragmented RNA using SuperScript III Reverse Transcriptase (Invitrogen). The first strand cDNA was further converted into double stranded (ds) cDNA. Ampure XP beads (Beckman Coulter) were used to separate the ds cDNA from the 2nd strand reaction mix as per the manufacturer's instructions, and the resultant ds cDNA was subjected to library preparation according to Illumina TruSeq™ RNA protocol. cDNA fragments were end-repaired and phosphorylated, followed by adenylation of 3'ends and adapter ligation. Twelve cycles of Polymerase Chain Reaction (PCR) amplification were then done, and the library was finally purified with AMPure XP beads (Beckman Coulter) according to the manufacturer's recommendations. A small aliquot (1 µl) was analyzed on Invitrogen Qubit and Agilent Bioanalyzer. The bar-coded cDNA libraries were pooled in equal concentrations before sequencing on Illumina HiSeq2000 using the TruSeq SR Cluster Generation Kit v3 and TruSeq SBS Kit v3. Data were processed with the Illumina Pipeline Software v1.82.

5.2.6 RNA-seq analysis

Similar to whole genome sequencing analysis, reads were assesed by FastQC, followed by quality trimming with Trimmomatic (18). Subsequently, trimmed reads were imported into the Lasergene 12 ArrayStar® software package (DNASTAR®, Inc. Madison, WI). H37Rv (GenBank NC000962.2) was used as a reference genome, and normalization by reads per kilobase per million reads (RPKM) was chosen. Scatterplots of the forward versus reverse reads, as well as biological replicates of each

sample were viewed, and R^2 statistics were used in validation, where samples with $R^2 < 0.9$ were discarded. Subsequently, the retained reads from each original isolate were grouped together in replicate sets. The two drug-susceptible isolates were used individually and together as controls in separate analyses. Drug-resistant strains were grouped together as experiments, based on phylogenetic relationships and the hypothesis that certain groups of strains posess a fitness advantage. Student's t-test was done for each experiment to determine statistical significance of up- or down-regulation, as well as the effect-size of the difference. Gene sets for analyses were created of all genes in an experiment that were at least two-fold up- or down-regulated, compared to the control, and at 99% confidence.

5.3 Results and discussion

5.3.1 Whole genome sequencing coverage and quality

To assess the coverage of WGS data, we utilised Qualimap (22) to analyse Novoalign-generated binary alignment map (BAM)-files. Coverage in excess of 100X was obtained for the majority of the isolates (Table 2). Only isolate TT564 had a mean coverage below 100. In this study, >90% of reads were mapped for all isolates, except TT50, TT147 and TT719, for which <79% of reads were mapped to the reference genome. Caution should be applied when interpreting the analysis of strains TT50, TT147, TT719 and TT564 as spurious results may occur due to the low sequencing quality. WGS data of the different strains were compared to identify SNPs and small insertions and deletions (indels, up to 50bp) that distinguish different groups of Atypical Beijing genotype isolates. Comparing all F31 isolates (isolates identified by IS*6110* RFLP to belong to Family 31, n= 29, including 27 EC and 2 WC strains) to the non-Atypical Beijing genotype strains in Figure 3, yielded 222 SNPs that were unique to F31, and present in all investigated F31 strains. The distribution of these SNPs in terms of functional category is shown in Figure 1. Genes involved in intermediary metabolism, respiration, the cell wall and cell processes were most polymorphic, followed by conserved hypothetical genes and intergenic regions.

Table 2: Quality and coverage values as reported by Qualimap

Isolate	No. of reads	% mapped reads	Mean depth of Coverage (Std dev)
TT46	21,165,367	98.21	400.87 (95.05)
TT48	18,282,314	98.25	346.69 (82.77)
TT50	11,378,772	78.92	191.04 (41.93)
TT57	20,173,554	98.23	381.93 (88.64)
TT61	20,868,422	98.23	395.77 (90.11)
TT62	20,623,626	98.20	392.57 (91.67)
TT98	19,076,063	98.18	362.07 (86.33)
TT99	17,921,992	98.20	341.67 (84.01)
TT135	21,115,670	98.20	394.39 (89.89)
TT145	18,411,02	98.27	348.95 (82.11)
TT147	9,407,868	65.08	127.47 (31.56)
TT154	18,529,430	98.26	352.57 (81.29)
TT160	15,656,017	98.25	293.61 (70.45)
TT219	18,767,531	98.24	356.27 (82.48)
TT248	16,477,473	98.26	311.15 (73.59)
TT321	10,993,557	96.74	224.66 (44.46)
TT372	7,474,841	97.42	153.72 (32.87)
TT508	6,427,782	97.59	131.88 (28.91)
TT545	10,083,571	97.71	208.15 (43.25)
TT564	4,349,139	95.63	89.10 (25.37)
TT574	10,210,547	97.76	212.43 (50.08)
TT589	9,020,067	98.16	187.63 (38.69)
TT606	9,966,248	98.25	206.94 (45.43)
TT607	12,216,968	97.45	247.33 (84.83)
TT622	20,866,766	98.26	396.31 (91.28)
TT648	5,637,053	94.50	112.32 (28.72)
TT679	11,056,373	97.68	228.13 (48.96)
TT700	8,885,047	97.78	182.53 (44.14)
TT719	9,596,258	74.97	152.23 (37.12)
Average	13,793,868	95.00	267.00 (63.00)
min	4,349,139	65.08	89.10
max	21,165,367	98.26	400.87

Further quality assurance was built into the analysis pipeline by the use of 3 different alignment algorithms (BWA (19), Novoalign (20) and SMALT (21)). Only variants that were called by all 3 algorithms (high confidence variants) were used in further analyses.

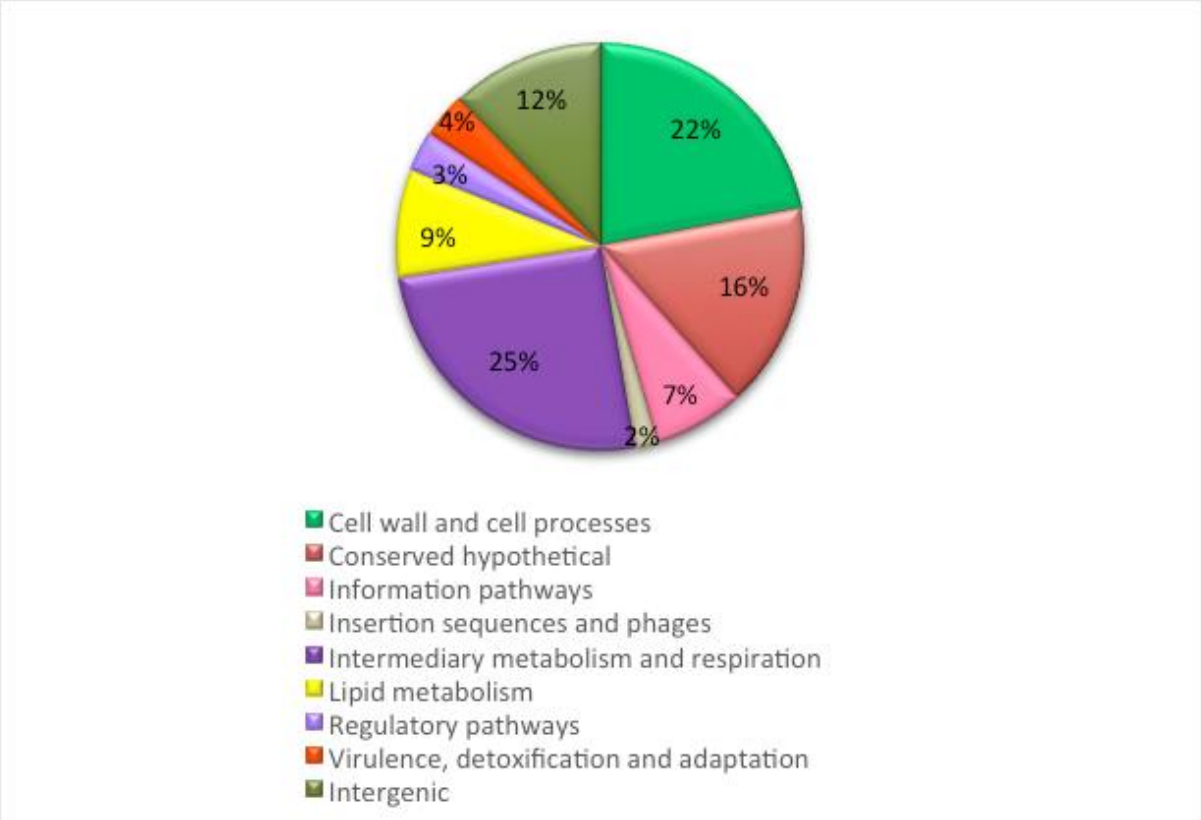

Figure 1: Distribution of SNPs that are unique to Atypical Beijing F31 strains and present in all these isolates

Each of the EC isolates was investigated for the number of SNPs relative to H37Rv, as well as the number of drug-resistance conferring SNPs (Table 1) present in each isolate. Drug-resistance associated SNPs were removed for phylogenetic analyses, as these SNPs could be acquired in parallel and could distort the phylogenetic interpretation. These results are presented in Table 3.

Considering indels only in isolates from the EC, a total of 5208 indels (excluding changes in PE/PPE genes and highly repetitive regions) were called in 29 isolates by Unified Genotyper. Of the 5208 indels, 3018 were called with high confidence (i.e. by all three alignment algorithms, namely, BWA, Novoalign and SMALT). Indels occurring in only 1-6 isolates were regarded as unlikely to confer any significant change in terms of evolution of drug resistance and were disregarded, given that no clear pattern in terms of drug resistance could be discerned (i.e. there was a mixture of drug-resistant and -susceptible isolates in each group of isolates with the same indel). Similarly, indels present in all isolates (n=117 indels) or in a proportion of isolates that represent both drug-sensitive and drug-resistant strains were disregarded, based on the observation that such events were not unique to drug-resistant isolates and thereby could not explain a fitness advantage.

5.3.2 Phylogeny

WGS of a selection of *Mycobacterium tuberculosis* complex (MTBC) isolates (n = 93) reflecting different strain families were included in a comprehensive phylogenetic reconstruction (Figure 2) to indicate the relative phylogenetic position of the Atypical Beijing genotype strains among other mycobacterial strains. As expected, this phylogeny demonstrates that Beijing genotype strains (IS*6110* families 27, 29 and 31, denoted F27, F29, and F31) are closely related, compared to strains from other genotypes. All of the included strains from the Eastern Cape belonged to the F31 group (marked in red, Figure 2), with the exception of two drug-susceptible strains - TT508 (F29) and TT564 (unassigned to a specific genotype). The latter two (blue boxes) were shown by RFLP as well as several methods of phylogenetic analysis to be outliers to the EC Atypical Beijing genotype.

The phylogeny depicted in Figure 2 was generated using RaxML v 8.0 (26). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model with a Gamma distribution (28). 1000 pseudo-replications were done. All of the EC Atypical Beijing genotype strains with resistance profiles ranging from pre-XDR to "TDR" cluster closely together. It should be noted that within the Atypical Beijing genotype sub-lineage the bootstrap values were low despite the use of different models and predictive algorithms (also see Figure 3 and Figure 4). This may be due to the high level of similarity as well as recent parallel evolution events. The notion of high similarity was supported by the observation that the number of SNPs that differentiated isolates from their nearest putative common ancestor was consistently below 23 (average 8.5). In addition, a detailed analysis of one branch (Group B, Figure 3), revealed 48 SNPs that were subjected to either reversion or homoplasy within these isolates. Interestingly, a subsequent investigation revealed that 40 of these events were also present in the non-Atypical Beijing genotype strains used in the phylogeny illustrated in Figure 2 (positions not indicated), indicating that these positions are prone to change. This needs further investigation and may contribute to our knowledge of positions that are not informative to the evolution of *M. tuberculosis* strains. Interestingly, this sub-lineage lacked the SNP 1548149 reported by Hanekom *et al*. (29) to be an evolutionary event that was specific to the Beijing genotype and common to both the Typical and Atypical sub-lineages. This implies that these strains branched off from the clade earlier than any of the strains investigated by those autors. The presence of SNP 909166, which was hypothesised to be more modern than SNP 1548149 suggests that the SNP 909166 is subject to homoplasy.

Table 3: Number of SNPs, non-drug-resistance SNPs and drug-resistance SNPs removed for phylogenies, relative to H37Rv

Group	Isolate	All SNPs	Non-DR SNPs	DR SNPs removed
Group A	TT46	1245	1238	7
	TT135	1264	1256	8
	TT622	1249	1242	7
Group B	TT48	1232	1225	7
	TT57	1240	1234	6
	TT61	1237	1230	7
	TT62	1247	1240	7
	TT98	1246	1239	7
	TT99	1235	1228	7
	TT145	1236	1229	7
	TT147	1211	1204	7
	TT154	1243	1236	7
	TT160	1235	1229	6
	TT219	1238	1230	8
	TT248	1227	1221	6
Group C	TT50	1239	1237	2
	TT321	1271	1268	3
	TT648	1189	1187	2
	TT372	1228	1228	0
	TT545	1249	1249	0
	TT574	1248	1248	0
	TT589	1243	1243	0
	TT606	1254	1254	0
	TT607	1210	1210	0
	TT679	1254	1254	0
	TT700	1240	1240	0
	TT719	1227	1227	0
Other	TT508	1309	1309	0
	TT564	1228	1228	0

SNP = single nucleotide polymorphism; DR= drug-resistance; Groups A, B, C derived from Figure 3.

Figure 3 shows the evolutionary history inferred using the Minimum Evolution (ME) method (30) as analysed in MEGA6 (25). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (31) and are in the units of the number

of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm (28). The Neighbor-joining algorithm (32) was used to generate the initial tree. The analysis included 35 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 3794 positions in the final dataset. This phylograph is the closest to what was expected, separating the strains according to known drug-resistance groups. Both Group A and Group B include strains that are resistant to more drugs than the strict definition of MDR-TB. Furthermore, each group has a distinct set of mutations relative to each other, including drug-resistance causing mutations (detailed in Table 8), suggesting clonal expansion of two variants of the Atypical Beijing genotype. Group A consists exclusively of strains that harbour the *pncA* 14 mutation and other associated mutations, while Group B harbours a distinct set of mutations in these same genes (Figure 4 and Table 8).

The phylograph depicted in Figure 4 was inferred by using the Maximum Likelihood method based on the General Time Reversible model (28) as analysed using MEGA6 (25). Bootstrap values are shown next to the branches (black figures). Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 28 nucleotide sequences, excluding the Typical Beijing genotype strains, and TT147 which had questionable sequencing quality (Table 2). All positions containing gaps and missing data were eliminated. There were a total of 2145 positions in the final dataset. The evolutionary position of the removed drug-resistance causing mutations are indicated on the tree in circled red figures. A legend of these changes is provided in Figure 4. *GyrA* mutations are not indicated, as there is a variety of mutations present among these strains, all occurring at the termini of the branches, suggesting independent acquisition. The assigned positions of TT607 (drug-susceptible) and TT160 (exactly the same pattern of mutations seen in Group B) are thought to be unlikely, although these positions are supported by high bootstrap values. This phylogenetic prediction suggest that *katG* mutation was deeply rooted implying that this event was the initial event causing drug resistance to isoniazid. This corresponds to the chronology in which patients were treated. During the period from the mid 1950's to the late 1960's patients were treated with acombination of SM, INH, PAS (33). The *rpoB* 531 and *inhA* promoter -15 mutations, which are seen both in strains with higher resistance (Group A), as well as unrelated MDR-TB strains (in Group C), suggesting that these mutation were acquired on different occasions.

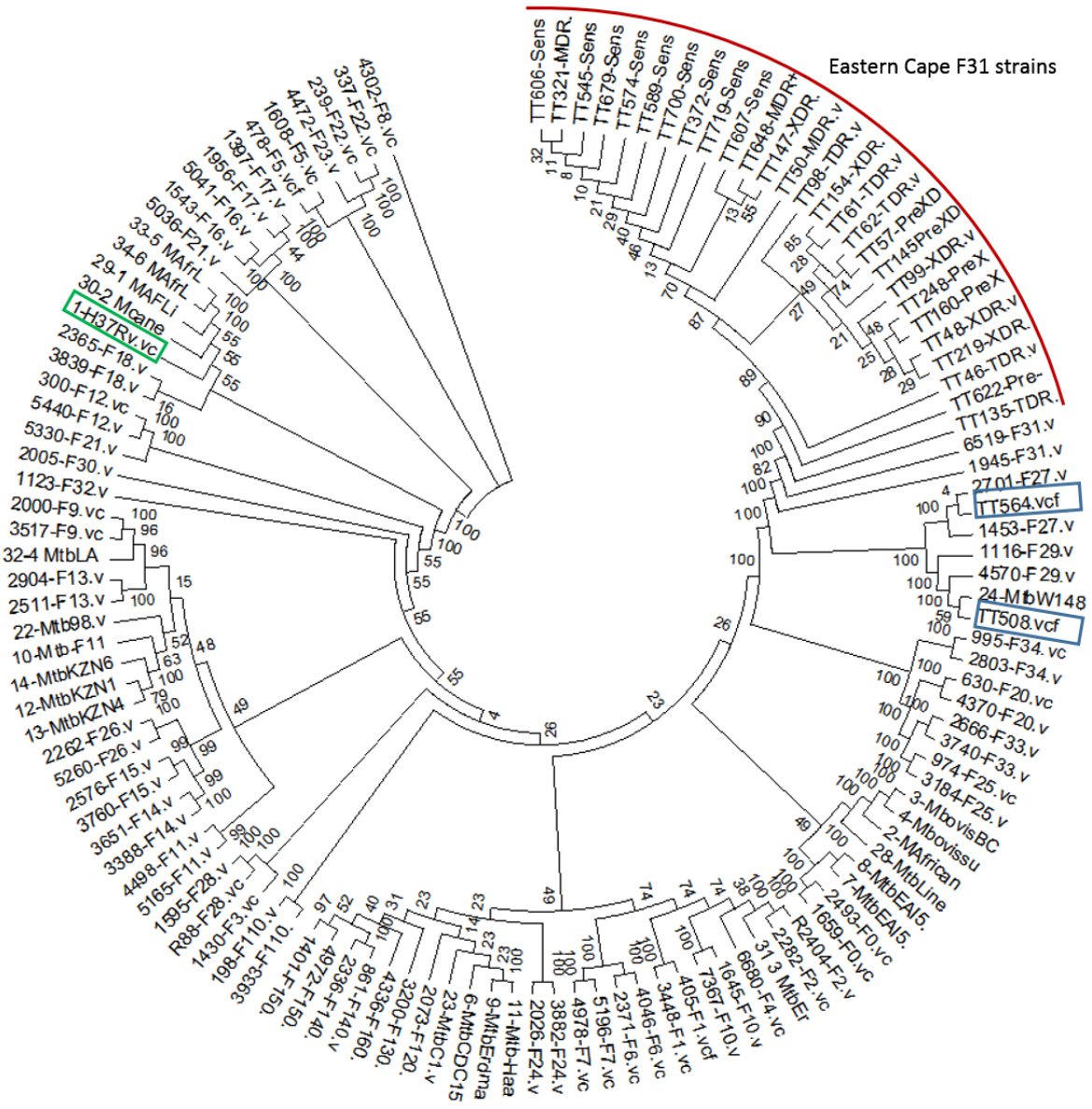

Figure 2: Maximum likelihood phylogeny of representative strains from a range of different strain families. Atypical Beijing strains of family 31 are indicated in red; Atypical Beijing strains of other sub-classifications are indicated in blue; H37Rv is indicated in green.

