Antibiotic resistance in *Mycobacterium tuberculosis*: The role of genetic mutations in resistance conferring genes and efflux transporters.

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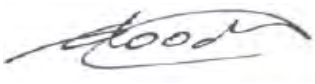
Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (Medicine) in the School of Laboratory Medicine and Medical Science, University of KwaZulu-Natal.

As the candidate's supervisor I agree to the submission of this thesis.

Signed:  Date: 06 April 2016

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For you,

Mum and Dad

Acknowledgements

To my creator, for granting me this incredible opportunity. "Ya Devi Saravabhuteshu Vidhya Rupen Sansthita, Namastasyai, Namastasyai, Namastasyai, Namo Namah…Om Shree Durgayai Namah…Jai Shree Hanuman."

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Presentations emanating from this research

1. Sequence analysis of known resistance conferring genes to fluoroquinolones & aminoglycosides amongst *Mycobacterium tuberculosis* isolates from KwaZulu-Natal, South Africa. **(3rd International Conference on Drug Discovery and Therapeutics in Dubai, United Arab Emirates)**
2. The A1401G polymorphism in the *rrs* gene of *Mycobacterium tuberculosis* correlates with high levels of resistance to Kanamycin, Amikacin & Capreomycin. **(4th FIDDSA -Federation of Infectious Diseases societies of Southern Africa Congress) – <u>Award: Best Abstract-Poster Presentation</u>**
3. Molecular analysis of the Quinolone Resistance-Determining Regions in *gyrA* and *gyrB* genes in clinical isolates of *Mycobacterium tuberculosis* from KwaZulu-Natal, South Africa. **(3RD SA TB conference 2012)**
4. Fluoroquinolone resistance in clinical isolates of *Mycobacterium tuberculosis.* **(College of Health Science Research Symposium 2012)**

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List of Abbreviations

ABC	ATP-Binding cassette family
ACP	Enoyl acyl carrier
AMIK	Amikacin
bp	Base pairs
CAP	Capreomycin
CPX	Ciprofloxacin
CTAB	Cetyltrimethyl ammonium bromide
DNA	Deoxyribonucleic acid
DS	Drug Susceptible
DST	Drug Susceptibility Testing
EMB	Ethambutol
EPI	Efflux Pump Inhibitor
ETH	Ethionamide
HIV	Human Immunodeficiency Virus
INH	Isoniazid
KAN	Kanamycin
LAM	Latin American Lineage
LIN	Linezolid

MATE	Multidrug and toxic compound extrusion
MDR	Multi Drug Resistant
MFS	Major facilitator superfamily
MGIT	Mycobacterial Growth Indicator Tube
MIC	Minimum Inhibitory Concentration
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units – Variable Number Tandem Repeats
MXF	Moxifloxacin
OADC	Oleic Acid-albumin-Dextrose-Catalase
OFX	Ofloxacin
PAS	Para-amino Salicylic Acid
PCR	Polymerase Chain Reaction
QRDR	Quinolone Resistance Determining Region
RES	Reserpine
RFLP	Restriction Fragment Length Polymorphism
RIF	Rifampicin
RNA	Ribonucleic acid
RND	Resistance Nodulation Division
RRDR	Rifampicin Resistance Determining Region

SMR Small Multidrug Resistant

STR Streptomycin

TB Tuberculosis

TDR Totally Drug Resistant

THIO Thioridazine

VERA Verapamil

WHO World Health Organization

XDR Extensively Drug Resistant

ZN Ziehl Neelsen

Abstract

Two decades after the World Health Organisation (WHO) declaration of tuberculosis (TB) as a global emergency, the disease remains a public health crisis of epic proportions. The emergence of drug resistant strains of *Mycobacterium tuberculosis*, the etiologic agent of TB, and the convergent human immunodeficiency virus (HIV) epidemic places a devastating burden on an already weakened public health care system in South Africa. Rapid and accurate detection of drug resistance to first and second line drugs to guide effective treatment of TB is central to control of the disease and in preventing further dissemination of drug resistant strains. Knowledge of the underlying resistance mechanisms driving drug resistance in *M.tuberculosis* is pivotal in the design of rapid molecular based assays and will impact of the development of novel drugs and regimens for the disease.

The manuscript in chapter 2 of this thesis, entitled **Dynamics of antimicrobial resistance in Multi-Drug and Extensively Drug resistant strains of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa,** demonstrated the diversity of the resistance mechanisms amongst the multidrug resistant (MDR) TB strains currently circulating in the KwaZulu-Natal province of South Africa by the analysis of the *rpoB*, *katG*, *inhA*, *pncA* and *embB* genes associated with resistance to key drugs used in the treatment of TB. Multiple drug resistance mechanisms in the MDR-TB isolates suggests that the strains emerged separately and acquired resistance mutations independently. The findings of this study also confirms the clonality of the XDR-TB epidemic demonstrated by the predominance of the F15/LAM4/KZN strain family and reveals that MDR-TB strains are evolving and spreading via transmission.

The manuscript in chapter 3 of this thesis, entitled **Streptomycin resistance in the F15/LAM4/KZN strain of *Mycobacterium tuberculosis* is mediated by lineage-specific**

alteration of the gidB gene, demonstrated that streptomycin (STR) resistance in the F15/LAM 4/KZN MDR and XDR-TB strains was mediated by a rare, 130bp deletion within the *gidB* gene of *M.tuberculosis* leading to a complete disruption of the gene. Classical mutations in the *rpsL* gene mediated STR resistance in the remaining strain families. Widespread STR resistance has resulted in the exclusion of the drug from current treatment regimens. The findings of this study support the decision of policymakers and cautions the application of the drug in the absence of drug susceptibility testing.

The manuscript in chapter 4 of this thesis, entitled **Moxifloxacin resistance in the F15/LAM4/KZN extensively drug-resistant strain of *Mycobacterium tuberculosis*,** demonstrated that the F15/LAM4/KZN XDR strain harboured the A90V *gyrA* mutation associated with high level ciprofloxacin (CPX) and ofloxacin (OFX) resistance and correlated with increased minimum inhibitory concentrations (MIC) for moxifloxacin (MXF). The results of this study cautions the utilization of MXF as part of empiric treatment protocols in the absence of moxifloxacin MIC data of the circulating XDR strains in an area. It also raises concerns regarding the regarding the use of moxifloxacin in KwaZulu-Natal. Furthermore, the current breakpoint defining resistance to MXF is of concern and requires revision.

The manuscript in chapter 5 of this thesis, entitled **Evaluation of Capreomycin in the treatment of the F15/LAM4/KZN extensively drug-resistant strain of *Mycobacterium tuberculosis*** demonstrated that the A1401G *rrs* mutation was the main mechanism mediating resistance to the aminoglycosides, kanamycin (KAN) and amikacin (AMIK); and to capreomycin (CAP). CAP was reintroduced into TB treatment protocols without prior drug susceptibility testing. This results of this study demonstrates high level resistance to CAP and urges careful consideration in the application of CAP the KwaZulu-Natal province. Furthermore, concerns regarding the high breakpoint value that defines CAP resistance as

compared to wild-type MICs for the drug results in misdiagnosis of resistance that results inadequate patient treatment and amplifies resistance.

The manuscript in chapter 6 of this thesis, entitled **KZN Multidrug and Extensively drug resistant strains of *Mycobacterium tuberculosis* remain susceptible to Linezolid and para-Amino salicylic Acid,** demonstrated that the mechanisms most commonly associated with resistance to the linezolid (LIN) and para-amino salicylic acid (PAS) were absent in the MDR and XDR-TB strains in this study. Mutations detected in the drug targets were lineage specific markers rather than resistance mechanisms. This study also highlights the poor understanding of resistance to these drugs and the need for further study to allow for resistance detection to be incorporated into diagnostic assays, thus prolonging the utility of these drugs.

The manuscript in chapter 7 of this thesis, entitled **Efflux mediated drug resistance in clinical isolates of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa,** demonstrated the role of efflux pumps in mediating low level resistance. The results of this study supports the hypothesis that efflux activity leads to decreased intracellular antibiotic concentrations, thereby allowing the survival of a sub-population of bacteria under the sub-inhibitory level of the antibiotic, from which resistant mutants emerge, leading to clinically significant levels of resistance. The results of this study strongly supports the application of efflux pump inhibitors as adjunctive to the current treatment protocols.

The results emanating from this thesis has contributed to the body of knowledge of drug resistance in *M.tuberculosis,* especially in the KwaZulu-Natal province of South Africa. Furthermore, the results can be used to guide treatment protocols and contributes to the future development of molecular based assays aimed at detecting resistance.

Chapter One

INTRODUCTION

1.1 Background

Tuberculosis has plagued mankind since antiquity. The discovery of *Mycobacterium tuberculosis*, the etiologic agent of TB, by German bacteriologist, Robert Koch was the cornerstone in history of the disease, which subsequently led to the development of modern chemotherapy.(1–3) However, soon after the discovery of STR, the first described antibiotic effective against *M.tuberculosis*, reports of resistance emerged. This was mainly due to the use of the drug as monotherapy. The realization that *M.tuberculosis* rapidly develops resistance when treated with only one drug, paved the path for modern combination regimens.(4)

M.tuberculosis has evolved over decades, and despite advances in the development of anti-TB drugs, the organism is now capable of causing severe untreatable forms of the disease. Multi-drug resistant (MDR-TB) strains of *M.tuberculosis* are resistant to isoniazid (INH) and rifampicin (RIF), the most effective drugs in the current multidrug regimen. MDR-TB strains that display additional resistance to the fluoroquinolones or the injectable second line drugs are classified as extensively drug resistant (XDR-TB).(5) More recently, resistance beyond XDR-TB has been described as to totally drug resistant (TDR-TB). These isolates display resistance to all available first and second line drugs available.(6)

Major advances in molecular based techniques and the whole genome sequence of *M.tuberculosis* has provided a wealth of information on the mechanisms mediating resistance to the key anti-TB drugs, leading to the development of rapid diagnostic assays with the

ability to detect *M.tuberculosis* infection and resistance to rifampicin (RIF) concurrently. (7,8) However, detection of resistance to second line drugs still depends heavily on mycobacterial culture methods in liquid or solid media. These techniques are most accurate in determining resistance to INH and RIF. Susceptibility testing for second line drugs is more complicated and less reliable. Furthermore, results from these methods are only available weeks to months later.(9) During this period, inadequate treatment allows for the development of further resistance and a population of individuals transmitting drug resistant strains of TB.(5)

Modern molecular diagnostics are not dependent on culture techniques that require the slow growth of *M.tuberculosis*. Instead, these methods depend on the association of genetic mutations in resistance conferring genes of the pathogen. The accuracy of these tests depend on the relationship of the mutation and phenotypic drug resistance in the isolate bearing the mutation.(9) This relationship is well established for RIF and INH, but remains vague in the case of subsequent drugs.(7,8) The mutations have also been reported to vary geographically and the diversity of mechanisms mediating resistance and drug targets has posed a further challenge. (10)

Prolonged treatment for drug resistant TB supports the adaptation and survival mechanisms in *M.tuberculosis*. Increased bacterial fitness resulting in accelerated transmission and the induction of efflux mechanisms has been described. Efflux pumps play a role in the extrusion of toxic substances and metabolites from the bacterial cell. However, prolonged antibiotic exposure has allowed *M.tuberculosis* to adapt efflux mechanisms to extrude vital drugs and escape the effects of the most potent drugs available for eradication of the disease.(11)

1.2 Epidemiology of Tuberculosis

According to the 2014 global TB report by the WHO, it has been estimated that 9.0 million (range, 8.6 million- 9.4 million) incident cases of TB were reported globally in 2013 and approximately 1.5 million deaths resulted from the disease. Of these, 0.4 million deaths occurred in HIV positive people and 210 000 deaths were attributed to MDR-TB. 3.5% of the incident cases and 20.5% of previously treated cases were estimated to be MDR-TB. (12)

WHO identified 22 high burden countries since 2000, accountable for 82% of all TB cases worldwide. The 6 countries recording the highest burden of disease include, India (2.0 million- 2.3 million), China (0.9 million- 1.1 million), Nigeria (340 000- 880 000), Pakistan (370 000- 650 000), Indonesia (410 000- 520 000) and South Africa (410 000- 520 000). (12)

South Africa, together with Indonesia, ranks 5th amongst the 22 high TB burden countries: 1.8% of the incident cases and 6.7% of previously treated cases are MDR-TB. The estimated epidemiological burden of TB in SA, includes a prevalence of 715 cases per 100 000 population and incidence of 860 per 100 000 population. (12)

1.3 Diagnosis of Tuberculosis & Drug Susceptibility Testing

Early diagnosis and drug susceptibility testing is central to the management of TB. Diagnosis of TB is still reliant on sputum smear microscopy and culture based technology.(13) Differential staining of mycobacteria is based on the acid fast staining of the organism, resulting from the mycolic acid structure of the bacterial cell wall. Sputum smear microscopy using the Ziehl-Neelsen (ZN) staining technique is applied to the detection of *M.tuberculosis*. The specimen is stained with phenol fuschin, followed by decolourisation with acid alcohol. Methylene blue or malachite green is applied as a counterstain. Auramine-rhodamine staining is an alternate to the ZN stain. This technique employs potassium permanganate as a counterstain following decolourisation with acid alcohol. The fluorescence of the bacteria is visible under UV illumination. (14)

Cultivation of *M.tuberculosis* is a sensitive and specific method for the diagnosis of TB. The basic types of culture media include; Lowenstein-Jensen (LJ), an egg-based medium; Middlebrook 7H10/11, agar-based media and liquid media such as Middlebrook 7H9. (15) Conventional culture based methods requires 3-6 weeks for growth and isolation. (16) Culture of *M.tuberculosis* in liquid media is the current reference method for the diagnosis of *M.tuberculosis.* This method requires adequate digestion and decontamination of samples prior to cultivation in media. Liquid media is associated with higher recovery of tubercle bacilli and shorter time to detection.(5) Colorimetric systems such as the BACTEC (Johnson Laboratories) and BACTEC-MGIT (Johnson Laboratories) are culture based assays that have improved the time to detection of *M.tuberculosis.*(16)

The recent Xpert MDR/RIF (Cepheid) assay is a polymerase chain reaction (PCR) based assay that utilizes molecular beacon technology. The test has a significantly higher sensitivity that sputum smear microscopy. The test is a fully automated, rapid diagnostic assay that simultaneously diagnoses TB and detects RIF resistance. This test allows for the rapid initiation of treatment for drug resistant TB pending confirmation of drug susceptibility testing. (17)

Drug susceptibility testing on solid media remains the gold standard for phenotypic determination of drug resistance. This is mainly due to its standardisation on solid media and reproducibility. This technique involves incorporating the drug into the media, thereafter inoculating the sputum specimen directly onto the medium or indirectly by inoculating the media with culture isolated from the sputum specimen. The 1% proportion method is the most common technique used to determine susceptibility. Microplate assays have been tested but their reproducibility remains unreliable, especially in the case of second line drugs.(18–21)

1.4 Molecular Typing of *M.tuberculosis*

1.4.1 Typing Methods

M.tuberculosis was initially believed to be a highly homogenous organism and variations in disease presentation were mediated by the host immune response. Modern molecular techniques have increased our capacity to distinguish between strains of *M.tuberculosis.* Restriction fragment length polymorphism (RFLP) typing is the most common technique adopted to differentiate between *M.tuberculosis* strains. The IS*6110* specific insertion sequence displays characteristic binding patterns that can be used to compare isolates. (22) PCR based typing methods such as mycobacterial interspersed repetitive units-variable number of tandem repeats (MIRU-VNTR), spacer oligonucleotide typing (spoliogotyping), strain specific PCR amplification have also been applied to distinguish between strain types. More recently, whole genome sequencing technology allows for a greater degree of discrimination of *M.tuberculosis* isolates.(23)

1.4.2 Circulating Strains

To date, seven major strain families have been described globally and are further divided into subfamilies. These include the East African-Indian (EAI), Haarlem (H), Central and Middle Eastern Asia (CAS), European Family X, Default Family T, W-Beijing and Latino-American and Mediterranean (LAM). In South Africa, strain families have been reported to vary geographically. The TB epidemic in the Western Cape province of South Africa is characterised by 4 strain types, the Beijing, F11, F28 and DRF 150. The Beijing strain family is the most prevalent strain family, endemic to China and neighbouring countries of Mongolia, South Korea and Thailand. This strain type is identified by spoligotype signature that lacks spacers 1 to 34 and the IS*6110* RFLP signature is the inverted IS*6110*copy within the DR region. The F11 strain family is characterised by the lack of spoliogotype spacers 9 to

11, 21 to 24 and 33 to 36 and the presence of 11 to 19 IS*6110* RFLP bands that serves as unique markers for identification of this family. The F28 family found in South Africa is identical to S strain family endemic to Sicily and Sardina. The DRF 150 genotype was identified in an outbreak of an emerging resistant non-Beijing strain. This genotype is characterised by 5 IS 6110 insertions and high level resistance to INH, RIF and STR. In KwaZulu-Natal, the F15/LAM4/KZN, Beijing, F28 and F11 families have been described. The F15/LAM4/KZN and Beijing are the predominant strains driving the drug resistant epidemic in the province. (24) The F15/LAM4/KZN strain has evolved from 1995 from a single phenotype to XDR-TB. (25) This strain family was also attributed as the strain responsible for the 2006 outbreak of XDR-TB in the province.(26) The F15 family belong to the major Latino-American and Mediterranean family and correspond to the LAM4 sub-family. The spoligotype pattern lacks spacers 21-24, 33-36 and 40 and has a unique RFLP pattern. Spoligotyping of XDR-TB strains from 7 provinces in South Africa revealed high genotypic diversity, which included 7 internationally recognised strain families (Beijing, LAM, EAI, the T, H, S and X3 families). The Beijing strain family represented majority of the isolates.

1.5 Treatment of Tuberculosis

1.5.1 Historical overview of tuberculosis treatmentThe era of antibiotic treatment of TB began in 1946, with the discovery of STR, the first antibiotic effective against *M.tuberculosis.* However, its ability to consistently cure TB was questioned when patients were relapsing after 3 months of STR monotherapy. This was confirmed by the discovery that *M.tuberculosis* rapidly develops resistance when treated with only one drug. (27–30)The next decade saw the development of multiple drugs with alternate mechanisms of action against *M.tuberculosis,* including INH, PAS, KAN and cycloserine. This gave rise to combination therapy of 18 months duration. The introduction of RIF in 1963, was a major

breakthrough in treatment, as it reduced treatment duration to 9 months. Empiric observation and the subsequent development of drugs with activity against *M.tuberculosis* over the next 4 decades gave rise to the current 4 drug regimen. The use of combination therapy reduced the occurrence of resistance and became a standard of care for TB therapy.(4,31) A further rationale for combination therapy is based on the premise that 3 population subtypes characterize cavitary TB disease; bacilli in the log phase of growth and slow replicating bacteria in hypoxic and acidic conditions. Multidrug regimens combining INH, RIF and PZA actively inhibits each of the population subtypes.(31,32)

1.5.2 Current Treatment

Drug Susceptible Treatment

Current treatment guidelines recommend a two-phase treatment plan for 6 months for drug susceptible (DS) TB. The first phase of 2 months includes a combination of INH, RIF, ethambutol (EMB) and pyrazinamide (PZA), known as the intensive phase. The second phase of 4 months is treatment with INH and RIF, known as the continuation phase. This regimen is currently applied to the treatment of pulmonary TB and most forms of extra pulmonary TB. Guidelines further differentiate between new cases (treatment naïve) and previously treated cases (previously treated for at least 1 month). Patients previously treated with anti-TB drugs are managed with caution due to the risk of inducing drug resistance.(33,34) Figure 1.1 depicts the clinical management algorithm for TB treatment.(34)

Drug Resistant Treatment

Multidrug Resistant TB

Tuberculosis programmes adopt a combination of standardised and individual based treatment regimens for the management of drug resistant TB. The application of standardised regimens for MDR-TB cases is confirmed using drug susceptibility testing for the patient or

generalised data representing a particular patient population. Individualised regimens utilize patient specific data, including prior anti-TB drug use and drug susceptibility testing to tailor a drug regimen. In the absence of, or limited drug susceptibility testing data, empirical regimens are applied.(5) Table 1.1 summarises the stepwise selection of drugs applied to the management of drug resistant TB.(34)

The recommended regimen for MDR-TB should comprise of at least 4 active second line drugs (Table 1.2 shows WHO grouping of anti-TB drugs), a fluoroquinolone (moxifloxacin (MXF), gatifloxacin or levofloxacin); an injectable aminoglycoside (CAP, AMIK, KAN); a first line drug to which the isolate is susceptible and one group 4 drug (cycloserine, PAS, terizidone, protionamide, or ethionamide). Drugs from group 5 are used if 4 active drugs are not available from the former groups. The intensive phase of treatment, using the injectable aminoglycosides is a minimum of 8 months, followed by a continuation phase of treatment of 12-18 months. WHO recommends that treatment should be guided in accordance to culture conversion and should generally continue for 18 months after the first negative sputum smear. (5)

Extensively Drug Resistant TB

XDR-TB is associated with poor treatment outcomes and significantly higher mortality when compared to MDR-TB, especially in people co-infected with HIV. Clinical data on the management of XDR-TB remains limited. (5) Whilst no defined regimen has been shown to be successful in the treatment of XDR-TB, reports suggest the use of at least 6 drugs in the intensive phase of treatment and 4 drugs in the continuation phase of treatment. (5)

The selection of these drugs, based on WHO recommendations includes the use of PZA or a first line drug that is effective against the infecting strain; a group 2 injectable aminoglycoside antibiotic, if the strain is susceptible to these drugs; a group 3 new generation

fluoroquinolone such as MXF or gatifloxacin. All the drugs belonging to group 4 may be used, only if these have not been used extensively in a previous treatment regime. Two or more drugs from group 5 may be used, bedaquiline and delamanid are recommended. Drugs that are approved for compassionate therapy may also be applied to the management of XDR-TB. High dose INH is only recommended for treatment in strains with low-level INH resistance or no documented genetic alteration in the *katG* gene.(5)

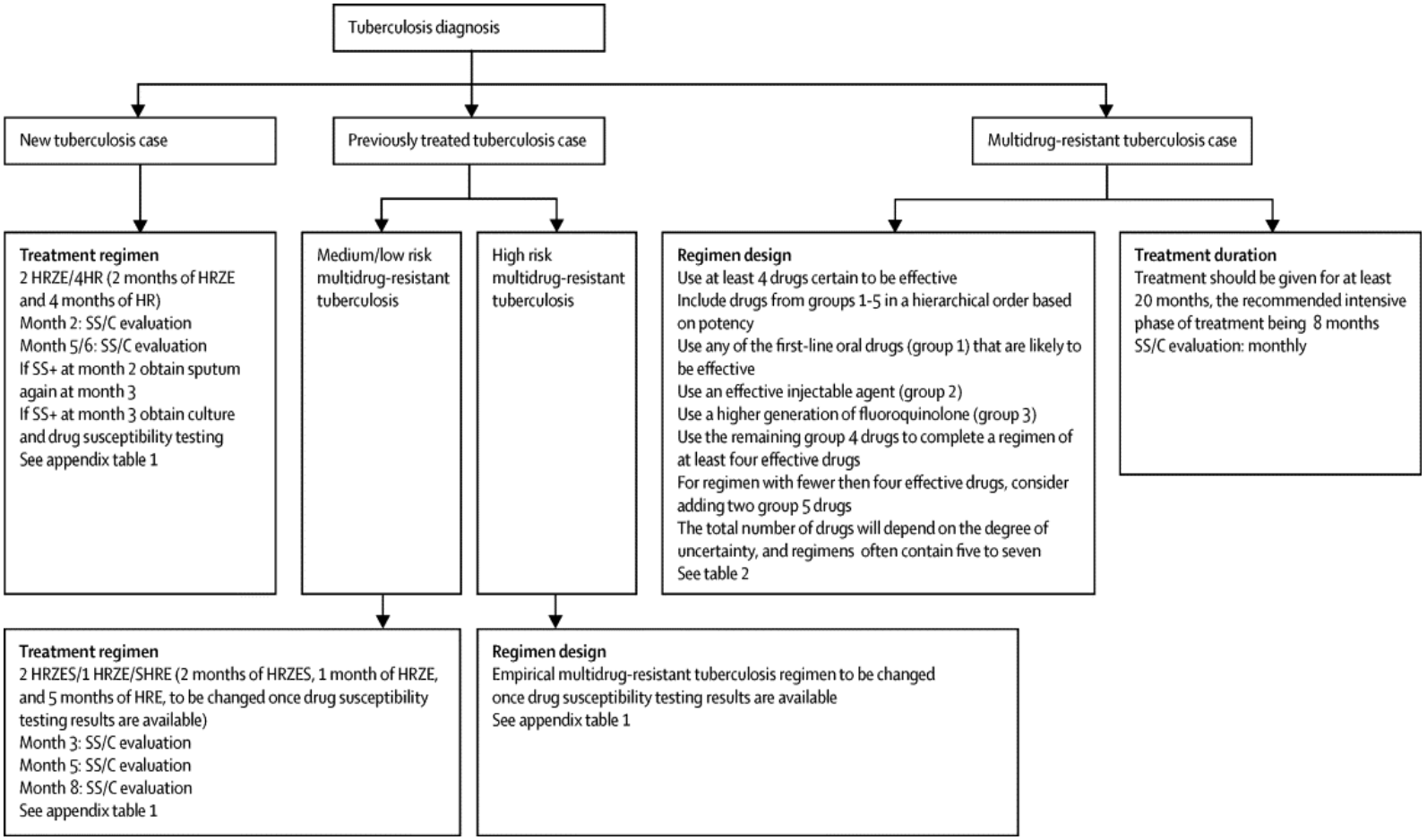

Figure 1.1: Treatment regimens and monitoring in new, previously treated, and MDR tuberculosis cases- Reproduced with permission from Lancet Respiratory Diseases. H=isoniazid. R=rifampicin. Z=pyrazinamide. E=ethambutol. S=streptomycin. SS/C=sputum smear/culture. SS+=sputum smear positive.

