Resistance to first-line anti-TB drugs

by gene mutation and gene modulation

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:……. Date:……

Summary

This study highlights the importance of rapid identification of drug-resistant tuberculosis and the complex network of mechanisms that regulates drug resistance in the bacteria. Tuberculosis control revolves around rapid detection and diagnosis of the disease to initiate effective treatment as soon as possible. The emergence and subsequent transmission of drug-resistant *Mycobacterium tuberculosis* strains has complicated controlling this disease. Various phenotypic and genotypic methods have been employed for the detection of resistance to anti-tuberculosis drugs. Phenotypic methods are technically demanding and time consuming due to the slow-growing bacterium. One example is to test for pyrazinamide resistance. Pyrazinamide is only active at low pH, thereby complicating susceptibility testing due to poor growth and subsequent death of mycobacteria in the test medium. For these technical reasons, pyrazinamide susceptibility testing is not done routinely in many countries. The extent of pyrazinamide resistance is therefore unknown in South Africa. Alarmingly, it was observed that more than 50% of patients infected with a drug-resistant tuberculosis strain are phenotypically and genotypically resistant to pyrazinamide. Genotypic resistance is mostly attributed to mutations scattered in the *pncA* gene. This makes it difficult to develop a molecular method to rapidly detect pyrazinamide resistance. Failure to detect resistance to this first-line drug will result in inappropriate therapy which may lead to prolonged treatment. This has negative implications for the Tuberculosis Control Program. Therefore, pyrazinamide testing is essential to ensure effective treatment for tuberculosis patients.

Another first-line drug, rifampicin, is promoted as a marker for the identification of multi-drug resistant tuberculosis. Various molecular methods have been employed to

identify mutations associated with rifampicin resistance in this pathogen. Recently, a newly identified nucleotide change (*Rv2629* 191A/C) was reported to confer rifampicin resistance. In contrast, we have shown that this nucleotide change was not associated with rifampicin resistance, but was significantly associated with the Beijing lineage of *M. tuberculosis*. This nucleotide change is a useful marker to identify the Beijing lineage that is widely distributed in various parts of the world.

This study has provided the first evidence that variation in the level of rifampicin resistance in closely related clinical isolates of *M. tuberculosis* is more complex than the central dogma suggests. Hyper-resistance can not be explained solely on the basis of mutations in the *rpoB* gene that confer resistance to rifampicin. We have provided evidence that exposing rifampicin resistant *M. tuberculosis* strains to efflux pump inhibitors, reserpine and verapamil, together with rifampicin, significantly reversed the rifampicin resistance phenotype to become rifampicin susceptible. This has far-reaching therapeutic implications for the treatment of tuberculosis when used in combination with anti-tuberculosis drugs. With this knowledge, we proposed a model that predicts the evolution of the level of rifampicin resistance. Subsequent transcriptomic analysis of numerous transporter genes identified potential candidates for functional assays and future drug design.

This innovative research enables scientists to re-investigate existing ideas, thereby shifting paradigms, challenging scientific dogmas and ultimately unraveling the mysteries regarding drug resistance in this pathogen.

Opsomming

Hierdie studie beklemtoon die belangrikheid van vinnige identifikasie van middelweerstandige tuberkulose en die komplekse netwerk van meganismes wat weerstandigheid in die bakterium reguleer. Vinnige diagnose is noodsaaklik om spoedige behandeling te verskaf en tuberkulose te beheer. Die verskyning van middelweerstandige *Mycobacterium tuberculosis* stamme bemoeilik die verdere beheer van hierdie siekte. Verskeie fenotipies en genotipiese metodes word gebruik om weerstandigheid teen anti-tuberkulose middels te bepaal. Fenotipiese metodes is egter tydrowend en vereis ‘n hoë mate van tegniese vaardigheid. Een voorbeeld is die toetsing vir weerstandigheid teen Pirasinamied. Hierdie middel is slegs aktief by ‘n lae pH. Dit bemoeilik die toets as gevolg van die swak groeiende bakterie in die toetsmedium. Weerstandigheid van Pirasinamied word om hierdie rede nie as roetiene gedoen nie. Die voorkoms van Pirasinamied weerstandigheid in Suid-Afrika is dus onbekend. Die studie maak die onstellende observasie dat meer as 50% van pasiente wat weerstandig is teen een of meer anti-tuberkulose middel ook fenotipies en genotipies weerstandig is teen Pirasinamied. Genotipiese weerstandigheid word toegeskryf aan mutasies wat verspreid in die *pncA* geen voorkom. Dit bemoeilik die ontwikkeling van molekulêre metodes vir vinnige diagnose. Die onvermoë om weerstandigheid teen hierdie eerste-linie middel te bepaal lei tot onvoldoede behandeling. Dit hou negatiewe gevolge vir die Tuberkulose Beheer Program in. ‘n Toets vir Pirasinamied weerstandigheid is dus noodsaaklik om te verseker dat tuberkulose pasiënte op effektiewe behandeling is.