Comparison of F31 strains of Groups A and B (higher drug-resistance) with drug-susceptible and MDR *sensu stricto* (Group C) as per Figure 3, yields 127 SNPs that were unique to the higher resistance group (i.e. not present in MDR*ss* or susceptible). Two of these SNPs were common to all

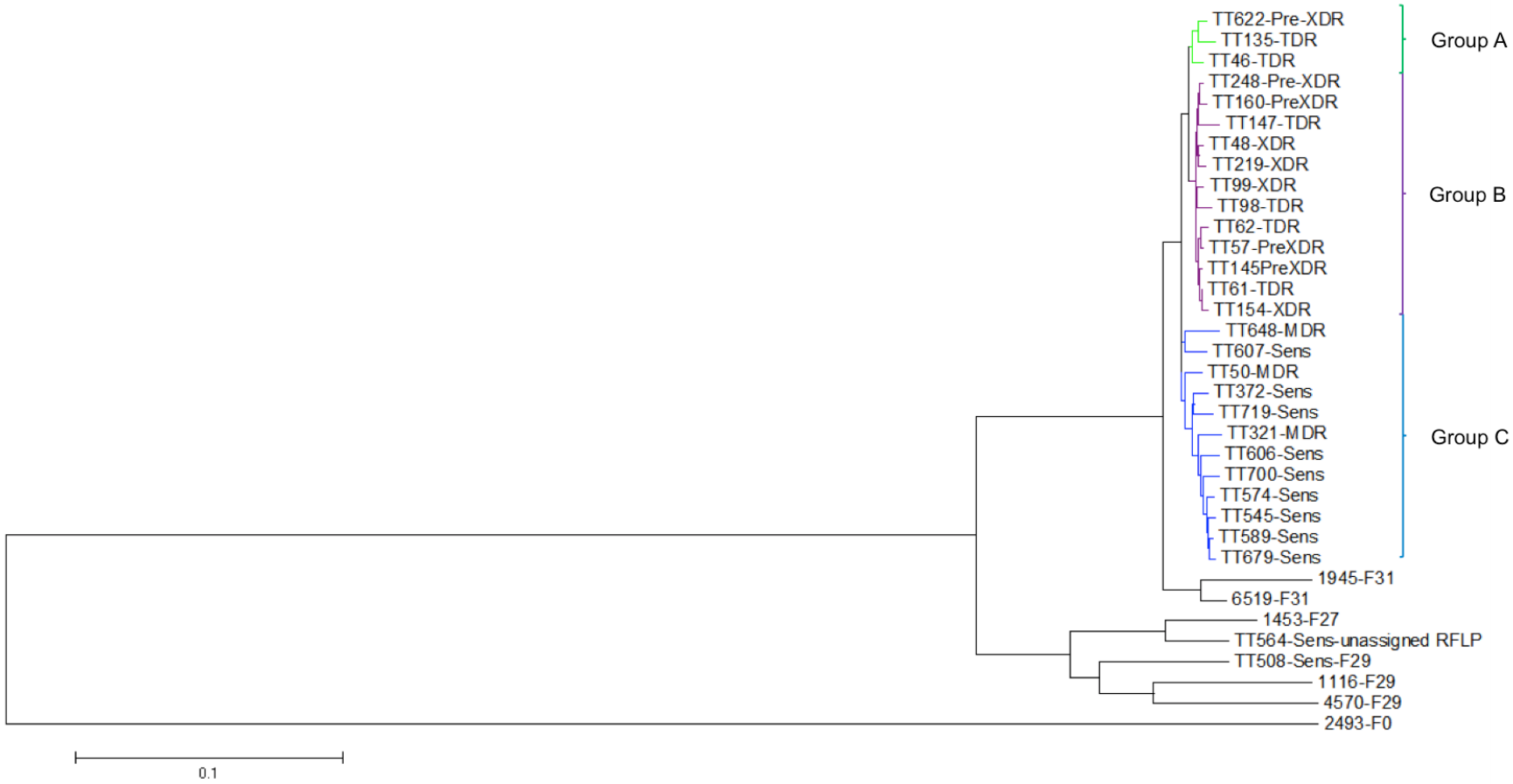

Figure 3: Evolutionary history of Beijing genotype strains, inferred by the Minimum evolution method. Group A, including isolates with *pncA* 14GCG mutation is indicated in green; Group B, consisting of isolates with *pncA* ins172G is indicated in purple; Group C, consisting of durg-sensitive and MDR*ss* isolates is indicated in blue

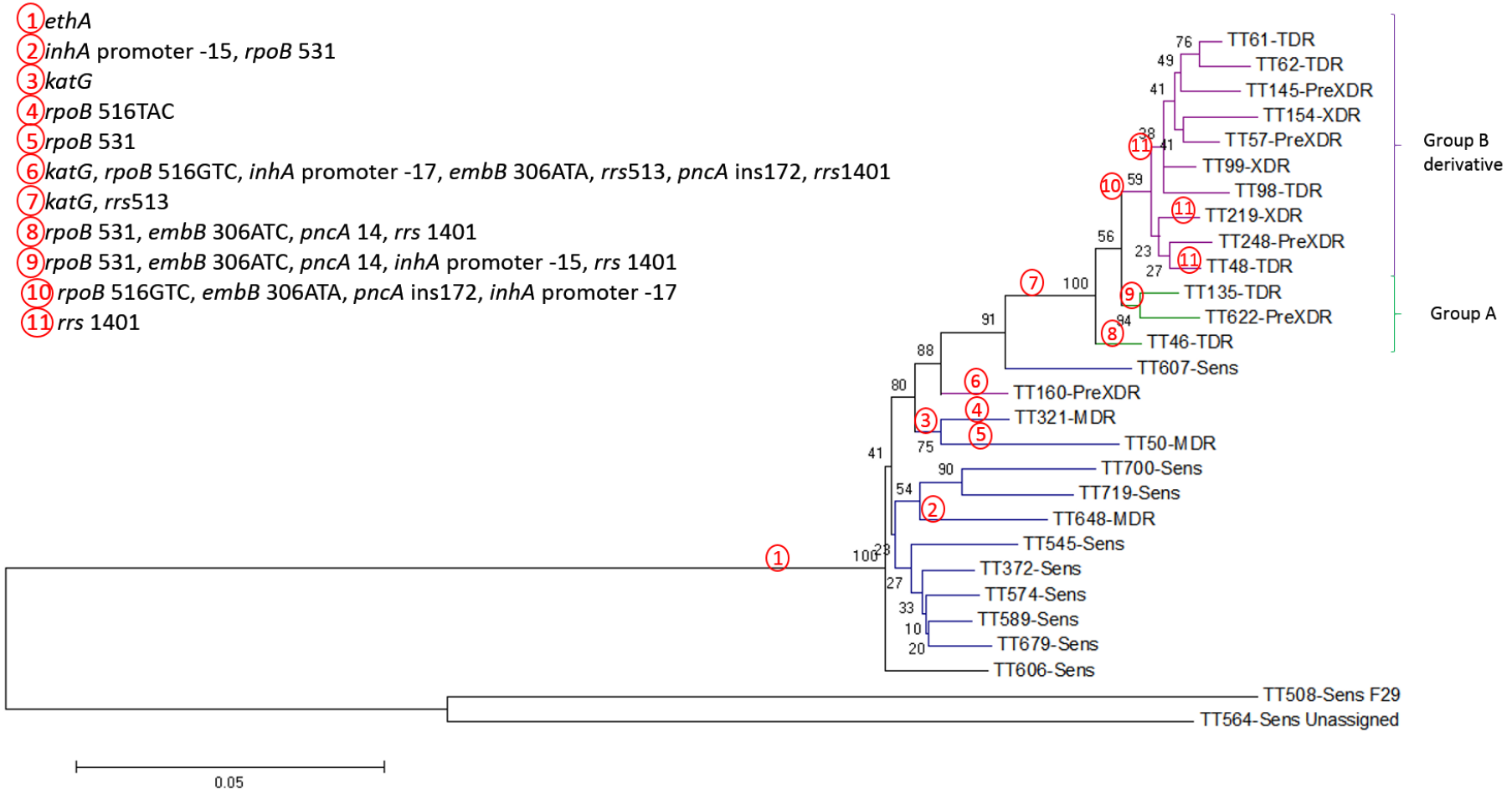

Figure 4: Evolutionary history of Beijing genotype strains inferred by the Maximum Likelihood method, without drug-resistance causing SNPs

to all isolates in the higher drug-resistance groups but not present in Group C, namely *rrs*513, conferring resistance to streptomycin (SM), and *rrs*1401, conferring resistance to amikacin, kanamycin, and capreomycin. These SNPs may have occurred early in the evolution of these strains, which is plausible for *rrs*513, given that SM was administered as monotherapy in the early years of TB chemotherapy and it is unlikely that many of these patients would have been exposed to SM. However, it is not possible to determine whether *rrs*1401 occurred early in the evolution of these strains, or whether it evolved in parallel, this being only one of two mutations conferring resistance to amikacin, kanamycin, and capreomycin. Furthermore, when these drugs are prescribed for TB it is only as second-line therapy, implying that exposure to the drug should have been after diagnosis of initial resistance, although aminoglycosides are also prescribed for non-tuberculous infections.

Additional resistance was acquired after transmission of the progenitor strains. This is evident from the presence of a variety of *gyrA* mutations (conferring fluoroquinolone resistance) in different isolates (Table 4). Furthermore, phenotypic PAS resistance was shown in some strains that are not directly related (not situated on the same sub-branch of the tree). No mutation that was called with high confidence was specific to all PAS resistant isolates. This may be explained by a few possible scenarios: PAS resistance may not be attributable to a single mutation, but rather any of a range of mutations involved in the metabolism or efflux of the drug, or epigenetic factors may play a role. Alternatively, some isolates may have been false-positives or false-negatives for PAS resistance, or PAS resistance has not fully emerged (i.e. only a portion of reads may have had the mutation) causing it to be missed by our stringent NGS data analysis algorithm. Our current knowledge of PAS resistance is limited to high confidence SNPs situated in *tlyA* (34), which was not observed in any of these isolates.

The number of SNPs that differentiate strains within a group from each other was investigated in order to determine transmission events. Previously published whole genome sequencing studies investigating transmission events suggest that a transmission event could be defined when the genome sequences of *M. tuberculosis* isolates differ by a maximum of 15 SNPs (35–37). The number of SNPs found in each isolate in this study, relative to other isolates within its group are shown in Table 5-7. Each cell represents the number of SNPs that are unique to the isolate in the header row, compared to the isolate in the leading column. Therefore, e.g. from Table 5, TT46 harbours 13 SNPs that are not present in TT135, whereas TT135 harbours 32 SNPs that are not present in TT46. Like-coloured cells represent isolates from the same health district in the header rows or leading columns, whereas the cells highlighted in yellow shows the variation among isolates taken from patients within the same in-patient facility within a month of each other (Table 6) or isolates taken

from the same patient on the same day (Table 7). The tables are organised according to groups based on drug-resistance mutations, and therefore such mutations are not necessarily reflected in the numbers. These results imply that the isolates selected for next generation sequencing are not all reflective of recent

Table 4: gyrA mutations observed in the selected isolates

Isolate	Genotypic classification	Phylogenetic relationship	*gyrA* mutation
TT46	TDR	Group A	91CCG
TT48	XDR	Group B	94AAC
TT62	TDR	Group B	94AAC
TT99	XDR	Group B	90GTG
TT98	TDR	Group B	90GTG
TT135	TDR	Group A	90GTG
TT154	XDR	Group B	94GGC
TT61, TT147	TDR	Group B	94GGC
TT219	TDR	Group B	91GCG/94AAC

Table 5: Number of SNPs in each isolate (column) relative to each other isolate (row) in Group A

	TT46 JP	TT135 SH	TT622 FH
TT46 JP	0	32	19
TT135 SH	13	0	10
TT622 FH	15	25	0

Health districts are colour coded
Abbreviations represent health care facilities

transmission events. Although direct transmission cannot be ruled out based on geographic location, it certainly decreases the likelihood of transmission, especially in this setting where rural areas are very remote and residents tend not to travel far from their hometowns (personal communication, Dr Kate Mekler, Beyond Zero). Therefore our results question the definition of transmission as isolates from patients from different regions of the EC in some instances showed less than 15 SNP variations. This was further exemplified when the genomes from isolates TT606 and TT607 were compared (Table 7). These isolates were taken from the same patient, on the same day. From our analysis it is clear that these isolates are evolving independently within a single host with TT607 gaining only 3 SNPs with respect to TT606, while TT606 gained 47 SNPs with respect to TT607.

Even considering the possibility of mixed infection, it is implied that with respect to the common progenitor, one strain gained many SNPs while the other gained only a few. Further examples of such unequal evolution are evident from Tables 5-7. These results are in accordance with studies which showed stability within the *M. tuberculosis* genome, as well as evolutionary bursts, concluding that genetic distance alone cannot be used to infer transmission (15,38). In addition, in an endemic setting such as the EC, inference of transmission may be even further confounded by extended networking among cases as well as patients not included in the study.

Table 6: Number of SNPs in each isolate (column) relative to each other isolate (row) in Group B.

	TT248 KzC 09/08	TT160 PMT 12/08	TT147 KxC 11/08	TT48 JP 08/08	TT219 PE 10/08	TT99 FG 09/08	TT98 GC 09/08	TT62 JP 08/08	TT57 JP 08/08	TT145 NC 12/08	TT61 WT 08/08	TT154 CC 12/08
TT248 KzC	0	11	10	8	16	14	25	25	17	14	15	22
TT160 PMT	3	0	10	8	14	13	24	23	15	12	13	20
TT147 KxC	26	34	0	31	37	35	47	43	35	30	32	39
TT48 JP	3	11	10	0	11	11	22	21	13	11	12	19
TT219 PE	5	11	10	5	0	10	22	18	10	12	12	19
TT99 FG	6	13	11	8	13	0	20	18	14	10	9	16
TT98 GC	6	13	12	8	14	9	0	20	13	9	10	17
TT62 JP	5	11	7	6	9	6	19	0	4	5	4	12
TT57 JP	4	10	6	5	8	9	19	11	0	5	5	13
TT145 NC	5	11	5	7	14	9	19	16	9	0	4	11
TT61 WT	5	11	6	7	13	7	19	14	8	3	0	8
TT154 CC	6	12	7	8	14	8	20	16	10	4	2	0

Health districts are colour coded
Abbreviations represent health care facilities
Cells representing data from patients from the same facility, and collected within one month are highlighted

5.3.3 Variants: SNPs

All F31 Atypical Beijing strains, including those that were thought to be pan-susceptible based on drug-susceptibility testing for first-line drugs, were found to harbour mutations in *ethA*, as well as in *Rv1772*. The mutation in *Rv1772* was a synonymous mutation in codon 61, and as such, has not been implicated in resistance. However, previous studies have reported an association between

Rv1772 mutations and INH resistance (39). The *ethA* mutation (A381P) has previously been associated with resistance to ethionamide (ETH) (40), a second-line TB drug that was historically used in dual therapy. This ETH resistance could also explain the vastly different distribution of Atypical Beijing genotype isolates seen in different classes of drug-resistance: 4% among "pan-susceptible" TB; 27% among MDR *s.s.*-TB and 93% among pre-XDR- and XDR-TB (4). ETH resistance would remain undetected among otherwise drug-susceptible isolates, without conferring a selective advantage. However, once INH and RIF resistance has been acquired (or with current guidelines, once RIF resistance is acquired (41)) and the patient was treated with an ETH-containing second-line regimen, these strains would acquire additional resistance more readily due to the regimen inadvertently being compromised. Preliminary DST results suggest that *ethA* mutations confer a relatively lower level of ETH-resistance (unpublished data), therefore the bacilli "require" an additional mutation in the *inhA* promoter for full resistance, which contributes to development of an XDR-TB phenotype (42).

Table 7: Number of SNPs in each isolate (column) relative to each other isolate (row) in the drug-susceptible and MDR *s.s.* group

	TT648 TC	TT607 PMT	TT50 SAP	TT372 KzC	TT719 LBD	TT321 SAP	TT606 PMT	TT700 GMC	TT574 LP	TT545 GP	TT589 FH	TT679 KzC
TT648 TC	0	50	69	58	59	101	85	69	78	79	74	84
TT607 PMT	29	0	50	41	38	80	47	55	57	59	54	61
TT50 SAP	19	21	0	25	30	44	45	39	38	40	36	43
TT372 KzC	19	23	36	0	22	56	39	22	27	29	21	31
TT719 LBD	21	21	42	23	0	62	37	35	42	40	35	45
TT321 SAP	19	19	12	13	18	0	19	17	19	15	12	16
TT606 PMT	20	3	30	13	10	36	0	17	20	19	12	20
TT700 GMC	18	25	38	10	22	48	31	0	24	23	15	24
TT574 LP	19	19	29	7	21	42	26	16	0	13	6	13
TT545 GP	19	20	30	8	18	37	24	14	12	0	8	12
TT589 FH	20	21	32	6	19	40	23	12	11	14	0	13
TT679 KzC	19	17	28	5	18	33	20	10	7	7	2	0

Health districts are colour coded
Abbreviations represent health care facilities
TT606 and TT607 were isolated from the same patient on the same day (highlighted)

When isolates from Group B was compared to Group A and Group C, seven SNPs were common to all strains of Group B and not present in any other strain. These SNPs are located in *mmaA4*, *rpoB*, *murA*, *Rv2019*, *viuB*, *embB*, and *inhA* promoter, as detailed inTable 8.

MmaA4

MmaA4 encodes methoxy mycolic acid synthase 4, which is involved in the production of hydroxymycolic acid, a component of the bacterial cell envelope. Mycolic acids have been shown to play a role in virulence through their influence on the permeability of the cell envelope – decreased permeability is hypothesised to restrict the entry of nutrients and antibiotics, thereby increasing intrinsic resistance. Furthermore, mycolic acids were shown to be essential for viability, drug resistance and cell wall integrity (43) and they play a role in immune modulation of IL-12 (44), affirming a different mechanism of virulence mediated by the same gene product. In vitro generated thiacetazone (a repurposed drug used in the treatment of XDR-TB) resistant mutants harboured a SNP in the *mmaA4* gene (45). However, none of the mutants had the specific mutation seen in this study. Taken together, this evidence provides support for further investigation of the functionality *mmaA4* and its products in these isolates. Even though the mutation seen in this instance is a synonymous SNP, it may be important, as recent findings have shown that synonymous SNPs may play a role in drug resistance, for instance, a silent mutation in *mabA* acts as an enhancer of *inhA* transcription, leading to isoniazid resistance (46).

MurA

The product of the gene *murA*, UDP-*N*-acetylglucosamine enolpyruvyle transferase (MurA) is involved in the first reaction in the biosynthesis of peptidoglycan. The coding region of the gene also contains the transcription starting point of the *rrnA* operon (47), and the two transcripts have been shown to form a hybrid (protein coding-rRNA coding) operon, although the patterns of expression during the growth cycle were different for *murA* and *rrnA* (47).

ViuB

ViuB encodes a probable mycobactin utilization protein, involved in iron acquisition, which is essential for mycobacterial survival and growth. This protein has been shown to be over expressed

in macrophages (48), although a recent study showed that the protein was dispensable to *M. tuberculosis* for growth (49).

Rv2019

A SNP was also present in *Rv2019*, encoding a conserved hypothetical protein. The role of this gene is discussed under large deletions (section 5.3.5).

Isolates from Group A harboured 5 unique SNPs, namely in *Rv0785* (encoding a conserved hypothetical protein); *pncA* (14GCG), associated with pyrazinamide resistance; *embB* (306ATC), associated with EMB resistance; *mez* and in an intergenic area located between *Rv3144c* (*PPE52*) and *nuoA* (Table 8) that was not present in any other group.

Mez

The protein encoded by *mez* is an NAD dependent malate oxidoreductase, which is involved in the formation of pyruvate, and as such forms part of the intermediary metabolism of *M. tuberculosis* (50).

NuoA and *PPE52*

The intergenic SNP at genomic position 3511368 lies 51 bp upstream from *PPE52* (reverse orientation), and 314 bp upstream from *nuoA* (forward orientation), and could therefore form part of either gene's promoter region. The function of PPE52 is currently unknown, and it is hypothesised to be non-essential (50). *NuoA* is a probable NADH dehydrogenase I, involved in respiration (50).

The variants in the *inhA* promoter (42,51,52), *embB* (53,54), *katG* (55), *rpoB* (56,57), *rrs* (58,59), and *pncA* (60,61) are well-described, and known to be involved in drug-resistance as indicated in Table 8.

These changes indicate a fundamental difference between Group A and B, and suggest their independent evolution. The two groups can also be distinguished by their *rpoB* mutations: all isolates in Group A have a mutation in codon 531, whereas all isolates in Group B have a mutation in

codon 516. However, both these mutations are also present in the MDR isolates from group C. Furthermore, an *rpoC* mutation in codon 483 is present in all Group A isolates, as well as in one of the MDR isolates (TT648, group C). This isolate also harbours the *rpoB* 531 mutation as well as the *inhA* promoter -15 mutation that is present in Group A. TT50 (*rpoB* 531) harbours a different *rpoC* mutation (codon 521), suggesting that *rpoB* 531 mutations predisposes the bacilli to acquire *rpoC* mutations (62). Neither TT321 (*rpoB* 516) nor any of the Group B isolates harbours *rpoC* mutations.

5.3.4 Variants: Indels

Two insertions (Table 8) were present in all isolates of Group B, and in no other isolate, warranting further investigation. The first was a known insertion in the *pncA* gene (ins172G), associated with pyrazinamide resistance. This insertion causes a frame-shift leading to the elongation of the transcript to include *Rv4042c*, a hypothetical conserved protein which is situated immediately downstream from *pncA*.

The second indel was a T-insertion in codon 12 of the *vapC22* gene, leading to a frame shift. This gene encodes a possible toxin involved in virulence, detoxification or adaptation (63). *VapC22* is part of a toxin-antitoxin system – one of many of its kind found in *M. tuberculosis*. The toxin is hypothesised to be induced under stress conditions, acting as a ribonuclease and leading to arrested cell growth, which has been implicated in dormancy and persistence (63). Co-expression of its cognate antitoxin (*vapB22*) ensures binding of the two proteins in a complex that inhibits the action of the toxin, while also inhibiting translation of the operon by binding to its promoter region. Although the exact function of this specific toxin is still unclear, evidence suggest that vapC22 inhibits cell growth in the absence of its cognate antitoxin (64).

Present in all Group A and B isolates, as well as two MDR *s.s.* isolates, was a 1bp deletion (del671G) in *Pks9*, thought to be involved in the synthesis of a polyketide molecule, which may be involved in secondary metabolism.

Table 8: Variants as called by GATK for three alignment algorithms (BWA, Novoalign and SMALT)

Gene	Function	Isolates affected	Nucleotide change	Amino acid change; drug-resistance
Nonsynonymous SNPs				
inhA promoter	promoter of inhA (NADH-dependent enoyl reductase)	Group B	G -17 A	(intergenic) Isoniazid and ethionamide resistance
rpoB	RNA polymerase	Group B	GAC516CTC	D516V Rifampicin resistance
embB	Arabinan synthesis	Group B	ATG306ATA	M306I Ethambutol resistance
murA	peptidoglycan synthesis	Group B	GGC385GAC	G385D
Rv2019	unknown	Group B	ATC109ACC	I109T
inhA promoter	promoter of inhA (NADH-dependent enoyl reductase)	TT135; TT622; TT648	C -15 A	(intergenic) Isoniazid and ethionamide resistance
rpoB	RNA polymerase	Group A; 2 MDR	TCG531TTG	S531L Rifampicin resistance
rpoC	RNA polymerase	Group A; 1 MDR	GTG483GGG	V483G Compensatory mutation
embB	Arabinan synthesis	Group A	ATG306ATC	M306I Ethambutol resistance
pncA	Converts amides to corresponding acid	Group A	AGC14CGC	C14R Pyrazinamide resistance
Rv0785	Unknown/probable membrane bound dehydrogenase	Group A	CAT350TAT	H350Y
mez	probable malate oxidoreductase	Group A	GCA15ACA	A15T
genomic position 3511368	intergenic	Group A	G-->T	unknown;51bp upstream of Rv3144c (PPE52) or 314bp upstream of *nuoA*
rrs	16S RNA	Group A, Group B	A513C	Streptomycin resistance
rrs	16S RNA	Group A, Group B	A1401G	Aminoglycoside and capreomycin resistance
katG	Catalase and pyroxidase	All DR isolates	AGC315ACC	S315T Isoniazid resistance
Synonymous SNPs				
mmaA4	mycolic acid cyclopropanation	Group B	CTG63CTA	L63L Possible Thiacetazone resistance
viuB	iron acquisition	Group B	CCG176CCA	P176P
Insertions				
pncA	Converts amides to corresponding acid	Group B	ins172C	Frame shift; extension by 265 amino acids; PZA resistance
vapC22	toxicity	Group B	ins12T	Frame shift
Deletions				
pks9	polyketide synthesis	All DR strains	del671G	truncation of protein at codon 673

Group A and Group B derived from Figure 3; All DR strains = all drug-resistant strains, including MDR

5.3.5 Variants: large deletions

GATK was used to identify areas of zero coverage, implying that these areas are deleted from the genome. Files of the different strains were inspected visually. These deletions were used as a starting point to verify the absence of coverage by viewing the alignments in Artemis (Sanger Istitute). Isolates from this study were also compared to three additional Atypical Beijing (F31) strains, 4 Typical Beijing (F29) strains and one non-Beijing strain. As the purpose of the study was to identify genetic elements that may confer an advantage to the strains of interest, deletions that were present in all of the above strains were disregarded.