Table 1.1: WHO-recommended stepwise approach to design a regimen for multidrug-resistant tuberculosis. Reproduced with permission from Lancet Respiratory Medicine.(34)

	Actions needed	Drug to consider	Notes
Step 1	Choose an injectable (group 2) drug based on drug-susceptibility testing and treatment history	Kanamycin; amikacin; capreomycin	Streptomycin is generally not used because of high rates of resistance in patients with MDR disease
Step 2	Choose a higher generation of fluoroquinolone (group 3)	Levofloxacin; moxifloxacin	If levofloxacin (or ofloxacin) resistance is documented, use moxifloxacin; avoid moxifloxacin if possible when using bedaquiline
Step 3	Add two or more group 4 drugs until there are at least four second-line antituberculosis drugs likely to be effective	Cycloserine/terizidone; para-aminosalicylic acid; ethionamide/protionamide	Ethionamide and protionamide are considered the most effective group 4 drugs; consider treatment history side-effect profile and cost; drug-susceptibility testing is not considered reliable for the drugs in this group
Step 4	Add group 1 drugs	Pyrazinamide; ethambutol	Pyrazinamide is routinely added in most regimens; ethambutol can be added if the criteria for an effective drug are met*; if susceptibility to isoniazid is unknown or pending it can be added to the regimen until drug-susceptibility testing results become available
Step 5	Consider adding group 5 drugs if four second-line antituberculosis drugs are not likely to be effective from groups 2–4	Bedaquiline; linezolid; clofazimine; amoxicillin/clavulanate; imipenem and cilastatin plus clavulanate; meropenem plus clavulanate; high-dose isoniazid; clarithromycin; thioacetazone	If drugs are needed from this group, two or more should be added; drug-susceptibility testing is not standardised for the drugs in this group

**An anti-tuberculosis drug is thought likely to be effective when the drug has not been used in a regimen that failed to cure the patient; drug-susceptibility testing done on the patient's M. tuberculosis strain shows that it is susceptible to the drug (drug-susceptibility testing for isoniazid, rifampicin, and group 2 and 3 drugs is deemed reliable; drug-susceptibility testing for all other drugs is judged not reliable enough for individual patient management); no known resistance to drugs with high cross-resistance; no known close contacts with resistance to the drug; and drug resistance surveys show that resistance is rare to the drug in patients with similar tuberculosis history. This final criterion is relevant in the absence of drug-susceptibility testing or for drugs in which individual drug-susceptibility testing is not reliable. Information from all five criteria is not always possible to be ascertained. Therefore, clinical judgment is often necessary on whether to count a drug as likely to be effective.*

Table 1.2: Classification of drugs used to treat drug susceptible and drug resistant tuberculosis. According to the grouping of the World Health Organisation(5)

Group	Drugs
Group 1: First Line-Oral Antibiotics	Isoniazid Rifampicin Ethambutol Pyrazinamide
Group 2: Second Line – Injectable Aminoglycosides	Kanamycin Amikacin Capreomycin Streptomycin
Group 3: Second Line – Oral and Injectable Fluoroquinolones	Levofloxacin Moxifloxacin Gatifloxacin
Group 4:Oral	Ethionamide Prothionamide Cycloserine Terizidone Para-amino salicylic acid
Group 5: Third Line Unclear efficacy/ undefined roles	Bedaquiline Delamanid Clofazamine Amoxicillin/Clavulanate Clarithromycin Linezolid Thioacetazone Imipenem/cliastatin Meropenem High dose of isoniazid

1.5.3 Future Treatment

New treatment regimens for the treatment of TB include repurposing of older TB drugs in new combinations as well as the introduction of new drugs.. Repurposed drugs include the use of LIN, meropenem/clavulanic acid and co-trimoxazole. Meropenenem/clavulanic acid has shown promising activity against *M.tuberculosis*, in combination with LIN. Co-trimoxazole has also been indicated for treating MDR-TB.(4,34)

High-dose rifapentine is currently under investigation to replace RIF and potentially reduce the current treatment duration. Rifapentine demonstrates similar activity to RIF but has a longer half-life that allows for better exposure. Furthermore, studies have revealed that the optimal dosage for RIF has never been established, thus leading to trials applying higher dosages of RIF. However, a major shortfall of rifamycin antibiotics is its interaction with antiretroviral therapy in HIV co-infected patients.(4,34)

Bedaquiline, a new antibiotic, is a diarylquinoline antibiotic with proven efficacy against drug susceptible and resistant strains of *M.tuberculosis*. After completion of phase IIb clinical trials the drug received conditional approval for the treatment of MDR TB. The drug is currently applied on the basis of compassionate therapy. This drug has been associated with toxicities and has not yet been applied to a phase III trial, due to the cardiotoxicity associated with the drug. The use of this drug is limited to patients with documented resistance to several other drugs. Reports of resistance to the drug have already emerged despite its recent introduction. (4,34)

Delamanid, a nitro-dihydro-imidazooxazole is also a new drug introduced to TB treatment. The drug is currently under evaluation in phase III clinical trials for the treatment of MDR TB. This drug has demonstrated efficacy against drug susceptible and resistant strains of *M.tuberculosis*, with its early bactericidal activity comparable to RIF.(4,34)

1.6 Mechanisms of Drug resistance in *Mycobacterium tuberculosis*

1.6.1 Genetic Resistance Mechanisms

Antibiotic resistance in *M.tuberculosis* occurs by the acquisition of mutations in genes that code for drug targets or those involved in drug activation, allowing for the selection of resistant strains mediated by sub-optimal therapy. Unlike other bacteria, resistance is not acquired via horizontal gene transfer by mobile genetic elements. Whilst no single mutation defines the MDR phenotype, an accumulation of mutations leads to the resistance of multiple drugs. It is also postulated that the presence of classical resistance mutations maybe the gateway to resistance to other drugs.(7,35)

There are 2 mechanisms resulting in drug resistant TB. (I) Primary resistance occurs by infection with an already drug resistant strain of *M. tuberculosis.* (II) Acquired (secondary) resistance occurs through inadequate treatment or poor treatment compliance that allows for the selection of drug resistant mutants within a patient's body ie: after infection.(5,7,35)

1.6.1.1 First Line Anti-TB Drugs

Isoniazid

INH was first introduced as an anti-TB drug in 1952 and, together with RIF forms the basis of the short-course regimen currently applied to the treatment of TB. INH is effective against metabolically active, replicating bacilli. INH is a pro-drug which is activated by the catalase/peroxidase enzyme, encoded by the *katG* gene. Once activated, INH inhibits mycolic acid synthesis via the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by the *inhA* gene. (7,8) The molecular basis of INH resistance is mediated by mutations in the *katG, inhA* gene or within the promoter region of the *inhA* gene. The most common resistance mechanism has been identified as the *katG* S315T mutation which leads to an inefficient INH-NAD product inhibiting the antimicrobial action of INH. This mechanism is associated

with high-level INH resistance in MDR isolates. (36–39) Mutations of the *inhA* promoter region, the most common at position, -15, results in an overexpression of *inhA*. This mechanism is associated with low-level resistance in INH mono-resistant isolates and has been implicated in cross resistance to a structural analogue, ethionamide. Mutations in the active region of the *inhA* gene result in a decreased affinity of the INH-NAD product. Such mutations are less frequent. (40,41) A recent study reported that mutations occurring in the *inhA* regulatory region and coding region, resulted in high-level INH resistance and cross resistance to ethionamide. (42) Mutations in the *dfrA* gene have been recently implicated in resistance to INH. The 4R isomer of the INH-NADH product inhibits dihydrofolate reductase, encoded by *dfrA.* However, studies have failed to demonstrate a correlation between mutations in the *dfrA* and INH resistance.(43) Mutations in the promoter region of the *ahpC* gene were proposed as proxy markers for INH resistance. The *ahpC* gene in *M.tuberculosis* codes for an alkyl hydroperoxidase reductase enzyme responsible for resistance to reactive oxygen and nitrogen derivatives. Further analysis of such mutations revealed that this is compensatory mechanism for the reduction or loss of activity of the catalase-peroxidase system and does not confer INH resistance.(44) Studies have also reported mutations in the *kasA, oxyR-ahpC* and *furA-katG* in INH resistant isolates of *M.tuberculosis*. However, their exact role in mediating INH resistance is yet to be demonstrated. (45,46) More recently, a silent mutation in the *mabA* gene, resulting in the upregulation of *inhA* resulted in INH resistance.(47)

Rifampicin

RIF, a rifamycin derivative, was first introduced as an anti-TB drug in1972. RIF is one of the most effective anti-TB drugs, effective against actively metabolizing and slow-metabolizing bacilli, making the drug a key component of the current first line treatment regimen applied to the treatment of TB.(7,8,32,48) In *M. tuberculosis*, RIF binds to the β sub-unit of the RNA

polymerase, resulting in the inhibition of elongation of messenger RNA. Resistance to RIF is mediated by mutations clustered in codons 507-533 of the gene coding for the RNA polymerase β sub-unit, *rpoB.* This region is known as the RIF resistance-determining region (RRDR), the target of modern molecular based assays. Codons 526 and 531 bear the most common mutations associated with RIF resistance.(7,8,49–52) Mutations outside the RRDR have been reported in RIF resistant isolates. (53) Studies have also demonstrated a lack of alteration in the *rpoB* gene in a fraction of RIF resistant isolates, suggesting an alternate mechanisms of RIF resistance.(53) RIF mono-resistance is rare as RIF resistance occurs in conjunction with resistance to other drug, most commonly INH, making RIF targets a surrogate marker of the MDR phenotype. (54)Recent, whole genome sequence analysis demonstrated mutations in the *rpoA* and *rpoC* genes which encode the α and β' subunits of the RNA polymerase as compensatory mechanisms in isolating bearing mutations in the *rpoB* gene. These mutations are associated with increased fitness and transmissibility of resistant strains.(11)

Pyrazinamide

PZA, a nicotinamide analogue, was first introduced as an anti-TB drug in 1952. PZA significantly reduced the duration of TB treatment to six months. A vital characteristic of PZA is its ability to inhibit semi-dormant bacilli located in acidic environments such as that of TB lesions. (32)PZA not only constitutes a part of the standard first line regimen to treat TB but is also key component of all current regimens undergoing evaluation in phase II and III clinical drug trials for the treatment of drug susceptible and resistant TB. (55)PZA is a pro-drug which is activated by the pyrazinamidase/nicotinamidase (PZase) enzyme, encoded by the *pncA* gene. (56,57)Once activated, pyrazinoic acid disrupts the bacterial membrane energetics thereby inhibiting membrane transport. PZA enters the bacterial cell by passive diffusion and is then converted into pyrazinoic acid. The pyrazinoic acid is the pumped out of

the bacterial cell by a weak efflux mechanism. In an acidic environment, the pyrazinoic acid is protonated allowing for reabsorption into the cell, resulting in cellular damage. (58) Pyrazinoic acid and its n-propyl ester have also been implicated in inhibition of fatty acid synthase I in *M.tuberculosis.* (59,60) It has been recently proposed that pyrazinoic acid is involved in inhibiting trans-translation in *M.tuberculosis*. Isolates lacking alteration in the *pncA* gene were reported to have mutations in the *rpsA* (ribosomal protein I) gene. Overexpression of *rpsA* has also been implicated in increased resistance to PZA.(61) However, there was no clear demonstration that mutations in *rpsA* was linked to PZA resistance.(62–64) Mutations in the *pncA* gene and its promoter region remains the most common mechanism mediating PZA resistance.(57) The mutations identified within this gene are diverse, with 600 unique mutations in 400 positions reported to date.(55) A small proportion of resistant isolates lack mutations in the *pncA* gene, suggesting an alternate mechanism of resistance exists.(65)

Ethambutol

EMB was first introduced as an anti-TB drug in 1966 and remains a part of the current first line regimen applied to the treatment of TB. EMB is active against actively multiplying bacilli, disrupting the biosynthesis of the arabinogalactan in the cell wall. The *embCAB* operon encodes the mycobacterial arabinosyl transferase enzyme. Resistance to EMB is mediated via mutations in the *embB* gene.(66,67) Alteration on codon 306 is the most prevalent mechanism reported.(68,69) It was further reported that this mutation predisposes the isolate to develop resistance to other drugs and is not necessarily involved in EMB resistance.(70) Allelic exchange experiments have demonstrated that only certain amino acid substitutions led to EMB resistance.(71) Studies have shown that mutations in the decaprenylphosphoryl-B-D-arabinose (DPA) biosynthetic and utilization pathway genes (Rv3806c and Rv379), that occur simultaneously with mutations in *embB* and *embC* result in

a variable MIC range for EMB. This depends on the type of mutation that is present. Furthermore, this implies that the *embB*306 mutation results in varying degrees of EMB resistance but does not cause high-level EMB resistance on its own. (72)Resistance in approximately 30% of EMB resistant isolates lack alteration in the *embB* gene, suggesting an alternate mechanism of resistance.(7,8)

Streptomycin

STR, an aminocyclotol antibiotic was the first drug to be applied to the treatment of TB in 1942. Due to the initial application of the drug as TB monotherapy, resistance rapidly emerged. (73)STR is active against slow growing bacilli and acts by irreversibly binding to the ribosomal protein S12 and 16S rRNA, which are the components of the 30S subunit of the bacterial ribosome. Through this interaction, STR blocks translation thereby inhibiting protein synthesis. (74,75)The main mechanism of resistance to STR is believed to be mediated via mutations in the *rpsL* and *rrs* genes, encoding the ribosomal protein S12 and the 16S rRNA, respectively, accounting for approximately 60-70% of STR resistance.(76) Recently, reports of mutations in the *gidB* gene, encoding a 7- methylguanosine methyltransferase specific for methylation of the G527 in loop of the 16S rRNA, has been implicated in low-level STR resistance.(77–80) Whole genome analysis has also demonstrated a 130bp deletion within the *gidB* gene mediating STR resistance.(81)

1.6.1.2 Second Line Anti-TB Drugs

Fluoroquinolones

Fluoroquinolones are potent bactericidal antibiotics currently applied as second line treatment for drug resistant tuberculosis. CIP and OFX, are older generation antibiotics, derivative of nalidixic acid.(82) New generation fluoroquinolones, MFX and gatifloxacin are currently under evaluation in clinical trials to be applied in first line regimens in an attempt to shorten

the duration of treatment. (4,34,83,84) This class of antibiotics target the DNA gyrase enzyme, thereby preventing transcription during cell replication. DNA gyrases are encoded for by the *gyrA* and *gyrB* genes. Resistance to the fluoroquinolones has been linked to mutations occurring in a conserved region known as the 'quinolone resistance-determining region' (QRDR) in the *gyrA* and *gyrB* genes.(7,85–87) Fluoroquinolone resistant strains of *M.tuberculosis* most frequently display mutations on codons 90, 91 and 94 of the *gyrA* gene. Mutations on codons 74, 88 and 91 have also been associated with fluoroquinolone resistance.(88–90) It has been reported that clinically significant resistance to ciprofloxacin and ofloxacin (MIC of 2µg/ml) is conferred by a single gyrase mutation, while double mutations in the *gyrA* or concomitant *gyrA* and *gyrB* mutations result in high-MICs. (90)A mutation detected on codon 95 of the *gyrA* is natural polymorphism that has no role in mediating fluoroquinolone resistance. (91) The complexity of fluoroquinolone resistance in *M.tuberculosis* has been demonstrated by the hyper susceptibility induced by the presence of mutations on codon 80 of the *gyrA* gene, especially when occurring with other resistance conferring mutations.(92) Efflux mechanisms have also been reported to mediate fluoroquinolone resistance.(93) Mutations in the *gyrB* gene are rare.(7)

Kanamycin, Amikacin, Capreomycin

The aminoglycosides KAN and AMIK and the cyclic polypeptide, CAP are second line injectable agents currently applied to the treatment of drug resistant tuberculosis. Although these belong to different classes of antibiotics, they all exert their effect via the same target. (7,8)All three drugs are protein synthesis inhibitors, which act by binding to the bacterial ribosome resulting in a modification of the 16S rRNA structure. High level resistance to all three drugs has been associated with mutations in the 1400bp region of the *rrs* gene and additional resistance to CAP has been associated with polymorphisms of the *tlyA* gene. This gene codes for rRNA methyltransferase required for 2'-O-methylation of ribose in rRNA.

(77,94)The A to G polymorphism at position 1401 of the *rrs* gene is the most common molecular mechanism of resistance to all three drugs associated with approximately 70-80% of CAP and AMIK resistance and 60% of KAN resistance, globally.(9) A recent study reported increased fitness in clinical isolates bearing the *rrs* A1401G mutation. This was demonstrated by the difference in MIC between the laboratory engineered strains and clinical isolates with the same mutation. This increased fitness is thought to occur due to the presence of compensatory mutations that restore bacterial fitness.(95) Cross-resistance between KAN, AMIK and CAP has also been reported. Each of the drugs acts by inhibiting translation and therefore cross resistance between them is likely to occur. Full cross-resistance between KAN and AMIK was initially assumed, however, other studies have demonstrated discordant resistance patterns between these two agents.(96) It has also been reported that CAP resistance varies according to the level of resistance to KAN, and high-level resistance to KAN was associated with cross-resistance to CAP.(97) More recently, mutations reported in the promoter region of the *eis* gene resulted in low level resistance to kanamycin. The *eis* gene encodes an aminoglycoside acetyltransferase. Polymorphisms at positions -10 and -35 of this gene resulted in an over expression of its protein product and low level kanamycin resistance. A study reported that 80% of the clinical isolates with low level resistance to KAN had genetic alterations in the promoter region of this gene.(98,99)

Ethionamide

Ethionamide (ETH), a derivative of isonicotinic acid is a structural analogue of isoniazid. ETH is a pro-drug which is activated by the mono-oxygenase enzyme, encoded by the *ethA* gene. Once activated, ETH inhibits mycolic acid synthesis during cell wall biosynthesis by inhibiting the enoyl-ACP reductase enzyme. Regulatory control of the *ethA* gene occurs via the transcriptional repressor, EthR.(100) Resistance to ETH is mediated by mutations in the *etaA/ethA*, *ethR* and *inhA* genes. Mutations in the *inhA* gene mediates co- resistance to both

INH and ETH.(101,102) A study has recently demonstrated the role of the *mshA* gene, encoding an enzyme essential to mycothiol biosynthesis as a target for ETH resistance using spontaneous INH- and ETH resistant mutants.(103)

Para-Amino Salicyclic Acid

PAS, an analogue of para-amino benzoic acid, was one of the first antibiotics used in the treatment of TB together with INH and STR. (28)PAS now forms a part of second line treatment regimens applied to the treatment of drug resistant TB. The mechanism of PAS resistance was only very recently elucidated. It is suggested that that PAS competes with p-amino benzoic acid for the enzyme dihydropteroate synthase, inhibiting folate synthesis. The main mechanism mediating PAS resistance has been identified as mutations occurring in the *thyA* gene, accounting for 40% of PAS resistance. (104,105) A recent study demonstrated that mutations in the *folC*, encoding dihydrofolate synthase conferred resistance in clinical isolates resistant to the drug. (106) The T202A mutation initially associated with PAS resistance was found to be a phylogenetic marker associated with the Latin American (LAM) strain families rather that resistance to PAS. Mechanisms to fully elucidate PAS resistance are lacking.(107)

Linezolid

LIN, an oxazolidinone, is the first antibiotic in its class to be approved for the treatment of TB.(108) Recent studies have found that treatment outcomes with regimens containing LIN for complicated cases of MDR-TB are equal to or better than those reported for uncomplicated MDR-TB and better than those reported among patients treated for XDR-TB.(109) LIN acts by binding to the V domain of the 50S ribosomal subunit, thereby inhibiting an early step in protein synthesis. (110) Resistance is mediated by mutations in the 23 S rRNA (*rrl*) gene. A study reported 1.9% of LIN resistant isolates in a cohort of 210

MDR isolates.(110) Analysis of *in vitro* selected mutants with mutations in the 23 S rRNA gene were associated with MICs of 16-32 mg/L while no mutations were detected in isolates with an MIC of 4-8 mg/L or in the LIN susceptible strains.(111) More recently, advances in sequencing technology identified mutations in the *rplC* gene, encoding the 50S ribosomal L3 protein, in in vitro selected mutants and clinical isolates.(112)

1.6.2 Efflux Mediated Resistance

Efflux mechanisms are now recognised as a significant factor in antibiotic drug resistance of *M.tuberculosis*. These efflux systems are involved in expelling antibiotics from the bacterial cell, thereby allowing for the acquisition of resistance mutations in the bacterial genome. *M.tuberculosis* presents with one of the largest number of putative efflux pumps with 148 genes coding for membrane transport proteins within its 4.4KB genome. The contribution of these efflux systems in acquiring multidrug resistance in *M.tuberculosis* has been demonstrated by numerous studies. (113,114)

1.6.2.1 Mechanisms of Efflux Mediated Resistance

The overexpression of efflux pumps is believed to mediate the build-up of resistance mutations, which confers high-level drug resistance allowing for *M.tuberculosis* to survive and persist at clinically relevant drug concentrations. The ability of the efflux pumps to extrude a diversity of compounds allows them to expel multiple drugs leading to the MDR phenotype. (113,114)

Efflux pumps have been classified into five superfamilies; the ATP-binding cassette (ABC), major facilitator super-family (MFS), resistance nodulation division (RND), small multidrug resistance (SMR) and multidrug and toxic-compound extrusion (MATE). The ABC superfamily is a primary transporter which utilizes ATP to pump to extrude drugs. The

remaining superfamilies are secondary transporters that utilize the trans-membrane proton motive force as a source of energy. (113,114)

The ABC superfamily comprises of efflux systems responsible for the uptake and efflux of various compounds including; drugs, sugars, amino acids carboxylates and peptides. These transporters have been associated with resistance to INH, RIF, STR, EMB and the fluoroquinolone antibiotics. A significant ABC efflux mechanism encoded by the genes *Rv2686c-Rv2687c-Rv2688c* has been associated with an eight fold MIC increase for CIP when the genes are expressed as an operon in *M.tuberculosis.*(113–115)

The MFS superfamily has been associated with resistance to almost all anti-tuberculosis drugs including INH, RIF, STR, EMB, the fluoroquinolones, aminoglycosides and ethionamide. The expression of MFS superfamily transporters operates under the regulatory control of inducer and repressor mechanisms that modulate gene expression. Significant MFS efflux mechanisms include the P55 pump, a multidrug efflux pump in *M.tuberculosis* encoded by *Rv1410c*. (113,114,116–118)

Efflux pumps of the RND family are predominant in Gram-negative bacteria. A significant RND efflux mechanism in *M.tuberculosis*, encoded by the *Mmpl7* gene, results in high level resistance to isoniazid.This phenomenon was reversed in the presence of efflux pump inhibitor (EPI) compounds.(113,114,119)

Transporters belonging to the MATE superfamily have not been reported in *M.tuberculosis* and no significant SMR efflux mechanisms have been demonstrated in *M.tuberculosis*.(113,114,120)

1.6.2.2 Efflux Pump Inhibitors

Efflux pump inhibitors (EPIs) are compounds capable of restoring the activity of antibiotics independent of the level of resistance. The inhibitor-antibiotic combination decreases the

concentration of antibiotics expelled by efflux pumps, thus decreasing the MIC of the antibiotic. The use of EPIs has been considered as an adjuvant in TB treatment and has the potential to reduce the duration of TB treatment. (11,114)

Thioridazine

Thioridazine (THIO), a phenothiazine compound used as an antipsychotic drug has been explored in patients infected with *M.tuberculosis*, with encouraging outcomes. The application of THIO as an adjuvant in TB treatment demonstrated cure in 10/12 patients with XDR TB and is currently applied to the treatment of drug resistant TB on the basis of compassionate use. (121)

The phenothiazines are calcium channel blockers that prevent calcium binding to calcium dependent enzymes. It has been demonstrated that THIO enhances the killing on newly phagocytosed bacteria and MDR and XDR TB. THIO acts by inhibiting the transport of calcium and potassium from the phagolyosome to the cytoplasm, resulting in an increased concentration of H^+ to activate hydrolases that lead to the degradation of phagocytosed bacteria.(122,123)

The utility of THIO in the management of newly diagnosed TB is reinforced by observations that this agent enhances the *in vitro* activity of RIF against resistant strains of *M.tuberculosis.* RIF resistance decreases significantly in the presence of EPIs. These observations suggest that the use of THIO may permit a reduction in the current dosage of anti-tuberculosis drugs and may potentially restore the activity of the current standard treatment regimen. Another prospective benefit of THIO is that genetic mutations that result in drug resistance are not expected as calmodulin, the calcium transport protein to which THIO binds and inactivates, is an imperative cell wall component of mycobacteria.(121–126)

Verapamil

Verapamil (VERA) is a calcium channel blocker currently applied to various disorders that include angina, hypertension and cardiac disorders. It has been demonstrated that VERA has the greatest ability to inhibit efflux of ethidium bromide as compared to THIO. VERA is also known to interfere with the proton motive force which is the energy source of most efflux pumps. VERA has demonstrated a significant reduction in the MICs for INH of up to 4 folds in *M.tuberculosis* isolates with the resulting MIC in the presence of verapamil similar to the corresponding wild type and INH susceptible isolates.(125,126)

Reserpine

Reserpine (RES), like verapamil is a calcium channel blocker. RES is a natural plant metabolite that was utilized as a hypertensive drug. RES demonstrated activity against the pyrazinoic acid pump, increasing the susceptibilities of *M.tuberculosis* isolates to PZA. It has also demonstrated activity in decreasing MICs of LIN and EMB. Due to the carcinogenicity associated with the compound, it is no longer utilised in treatment protocols.(126)

1.6.3 Alternate Mechanisms of Resistance

DNA Repair Systems

The role of DNA repair systems in antibiotic resistance is of growing interest. DNA repair systems are believed to influence the type and frequency of mutations that are involved in drug resistance. Mutations within the genes that code for repair systems, result in inefficient repair systems that allow for the development of further mutations. This provides the pathogen with a selective advantage over its host.(127,128) This was demonstrated in the Beijing strain family, where mutations in the antimutator (*mut*) genes were associated with increased ability to acquire resistance. The role of these systems requires further elucidation. (11,129,130)

Increased Mutation Rates

An increase in mutation rates has been in observed in *M.tuberculosis* when exposed to sub-inhibitory levels of antibiotics. This effect is most predominant in antibiotics that target DNA.(131) The fluoroquinolone antibiotics serve as a prime example. They act by binding to DNA gyrases. It has been demonstrated that *M.tuberculosis* has the ability to induce breaks in its double-stranded DNA resulting in transcriptional changes in genes that encode vital DNA repair mechanisms such as the *SOS* repair system. (11,132,133)

The Role of Compensatory Mutations

It has been postulated that resistance mutations bear a fitness cost to the bacterium. However, recent studies have demonstrated the presence of secondary mutations that act as compensatory mechanisms for the impaired fitness of the pathogen.(11) Sherman *et al*. demonstrated this phenomenon in INH resistant isolates of *M.tuberculosis* with an inactivated *katG* gene. The absence of *katG* catalase-peroxidase activity resulted in mutations in the regulatory region of the *ahpC* (alkyl hydroperoxidase reductase) gene, leading to overexpression of this gene. Mutations of the *ahpC* gene are believed to be compensatory for the loss of *katG* activity.(134) More recently, whole genome analysis showed that mutations occurring in RNA polymerases *rpoA* and *rpoC* were compensatory for the loss of fitness mediated by mutation in the *rpoB* gene in RIF resistant isolates.(135,136) A recent report also demonstrated varying levels of CAP resistance amongst A1401G laboratory mutants and clinical isolates bearing the same mutation, implying a possible role of compensatory mutations that restore fitness in isolates bearing resistance mutations. (95)

1.7 Problem Statement

Antibiotic therapy remains the key tool in the control of TB and understanding the mechanisms of drug resistance in *M.tuberculosis* is a significant component of the disease control strategy. Mutations in genomic drug targets have been identified as the principal

mechanism mediating antibiotic resistance. However, recent reports demonstrate the interplay of additional mechanisms. The role of the efflux pumps is believed to play a significant role in mycobacterial drug resistance. However, this mechanism has never been fully elucidated in our setting. This study, through amplification and sequencing of genes associated with resistance to first, second and third line drugs and by investigating the role of putative efflux pumps provides additional novel data on antibiotic resistance mechanisms in *M.tuberculosis.*

1.8 Aims

The aim of the study was 2-fold:

1. To characterize genetic mutations associated with resistance to anti-TB drugs and correlate these to the MIC of anti-TB drugs in clinical isolates of *M.tuberculosis*.
2. To investigate the role of efflux transporters mediating drug resistance in clinical isolates of *M.tuberculosis*.

1.9 Objectives and Thesis Layout

Layout of this thesis with specific objectives of the study:

- To determine the MICs of the key first line drugs; INH, RIF and EMB and to amplify and sequence the genes associated with resistance to each of the drugs: *inhA, katG, rpoB* and *embB* ***(Manuscript I, Chapter 2)***.
- To sequence the *pncA* gene for mutations associated with resistance to PZA ***(Manuscript I, Chapter 2)***.
- To determine the MICs for STR and sequence the *rrs* 500 region, *rpsL* and *gidB* genes ***(Manuscript II, Chapter 3)***.
- To determine the MICs of the fluoroquinolones: CIP, OFLOX and MOXI and sequence the *gyrA* and *gyrB* genes ***(Manuscript III, Chapter 4)***.

- To determine the MICs of the second line injectable drugs; KAN, AMIK and CAP and to sequence the *rrs*, *thyA* and *eis* genes ***(Manuscript IV, Chapter 5)***
- To sequence the *rrl* and *thyA* genes associated with resistance to LIN and PAS ***(Manuscript V, Chapter 6)***
- Determine the MICs in presence of EPIs ***(Manuscript VI, Chapter 7).***
- Summary of Findings ***(Chapter 8)***

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Chapter Two

Manuscript I:

Dynamics of antimicrobial resistance in Multi-Drug and Extensively Drug resistant strains of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa.

Submitted to BMC Infectious Diseases

Abstract

Background: In South Africa, drug resistant tuberculosis is a major public health crisis in the face of the colossal HIV pandemic.

Methods: In an attempt to understand the dynamics of drug resistance in our setting, we analysed the *rpoB*, *katG, inhA*, *pncA* and *embB* genes associated with resistance to key drugs used in the treatment of tuberculosis in clinical isolates of *Mycobacterium tuberculosis* in the KwaZulu-Natal province.

Results: Classical mutations were detected in the *katG, inhA* and *embB* genes associated with resistance to isoniazid and ethambutol. Diverse mutations were recorded in the multidrug resistant (MDR) and extensively drug resistant (XDR) isolates for the *rpoB* and *pncA* gene associated with resistance to rifampicin and pyrazinamide.

Conclusions: Multiple drug resistance mechanisms in the MDR-TB isolates suggests that the strains emerged separately and acquired resistance mutations independently. The similarity of mutations detected in the XDR-TB strains confirms reports of the clonality of the XDR epidemic. The successful dissemination of the drug resistant strains in the province underscores the need for rapid diagnostics to effectively diagnose drug resistance and guide treatment.

Keywords: Drug-resistance, first-line drugs, multi-drug resistant-TB, extensively drug-resistant-TB, clonality

Background

Tuberculosis (TB) remains one of the greatest public health concerns of our time, exacerbated by co-infection with human immunodeficiency virus (HIV) and drug resistance. In its latest global TB report in 2014, the world health organization (WHO) estimated that 3.5% of new cases and 20.5% previously treated patients have multi-drug resistant (MDR) TB; i.e. resistance to isoniazid (INH) and rifampicin (RIF). Of these cases, 9% have extensively drug resistant (XDR) TB. XDR-TB strains display resistance to INH, RIF and additional resistance to a fluoroquinolone antibiotic and one of the three injectable second line agents: amikacin (AMIK), kanamycin (KAN) and capreomycin (CAP). [1]

Despite the implementation of therapeutic regimes combining INH, RIF, Ethambutol (EMB) and pyrazinamide (PZA)[2], the escalation of MDR-TB strains has compromised the utility of this drug combination. The morbidity and mortality rates associated with drug resistant TB is several times higher than drug susceptible forms.[1] Treatment of drug resistant TB is further complicated by the decreased efficacy and higher toxicity associated with the second line drugs as well as the inability to provide early diagnostic data to guide treatment. [3]

Conventional drug susceptibility testing relies on mycobacterial culture methods, providing results after weeks or months. Molecular based diagnostics, such as the GeneXpert MTB/RIF (Cepheid) assay that do not rely on culture, are central to the future management of drug resistant tuberculosis. Their accuracy, however, is dependent on the association between a specific gene mutations and phenotypic drug susceptibility results. Drug susceptibility testing to second-line anti-TB drugs are more complicated that first line drugs. This is mainly due to

the technical difficulty associated with drug susceptibility testing for second line agents and the geographic diversity of the associated resistance conferring mutations. [4]

To date, the genes associated with resistance to the first line drugs in *Mycobacterium tuberculosis* have been identified based on the mode of action of each of the drugs and their demonstrated association with drug resistance. These include *ropB* (RIF), *katG* and *inhA* (INH), *pncA* (PZA) and *embB* (EMB). [5, 6]

In the study described in this report, we analysed mutations in the *ropB*, *katG, inhA*, *pncA* and *embB* genes and their association to resistance to the key first line antimicrobials in clinical isolates from the KwaZulu-Natal (KZN) province of South Africa.