Nog ’n eerste-linie middel, Rifampisien, word beskou as ’n merker vir die identifikasie van multi-weerstandige tuberkulose. Verskeie molekulêre metodes word gebruik om mutasies wat met rifampisien weerstandidgheid geassosieer word, te identifiseer. Onlangs is ’n nuwe nukleotied verandering (*Rv2629* 191A/C) gerapporteer wat weerstandigheid teen Rifampisien veroorsaak. In teenstelling, het ons bewys dat hierdie nukleotied verandering nie geassosieer word met rifampisien weerstandigheid nie, maar dat dit wel beduidend geassosieer word met die Beijing stamboom van *M. tuberculosis*. Hierdie verandering is dus ’n bruikbare merker vir identifikasie van die Beijing stamboom wat in verskeie dele van die wêreld voorkom.

Die studie het eerste bewyse gelewer dat variasie in die vlakke van rifampisien weerstandigheid in verwante kliniese isolate van *M. tuberculosis* meer kompleks is as wat sentrale dogma beweer. Hiper-weerstandigheid kan nie net verduidelik word op grond van mutasies in die *rpoB* geen wat rifampisien weerstandigheid veroorsaak nie. Blootstelling van rifampisien weerstandige *M. tuberculosis* stamme aan middels (reserpine en verapamil) wat sal verhoed dat rifampisien deur die bakterie uitgepomp word, het getoon dat hierdie middels die vlakke van rifampisien sensitiwiteit beduidend beïnvloed. Dit het verrykende terapeutiese implikasies vir die behandeling van tuberkulose as dit in kombinasie met anti-tuberkulose middels gebruik kan word. Op grond van hierdie kennis is ’n model voorgestel van die evolusie wat die variërende vlakke van rifampisien weerstandigheid in die bakterie verduidelik. Deur transkriptomiese analise is ‘n aantal transporter gene geïdentifiseer wat as potensiële kandidate vir funksionele toetse en toekomstige middel ontwikkeling kan dien.

Hierdie innoverende navorsing bemagtig wetenskaplikes om bestaande idees weer te ondersoek en daardeur paradigmas te verskuif, wetenskaplike dogmas uit te daag en uiteindelik die raaisel omtrent weerstandigheid in die patogeen te ontrafel.

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CHAPTER 1

General Introduction

1.1 BACKGROUND

Mycobacterium tuberculosis (*M. tuberculosis*), the causative bacteria of tuberculosis (TB) in humans, is the leading cause of death by an infectious pathogen globally (3,15). The resurgence of TB can in part be attributed to the ability of the tubercle bacillus to adapt, evolve and become resistant to various anti-TB drugs. Multi-drug resistant (MDR) TB is defined as *M. tuberculosis* strains that are resistant to the two most important first-line drugs, Isoniazid (INH) and Rifampicin (RIF). Recently, extreme drug resistant (XDR) TB, the more severe and often frequently fatal form of drug resistance, has been identified in various parts of the world, including in South Africa (5,6). XDR-TB is defined by the World Health Organization (WHO) as *M. tuberculosis* strains that are MDR and in addition, also resistant to a Fluoroquinolone and one of the injectable drugs i.e. Kanamycin, Amikacin and Capreomycin. The emergence of MDR and now XDR *M. tuberculosis* strains is alarming and represents a worldwide threat to TB control (16,17).

Recent, surveillance data reported that the TB incidence rate in South Africa is 600/100 000 per annum (18). The WHO has also declared South Africa as one of the hot spots for MDR-TB (17). In 2001, the WHO reported that MDR-TB was identified amongst 1.75% and 6.95% for new and previously treated TB cases in South Africa (17). Findings from our laboratory indicate that more than 60% of drug resistance is due to transmission of an

already resistant strain (10-12). This is alarming and indicates that current control measures are inadequate to control the transmission of drug resistant TB strains. Furthermore we, and others have shown that resistance is due to a number of chromosomal mutations in specific genes of *M. tuberculosis* (14). Evidence of drug resistant mechanisms have been found through cloning and characterization of these specific genes in drug resistant isolates (7). Drug susceptible isolates lack these corresponding mutations. Thus the identification of these resistance conferring mutations has been proposed as means to genetically identify drug resistance, in particular MDR-TB, thereby improving the time to diagnosis and preventing the transmission of drug-resistance.