A number of areas were deleted from all F31 isolates but not any of the other strains. These include 1207bp from the region *GlnA* - *Rv1879* (previously described as RD163 (65)); 80bp from *Rv1841c* and 7bp from *esxP*. A 2390bp deletion starting in *Rv2016* and ending in *Rv2019* includes the previously described RD175a (65), and is also found in all F31 isolates, but none of the other isolates. *Rv2016*, *Rv2018* and *Rv2019* are conserved hypothetical proteins that are deemed to be non-essential in *M. tuberculosis* H37Rv. *Rv2019* is postulated to be involved in information pathways (50), while *Rv2017* is thought to be a transcriptional regulator, and essential for *in vitro* growth (50). This observation questions the definition of essentiality defined by Himar1 transposon mutagenesis (66). These four deletions may prove useful markers for the classification of these Atypical Beijing strains. The physiological impact of these deletions remains to be determined.

Deletions constituting other previously described Beijing-specific RDs include RD105 (*Rv0071-Rv0074*), RD131e (*Rv0795-Rv0796*), RD149 (*Rv1573-Rv1588c*), RD152 (*Rv1754c-Rv1765c*). Deleted in all strains (including non-Beijing) was *Rv2278-Rv2279*, which falls within RD182 and *Rv2648-Rv2649*, within RD198a (65).

5.3.6 RNAseq analysis

In order to identify differential gene expression (≥2-fold change in RNA abundance with a 99% confidence interval) of the Group A or Group B isolates we compared RNA abundance relative to the pan-susceptible Atypical Beijing genotype strains.

Table 9: Table of the top 10 up-regulated genes for Group A and Group B, relative to drug-susceptible TT372

Group A					Group B				
Gene name	**Function**	**Fold change**	**p-value**	**Range**	**Gene name**	**Function**	**Fold change**	**p-value**	**Range**
proT	-	55.9 up	0.048	12.4-162.4 up	*vapB22*	Toxin	28.9 up	<0.001	11.2-52.2 up
Rv0699	Probable hydrolytic enzyme	22.9 up	0.010	7.2-63.5 up	*vapC22*	Antitoxin	17.8 up	<0.001	7.6-27.5 up
inhA	NADH-dependent enoyl reductase	22.1 up	0.002	12.8-30.1 up	*lppN*	Probable lipoprotein	14.8 up	0.004	8.2-30.8 up
moaD1	Molybdenum cofactor synthesis	21.9 up	0.039	14.9-30.5 up	*Rv1669*	Unknown	12.5 up	0.018	5.8-20.2 up
fabG1	Fatty acid biosynthesis	14.4 up	0.020	3.6-29.5 up	*Rv2828c*	Unknown	11.2 up	0.026	3.4-20.0 up
esxS	ESAT-6-like protein	14.3 up	0.039	10.3-22.6 up	*fadD18*	Lipid degradation	10.8 up	0.005	1.0-49.7 up
Rv1138c	Possible oxidoreductase	12.8 up	0.026	11.8-13.4 up	*lppQ*	Probable lipoprotein	9.8 up	0.015	6.0-12.6 up
hemZ	Protoheme biosynthesis	12.2 up	0.002	8.3-17.0 up	*echA16*	Fatty acid oxidation	9.4 up	<0.001	5.6-16.0 up
PE22	Unknown	11.8 up	0.037	9.0-14.6 up	*Rv1138c*	Possible oxidoreduc-tase	9.2 up	0.016	2.5-19.6 up
leuT	-	9.6 up	0.010	6.0-17.0 up	*esxS*	ESAT-6-like protein	8.3 up	0.046	1.2-42.6 up

When comparing Group A or Group B to TT372 (drug-susceptible), 423 and 721 genes were differentially expressed, respectively. For each of these two groups, the top-ten up-regulated genes are presented in Table 9. Very similar results were obtained when the groups were compared to TT700 (drug-susceptible). This suggests that both groups of drug resistant isolates differs fundamentally from the respective drug-susceptible isolates, and each drug-resistance group differs from each other. Interestingly, in Groups A or B compared to drug-susceptible, only 2 genes were down-regulated (*Rv3395c* and *moaC1*), as opposed to the many genes that were up-regulated (423 and 719, respectively).

Table 10: Top 10 genes significantly up-regulated in both Group A and Group B

Name	Function	Group A	p-value	Range	Group B	p-value	Range
esxS	ESAT-6-like protein	14.3 up	0.039	10.3-22.6 up	8.3 up	0.046	1.2-42.6 up
fabG1	Fatty acid biosynthesis	14.4 up	0.020	3.6-29.5 up	7.2 up	<0.001	5.1-11.4 up
hemZ	Protoheme biosynthesis	12.2 up	0.002	8.3-17.0 up	4.3 up	<0.001	3.4-6.2 up
inhA	NADH-dependent enoyl reductase	22.1 up	0.002	12.8-30.1 up	7.4 up	<0.001	5.3-10.0 up
lppQ	Probable lipoprotein	9.4 up	0.044	9.7-11.6 up	9.8 up	0.015	6.0-12.6 up
PE22	Unknown	11.8 up	0.037	9.0-14.6 up	6.8 up	0.046	1.1 down-13.5 up
Rv0699	Probable hydrolytic enzyme	22.9 up	0.010	7.2-63.5 up	6.7 up	<0.001	None-70.9 up
Rv1138c	Possible oxidoreductase	12.8 up	0.026	11.8-13.4 up	9.2 up	0.016	2.5-19.6 up
Rv3638	Possible transposase	6.1 up	0.035	5.6-7.0 up	6.2 up	0.010	3.6-8.6 up
thiS	Thimine biosynthesis	9.0 up	0.039	6.7-11.6 up	5.9 up	0.037	3.6-10.7 up

In order to investigate similarities between the drug-resistance groups (Group A and B) relative to drug-susceptible (TT372), the 34 most differentially expressed genes (at statistical significance) in each of the groups was compared. The overlap consisting of 10 genes is presented in Table 10.

Genes affected by the variants listed in Table 8 were investigated to identify an association with RNA abundance (presented in Table 11). Interestingly, the genes with known drug-resistance causing mutations were all up-regulated in Groups A or B, but no more than 1.8 fold, with the notable exception of *embB* (2.1 and 2.4 fold up) and *inhA* (7.4 and 22.1 fold up) (all statistically significant ($p<0.05$), except *rrs*). In fact, *inhA* was one of the most severely affected genes in both Group A and B, but more pronounced in Group A. Further investigation revealed that the genes surrounding *inhA* were also up-regulated. The effect was most pronounced in *fabG1* and *hemZ* – the genes immediately up- and down-stream from inhA (Figure 5), and part of the same operon. The up-regulation of genes within the same operon in isolates with *inhA* promoter mutations is not surprising, as promoter mutations have the potential to alter expression levels of the genes they regulate. It is, however, unexpected that genes adjacent to the operon is also up-regulated, possibly suggesting their co-regulation, although these genes were not up-regulated to the same extent as the *fabG1-hem*Z operon. It is unclear why these three genes are regulated as an operon, as *fabG1*

Table 11: RNA abundance in the relevant groups for genes affected by small mutations (SNPs or indels)

	Group A				Group B			
Gene name	**Mutation**	**Fold change**	**p-value**	**Range**	**Mutation**	**Fold change**	**p-value**	**Range**
inhA	C-15A	22.1 up	0.002	12.8-30.1 up	G-17A	7.4 up	<0.001	5.3-10.0 up
rpoB	TCG531TTG	1.8 up	0.010	1.6-2.2 up	GAC516CTC	1.4 up	0.003	1.0 down-2.1 up
embB	ATG306ATC	2.1 up	0.045	1.4-3.1 up	ATG306ATA	2.4 up	<0.001	1.8-3.4 up
murA	N/A	2.2 up	0.006	2.0-2.8 up	GGC385GAC	1.7 up	<0.001	1.4-2.0 up
Rv2019	N/A	2.3 up	0.028	1.7-3.0 up	ATC109ACC	1.9 up	0.011	1.1 down-2.9 up
rpoC	GTG483GGG	2.1 up	0.007	1.8-2.6 up	N/A	1.6 up	<0.001	1.1-2.1 up
pncA	AGC14CGC	1.1 up	0.624	1.1 down – 1.2 up	is172C	1.4 up	0.002	1.0-1.9 up
Rv0785	CAT350TAT	1.5 up	0.190	1.2-2.0 up	N/A	1.8 up	0.008	1.0 down-2.3 up
mez	GCA15ACA	1.3 up	0.080	1.1-1.4 up	N/A	1.5 up	<0.001	1.1-1.9 up
PPE52	pos 3511368 G->T	2.4 up	0.013	1.9-3.2 up	N/A	2.1 up	<0.001	1.1-2.9 up
nuoA	pos 3511368 G->T	1.9 up	0.045	1.4-2.6 up	N/A	1.7 up	0.006	1.2-2.0 up
rrs	A513C; A1401G	1.4 up	0.105	1.0-1.8 up	A513C; A1401G	1.3 up	<0.001	1.1 down-1.8 up
katG	AGC315ACC	1.6 up	0.009	1.5-1.8 up	AGC315ACC	1.3 up	0.027	1.2 down-1.4 up
mmaA4	N/A	2.0 up	0.022	1.4-2.5 up	CTG63CTA	1.9 up	<0.001	1.4-2.8 up
viuB	N/A	1.4 up	0.161	1.2-1.8 up	CCG176CCA	1.6 up	0.011	1.0-2.3 up
vapC22	N/A	1.3 up	0.238	1.1-1.7 up	ins12T	17.8 up	<0.001	7.6-27.5 up
pks9	del671G	1.7 up	0.028	1.3-2.1 up	del671G	1.6 up	<0.001	1.2-2.6 up

and *inhA* are components of the fatty acid synthase (FAS) II enzyme system, involved in mycolic acid synthesis (67), whereas *hemZ* is a ferrochelatase (68). It is also interesting to note that both the -15 and -17 mutations in the promoter-region leads to up-regulation of the operon, but that the -15 mutation has a much greater effect. This needs further investigation, as the apparent effect may be the result of pooling of data.

Of the genes that were shown by WGS to harbour SNPs or indels but are not known to cause drug-resistance, only *vapC22* was significantly up-regulated, in the relevant group (Group B). Similar to *inhA*, this was one of the most highly up-regulated genes in the group, and again, other genes in close proximity were also highly up-regulated, reaching statistical significance in each case (Figure 6). Again, several genes up-and down-stream from this operon (*vapBC*) were similarly up-regulated, possibly suggesting some common involvement.

Under normal conditions, formation of the vapBC complex inhibits the action of the toxin, and binds to the promoter of the operon, inhibiting translation of both proteins. In the case of the Group B isolates, we hypothesise that the disruption of the vapC22 protein through the nucleotide insertion prevents complex formation with vapB22. This in turn prevents the inhibition of translation through binding with the promoter, explaining the massive up-regulation of *vapB22* and *vapC22*. Furthermore, we hypothesise that the alteration of the protein abrogates its putative toxic effect, allowing the cells to be fully functional despite the inability of vapB22 to inhibit the toxin. However, this needs validation through protein studies.

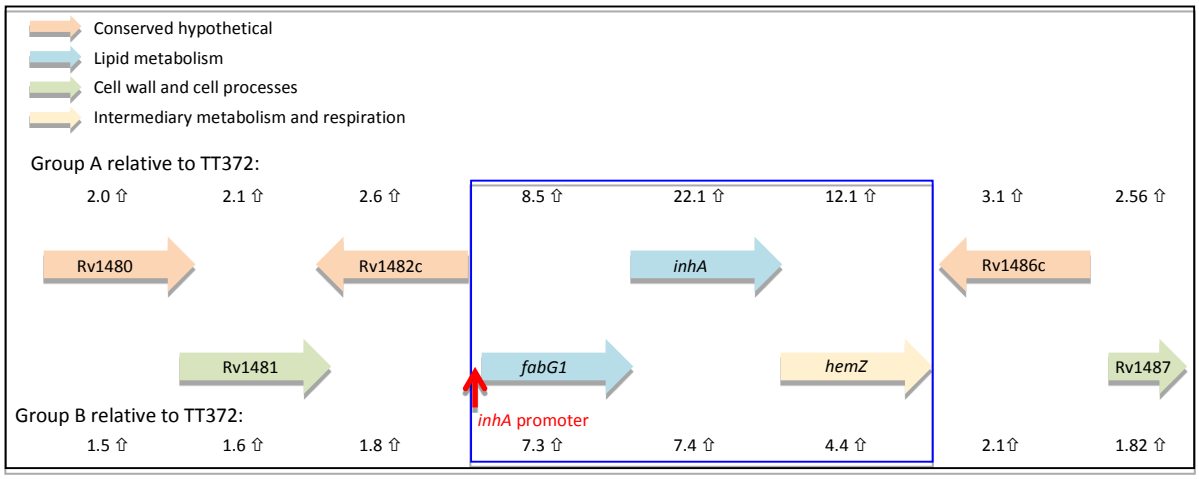

Figure 5: inhA operon showing relative upregulation in relation to TT372, for both Group A and Group B isolates. Genes immediately up- and downstream of the operon were also up-regulated.

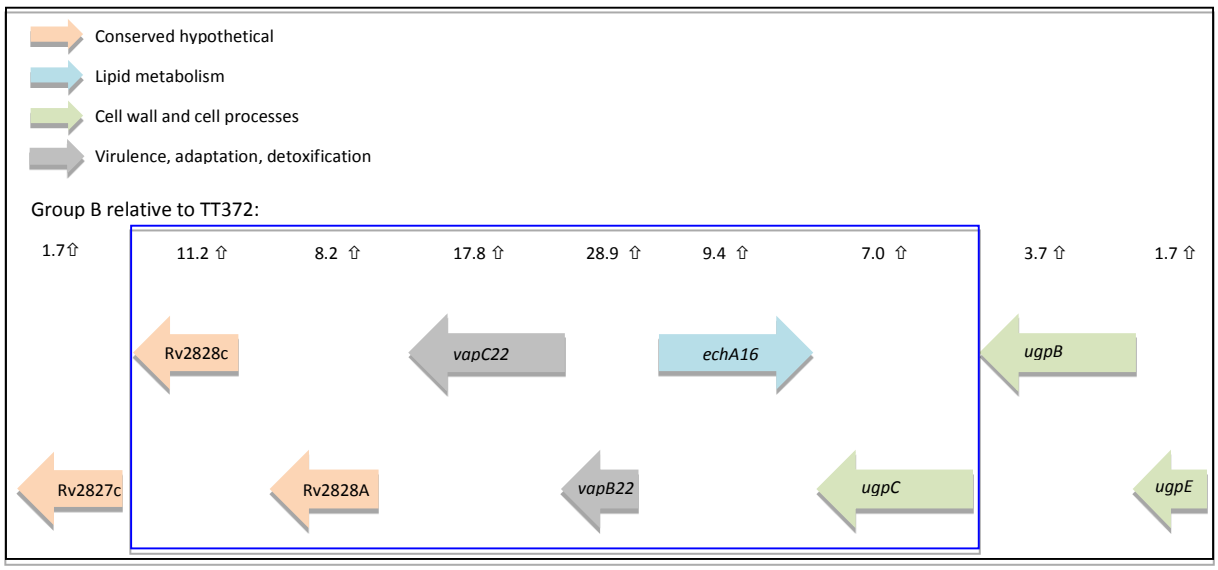

Figure 6: vapBC operon and surrounding genes upregulated in Group B isolates, compared to TT372

5.3.7 Limitations

It is unknown at what point in the evolution of atypical Beijing strains the development of increased fitness occurred – if at all. Therefore, by looking for variants that are only present in drug-resistant strains, those changes that are also present in (some) drug-susceptible isolates will be missed. More isolates, preferably serial isolates from the same patient, and powerful statistics will be needed to elucidate this.

5.4 Conclusion

Our molecular epidemiological data reveals that the Atypical Beijing genotype strains from the Eastern Cape Province are closely related, and that the majority of the selected strains belong to the F31-subgroup. The whole genome sequencing data suggest that existing phylogenies of Beijing genotype strains need to be revisited as these isolates lack a specific SNP that was thought to be common to all Atypical and Typical Beijing genotype strains, while possessing another SNP that was thought to be evolutionarily more recent. Furthermore, the presence of certain large deletions could be applied as markers to distinguish between F31 and other Atypical and Typical Beijing genotype strains. However, the deletion of *Rv2017*, which was thought to be essential by Himar1-transposon mutagenesis, questions the definition of essentiality.

SNP and indel data show that apparently two separate genotypes (Group A and Group B) of drug-resistant Atypical Beijing genotype strains have evolved historically, each with distinctive features, including specific drug-resistance mutations. However, careful consideration of additional data suggest that Group A may be more diverse than what is evident by the WGS of only three strains. Both these genotypes have subsequently spread throughout the Province and is currently endemic. Relatively little variation is observed between some isolates that are not epidemiologically linked, suggesting a low mutation rate. In contrast, more variation exist between other isolates with proven epidemiological links than what is considered the maximum amount indicative of a transmission event. This confirms findings of other studies (15,38) that although the genome seems to be stable in most cases, the number of SNPs difference alone cannot be used to infer transmission events and

that the rate of mutation of *M. tuberculosis* may not be fixed. Taken together, these results question the validity of quantifying transmission events in terms of SNPs.

RNA abundance studies confirmed that both Group A and Group B are distinct from drug-susceptible isolates, as well as from each other. Further investigation into the roles of other abundantly expressed RNA, especially those of the altered vapBC22 is needed to determine their role in the metabolism, virulence and adaptation of these highly resistant strains.

However, some similarities between the drug-resistance groups exist. For example, the the *inhA* promoter mutations in both Group A and Group B-isolates has a profound effect on the RNA abundance of the genes that forms part of the operon regulated by that promoter. InhA is the target of both ETH and INH (in their activated forms). The massive up-regulation of InhA ensures that there is an over-abundance of the target of these drugs, depleting the drug before all of the target is bound, effecting drug-resistance. This is in accordance with the INH and ETH resistant phenotypes observed when InhA is overexpressed (69)

A concerning finding was the presence of a mutation in *ethA* in all F31 isolates, including those that were regarded pan-susceptible, based on standard first line DST and Sanger sequencing of *katG* and *rpoB*. This mutation has previously been linked with resistance to ETH (40). Preliminary quantitative DST results suggest that these isolates are indeed ETH resistant. This implies that the standard second-line regimen may be compromised for individuals infected with Atypical Beijing genotype strains. Further validation is required to prove the causality of the mutation for ETH resistance.

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Chapter 6 Literature review

This literature review will be presented to the WHO Bulletin for publication.

Diagnosis and management of drug-resistance beyond XDR-TB: lessons from the South African context

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My contribution to this work included initiation of the review, literature search and writing of the manuscript

He is a man of courage who does not run away, but remains at his post and fights against the enemy. – Socrates

Management of drug-resistance beyond XDR-TB: lessons from the South African context

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Abstract

Drug-resistant Tuberculosis (TB) is a world-wide problem that is growing not only in numbers of cases affected, but also in numbers of drugs lost to resistance. Amplification of drug-resistance, where the bacilli become resistant to an increasing number of drugs, is facilitated through inadequate drug-susceptibility testing (DST), concomitant with inappropriate treatment. In South Africa, several iterations of testing algorithms and treatment regimens have not improved the situation due to a knowledge gap in understanding the repertoire of resistance circulating. Currently, many patients are infected with TB strains that are resistant to all drugs used in the standardised MDR-TB regimen as well as drugs used to treat XDR-TB. These patients are put on salvage regimens without knowing their resistance profiles, which often leads to treatment failure and death. When all treatment options are exhausted, these infectious patients may be sent home, potentially perpetuating the epidemic by allowing other members of the community to be infected with these highly resistant TB strains. This problem can only be addressed by ensuring adequate treatment regimens consisting of at least 4 effective drugs are always given. With the available anti-TB drugs, this can only be achieved by testing all drugs intended for inclusion in the regimen before treating. Effective treatment of highly resistant TB will depend on the development of new drugs, new drug combinations in association with comprehensive rapid DST. In the interim, palliative care facilities for patients without adequate treatment options need to be built within the communities where such patients reside.

6.1 Introduction

Tuberculosis (TB) control is a global problem, most severely affecting resource-poor countries. A key concern has been the rise in drug-resistant TB (DR-TB), which first emerged shortly after the introduction of the first anti-TB drug. Since its emergence, it has been a constant battle to keep ahead of the evolution of DR-TB. In the mid-1970s, short course treatment regimens including rifampicin (RIF) were initiated and have subsequently been adopted by the WHO to threat TB on a global scale. However, this strategy is threatened by the emergence and spread of multi-drug resistance (MDR-TB, resistant to isoniazid (INH) and RIF). The loss of these two most potent anti-TB drugs severely compromises treatment outcome and has become a measure of the efficacy of TB control programs worldwide. Globally, there were estimated to be 480,000 incident cases of MDR-TB in 2013 (1). Only 97,000 detected MDR-TB cases were started on treatment and less than half of these patients have favourable outcomes.

In 2006, the severity of the drug resistant TB epidemic was brought to the attention of the world by a report documenting the spread and high mortality of a highly resistant form of TB in HIV-infected patients in South Africa. This culminated in the WHO definition of extensively drug-resistant (XDR-) TB – resistance to INH, RIF, second-line injectables and fluoroquinolones (FQ) (2). Global surveillance now reports XDR-TB in 100 countries, affecting an estimated 43,000 patients (1). This indicates that XDR-TB is a problem that is not restricted to high TB-burden countries, and that it is emerging repeatedly.

The application of comprehensive drug-susceptibility testing (DST) has identified patients with TB that was resistant to more drugs than those that strictly define XDR-TB. These cases have been controversially referred to as XXDR-TB, totally drug-resistant (TDR)-TB or super-XDR-TB (3–7). In addition, several publications from around the world report TB with resistance beyond XDR, but do not differentiate such resistance from XDR by the use of a special term (8–12).

6.2 Historic drivers of DR-TB in South Africa

Chemotherapy of TB in South Africa started soon after the discovery of streptomycin (SM) and largely followed recommended regimens used throughout the world. In 1965, surveillance showed that primary and acquired resistance to INH was as high as 14% and 54% respectively, in newly admitted adult black tuberculosis patients. By 1988, these figures had declined to 9% and 15%, respectively (13). This was ascribed to the implementation of treatment guidelines and the

introduction of RIF into the regimen. Although the emergence of MDR-TB was first noted in the early 1980's, the 1992-1993 Western Cape drug resistance survey (14) highlighted the emergence of the MDR-TB epidemic in South Africa (1.1% in new cases and 4.0% in retreatment cases). These statistics reflect the drug-resistant TB epidemic prior to the rise of the HIV pandemic.

The first national drug-resistance survey, conducted in 2002, showed MDR-TB rates of 1.6% in new and 6.6% in retreatment cases (15). Resistance to ethambutol (EMB) and SM was present in 25% of these cases (15). By 2011, MDR-TB had increased dramatically to 7.8% of new TB cases while 5.7% of all MDR-TB cases were found to be XDR-TB (16).