Methods

M. tuberculosis Clinical Isolates

M. tuberculosis clinical isolates were selected from the storage collection of the Infection Control laboratory, University of KwaZulu-Natal. The isolates were from sputum specimens obtained from patients presenting to the Church of Scotland Hospital in the Tugella Ferry region of KZN, South Africa from 2005 to 2009. At initial isolation, the drug susceptibility profiles of the isolates were established in our laboratory using the 1% proportion method. [7] Sixty isolates were selected for the study: 10 drug susceptible (DS), 20 multi-drug resistant (MDR-TB) and 30 extensively drug resistant (XDR-TB). The H37Rv laboratory strain was included as a control. Ethical approval for the study was obtained from Biomedical Research Ethics Committee of the University of KwaZulu-Natal (BREC 247/09).

Determination of the Minimum Inhibitory Concentration (MIC)

MIC was established using a multipoint inoculation technique on Middlebrook 7H10 agar medium supplemented with oleic acid-albumin-dextrose-catalase (OADC). Test plates

contained INH, RIF and EMB at concentrations of 0.125; 0.25; 0.5; 1; 2; 4; 8; 16; 32; 64 and 128 mg/L. The plates were seeded with *M.tuberculosis* at an optical density (600nm) reading of 1. Test plates were incubated at 37ºC for 21 days in the presence of 5% CO_2. The MIC of an isolate was recorded as the lowest antibiotic concentration that inhibited growth of the organism. Resistance to INH, RIF and EMB was defined as concentrations of 0.2, 1.0 and 5.0 mg/L respectively, in accordance to WHO guidelines. [8] Isolates were tested in triplicate to ensure test accuracy and reproducibility. Due to the technical difficulty associated with conducting susceptibility testing with PZA, MICs were determined for the drug.

Genomic DNA Extraction & Amplification

Genomic DNA was extracted from cultures grown on Middlebrook 7H11 media using the CTAB-NaCl (Cetyl-trimethyl-ammonium Bromide-Sodium Chloride) method, as described previously.[9] The integrity and concentration of the DNA was determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific). PCR amplification assays were carried out for the *inhA, katG, rpoB, pncA* and *embB* genes. Primers for each of the genes were selected from published literature or designed using Primer3 design software.[10] The Expand Hi Fidelity PCR kit (Roche) was used in accordance to the guidelines set out by the manufacturer. Table 2.1 contains specific annealing temperatures and primer sequences used for amplification.

DNA Sequencing & Analysis

Prior to sequencing, the quality of PCR amplicons were determined on a 1% agarose gel. Amplicons were purified using the Invitrogen PureLink PCR purification kit (Applied Biosystems) and sequenced using ABI Prism Big Dye Terminator cycle sequencing kit V3.1 (Applied Biosystems) together with the forward primers selected for PCR amplification.

Nucleotide sequences were aligned to the H37Rv reference strain using Genious V5.5.7 (Biomatters) sequence analysis software.[11]

Genotyping

The genotypes of the isolates were established using the IS*6110* restriction fragment length polymorphism (RFLP) method, as described previously. [12]

Results

RFLP Analysis

RFLP analysis revealed that most of the DS isolates belonged to the Beijing family of strains. Three DS isolates had a unique profile and 1 isolate was a variant of the F11 strain family. The F28 strain family was the predominant genotype of the MDR-TB isolates, whereas the remaining isolates belonged to the F15/LAM4/KZN (KZN) strain family. One MDR-TB isolate recorded a unique profile and 1 was a variant of the F28 strain family. All of the XDR-TB isolates analysed in the study belonged to the KZN strain family. Genotypes, mutations and associated phenotypes are shown in tables 2.2 and 2.3.

rpoB mutations and RIF resistance

Of the sixty *M.tuberculosis* isolates initially selected, 2 MDR and 3 XDR-TB isolates failed multiple attempts at amplification of the *rpoB* gene and were excluded from further analysis. Majority (73%) of the RIF resistant isolates had at least 1 mutation in the *rpoB* gene, while 5 RIF resistant (3 MDR-TB, 2 XDR-TB) isolates had no alteration within the *rpoB* gene. The *rpoB* mutations were of various types: (1) A→G substitution on codon 435 (A1304G, D435G); detected in 3 XDR-TB isolates with an MIC range of 64-128 mg/L, belonging to the KZN family of strains. (2) T→ C substitution on codon 452 (T1355C, L452P); detected in 1 MDR-TB isolate of the KZN family strain family with an MIC of 8 mg/L. (3) C→T

substitution on codon 450 (C1349T, S450L); detected in 7 MDR-TB isolates which belonged to the KZN and F28 strain families and 1 XDR-TB isolate of the KZN strain family with an MIC range of 32-128 mg/L. Double mutants included (4) the D435Y together with an C→ A substitution on codon 454 (C1360A, P454T); detected in 1 MDR-TB isolate of the KZN strain family with an MIC of 128 mg/L. (5) A → T substitution on codon 445 (A1334T, H445L) and a G → C substitution on codon 491 (G1473C, I491M); detected in 1 MDR-TB isolate, a variant of the F28 strain family with an MIC of 128 mg/L. (6) The S450L and L452P detected in 2 MDR-TB isolates of the KZN and F28 strain families with an MIC of 64 mg/L; (7) the D435G and L452P detected in 22 XDR-TB isolates belonging to the KZN strain family with an MIC range of 32-128 mg/L. (8) The S450L and a T → C substitution on codon 564 (T1690C, Y564H), detected in 2 MDR-TB isolates of the KZN strain family with an MIC of 128 mg/L. No mutations were detected in any of the DS isolates screened. The *rpoB* mutations, with the corresponding MIC and genotypes of the isolates are shown in table 2.2.

<u>*inhA; katG* mutations and INH resistance</u>

No mutations were detected in the *inhA* gene or its promoter region amongst the isolates screened in the study. The *katG* mutations were of 3 types, (1) G→T substitution on codon 473 (G1388T; no amino acid alteration); detected in 5 DS isolates, all belonging to the Beijing family of strains. (2) G→C substitution on codon 315 (G944C, S315T); detected in all of the MDR and XDR-TB isolates studied. The MIC range of the isolates was 4-16 mg/L. The mutation was detected across all genotypes detected in the study. (3) One MDR-TB isolate, unique in its genotype had double mutations within the *katG* gene. In addition to the S315T mutation, an A→C substitution on codon 468 (A1343C, N468A) was detected, associated with MIC of 16 mg/L.

The remaining DS isolates had no alteration in the *katG* gene.

pncA **mutations and PZA resistance**

Of the sixty *M.tuberculosis* isolates initially selected, 1 MDR and 2 XDR-TB isolates failed multiple attempts at amplification of the *pncA* gene and were excluded from further analysis. *pncA* gene mutations were of 3 types, (1) T→G substitution on codon 34 (T100G, Y34D); detected in 6/15 MDR-TB isolates, of which 5 belonged to the F28 family and 1 to the KZN family of strains. (2) T→G substitution on codon 139 (T416G, L139G); detected in 2/15 MDR-TB isolates, belonging to the KZN family of strains. (3) Insertion of a cytosine at position 457, present in 1 MDR-TB and all the XDR-TB isolates screened, all belonging to the KZN strain family, resulting in a frameshift. The remaining MDR-TB isolates and DS isolates had no alteration in the *pncA* gene.

embB **mutations and EMB resistance**

The *embB* mutations were of 2 types, (1) A→G substitution on codon 306 (A916G, M306V); detected in 14 MDR-TB and 30 XDR isolates, associated with an MIC range of 2-16 mg/L. The mutation was detected across all genotypes of the study. (2) C→A substitution on codon 506 (C1489A, Q506K); detected in 2 MDR-TB isolates, belonging to the F28 family of strains and associated with an MIC of 16 mg/L. The DS isolates had no alteration in the *embB* gene.

Mutations of the *inhA, katG, embB* and *pncA* gene, MIC and associated genotypes are shown in table 2.3.

Discussion

In this study, we report on the dynamics of drug resistance amongst clinical isolates from KwaZulu-Natal, South Africa. South Africa ranks amongst the top ten high burden countries

of drug resistant tuberculosis worldwide. The overall incidence rate in the KwaZulu-Natal province alone currently exceeds the incidence rates for all types of TB in some low incidence countries, like the USA.[13] In an attempt to understand the molecular basis of drug resistance in our setting, we sequenced the *ropB*, *inhA*, *katG, pncA* and *embB* genes associated with resistance to key drugs used in the treatment of tuberculosis.

Analysis of the *inhA*, *katG* and *embB* genes demonstrated classical mechanisms that have been associated with resistance to INH and EMB.[5, 6] No mutations were detected in the *inhA* gene or its promoter region in the isolates screened. This is in keeping with previous reports.[14] Common *inhA* mutations that have been reported occur in the *inhA* promoter region at position -15, its correlation, however, is strongest with INH mono-resistant isolates or isolates with low-level INH resistance.[5, 6] However, a recent study demonstrated that double mutations in the *inhA* gene, in the promoter and coding regions, resulted in high-level INH resistance.[15] A large number of RIF resistant isolates bear mutations in the *inhA* and its promoter region making these mutations high predictors of RIF resistance, despite being absent in a subset of INH resistant isolates.

The main mechanism mediating INH resistance in the isolates studied is the *katG* S315T mutation that was detected in all the MDR and XDR-TB isolates. Numerous reports have found this mutation to be the most common mutation associated with INH resistance. One MDR-TB isolate with unique genotype had double mutations in the *inhA* gene:the S315T mutation occurred in conjunction with the N468A mutation. The double mutant did not record a higher MIC as compared to the other isolates bearing the S315T mutation only. The N468A appears to be novel, but may represent a natural polymorphism or phylogenetic marker of the unique genotype of the isolate. The G1388T mutation detected in the 5 DS isolates is natural polymorphism associated with the Beijing genotype that has no bearing on resistance.

Approximately 96% of RIF resistance is attributed to mutations contained in an 81bp hot-spot region known as the RIF resistance determining region (RRDR) which encompasses codons 507-533 of the *rpoB* gene.[5, 6] Mechanisms of resistance for the MDR and XDR-TB isolates varied in the case of RIF resistance, despite the MICs falling within a similar range. Various mutation types were described amongst the MDR-TB isolates. The S450L (corresponding to codon 531 in *E.coli*) mutation was the most common, accounting for resistance in 7 MDR-TB isolates, in keeping with various reports. Four MDR-TB Isolates had the S450L mutation together with L452P (corresponding to codon 533 in *E.coli*) or the T564H mutation. One MDR-TB isolate had the L452P mutation only. One isolate had the H445L (corresponding to codon 526 in *E.coli*) and the I491M mutation. To the best of our knowledge, the I491M and T564H mutations are novel and appear to be involved in mediating RIF resistance.

The main mutation mediating RIF resistance in the XDR-TB isolates was the D435G mutation together with the L452P mutation. This was detected in all the XDR-TB isolates and 1 MDR-TB isolate. Three isolates had the D435G mutation only and one isolate had the S450L mutation. Although the mutations and genotypes of the isolates were diverse, all mutations correlated with high level RIF resistance. Interestingly, the MDR and XDR-TB isolates both had mutations on the codon 435 but each resulted in different amino acid substitutions. Similar findings were described for KZN MDR and XDR-TB isolates in a study by Ioger *et al.* [14]analysing the whole genome sequences of drug resistant isolates KZN strain family. The main mechanism mediating resistance in the KZN MDR-TB isolate was attributed to the D435Y mutation and the D435G and L452P mutations in the KZN XDR-TB isolates. The study only analysed the KZN strain family.[14] Our study shows a greater diversity in the MDR-TB RIF resistance mechanisms and the isolates represented various strain families. In our study, the mutation on codon 435 was responsible for

resistance in most of the MDR-TB isolates. In contrast, Ioger *et al.* [14] reported that this mutation was only responsible for 9% of RIF resistance. [14]

Resistance to PZA, as in the case of RIF was represented by diverse mutations in the MDR-TB and XDR isolates. The MDR-TB isolates had a mutation either on codon 34 (nucleotide 100) or codon 139 nucleotide 416), while XDR-TB isolates had an insertion of a cytosine at position 457, leading to a frameshift in the amino acid translation. The insertion was also detected in one MDR-TB isolate, possibly with a higher level of resistance to PZA. Due to technical difficulty associated with PZA susceptibility testing, no MICs were done for the drug. Instead, we sought to identify mutations in the MDR and XDR-TB groups and compare them with published literature. Mutations at position 100 have been described in isolates in Japan and Peru while mutations at position 416 have been reported South Africa, Thailand, China, USA, Portugal, Spain and Singapore. [16–24] The insertion at position 457 has been described in isolates from Brazil. [25]

Mutations within the *pncA* gene are highly diverse, with 600 unique mutations at 400 different positions reported to date.[24] In keeping with this diversity, the study by Ioger *et al.* showed different mechanisms of PZA resistance in the MDR and XDR-TB isolates from the KZN strain family as compared to the mechanisms described in this report.[14] This highlights the difficulty associated with detection of PZA resistance. The diversity of the mutations detected in the isolates vary greatly, making it impossible to apply to molecular diagnostic assay. PZA susceptibility testing poses a further challenge. The PZase enzyme required for the conversion of PZA to its active form is only functional at an acid pH, making it difficult to test the drug using conventional media. With a prevalence of approximately 60.5% in patients with confirmed MDR-TB, PZA resistance testing is of utmost importance as PZA forms an integral role in current multidrug regimens and is also a key component of new treatment regimens undergoing evaluation in phase II or III clinical trials. [24]

A few isolates resistant to RIF and PZA did not display any mutations in the related genes. This phenomena has been previously described.[5, 6] This suggests that alternate resistance mechanisms may exist that remain to be identified. The differences in resistance mechanisms in the MDR and XDR-TB isolates suggest that the strains emerged separately and acquired resistance mutations independently. This is against the premise that the XDR phenotype had evolved directly from the MDR phenotype.

Conclusion

The multiple MDR-TB strains circulating in the KZN province suggests that the MDR-TB strains are evolving and spreading by transmission, demonstrated by the diversity of resistance mechanisms in the isolates. In contrast, the XDR-TB strains have disseminated by clonal expansion, demonstrated by the high level of similarity in the mutations detected. The successful dissemination of these resistant strains demonstrates that the resistance mutations come with a lower/no fitness cost than previously assumed; compensatory mechanisms might play a role in maintaining the fitness of this pathogen. The results of the current study underscores the need for novel assays to rapidly detect resistance to all drugs in order to effectively guide individualised treatment.

Competing Interests

None to declare.

Authors Contributions

ND was involved in the conception and design of the study, conducted the molecular assays, sequence alignment and drafted the manuscript. PM and AWS were involved in the conception and design of the study, participated in the co-ordination of the study and reviewed the manuscript for publication. All authors have read and approved the final manuscript.

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Tables

Table 2.1: Primer sequences and annealing temperatures used for PCR and sequencing.

Gene	Primer	Nucleotide Sequence (5'→3')	Annealing Temp (◦C)	Amplicon Size	Associated Drug Resistance	Ref
rpoB	*rpoB* F	TGTTGGACATCTACCGCAAG	54◦C	916bp	Rifampicin	*
	rpoB R	CGAGACGTCCATGTAGTCCA				
inhA	*inhA* F	CTACATCGACACCGATATGAC	55◦C	700bp	Isoniazid	[26]
	inhA R	GACCGTCATCCAGTTGTAG				
katG	*katG* F	GGTCGACATTCGCGAGACGTT	57◦C	987bp	Isoniazid	[27]
	katG R	TTGTTCCTGCGACGCATCGTG				
pncA	*pncA* F	GCTGGTCATGTTCGCGATCG	59◦C	561bp	Pyrazinamide	[28]
	pncA R	GCTTTGCGGCGAGCGCTCCA				
embB	*embB* F	AAGCTGGCGCACCTTCAC	55◦C	833bp	Ethambutol	*
	embB R	ATAGCGCGGTGATCAAAAA				

***Designed using Primer 3 Software**

Table 2.2: Mutations in the *rpoB* gene with the associated MICs, phenotypes and genotypes.

rpoB **Mutation**	**Associated MIC (mg/L)**	**Number Of Isolates**	**Associated Genotype**
D435G	64- 128	3 XDR	KZN
S450L	32-128	7 MDR 1 XDR	F28 / KZN
L452P	8	1 MDR	KZN
D435Y & P454T	128	1 MDR	KZN
H445L & I491M	128	1 MDR	F28V
S450L & L452P	64	2 MDR	F28/ Unique
D435G & L452P	32-128	22 XDR	KZN
S450L & T564H	128	2 MDR	F28/KZN
No Mutations	2-128	10 DS 3 MDR 2 XDR	Various

Table 2.3: Mutations of the *inhA*, *katG*, *embB* and *pncA* gene, associated MICs, phenotypes and genotypes.

Drug	Gene	Mutation	Associated MIC (mg/L)	Number of Isolates	Associated Genotype
INH	***inhA***	None	0.125-16	All study isolates	Various
	katG	G1388T	<0.125	5 DS	Beijing
		S315T	4-16	16 MDR 30 XDR	Various
		S315T + N468T	16	1 MDR	Unique
PZA	***pncA***	Y34D	*	6 MDR	F28/ KZN
		L139G	*	2 MDR	KZN
		Ins C	*	1 MDR 30 XDR	KZN
EMB	***embB***	M306V	2-16	14 MDR 30 XDR	Various
		Q506K	16	2 MDR	F28

*MICs for PZA not done

Chapter Three

Manuscript II:

Streptomycin resistance in the F15/LAM4/KZN strain of *Mycobacterium tuberculosis is mediated by lineage-specific alteration of the gidB gene.*

Currently under review by the Journal of Medical Microbiology (Manuscript number: JMM-D-15-00568)

Abstract

Widespread streptomycin resistance in *Mycobacterium tuberculosis*, resulting from the historical application of the drug as tuberculosis monotherapy has impelled policymakers to exclude the drug from treatment regimens. Streptomycin resistance is believed to be mediated via mutations in the r*psL, rrs* and *gidB* genes. This study aimed to investigate the mechanisms of streptomycin resistance in clinical isolates from KwaZulu-Natal, South Africa and to determine if the drug has any further clinical relevance in tailored treatment and retreatment regimens. A mutational analysis was conducted on the *rpsL, rrs* and *gidB* genes by PCR amplification and sequencing. MIC's for streptomycin were determined using a multipoint inoculation technique on Middlebrook 7H10 medium and linked to the mutation profiles and IS*6110* RFLP genotype pattern of clinical isolates. The circulating MDR and XDR F15/LAM4/KZN strains had a rare 130bp deletion in the *gidB* locus resulting in streptomycin resistance. The K43R *rpsL* mutation was linked to the F28 family in the MDR-TB isolates. The *gidB* L16R mutation linked to KZN family was detected in all KZN strains whilst the E92D mutation associated with the Beijing lineage was detected in the F28 strains. None of the mutations could be linked to the phenotypic level of streptomycin resistance that the isolates displayed. A 130bp deletion in the *gidB* gene in all MDR-TB and XDR-TB

strains of the F15/LAM4/KZN family, is the most significant mechanism mediating streptomycin resistance in the province.

Introduction

The discovery of streptomycin (STR), an aminoglycoside antibiotic, was a turning point in the treatment of tuberculosis (TB) in the 1940's. STR was the first chemical compound effective in the treatment of TB. Historically, the drug was applied as TB monotherapy and early studies indicated a decline treatment response after three months of treatment, resulting from the rapid development of drug resistance when a single drug is used for the treatment of TB. In the 1950's, several new anti-TB drugs were developed and applied as combination therapy. (Zumla et al., 2013) STR remained an integral part of treatment regimens, *playing* an important role in combination and re-treatment therapy for the disease. However, the high rates of STR resistance, increased toxicity associated with the drug and decreased efficacy in comparison to succeeding drugs, lead to a gradual decrease in the application of the drug in TB treatment.(Jagielski et al., 2014; Zumla et al., 2013)

In South Africa, the first line treatment for TB includes a combination of isoniazid, rifampicin, pyrazinamide, and ethambutol during the intensive phase of treatment, followed by isoniazid and rifampicin in the continuation phase. STR has been excluded , from all treatment protocols since 2014, mainly due to the emergence and spread of drug resistant strains of *Mycobacterium tuberculosis.*(National Department of Health, 2014)

The mechanism of action of STR in *M. tuberculosis*, involves irreversible binding to the ribosomal protein S12 and 16S rRNA, which are the components of the 30S subunit of the bacterial ribosome. Through this interaction, STR blocks translation thereby inhibiting protein synthesis. The main mechanism of resistance to STR is believed to be mediated via mutations in the *rpsL* and *rrs* genes, encoding the ribosomal protein S12 and the 16S rRNA, respectively. Amino acid substitutions in the S12 protein affect the affinity of STR binding

by altering the higher-order structure of 16S rRNA. Mutations occurring within the16 S rRNA gene itself, results in a decreased affinity for STR. Mutations in the *rpsL* gene have been associated with a high-level of STR resistance, whereas mutations described in the *rrs* gene have been shown to confer lower levels of resistance (Jagielski et al., 2014; Okamoto et al., 2007; Verma et al., 2014)

More recently, reports of mutations in the *gidB* gene, encoding a 7- methylguanosine methyltransferase specific for methylation of the G527 in loop of the 16S rRNA, has been implicated in low-level STR resistance (Da Silva and Palomino, 2011; Jagielski et al., 2014; Okamoto et al., 2007; Spies et al., 2011, 2008; Verma et al., 2014; Wong et al., 2011). The G527 site lies within the 530 loop, a hotspot region for STR resistance. The S12 protein binding site lies in close proximity to the 530 loop and assists in proofreading during translation. Lack of methylation by *gidB,* in the presence of mutations mediates STR resistance. (Jagielski et al., 2014; Okamoto et al., 2007; Verma et al., 2014). Reports on the deletion of the *gidB* gene in *Salmonella* species has been implicated with decreased bacterial fitness and has been associated high-level resistance to aminoglycoside antibiotics. (Mikheil et al., 2012)

The rapid determination of drug resistance patterns in clinical isolates of *M.tuberculosis* is an essential prerequisite for optimal treatment and decreased transmission of the disease. Currently, screening for mutations in well-defined genes associated with drug resistance is the most promising tool.(Da Silva and Palomino, 2011) In the case of STR, the association between mutations in the *rpsL* and *rrs* genes and drug-resistant phenotypes has been well characterized, whilst reports of mutations in the *gidB* gene have been inconsistent (Da Silva and Palomino, 2011; Okamoto et al., 2007; Palomino and Martin, 2014). Furthermore, STR resistance mutations have been reported to vary depending on the population and

geographical area studied. (Dobner et al., 1997; Jagielski et al., 2014; Katsukawa et al., 1997; Ramaswamy et al., 2004; Shi et al., 2007; Tudo et al., 2010)

The aim of this study was to investigate the role of mutations in the *rpsL, rrs*, and *gidB* genes in clinical isolates of *M. tuberculosis* from KwaZulu-Natal, South Africa.

Materials and Methods

Clinical isolates

Clinical isolates for the study were obtained from the culture collection of the Infection Control Laboratory, University of KwaZulu-Natal, South Africa. Stored isolates were cultured onto Middlebrook 7H11 medium supplemented with oleic acid-albumin-dextrose-catalase (OADC). A total of 60 isolates were included in the study: 10 drug susceptible (DS), 20 multi-drug resistant (MDR-TB) and 30 extensively drug resistant (XDR-TB). The H37Rv laboratory reference strain was included as an experimental control. The primary isolation, species identification, genotyping and drug susceptibility testing were established in previous studies conducted in our laboratory, using standard mycobacterial protocols. The study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (BREC 247/09).

Minimum Inhibitory Concentration Determination

STR MIC's were established using a multipoint inoculation method on Middlebrook 7H10 agar medium supplemented with OADC. Plates containing STR at concentrations of 0.125; 0.25; 0.5; 1; 2; 4; 8; 16; 32; 64 and 128 mg/L were inoculated with bacterial cultures grown to an optical density (600nm) reading of 1 . Plates were then incubated in a CO_2 enriched atmosphere at 37ºC for 21 days. The MIC of an isolate was recorded as the lowest antibiotic concentration that resulted in a complete inhibition of growth. Resistance to STR was defined at a break point concentration of 2 mg/L, according to guidelines set out by WHO (World

Health Organisation, Geneva, 2009). All isolates were tested in triplicate to ensure test accuracy and reproducibility.

DNA Extraction

Genomic DNA was extracted from *M.tuberculosis* cultures using the CTAB-NaCl (Cetyl-trimethyl-ammonium Bromide-Sodium Chloride) method (Van Soolingen et al., 1991). A NanoDrop spectrophotometer was used to estimate the quantity and purity of the isolated DNA.

PCR Amplification & Sequencing Analysis

PCR amplification assays were conducted for *rpsL*, *rrs* and *gidB* genes and sequenced to determine genetic alteration. The oligionucleotide primers for each gene (Table 1) was selected from published literature and applied to the Expand High Fidelity PCR kit (Roche) in accordance to the kit guidelines with annealing temperatures of 56◦C for the *rrs* gene and 55◦C for *rpsL and gidB* genes. The quality and integrity of the PCR amplicons was analysed using agarose gel electrophoresis and purified using the Invitrogen PureLink PCR purification kit (Applied Biosystems). Direct single stranded sequencing of the amplicons was performed using ABI Prism Big Dye Terminator cycle sequencing kit V3.1 (Applied Biosystems) with the forward primers used for PCR amplification. Sequence data was analysed using Geneious V5.5.7 sequence analysis software and final nucleotide sequences were aligned with corresponding reference sequence of the H37Rv reference strain.

Results

Of the sixty *M.tuberculosis* isolates selected, 4 MDR-TB isolates failed to grow sufficiently on sub- culture medium and 2 MDR-TB isolates failed numerous attempts at PCR amplification. Therefore, we report MIC and sequencing data for 10 SUS, 14 MDR-TB and 30 XDR-TB *M.tuberculosis* isolates. 34/54 (81.0%) of the *M.tuberculosis* isolates included in the final analysis were classified as STR resistant. This included all the MDR-TB and XDR-

TB isolates and 1 DS isolate. All 54 *M.tuberculosis* isolates were screened for mutations in the *rrs*, *rpsL* and *gidB* genes.

Mutations of the *rpsL* gene were of 2 types. An A → G substitution on codon 43 (A128G, Lys→Arg) and a silent mutation (A→G) on codon 121. The A → G substitution on codon 43, commonly referred to as the K43R mutation was found in 7/54 (7 MDR-TB) isolates. The silent mutation on codon 121 was observed in 30/54 (30 XDR-TB) isolates. Both mutation types were associated with an MIC range of 4 to 128 mg/L. No mutations in the *rpsL* gene were observed in any of the drug susceptible isolates tested.

The *gidB* mutations were of 5 types: T → G substitution on codon 16 (T47G, Lys→Arg). The T → G substitution on codon 16, commonly referred to as the L16R mutation was found in 35/54 (1 DS, 4 MDR and 30 XDR) isolates. An A → C substitution on codon 92 (GAA-GAC, Glu→Asp) was found in 13/54 (3 DS and 10 MDR-TB) isolates commonly referred to as the E92D mutation. Both these mutations were associated with an MIC range of 0.5 to 128 mg/L. All 54 isolates displayed an alteration on codon 100 (TCT-TTT, Ser→Phe), irrespective of the MIC or resistance profile. This mutation is reffered to as the S100F mutation. One DS isolate had a silent mutation on codon 135 (GTG-GTT), associated with an MIC of 0.5 mg/L. A 130bp deletion within the gene encompassing codons 50-93 was also observed in 34/54 (4 MDR-TB and 30 XDR-TB) isolates tested, associated with an MIC range of 4 to 128 mg/L. The 130bp deletion of the *gidB* gene is shown in figure one. All the isolates in this study displayed alteration in the *gidB* gene. Seven of the STR resistance isolates of the MDR phenotype had mutations in the *gidB* gene only.

None of the isolates tested displayed any mutations in the *rrs* gene region linked to STR resistance. The relationship between the MIC's of STR and mutations in the *rrs, rpsL* and *gidB* genes are shown in Table 3.2.

IS*6110* genotyping revealed that a majority of drug resistant isolates belonged F15/LAM4/KZN (KZN) lineage, whereas the remaining isolates belonged to F28/F11 and Beijing strain families. Four isolates used in the study displayed a unique RFLP profile. The *rpsL* K43R mutation was found exclusively in the F28 strain family and the A363G mutation was identified in only the KZN XDR-TB strains. The 130bp deletion and L16R mutation in the *gidB* gene were associated with the KZN family. One strain, a variant of the F11 family displayed the L16R mutation in the *gidB* gene. The E92D *gidB* mutation was identified in all strains of Beijing and F28 families, as well as variants of the F28 family. RFLP profiles, lineage specific mutations and associated phenotypes are shown in Table 3.3.