By application of molecular epidemiological approaches, we have shown that groups of closely related strains, [Beijing/W-like (28%), the IS*6110* Low-Copy-Clade (LCC) (26%), F11 (12%) and F28 (5%)], represent 71% of the drug resistant isolates in the local study communities (10). Although the Beijing/W-like and LCC strain families are dominant amongst drug resistant isolates, it is striking that these strain families are not the most dominant amongst drug susceptible isolates from local communities. Furthermore it is unclear why certain family specific drug resistant strains transmit better than other strains from within the same strain family, despite the fact that the control program has remained constant. In this regard it is possible that prolonged exposure to sub-lethal levels of anti-TB drugs, such as in many TB cases in South Africa that do not adhere to anti-TB treatment (13), may render the organism increasingly resistant (as defined by minimum inhibitory concentrations) to one or more drugs during treatment. This prolonged exposure might aid in the evolution and adaptation of survival mechanisms. Under such conditions clinical

strains may become more fit and transmissible. Long term drug exposure might result in the induction of additional mechanisms which may include efflux pumps (3). Before the introduction of anti-tuberculosis drugs, the physiological role of influx and active efflux systems was to protect the bacterial cell from toxic compounds or metabolites. However, the continuous exposure of *M. tuberculosis* to anti-TB drugs provides an evolutionary pressure for the selection of mutants in which alterations in drug efflux/influx mechanisms confer drug-resistance. The completion of the whole genome sequence of *M. tuberculosis* H37RV has fuelled attempts for other clinical strains to be sequenced. The Family 11 MDR strain is the first drug resistant clinical strain that has been sequenced, followed by other MDR and XDR TB strains from our drug resistant strain bank (http://www.broad.mit.edu). Analysis of these genome sequences has shown the presence of multiple putative efflux pumps (2). These influx/efflux pumps are grouped into different transporter types based on bio-energetics and direction of drug translocation. One such example is the class ATP-binding cassette (ABC) transporters that hydrolyse ATP to extrude the drugs out of the cell. The exposure of mycobacteria to various efflux pump inhibitors (EPI's) such as Reserpine, Verapamil and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) has been shown to reduce the resistance levels of anti-TB drugs (1,9). Recently, it was also shown that putative efflux pump genes were upregulated in clinical isolates when exposed to anti-TB drugs, such as INH, RIF and Ethambuthol (4,8). Therefore, it is evident that prolonged exposure of *M. tuberculosis* to drugs may enable the bacteria to evolve and adapt mechanisms, such as efflux pumps, that may modulate the level of drug resistance.

1.2 PROBLEM STATEMENT

Our observations indicate that certain drug resistant genotypes transmit better than others. It is possible that these genotypes have become increasingly resistant to anti-TB drugs due to mechanisms that are still undetermined. The inability to detect resistance to certain drugs may lead to transmission. In addition, unidentified mechanisms that may possibly promote strain fitness, also enables these genotypes to be transmitted. This study was designed to enhance the ability to detect drug resistance and to optimize the identification of mechanisms, in addition to gene mutations that would render the bacilli hyper-resistant to anti-TB drugs.

1.3 OVERALL HYPOTHESIS:

In this study we hypothesize that transmission of drug resistant strains is due to a combination of shortcomings in the TB control program and adaptive mechanisms that cause *M. tuberculosis* strains to become hyper-resistant to anti-TB drugs.

1.4 OVERALL AIM:

To identify the mechanisms that enable drug-resistant strains of *M. tuberculosis* to be transmitted.

Structure of Thesis:

Each chapter is structured for potential publication. If the results in a specific chapter have not been published yet, the references were formatted according to instructions of the Journal of Clinical Microbiology.

Chapter 1: General Introduction

Chapter 2: A balancing act: The role of efflux/influx in the evolution of mycobacterial drug-resistance

Chapter 3: Frequency and implications of Pyrazinamide resistance in managing previously treated tuberculosis patients

Chapter 4: *Rv2629* 191A/C nucleotide change is not associated with
Rifampicin resistance

Chapter 5: Evidence that the level of Rifampicin resistance in *Mycobacterium tuberculosis* evolves through a multi-step mechanism.