6.3 The South African response plan

Since the recognition of MDR-TB, control policies, including that of South Africa, have been a few steps behind the epidemic due to inadequate data, insufficient resources and the dogma that drug-resistant strains were less likely to transmit. Despite these shortcomings, and in response to the 2002 drug-resistance surveillance data, a standardized MDR-TB treatment regimen was implemented. At that time, financial constraints limited drug-resistance testing to high-risk cases and treatment failures (17). These patients received DST for INH, RIF and EMB only, despite the unreliability of EMB DST. All patients were placed on standard first-line treatment prior to obtaining DST results. New cases received INH, RIF, EMB and pyrazinamide (PZA) during the intensive phase and INH and RIF during the continuation phase. Retreatment cases received an additional drug (SM) during the intensive phase and the treatment duration was extended. This strategy created an opportunity for amplification of resistance to include PZA and EMB resistance if undiagnosed MDR-TB was present. Once diagnosed with MDR-TB, standardised MDR treatment was initiated. However, this MDR-TB regimen failed to recognize the fact that the MDR-TB strains may have been resistant to EMB and PZA (18–20). This, together with high default rates, could explain the poor treatment outcomes (treatment success rate below 48%) (21).

The Tugela Ferry outbreak in Kwa-Zulu Natal (KZN), which lead to the definition of XDR-TB, was ascribed at the time to a failing health system and poor infection control (2). The South African Department of Health's response was to bolster the treatment regimen by the inclusion of two additional drugs for the treatment of XDR-TB, offering second-line DST for all MDR-TB cases and improving infection control in health care facilities (22). Despite the introduction of these measures, amplification of resistance continues to occur (11). In KZN, the proportion of XDR-TB cases resistant to six drugs increased from 45% in 2005 to 100% in 2009. Sixty-eight percent of these were resistant

to eight anti-TB drugs (11). This was the first indication that drug-resistance "beyond XDR" was present in South Africa. In 2011, new MDR-treatment guidelines were implemented, whereby ofloxacin was replaced with moxifloxacin (MOX), and EMB with terizidone (TZD) (23). However, these measures continue to ignore the high prevalence of PZA resistance and cross-resistance between INH and ethionamide (ETH) in some patients (24).

In 2011, the Department of Health introduced molecular based *M. tuberculosis* diagnosis and RIF DST in the form of GeneXpert to improve case detection. 2014 diagnostic guidelines focus only on identifying RIF resistant cases implying that INH mono-resistance will be missed. The consequence of this strategy has not been fully realised as a recent study revealed that the relative risk of acquired RIF resistance was significantly increased in patients with undetected INH mono-resistance resulting in the emergence of MDR-TB (25). These guidelines stipulate that patients with RIF resistant TB should be placed on standardized MDR-TB treatment (see below). This implies treating blindly for one to two months (26) until the treatment regimen can be adjusted according to the DST profile. However, culture-based DST is restricted to the detection of resistance towards FQ and aminoglycosides. DST for EMB, PZA and ETH is not routinely done. This potentiates the risk for the amplification of drug resistance in cases that have resistance beyond MDR-TB, potentially negatively impacting on treatment outcome.

6.4 Current treatment guidelines

The latest management guidelines recommend that any RIF resistant TB should be treated with a standardized MDR regimen (Kanamycin (KAN), MOX, ETH, TZD, PZA), regardless of INH susceptibility (23,27). INH mono-resistant TB will be treated as drug susceptible TB unless subsequent DST is done should the patient fail to respond after two months of treatment. Therefore, INH mono-resistant cases are treated with only three effective drugs and one drug during the continuation phase if undiagnosed. This is in contrast to the WHO recommendation of treatment with at least 4 effective drugs (28), as well as the South African 2004 guidelines, which states that one of the most common errors leading to the selection of resistant bacilli is prescription of inadequate chemotherapy (e.g. three drugs during the initial phase of treatment) (17). However, if INH mono-resistant TB is diagnosed, the patient should be treated with the standard first line combination (INH, RIF, EMB, PZA) for 6 months, according to the 2014 guidelines (27). In the case of RIF mono-resistance, the current guidelines stipulate that the patient should be treated with a standardized MDR-TB regimen. No provision is made for the inclusion of INH in that regimen. Both of these recommendations subject the patient to suboptimal therapy. The impact of these guidelines on the amplification of

resistance remains to be determined.

Proven XDR-TB cases are treated individually, taking into account known resistance and treatment history (27). When the treatment options of the standard regimens are depleted, clinicians turn to so-called salvage drugs. These drugs are not included in standard regimens due to inferior efficacy, decreased tolerability, insufficient evidence of their safety, or they may be unavailable or too expensive. Under these conditions, individualized treatment is implemented, often without evidence of its efficacy, because funding to conduct extensive testing is not available (29). However, treating a patient with ineffective drugs is a waste of resources and may further compromise the efficacy of other drugs. No drug should be administered without good evidence that the bacilli are susceptible to it, and mono therapy is to be avoided. In treating patients with limited treatment options, clinicians are faced with many challenges. Most importantly, several drugs have no standardized test available, preventing accurate diagnosis. Furthermore, some drugs are frequently used for indications other than TB (e.g. FQ, SM, amoxicillin-clavulanic acid), increasing the risk of resistance. Additional considerations include interactions between TB drugs and ARVs where HIV co-infection is treated (30), as well as between certain TB drugs with cross-resistance (31–33) or which may act synergistically to exacerbate adverse reactions (34,35). The latest additions to the arsenal of anti-TB drugs in South Africa are bedaquiline, which has recently been approved for use in the treatment of MDR-TB, and linezolid, a repurposed drug which shows promise to improve treatment outcomes (36). Several clinical trials of further novel drugs are underway. It is paramount that these drugs should be used only as part of a robust regimen, in order to prevent development of resistance to these drugs. Among newer generation drugs, there is concern over the simultaneous administration of bedaquiline, clarithromycin, delaminid (not available in South Africa yet) and/or MOX, as all of these may cause heart QT prolongation (37–40). Surgery, as an adjunctive therapy, is only used in cases that meet strict criteria and should be evaluated on a patient-by-patient basis (41).

This situation of exhausted treatment options is common in South Africa. For example, in the Eastern Cape Province of South Africa, an inadequate treatment strategy, along with under-resourced and poorly managed health systems, has led to practically the worst possible form of TB emerging. The Eastern Cape is one of the most adversely affected South African Provinces in terms of TB prevalence, MDR-TB (6.8% among new culture positive patients) and XDR-TB (11.9% of new MDR-TB cases) (16). This is emphasized by a report which showed that almost all XDR-TB patients (91.6%) in Jose Pearson TB Hospital (Nelson Mandela Metropole) failed to achieve culture

conversion within one year (42). Furthermore, on average, patients in that study received only 1.7 drugs that were deemed effective (42), assuming that capreomycin (CAP) was effective for treatment of XDR-TB patients. However, a recent study demonstrated the contrary (31), highlighting cross-resistance between amikacin (AMK) and CAP. Clearly, treatment options for these patients was inadequate according to WHO recommendations (28). Not surprisingly, mortality was high: 58% of XDR-TB patients died within one year of diagnosis or treatment initiation (42).

High rates of mortality are not limited to the Eastern Cape and have been reported for the Western and Northern Cape Provinces, as well as Gauteng and Kwa-Zulu Natal (2,43). This situation will continue to occur – as supported by other studies (6,42,44) - if treatment regimens fail to be built on a comprehensive analysis of the resistance profile of the pathogen and in the absence of novel treatment regimens.

Under these conditions, cases that do not achieve culture conversion within a period of 12 to 18 months, and have high-level drug-resistance with no options of adding further drugs or surgery are considered treatment failures. These cases are sent home after investigation by a review board, with no further anti-TB treatment (23). This is controversial, as it puts community contacts at risk, especially given that such patients may survive in the community for several years. A recent study demonstrated the transmission of XDR-TB from discharged patients to their close contacts, resulting in the death of several members within these families (44). This has created an ethical dilemma: the right of the individual vs. the right of the community. Some argue that transmission occurs before hospitalization and therefore the discharged patients are not an additional threat to the community. However, these patients are often still smear positive at discharge, indicating a high transmission potential (44). Therefore, the rights of the community dictate that infectious patients should have no or very limited contact with the community. In our opinion, there is a need to develop TB palliative care facilities in all high TB prevalence communities.

6.5 Considerations for optimized control

It is clear that highly resistant forms of TB are developing spontaneously in response to inadequate treatment and are subsequently spreading. These problems can be addressed by two possible strategies. The first is to treat all patients initially with a “super regimen”, containing as many drugs as possible. This regimen would then be tailored down according to DST as it becomes available. However, adverse side effects is likely to prevent this strategy from being adopted widely. Furthermore, careful investigation would be needed to determine the effect of this strategy on the

acquisition of drug-resistance. The second strategy is to expand the use of rapid, molecular DST as well as expanding the set of routinely done tests to include all drugs that are prescribed. The major problem with this strategy, apart from cost and lab capacity, is the unavailability of rapid, reliable tests for certain drugs. The WHO has endorsed the MTBDR*plus* line probe assay (Hain Lifescience, Nehren, Germany) and Xpert MTB/RIF assay (GeneXpert) for detecting *M. tuberculosis* complex. The efficiency of MTBDR*plus* is comparable to that of GeneXpert (45), with the additional benefit of rapidly determining INH resistance, thereby identifying INH mono-resistant cases who are at risk of developing MDR-TB (26). We advocate that further resistance testing should be undertaken immediately with GenoType MTBDR*sl* (Hain Lifescience, Nehren, Germany) upon identification of RIF resistance using GenoType MTBDR*plus*. It has been shown by several studies (46) (meta-analysis), (47), (48) that this assay can be used as a rule-in test to detect resistance to second-line drugs (FQ, AMK, KAN and Cap). However, due to concern over sensitivities and specificities for the various drugs, it has been suggested that this assay be used in conjunction with culture-based DST (48). A further concern is that this test does not detect resistance to PZA and sensitivity and specificity is very low for EMB. This implies that resistance to any of several drugs may still be missed, leading to inappropriate treatment and the consequent amplification of resistance. Although not standardized, it is possible to detect PZA resistance with targeted gene sequencing, offering a faster alternative to culture-based DST (49). Several new diagnostic technologies are currently in development that may offer improved methods in future (50). MDR-TB treatment regimens should subsequently be strengthened to ensure that at least 4 effective drugs are always present.

In addition to these knowledge-based strategies, heightened infection control in health care facilities as well as specialized TB hospitals is emphasised by nosocomial transmission of drug-resistant TB (2). Infection control also need to be extended to community based care, especially for therapeutically destitute cases (51,52). In the face of constrained resources, this goes hand-in-hand with improved education of household contacts which is vital to curb the epidemic.

Precise classification of highly resistant TB is needed in order to improve targeted treatment, and to protect the few drugs that may still be effective. Currently, lack of reliable testing for some drugs precludes precise definitions, indicating an urgent need to focus research on improved diagnostics.

It is imperative that we look on the lessons learned from the past: a weak regimen will be easily overcome by TB bacilli. For this reason, it is crucial to not treat a patient without knowing with

reasonable certainty that the regimen given contains sufficient effective drugs. Failing to learn this lesson will result in losing new drugs as fast as they become available.

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Chapter 7 Conclusions

Oh, the depth of the riches of the wisdom and knowledge of God!
How unsearchable his judgments,
and his paths beyond tracing out!
"Who has known the mind of the Lord?
Or who has been his counselor?"
"Who has ever given to God,
that God should repay them?"
For from him and through him and for him are all things.
To him be the glory forever! Amen.

- Romans 11:33-36

Conclusions

This work was the first in-depth molecular epidemiological study of the drug-resistant TB epidemic in the Eastern Cape (EC) of South Africa.

We have shown that the population structure of TB in the EC differs dramatically among drug-susceptible, MDR- and pre-XDR- and XDR-TB strains. The Atypical Beijing genotype is significantly overrepresented among MDR-TB strains, and even more so among Pre-XDR- and XDR-TB strains, suggesting a selective advantage among strains with higher resistance. Our phylogenetic analysis concludes that these strains have evolved from a common progenitor, and may be resistant to up to 11 drugs. According to the definition of Udwadia et al (1), we demonstrate the evolution and spread of "totally" drug-resistant TB in a Province with a failing health care system.

This work was framed in the South African context, investigating the population structure of MDR-TB strains in three additional Provinces. This analysis showed that the MDR-TB population structure was distinct for each Province. Furthermore, in keeping with EC data, a negative correlation between strain diversity and the number of resistance markers was observed. It was demonstrated that the population structure of XDR-TB in the Western Cape was strongly influenced by strains originating from the EC, probably through migration of patients seeking better employment opportunities and better health care. This shows how sub-optimal health care management and poor socioeconomic conditions in one setting can contribute to the burden of disease in another. This emphasises the need for access to appropriate health-care in all provinces as well as a functioning National electronic database.

Our findings highlight a high degree of clustering of drug resistance-causing mutation patterns, indicating transmission of highly drug-resistant strains. This indicates the failure of standardised regimens to prevent the acquisition of higher resistance, as well as the failure to provide adequate treatment that would result in culture conversion, and therefore limit transmission. This calls for a change in the policy to allow the initiation of individualised treatment based on knowledge of the exact resistance profile for each patient.

We have shown that *inhA* promoter mutations in MDR-TB strains appear to be instrumental in the acquision of further mutations leading to the development of XDR-TB. Importantly, these mutations

are not detected by the primary screening tool, GeneXpert MTB/RIF assay. However, these mutations are detectable by the GenoType® MTBDR*plus* line probe assay which is currently used to confirm rifampicin resistance detected by Xpert MTB/RIF. Using this diagnostic data will allow for the rapid identification of patients at risk of XDR-TB and thereby will facilitate further triaging for second-line DST, followed by adjustment of the regimen. We suggest that further studies are needed to investigate the usefulness of this test to detect *inhA* promoter mutations as a risk screen for XDR-TB.

In the face of extremely limited treatment options for XDR-TB, we investigated cross-resistance between capreomycin (CAP) and amikacin (AMK), to determine its potential value in the treatment of XDR-TB. High levels of cross-resistance was observed between CAP and AMK in strains harbouring the *rrs* A1401G mutation. Furthermore, this study showed that CAP resistance is missed by routine DST which led to a change in the methods of CAP testing at the National Health Laboratory Services in Port Elizabeth. Recent evidence presented at the 2014 MDR/XDR TB Conference in Cape Town confirmed that treatment with CAP had no therapeutic value in patients with isolates harbouring the A1401G mutation.

We noted that incomplete cross-resistance exist between RIF and rifabutin (RFB) (2,3) in isolates from our cohort which were characterised by the *rpoB* 516 mutation which was present in the majority of Atypical Beijing genotype isolates from the EC. Accordingly, RFB may be an available option for the treatment of a large proportion of drug-resistant TB cases in this setting and other settings (e.g. Brazil (4)) where MDR-TB is characterised by *rpoB* 516 mutations. However, it is important that knowledge about the exact mutation is available before inclusion of this drug in a treatment regimen. Clinical studies on the effectiveness of both CAP and RFB are needed in this setting.

Whole genome sequencing (WGS) analysis of a selection of Atypical Beijing genotype isolates supported the notion of an endemic presence of these strains. It was shown that two important sub-groups evolved independently from a common progenitor, and subsequently spread throughout the region. Each of these groups harboured group-specific mutations, including a distinct set of drug resistance-causing mutations. Our study also revealed that all Atypical Beijing F31-genotype isolates, including those diagnosed as pan-susceptible, harbour an *ethA* mutation conferring ethionamide (ETH) resistance. Our phylogenetic analysis suggested that the *katG, rrs*513 and *ethA* mutations were acquired historically before the development of standardised 4-drug regimens.

With modern standardised regimens, the *ethA* mutation is hypothesised to not confer a selective advantage to otherwise drug-susceptible strains, as ETH forms part of the second-line regimen. However, once a patient is diagnosed with MDR-TB, or RIF resistant TB according to current guidelines (5), he or she will receive treatment with a regimen containing ETH. The hidden resistance to ETH weakens the regimen, leading to acquisition of additional resistance. This strengthens our recommendation that comprehensive DST should be available for all suspected MDR-TB cases, before the commencement of treatment. Further research is needed to determine the difference in MICs between strains with different permutations of *inhA* promoter and *ethA* mutations, and whether such a difference will have any clinical relevance.

The distinct nature of the two groups of the Atypical Beijing genotype was also supported by RNAseq analysis. A large number of genes were significantly differentially regulated, compared to drug-susceptible isolates in each group: in Group A, 423 genes were up-regulated and one down-regulated, while 721 genes were up-regulated and two down-regulated in Group B. Significant up-regulation of the *inhA* operon was shown in all isolates that harboured *inhA* promoter mutations. This confirms that the mechanism of resistance is through over-production of the target of INH and ETH, resulting in low-level INH resistance, as well as high-level ETH resistance.

Cases of TB with resistance beyond XDR are arising around the world and we have shown that these highly resistant strains can spread. The problem is exacerbated when infectious patients without any further treatment options are sent home. We propose that nomenclature be developed that adequately describe cases with resistance beyond XDR-TB in order to provide the correct treatment and to protect drugs that may still be useful. Such nomenclature will also aid in epidemiological studies to understand the severity of the problem. We further strongly propose that individualised treatment regimens based on known drug susceptibility be designed for all MDR-TB cases.

This study was conducted before the roll-out of GeneXpert and the associated guidelines for treatment management. We hypothesise that this roll-out will have had a major impact on the epidemiology of drug-resistant TB. According to the 2014 TB treatment guidelines (5), all patients with a RIF resistant GeneXpert result are immediately started on MDR-treatment, regardless of the spectrum of drug resistance. This implies that patients infected with strains that are resistant beyond MDR (e.g. Atypical Beijing genotype strains) are at increased risk of developing resistance to additional first- as well as second-line drugs. This strategy has the potential to increase mortality as

well as to exacerbate transmission. Further studies are needed to determine the effect these guidelines will have in various settings.

It is currently unknown whether Atypical Beijing genotype strains with different levels of drug resistance also differ in terms of virulence. Studies in *in vivo* infection models may help to elucidate this. This knowledge will improve our understanding of what genetic changes contribute to virulence.

The findings of this thesis pertaining to Atypical Beijing genotype strains, their drug-resistance patterns and prevalence in the Eastern Cape Province may not be generalizable to the rest of the country. However, its increasing prevalence in the Western Cape Province is reason for concern, and these findings may in future prove valuable in the management of cases infected with Atypical Beijing genotype strains. It also serves as a warning that regular epidemiological studies are needed, as the epidemic is dynamic, implying that strategies for diagnosis and treatment need to be adapted over time. Furthermore, the Atypical Beijing genotype and its devastating effect in the Eastern Cape may serve as an example and the knowledge gained may be applied to study the dominant strains in other regions. This work also shows that considerable diversity exist within the country, and therefore a different diagnostic and treatment approach may be needed for each region according to the most dominant strain type and prevailing drug-resistance patterns.

Together these findings have challenged the use of standardised MDR-TB treatment without comprehensive DST. This widely recognised view has not influenced the South African TB guidelines (2014) which promote treatment of rifampicin resistance without relevant knowledge of additional drug resistance. Drug-resistance beyond XDR-TB is present in the EC, and is spreading. This situation will continue to occur unless the way patients are diagnosed and treated is adapted. We propose that the effective treatment of highly resistant TB can only be achieved with the development of new drugs, new drug combinations and comprehensive rapid DST.

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Chapter 8 Appendix

The appendices include further work that was published using data generated during this project.

1. **Emergence and treatment of multidrug resistant (MDR) and extensively drug-resistant (XDR) tuberculosis in South Africa.**

Streicher EM, Müller B, Chihota V, Mlambo C, Tait M, Pillay M, Trollip A, Hoek KG, Sirgel FA, Gey van Pittius NC, van Helden PD, Victor TC, Warren RM.

Published in Infection, Genetics and Evolution. 2012 Jun;12(4):686-94.

2. **Emergence and treatment of Drug Resistant Tuberculosis: A Comedy of Errors.**

Robin M. Warren, Kim Hoek, Frik Sirgel, Marisa Tait, Nicolaas C. Gey van Pittius, Borna Muller, Elizabeth M. Streicher, Thomas C. Victor, Paul D van Helden

Published in SA Respiratory Journal 2010 Dec 16(4):112-116

3. **Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis.**

Borrell S, Teo Y, Giardina F, Streicher EM, Klopper M, Feldmann J, Muller B, Victor TC, Gagneux S.

Published in Evolution, Medicine, and Public Health. 2013. Issue 1, Pp65-74

8.1 Emergence and treatment of M(X)DR-TB in South Africa.

Published in Infection, Genetics and Evolution. 2012 Jun;12(4):686-94.

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Abstract

Drug resistant tuberculosis (TB) has reached alarming proportions in South Africa, draining valuable resources that are needed to fight drug susceptible TB. It is currently estimated that 9.6% of all TB cases have multi-drug resistant (MDR)-TB, thereby ranking South Africa as one of the highest MDR-TB burden countries in the world. Molecular epidemiological studies have demonstrated the complexity of the epidemic and have clearly shown that the epidemic is driven by transmission as a consequence of low case detection and diagnostic delay. The latter has in turn fuelled the amplification of drug resistance, ultimately leading to the emergence of extensively drug resistant (XDR)-TB. Despite the introduction of new drugs to combat this scourge, culture conversion rates

for XDR-TB remain below 20%. Failure to achieve cure may be explained from DNA sequencing results which have demonstrated mutations in 7 genes encoding resistance to at least 8 anti-TB drugs. This review shows how molecular epidemiology has provided novel insights into the MDR-TB epidemic in South Africa and thereby has highlighted the challenges that need to be addressed regarding the diagnosis and treatment of MDR-TB. An important step towards for curbing this epidemic will be collaboration between clinicians, laboratories and researchers to establish scientific knowledge and medical expertise to more efficiently guide public health policy.

Global MDR-TB epidemic

Recent statistics released by the World Health Organization (WHO) have raised concern about the ever-increasing global drug resistant tuberculosis (TB) epidemic. It is now estimated that 440 000 cases of multidrug-resistant (MDR)-TB (resistant to at least isoniazid and rifampicin) were diagnosed in 2008. Of these, approximately 40 000 had extensively drug resistant (XDR)-TB (MDR-TB with additional resistance to a fluoroquinolone and one of the injectables, i.e. kanamycin, amikacin or capreomycin) (WHO media report, 2006; WHO-IUATLD Global Project on anti-tuberculosis drug resistance surveillance, 2008). By January 2010 XDR-TB cases had been reported in 58 countries around the world (WHO, 2010a).