Discussion

We report on STR resistance in clinical isolates of *M.tuberculosis* from the KwaZulu-Natal province of South Africa in relation to the mutation profiles of the *rrs, rpsL*, and *gidB* genes. In *M. tuberculosis,* resistance to STR is associated with alterations within the *rrs, rpsL*, and *gidB* genes which encode constituents of the 30S ribosomal subunit. Mutations occurring in the *rpsL* and *rrs* genes have been well characterized, accounting for 70% of STR resistance (Da Silva and Palomino, 2011; Spies et al., 2011). Reports on *gidB* gene mutations remain inconsistent, associated with low-level STR resistance and are most predominant in isolates with mutations in the *rrs* or *rpsL* genes (Jagielski et al., 2014; Okamoto et al., 2007; Verma et al., 2014). Furthermore, the relationship between the mutations and the level of STR remains unclear (Jagielski et al., 2014; Shi et al., 2007; Verma et al., 2014).

Numerous studies report that the amino acid substitution on codon 43 (AAG-AGG, Lys→Arg) of the *rpsL* gene is the most common mechanism conferring resistance in STR resistant clinical isolates of *M.tuberculosis* (Da Silva and Palomino, 2011; Dobner et al., 1997; Fakuda et al., 1999; Honore and Cole, 1994; Jagielski et al., 2014; Verma et al., 2014). In our study, this mutation was found in 7 MDR-TB isolates, representing only 18.9% of the

rpsL mutations in the STR resistant isolates from this study. This mutation has been associated with varying detection rates across geographical areas, ranging between 13.2% and 80.4%. This mutation has been correlated with isolates of the Beijing genotype and with isolates of an MDR-TB phenotype (Jagielski et al., 2014; Spies et al., 2011). In our study, this mutation was associated with the F28 family, a predominant strain responsible for MDR-TB in our setting and in the Western Cape region of South Africa. The A363G silent mutation was present in 30 isolates, representing 81.1% of the *rpsL* mutations in this study. This mutation was found exclusively in the XDR-TB isolates, with no additional mutations in hotspot regions of the gene. Verma *et al.* described this mutation in 30/75 STR resistant isolates with or without additional alteration on codon 43 or 88 of the *rpsL* gene (Verma et al., 2014). We postulate that this mutation maybe a natural polymorphism, with no link to resistance. Furthermore, MDR-TB isolates that did not display the mutation at position 363 of the *rpsL* gene but had a *gidB* deletion recorded similar MICs to that of the XDR-TB isolates with alteration in both the *rpsL* and *gidB* genes.

The *gidB* gene, a highly conserved gene present in all species sequence to date, is associated with low level STR resistance in *M.tuberculosis* (Verma et al., 2014). In our study, the most significant finding was a 130bp deletion within the *gidB* gene. This mutation does not occur with any of the classical *rpsL* mutations. We believe that this mutation which results in the loss of function of the *gidB* gene, mediates STR resistance in KZN family of strains. Whole genome analysis conducted on the KZN family strains by Ioerger *et al.* described this deletion which includes amino acids 50-93, encompassing the SAM binding site and results in a frameshift of the C terminal remainder, completely disrupting the function of the *gidB* gene (Ioerger et al., 2009). In our study, this deletion was exclusive to the MDR and XDR-TB strains of the KZN family. Ioerger *et al*. detected this mutation in on DS isolate, but attributed this finding to mischaracterization of the strain (Ioerger et al., 2009). Whole

genome sequencing analysis of strains from Panama, described to be closely related to the KZN family of strains, did not display this 130bp deletion. However it was reported that a unique 16 bp deletion in the *gidB* gene was present in both STR resistant and susceptible isolates (Lanzas et al., 2013).

The L16R and E92D mutations have been described as phylogenetic markers rather than resistance mediating mutations. The L16R mutation is specific for the Latin American-Mediterranean (LAM) family (Spies et al., 2011). Our results are in keeping with these reports, as this mutation was present in all 34 KZN isolates. One isolate, a variant of the F11 family of strains, had this alteration. It is possible that the strain maybe closely related to the KZN family as it is classified within the Latin America Mediterranean (LAM 3) lineage. The E92D mutation, reported to be specific for the Beijing family was detected in only 3/5 Beijing isolates in our study. It may be possible that the 2 isolates were misclassified. Interestingly, this mutation was detected in all isolates of the F28 family and variants of the family. To the best of our knowledge, this mutation has not been reported in the F28 family and therefore may not be a representative marker of the Beijing family as previously reported.

The S100T alteration was present in all the isolates tested. It has been described by Verma *et al*. in all 75 isolates analysed in the study, with no link to resistance and is thus a naturally occurring polymorphism. Furthermore, protein modelling and MSA mutation mapping of codon 100, revealed that substitution was a spontaneous alteration, also identified in the H37Rv laboratory strain (Verma et al., 2014). The G405T silent mutation found in one DS isolate did not have any link to resistance. Analysis of the binding sites on codon 16 revealed that this alteration results in a change in binding cavity morphology (Verma et al., 2014). This implies that the KZN family of strains with the L16R alteration will naturally display STR resistance without the presence of additional mutations.

Whilst many reports associate the mutations found in the *rpsL* gene with high-level STR resistance and mutations in *rrs* gene with low to intermediate resistance level (Cooksey et al., 1996; Meier et al., 1996; Tudo et al., 2010), we observed that the mutations on the different *loci* did not discriminate between the levels of STR resistance in the current study. The MICs of isolates bearing mutations in the various genes did not differ from each other. Jagielski et al. compared the MIC_{50}, MIC_{90} and median MIC values for STR resistant and susceptible isolates to establish a relationship between the mutations and levels of STR resistance but found no difference in the MICs amongst isolates (Jagielski et al., 2014). Shi et al. analysed 215 clinical isolates using denaturing HPLC analysis to establish if the various mutations types could be linked resistance levels but found no close correlation in MICs and mutation types (Shi et al., 2007).

In this study, 4 isolates (8.8%) with MICs in the STR resistant range, did not have any mutations that are associated with STR resistance. Three MDR-TB isolates and 1 DS isolate had no mutations in the *rpsL* or *gidB* genes, while the 3 MDR-TB isolates had only the E92D *gidB* phylogenetic marker. This is in close comparison to other studies that analysed all 3 genes, ranging from 6.9-22% (Jagielski et al., 2014). Furthermore, none of the isolates had any alteration in the *rrs* gene related to STR resistance, in keeping with the whole genome analysis of KZN strains (Ioerger et al., 2009). Wong *et al.* reports similar findings. Detection of the *rrs* A1401G mutation was associated with kanamycin resistance and had no bearing on STR resistance (Wong et al., 2011). Verma et al. suggests that alteration in the *rrs* gene at position 516 is a compensatory mechanism to overcome the loss of *gidB* function (Verma et al., 2014).

Alternate mechanisms such as the role of efflux pumps are believed to mediate resistance in the absence of mutations. A report by Spies et al. demonstrated a reduction in the STR MIC in the presence of efflux pump inhibitors in STR resistant isolates with mutations in the various *loci*. However, the changes were most significant in isolates that displayed *gidB* mutations. Thus, *gidB* mutations act in synergy with efflux pumps to confer low level STR resistance. Further confirmation is required as well as the identification of novel mechanisms that mediate STR resistance (Spies et al., 2008).

In conclusion, we provide evidence that the main mechanism mediating STR resistance in the predominant KZN strain is a rare 130bp deletion within the *gidB* gene while the classical K43R mutation of the *rpsL* gene is linked to resistance in the F28 MDR-TB strain. The low detection rate of the *rpsL* and *rrs* mutation in this subset of isolates questions the utility of these mutations as accurate predictors of STR resistance. Whilst previous findings suggest the role of *gidB* gene mutations as phylogenetic markers, results of this study show that the E92D mutation was not exclusive to the Beijing lineage. Furthermore, none of the resistance conferring mutations detected in this study could predict the level of STR resistance. Our results suggest that STR should not be applied to empiric TB treatment regimens and should not be applied to tailored regimens in the absence of drug susceptibility testing.

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Tables

Table 3.1: Oligonucleotide Primers used for PCR & Sequencing

Gene	Primer	Nucleotide Sequence (5'→3')	Amplicon Size (bp)	Reference
rpsL	*rpsL* F	GGCCGACAAACAGAACGT	502	(Sreevatsan et al., 1996)
	rpsL R	GTTCACCAACTGGGTGGAC		
Rrs	*rrs* F	TGCTTAACACATGCAAGTCG	920	(Jugheli et al., 2009)
	rrs R	TCTCTAGACGCGTCCTGTGC		
gidB	*gidB* F	GTCCCTCCACTCGCCATC	675	(Spies et al., 2008)
	gidB R	GCGGAGTGCGTAATGTCTC		

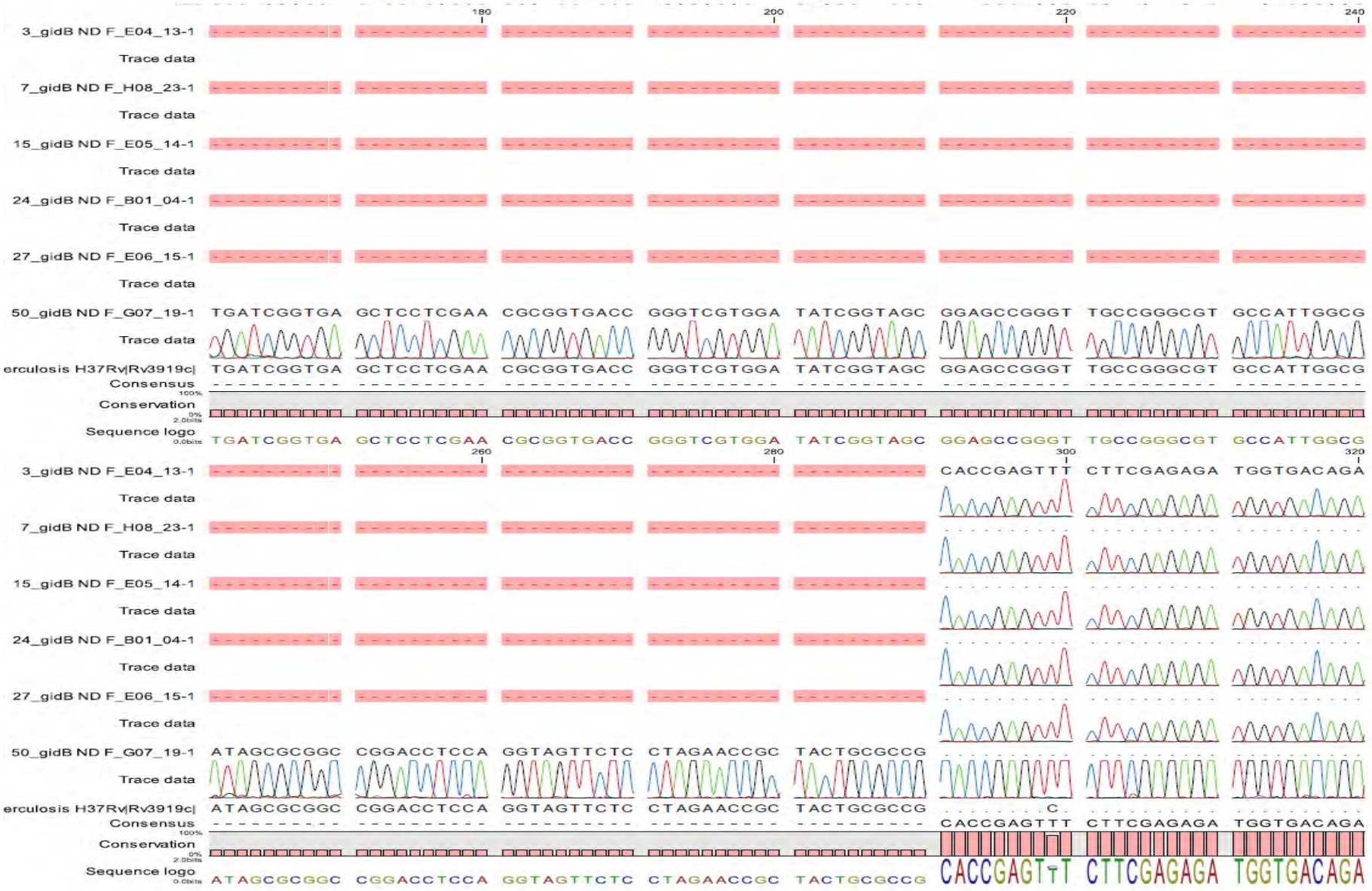
Table 3.2: Relationship between the STR MIC, RFLP analysis and mutations in the *rpsL*, *rrs* and *gidB* genes in 54 clinical isolates of *Mycobacterium tuberculosis*

Phenotype	RFLP Classification	Mutations			Number of Strains	STR MIC mg/L
		Rrs	*rpsL*	*gidB*		
DS	2 Beijing 2 Unique	None	None	S100F only	4	0.5
DS	Beijing	None	None	S100F only	1	4
DS	F11V	None	None	L16R + S100F	1	0.5
DS	Unique	None	None	S100F + G405T	1	0.5
DS	Beijing	None	None	E92D + S100F	3	0.5
MDR	F28 F28V	None	None	E92D + S100F	2	4
MDR	F28	None	None	E92D + S100F	1	8
MDR	KZN	None	None	L16R + S100F + 130 bp Deletion	1	32
MDR	KZN	None	None	L16R + S100F + 130 bp Deletion	3	128
MDR	F28	None	K43R	E92D + S100F	6	4
MDR	F28	None	K43R	E92D + S100F	1	128
XDR	KZN	None	A363G	L16R + S100F + 130 bp Deletion	10	4
XDR	KZN	None	A363G	L16R + S100F + 130 bp Deletion	6	32

Figure 3.1

Figure Legend

Figure one depicts the 130bp deletion of the gidB gene which includes codons 50-93, encompassing the SAM binding site and results in a frameshift of the C terminal remainder, completely disrupting the function of the *gidB* gene. The first 3 chromatograms respresent the F15/LAM4/KZN XDR strains, the fourth represents an MDR strain of the same strain family and the last chromatogram represents a DS isolate, all in comparison to the H37Rv laboratory strain sequence.


180
200
220
240
3_gidB ND F_E04_13-1
Trace data
7_gidB ND F_H08_23-1
Trace data
15_gidB ND F_E05_14-1
Trace data
24_gidB ND F_B01_04-1
Trace data
27_gidB ND F_E06_15-1
Trace data
50_gidB ND F_G07_19-1
TGATCGGTGA GCTCCTCGAA CGCGGTGACC GGGTCGTGGA TATCGGTAGC GGAGCCGGGT TGCCGGGCGT GCCATTGGCG
Trace data
erculosis H37Rv|Rv3919c|
TGATCGGTGA GCTCCTCGAA CGCGGTGACC GGGTCGTGGA TATCGGTAGC GGAGCCGGGT TGCCGGGCGT GCCATTGGCG
Consensus
100%
Conservation
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Sequence logo
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TGATCGGTGA GCTCCTCGAA CGCGGTGACC GGGTCGTGGA TATCGGTAGC GGAGCCGGGT TGCCGGGCGT GCCATTGGCG
260
280
300
320
3_gidB ND F_E04_13-1
CACCGAGTTT CTTCGAGAGA TGGTGACAGA
Trace data
7_gidB ND F_H08_23-1
Trace data
15_gidB ND F_E05_14-1
Trace data
24_gidB ND F_B01_04-1
Trace data
27_gidB ND F_E06_15-1
Trace data
50_gidB ND F_G07_19-1
ATAGCGCGGC CGGACCTCCA GGTAGTTCTC CTAGAACCGC TACTGCGCCG
Trace data
erculosis H37Rv|Rv3919c|
ATAGCGCGGC CGGACCTCCA GGTAGTTCTC CTAGAACCGC TACTGCGCCG
Consensus
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CACCGAGTTT CTTCGAGAGA TGGTGACAGA
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ATAGCGCGGC CGGACCTCCA GGTAGTTCTC CTAGAACCGC TACTGCGCCG
CACCGAGTTT CTTCGAGAGA TGGTGACAGA

Chapter Four

Manuscript III:

Moxifloxacin resistance in the F15/LAM4/KZN extensively drug-resistant strain of *Mycobacterium tuberculosis.*

Published in the Journal of Infection and Drug Resistance:

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Abstract

Objectives: Moxifloxacin (MXF) has been advocated for the treatment of extensively drug-resistant (XDR) tuberculosis despite resistance to older-generation fluoroquinolones. We investigated the relationship between the minimum inhibitory concentration (MIC) of MXF and mutations in the *gyrA* and *gyrB* genes in *Mycobacterium tuberculosis* (MTB) isolates from KwaZulu-Natal (KZN) Province of South Africa.

Materials and methods: MICs of 56 MTB isolates were compared to the mutations in the quinolone resistance-determining region known to confer fluoroquinolone resistance. Isolates were genotyped by IS*6110* restriction fragment length polymorphism analysis.

Results: The circulating F15/LAM4/KZN XDR strain circulating in KZN Province harbored the A90V mutation and displayed high-level resistance with MICs of 8 mg/L for ciprofloxacin and ofloxacin and ≥1 mg/L for MXF.

Conclusion: The inclusion of MXF in XDR-TB treatment regimens requires careful consideration in our setting, where clinical outcome data in MXF-containing regimens are unavailable.

Keywords: fluoroquinolones, susceptibility testing, strain typing, drug-resistance

Introduction

The human immunodeficiency virus (HIV) pandemic has fuelled the tuberculosis (TB) epidemic by creating a population of immunosuppressed individuals that are highly susceptible to *Mycobacterium tuberculosis* (MTB) infection. The last decade has seen an unprecedented increase in antimycobacterial drug resistance. Of the estimated 1.3 million deaths resulting from TB globally in 2012, 13.1% of these deaths were due to drug resistance. Appropriate treatment of patients with drug-resistant strains of MTB is of vital importance in limiting the transmission of the disease and reducing mortality rates.[1]

Fluoroquinolones are potent antibiotics that have been used in clinical practice since the early 1980s.[2] They display broad-spectrum antimicrobial activity, and have been used extensively in the treatment of bacterial infections of the respiratory, gastrointestinal, and urinary tracts, as well as in sexually transmitted diseases and chronic osteomyelitis.[3] The fluoroquinolones have been advocated for the treatment of patients with multidrug-resistant (MDR) MTB, defined as resistance to at least isoniazid and rifampicin. Patients with extensively drug-resistant (XDR) MTB harbor MDR TB strains with additional resistance to fluoroquinolones and one of the second-line antimycobacterial injectable (kanamycin, amikacin, and capreomycin) agents. However, moxifloxacin (MXF), a new-generation fluoroquinolone, has been recommended by the World Health Organization (WHO) for the treatment of XDR. Studies that have explored the efficacy of MXF against XDR strains of MTB have concluded that the drug may be used in XDR cases provided that the infecting isolate has a minimum inhibitory concentration (MIC) of <2 mg/L for MXF.[1,4]

MXF differs in structure when compared to ofloxacin (OFX) and ciprofloxacin (CPX). The structural difference, which includes a methoxy group in the C-8 position of MXF, results in

increased bactericidal activity of the drug, lower MICs, and a lower propensity for the development of resistance to the drug.[5] Although cross-resistance has been reported, it has been argued that the increased bactericidal activity of MXF and the lower MIC allow for this drug to be effective against XDR isolates where CPX and OFX are ineffective.[3,4]

The fluoroquinolones inactivate the deoxyribonucleic acid (DNA) gyrase enzyme, thereby preventing transcription during cell replication. DNA gyrases are encoded by the *gyrA* and *gyrB* genes. Fluoroquinolone-resistant strains of MTB most frequently display mutations on codons 90, 91, and 94 of the *gyrA* gene.[6–9] Additionally, double mutations in the *gyrA* or concomitant *gyrA* and *gyrB* mutations have been reported.[6,8] The level of fluoroquinolone resistance is dependent on the mutation in the resistance-conferring gene and the fluoroquinolone tested.[10] Studies have demonstrated that MIC levels of resistant isolates are higher for older-generation fluoroquinolones than for MXF.[6–9]

The use of MXF in XDR treatment regimens was introduced without prior testing for susceptibility against the circulating XDR isolates in KwaZulu-Natal (KZN) Province. The aim of this study was to correlate the minimum inhibitory concentration (MIC) levels of the fluoroquinolones with mutations in the *gyrA* and *gyrB* genes in a subset of clinical isolates from the KZN Province of South Africa.

Materials and methods

Clinical isolates

The isolates used in this study were retrieved from the culture collection in the Department of Infection Prevention and Control, Nelson R Mandela School of Medicine, School of Laboratory Medicine and Medical Science, College of Health Science, University of KwaZulu-Natal. We included the following phenotypes: ten fully drug-susceptible (DS), 20 MDR, and 30 XDR. The isolates were collected between 2005 and 2008 from patients in

Umzinyathi District, KZN, South Africa. H37Rv was included as the reference strain. Ethical approval for the study (BREC 247/09) was granted by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal.

MIC determination

MIC determination of the drugs was performed by means of the agar dilution method using Middlebrook 7H10 (BD, Franklin Lakes, NJ, USA) media supplemented with oleic acid–albumin–dextrose–catalase (BD). The drug concentrations used ranged from 0.03 to 8 mg/L for CPX, OFX (Sigma-Aldrich, St Louis, MO, USA), and MXF (Bayer, Leverkusen, Germany). Following inoculation, plates were incubated in a CO_2 (5%)-enriched atmosphere at 37°C for 21 days. MIC values were recorded as the lowest concentration of the drug that resulted in complete inhibition of growth. The cutoff value for resistance ≥2 mg/L for CPX and OFX according to WHO recommendations and ≥0.5 mg/L for MXF, as described by Angeby et al.[11,12] All MIC experiments were carried out in triplicate.

DNA extraction and PCR

DNA was extracted using cetyl-trimethyl-ammonium bromide-sodium chloride, as previously described.[13] The quinolone resistance-determining region (QRDR) and flanking regions of the *gyrA* and *gyrB* genes were amplified using primer pairs designed for this study: *gyrA* forward (CGATTGCAAACGAGGAATAG), *gyrA* reverse (GGCCAGTTTTGTAGGCATCA), and *gyrB* forward (ATCAACCTGACCGACGAGAG), *gyrB* reverse (GCCGAGTCACCTTCTACGAC).[14] Polymerase chain reaction (PCR) was performed using the Expand high-fidelity PCR system (dNTPack; Hoffman-La Roche, Basel, Switzerland).

Cycling conditions were as follows: initial denaturation at 94°C for 2 minutes; 40 cycles of denaturation at 94°C for 45 seconds, annealing at 53°C (*gyrA)* or 56°C (*gyrB)* for 45 seconds

and extension at 72°C for 45 seconds; and a final extension of 7 minutes at 72°C. The quality of PCR amplicons was checked on a 1% agarose gel.

Sequencing PCR products were purified using the Invitrogen PureLink® PCR purification kit (Thermo Fisher Scientific, Waltham, MA, USA). The sequencing reactions were performed using an ABI Prism BigDye® Terminator cycle-sequencing kit 3.1 (Thermo Fisher Scientific) with the same forward primers as used for PCR amplification. Geneious version 5.5.7 sequence-analysis software was used to identify mutations in the final nucleotide sequences in comparison to the MTB H37Rv reference strain.[15]

Genotyping

Genotyping was performed by IS*6110* restriction fragment length polymorphism (RFLP) analysis using the Southern blot hybridization method, as previously described.[16]

Results

Isolates

Of the 60 isolates selected, four MDR isolates did not grow sufficiently on retrieval subculture and were excluded from further analysis. MIC and sequencing data were therefore obtained for ten DS, 16 MDR, and 30 XDR isolates.

MIC results

The DS and MDR isolates displayed MICs for CPX, OFX, and MXF in the susceptible range. All 30 XDR isolates tested displayed MICs for CPX, OFX, and MXF that were in the resistant range (Table 4.1).

Sequencing of the gyrA *and* gyrB *genes*

Three mutations were observed in the nucleotide sequence of the *gyrA* gene: E21Q (GAA→CAA), S95T (ACG→ACC), and A90V (GCG→GTG). E21Q and S95T were present in all 56 isolates, regardless of MIC values (Table 1). The A90V mutation was present only in the 30 XDR isolates, correlating with an MIC value of 8 mg/L for CPX and OFX. In the case of MXF, 23 of the 30 XDR isolates had an MIC value of 2 mg/L, while seven had an MIC value of 1 mg/L. Ten MDR isolates with an MIC for MXF at the proposed breakpoint for resistance, ie, 0.5 mg/L, did not display the A90V mutation. No mutations were detected in the *gyrB* genes of the isolates tested.

*IS*6110 *restriction fragment length polymorphism analysis*

IS*6110* RFLP analysis revealed that 35 of the 56 isolates belonged to the F15/LAM4/KZN family of strains. This included all 30 XDR isolates and five MDR isolates. Seventeen of the remaining isolates belonged to recognized strain families (F28, F11, and Beijing) while four showed a unique RFLP profile.

Discussion

We report on the correlation between MICs of MXF and mutations in the *gyrA* gene in a selection of clinical isolates in KZN, South Africa. Fluoroquinolone resistance in MTB is most frequently attributed to mutations occurring in the QRDR of the *gyrA* gene. The QRDR of the *gyrA* gene consists of a short region, coding for amino acids 74–113. In our study, we sequenced the QRDR of both the *gyrA* and *gyrB* genes, as well as flanking regions. We found the C269T mutation within the QRDR of the *gyrA* gene, which corresponds with the amino acid change A90V, correlated with the high MICs seen in the XDR MTB isolates that we studied. The A90V mutation in *gyrA* has been described as one of the most frequent mutations associated with fluoroquinolone resistance.[17] In our study, based on WHO-

recommended breakpoints, the A90V mutation in XDR isolates was linked to resistance in all three fluoroquinolones tested.[11]

We did not find mutations in *gyrB* in any of the isolates tested. This supports previous observations that mutations within the *gyrB* gene are rare in MTB.[8,17] Maruri et al conducted a systematic review to evaluate gyrase mutations associated with fluoroquinolone resistance in MTB. The study reported on 534 fluoroquinolone-resistant MTB isolates, of which 17 (3%) harbored mutations within the QRDR of the *gyrB* gene. In addition, four different numbering systems were used to report on mutations in the *gyrB* gene, resulting in major discrepancies. The authors proposed a uniform numbering system in an attempt to improve the molecular detection in the gyrases.[18] The significance of mutations within the QRDR of the *gyrB* gene cannot be ignored, and is thought to play an important role in resistance.[9]

WHO guidelines propose 0.5 mg/L as the breakpoint MIC for susceptibility testing of MXF in the BACTEC™ 460 system (BD) and 0.25 mg/L in the BACTEC MGIT 960 (BD) system.[11] Breakpoints for other test systems are not proposed. Angeby et al demonstrated comparable MIC results for MXF on Middlebook 7H10 agar and the BACTEC 460 system.[12] With Middlebrook 7H10 plates used for MIC determination, all our XDR isolates had MICs of >0.5 mg/L. As per the WHO definition, these are classified as MXF-resistant, and this implies that MXF should not be recommended for treatment of cases harboring such isolates. However, there are reports of MXF efficacy in isolates with MXF MICs <2 mg/L.[3,4,6,10,19]

Poissy et al used the murine model to demonstrate that MXF is effective against OFX-resistant strains. They reported that MXF was most effective on MTB strains with MICs ≥0.5 mg/L. Reduced mortality was observed in mice infected with strains, with MICs ≥2 mg/L compared to untreated controls.[4] Fillion et al demonstrated similar findings using the murine model to determine the effect of a multidrug regimen containing MXF. The sterilizing

activity of the multidrug regimen decreased in strains with increased MICs to MXF. The impact of the sterilizing activity of the most effective second-line treatment regimen (ie, ethionamide, pyrazinamide, amikacin, and MXF) is dependent on the MIC of MXF, and thus the MIC of MXF has to be determined for all strains resistant to OFX. Mice infected with strains with MXF MICs of 0.5 mg/L and 4 mg/L recorded relapse rates of 50% and 86%, respectively, compared to the wild type.[10]

Sirgel et al proposed that MXF and OFX are possibly not equally affected by mutations associated with fluoroquinolone resistance.[6] They concluded that the use of MXF for the treatment of infection with OFX-resistant strains is justified when combined with other drugs. They further suggested that the low recommended breakpoint of 0.5 mg/L determining MXF resistance may therefore give a false impression of clinical inactivity. Poissy et al and Sirgel et al support WHO recommendations on the use of MXF for the treatment of XDR provided that the infecting isolate has an MXF MIC of <2 mg/L.[4,6]

Feasey et al reported on a case where high-dose MXF (600 mg/day) in combination with PZA, CAP, LIN, PAS, and amoxicillin clavulanic acid for 22 months successfully treated a case with an infecting isolate that was resistant to isoniazid, rifampicin, ethambutol, prothionamide, OFX, streptomycin, and MXF (MIC of 2 mg/L). While high-dose MXF treatment increases the peak plasma (MXF), resulting in levels that remain constantly above the MIC, it is difficult to assess the exact role of MXF in the successful management of this patient, since other drugs with known efficacy were also included in the treatment regimen.[19]

Jacobson et al conducted a meta-analysis to assess treatment outcomes in patients with XDR. The report summarized 13 studies conducted mainly amongst HIV-uninfected people, who received a new-generation fluoroquinolone together with other anti-TB drugs. They

concluded that the addition of MXF to XDR regimens may improve outcomes, but further evaluation in clinical trials is warranted.[3]

In KZN, the regimen used for XDR treatment is a combination of drugs with proven and putative efficacy. The National Department of Health in South Africa recommends the use of MXF as part of XDR treatment in the presence of OFX resistance. Fluoroquinolones are added despite in vitro reports of resistance in the hope that there may be some residual activity. It is debatable whether the perceived benefit of using MXF under these circumstances outweighs the risks caused by side effects of this drug or the increased exertion of antibiotic pressure in the era of ever-increasing drug resistance.[20] Mendel and Springsklee[21] warned that the use of newer-generation fluoroquinolones in patients that display low-level resistance will be disastrous from a public health perspective. The use of MXF in such cases will result in the ready emergence of highly resistant strains unless drug concentrations are sustained above mutant-prevention concentrations at all infection sites. The latter is extremely difficult to achieve, and thus the use of MXF in patients with resistance to older-generation fluoroquinolones will only further drive resistance among XDR strains of MTB.