MDR-TB epidemic in South Africa

South Africa is ranked as a high burden MDR-TB country with an estimated 13 000 cases being diagnosed in 2008 (WHO, 2010a). In a nationwide survey in South Africa in 2008, 20.2% of all notified TB cases showed resistance to isoniazid and nearly half of these (9.6% of all cases) were MDR-TB (WHO, 2010a). This represents a dramatic increase (3-fold) since 2002, when it was shown that 3.1% of all TB cases (new and retreatment) had MDR-TB (WHO-IUATLD Global Project on anti-tuberculosis drug resistance surveillance, 2008). These findings are supported by a report from the National Health Laboratory Service (NHLS) which showed a steady increase in the number of MDR-TB cases since 2004 (Table 1), (National Health Laboratory Services, 2010) and a study in Khayelitsha

which showed that MDR-TB was diagnosed in 4.4% of all TB cases in 2008 (Cox et al., 2010). NHLS data suggest that 6.3% of the diagnosed MDR-TB cases are XDR-TB (after second-line drug susceptibility testing, DST) (Table 2), while WHO estimates for South Africa are even more pessimistic with an estimate of 10.5% of XDR-TB among MDR-TB cases (National Health Laboratory Services, 2010; WHO, 2010a).

These statistics appear significantly worse if we consider that only 8 200 of the projected 13 000 patients were diagnosed with MDR-TB or XDR-TB by the NHLS in 2008 – suggesting a case detection rate of 63%. More worrying is the fact that no more than 50% of diagnosed cases were placed on MDR treatment in 2009 (Dr. Norbert Ndjeka, Director Drug-Resistant TB, TB and HIV, National Department of Health). In South Africa the cure rates for MDR-TB cases receiving treatment are in the order of 49% (Department of Health Government South Africa, 2007; Shean et al., 2008). The culture conversion rate for XDR-TB is even more dismal, with an almost 100% mortality reported for the Tugela Ferry outbreak among human immunodeficiency virus (HIV) co-infected individuals in KwaZulu-Natal (Gandhi et al., 2006). Similarly, culture conversion rates for XDR-TB cases from the Western Cape, Eastern Cape and Gauteng provinces were less than 19% among HIV infected and uninfected patients (Dheda et al., 2010). A recent study from the Eastern Cape suggested that culture conversion at month 12 was only in the order of 8%, while mortality was 43% (Kvasnovsky et al., 2011).

The ever increasing MDR-TB epidemic in South Africa places significant constraints on the National TB Control Program budget (World Health Oganization, 2009). It has been estimated that treatment of MDR-TB cases consumes nearly 70% of the budget allocated to fight the entire TB epidemic in South Africa (World Health Oganization, 2009). Thus, valuable resources are directed away from combating the drug susceptible TB epidemic which has now reached alarming proportions with an estimated 500 000 cases being diagnosed each year in South Africa (WHO-IUATLD Global Project on anti-tuberculosis drug resistance surveillance, 2008). The above statistics suggest that the current TB control strategy is unable to curb the emergence and spread of MDR-TB, despite the implementation of the DOTS program in 1996. This may in part be explained by the poor implementation of the DOTS program in certain regions of South Africa. A recent study in KwaZulu-Natal showed that only 18% of patients diagnosed with smear-positive pulmonary disease completed treatment thereby potentially fuelling the emergence of drug resistance in that province (Loveday et al., 2008).

Table 1. Number of MDR-TB patients diagnosed by the National Health Laboratory Service by province per year (National Health Laboratory Services, 2010).

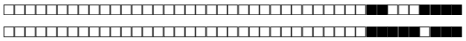
Province	2004	2005	2006	2007	2008	2009	Total
Eastern Cape	379	545	836	1092	1501	1858	6211
Free State	116	151	198	179	381	253	1278
Gauteng	537	676	732	986	1028	1307	5266
Kwazulu-Natal	583	1024	2200	2208	1573	1773	9361
Limpopo	59	40	77	91	185	204	656
Mpumalanga	162	134	139	506	657	446	2044
North West	130	203	225	397	363	520	1838
Northern Cape	168	155	188	199	290	631	1631
Western Cape	1085	1192	1179	1771	2220	2078	9525
Total	3219	4120	5774	7429	8198	9070	37810

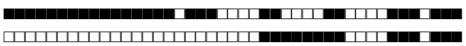
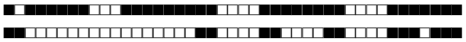
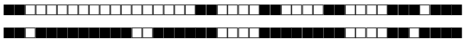
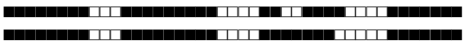
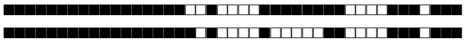
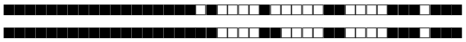
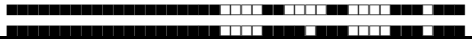
Table 2. Number of XDR-TB patients diagnosed by the National Health Laboratory Service by province per year (National Health Laboratory Services, 2010).

Province	2004	2005	2006	2007	2008	2009	Total
Eastern Cape	3	18	61	108	175	123	488
Free State	1	6	3	4	3	3	20
Gauteng	5	14	19	38	30	65	171
Kwazulu-Natal	59	227	336	241	181	254	1298
Limpopo		2	5	2	2	6	17
Mpumalanga				12	14	18	44
North West	1	5	9	4	4	13	36
Northern Cape	4	10	3	7	19	40	83
Western Cape	12	16	28	42	60	72	230
Total	85	298	464	458	488	594	2387

Drug resistance surveillance studies in South Africa have in the past quantified the relative contribution of resistance in new patients (transmitted) and resistance in previously treated patients (acquired) with the view to direct policy to most effectively tackle the drug resistance TB epidemic (Weyer K, 2001; Weyer et al., 1995; Weyer and Kleeberg, 1992). These surveys have highlighted a significant association between retreatment and drug resistance, thereby suggesting that such patients acquired resistance. As an example, in a nationwide survey in South Africa in 2002, the proportion of MDR-TB in all new cases was estimated at 1.8%, while among previously treated cases this estimate rose to 6.7% (WHO-IUATLD Global Project on anti-tuberculosis drug resistance surveillance, 2008). The association between retreatment and drug resistance informed the diagnostic policy to be targeted towards high risk patients (retreatment cases, treatment failure cases and contacts of drug resistant cases). In our opinion this was largely a cost saving strategy which has had a significant impact on the amplification of drug resistance.

Table 3. Spoligotype patterns of MDR-TB isolates culture from patients resident in the Western Cape Province of South Africa (2008 to 2010).

Lineage	nr. of isolates in each lineage	ST	International family according to SpolDB4	Spoligotypes	nr. of isolates
BEIJING	1842	1	BEIJING		1833
		541	BEIJING		4
		255	BEIJING		2
					1
		190	BEIJING		1
		1674	BEIJING		1
X-family	366	119	X1		154
		92	X3		71
		1329	X1		32
					31
		137	X2		20
		18	X2		9
		336	X1		9
					8
		347	X2		7
					4
		348	X1		4
					4
					3
					3
					2
					1
					1
					1
					1
					1
LAM	359	33	LAM3		146
		60	LAM4		84
		719	T1		28
		1241	U		14
		42	LAM9		14
		811	LAM4		8
		20	LAM1		7
		130	LAM3		7
		4	LAM3		5
					4
					4
		1293	LAM3		3
					3
					3

		1873	LAM11_ZWE		3
					2
					2
		125	T2		1
		177	LAM9		1
		1354	LAM3		1
					1
					1
		1321	LAM1-LAM4		1
					1
		30	LAM9		1
					1
		1528	LAM9		1
					1
		1830	LAM3		1
					1
					1
		1841	U		1
					1
					1
		1324	T1		1
					1
					1
		815	LAM11_ZWE		1
		95	LAM6		1
S family	83	34	S		40
		790	U		21
		71	S		7
		789	S		4
		1334	S		2
					1
		1253	S		1
					1
					1
					1
					1
					1
		1783	LAM10_CAM-S		1
					1
		721	U		1
Haarlem	34	50	H3		12
					9
		47	H1		6
		62	H1		4
		36	H3-T3		3
CAS	9	21	CAS1_KILI		3
		26	CAS1_DELHI		3
		1092	CAS1_DELHI		2
					1
EAI	7	48	EAI1_SOM		4
		8	EAI5 or EAI3		2
		806	EAI1_SOM		1
T-Family	245	53	T1		110
		244	T1		9
		462	T1		8
		926	T1		7
		501	T1		6
		521	T1		6
		803	T1		4
		766	T1		3
		373	T1		3
		136	T1		2
		498	T1		2
		1202	T1		1
		649	T1		1
		1574	T1		1
		1067	T1		1
		156	T1		1
		913	T1		1
		732	T1		1
		880	T1		1
		1688	T1		1
		1147	T1		1
		888	T1		1
		245	T1		1
		628	T1		1
		52	T2		5
		73	T2-T3		2
		37	T3		14
		565	T3		4
		158	T3		1
		39	T4_CEU1		12

		44	T5		32
		254	T5_RUS1		2
OTHER	63	100	MANU1		1
		54	MANU2		7
		226	MANU2		3
		1247	MANU2		2
		1634	MANU2		1
		519	U		1
		237	U (likely H3)		1
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				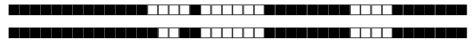	1
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Treatment guidelines between 2002 and 2010

In order to curb the MDR-TB epidemic in South Africa, the National TB Control Program implemented standardized treatment guidelines for MDR-TB in 2002 (Department of Health, 2000). At the same time, routine DST was limited to testing for susceptibility to isoniazid, rifampicin and ethambutol. This strategy was based on drug surveillance data which demonstrated that MDR-TB associated with ethambutol resistance was rare. DST for pyrazinamide was not done in that survey. Additional DST was only done on request.

The standardized treatment regimen consisted of a four-month intensive phase with five anti-TB drugs (kanamycin, ethionamide, pyrazinamide, ofloxacin, and cycloserine or ethambutol), followed by a 12-18 month continuation phase with three drugs (ethionamide, ofloxacin and cycloserine or ethambutol). It was recommended that these anti-TB drugs should be administered five times per week in out-patient clinics and seven times per week in hospitals. The continuation period could be shortened provided that 12 months of treatment had been given after sputum culture conversion as

demonstrated by three consecutive monthly negative cultures. These treatment guidelines were revised in 2007 following the outbreak of XDR-TB in KwaZulu-Natal (Department of Health Government South Africa, 2006; Department of Health Government South Africa, 2007). These revisions included DST of second-line anti-TB drugs (ofloxacin and amikacin) for all MDR-TB cases (Department of Health Government South Africa, 2007). In addition, capreomycin and p-aminosalicylic acid were made available for treatment of XDR-TB (Department of Health Government South Africa, 2006).

Molecular Epidemiology

Re-examination of the surveillance data clearly showed that although the percentage of MDR-TB was lower in new cases, the vast majority of MDR-TB cases had primary MDR-TB in South Africa (estimated 8 238 new cases vs 5 795 retreatment cases based on the 2002 drug resistance survey) confirming that MDR-TB is also efficiently transmitted (WHO-IUATLD Global Project on anti-tuberculosis drug resistance surveillance, 2008). Analysis of this data using a very simple model suggests that approximately 80% of MDR-TB results from ongoing transmission in South Africa (P. Uys et al. submitted). This results as a consequence of the reclassification of new primary MDR-TB patients as retreatment cases when failing first-line anti-TB therapy. Similar conclusions were drawn from a drug surveillance study in Khayelitsha, Cape Town, which estimated that 81% of MDR-TB was transmitted (Cox et al., 2010).

The importance of transmission as a major driver of the MDR epidemic in South Africa is supported by molecular genotypic analysis of MDR-TB and XDR-TB strains. DNA fingerprinting and DNA sequencing of target genes conferring resistance clearly demonstrated that MDR-TB strains are clustered, implying ongoing transmission (Johnson et al., 2010; Pillay and Sturm, 2007; Strauss et al., 2008; Streicher et al., 2004; van Rie et al., 1999; Victor et al., 2006). The mechanism fuelling these outbreaks may be numerous; however, two recent studies have clearly demonstrated that poor infection control leads to nosocomial spread of MDR-TB and XDR-TB (Calver et al., 2010; Gandhi et al., 2006). However, the extent to which noscomial spread contributes to the MDR and XDR-TB epidemic has not been quantified. Furthermore, it is not known whether the current infection control strategies are sufficient to curb transmission – especially to highly vulnerable individuals.

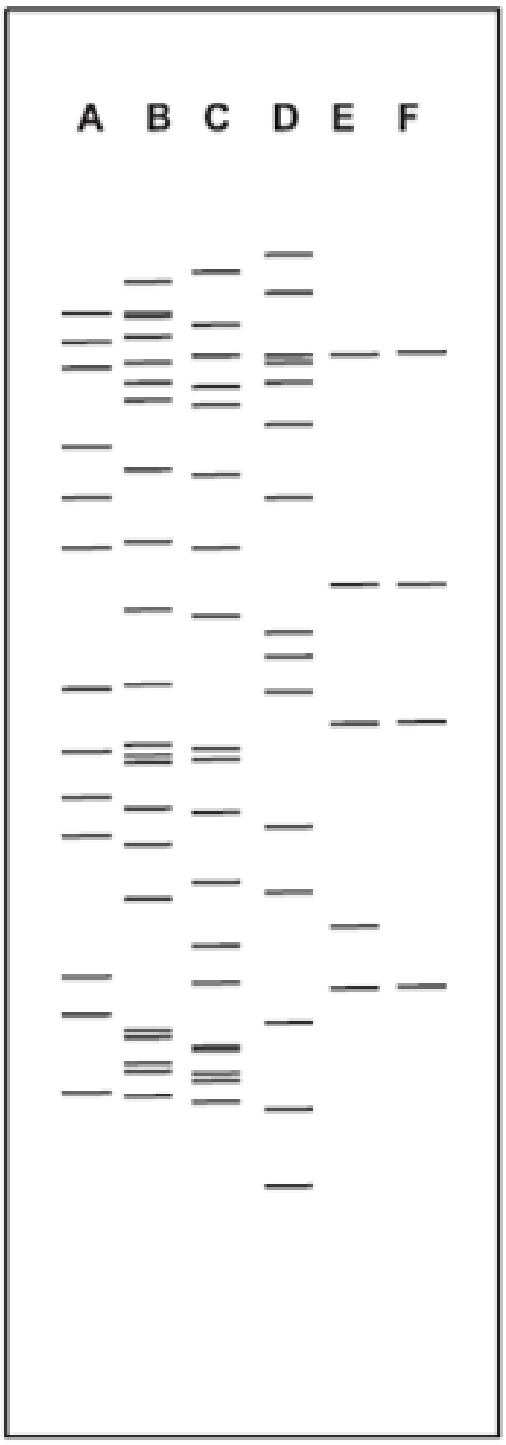

Figure 1. IS*6110* DNA fingerprint patterns of outbreak MDR-TB strains identified in South Africa. A – Atypical cluster 86 strain (Strauss et al., 2008), B – Beijing R220 strain (Johnson et al., 2010), C – Bejing cluster 213 strain (van Rie et al., 1999), D - F15/LAM4/KZN strain (Pillay and Sturm, 2007), E – Low Copy Clade cluster DRF150 strain (Victor et al., 2007), and F – Low Copy Clade 140 strain (Calver et al., 2010).

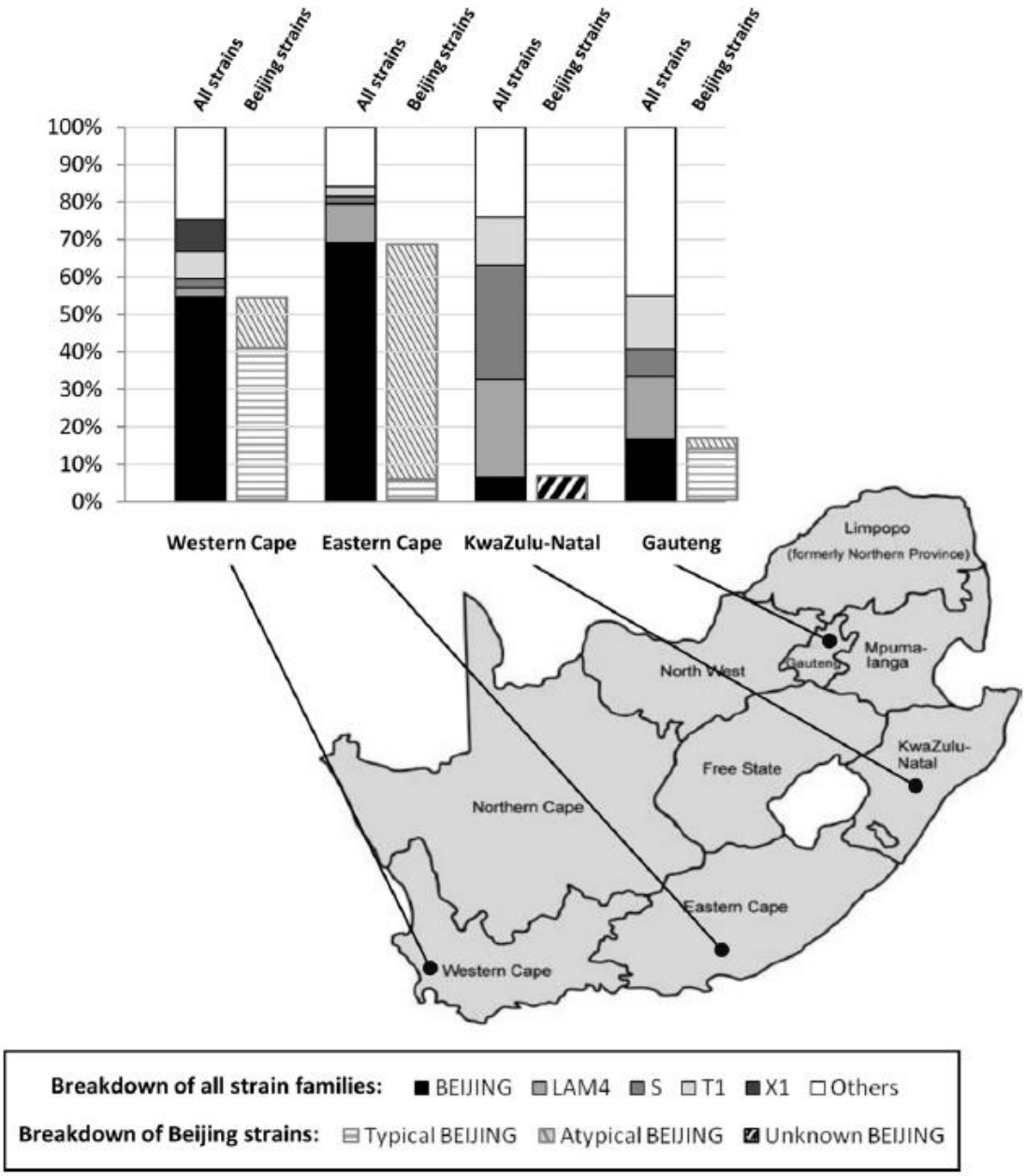

Figure 2. Distribution of major MDR genotypes in four South African provinces. The frequency distribution of MDR-TB isolates with spoligotype patterns corresponding to Beijing, LAM4, S, T1, X1 or other genotypes are indicated for the Western Cape, Eastern Cape, KwaZulu-Natal and Gauteng Provinces (manuscript in preparation). Isolates from the Western Cape, Eastern Cape and Gauteng belonging to the Beijing genotype, were further differentiated into typical and atypical Beijing strains (Beijing isolates from KwaZulu-Natal were not available for further characterization)

Table 3 shows the spoligotypes of MDR-TB isolates cultured from patients resident in the Western Cape. Multiple recent studies reported MDR-TB outbreaks caused by clones of drug resistant strains (Calver et al., 2010; Johnson et al., 2006b; Johnson et al., 2010; Pillay and Sturm, 2007; Strauss et al., 2008; van Rie et al., 1999; Victor et al., 2007) (See Figure 1 for IS*6110* DNA fingerprint patterns of major outbreak MDR-TB strains). For example, the Beijing cluster R220 strain is widely spread in the Western Cape province of South Africa and responsible for 42% of the increase in the incidence of MDR-TB in the Western Cape Province between 2001 and 2006. This translates to a doubling time of 2.38 years (Johnson et al., 2010). In contrast, the MDR-TB epidemic in the Eastern Cape was characterized by the over-abundance of an atypical Beijing strain (Cluster 86), which accounts for most of the transmission of MDR-TB in that province (Figure 2) (Strauss et al., 2008). This differs from the KwaZulu-Natal Province of South Africa where it has been shown that the F15/LAM4/ KZN strain is the principle MDR-TB strain (Figure 2) (Pillay and Sturm, 2007). In line with this, a comprehensive analysis suggests that the population structure of MDR-TB in South Africa is largely distinct in all provinces surveyed with certain lineages being significantly overrepresented in different regions (Chihota et al. submitted). Interestingly, genotyping of MDR-TB isolates from Gauteng show a spectrum of different genotypes representing the strains found in the other provinces, thereby probably reflecting human migration (Figure 2) (Kok et al., 2003).

Diagnostic Delay

The targeted diagnostic approach adopted by the National TB Control Program implies that the vast majority of MDR-TB cases would receive regimen 1 treatment (2 month intensive phase: isoniazid, rifampicin, ethambutol and pyrazinamide, and a 4 month continuation phase: isoniazid and rifampicin) until treatment failure is noted. Similarly, retreatment cases with undiagnosed MDR-TB would receive regimen 2 (2 month intensive phase: isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin, and 4 month continuation phase: isoniazid and rifampicin) until the diagnosis of

MDR-TB is made. In both instances, this deficient treatment regimen was given thereby increasing the risk of amplification of resistance. Furthermore, the prolonged infectious period will perpetuate the drug resistant TB epidemic through transmission to close contacts. The amplification of resistance during inadequate treatment has been illustrated by two recent publications (Calver et al., 2010; Pillay and Sturm, 2007). The study by Calver et al. demonstrated the emergence of ethambutol, pyrazinamide and ofloxacin resistance in a well-functioning TB control program as a consequence of delayed diagnostics and an inadequate treatment regimen. This occurred in a setting where treatment was hospital-based and adherence was excellent, a disturbing picture when compared to the rest of the country where adherence is much less likely. The extent to which amplification of resistance has occurred in South Africa has been illustrated by another study which documented that approximately 20% of MDR-TB cases also displayed resistance to ethambutol in 2006 (Johnson et al., 2006a). Subsequently, a follow up study showed that more than 50% of MDR-TB cases had isolates which harbored mutations in the *embB* gene (which are known to confer ethambutol resistance) (Hoek et al., 2009). These findings are in stark contrast with routine DST data as well as surveillance data which suggested that ethambutol resistance was rare (Weyer K, 2001). This may be explained by the technical difficulties associated with culture-based ethambutol resistance testing. Similarly, DST for pyrazinamide is technically challenging and is not routinely done in South Africa. For this reason the extent of pyrazinamide resistance is largely unknown and was assumed to be rare. Two recent molecular epidemiological studies confirm that pyrazinamide resistance is strongly associated with MDR-TB (Louw et al., 2006; Mphahlele et al., 2008). In both studies, more than 50% of MDR-TB isolates harbored mutations in the *pncA* gene known to confer pyrazinamide resistance. Together, this suggests that at least 50% of MDR-TB cases have resistance to ethambutol and/or pyrazinamide. This will undoubtedly have impacted significantly on the effectiveness of MDR-TB treatment in South Africa over the past few years.