Although all isolates used in this study were from different patients, the XDR isolates displayed a high degree of similarity and belonged to a single genotype, ie, F15/LAM4/KZN. To date, all reports from KZN have attributed XDR to this strain. Ramtahal showed that the spread of XDR in KZN was clonal with the F15/LAM4/KZN strain.[22] Clonal spread of this strain has been ongoing since at least 2005.[23] During this period, further acquisition of resistance may have occurred. We therefore performed susceptibility testing and sequencing on 30 XDR isolates belonging to the only XDR strain family currently in KZN. Seven of the isolates had MICs of 1 mg/L, and 23 had MICs of 2 mg/L. Basic microbiological principles regarding in vitro determination of MICs allows for one twofold MIC variation between

tests. This implies that our XDR isolates may in their most susceptible form have MICs between 0.5 and 1 mg/L and in their most resistant form from 2 to 4 mg/L.[24] Gandhi et al found that a large variety of strains were associated with DS, and this decreased as the degree of resistance increased. The low diversity of strains driving the MDR and XDR epidemics supports the theory of clonal expansion of drug-resistant phenotypes in KZN. This picture is different in other parts of South Africa. In the Eastern and Western Cape Provinces, the Beijing strain is accountable for the majority of XDR. Strains responsible for MDR and XDR in other provinces include the S, T1, and other families. The reasons for geographic differences remain uncertain.[25]

Conclusion

Regardless of the strain family implicated in infection with XDR, the breakpoint for resistance to MXF remains the subject of debate. Our results support concerns regarding the use of MXF in KZN. While there may be a role for MXF as part of individualized XDR treatment regimens, this cannot be advocated as part of empiric treatment protocols in the absence of MXF MIC data of the circulating XDR strains in an area. In addition, validation from larger population-based studies using MXF in combination with various other antidrug regimes must be conducted. Early bactericidal assays with MXF will also give useful data to inform our practice.

Disclosure

The authors report no conflicts of interest in this work.

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Table 4.1 RFLP strain families of *Mycobacterium tuberculosis* isolates stratified by MIC and resistance conferring mutations in the *gyrA* gene

Strain family	Isolates, n (phenotype)	Mutations associated with resistance	MIC (mg/L)[a]		
			CPX	OFX	MXF
F15/LAM4/KZN	23 (XDR)	A90V	8	8	2
	7 (XDR)	A90V	8	8	1
	4 (MDR)	None	1	1	0.5
	1 (MDR)	None	1	1	0.25
F28	5 (MDR)	None	1	1	0.5
	3 (MDR)	None	1	1	0.25
	1 (MDR)	None	0.5	0.5	0.5
	1 (MDR)	None	0.5	0.5	0.25
LAM3/F11	1 (DS)	None	0.5	0.5	0.125
Beijing family	6 (DS)	None	0.5	0.5	0.125
Unique	3 (DS)	None	0.5	0.5	0.125
	1 (MDR)	None	0.5	0.5	0.25

Note: [a]Resistance defined as 2 mg/L for CPX and OFX and 0.5 mg/L for MXF.

Abbreviations: RFLP, restriction fragment length polymorphism; MIC, minimum inhibitory concentration; CPX, ciprofloxacin; OFX, ofloxacin; MXF, moxifloxacin; XDR, extensively multidrug-resistant; MDR, multidrug-resistant; DS, drug-susceptible.

Chapter Five

Manuscript IV:

Evaluation of Capreomycin in the treatment of the F15/LAM4/KZN extensively drug-resistant strain of *Mycobacterium tuberculosis*.

Currently under review by the Journal of Chemotherapy (Manuscript number: JOC 745)

Abstract:

Capreomycin has been advocated for the treatment of extensively drug resistant tuberculosis without drug susceptibility testing. We investigated the relationship between the minimum inhibitory concentration of CAP and mutations in the *rrs, tlyA* and *eis* genes in *Mycobacterium tuberculosis* isolates from KwaZulu-Natal, South Africa. MIC data of 56 isolates were compared to mutations in the *rrs*, *tlyA* and *eis* genes. Mutational analysis identified the A1401G mutation in the *rrs* gene in 30 XDR-TB isolates with an MIC of ≥ 16 mg/L for CAP and ≥ 128 mg/L for kanamycin and amikacin. Genotypic analysis revealed that the XDR-TB strains were clonal belonging to the F15/LAM4/KZN strain family. The MICs of CAP reveal that the use of the drug in XDR-TB treatment protocols requires careful re-consideration in the KZN setting. A review of the current breakpoint value for CAP will be invaluable in improving the quality of drug susceptibility testing against MTB.

Introduction

In 2013, 9.0 million new cases of tuberculosis (TB) and 1.5 million TB-related deaths were reported worldwide. While the global incidence of TB has decreased, multidrug-resistant (MDR-TB) and extensively drug resistant (XDR-TB) remain a major public health concern in several countries. MDR-TB, as defined by World Health Organisation (WHO), are *Mycobacterium tuberculosis* (MTB) strains that are resistant to isoniazid (INH) and

rifampicin (RIF). XDR-TB strains are characterized by resistance to INH and RIF (ie MDR-TB) with additional resistance to a fluoroquinolone and one of the three injectable agents: amikacin (AMIK), kanamycin (KAN) and capreomycin (CAP).[1]

The treatment of XDR-TB is a major challenge given the paucity of effective drugs available. Antimicrobial regimens that are currently used to treat XDR-TB, often do not take cross resistance among drug classes into consideration. In addition, inclusion of anti-TB drugs into the XDR-TB treatment protocols are often advocated without appropriate drug susceptibility testing (DST) of locally prevalent XDR-TB strains.

In 2006, a report by Gandhi *et al.*[2] underscored the drug resistance TB problem in which the largest ever outbreak of XDR-TB was described in Tugela Ferry, KwaZulu-Natal, South Africa. The isolates from TB cases described in this outbreak were resistant to INH, RIF, KAN, and ofloxacin (OFX). [2] This prompted the Department of Health in South Africa to look at alternatives for the treatment of patients with XDR-TB. It was recommended that two drugs that were previously used to treat TB, be reintroduced for the management of XDR-TB cases.[3]

The drugs recommended were CAP (a polypeptide with a similar mode of action to aminoglycosides) and para-amino salicylic acid (PAS). The Medicines Control Council (MCC) registration for the latter was still in effect. However, MCC registration for CAP had expired, and a new approval was sought and granted. It was presumed that strains of XDR-TB would be susceptible to CAP and PAS, and susceptibility testing for these drugs against XDR-TB strains was therefore not performed at the outset. [3]

The treatment of MDR-TB in South Africa includes an aminoglycoside i.e. KAN or AMIK. With the emergence of XDR-TB strains in KZN, CAP became an integral part of treatment regimens amid extensive aminoglycoside resistance. [4] The aminoglycosides (KAN and

AMIK) and cyclic polypeptides (CAP) are protein synthesis inhibitors that act by binding to the bacterial ribosome resulting in a modification of the 16S rRNA structure.[5] Resistance to all three of these antibiotics have b4een linked to mutations in the *rrs* gene coding for 16S rRNA and the *tlyA* gene which encodes an rRNA 2' –O-methyltransferase responsible for methylation of nucleotide C1409 in helix 44 of the 16S rRNA. In addition, cross resistance amongst these agents has been reported.[6-9] Recent reports have demonstrated that mutations in the *eis* promoter, a putative enhanced intracellular survival protein in *M.Smegmatis*, is linked to KAN resistance. [5, 9, 10]

Rapid molecular diagnostic assays used to identify drug resistance in clinical isolates rely on the identification of mutations in known resistance conferring genes. Whilst this phenomenon is well established for first line agents, molecular based testing for resistance to second line agents has not been well characterized. A greater understanding of these mechanisms is imperative to appropriately guide treatment in patients with MDR-TB and XDR-TB. [5, 6, 8]

The aim of this study was to correlate the Minimum Inhibitory Concentration (MIC) levels of the aminoglycosides and CAP with mutations in the genes known to confer drug resistance in a subset of clinical isolates from the KZN province of South Africa.

Materials and Methods

Clinical Isolates

A total of 60 isolates were included in this study: 10 drug susceptible (DS), 20 MDR and 30 XDR. The isolates were collected during 2005-2008 from in and out patients at the Church of Scotland Hospital, Tugela Ferry, KZN, South Africa. H37Rv was included as the reference strain. Ethical approval for the study (BREC 247/09) was granted by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal.

MIC Determination

We established the MIC on Middlebrook 7H10 agar (BD, Difco Laboratories, USA) medium supplemented with oleic acid-albumin-dextrose-catalase (OADC) (BD, Difco Laboratories, USA) using the agar dilution method. Bacterial cultures were prepared by adjusting the bacterial suspension to give a turbidity equivalent to that of a 1.0 McFarland standard and then diluted to obtain a final bacterial density of 1 x 10^{-3} colony forming units per ml. 0.1ml was used to inoculate each quadrant of the Middlebrook 7H10 agar plates with dilutions ranging from 0.06 – 16 mg/L for CAP and 0.5 – 128 mg/L for KAN and AMIK. All drugs were obtained from Sigma-Aldrich (Capital Lab Supplies, South Africa). Plates were incubated in a CO_2 enriched atmosphere at 37ºC for 21 days. The MIC was defined as the lowest antibiotic concentration that resulted in a complete inhibition of growth. Resistance was determined at a critical concentration of 5 mg/L for KAN, 4 mg/L for AMIK and 10mg/L for CAP. [11] All experiments were carried out in triplicate to ensure accuracy and reproducibility.

Gene Amplification and Sequencing

Genomic DNA was isolated using the CTAB-NaCl (Cetyl-trimethyl-ammonium Bromide-Sodium Chloride) as described previously. [12]

PCR amplification of all 56 isolates for the *rrs* gene was performed using the Expand High Fidelity PCR system, dNTPack (Roche Diagnostics, Mannheim, Germany) and primers KM-SA, KM-RA, P1 and P2 and cycling protocol as described previously. [6] The *tlyA* gene was amplified using newly designed primers: *tlyA*F (AAGGCATCGCACGTCGTCTTTCC) and *tlyA*R (TGTCGCCCAATACTTTTTCTACGC). The *eis* gene was amplified using primers AZ80 and AZ87.[7] The cycling protocol utilised was an initial denaturation at 94ºC for 2 mins followed by 40 cycles of denaturation at 94ºC, annealing at 60ºC *(tlyA)* and 58 ºC *(eis)*;

extension at 72ºC for 45secs each and a final extension of 10 min at 72ºC. The quality of PCR products were checked on a 1% agarose gel purified using the Invitrogen PureLink PCR purification kit (Applied Biosystems, South Africa), matching 831bp and 920bp for the 2 regions of the *rrs* gene. The *tlyA* gene yielded a 981bp product and the *eis* gene 567 bp product.

Direct single stranded sequencing of the *rrs* and *tlyA* amplicons was performed using ABI Prism Big Dye Terminator cycle sequencing kit V3.1 (Applied Biosystems, South Africa) with the forward primers used for PCR amplification. Geneious V5.5.7 [13] sequence analysis software was used to detect mutations in the final nucleotide sequences in comparison to the H37Rv reference strain.

RFLP Analysis

Isolates were genotyped using the IS*6110* restriction fragment length polymorphism (RFLP) method as described previously. [14] The Bionumerics version 3.5 software (Applied Maths, Kortijk, Belgium), was used for the analysis of IS*6110* RFLP patterns.

Results

Of the sixty isolates selected for analysis, 4 MDR-TB isolates failed to grow sufficiently on sub- culture and had to be excluded. Therefore, we report MIC and sequencing data for 10 SUS, 16 MDR-TB and 30 XDR-TB isolates.

MIC Results

The 30 XDR-TB isolates were found to be resistant to KAN, AMIK and CAP with MIC's of > 128mg/L for KAN and AMIK and ≥ 16 mg/ L for CAP. The isolates classified as SUS and MDR-TB displayed MICs for KAN, AMIK and CAP in the susceptible range. The MIC profiles for KAN, AMIK and CAP are shown in Table 5.1.

Sequencing of the of the *rrs* and *tlyA* genes

Sequencing of the 56 isolates revealed 2 mutations: The *rrs* A1401G and the *tlyA* A33G. The *rrs* A1401G mutation was found in all 30 XDR-TB isolates, correlating with MIC values ≥128 µg/ml for KAN and AMIK and ≥16µg/ml for capreomycin. The SUS and MDR-TB isolates did not display any mutations in this gene. The *tlyA* A33G mutation was present in all 56 isolates, regardless of MIC values. No mutations were detected in the *eis* genes in the isolates tested. Sequencing results are shown in Table 5.2.

RFLP Genotyping Results

RFLP analysis revealed that the majority of the isolates belonged to the F15/LAM4/KZN lineage, whereas the remaining isolates belonged to recognised strain families (F28/F11 and Beijing). Four isolates used in the study displayed a unique RFLP profile. The A33G *tlyA* mutation was found in all isolates whilst the A1401G *rrs* mutation was found only in the F15/LAM4/KZN lineage. RFLP profiles, mutations and associated phenotypes are shown in Table 5.2.

Discussion

In this study, we report on the association between MICs of CAP and the aminoglycoside antibiotics and mutations in the *rrs*, *tlyA* and *eis* genes in clinical isolates of MTB from our setting. Resistance to the injectable antibiotics are commonly associated with the A1401G mutation in the *rrs* gene. [5-9] In our study, sequencing revealed the A1401G mutation in the *rrs* gene correlated with high level resistance in the F15/LAM4/KZN XDR-TB strains. The five F15/LAM4/KZN MDR-TB strains susceptible to CAP did not carry the *rrs* A1401G mutation. It has been reported that the A1401G *rrs* mutation alone detected approximately 70-80% of CAP and AMIK resistance and 60% of KAN resistance, globally. [9] Our findings

show that the A1401G mutation in XDR-TB isolates confers high level resistance to KAN and AMIK and a decreased susceptibility to CAP, corroborating previous reports. [5, 6, 8, 9]

Mutations in the *tlyA* gene have been implicated in CAP resistance. Sequencing of the *tlyA* gene revealed the A33G mutation in all the isolates tested. Sowajassatakul *et al*.[15] recently reported that the *tlyA* A33G substitution does not result in any amino acid changes, suggesting that this mutation is merely a natural polymorphism with no link to the resistant phenotype. [15] The results of this study corroborates this finding. Although mutations conferring resistance in the *tlyA* gene have been reported in only 1-3% of CAP resistant strains, there are no reports of these mutations in CAP sensitive strains therefore, the significance of *tlyA* mutations should not be ignored as they have potential to serve as high predictor of CAP resistance.[9]

Mutations in the promoter region of the *eis* gene, coding for an aminoglycoside acetyltransferase, are associated with low-level KAN resistance.[9] In our study, no mutations were detected in any of the isolates studied. Common mutations associated with resistance occur at positions -10, -14 and -37. *eis* promoter mutations are reported to occur exclusively in KAN resistant isolates with no additional mutation in *rrs* gene, therefore, they have a greater predictive potential for detecting KAN resistance than using the A1401G *rrs* mutation alone. [9, 10]

The emergence of MDR-TB and XDR-TB has created a higher demand for antimicrobial DST. Current DST against *M.tuberculosis* is based on the testing of an antimicrobial against a set concentration known as the breakpoint concentration; defined as the lowest concentration of drug that will inhibit 95% (90% for pyrazinamide) of wild-type strains that have never been exposed to the drug, while not inhibiting clinical strains that display resistance to the drug. Angeby *et al.*[16] considers this definition to be flawed as it categorises

5% of the wild-type strains to be resistant. In addition, combination therapy is mandatory for the treatment of *M.tuberculosis* and therefore clinical outcome data for individual drugs are not readily available. They propose the use of wild-type MIC distributions for indicating susceptibility breakpoints for *M.tuberculosis.* The highest MIC value within the wild-type MIC range is defined as the epidemiologic cut-off value (ECOFF), is considered a more accurate tool for indicating susceptibility breakpoints for *M.tuberculosis.* [16] The tentative ECOFF value for CAP is 4 mg/L whilst current WHO guidelines on DST recommend a breakpoint concentration of 10 mg/L for CAP on Middlebrook agar. [11,16] By applying the ECOFF value of 4mg/L for CAP, all SUS and MDR-TB isolates used in our study with MICs of 4mg/L display low-level resistance to CAP. Reeves *et al.*[17] reported on the disparities associated with the *rrs* A1401G mutation. They conducted MIC's in clinical isolates and genetically engineered mutants that had clean genetic backgrounds and no prior exposure to the drug. The MIC's for the clinical isolates ranged from 8 mg/L to 40 mg/L. All the genetically engineered mutants had an MIC of 40 mg/L. They suggest a re-evaluation of the current breakpoint value for CAP and that the differences in resistance levels are due to compensatory or second site mutations.[17]

CAP resistance was reported in the F15/LAM4/KZN strain prior to the use of the antibiotic in South Africa.[18] This phenomenon was also reported by Sirgel *et al*. [8] in the Eastern Cape region of South Africa and Jugheli *et al*. [6] in isolates from Georgia. This suggests that CAP resistance occurs as a result of cross resistance to KAN and AMIK and that the drug therefore has no clinical relevance in treatment regimens for XDR-TB. [6,8]

Sirgel *et al*. [8] suggests that CAP MIC's in the range of 10-15 mg/L warrants the clinical use of CAP in treatment of patients infected with strains resistant to low level CAP based on the fact that it is below achievable serum levels. [11] In our subset of isolates, all XDR-TB isolates displayed MIC's of ≥16 mg/L. This MIC could in fact be much higher as basic

microbiological principles for *in vitro* MIC determination allows for one twofold variation of the real end point. Thus, our XDR-TB isolates could in their most resistant form have MIC's of 32-64 mg/L which exceed the peak serum concentrations levels of 20-47 mg/L for CAP. Therefore, the concentration of the drug will be inadequate for patient's harbouring strains resistant to CAP. Furthermore, we believe that the use of CAP in the treatment of patients with low-level CAP resistance will only drive further resistance in these isolates.

Although all isolates used in this study were from different patients, the XDR-TB isolates belonged to the F15/LAM4/KZN genotype. Thus far, all reports of XDR-TB in the KZN province of South Africa have been attributed to this genotype. [19] The strains analysed in this study are highly resistant, presenting clinicians with few therapeutic options. The aminoglycosides, CAP and the fluoroquinolones remain the most effective agents in the treatment of drug-resistant strains of MTB. The present study has, consequently, important implications for the treatment of XDR-TB in South Africa. The re-introduction of CAP in drug regimens for treatment in patients with resistance to KAN or AMIK in the absence of drug susceptibility testing possibly amplified this drug resistance. We suggest that the current breakpoint concentration set out for CAP needs to be revised in order to prevent patients from receiving inadequate treatment that could lead to the development of further resistance, continued transmission of drug resistant strains as well as death of the patient. However, this can only be determined if CAP is assessed in combination with suitable companion drugs, which may not be available. Therefore, the clinical use of CAP needs careful re-consideration for treatment of XDR-TB cases in KZN province.

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Tables

Table 5.1: MIC profiles for KAN, AMIK and CAP in 56 isolates from patients from KwaZulu-Natal, South Africa

No of Isolates	MIC (mg/L)		
	KAN	AMIK	CAP
30 (XDR-TB)	>128	>128	≥16
1 (MDR-TB)	4	2	8
5 (1 SUS, 4 MDR-TB)	4	2	4
11(5 SUS, 6 MDR-TB)	2	2	4
9 (4 SUS, 5 MDR-TB)	2	1	4

Table 5.2: RFLP analysis of *M. tuberculosis* isolates with mutations and their associated phenotypes

Strain Family	Mutations			No of Isolates
	rrs	*tlyA*	*eis*	(Phenotype)
F15/LAM4/KZN	A1401G	A33G	None	30 (XDR-TB)
	None	A33G	None	5 (MDR)
F28	None	A33G	None	10 (MDR)
LAM3/ F11	None	A33G	None	1 (SUS)
Beijing Family	None	A33G	None	6 (SUS)
Unique	None	A33G	None	3 (SUS)
	None	A33G		1(MDR-TB)

Chapter Six

Manuscript V:

KZN Multidrug and Extensively drug resistant strains of Mycobacterium tuberculosis remain susceptible to Linezolid and para-Amino salicylic Acid.

Submitted to the South African Medical Journal

Abstract

Background: Strategic application of Linezolid (LIN) and para-Amino salicylic acid (PAS) may play an important role in the management of drug resistant tuberculosis in our setting.

Objectives: The aim of this study was to screen clinical isolates of *M.tuberculosis* for resistance LIN and PAS.

Methods: In an attempt to detect resistance to LIN and PAS, we screened the *rrl* and *thyA* genes of drug susceptible, multi drug-resistant (MDR) and extensively drug-resistant (XDR) clinical isolates of *Mycobacterium tuberculosis.*

Results: No resistance mutations were detected in the *thyA* and *rrl* genes in the isolates screened. The presence of the Thr202Ala mutation in *thyA* and the G2399A mutation in the *rrl* gene was associated with strains of Latin American Lineage and played no role in mediating resistance to the drugs.

Conclusions: Failure to associate LIN or PAS resistance to the most common mechanisms described underscores the need for more effective screening of novel targets and detection of resistance mechanisms that can be added to rapid diagnostic assays to effectively diagnose and detect drug resistance concurrently.

Background

Drug resistant tuberculosis (TB) is major public health concern, associated with significant morbidity and mortality. The recent emergence of totally drug resistant (TDR) TB, defined as strains resistant to all the current first and second line drugs in TB treatment regimens, has the potential to completely disrupt TB control programs.(1) Treatment of multidrug resistant (MDR) TB; i.e. strains resistant to isoniazid and rifampicin, relies on the use of second line anti-TB drugs such as the fluoroquinolones and injectable aminoglycosides which are less effective and have higher toxicities. Additional acquisition of resistance to the second line anti-TB drugs defines extensively drug resistant (XDR) TB, associated with poor treatment outcomes.(2,3). Given the paucity of new drugs available for the treatment of XDR-TB, it is imperative that *Mycobacterium tuberculosis* isolates from patients must be tested for susceptibility to anti-TB drugs to ensure appropriate treatment choices are made and to prevent further resistance.(4) Two such drugs, para-amino salicylic acid (PAS) and Linezolid (LIN) are treatment options for XD-TB in our setting. Resistance to each of these drugs, although rare, are believed to be mediated through mutations in the *thyA* (5,6) and *rrl* (7) genes, respectively. Recent studies have demonstrated improved treatment outcomes in patients with complicated MDR and XDR when treated with LIN.(8) The aim of this study was to screen clinical isolates of *M.tuberculosis* for mutations in the *rrl* and *thyA* genes associated with resistance LIN and PAS respectively.

Materials and Methods:

Clinical Isolates

The *M. tuberculosis* H37Rv laboratory strain and 60 stored clinical isolates, collected from 2005-2009 at the Church of Scotland Hospital in the Tugela Ferry region of KwaZulu-Natal were selected from the culture collection from the Infection Control laboratory, University of

KwaZulu-Natal. The samples represented varying antibiotic resistance patterns, comprising 10 drug susceptible (DS), 17 multi-drug resistant (MDR-TB) and 30 extensively drug resistant (XDR-TB) isolates. Genotyping and drug susceptibility profiles were determined previously in our laboratory, using standard mycobacteriology protocols. Ethics approval for the study was obtained from the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (BREC 247/09). DNA was isolated from the cultures using the CTAB-NaCl (Cetyl-trimethyl-ammonium Bromide-Sodium Chloride) method, as previously described. (9)

PCR Amplification and Sequencing

PCR assays for the *rrl* gene was conducted as previously described.(7) Primers for *thyA* gene; FWD GCCTCCGTTGTACTCCTGTG and REV TGTCGCCCAATACTTTTTCTACGC; were designed for the current study and amplified using the Expand High Fidelity PCR kit (Roche) according to the kit guidelines, with annealing temperature of 60◦C. Each PCR yielded amplicons of 835bp and 850bp, respectively. PCR amplicons were verified on a 1% agarose gel and purified using the Invitrogen PureLink PCR purification kit (Applied Biosystems). Direct single stranded sequencing of the amplicons were conducted using ABI Prism Big Dye Terminator cycle sequencing kit V3.1 (Applied Biosystems) with the forward primers used for PCR amplification. The reactions were cycled in accordance with the manufacturer's guidelines. Sequence chromatograms were analysed for the presence of mutations by comparison of the corresponding reference sequence of the H37Rv reference strain using Geneious V5.5.7 sequence analysis software program.

Results and Discussion

Three XDR-TB isolates failed multiple attempts at amplification of the *rrl* gene and were excluded from the analysis.

The mutations detected in the study were of 2 types: (1) A → G substitution at position 604 of the *thyA* gene (Thr202Ala) and (2) G → A substitution at position 2399 of the *rrl* gene. Both these mutations were detected in 5 MDR-TB isolates and all the XDR-TB isolates screened. All isolates with the mutations belonged to the F15/LAM4/KZN strain. Table 6.1 shows the frequency of the mutations detected and the associated genotypes. The former, Thr202Ala, initially implicated in PAS resistance, is now believed to be a phylogenetic marker for the Latin American Lineage (LAM) strain.(10) The results of this study support the latter findings. The G2399A mutation of the *rrl* gene has been reported in isolates from Brazil and South Africa in 2004 (Listed on the Broad Institute TB mutation database). More recently, a study conducted in our laboratory reported this mutation in drug susceptible (DS) and resistant isolates demonstrating that this mutation does not mediate LIN resistance.(11) The isolates analysed in the study all belonged to the F15/LAM4/KZN strain. We postulate that this mutation maybe a marker for this strain type rather than a resistance mutation.

Moodley *et al.* also demonstrated that the KZN XDR-TB isolates are still eligible for treatment by the drugs. Time kill data demonstrated that PAS was effective against 50% of the drug resistant isolates while LIN was effective against 80% of the drug resistant isolates.(11) However, the use of these drugs should be applied in the presence of drug susceptibility tests. Alternate mechanisms of drug resistance such as efflux pumps and mutations in other target genes have been proposed in the resistance of LIN. Richter *et al*. demonstrated a reduction in the MIC of LIN in the presence of efflux pump inhibitor, reserpine.(7) Mutations in the *rplC* gene, encoding the 50S ribosomal protein L3 in *in-vitro* selected mutants and clinical isolates have also been implicated in LIN resistance. These mechanisms require further validation.(1)

In the case of PAS, no resistance mutations were detected in the *thyA* gene amongst isolates of the KZN strain family. (11) This is in contrast to the report where the Thr202Ala mutation

was reported as a specific marker of the LAM lineage. The role of *folC* gene mutations has been implicated in PAS resistance and requires further validation. (12)Resistance mechanisms to PAS remains uncertain, with about 40% of resistance attributed to *thyA* mutations.(1,13) LIN is currently included in newer drug resistant TB treatment regimens under evaluation in clinical trials. With proven clinical efficacy against MDR and XDR-TB, the utility of these drugs need to be preserved in order to effectively treat drug resistant tuberculosis.(8)

Conclusion

The absence of proven resistance mechanisms of LIN and PAS underscores the need for more effective screening of novel targets and detection of resistance mechanisms that can be added to rapid diagnostic assays to effectively diagnose and detect drug resistance concurrently.

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Table 6.1: Mutations in the *thyA* and *rrl* genes and their associated genotypes in clinical isolates of *M. tuberculosis*.

Strain Classification	*thyA*		*Rrl*		Associated Genotype of Isolate with Mutation.
	No Mutation	Thr202Ala (A604G)	No Mutation	G2399A	
DS	10/10	0	10/10	0	-
MDR	12/17	5/17	12/17	5/17	F15/LAM4/KZN
XDR	0	30/30	0	27/27	F15/LAM4/KZN

Chapter Seven

Manuscript VI:

Efflux mediated drug resistance in clinical isolates of *Mycobacterium tuberculosis* in KwaZulu-Natal, South Africa.

Short Communication

Abstract

The global dissemination of drug resistant phenotypes of *Mycobacterium.tuberculosis* has turned the most effective anti-tuberculosis drugs currently available for the treatment of tuberculosis, ineffective in a subset of patients. Efflux mediated resistance in *M.tuberculosis* has emerged as a significant factor in drug resistance. Efflux pumps mediate the extrusion of antibiotics from the bacterial cell, allowing for the establishment of resistance conferring mutations, resulting in selection of resistant mutants. In this study, we assessed the role of efflux pumps in drug resistance of *M.tuberculosis*. We analysed a panel of clinical isolates that displayed low-level resistance to isoniazid, rifampicin, ciprofloxacin, moxifloxacin and kanamycin. In the presence of the efflux pump inhibitors reserpine, verapamil and thioridazine, a reduction in the MICs was observed. We provide evidence that efflux pumps mediate low-level drug resistance in clinical isolates from our setting. The results of this study support further studies to assess the effect of efflux pump inhibitor compounds as adjunctive treatment for drug resistant tuberculosis.

Text

Antimicrobial resistance in *M.tuberculosis* has been attributed to several mechanisms including mutations in genes coding for drug targets, decreased cell wall permeability and increased efflux pump activity. The role of efflux pump activity has recently been recognized

as a significant factor in the natural resistance in mycobacteria.[1,2] In contrast to the high-level resistance resulting from genetic mutations leading to altered drug targets, increased efflux activity has been associated with low-level resistance.[3] The decreased intracellular antibiotic concentration as a result of efflux, promotes the survival of a sub-population of bacteria exposed to sub-inhibitory levels of the antibiotic. This sub-population acquires resistance conferring mutations, leading to clinically significant levels of resistance.[4–6]Studies have reported the association of efflux pumps in low-level resistance to isoniazid (INH), rifampicin (RIF), aminoglycosides, fluoroquinolones, streptomycin, linezolid, tetracycline and chloramphenicol.[7–15] Efflux pump inhibitor (EPI) compounds, like reserpine (RES), verapamil (VERA) and thioridazine (THIO) have the ability to restore the activities of antibiotics.[13,16–20] They also have the ability to reduce the minimum inhibitory concentration (MIC) of anti- tuberculosis (TB) drugs, potentially reducing the dosage requirements for treatment. This could assist in decreasing the toxicity associated with second line anti- TB drugs.[21] The value of EPIs as adjunct therapy in TB treatment has been tested in patients and has shown potential to reduce the current treatment duration and has been effective in the treatment of multidrug resistant (MDR) and extensively drug resistant (XDR) TB.[16] The aim of this study was to assess the potential of RES, VERA and THIO in reducing the MICs of the key anti-TB drugs in *M.tuberculosis* isolates from KwaZulu-Natal, South Africa. *M. tuberculosis* H37Rv and stored clinical isolates were selected from the culture collection of the Infection Control laboratory, University of KwaZulu-Natal. The isolates selected for the study were MDR-TB isolates that displayed an increased MIC when compared to the DS isolates, but lacked the mutations in drug targets that were responsible for drug resistance in the XDR-TB isolates. These isolates subjected to MIC determination in the presence of RES (80mg/L)[22], VERA (50mg/L)[22] and THIO (16mg/L)[17] together with each of the antibiotics. The selected range of drug concentrations was based on the MIC of the isolates in the

absence of EPIs and reflects one concentration above and four below the MIC of each of the drugs. The MICs were determined in Middlebrook 7H10 agar medium supplemented with oleic acid-albumin-dextrose-catalase (OADC) using the agar dilution technique in quadrant petri dishes. Bacterial cultures were prepared by adjusting the bacterial suspension to give a turbidity equivalent to that of a 1.0 McFarland standard and then diluted to obtain a final bacterial density of 1 x 10^3 colony forming units per ml. One hundred microliters was inoculated on each quadrant of the Middlebrook 7H10 agar plates with drug concentrations ranging from 0.25 to 8 mg/L for INH, RIF and kanamycin (KAN) and from 0.06 to 2 mg/L for ciprofloxacin (CPX) and moxifloxacin (MXF). EPIs were added at their respective concentrations to detect a change in MIC. Plates were incubated in a CO_2 enriched atmosphere at 37ºC for 21 days. The MIC was defined as the lowest antibiotic concentration that resulted in a complete inhibition of growth. In the case of RIF, one of the isolates tested failed to grow and the second isolate tested recorded an MIC higher than its initial RIF MIC, thereby displaying growth at all concentrations. Therefore, we could not assess the change in MIC in this isolate. The INH MIC was reduced 3 to 4 fold in the presence of RES, 2 fold in the presence of VERA and 3 fold in the presence of THIO. In the case of the H37Rv laboratory strain, growth was only observed in the drug free medium control, indicating that the MIC for INH was < 0.25mg/L. All 3 EPIs reduced the MIC of KAN by 1 fold for the 2 clinical isolates tested. The MIC of the H37Rv lab strain was decreased by 1 fold in the presence of RES and VERA and 3 fold or more in the presence of THIO. RES failed to alter the MIC for MXF, whilst VERA reduced the MIC by 1 fold. The MIC for THIO were reduced 3 fold or more. In the case of H37Rv, growth was only observed in the drug free medium control, indicating that the MIC for MXF was < 0.06mg/L. In the case of CPX, one isolate failed to grow when sub cultured onto the MIC media. The MIC of the remaining clinical isolate, was decreased 3 folds in the presence of VERA and RES and 1 fold for

THIO. The MIC of H37Rv was decreased more than 3 folds in the presence of RES and 1 fold with VERA and THIO. The change in MIC for each of the drugs tested is shown in table 7.1. The results of this study demonstrate that efflux pumps, in addition to genetic mutations play a role in drug resistance in the clinical isolates from our setting. Studies have demonstrated that efflux pump activity results from exposure to decreased concentrations of a drug allowing for *M.tuberculosis* bacilli to survive. This subsequently leads to the selection of mutants resulting in high-level resistance.[23–25] The isolates analysed in the current study displayed low-level resistance in the absence of mutations. Based on WHO guidelines, we also demonstrated the ability of EPIs to reverse the resistance induced by efflux activity.[26] RES restored INH susceptibility, all 3 EPIs restored KAN and CPX, and VERA and THIO restored MXF susceptibility. Given the current paucity of effective drugs available for treatment of drug resistant TB, the possible role of these agents as adjunct therapy is encouraging. THIO has shown effective activity against MDR and XDR TB strains *in vitro* and in mice.[27] THIO in combination with MXF and LIN was recently evaluated in XDR TB patients on the basis of compassionate use. The combination was associated with cure, free of relapse. The addition of THIO was also associated with earlier bacteriological sputum conversion. [16] Recent reports have provided compelling evidence that the addition of verapamil as an adjunct to treatment with bedaquiline, a newly introduced anti-TB drug will aid in reducing the MIC of bedaquiline. As a result the amount of the drug to be administered per dose could be decreased decreasing the chance of cardiac morbidity associated with the drug. [21,28] The application of EPIs to current regimens has been reported to shorten the duration of treatment. This has important implications for patient adherence and will significantly impact on treatment outcomes. [17,29,30] Of significance, EPIs can be applied to TB treatment, independently of the level of resistance of the organism.[17] In conclusion, we

provide proof of concept that efflux pumps mediate low-level resistance in our setting and support further exploration of efflux pump inhibitors for treatment of TB.

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Table 7.1: MICs in the presence of efflux pump inhibitors.

Isoniazid MICs (mg/L)				
Isolate	INH	RES	VERA	THIO
MODS 39	2	< 0.25	0.5	0.25
MODS 11	4	< 0.25	1	0.5
H37Rv	< 0.25	< 0.25	< 0.25	< 0.25
Rifampicin MICs (mg/L)				
Isolate	RIF	RES	VERA	THIO
TF44949	> 8	> 8	> 8	> 8
MODS11	NG	NG	NG	NG
H37Rv	< 0.25	< 0.25	< 0.25	< 0.25
Kanamycin MICs (mg/L)				
Isolate	KAN	RES	VERA	THIO
TF 44949	4	2	2	2
MODS 11	4	2	2	2
H37Rv	2	2	2	< 0.25
Ciprofloxacin MICs (mg/L)				
Isolate	CPX	RES	VERA	THIO
TF 64747	1	0.125	0.125	0.25
TF 3228	2	NG	NG	NG
H37Rv	0.5	< 0.06	0.25	0.25
Moxifloxacin MICs (mg/L)				
Isolate	MXF	RES	VERA	THIO
MODS 11	0.5	0.5	0.25	< 0.06

TF 3181	0.5	0.5	0.25	< 0.06
H37Rv	< 0.06	< 0.06	< 0.06	< 0.06

Chapter Eight

Summary

Drug resistant TB is one of the key public health challenges of modern times. The devastating emergence of drug resistant forms of the disease is attributed to the ability of *M.tuberculosis* to adapt and evolve under antimicrobial pressure. To tackle the management of patients with drug resistant TB, we must understand the dynamics of antimicrobial resistance and the complexity of the mechanisms mediating resistance in *M.tuberculosis*.

Analysis of the various genes linked to drug resistance in *M.tuberculosis*, has revealed that the strains circulating in our setting display a combination of previously observed mutations. Each of these mutations results in resistance to a different drug. This supports previous reports that there is no single pleiotropic mutation resulting in the MDR phenotype, but rather an accumulation of mutations in the genome of *M.tuberculosis*. However, a complex association between resistance mutations are believed to exist. Classical mutations associated with resistance to one drug is thought to be the initial step that leads to resistance to other drugs. (1)

The MDR and XDR-TB strains analysed in this study displayed classical mutations in the *katG, embB, gyrA* and *rrs* genes, responsible for resistance to INH, EMB, fluoroquinolones and aminoglycosides respectively. The diversity of the mutations in the *rpoB* and *pncA* genes, responsible for resistance to RIF and PZA, demonstrates that MDR phenotype is due to de novo resistance.(2) The high level of similarity amongst the XDR-TB strains, with the predominance of the F15/LAM4/KZN strain supports previous reports regarding the clonality of the XDR epidemic in KZN. The F15/LAM4/KZN strain is a highly virulent strain, endemic to our setting. The strain has successfully disseminated amongst immune compromised patients with HIV co-infection.(3,4)

The results of this study demonstrates that the rare 130bp deletion of the *gidB* gene is lineage specific, observed in only the F15/LAM4/KZN strain family. Whilst the deletion has been previously described, we demonstrate this deletion exclusively in MDR and XDR-TB strains of the KZN lineage from our setting.(2) In contrast to reports that attribute low-level STR resistance induced by mutations in the *gidB* gene, our results demonstrate that alteration within this gene mediates high-level STR in this subset of clinical isolates.(5–7) Furthermore, classical mutations in the *rpsL* and *rrs* genes previously described, were only found in 18.9% of the STR resistant isolates in this study.(5,7–11) Our results also demonstrates the inability of the mutations to discriminate between various levels of STR resistance. The low detection rate of the *rpsL* and *rrs* mutations in this subset of isolates questions the utility of these mutations as accurate predictors of STR resistance.

Whilst the WHO does not define resistance beyond XDR-TB, reports of isolates resistant to all first and second line drugs has been termed TDR-TB.(12–14) The XDR TB isolates analysed in this study are characteristic of TDR-TB. The presence of the A90V *gyrA* mutation correlates with high-level resistance to CPX and OFX, and an increased MXF MIC in the XDR TB clinical isolates analysed. Although the breakpoint concentration for MXF remains debatable, we raise concerns regarding the application of the drug to XDR-TB treatment protocols. MXF is added to treatment protocols despite reports of *in vitro* resistance, with the hope that there may be some residual activity. This is disastrous from a public health perspective, in the era of ever-increasing drug resistance.

We also demonstrate the presence of the A1401G *rrs* mutation and its correlation with high level resistance to KAN and AMIK and a decreased susceptibility to CAP in the isolates analysed. We support the WHO revised breakpoint concentration of 4mg/L for CAP, this will prevent patients from receiving inadequate treatment that could lead to the development of further resistance as well as continued transmission.(12)

Screening of drug drugs of LIN and PAS revealed no genetic alteration that indicates drug resistance. Therefore, the strategic application of LIN and PAS is central to the treatment of drug resistant tuberculosis in our setting, as the strains may still be accessible for treatment with these drugs.

The XDR-TB isolates analyzed here displayed resistance to the fluoroquinolones and aminoglycosides, the most effective second line anti-TB drugs available for the treatment of drug resistant TB. Therefore, appropriate application of these agents is central to the effective management of MDR-TB and prevention of XDR-TB. It is imperative that MDR-TB patients are first tested to ensure that they are eligible for treatment to ensure appropriate treatment choices are made and to prevent the further amplification of drug resistance.(15)

We also demonstrate that efflux mediated resistance results in low-level resistance in the absence of mutations. Efflux mediated resistance in *M.tuberculosis* has emerged as a significant factor mediating drug resistance. Efflux pumps allow the extrusion of antibiotics from the bacterial cell, resulting in selection of resistant mutants. We also demonstrated the ability of EPIs to reverse the resistance induced by efflux activity, providing proof of concept that efflux pumps mediate low-level resistance in our setting and support further exploration of efflux pump inhibitors for treatment of TB.

Antibiotic exposure induces a complex response in *M.tuberculosis*, including changes in metabolic state and activity that contributes to resistance. Recent reports have implicated a number of mechanisms in resistance, demonstrating the complexity of resistance in the organism. Alteration in DNA repair systems, resulting in a reduced ability to repair DNA, results in increased mutation rates. Such mutator phenotypes have a selective advantage under antibiotic pressure. Compensatory evolution has allowed the organism to adapt by eliminating the fitness cost associated with mutations. Recent studies have demonstrated that

laboratory generated mutants were less fit than clinical strains, demonstrating the role of compensatory mechanisms.(16)

In South Africa, the public health service faces major barriers in the control of TB. The active transmission of drug resistant phenotypes, HIV co-infection, complex treatment regimens associated with higher toxicity and the duration of treatment have impacted on the poor control of TB. (17)Although drug resistance accounts for approximately 3% of all TB cases, it consumes more than a third of the national budget for TB, which is unsustainable and further threatens to destabilize control.(18)

Early diagnosis and treatment with antimicrobials known to be effective against the infecting strain, coupled with infection prevention measures remain the primary strategy in TB control. The Xpert MTB/RIF assay has assisted somewhat in this strategy. However, susceptibility is only obtained for one drug. Expansion of this test platform to include other drugs would assist in making an early diagnosis as well as allowing for patient individualized selection of antimicrobial agents. Whole genome sequencing technology has increased our capacity to understand the disease and may be the only hope in detecting resistance to multiple drugs. (19) There has been no greater need for new rapid diagnostic tests, antimicrobial tests and anti-TB drugs or regimens than the present. Recent focus has shifted to investigating adjunct treatment options, known as host directed therapy with the aim to enhance host immune responses against *M.tuberculosis* infection, reduce excessive inflammation, prevent and repair tissue damage and enhance the effectiveness of current treatment regimens.(17)

Whilst new developments offer a ray of hope, they are years away from integration into TB programmes. Thus strengthening the public health systems and strategic use of current anti-TB drugs remain critical in TB control efforts.

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Appendix 1

DNA Extraction Solutions

1.1 10x TE Buffer

Trizma base (Sigma-Aldrich, USA) 1.21g

EDTA (Sigma-Aldrich, USA) 0.37g

Weigh out the required amounts of reagent powders and dissolve the trizma base in 80ml of distilled water, pH to 8 using concentrated HCl (Merck). Add the EDTA and dissolve. Check the final pH and adjust to a final volume of 100ml. Autoclave at 121°C for 15 minutes.

1.2 Lysozyme (10mg/ml) (Sigma-Aldrich, USA)

Add 1ml of distilled water to 10mg of lysozyme powder. Store at 4°C until

1.3 Proteinase K (10mg/ml) (Roche Diagnostics)

Add 10ml of distilled water to 100mg of proteinase K powder. Store at 4°C until.

1.4 10% Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA)

Weigh 10g of SDS powder and dissolve in 100ml of distilled water.

1.5 5M Sodium Chloride (NaCl) (Sigma-Aldrich, USA)

Weigh 14.6 g of NaCl powder and dissolve in 50ml of distilled water, autoclave at 121°C for 15 minutes.

1.6 CTAB-NaCl Solution

NaCl (Sigma-Aldrich, USA)	4.1g
CTAB (Sigma-Aldrich, USA)	10g

Weigh out the required amounts of reagent powders and dissolve in 100ml of distilled water, the solution is heated to 65°C until powders are completely dissolved.

1.7 Chloroform: Isoamyl alcohol (24:1)

Add 1ml of isoamyl alcohol (Sigma-Aldrich, USA) to 24ml of chloroform (Sigma-Aldrich, USA.

1.8 70% ethanol

Add 35ml of absolute ethanol (Merck, SA) to 15ml of distilled water. Store at -20°C.

1.9 1% agarose gel (140ml)

Agarose powder (Lonza, USA)	1.4g
1x TBE buffer	140ml
Ethidium bromide	140µl

The agarose powder was weighed and added to a flask containing 1x TBE buffer. The mixture was boiled using a microwave until the powder dissolved. It was allowed to cool and ethidium bromide was added.

1.10 10xTBE Buffer

Trizma base (Sigma-Aldrich, USA)	108g
Boric acid (Sigma-Aldrich, USA)	55g
EDTA (Sigma-Aldrich, USA)	9.3g

Weigh out the required amounts of the reagents and dissolve in 1000ml of distilled water.

1.11 Sample loading dye

1% Double dye

Bromphenol Blue (Sigma-Aldrich, USA)	1g
Xylene cyanole (Sigma-Aldrich, USA)	1g

Weigh out the required amounts of reagent powders and dissolve in 100ml of distilled water.

50 ml of Loading Dye from 1% Double Dye (DD) stock:

10x TBE	5 ml
Glycerol (Merck, SA)	25 ml
1% Double dye	5 ml

Measure the required volumes of reagents and dissolve in 15ml of distilled water.

Appendix 2

Media and Reagents

2.1 Middlebrook 7H9 broth (Difco)

4.7g Middlebrook 7H9 powder

100ml OADC (BD)

10ml 50% (w/v) glycerol

2.5ml 20% Tween 80

4.7g of Middlebrook 7H9 powder was dissolved in approximately 800ml of autoclaved distilled water together with 10ml of 50% (w/v) glycerol, 2.5ml of 20% Tween 80 and was autoclaved at 121ºC for 15 minutes. The solution was placed in preheated water bath set to 50ºC to cool with gentle swirling for approximately 30 minutes and 100ml of OADC was added.

2.2 Middlebrook 7H11 solid agar (Difco)

21g Middlebrook 7H11 powder

100ml OADC (BD)

10ml 50% (w/v) glycerol

Twenty-one grams of Middlebrook 7H11 powder was dissolved in 900ml of triple distilled water and autoclaved at 121ºC for 15 minutes. The solution was placed in preheated water bath set to 50ºC to cool with gentle swirling for approximately 30 minutes.

100ml of OADC and 10ml of 50% (w/v) of glycerol were added and decanted into sterile

petri dishes.

2.3 Phosphate buffered saline (PBS) (Oxoid)

10 PBS tablets

1000ml distilled water

Ten PBS tablets were dissolved in 1000ml autoclaved distilled water. The PBS was autoclaved at 121ºC for 15 minutes, thereafter decanted into 20ml aliquots and refrigerated at 4ºC until use.

2.4 20%Tween 80 (Fisher)

20ml Tween 80

80ml distilled water

20ml of Tween 80 (Fisher) was added to 80ml of autoclaved distilled water. The solution was placed in a pre-heated waterbath set to 56ºC and then sterilized by filtration through a 0.22µm membrane.

Appendix 3: IS*6110* RFLP patterns

Figure A 3.1 is a scanned hyperfilm showing the IS*6110* RFLP patterns obtained in the study

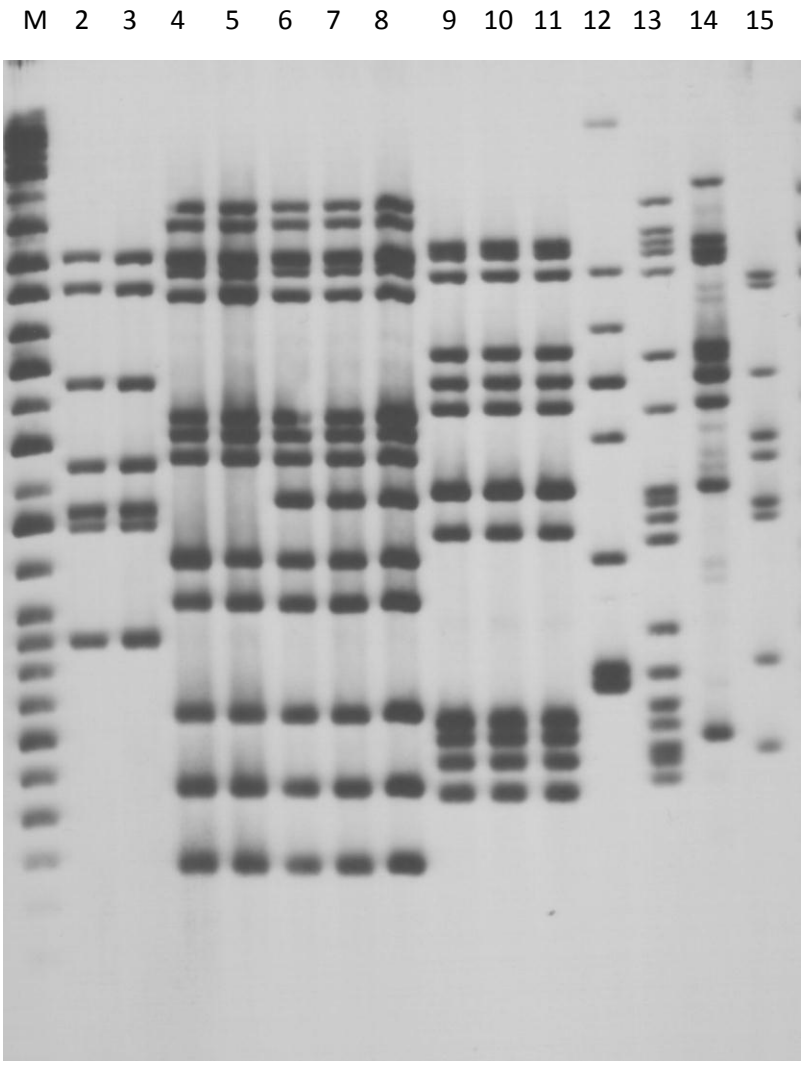
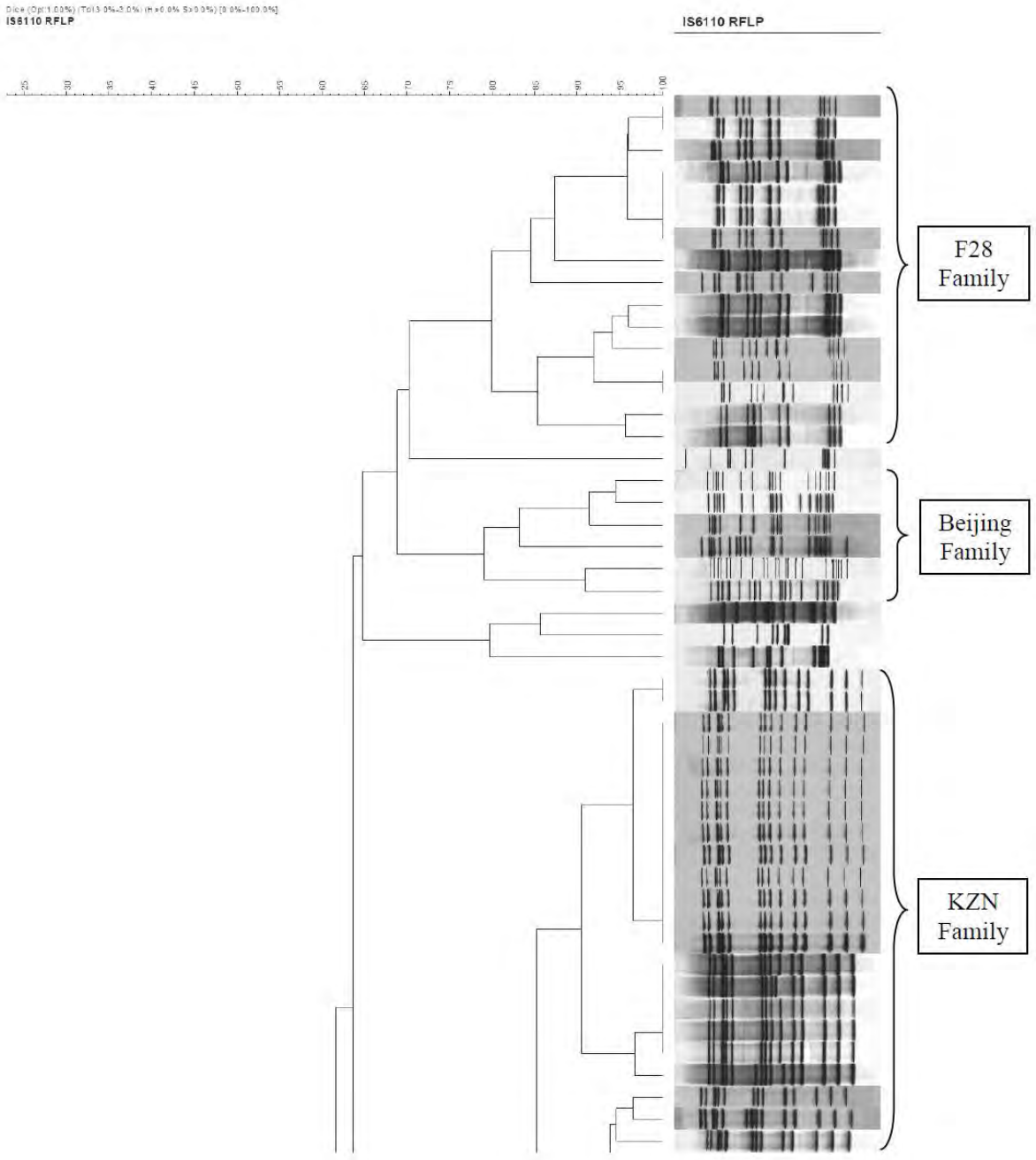


Figure 1: IS*6110* RFLP patterns for (2005-2006) and (2008-2009)

Lane 1: Jack's standard molecular weight marker (0.7-15kbps)
(2005-2006): Lane 2-3: unique cluster
Lane 4-8: F15/LAM4/KZN family patterns
Lane 9-11: F28 family patterns
Lane 12: unique patterns
Lane 13: Beijing family pattern
(2008-2009): Lanes 14-15: unique patterns

Figure A 3.2 Dendogram generated for the IS*6110* RFLP patterns. The predominant families and unique patterns are labelled accordingly.