XDR-TB in South Africa

The identification in 2006 of XDR-TB in Tugela Ferry, KwaZulu-Natal, South Africa, highlighted the inadequacies of the National TB Control program and emphasized the importance of infection control (VanRie and Enarson, 2006). In that study, 53 cases of XDR-TB were diagnosed between January 2005 and March 2006. The median time to death was 16 days from collection of initial sputum specimen (Gandhi et al., 2006). Genotyping suggested an outbreak, raising fears of the spread of a "super bug" which primarily infected HIV infected individuals. However, a subsequent study demonstrated that country-wide, 6% of MDR-TB cases had XDR-TB (Mlambo et al., 2008), that strains outside KwaZulu-Natal differed from the Tugela Ferry outbreak and that they were largely associated with acquisition given that unique spoligotype patterns were found in XDR-TB isolates from different geographical regions (Mlambo et al., 2008). Genotyping of XDR-TB isolates cultured from patients resident in the Western Cape showed a strong association with the Beijing R220 cluster and the atypical Beijing cluster 86 suggesting ongoing transmission (Streicher et al. unpublished data). DNA sequencing of the *inhA* promoter, *katG*, *rpoB*, *embB* and *pncA* genes (conferring resistance to isoniazid, rifampicin, ethambutol and pyrazinamide) confirmed clonality. However, sequencing of the *rrs*- and *gyrA* genes demonstrated that resistance to amikacin and ofloxacin, respectively, had been acquired on a number of different occasions. This suggests that poor management of circulating MDR-TB strains was one of the main reasons for the emergence of XDR-TB in the Western Cape (Dheda et al., 2010). The identification of the atypical Beijing cluster 86 strain (originally identified in the Eastern Cape (Strauss et al., 2008)) suggests migration of patients to the Western Cape as this strain was only rarely found in MDR-TB cases in that province (Hanekom et al., 2007). Unpublished genotypic data shows that the Beijing R220 cluster and atypical Beijing cluster 86 accounts for 35% and 55% of XDR-TB cases in the Western Cape, while the atypical Beijing cluster 86 accounts for >90% of all XDR-TB cases in the Eastern Cape (Tait et al. unpublished data). DNA sequencing of the *inhA* promoter, *katG*, *rpoB*, *embB*, *pncA*, *rrs* and *gyrA* genes identified

mutations causing resistance to isoniazid, ethionamide, rifampicin, ethambutol, pyrazinamide, streptomycin, amikacin, capreomycin and ofloxacin, suggesting the emergence of totally drug resistant (TDR)-TB. DST for terizidone/cycloserine and p-aminosalicylic acid has not been done. This in part may explain the high mortality and low culture conversion rates reported for XDR-TB in the Eastern Cape (Kvasnovsky et al., 2011).

Table 4. 2010 treatment guidelines of MDR-TB and XDR-TB (adapted from http://dev.tbsouthafrica.org/Documents/ACSM/TB_job_aids_10_08.pdf).

MDR TB		XDR TB	
Intensive Phase	**Continuation Phase**	**Intensive Phase**	**Continuation Phase**
Kanamycin (IM)		Capreomycin (IM)	
Ethionamide	Ethionamide	Ethionamide	Ethionamide
Pyrazinamide	Pyrazinamide		
		p-aminosalicylic acid	*p*-aminosalicylic acid
Ofloxacin	Ofloxacin	Moxifloxacin	Moxifloxacin
Terizidone or Cycloserine	Terizidone or Cycloserine	Terizidone or Cycloserine	Terizidone or Cycloserine

Treatment guidelines for MDR-TB and XDR-TB

In 2010 the Department of Health released new guidelines for the treatment of MDR-TB and XDR-TB (Table 4). Accordingly, ethambutol was replaced by terizidone for the treatment of MDR-TB, while capreomycin replaced amikacin/kanamycin, p-aminosalicylic acid replaced pyrazinamde, and moxifloxacin replaced ofloxacin for the treatment of XDR-TB. These changes were implemented based on the assumption that both capreomycin and *p*-aminosalicylic acid have not been used extensively in South Africa and therefore resistance to these drugs should be rare. However, it is unclear whether recent molecular epidemiological data was considered when this decision was

made, as a recent study in Korea has conclusively demonstrated cross-resistance between the aminoglycosides (amikacin and kanamycin) and capreomycin (Via et al., 2010). In an attempt to replicate this finding we have sequenced the rrs gene from clinical isolates which demonstrated both susceptibility and resistance to amikacin. Susceptibility testing in MGIT 960 medium showed that the A1401G *rrs* mutation was associated with high level amikacin resistance (>20µg/ml) and resistance to capreomycin (5 µg/ml) (Sirgel et al. submitted). This is double the critical concentration recommended by the WHO for DST in MGIT 960 media implying a laboratory diagnosis of capreomycin resistance (World Health Organization, 2008). This raises concern as to whether patient isolates which show resistance to amikacin will benefit from a regimen which includes capreomycin. Clinical trials are desperately needed to determine the therapeutic value of capreomycin, especially since capreomycin may lead to adverse events (Dheda et al., 2010).

The inclusion of moxifloxacin in the treatment regimen for XDR-TB was informed by improved treatment outcomes in XDR-TB cases (Jacobson et al., 2010). However, it is well known that cross-resistance occurs between different fluoroquinolones (Devasia et al., 2009). A study by (Kam et al., 2006) showed a correlation between specific *gyrA* mutations and the level of ofloxacin and moxifloxacin resistance. In that study the author suggested that patient isolates with mutations conferring a moxifloxacin minimum inhibitory concentration of ≤2µg/ml may benefit from the inclusion of moxifloxacin. However, this is significantly higher than the critical concentration (0.25µg/ml) recommended by the WHO to differentiate between resistant and susceptible isolates (World Health Organization, 2008). Provisional data from a study being conducted in South Africa confirmed the above findings – all isolates which harbor a mutation in the quinolone resistance determining region (QRDR) were resistant to 0.5µg/ml moxifloxacin (Sirgel et al. unpublished data). Again, it will be important to quantify the benefit of including moxifloxacin in the treatment regimen for XDR-TB given that these isolates are resistant to fluoroquinolones according to WHO definitions.

A further concern about the efficacy of these treatment guidelines has been highlighted (Hoek et al., 2009; Müller et al., 2011). This regimen may be weakened by previously unsuspected cross-resistance. A number of studies have demonstrated a positive correlation between *inhA* promoter mutations and high level ethionamide resistance (50 to 100 µg/ml) (Abe et al., 2008; Baulard et al., 2000; Morlock et al., 2003). The implementation of genetic-based DST in the Western Cape Province of South Africa demonstrated that 58.5% of MDR-TB isolates harbored mutations in the *inhA* promoter using the MTBDR*plus* line probe assay (Barnard et al., 2008). More recently, DNA sequencing showed that in the Western and Eastern Cape Provinces, respectively, 59% and 71% of all MDR-TB isolates and 86% and 92% of all XDR-TB isolates harbored an *inhA* promoter mutation (Müller et al., 2011). This implies that in the Western and Eastern Cape Provinces, treatment of MDR-TB with a regimen containing ethionamide against a background of *Mycobacterium tuberculosis* strains with resistance to isoniazid, rifampicin, ethambutol, pyrazinamide and ethionamide may enhance the risk of the emergence of XDR-TB. In fact, the strong association observed between *inhA* promoter mutations and XDR-TB noted in both provinces raises the possibility that in these settings, strains with an *inhA* promoter mutation have been selected to become XDR-TB due to the cross-resistance conferred by this mutation. Thus, the presence of an *inhA* promoter mutation may represent a gateway to the evolution of XDR-TB (Müller et al., 2011). Moreover, the predominance of certain transmitted strain groups causing the majority of the MDR-TB cases in these settings and characteristically harboring *inhA* promoter mutations will not be reduced by the newly implemented standardized treatment regimen for MDR-TB.

A central step in the emergence of XDR-TB is the acquisition of fluoroquinolone resistance. The reasons for the emergence of resistance to these anti-TB drugs is as yet unknown but is of considerable concern, as fluoroquinolones are the backbone of the current MDR-TB treatment regimen in South Africa. A study from Uzbekistan showed the development of ofloxacin resistance under well-controlled conditions and suggested that an association between pre-existing second-line drug resistance and a severe clinical condition (at baseline) was a risk factor for the development of

ofloxacin resistance (Cox et al., 2008). Similar findings were recently reported in a mine setting in South Africa (Calver et al., 2010). As mentioned above, treatment of MDR-TB in South Africa included (until recently) a fluoroquinolone (mostly ofloxacin), an aminoglycoside, pyrazinamide, ethambutol or cycloserine, and ethionamide. Considering the frequently observed resistance to pyrazinamide, ethambutol and cross-resistance conferred to ethionamide by the *inhA* promoter mutation, one explanation for the emergence of ofloxacin resistance could be the inadvertent use of ofloxacin mono-therapy during the continuation phase of second-line treatment when aminoglycosides are removed from the treatment regimen (Hoek et al., 2009). This implies that highly resistant and transmissible forms of *M. tuberculosis* may develop even under wel- controlled conditions with stringent adherence. This may have significant implications for MDR-TB treatment on a global scale, especially in settings where patient management may be suboptimal (Cox et al., 2008).

Conclusion

The majority of MDR-TB cases in South Africa are probably a result of the poor programmatic treatment of specific and efficiently transmitting MDR strains. The high incidence of TB and MDR-TB, low treatment and cure rates and the use of empiric regimens in the context of delayed DST are fuelling the continual dissemination and amplification of resistance, thus, perpetuating the emergence of XDR-TB. If the molecular epidemiology results are correct then treatment regimen options for XDR-TB are seriously curtailed. In certain patients only two of the five drugs will be active which may reflect currently observed treatment outcomes. This means that if we continue to treat XDR-TB patients with inadequate regimens we will probably generate pan-resistant-TB. Anecdotal evidence from clinicians treating these patients seems to suggest that we are already entering a stage of non-treatable tuberculosis disease or “TDR-TB” (“totally drug resistant TB”). At this rate, the discovery, evaluation and registration of new classes of anti-TB drugs will not be able to

keep up with the organism's ability to subvert our uncoordinated attacks. Thus, it is imperative that strategies are developed to prevent the amplification of drug resistance. Ideally, this can be achieved by only initiating MDR-TB treatment once the spectrum of drug resistance makers has been identified. However, practically this may not be achievable as patients with HIV co-infection will often require an immediate intervention to lower the risk of mortality. This emphasizes the need for the development of rapid diagnostics which can immediately guide treatment. The WHO recommended genetic based tests represent an important step forward in reducing the time to diagnosis and concomitantly enhancing the sensitivity of diagnosis (WHO, 2010b; WHO media report et al., 2008). However, these tests currently only target first-line anti-TB drugs and thus cannot guide treatment with second-line anti-TB drugs.

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8.2 Emergence and treatment of Drug Resistant Tuberculosis: A Comedy of Errors.

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Abstract

South Africa is ranked 4th among the high burden MDR-TB countries in the world. Recent statistics suggest that the current National TB Control Program is unable to curb the emergence and spread of this difficult to treat epidemic. Numerous factors have contributed to the perpetuation of the MDR-TB epidemic in South Africa. Many of which are errors, which in hindsight and with more modern technology could have been avoided. It is therefore essential that we learn from these mistakes and use current knowledge to design diagnostic algorithms and treatment guidelines that prevent acquisition of resistance and ensure improved treatment outcomes.

Introduction

Recent statistics released by the World Health Organization (WHO) have raised concern about the ever increasing global drug resistant tuberculosis (TB) epidemic. It is now estimated that 440 000 cases of multi drug resistant (MDR)-TB (resistant to at least isoniazid and rifampicin) were diagnosed in 2008.[1] Of these approximately 40 000 had extensively drug resistant (XDR)-TB (MDR-TB with additional resistance to a fluoroquinolone and one of the injectables kanamycin, amikacin or capreomycin[2]).[3] By January 2010 XDR-TB cases had been reported in 58 countries.[1] South Africa is ranked as a high burden MDR-TB country with an estimated 13000 cases being diagnosed annually.[1] It is calculated that approximately six percent of these cases will be subsequently diagnosed with XDR-TB. Cure rates for MDR-TB are of the order of 49%[4] in South Africa, while treatment outcome for XDR-TB is exceptionally poor as demonstrated by the high mortality rate among human immunodeficiency virus (HIV) co-infected individuals in KZN.[5] Similarly, culture conversion rates for XDR-TB cases from the Western Cape, Eastern Cape and Gauteng provinces were less than 19% among HIV infected and uninfected patients.[6] These statistics are significantly worsened if we consider that only 8200 of the projected 13000 patients were diagnosed with MDR-TB or XDR-TB by the National Health Laboratory Service in 2008 – suggesting a case detection rate of 63% (National Health Laboratory Service (NHLS) annual report 2010). More worrying is the fact that only 50% of diagnosed cases were placed on treatment. Furthermore, analysis of the NHLS annual report suggests that the drug resistant TB epidemic has increased from 3200 detected cases in 2003 to 8200 detected cases in 2008. This trend is supported by a recent molecular epidemiological study conducted in the Western Cape which estimated that the doubling time for MDR-TB was 4 years.[7] Even more alarming is the fact that 42% of the increase was due to a single Beijing genotype with a doubling time of 2.38 years.[7] This implies extensive transmission of MDR-TB probably due to diagnostic delay, thereby emphasizing the need for rapid diagnostics and comprehensive contact tracing. It has been estimated that treatment of MDR-TB cases consumes nearly 70% of the National Tuberculosis Program budget allocated to fight the entire TB epidemic in South Africa.[8] Thus, valuable resources are directed away from combating the drug susceptible TB epidemic which has now reached alarming proportions with an estimated 500,000 cases being diagnosed each year in South Africa.[3]

The above statistics suggest that the current TB control strategy is unable to curb the emergence and spread of MDR-TB, despite implementation of the DOTS program in 1996. Drug resistance surveillance studies in South Africa have in the past quantified the relative contribution of resistance

in new patients (transmitted) and resistance in previously treated patients (acquired) with the view to direct policy to most effectively tackle the drug resistance TB epidemic.[9-11] These surveys have highlighted a significant association between retreatment and drug resistance, thereby suggesting that such patients acquired resistance. As an example, in South Africa, the proportion of MDR-TB in all new cases is estimated at 1.8%, while among previously treated cases it goes up to 6.7%.[3] The observation is explained by the premise that drug resistance develops spontaneously and that prior treatment may select for the resistant population such that a subsequent diagnosis will lead to a classification of resistant disease. This conclusion may also be based on the prior understanding that drug resistant strains are attenuated and thus less likely to transmit.[12] However, a re-examination of the surveillance data clearly showed that although the percentage was lower in new cases, the vast majority of MDR-TB cases had primary MDR-TB in South Africa (8238 new cases vs 5795 retreatment cases) confirming that MDR-TB is also efficiently transmitted.[3] This together with molecular epidemiological data from other settings demonstrates that the long standing dogma that drug resistant strains are less virulent was largely incorrect.[13;14] These findings have important implications for the way that health-care providers perceive their patients. In most instances in South Africa, patients with MDR-TB were infected with an MDR-TB strain through transmission and therefore the patients should not be blamed for having drug resistant TB (legacy of the dogma that drug resistant TB is primarily acquired). In fact greater support is needed to ensure compliance during the prolonged treatment period. Concerns about the lack of patient support from health-care workers in South Africa have been stated and it has been suggested that this may in part account for treatment failure in MDR-TB patients.[15]

The strong association between retreatment and drug resistance has informed the diagnostic policy to be targeted towards high risk patients (retreatment cases, treatment failure cases and contacts of drug resistant cases). This was largely a cost saving strategy which has had a significant impact on amplification of drug resistance. Given our knowledge that drug resistance is efficiently transmitted in South Africa, new cases with undiagnosed/unsuspected MDR-TB will receive regimen 1 treatment (2 month intensive phase: isoniazid, rifampicin, ethambutol and pyrazinamide, and 4 month continuation phase: isoniazid and rifampicin) until treatment failure is noted. Similarly, retreatment cases with undiagnosed MDR-TB will receive regimen 2 (2 month intensive phase: isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin, and 4 month continuation phase: isoniazid and rifampicin) until the diagnosis of MDR-TB is made. In both instances, a weak treatment regimen was given thereby increasing the risk of amplification of resistance. Furthermore, the prolonged infectious period will perpetuate the drug resistant TB epidemic through transmission to close

contacts. The amplification of resistance during treatment has been illustrated by two recent publications.[16;17] The study by Calver *et al.* illustrated the emergence of ethambutol, pyrazinamide and ofloxacin resistance in a well-functioning TB control program as a consequence of delayed diagnostics and an inadequate treatment regimen. This occurred in a setting where treatment was hospital-based and adherence was excellent, a disturbing picture when compared to the rest of the country where adherence is much less stringent. The extent to which amplification of resistance has occurred in South Africa has been illustrated by another study which documented that approximately 20% of MDR-TB cases also displayed resistance to ethambutol in 2006.[18] Subsequently, a follow up study showed that more than 50% of MDR-TB cases had isolates which harbored mutations in the *embB* gene (which are known to confer ethambutol resistance).[19] These findings are in stark contrast with routine drug susceptibility testing (DST) data as well as surveillance data which suggested that ethambutol resistance was rare.[11] This may be explained by the technical difficulties associated with culture-based ethambutol resistance testing. Similarly, DST for pyrazinamide is technically challenging and is not routinely done in South Africa. For this reason the extent of pyrazinamide resistance is largely unknown and was assumed to be rare. Two recent molecular epidemiological studies confirm that pyrazinamide is strongly associated with MDR-TB.[20;21] In both studies, more than 50% of MDR-TB isolates harbored mutations in the *pncA* gene known to confer pyrazinamide resistance. Together this suggests that at least 50% of MDR-TB cases have resistance to ethambutol and/or pyrazinamide. This will undoubtedly have significantly impacted on the effectiveness of MDR-TB treatment in South Africa over the past few years. In 2002, standardized MDR-TB treatment was implemented in order to combat the emergence and spread of drug resistant disease. The standardized regimen for MDR-TB treatment consisted of five anti-TB drugs; three second-line drugs (amikacin/kanamycin, ofloxacin and ethionamide) and two first-line drugs (ethambutol and pyrazinamide – included because resistance was thought to be rare). This regimen has been in use in South Africa from 2002 to 2010. In view of the above molecular epidemiological data it must be concluded that this was a very weak regimen as a significant proportion of these patients would have only received three effective drugs during the intensive phase (kanamycin/amikacin, ofloxacin and ethionamide) and only 2 drugs (ofloxacin and ethionamide) during the continuation phase of therapy. Knowledge about the resistance to these three drugs remained sparse as routine DST was curtailed to testing for isoniazid, rifampicin and ethambutol during the period 2002 to 2007. Only after the disclosure of the outbreak of XDR-TB in 2006[5] was routine DST expanded to include amikacin/kanamycin, ofloxacin and ethionamide.

In response to the technical difficulties associated with phenotypic testing for ethambutol, the WHO recommended a revised MDR-TB treatment regimen.[22] They advocated the use of at least 4 active drugs. Pyrazinamide was only included in the regimen if the culture was susceptible to this drug, but was not counted as one of the 4 effective anti-TB drugs. Subsequently, the National TB Control Program issued a draft policy guideline for the treatment of MDR-TB. This was criticized as again being a weak regimen with the risk of amplification of drug resistance.[19] The draft guidelines proposed a treatment regimen based on the outcome of ethambutol DST. If an isolate was susceptible to ethambutol, it was included as the 5th drug in the regimen (disregarding the inaccuracy of DST for ethambutol), whereas if the results of DST for ethambutol showed resistance, it was replaced with terizidone/cycloserine. Pyrazinamide was included as one of the 4 effective drugs.

In 2010 new guidelines for the standardized treatment of MDR-TB and XDR-TB were issued by the Department of Health in South Africa. In the guidelines for MDR-TB treatment, ethambutol was replaced by terizidone/cycloserine (irrespective of the DST results, however, pyrazinamide was retained as one of the five drugs thereby fulfilling the criteria as stipulated by the WHO - at least 4 effective drugs – usually kanamycin/amikacin, ofloxacin, terizidone/cycloserine and ethionamide). However, this regimen may be weakened by previously unsuspected cross-resistance. Recent studies have demonstrated a positive correlation between *inhA* promoter mutations and high level ethionamide resistance (50 to 100 µg/ml).[23-25] The implementation of genetic-based DST in the Western Cape Province of South Africa demonstrated that 58.5% of MDR-TB isolates harbored mutations in the *inhA* gene using the MTBDR*plus* line probe assay.[26] More recently, DNA sequencing showed that in the Western and Eastern Cape Provinces, respectively, 59% and 71% of all MDR-TB isolates and 86 and 92% of all XDR-TB isolates harbored an *inhA* promoter mutation.[27] This implies that in the Western and Eastern Cape Provinces treatment of MDR-TB with a regimen containing ethionamide in a background of *Mycobacterium tuberculosis* strains with resistance to isoniazid, rifampicin, ethambutol, pyrazinamide and ethionamide may enhance the risk of the emergence of XDR-TB. In fact, the strong association observed between *inhA* promoter mutations and XDR-TB noted in both provinces raises the possibility that in these settings, strains with an *inhA* promoter mutation have been selected to become XDR-TB due to cross-resistance conferred by this mutation. Thus, the presence of an *inhA* promoter mutation is a gateway to the evolution of XDR-TB.[27] This hypothesis implies that in these two provinces XDR-TB is emerging. Moreover, the predominance of certain transmitted strain groups causing the majority of the MDR-TB cases in these settings and

characteristically harboring *inhA* promoter mutations will not be reduced by the newly implemented standardized treatment regimen for MDR-TB.

Genotypic analysis of MDR-TB and XDR-TB strains by DNA fingerprinting and DNA sequencing of target genes conferring resistance clearly demonstrates that MDR-TB is transmitted[16;28-31] and that in this early stage of the XDR-TB epidemic, XDR-TB emerges from the transmitted MDR-TB strains through acquisition, probably as a result of inadequate treatment as described above (unpublished results). In the Western Cape Province, strains from the Beijing cluster R220,[7] the atypical Beijing family[31] and the LCC family[32] mostly contribute to the transmission of MDR-TB while the atypical Beijing family accounts for most of the transmission of MDR-TB in the Eastern Cape Province. This differs from the KwaZulu-Natal Province of South Africa where it has been shown that the KZN/LAM4/F15 strain is primarily transmitted.[5;33] In support of the notion of emergence of resistance, we have documented the evolution of ofloxacin resistance during treatment (unpublished results). The reasons for the emergence of resistance to this anti-TB drug is as yet unknown but is of considerable concern as ofloxacin is the backbone of the current MDR-TB treatment regimen in South Africa. A study from Uzbekistan showed the development of ofloxacin resistance under well controlled conditions and suggested that an association between pre-existing second-line drug resistance and a severe clinical condition (at baseline) was a risk factor for the development of ofloxacin resistance.[34] Similar findings were recently reported in a mine setting in South Africa.[17] As mentioned above, treatment of MDR-TB in South Africa included (until recently) a fluoroquinolone (mostly ofloxacin), an aminoglycoside, pyrazinamide, ethambutol or cycloserine, and ethionamide. Considering the frequently observed resistance to pyrazinamide, ethambutol and cross-resistance conferred to ethionamide by the *inhA* promoter mutation, one explanation for the emergence of ofloxacin resistance could be the inadvertent use of ofloxacin mono-therapy during the continuation phase of second-line treatment.[19] This implies that highly resistant and transmissible forms of *M. tuberculosis* may develop even under well controlled conditions with stringent adherence. This may have significant implications for MDR-TB treatment on a global scale, especially in settings where patient management may be suboptimal.[34]

The 2010 Department of Health guidelines attempted to improve the treatment outcome of XDR-TB. Accordingly, capreomycin replaced amikacin/kanamycin, *p*-aminosalicylic acid (PAS) replaced pyrazinamde, and moxifloxacin replaced ofloxacin. These changes have been implemented based on the assumption that both capreomycin and PAS have not been used extensively in South Africa and therefore resistance to these drugs should be rare. However, it is unclear whether recent molecular

epidemiological data has been adequately considered when this decision was made, as a recent study in Korea has conclusively demonstrated cross-resistance between the aminoglycosides (amikacin and kanamycin) and capreomycin.[35] In an attempt to replicate this finding we have sequenced the *rrs* gene from clinical isolates which demonstrated both susceptibility and resistance to amikacin. Susceptibility testing in MGIT 960 media showed that in all instances the A1401G *rrs* mutation correlated with high level amikacin resistance (>20µg/ml) (unpublished results). Similarly all amikacin resistant isolates displayed phenotypic resistance to capreomycin at a concentration of 5µg/ml (unpublished results), which is twice the critical concentration recommended by the WHO for DST in MGIT 960 media.[36] According to this criterion a laboratory diagnosis of resistant to capreomycin would be given. This raises concern as to whether patient isolates which show resistance to amikacin will benefit from a regimen which includes capreomycin. Clinical trials are desperately needed to determine the therapeutic value of capreomycin, especially since capreomycin may lead to adverse events.[6]

The inclusion of moxifloxacin in the treatment regimen for XDR-TB was informed by improved treatment outcomes in XDR-TB cases.[37] However, it is well known that cross-resistance occurs between different fluoroquinolones.[38] A study by Kam in 2006 showed a correlation between specific *gyrA* mutations and the level of ofloxacin and moxifloxacin resistance.[39] The author suggested that patient isolates with mutations conferring a moxifloxacin minimum inhibitory concentration of ≤2µg/ml may benefit from the inclusion of moxifloxacin. However, this is significantly higher than the current critical concentration recommended by the WHO to differentiate between resistant and susceptible isolates.[36] Provisional data from a study being conducted in South Africa confirmed the above findings – all isolates which harbor a mutation in the quinolone resistance determining region (QRDR) were resistant to 0.5µg/ml moxifloxacin. Again, it will be important to quantify the benefit of including moxifloxacin in the treatment regimen for XDR-TB given that these isolates are resistant to fluoroquinolones according to WHO definitions.

If the molecular epidemiology results are correct then treatment regimen options for XDR-TB are seriously curtailed. In certain patients only two of the five drugs will be active which may reflect currently observed treatment outcomes. This means that if we continue to treat XDR-TB patients with weak regimens we will probably generate pandrug-resistant-TB - or have we already reached this point? Anecdotal evidence from clinicians treating these patients seem to suggest that we are already entering a stage of non-treatable tuberculosis disease or “TDR-TB” (“totally drug resistant TB”), only 12 years after observing the first documented XDR-TB case in South Africa. At this rate,

the discovery, evaluation and registration of new classes of anti-TB drugs will not be able to keep up with the organism's ability to subvert our uncoordinated attacks. It is therefore important to acquire knowledge that can be translated into practical applications to formulate, implement and review policies and programs. To achieve this goal, collaboration between clinicians, laboratories and researchers needs to be improved to establish specialised scientific knowledge and medical expertise. A comedy of errors typically culminates in a happy resolution of the thematic conflict caused by the foolish mistakes of the characters in the play. Whether this will hold true for drug resistant tuberculosis remains to be seen.

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8.3 Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis

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ABSTRACT

Background and objectives: Multi-drug resistant (MDR) bacteria are a growing threat to global health. Studies focusing on single antibiotics have shown that drug resistance is often associated with a fitness cost in the absence of drug. However, little is known about the fitness cost associated with resistance to multiple antibiotics.

Methodology: We used *Mycobacterium smegmatis* as a model for human tuberculosis (TB) and an *in vitro* competitive fitness assay to explore the combined fitness effects and interaction between mutations conferring resistance to rifampicin and ofloxacin; two of the most important first- and second-line anti-TB drugs, respectively.

Results: We found that four out of 17 *M. smegmatis* mutants (24%) resistant to rifampicin and ofloxacin showed a statistically significantly higher or lower competitive fitness than expected when assuming a multiplicative model of fitness effects of each individual mutation. Moreover, six of the 17 double drug-resistant mutants (35%) had a significantly higher fitness than at least one of the corresponding single drug-resistant mutants. The particular combinations of resistance mutations associated with no fitness deficit in *M. smegmatis* were the most frequent among 151 clinical isolates of MDR and extensively drug-resistant (XDR) *M. tuberculosis* from South Africa.

Conclusions and implications: Our results suggest that epistasis between drug resistance mutations in mycobacteria can lead to MDR strains with no fitness deficit, and that these strains are positively selected in settings with a high burden of drug-resistant TB. Taken together, our findings support a role for epistasis in the evolution and epidemiology of MDR- and XDR-TB.

INTRODUCTION

Epistasis refers to the phenomenon where the phenotypic effect of one mutation differs depending on the presence of another mutation (1). The importance of epistasis for our understanding of biology is increasingly recognized; it has been implicated in many processes, ranging from pathway organization, the evolution of sexual reproduction, mutational load, genomic complexity, to speciation and the origin of life (2). Moreover, recent studies have reported a role for epistasis in the evolution of antibiotic resistance (3–6). Multidrug-resistant (MDR) bacteria are emerging worldwide, in some cases leading to incurable disease. While new antibiotics are urgently needed, a better understanding of the forces that lead to the emergence of drug resistance would help prolong the lifespan of existing drugs.

Studies in various bacterial species have shown that the acquisition of antibiotic resistance often imposes a physiological cost on the bacteria in absence of the drug (7–9). However, some drug-resistance conferring mutations have been associated with low- or no fitness cost, and compensatory evolution can mitigate some of the initial fitness defects associated with particular drug resistance conferring mutations (10). Most of these studies have focused on resistance to a single drug. Given the public health threat posed by MDR bacteria, there is a need to understand the factors that influence the emergence of resistance to multiple drugs.

Recent studies in model organisms have shown that mutations conferring resistance to different drugs can interact epistatically. A study in *Pseudomonas aeruginosa* found that the relative fitness of certain strains resistant to streptomycin and rifampicin (RIF) (4,6) was lower than expected based on the fitness of the corresponding single-resistant mutants. Similarly, a study in *Escherichia coli* (3) showed that strains resistant to two drugs can have a higher fitness than strains resistant to only one drug; a phenomenon referred to as ‘sign epistasis‘ (11). However, whether such epistatic interactions play any role in the emergence and spread of MDR bacteria in clinical settings has not been determined.

Multi-drug resistance is a particular problem in human tuberculosis (TB) (12). Recent surveillance data showed the highest rates of resistance ever documented with some Eastern European countries

reporting up to 50% of TB cases as MDR (13). In *M. tuberculosis*, the main causative agent of human TB, drug resistance is chromosomally encoded and results from *de novo* acquisition of mutations in particular genes (14). These mutations are acquired sequentially, giving rise to MDR and extensively drug-resistant (XDR) strains (15,16). MDR-TB is defined as strains resistant to at least RIF and isoniazid, the two most important first-line anti-TB drugs. XDR-TB is caused by strains that, in addition to being MDR, are also resistant to ofloxacin (OFX), or any other fluoroquinolone, and to at least one of the injectable second-line drugs (17).

In this study, we used *Mycobacterium smegmatis* as a model for *M. tuberculosis* to investigate putative epistatic interactions between mutations conferring resistance to RIF and OFX, two of the most widely used first- and second-line anti-TB drugs, respectively. *M. smegmatis* is used widely in the TB research community because it is non-pathogenic, in contrast to *M. tuberculosis*, which requires biosafety-level 3 containment. Moreover, *M. smegmatis* forms visible colonies in 2-3 days, compared to 3-4 weeks for *M. tuberculosis*. We then compared our experimental data generated with *M. smegmatis* to the clinical frequency of particular combinations of RIF- and OFX- resistance-conferring mutations in a panel of MDR and XDR *M. tuberculosis* clinical strains from South Africa.

MATERIAL AND METHODS

Bacterial strains and growing conditions

All strains used for the competitive fitness experiments were derived from the wild-type *Mycobacterium smegmatis* strain mc^2155. Bacteria were grown in Middlebrook 7H9 broth supplemented with ADC or on Middlebrook 7H11 agar plates supplemented with OADC. The culture tubes were incubated in standard conditions and the optical density (OD_{600}) was recorded daily to measure the growth.

Selection of single and double-resistant *M. smegmatis* mutants

Independent RIF- and OFX- resistant *M. smegmatis* single mutants were isolated using an approach similar to the classic fluctuation tests of Luria and Delbrück (1943) to ensure that the mutants selected arise from independent mutational events. A starting culture of *M. smegmatis* mc^2155 was prepared from wild-type *M. smegmatis* and adjusted to approximately 300 bacilli per ml (OD_{600} ~ 0.01). 10ml of culture was transferred into 14 individual 50ml falcon tubes. When the bacteria reached end of log-

phase (OD_{600} ~ 3.00), the cultures were concentrated by centrifugation at 1500rpm for 5 minutes, the supernatant discarded, and the bacteria resuspended in 500µl 7H9 media. This concentrated bacterial culture was plated onto Middlebrook 7H11 media containing 200µg RIF per ml for the isolation of RIF-resistant colonies, and 2 µg OFX per ml for the isolation of OFX-resistant colonies. The plates were incubated for 3-5 days at 37˚C until colonies became visible. One colony from each plate was picked and sub-cultured in antibiotic free Middelbrook 7H9 broth. For the isolation of double-resistant mutants, different *rpoB*- and *gyrA*-mutants were used to generate different combinations of mutations conferring resistance to both antibiotics. Some double-resistant mutants were selected by plating on Middlebrook 7H11-OADC media containing both 200µg/ml of RIF and 2µg/ml of OFX.

Mutation identification

The main target genes for resistance to RIF and OFX are *rpoB* and *gyrA*, respectively. To detect the relevant drug resistance-conferring mutations, the *rpoB* and *gyrA* genes were amplified by PCR using DNA extracted from the single- and the double-resistant mutants. The primers used to amplify the portion of the *rpoB* gene encoding the main set of mutations conferring resistance to RIF were: 5' GGA CGT GGA GGC GAT CAC ACC 3'. For amplification of the *gyrA* gene, the primers 5' CAT GAG CGT GAT CGT GGG CCG and 5' CAG AAC CGT GGG CTC CTG CAC 3' were used. The same primers were used for direct DNA sequencing from the PCR product.

Fitness assay and calculation of fitness ratio

The *rpoB*-, *gyrA*- and *rpoB-gyrA*-mutants were competed against the wild-type antibiotic-susceptible strain in antibiotic-free Middlebrook 7H9 media. A total of 100 CFU of bacteria per ml were inoculated in 10ml of Middlebrook 7H9 media in a 1:1 ratio. For each wild type-mutant pair, between four and eight replicate competition assays were performed. At the start of the experiment (t=0 hours), 50µl from each competition culture was plated on both antibiotic-free- and antibiotic-containing–Middlebrook 7H11 plates in triplicates to estimate the baseline CFU counts. The competition cultures were incubated at standard conditions on a shaking incubator at 100rpm, and the optical densities (OD_{600}) were recorded daily. After 72 hours, the same competition cultures were diluted 10^5 to 10^6-fold and plated on both selective and non-selective Middlebrook 7H11 media to obtain the endpoint CFU counts. For both competing strains, the Malthusian parameters were calculated by taking the natural log of the endpoint CFU over the baseline CFU (7). The mean CFU count of the three replicates was used for the calculation of the relative competitive fitness. This gave the Malthusian parameters

(m_s and m_r) for both strains, which correspond to the number of doublings (generations) that each strain went through during the observed time period. Finally, the relative fitness of the drug-resistant strain relative to the wild-type was determined using: $W_{rs} = m_r / m_s$ (7). Shapiro-Wilk test evidenced the normality of the fitness data (p=0.3). Student's t-test was used to detect differences in the mean fitness and the limit for statistical significance was set at p=0.05. Test statistics and estimates

were based on 1000 bootstrap replicates. Statistical analysis was performed with STATA SE/10.

Measuring Epistasis

To explore putative genetic interactions between drug resistance mutations, pairwise epistasis (ε) was measured assuming a multiplicative model in which $\varepsilon = W_{AB}W_{ab} - W_{Ab}W_{aB}$, where W_{ab} is the fitness of the clone carrying alleles *a* and *b,* and capital letters represent the wild-type sensitive alleles (3). Following this model, values of $\varepsilon > 0.0$ indicate that the fitness of the double-mutant is higher than expected based on the fitness values of the individual single mutants. Similarly, values of $\varepsilon < 0.0$ indicate that the fitness of the double-mutant is lower than expected based on the fitness values of the individual single-mutants. We tested the normality of the epistasis data with a Shapiro-Wilk test. To test if epistasis values were significantly different from zero, we used the error-propagation method described by Trindade *et al*. (3). We considered that alleles *a* and *b* showed significant epistasis whenever the calculated error was smaller than the average value of ε (Figure 3).

An important type of epistasis is sign epistasis. Sign epistasis exists when a mutation is deleterious on some genetic backgrounds but beneficial on others (11). To detect the presence of sign epistasis from our experimental data, we performed pairwise comparisons between the fitness of each double-resistant mutant and the corresponding single-resistant mutants using a one-sided bootstrap Student's t-test with 1000 replicates (Figure 5). The combined p-values were obtained using Fisher's method.

Clinical frequency of *rpoB*- and *gyrA* mutation combination in *M. tuberculosis*

A total of 151 clinical MDR- and XDR-TB *M. tuberculosis* isolates were included in this study. These were collected in the Eastern (N=99) and Western Cape (N=52) Provinces of South Africa between 2008-2009 and 2001-2008, respectively. RIF and OFX resistance determining regions in the *rpoB* and *gyrA* genes were analysed using standardized PCR and sequencing (18,19). Amplification products were sequenced using an ABI 3130XL genetic analyzer, and the resulting chromatograms were

analysed using Chromas software. Only isolates harbouring a combination of resistance mutations in the *rpoB* and *gyrA* genes analyzed within our *M. smegmatis* study were included.

RESULTS

Fitness cost of single drug-resistant mutants

We first determined the relative fitness of *M. smegmatis* mutants resistant to a single drug. To this end, we selected a series of spontaneous *M. smegmatis* mutants resistant to RIF or OFX. From the RIF-selected mutants, we used five clones with *rpoB* mutations for further analysis (H526R; H526P; H526Y; S531W; S531L) (Table S1). These mutants were competed *in vitro* against their RIF-susceptible ancestor as described previously (7). We found that S531L, S531W and H526Y showed no difference in relative fitness compared to the ancestor (Figure 1A), while H526R and H526P showed a significantly lower relative fitness (Bootstrap p=0.02 and p < 0.01, respectively). Similar to previous work in *M. tuberculosis* (7), we found a strong correlation between fitness cost of *rpoB* mutations in *M. smegmatis* and the frequency of these mutations in clinical isolates of *M. tuberculosis* (Spearman Rank coefficient 0.9, p=0.04; Table S1). Individually, S531L and H526Y which showed no fitness cost in our *M. smegmatis* model are the most frequent RIF resistance-conferring mutations in clinical settings, whereas S526P, which had the lowest relative fitness of all mutants occurs only in 0.1% of clinical strains (Table S1). We found no correlation between the spontaneous mutation frequency of *rpoB* mutations and the clinical frequency of these mutations (Table S1).

From the OFX-selected mutants, we selected four that carried distinct *gyrA* mutations for further analysis (D94G; G88C; D94N; D94Y). *In vitro* competition against the OFX-susceptible ancestor revealed that mutants carrying D94G and D94Y had no fitness defect, while D94N and G88C had a significantly lower relative fitness (Bootstrap p = 0.02 and p < 0.01, respectively) (Figure 1B). We compared our fitness measures with the frequency of *gyrA* mutations found in *M. tuberculosis* clinical isolates using data from a recently published review based on 1,220 OFX-resistant *M. tuberculosis* isolates (20) (Table S2). Similar to our findings with RIF-resistant mutants, we found that mutations at codon position 94 of *gyrA*, which showed overall the highest *in vitro* fitness in *M. smegmatis*, were the most common mutations in *M. tuberculosis* clinical strains. By contrast, *gyrA* G88C which had the lowest fitness is only rarely (1.6%) found in clinical settings (Table S2). In contrast to the *rpoB* mutations, mutations at codon position 94 of *gyrA* were also the most frequent during the *in vitro* selection (Table S2).

Evidence for epistasis between *rpoB and gyrA* mutations

To test for possible epistatic interactions between mutations conferring RIF- and OFX- resistance, we selected for spontaneous mutants resistant to both drugs. These double-mutants harbouring a mutation in *rpoB* and *gyrA* were selected starting from the available single drug-resistant mutants. A total of 17 *rpoB-gyrA* double-mutants were generated out of the 20 possible combinations (Table S3). The relative fitness of the double-mutants was determined by standard competition assays against the pan-susceptible ancestor strain and compared to the fitness of the corresponding single-resistant mutants. We compared the observed fitness of each double-mutant to the expected fitness assuming no epistasis based on a multiplicative model (Figure 2, see Material and Methods for details). We found that in 11/17 (65%) of the double-mutants, the observed fitness was different from the expected, suggesting either negative or positive epistasis between particular RIF- and OFX resistance-conferring mutations (Figure 2A).

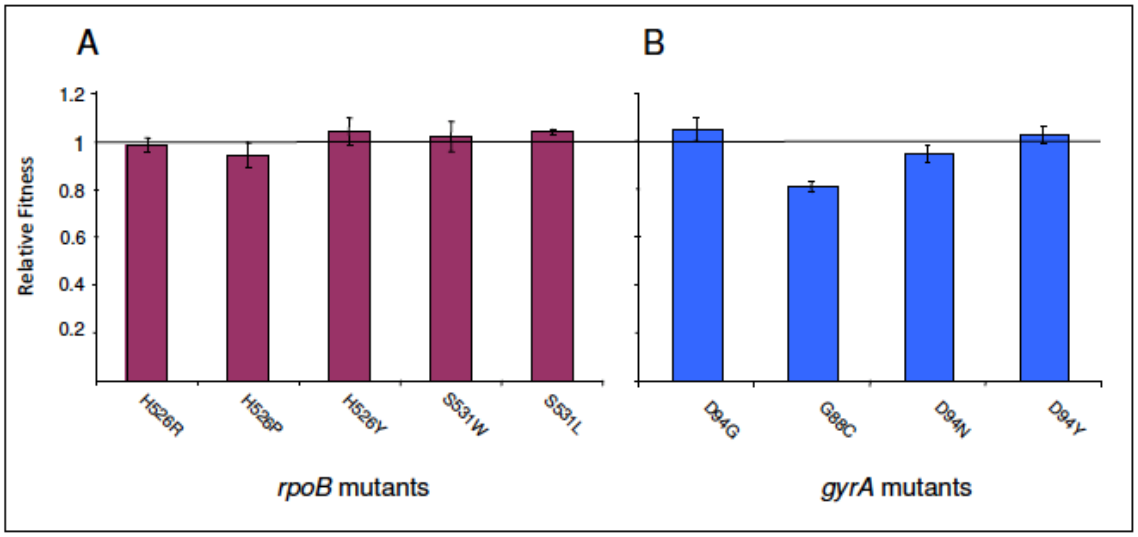

Figure 1. Relative fitness of *M. smegmatis* mutants resistant to a single drug compared to their pan-susceptible ancestor. Bars represent 95% confidence intervals. A. Relative fitness of *rpoB* single mutants resistant to RIF. B. Relative fitness of *gyrA* single mutants resistant to OFX

To measure epistasis quantitatively, we measured pairwise epistasis (ε) between all the different single-mutant pairs we had fitness data for, assuming a multiplicative model (Table S4); positive and negative values of ε indicate positive or negative epistasis, respectively (3). Overall, the ε values across all mutant pairs followed a normal distribution (Shapiro-Wilk, p-value=0.062) with an average positive value of 0.027 (95% confidence interval -0.02, 0.08) (Table S4). Four out of 17 (24%) double-mutants

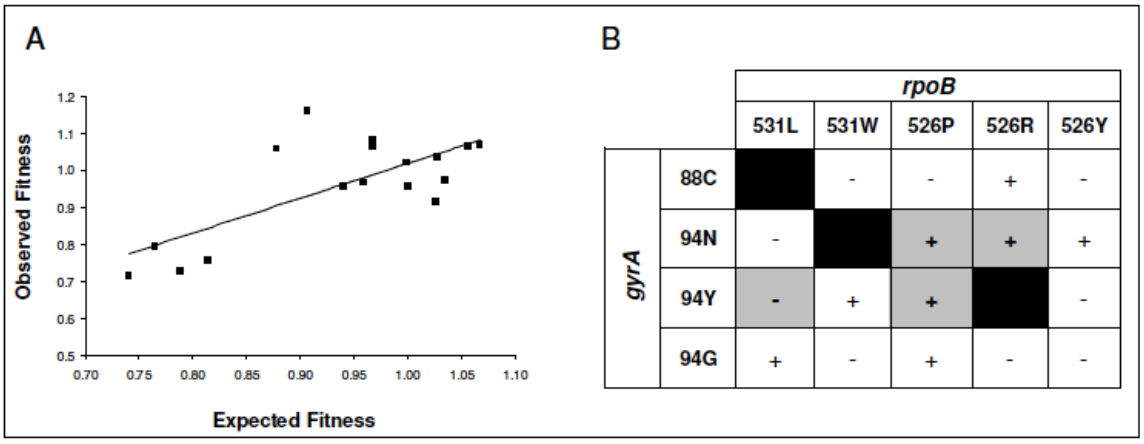

Figure 2. Evidence of epistasis between mutations conferring resistance to RIF and OFX. A. Relationship between observed and expected multiplicative fitness for the 17 double-resistant mutants (data point over/below the bar). The solid line represents the null hypothesis of multiplicative fitness effects. Deviations from this line arise as a consequence of epistatic fitness effects. B. Allelic combination analysed and the corresponding sign of epistasis. The grey squares correspond to the pairs of mutation showing statistically significant epistasis.