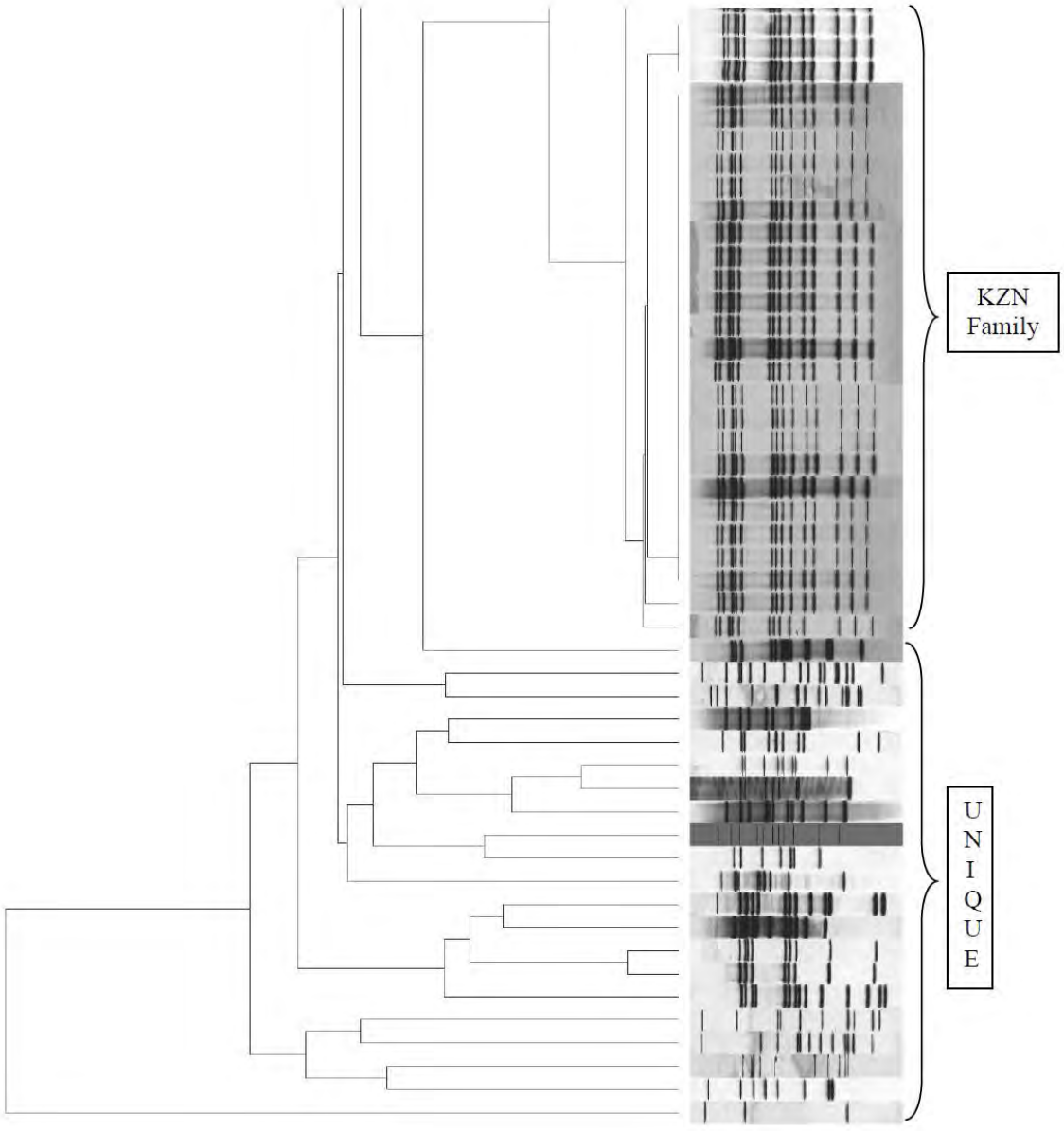
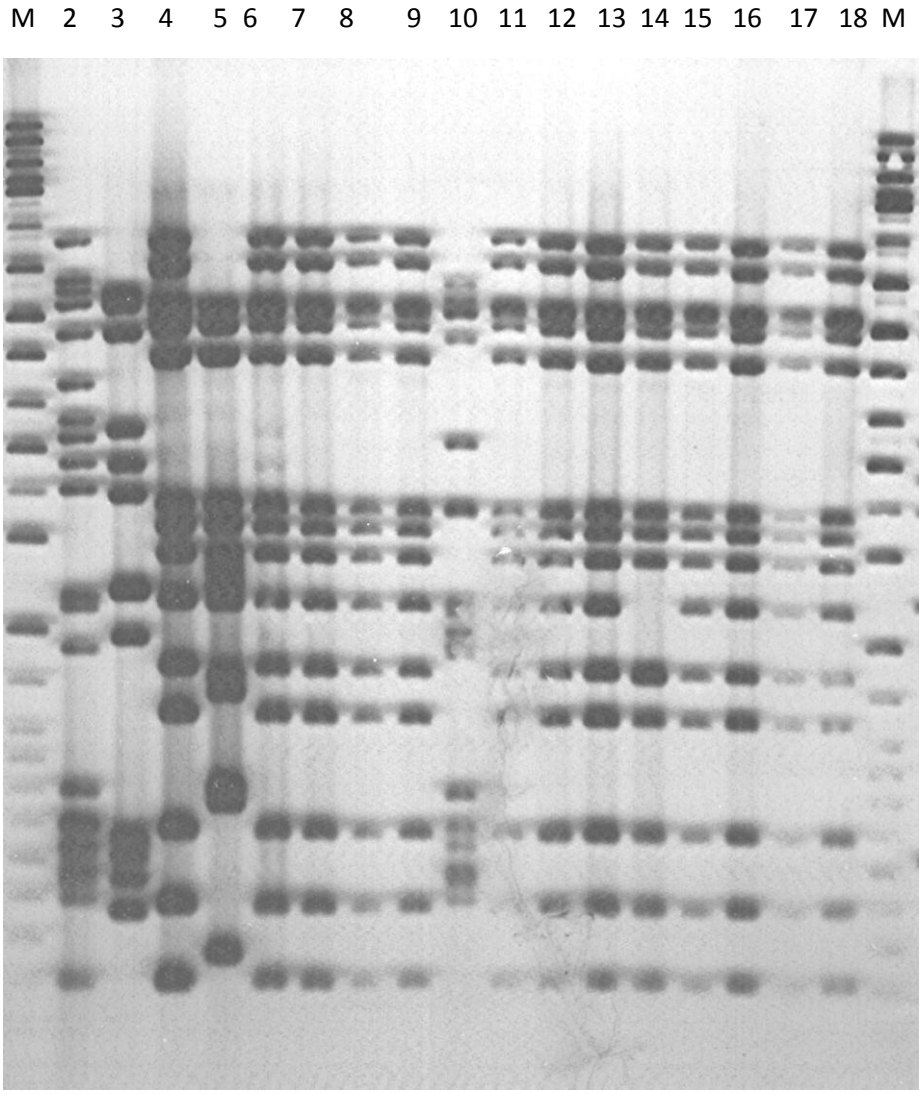

KZN
Family
UNIQUE

Figure A 3.2.1: IS*6110* RFLP patterns (2005-2006)



IS*6110* RFLP patterns (2005-2006)

Lane 1 & 19: Jack‘s standard molecular weight marker (0.7-15 kbps)

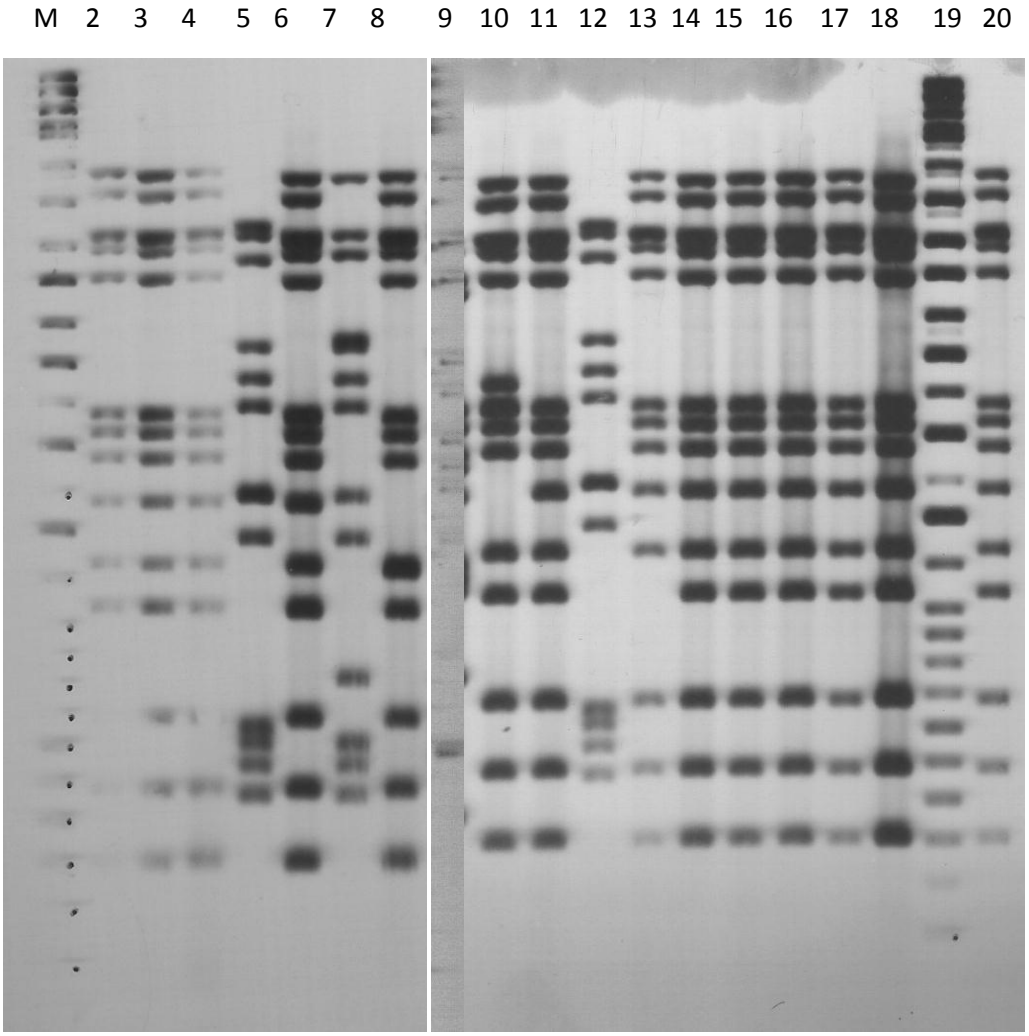
Lane 2 & 10: Beijing family patterns

Lane 3: F28 family pattern

Lane 5: unique pattern

Lane 6-9, 11-18: F15/LAM4/KZN family patterns

Figure A 3.2.2: IS*6110* RFLP patterns (2005-2006)



IS*6110* RFLP patterns (2008-2009)

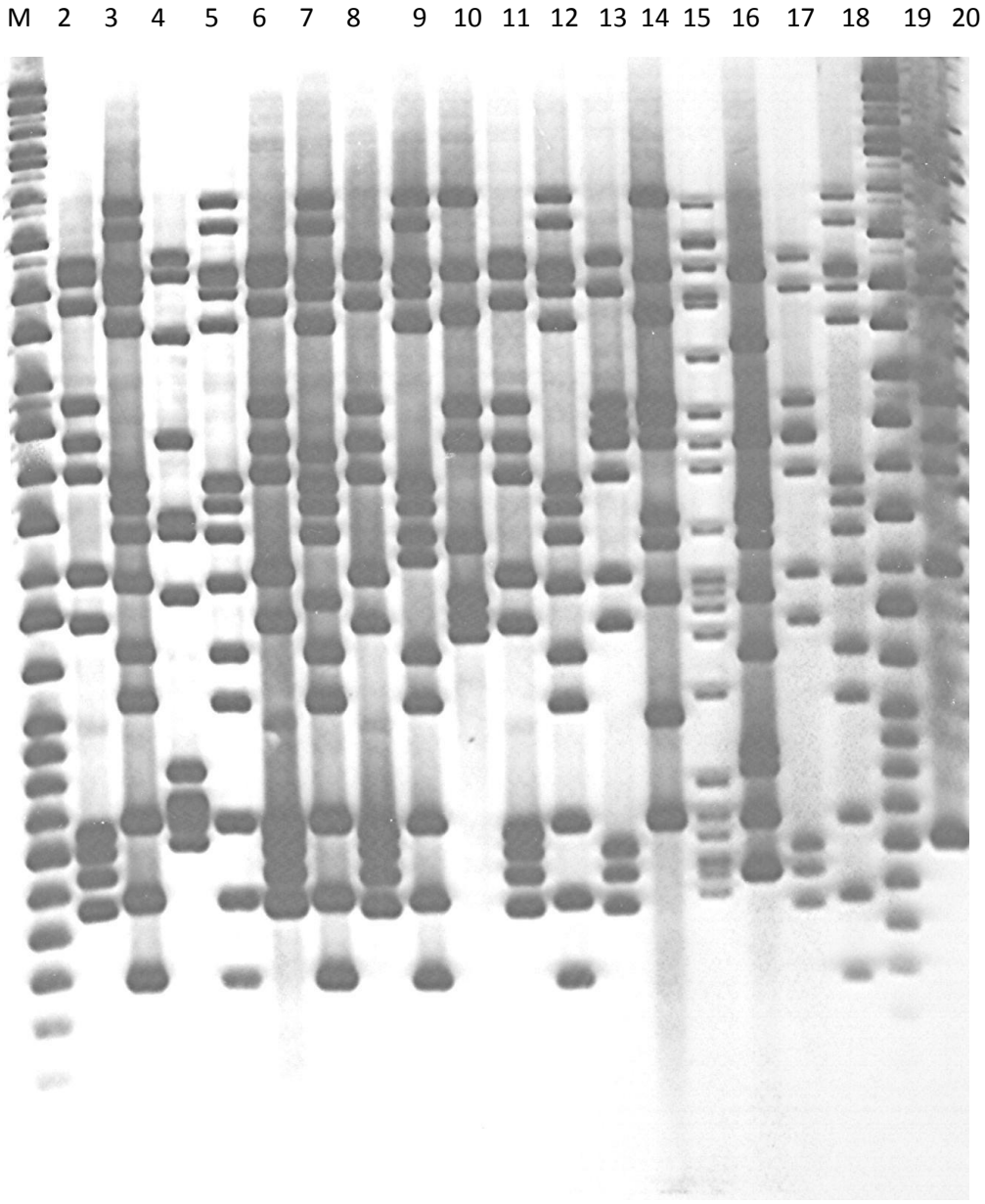
Lane 1 & 19: Jack‘s standard molecular weight marker (0.7-15 kbps)

Lane 2-4, 6, 8, 10-11, 13-18, 20: F15/LAM4/KZN family patterns

Lane 5, 7, 12: F28 family patterns

Lane 9: unique pattern

Figure A 3.2.3: IS*6110* RFLP patterns (2005-2006)



IS*6110* RFLP patterns (2005-2006)

Lane 1 & 19: Jack‘s standard molecular weight marker (0.7-15 kbps)

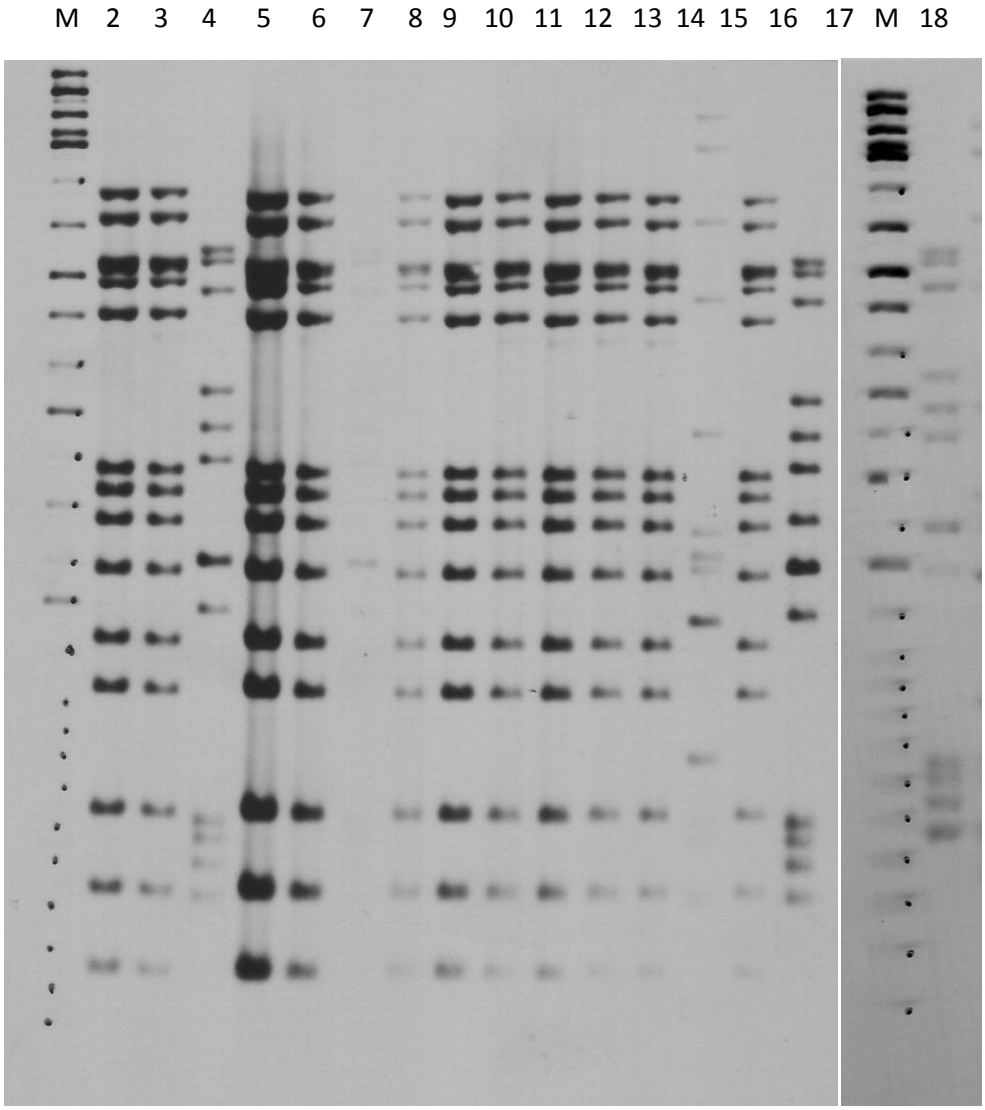
Lane 2, 6, 8, 11: F28 family patterns

Lane 3, 5, 7, 9, 12, 18: F15/LAM4/KZN family patterns

Lane 4, 10, 13, 14, 16, 17, 20: unique patterns

Lane 15: Beijing family patterns.

Figure A 2.3.4: IS*6110* RFLP patterns (2005-2006)



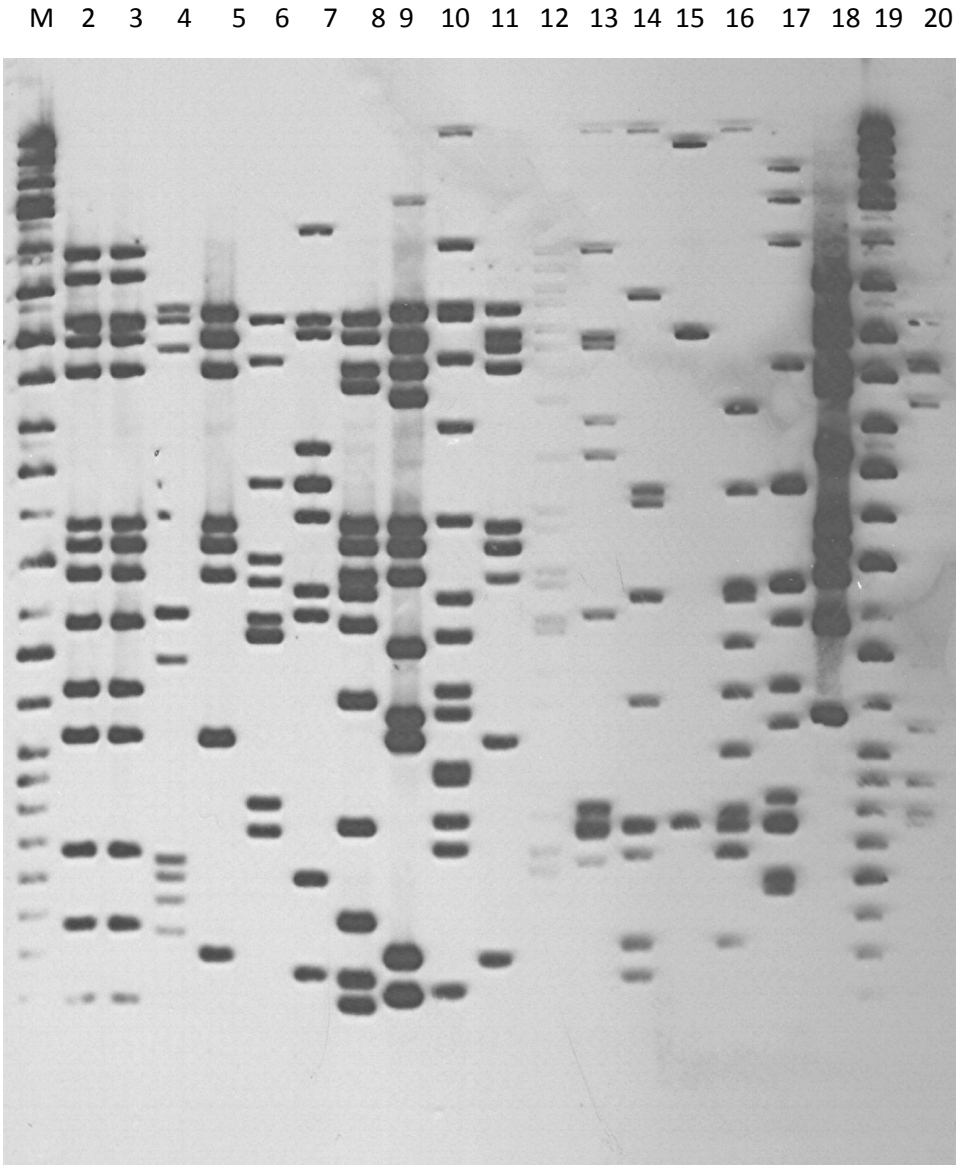
IS*6110* RFLP patterns (2005-2006)

Lane 1& 17: Jack‘s standard molecular weight marker (0.7-15 kbps)

Lane 2-3, 5-6, 8-13, 15: F15/LAM4/KZN family patterns

Lane 4 & 16, 18: F28 family patterns

Figure 3e: IS*6110* RFLP patterns for (2005-2006) and (2008-2009)



IS*6110* RFLP patterns for (2005-2006) and (2008-2009)

Lane 1 & 19: Jack‘s standard molecular weight marker (0.7-15 kbps)
(2005-2006): lane 2-3 F15/LAM4/KZN family patterns

Lane 4: F28 family patterns,

Lane 5-7: unique patterns
(2008-2009):

Lane 8-18, 19: unique pattern

Appendix 4: Data tables of MIC, PCR and Sequence Results

Table A 4.1: MIC, RFLP and sequencing data for INH, EMB and PZA

MIC's for PZA was not done

ΔC = insertion of cytosine

- = no mutation

Red spaces = failed reactions

SUSCEPTIBLE	**RFLP**	**MIC mg/L**		***inhA* Promoter**	***inhA***	***katG***			***embB***		***pncA***		
		INH	**EMB**			**944**	**1343**	**1388**	**916**	**1489**	**100**	**416**	**457**
TF1538	U	≤0.125	2	-	-	-	-	-	-	-	-	-	-
TF1413	B	16	2	-	-	-	-	G-T	-	-	-	-	-
TF1582	B	≤0.125	2	-	-	-	-	G-T	-	-	-	-	-
TF832	F11V	≤0.125	2	-	-	-	-	-	-	-	-	-	-
TF1519	B	≤0.125	2	-	-	-	-	-	-	-	-	-	-
TF1001	U	≤0.125	2	-	-	-	-	-	-	-	-	-	-
TF933	U	≤0.125	2	-	-	-	-	-	-	-	-	-	-
P090811	B	≤0.125	2	-	-	-	-	G-T	-	-	-	-	-
P090802	B	≤0.125	2	-	-	-	-	G-T	-	-	-	-	-
P090804	B	≤0.125	2	-	-	-	-	G-T	-	-	-	-	-
H37Rv				-	-	-	-	-	-	-	-	-	-
MDR	**RFLP**	**INH**	**EMB**			**944**	**1343**	**1388**	**916**	**1489**	**100**	**416**	**457**
MODS11	KZN	8	8	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS688	KZN	8	8	-	-	G-C	-	-	A-G	-	-	T-G	-
TF44949	F28	16	8	-	-	G-C	-	-	A-G	-	-	-	-
TF3251	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	-
TF78838	F28	16	8	-	-	G-C	-	-	A-G	-	T-G	-	-
TF2063	F28	16	16	-	-	G-C	-	-	A-G	-	T-G	-	-
TF3203	F28	16	16	-	-	G-C	-	-	A-G	-	T-G	-	-
TF1951	F28V	16	16	-	-	G-A	-	-	A-G	-	-	-	-
TF64747	KZN	16	8	-	-	G-C	-	-	A-G	-	-	T-G	-
MODS644	F28	16	8	-	-	G-C	-	-	A-G	-			

TF2889	F28	16	8	-	-	G-C	-	-	-	C-A	-	-	-
TF2040	F28	16	16	-	-	G-C	-	-	-	C-A	T-G	-	-
MODS682	F28	16	16	-	-	G-C	-	-	-	-	-	-	-
MDR	**RFLP**	**MIC mg/L**		***inhA* Promoter**	***inhA***	***katG***			***embB***		***pncA***		
		INH	**EMB**			**944**	**1343**	**1388**	**916**	**1489**	**100**	**416**	**457**
TF36480	KZN	16	8	-	-	G-C	-	-	A-G	-	T-G	-	-
TF2034	U	16	16	-	-	G-C	A-C	-	A-G	-	-	-	-
TF2153	F28	16	16	-	-	G-C	-	-	A-G	-	T-G	-	-
XDR						**944**	**1343**	**1388**	**916**	**1489**	**100**	**416**	**457**
TF1762	KZN	8	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS141	KZN	8	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS39	KZN	4	2	-	-	G-C	-	-	A-G	-			
MODS387	KZN	16	8	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS338	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS667	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS642	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS513	KZN	8	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS143	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF1824	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF1925	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF66937	KZN	16	8	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF3334	KZN	16	8	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF80198	KZN	16	8	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF80164	KZN	16	16	-	-	G-C	-	-	A-G	-			
TF1497	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF75549	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC

TF31066	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF739	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS370	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF3181	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
XDR	**RFLP**	**MIC mg/L**		***inhA* Promoter**	***inhA***	***katG***			***embB***		***pncA***		
		INH	**EMB**			**944**	**1343**	**1388**	**916**	**1489**	**100**	**416**	**457**
TF37806	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF2981	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS334	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF2038	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF3228	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF25027	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF51648	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
TF49127	KZN	16	16	-	-	G-C	-	-	A-G	-	-	-	ΔC
MODS 195	KZN	16	8	-	-	G-C	-	-	A-G	-	-	-	ΔC

Table A 4.2: MIC and sequencing data for RIF

SUSCEPTIBLE	RFLP	MIC mg/L	*rpoB*						
			1303	**1304**	**1334**	**1349**	**1355**	**1360**	**1473**
TF1538	U	1	-	-	-	-	-	-	-
TF1413	B	1	-	-	-	-	-	-	-
TF1582	B	1	-	-	-	-	-	-	-
TF832	F11V	1	-	-	-	-	-	-	-
TF1519	B	1	-	-	-	-	-	-	-
TF1001	U	1	-	-	-	-	-	-	-
TF933	U	1	-	-	-	-	-	-	-
P090811	B	1	-	-	-	-	-	-	-
P090802	B	1	-	-	-	-	-	-	-
P090804	B	1	-	-	-	-	-	-	-
H37Rv			-	-	-	-	-	-	-
MDR	**RFLP**		**1303**	**1304**	**1334**	**1349**	**1355**	**1360**	**1473**
MODS11	KZN	8	-	-	-		T-C	-	-
MODS688	KZN	128	-	-	-	C-T	-	-	-
TF44949	F28	32	-	-	-	C-T	-	-	-
TF3251	KZN	128	G-T	-	-		-	C-A	-
TF78838	F28	128	-	-	-	C-T	-	-	-
TF2063	F28	128	-	-	-	C-T	-	-	-
TF3203	F28	128	-	-	-	C-T	-	-	-
TF1951	F28V	128	-	-	A-T		-	-	G-C
TF64747	KZN	128	-	-	-	C-T	-	-	-
MODS644	F28	2							

MDR	RFLP	MIC mg/L	*rpoB*						
			1303	**1304**	**1334**	**1349**	**1355**	**1360**	**1473**
TF2889	F28	64	-	-	-	C-T	T-C	-	-
TF2040	F28	128	-	-	-	C-T	-	-	-
MODS682	F28	128	-	-	-	-	-	-	-
TF36480	KZN	128							
TF2034	U	64	-	-	-	C-T	T-C	-	-
TF2153	F28	128	-	-	-	-	-	-	-
XDR	**RFLP**		**1303**	**1304**	**1334**	**1349**	**1355**	**1360**	**1473**
TF1762	KZN	16	-	A-G	-	-	T-C	-	-
MODS141	KZN	128	-	A-G	-	-	T-C	-	-
MODS39	KZN	32	-	A-G	-	-	T-C	-	-
MODS387	KZN	128	-	A-G	-	-	T-C	-	-
MODS338	KZN	128	-	A-G	-	-	T-C	-	-
MODS667	KZN	128	-	A-G	-	-	T-C	-	-
MODS642	KZN	-							
MODS513	KZN	128	-	A-G	-	-	T-C	-	-
MODS143	KZN	128	-	A-G	-	-	T-C	-	-
TF1824	KZN	128	-	A-G	-	-	T-C	-	-
TF1925	KZN	128	-	A-G	-	-	T-C	-	-
TF66937	KZN	128	-	A-G	-	-	T-C	-	-
TF3334	KZN	-							
TF80198	KZN	128	-	A-G	-	-	T-C	-	-
TF80164	KZN	128	-	A-G	-	-	T-C	-	-
TF1497	KZN	128	-	A-G	-	-	T-C	-	-
TF75549	KZN	128	-	A-G	-	-	T-C	-	-

XDR	RFLP	MIC mg/L	*rpoB*						
			1303	**1304**	**1334**	**1349**	**1355**	**1360**	**1473**
TF31066	KZN	128	-	A-G	-	-	T-C	-	-
TF739	KZN	128	-	A-G	-	-	T-C	-	-
MODS370	KZN	128	-	A-G	-	-	T-C	-	-
TF3181	KZN	128	-	A-G	-	-	T-C	-	-
TF37806	KZN	128	-	A-G	-	-	T-C	-	-
TF2981	KZN	128	-	A-G	-	-	T-C	-	-
MODS334	KZN	128	-	-	-	-	-	-	-
TF2038	KZN	128		A-G	-	-	-	-	-
TF3228	KZN	128	-	-	-	-	-	-	-
TF25027	KZN	128	-	A-G	-	-	-	-	-
TF51648	KZN	128	-	A-G	-	-	-	-	-
TF49127	KZN	64	-	A-G	-	-	-	-	-
MODS 195	KZN	32	-	-	-	C-T	-	-	-

Table A 4.3: MIC and sequencing data for STREP

SUSCEPTIBLE	RFLP	MIC mg/L	*gidB*					*rpsL*	
			Del	**47**	**274**	**299**	**405**	**128**	**363**
TF1538	U	0.5	-	-	-	C-T	-	-	-
TF1413	B	4	-	-	-	C-T	-	-	-
TF1582	B	0.5	-	-	-	C-T	-	-	-
TF832	F11V	0.5	-	T-G	-	C-T	-	-	-
TF1519	B	0.5	-	-	-	C-T	-	-	-
TF1001	U	0.5	-	-	-	C-T	G-T	-	-
TF933	U	0.5	-	-	-	C-T	-	-	-
P090811	B	0.5	-	-	A-C	C-T	-	-	-
P090802	B	0.5	-	-	A-C	C-T	-	-	-
P090804	B	0.5	-	-	A-C	C-T	-	-	-
H37Rv		0.5	-	-	-	-	-	-	-
MDR	**RFLP**		**Del**	**47**	**274**	**299**	**405**	**128**	**363**
MODS11	KZN	128	Present	T-G	Del	C-T	-	-	-
MODS688	KZN	128	Present	T-G	Del	C-T	-	-	-
TF44949	F28	128	-	-	A-C	C-T	-	A-G	-
TF3251	KZN	128	Present	T-G	Del	C-T	-	-	-
TF78838	F28	4	-	-	A-C	C-T	-	A-G	-
TF2063	F28	4	-	-	A-C	C-T	-	A-G	-
TF3203	F28	4	-	-	A-C	C-T	-	A-G	-
TF1951	F28V	4	-	-	A-C	C-T	-	-	-
TF64747	KZN	32	Present	T-G	Del	C-T	-	-	-
MODS644	F28	4	-	-	A-C	C-T	-	-	-