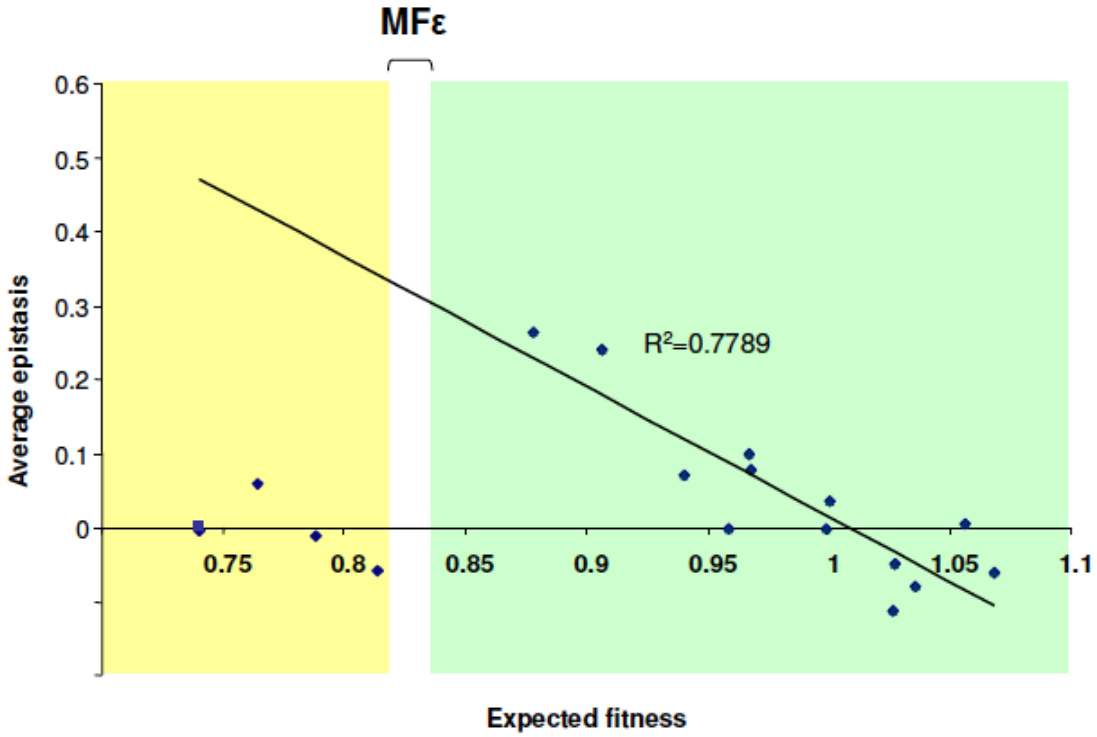

Figure 3. Correlation between the average expected fitness and the strength of epistasis. Average epistasis was measured as deviation from a multiplicative model of double-resistant mutant fitness scores estimated by head-to-head competition in Middlebrook 7H9 broth. MFε: Minimum Fitness for ε.

showed statistically significant positive or negative epistasis between RIF- and OFX resistance-conferring mutations. Moreover as shown in Figure 2B, these epistatic interactions were allele-specific, showing differences in the sign (i.e. positive *vs*. negative) of the ε value depending on the specific amino acid change at a particular codon position.

Theoretical and experimental evidence predicts a correlation between the average deleterious effect of a single mutation and the strength of epistasis (21–23). Hence, we tested whether this relationship holds for drug-resistant mycobacteria. In agreement with these predictions, we found a strong correlation between the expected fitness of our double-mutants and the strength of epistasis between the respective RIF- and OFX resistance-conferring mutations (R^2=0.78; T-test, p<0.001) (Figure 3). However, this correlation was only observed above a particular threshold of expected fitness, which we refer to as "Minimal Fitness for epistasis" (MFε). Above MFε, epistasis tended to be positive when individual mutations were costly and negative when individual mutations were beneficial (21,23). Below MFε, the correlation was lost, likely because these data points were all derived from mutants carrying the G88C mutation in *gyrA*, which was associated with a high fitness defect. The correlation when all datapoints were included show no significance (R^2=0.07; T-test, NS)

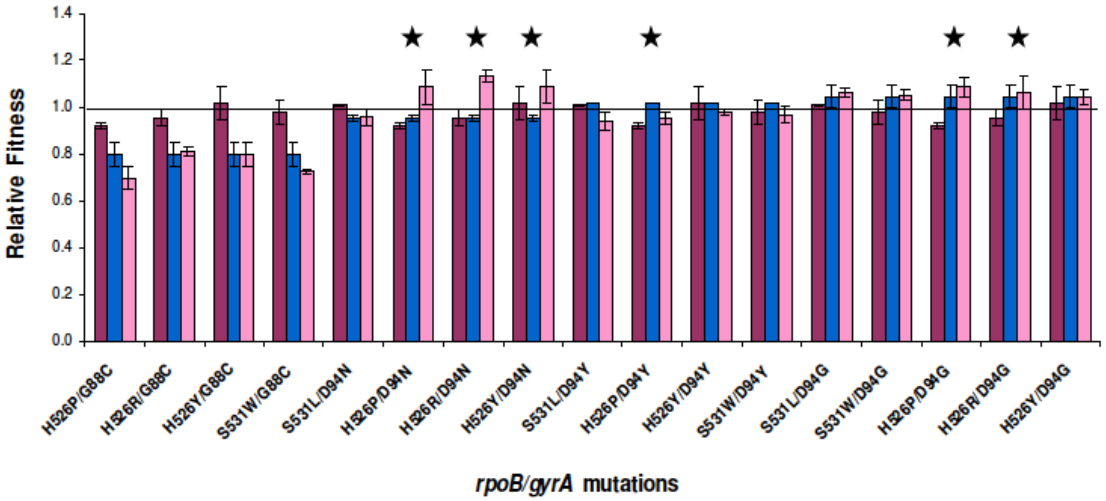

Figure 4. Evidence for sign epistasis between mutations conferring resistance to RIF and OFX. Sign epistasis occurs when the fitness of the double-resistant mutant (pink bar) is greater than the fitness of at least one corresponding single-resistant mutant [purple- (RIF) and blue- (OFX) bars]. The bars represent the standard deviation of the values. Double-resistant mutants with a bootstrapped p-value<0.05 are highlighted with a star.

Evidence for sign epistasis in *rpoB/gyrA* double mutants

Sign epistasis refers to the case where a particular mutation that is deleterious on its own is beneficial in the presence of another mutation (3). In the context of drug resistance, sign epistasis occurs when the fitness of the double-resistance mutant is higher than at least one of the corresponding single-resistance mutants. We found that 6 out of 17 double-mutants (35%) showed statistically significant evidence of sign epistasis (Figure 4). In addition, the observed sign epistatis was allele specific, i.e. the epistasis effects varied according to the specific alleles of the same gene. For example, D94N in *gyrA* led to the conversion of the fitness sign in the S526P RIF-resistant background but not in the S531L RIF-resistant background (Figure 4).

Role of epistasis in clinical XDR-TB

Given the evidence for epistasis between RIF- and OFX resistance mutations in *M. smegmatis*, we investigated how fitness changes along the mutational pathway leading from MDR-TB to XDR-TB might be influenced by corresponding epistatic interactions in *M. tuberculosis* (Figure 5A). In the standard treatment protocols for TB (17), RIF is an essential part of the first-line regimen for drug-susceptible disease, and OFX is part of the second-line regimen when resistance against first-line drugs has developed. Thus, *rpoB* mutations are generally acquired first and *gyrA* mutations second. Following this trajectory, selection by RIF will occur first, and the RIF-resistant mutants that survive will exhibit heterogeneous fitness in the absence of the drug depending on their *rpoB* mutations (Figure 1) (7,24). At this point, MDR-TB has developed and second-line treatment is initiated. Selection for OFX resistance begins, but the fitness levels of the emerging double-mutants can still be positively or negatively affected depending on which *gyrA* mutation is acquired. Our *M. smegmatis* data showed that the *gyrA* D94G mutation was associated with improved fitness in all of the double-mutants, irrespective of the *rpoB* mutation (pink bars compared to purple bars in Figure 4). This was statistically significant in two of the five corresponding double-mutants tested. Hence, based on the most likely clinical scenario of moving from MDR- to XDR-TB (Figure 5A), we would expect the *gyrA* D94G mutation to be the most commonly found mutation in XDR-TB strains, and also to be found in combination with many different *rpoB* mutations. By contrast, we would expect *gyrA* G88C, which was consistently associated with negative epistasis in our *M. smegmatis* model (Figure 3, 4 and 5A) to show the opposite trend. To test these predictions, we analyzed 151 MDR-and XDR-TB clinical isolates from South Africa. Sequencing of the relevant genes revealed that 71/151 (47%) harbored *gyrA* D94G while G88C occurred only once (0.7%). Moreover *gyrA* D94G was the only mutation that occurred in

combination with all three *rpoB* mutations detected among these clinical dataset (Figure 5B). Taken together, our results show that experimental fitness data generated with *M. smegmatis* can be predictive of clinical TB. Moreover, these findings support a role for epistasis in the progression of *M. tuberculosis* from MDR to XDR.

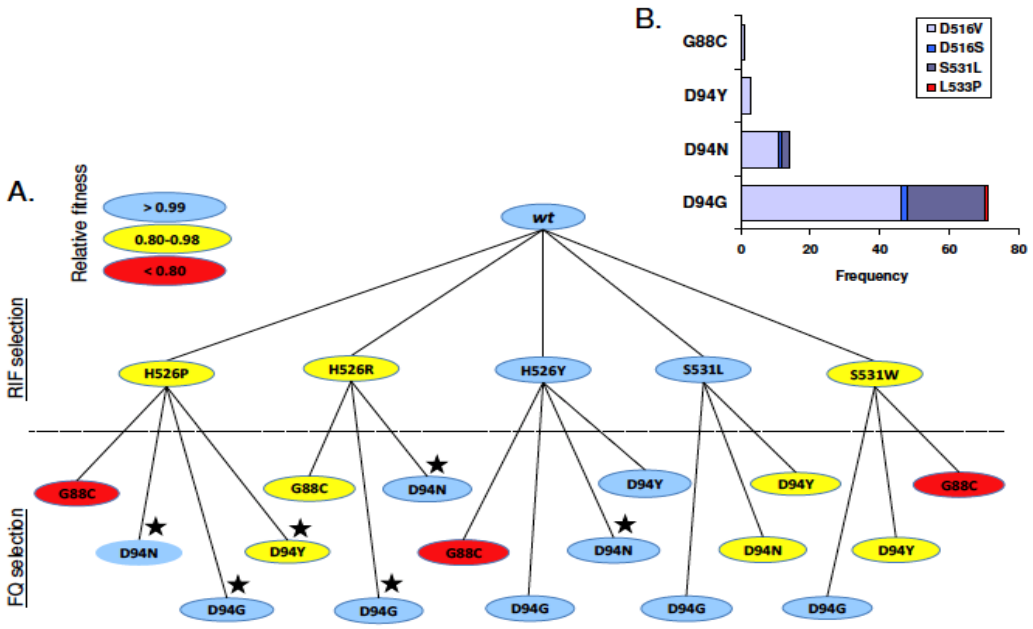

Figure 5. A. Mutational pathway leading to *rpoB-gyrA* double mutants when a patient undergoes standard TB treatment. *RpoB* mutations are generally acquired first, followed by *gyrA* mutations. The relative fitness of the various double-resistant mutants is indicated as determined by *in vitro* competition using the *M. smegmatis* model. *wt* - drug-susceptible wild-type strain; *rpoB* - point mutations in *rpoB* conferring RIF resistance; *gyrA* - point mutations in *gyrA* conferring OFX resistance B. Frequency of *rpoB-gyrA* mutation pairs found in 151 MDR- and XDR-TB clinical isolates from the Eastern Cape and Western Cape Provinces of South Africa.

DISCUSSION

In the present study, we used *M. smegmatis* as a model to show that epistasis can occur between mutations conferring resistance to RIF and OFX, which are two of the most important anti-TB drugs. Specifically, in several of the mutants resistant to both of these drugs, some of the mutations conferring resistance to one drug mitigated the negative fitness effects of some the mutations conferring resistance to the other drug (or vice-versa). Moreover, we found clear evidence of sign epistasis, showing that in some cases, the double-resistant mutants had a higher relative fitness than at least one of the corresponding single-resistant mutants. In the context of MDR, sign epistasis between different drug resistance-conferring mutations represent the worst case scenario; instead of accumulating fitness defects with each additional drug resistance, MDR strains manage to increase their relative fitness by acquiring additional drug resistance determinants. One limitation of our study

is that we cannot exclude the possibility that additional mutation(s) could have arisen during the selection of our mutants, which may compensate for the initial fitness defects associated with the individual resistance mutations.

More work is needed to elucidate the mechanisms involved in the interaction between mutations in *rpoB* and *gyrA*. Yet, several features make such interactions biologically plausible. *GyrA* encodes one of the subunits of DNA gyrase which is involved in the introduction of negative supercoiling to double stranded DNA, thereby relaxing the positive supercoils that form during DNA replication (25). RpoB encodes part of the RNA polymerase and therefore important for the transcription of DNA to RNA (26). Although these two pathways are separate (27,28), GyrA and RpoB are both involved in the fundamental flow from DNA to RNA. Intriguingly, Gupta *et al*. isolated an 'RNA-polymerase-DNA gyrase complex' in *M. smegmatis* that exhibited both DNA super-coiling and transcriptional activities. The authors also found that DNA gyrase inhibitors not only reduced DNA gyrase activity, but also reduced transcriptional activity indicating a role of DNA gyrase in transcription (29). Finally, it has been shown that during transcription, RNA polymerase introduces positive supercoiling ahead as it slides along its template DNA. This leads to a reduced accessibility as supercoiling increases, further supporting a potential role for DNA gyrase in transcription (25).

Our study also showed that experimental data obtained from *M. smegmatis* is relevant for our understanding of clinical TB. Not only did we observe the same drug resistance-conferring mutations in *M. smegmatis* as routinely encountered in clinical strains of *M. tuberculosis*, but similar to previous studies, we found a good correlation for both RIF and OFX between the fitness cost observed *in vitro* in *M. smegmatis* mutants and the relative clinical frequency of the corresponding mutations in *M. tuberculosis* (20,24). Our *M. smegmatis* data showed particular relevance when focusing on MDR- and XDR-TB. Based on the most probable mutational pathway leading from MDR to XDR, our *M. smegmatis* fitness data predicted particular combinations of *rpoB* and *gyrA* mutations to be more frequent than others in clinical settings. This prediction was confirmed when screening a large panel of MDR and XDR *M. tuberculosis* clinical strains from South Africa, which is one of the regions with the highest burden of XDR-TB in the world (17).

Our mutational pathway analysis also showed that in some cases, if certain mutations are acquired first, the fitness of these drug-resistant strains are permanently set at a high baseline that cannot be drastically affected regardless of the individual fitness cost associated with the second mutation. Moreover, some *gyrA* mutations can act as 'fitness safety nets' offering the bacteria the possibility to recover from loss of fitness caused by any of the initial *rpoB* mutations. Taken together, our results suggest that although evolution towards MDR- and XDR-TB can follow multiple trajectories, these are

likely to be influenced by epistatic interactions between the particular drug resistance-conferring mutations. This will constrain the particular mutational combinations to those that either increase or at least maintain fitness at a minimum level (Figure 4). Above this minimum level of fitness, our study indicates that the strength of epistasis between *gyrA* and *rpoB* will be stronger when the individual mutations are associated with large fitness defects. Whilst the fitness measures reported here were generated during *in vitro* growth, *M. tuberculosis* is facing harsher environments during human infection. The fitness effects of drug resistance mutations have been shown to vary in different environments (6,30). Hence, it would be interesting to explore how host immune pressure, oxidative and other stresses might influence epitasis between drug resistance mutations.

Our finding that a specific *gyrA* mutation (i.e. D94G; Figure 4 and Figure 5A) can restore the fitness of strains carrying different *rpoB* mutations has implications for the development of new TB treatment regimens. So far, OFX and other fluoroquinolones have primarily been used as second-line drugs to treat MDR-TB (31). However, because of their potential to shorten TB chemotherapy, they are currently being evaluated in the context of new first-line treatment regimens for drug-susceptible TB (32). Our results highlight that using fluoroquinolones as first-line treatment is likely to result in the early selection of fluoroquinolone resistance-conferring mutations like D94G *gyrA* that not only confer resistance, but might promote the acquisition of additional drug resistance while maintaining bacterial fitness at an advantageous level, either through positive epistasis with mutations conferring resistance to RIF or other drugs, or by establishing a higher baseline fitness (33). Moreover, exposure to fluoroquinolones induces the bacterial SOS response which leads to the induction of error-prone DNA polymerases, thereby increasing the bacterial mutation rate and the propensity of acquiring additional drug resistance-conferring mutations (34). Interestingly, we found that resistance mutations at codon position 94 of *gyrA* were also most frequent during *in vitro* selection, suggesting that in addition to epistatic interactions between *rpoB* and *gyrA* mutations, other mechanisms might influence the frequency of particular combinations of drug resistance mutations in clinical settings.

In conclusion, our study together with previous findings demonstrates that epistasis between different drug resistance-conferring mutations occurs across several bacterial species. While our study focused on the interaction between mutations in *rpoB* and *gyrA*, further work should explore possible similar effects in resistance to other anti-TB drugs, both existing as well as those currently under development (35) (http://www.newtbdrugs.org/pipeline.php). Three new drug candidates have shown promising results in recent clinical trials of MDR-TB treatment (32). However, how these new compounds should best be deployed, and in what combinations, remains unclear. Our study suggests that considering

putative epistasis between the relevant drug resistance-conferring mutations could help optimize treatment regimens. For example, combining drugs in which the resistance-conferring mutations interact negatively would reduce the probability of resistance emerging.

Declarations of funding and conflicts of interest

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No conflicts of interest to declare.

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SUPPORTING INFORMATION

Suppl Table 1. Rifampicine (RIF) single-resistant mutants used in the study.

Sample name	RIF resistance					
	Conc (µg/ml)	*rpoB* SNP	Rel. Fitness	Stdev	% in Clinical TB*	Freq. *in vitro* mutants (N=15)
S164	200	S531L	1.00	0.002	54.3	6.7%
S021	200	H526Y	1.01	0.071	10.6	13.3%
S025	200	S531W	0.98	0.052	4.0	6.7%
S016	200	H526R	0.95	0.037	2.8	60.0%
S018	200	H526P	0.92	0.014	0.1	13.3%

* Based on data extracted from 840 clinical isolates with a single *rpoB* mutation summarized in ref. 24. Spearman's rho = 0.9 p-value= 0.04

Suppl Table 2. Ofloxacine (OFX) single-resistant mutants used in the study.

Sample name	OFX resistance					
	Conc (µg/ml)	*gyrA* SNP	Rel. Fitness	Stdev	% in Clinical TB*	Freq. *in vitro* mutants (N=19)
S002	2	D94G	1.04	0.047	64.7	89.5%
S013	2	D94Y	1.01	0.004		
S004	2	D94N	0.95	0.011		
S003	2	G88C	0.8	0.051	1.6	10.5%

* Based on data extracted from 691 clinical isolates with a single *gyrA* mutation summarized in ref 20. That metanalysis was based on codons, no allele-specific information was included.

Suppl Table 3. Competitive fitness of mutants resistant to rifampicine (RIF) and ofloxacine (OFX).

Sample name	RIF-OFX resistance			
	rpoB SNP	*gyrA* SNP	Rel. Fitness	Stdev
S118	H526R	G88C	0.80	0.018
S101	H526P	G88C	0.71	0.051
S128	H526Y	G88C	0.76	0.051
S148	S531W	G88C	0.72	0.01
S114	H526R	D94N	1.16	0.025
S099	H526P	D94N	1.06	0.074
S137	H526Y	D94N	1.06	0.068
S071	S531L	D94N	0.97	0.035
S136	H526Y	D94Y	0.97	0.013
S142	S531W	D94Y	0.96	0.034
S107	H526P	D94Y	0.95	0.026
S094	S531L	D94Y	0.91	0.037
S097	H526P	D94G	1.08	0.041
S127	H526Y	D94G	1.07	0.032
S048	S531L	D94G	1.06	0.016
S138	S531W	D94G	1.03	0.022
S117	H526R	D94G	1.01	0.071

Suppl Table 4. Epistasis (ε) in mutants resistant to rifampicine (RIF) and ofloxacine (OFX).

Sample name	RIF-OFX resistance		ε	Stdev ε	95% CI *	
	rpoB SNP	*gyrA* SNP			Lower	Upper
S101	H526P	G88C	-0.004	0.070	-0.145	0.136
S118	H526R	G88C	0.059	0.060	-0.060	0.178
S128	H526Y	G88C	-0.059	0.093	-0.244	0.126
S148	S531W	G88C	-0.011	0.066	-0.143	0.120
S071	S531L	D94N	-0.001	0.037	-0.075	0.072
S099	H526P	D94N	0.263	0.075	0.112	0.414
S114	H526R	D94N	0.240	0.044	0.151	0.329
S137	H526Y	D94N	0.077	0.097	-0.117	0.270
S094	S531L	D94Y	-0.113	0.037	-0.188	-0.039
S107	H526P	D94Y	0.071	0.030	0.012	0.131
S136	H526Y	D94Y	-0.081	0.074	-0.229	0.067
S142	S531W	D94Y	0.036	0.063	-0.089	0.162
S048	S531L	D94G	0.006	0.050	-0.095	0.107
S138	S531W	D94G	-0.050	0.075	-0.200	0.099
S097	H526P	D94G	0.099	0.062	-0.025	0.223
S117	H526R	D94G	-0.003	0.093	-0.188	0.182
S127	H526Y	D94G	-0.062	0.095	-0.251	0.127

RIF; rifampicine, OFX; ofloxacine, CI; Confidence Interval