TF2889	F28	-							
MDR	**RFLP**	**MIC mg/L**	***GidB***					***rpsL***	
			Del	**47**	**274**	**299**	**405**	**128**	**363**
TF2040	F28	4	-	-	A-C	C-T	-	A-G	-
MODS682	F28	8	-	-	A-C	C-T	-	-	-
TF36480	KZN	4	-	-	A-C	C-T	-	A-G	-
TF2034	U	-							
TF2153	F28	4	**-**	**-**	**A-C**	C-T	**-**	**A-G**	**-**
XDR			**Del**	**47**	**274**	**299**	**405**	**128**	**363**
TF1762	KZN	12328	Present	T-G	Del	C-T	-	-	A-G
MODS141	KZN	1284	Present	T-G	Del	C-T	-	-	A-G
MODS39	KZN	4128	Present	T-G	Del	C-T	-	-	A-G
MODS387	KZN	44	Present	T-G	Del	C-T	-	-	A-G
MODS338	KZN	432	Present	T-G	Del	C-T	-	-	A-G
MODS667	KZN	4	Present	T-G	Del	C-T	-	-	A-G
MODS642	KZN	128	Present	T-G	Del	C-T	-	-	A-G
MODS513	KZN	128	Present	T-G	Del	C-T	-	-	A-G
MODS143	KZN	4	Present	T-G	Del	C-T	-	-	A-G
TF1824	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF1925	KZN	4	Present	T-G	Del	C-T	-	-	A-G
TF66937	KZN	32	Present	T-G	Del	C-T	-	-	A-G
TF3334	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF80198	KZN	32	Present	T-G	Del	C-T	-	-	A-G
TF80164	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF1497	KZN	32	Present	T-G	Del	C-T	-	-	A-G
TF75549	KZN	128	Present	T-G	Del	C-T	-	-	A-G

TF31066	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF739	KZN	4	Present	T-G	Del	C-T	-	-	A-G
XDR	**RFLP**	**MIC mg/L**	***gidB***					***rpsL***	
			Del	**47**	**274**	**299**	**405**	**128**	**363**
MODS370	KZN	32	Present	T-G	Del	C-T	-	-	A-G
TF3181	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF37806	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF2981	KZN	128	Present	T-G	Del	C-T	-	-	A-G
MODS334	KZN	4	Present	T-G	Del	C-T	-	-	A-G
TF2038	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF3228	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF25027	KZN	128	Present	T-G	Del	C-T	-	-	A-G
TF51648	KZN	4	Present	T-G	Del	C-T	-	-	A-G
TF49127	KZN	128	Present	T-G	Del	C-T	-	-	A-G
MODS 195	KZN	4	Present	T-G	Del	C-T	-	-	A-G

Table A 4.4: MIC and sequencing data for KANA, AMIK and CAP

SUSCEPTIBLE	RFLP	MIC mg/L			*Rrs*		*tlyA*	*eis promoter*	*eis*
		KANA	AMI	CAP	500	1400	33		
TF1538	U	2	1	4	-	-	A-G	-	-
TF1413	B	2	2	4	-	-	A-G	-	-
TF1582	B	2	2	4	-	-	A-G	-	-
TF832	F11V	2	2	4	-	-	A-G	-	-
TF1519	B	2	2	4	-	-	A-G	-	-
TF1001	U	2	2	4	-	-	A-G	-	-
TF933	U	4	2	4	-	-	A-G	-	-
P090811	B	2	1	4	-	-	A-G	-	-
P090802	B	2	1	4	-	-	A-G	-	-
P090804	B	2	1	4	-	-	A-G	-	-
H37Rv		2	1	4	-	-	-	-	-
MDR	**RFLP**	**KANA**	**AMI**	**CAP**	**500**	**1400**	**33**		
MODS11	KZN	4	2	4	-	-	A-G	-	-
MODS688	KZN	4	2	4	-	-	A-G	-	-
TF44949	F28	2	1	4	-	-	A-G	-	-
TF3251	KZN	4	2	8	-	-	A-G	-	-
TF78838	F28	2	2	4	-	-	A-G	-	-
TF2063	F28	4	2	4	-	-	A-G	-	-
TF3203	F28	2	1	4	-	-	A-G	-	-
TF1951	F28V	2	1	4	-	-	A-G	-	-
TF64747	KZN	2	2	4	-	-	A-G	-	-
MODS644	F28	2	2	4	-	-	A-G	-	-

TF2889	F28	2	2	4	-	-	A-G	-	-
MDR	**RFLP**	**MIC mg/L**			***Rrs***		***tlyA***	***eis promoter***	***eis***
		KANA	**AMI**	**CAP**	**500**	**1400**	**33**		
TF2040	F28	2	2	4	-	-	A-G	-	-
MODS682	F28	2	1	4	-	-	A-G	-	-
TF36480	KZN	2	1	4	-	-	A-G	-	-
TF2034	U	4	2	4	-	-	A-G	-	-
TF2153	F28	2	2	4	-	-	A-G	-	-
XDR		**KANA**	**AMI**	**CAP**	**500**	**1400**	**33**		
TF1762	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS141	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS39	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS387	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS338	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS667	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS642	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS513	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS143	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF1824	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF1925	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF66937	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF3334	KZN	>128	>128	16	-	A-G	A-G	-	-
TF80198	KZN	>128	>128	16	-	A-G	A-G	-	-
TF80164	KZN	>128	>128	16	-	A-G	A-G	-	-
TF1497	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF75549	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF31066	KZN	>128	>128	>16	-	A-G	A-G	-	-

TF739	KZN	>128	>128	16	-	A-G	A-G	-	-
XDR	**RFLP**	**MIC mg/L**			***Rrs***		***tlyA***	***eis promoter***	***eis***
		KANA	**AMI**	**CAP**	**500**	**1400**	**33**		
MODS370	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF3181	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF37806	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF2981	KZN	>128	>128	>16	-	A-G	A-G	-	-
MODS334	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF2038	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF3228	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF25027	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF51648	KZN	>128	>128	>16	-	A-G	A-G	-	-
TF49127	KZN	>128	>128	>16	-	A-G	A-G	-	-

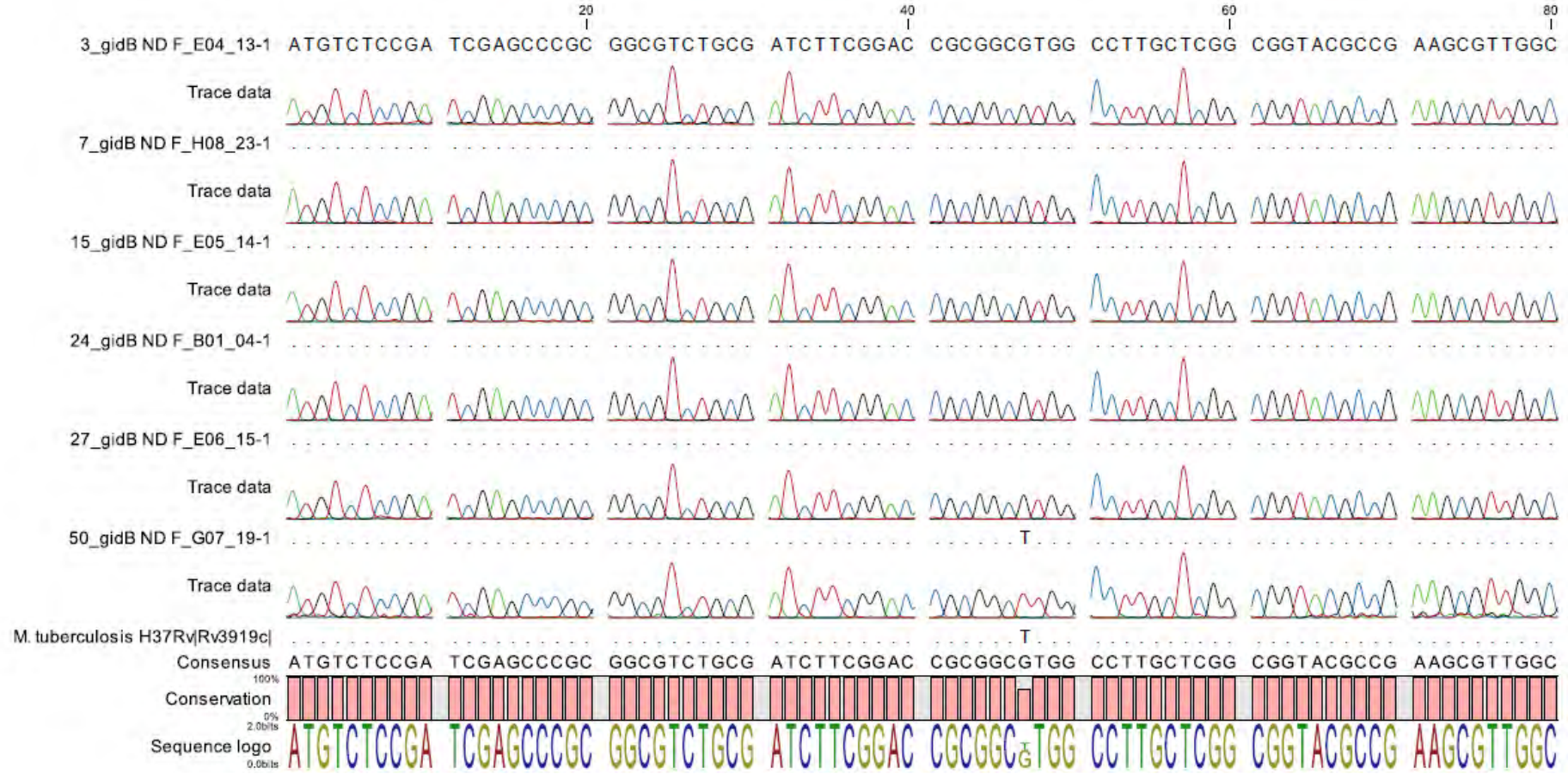
Table A 4.5: MIC and sequencing data for the fluoroquinolones, PAS and linezolid

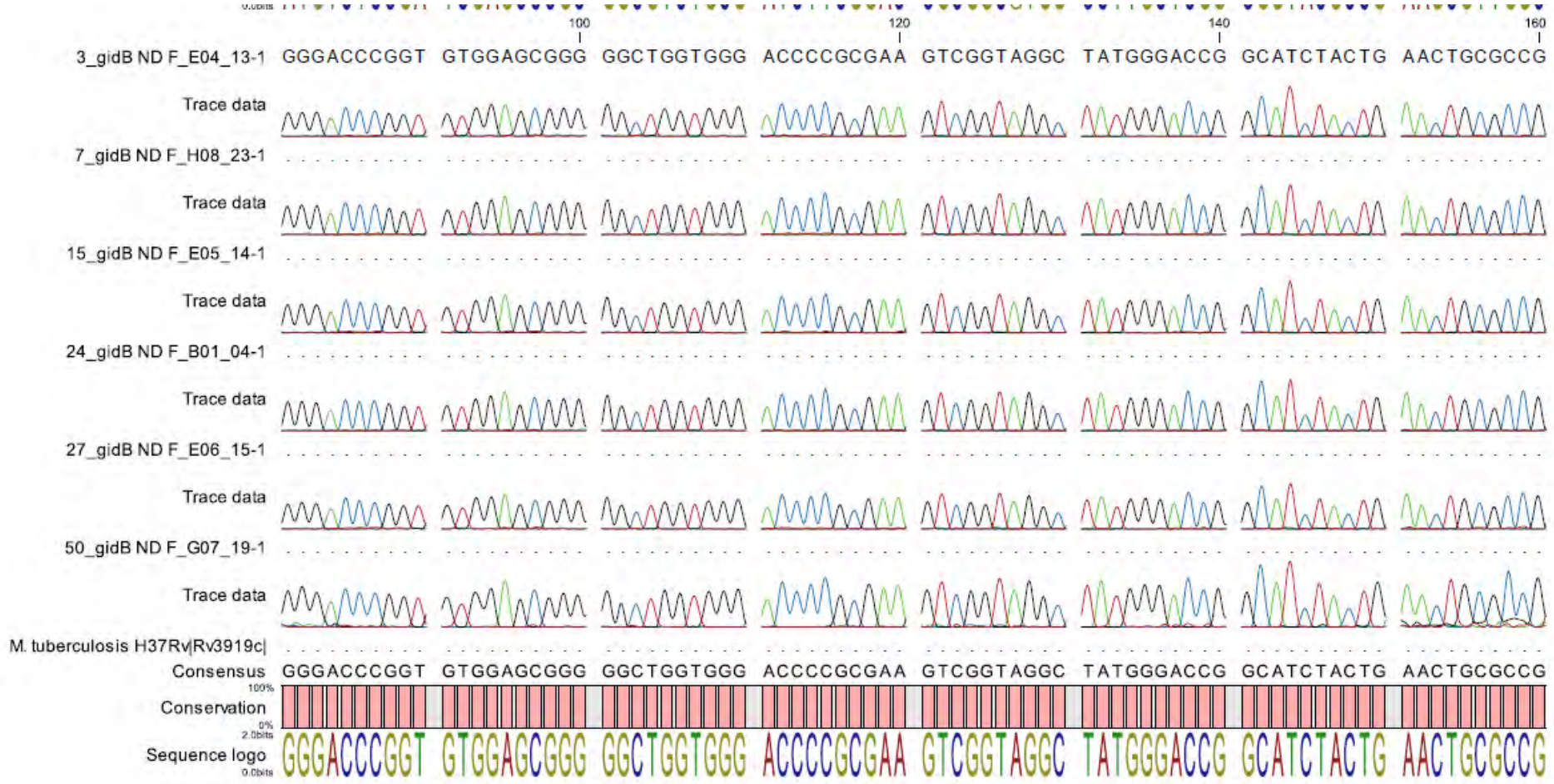
SUSCEPTIBLE	RFLP	MIC mg/L			*gyrA*			*gyrB*	PAS *thyA*	LIN *rrl*
		CIP	OFX	MFX	61	269	284		604	2399
TF1538	U	0.5	0.5	0.125	G-C	-	G-C	-	-	-
TF1413	B	0.5	0.5	0.125	G-C	-	G-C	-	-	-
TF1582	B	0.5	0.5	0.125	G-C	-	G-C	-	-	-
TF832	F11V	0.5	0.5	0.125	G-C	-	G-C	-	-	-
TF1519	B	0.5	0.5	0.125	G-C	-	G-C	-	-	-
TF1001	U	0.5	0.5	0.125	G-C	-	G-C	-	-	-
TF933	U	0.5	0.5	0.125	G-C	-	G-C	-	-	-
P090811	B	0.5	0.5	0.125	G-C	-	G-C	-	-	-
P090802	B	0.5	0.5	0.125	G-C	-	G-C	-	-	-
P090804	B	0.5	0.5	0.125	G-C	-	G-C	-	-	-
H37Rv		0.5	0.5	0.125	G-C	-	G-C	-	-	-
MDR	**RFLP**	**CIP**	**OFX**	**MFX**	**61**	**269**	**284**		**PAS *thyA***	**LIN *rrl***
MODS11	KZN	1	1	0.5	G-C	-	G-C	-	A-G	G-A
MODS688	KZN	1	1	0.5	G-C	-	G-C	-	A-G	G-A
TF44949	F28	1	1	0.5	G-C	-	G-C	-	-	-
TF3251	KZN	1	1	0.5	G-C	-	G-C	-	A-G	G-A
TF78838	F28	1	1	0.5	G-C	-	G-C	-	-	-
TF2063	F28	1	1	0.5	G-C	-	G-C	-	-	-
TF3203	F28	0.5	0.5	0.5	G-C	-	G-C	-	-	-
TF1951	F28V	0.5	0.5	0.125	G-C	-	G-C	-	-	-
TF64747	KZN	1	1	0.5	G-C	-	G-C	-	A-G	G-A
MODS644	F28	1	1	0.5	G-C	-	G-C	-	-	-

TF2889	F28	1	1	0.5	G-C	-	G-C	-	-	-
MDR	**RFLP**	**MIC mg/L**			***gyrA***			***gyrB***	**PAS *thyA***	**LIN *rrl***
		CIP	**OFX**	**MFX**	**61**	**269**	**284**		-	-
TF2040	F28	1	1	0.25	G-C	-	G-C	-	-	-
MODS682	F28	1	1	0.25	G-C	-	G-C	-	-	-
TF36480	KZN	1	1	0.25	G-C	-	G-C	-	A-G	G-A
TF2034	U	1	1	0.25	G-C	-	G-C	-	-	-
TF2153	F28	1	1	0.25	G-C	-	G-C	-	-	-
XDR		**CIP**	**OFX**	**MFX**	**61**	**269**	**284**		**PAS *thyA***	**LIN *rrl***
TF1762	KZN	8	8	1	G-C	C-T	G-C	-	A-G	G-A
MODS141	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS39	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS387	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS338	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS667	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS642	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS513	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS143	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF1824	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF1925	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF66937	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF3334	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF80198	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF80164	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF1497	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF75549	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A

TF31066	KZN	8	8	1	G-C	C-T	G-C	-	A-G	G-A
TF739	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
XDR	**RFLP**	**MIC mg/L**			***gyrA***			***gyrB***	**PAS *thyA***	**LIN *rrl***
		CIP	**OFX**	**MFX**	**61**	**269**	**284**		A-G	G-A
MODS370	KZN	8	8	1	G-C	C-T	G-C	-	A-G	G-A
TF3181	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF37806	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF2981	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS334	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF2038	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF3228	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF25027	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF51648	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
TF49127	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A
MODS 195	KZN	8	8	2	G-C	C-T	G-C	-	A-G	G-A

Appendix 5: *gidB* sequence analysis

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Trace data
7_gidB ND F_H08_23-1
Trace data
15_gidB ND F_E05_14-1
Trace data
24_gidB ND F_B01_04-1
Trace data
27_gidB ND F_E06_15-1
Trace data
50_gidB ND F_G07_19-1
Trace data
M. tuberculosis H37Rv|Rv3919c|
Consensus
GGGACCCGGT GTGGAGCGGG GGCTGGTGGG ACCCCGCGAA GTCGGTAGGC TATGGGACCG GCATCTACTG AACTGCGCCG
100%
Conservation
0%
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Sequence logo
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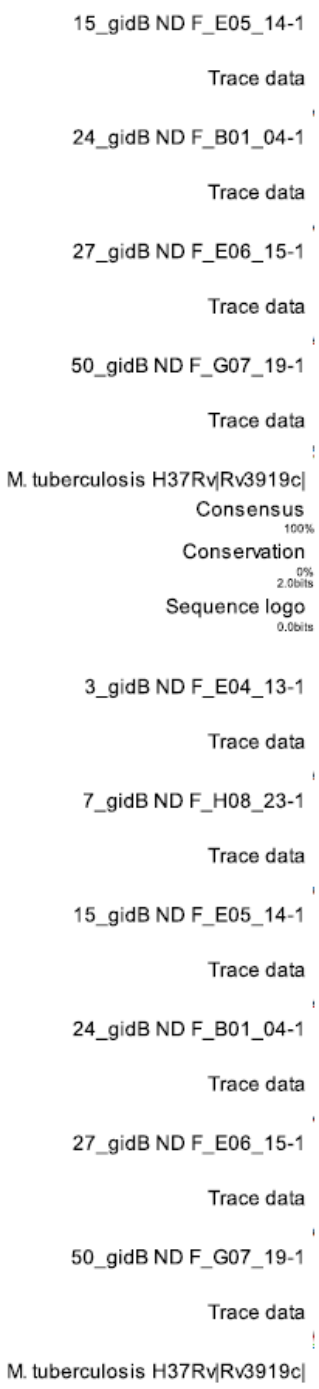
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Trace data
M. tuberculosis H37Rv|Rv3919c| 80
Consensus
100%
Conservation
0%
2.0bits
Sequence logo
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Trace data
7_gidB ND F_H08_23-1 160
Trace data
15_gidB ND F_E05_14-1 160
Trace data
24_gidB ND F_B01_04-1 160
Trace data
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Trace data
50_gidB ND F_G07_19-1 160
Trace data
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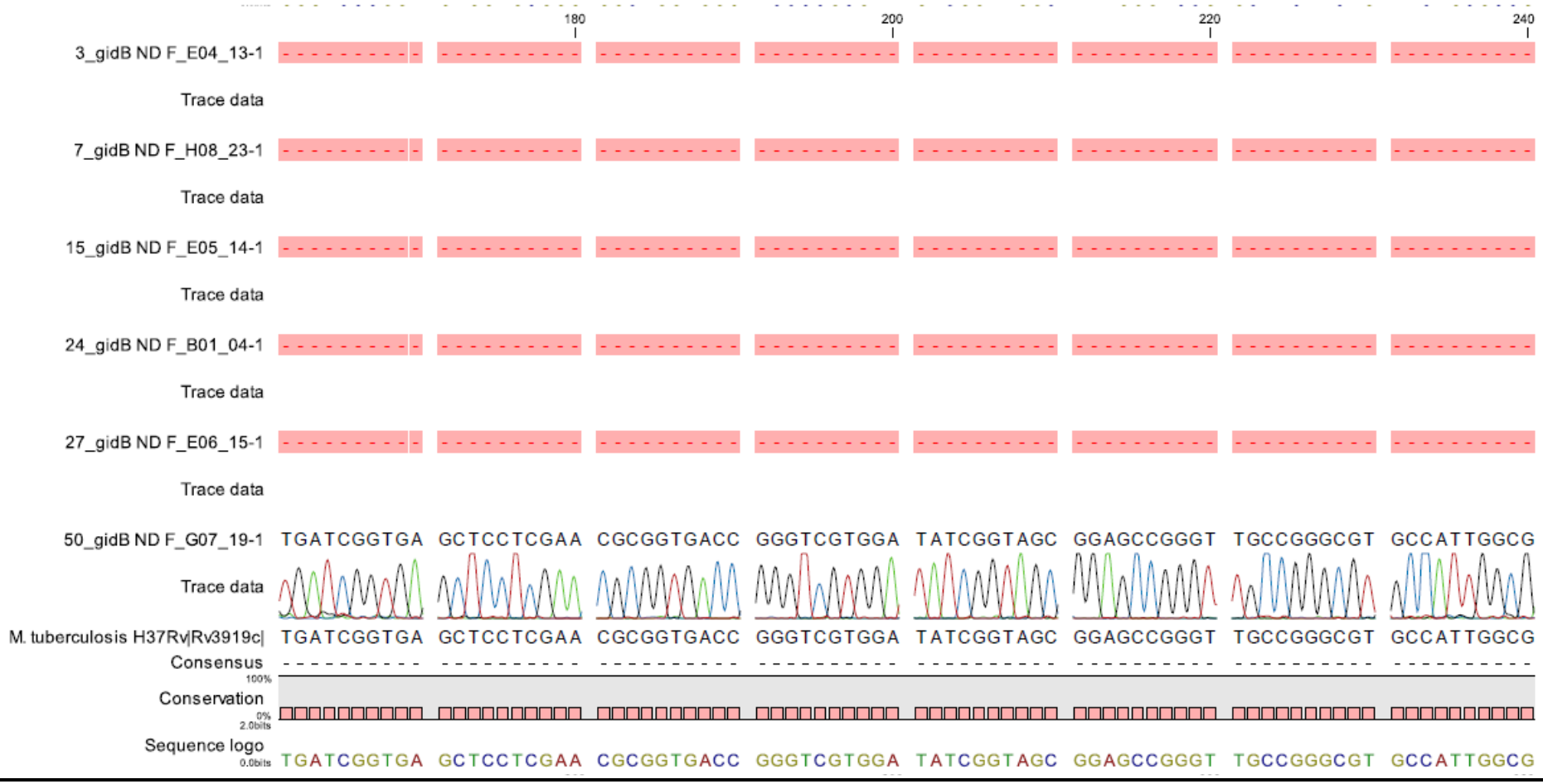
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220
240
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Trace data
7_gidB ND F_H08_23-1
Trace data
15_gidB ND F_E05_14-1
Trace data
24_gidB ND F_B01_04-1
Trace data
27_gidB ND F_E06_15-1
Trace data
50_gidB ND F_G07_19-1
TGATCGGTGA GCTCCTCGAA CGCGGTGACC GGGTCGTGGA TATCGGTAGC GGAGCCGGGT TGCCGGGCGT GCCATTGGCG
Trace data
M. tuberculosis H37Rv|Rv3919c|
TGATCGGTGA GCTCCTCGAA CGCGGTGACC GGGTCGTGGA TATCGGTAGC GGAGCCGGGT TGCCGGGCGT GCCATTGGCG
Consensus
100%
Conservation
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2.0bits
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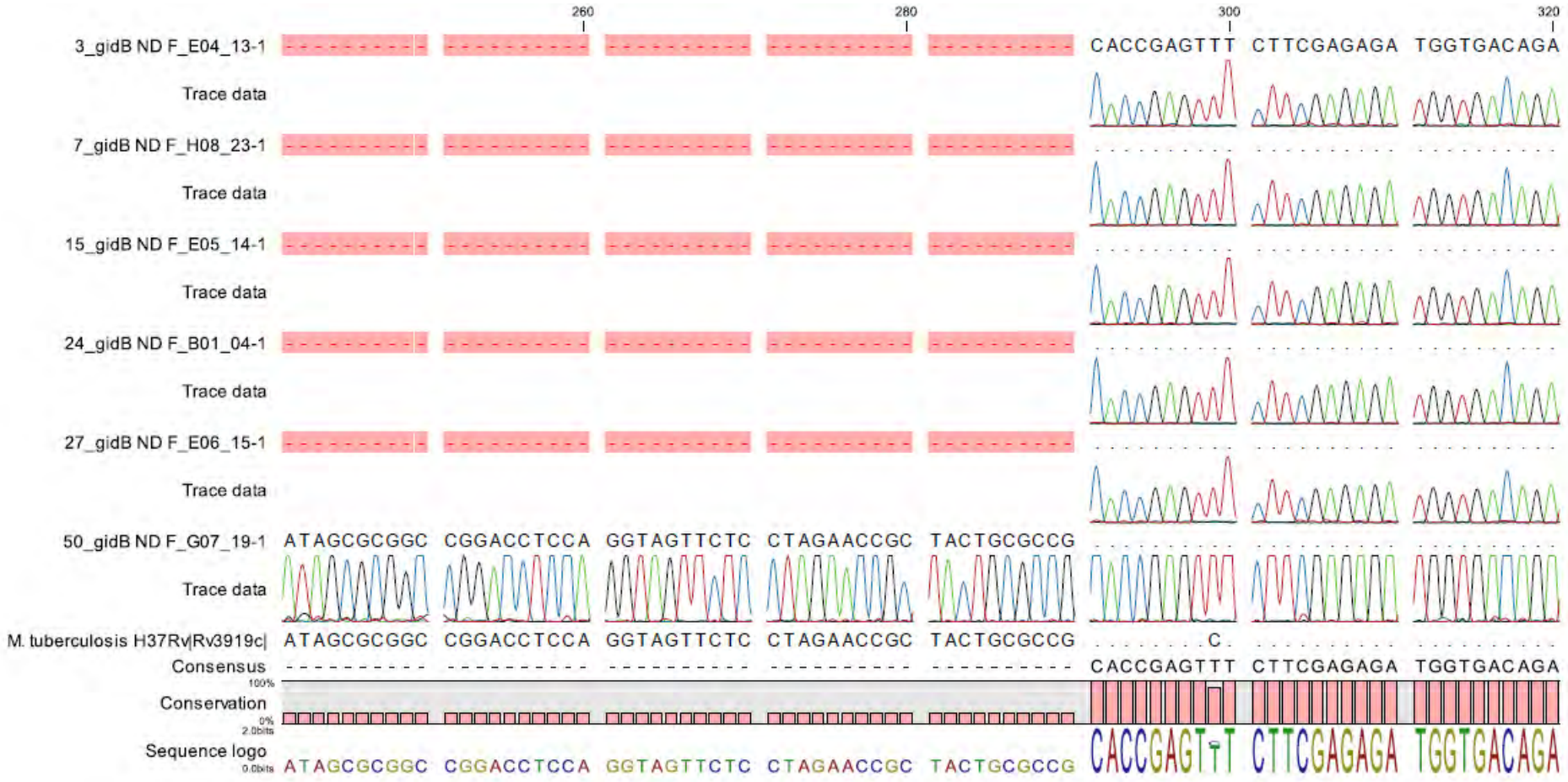
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280
300
320
3_gidB ND F_E04_13-1
Trace data
7_gidB ND F_H08_23-1
Trace data
15_gidB ND F_E05_14-1
Trace data
24_gidB ND F_B01_04-1
Trace data
27_gidB ND F_E06_15-1
Trace data
50_gidB ND F_G07_19-1
Trace data
M. tuberculosis H37Rv|Rv3919c|
Consensus
Conservation
Sequence logo
ATAGCGCGGC CGGACCTCCA GGTAGTTCTC CTAGAACCGC TACTGCGCCG
CACCGAGTTT CTTCGAGAGA TGGTGACAGA
C
100%
0%
2.0bits
0.0bits