Chapter 6: Rifampicin induces differential expression of putative transporter genes in *Mycobacterium tuberculosis*

Chapter 7: Conclusion

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CHAPTER 2

A Balancing Act:

The role of efflux/influx in the evolution of mycobacterial drug-resistance

My contribution:	Research of data
	Literature searches
	Planning of manuscript
	Writing and editing of manuscript

ABSTRACT

Many aspects of mycobacterial drug-resistance mechanisms remain unknown despite the availability of a number of whole genome sequences of drug-resistant and drug-susceptible clinical and laboratory strains of *Mycobacterium tuberculosis*. Recent research has primarily focused on the identification of gene mutations conferring drug-resistance. Those research activities largely ignore cellular processes that are in homeostasis and that influx is in balance with efflux. Before the introduction of anti-tuberculosis drugs, the physiological role of the intrinsic hydrophobic cell membrane and active efflux systems was to protect the bacterial cell from toxic compounds or metabolites. These mechanisms conferred an intrinsic level of resistance to modern day anti-tuberculosis drugs. Continuous exposure of *Mycobacterium tuberculosis* to anti-tuberculosis drugs provides an evolutionary pressure for the selection of mutants in which alterations in drug efflux/influx mechanisms confer drug-resistance. Understanding these mechanisms will help in developing tools to identify and combat drug-resistance. This review focuses on the importance of understanding the evolution of efflux/influx mediated drug resistance in mycobacteria.

1. Introduction

Since the discovery of the tubercle bacillus by Robert Koch in 1882 (93), understanding the dynamics and survival mechanisms of this pathogen has led to more questions than answers. Despite stringent control strategies and many advances in our knowledge of the epidemiology of tuberculosis (TB) and the biology of the causitive agent *Mycobacterium tuberculosis (M. tuberculosis),* TB still remains one of the most common and deadly infectious diseases worldwide. The emergence of multi-drug resistant (MDR) TB (resistance to the first-line drugs Isoniazid (INH) and Rifampicin (RIF)) (36) and extensively drug resistant (XDR) TB (additional resistance to a Fluoroquinolone (FQ) and any one of the injectable drugs, Kanamycin (KANA), Amikacin (AMI) or Capreomycin (CAP)) (1,40,57) are a major concern in the control of the global TB epidemic.

Resistance to anti-tuberculosis drugs develops by spontaneous mutation and the resulting resistant mutants are selected by subsequent treatment with anti-tuberculosis drugs for which the mutants are resistant. Resistance to various first-line anti-TB drugs such as INH, RIF, Pyrazinamide (PZA), Ethambutol (EMB), and classes of second-line drugs (FQs, aminoglycosides, thionamides, peptides and cycloserines) is attributed to specific mutations in target genes or regulatory domains (10,11,28,59,90-92) (Table1). It is thus believed that a specific gene alteration (mutations, insertions or deletions) will alter the structure of the target protein, thereby influencing the degree of susceptibility to the drug (99). For example, the *katG* gene encodes for both catalase and peroxidise enzyme activity which is essential for the conversion of INH to its active form. Mutations in the *katG* gene lead to a

decrease in catalase activity, thereby resulting in less INH being activated and *M. tuberculosis* being resistant to high levels of INH (37). This relationship was confirmed by Ramaswamy *et al.* who showed that INH resistant isolates with a minimum inhibitory concentration (MIC) of >256μg/ml INH all had low or no catalase activity levels (74). In contrast, mutations in the regulatory or structural regions of the *inhA* gene results in low level resistance to INH in *M. tuberculosis* (38,74). Interestingly, mutations within the promoter and the coding region of *inhA* were found to also confer ETH resistance (7,59). This demonstrates that mutations in the same genes or regulatory domain can result in different drug resistance phenotypes.

However, resistance in a proportion of clinical *M. tuberculosis* isolates cannot be explained by classical gene mutations such as those described above. For example, approximately 20-30% of clinical INH resistant *M. tuberculosis* isolates do not have mutations in any of the known genes (Table1) associated with INH resistance (73,74). Similarly, approximately 5% of clinical RIF resistant *M. tuberculosis* isolates do not harbour mutations in the Rifampicin Resistance Determining Region (RRDR) of the *rpoB* gene (95). Therefore, it is evident that other more esoteric mechanisms could play a role in drug resistance.

Additional mechanisms of drug resistance in mycobacteria include the production of drug modifying and inactivating enzymes. These mechanisms include resistance associated with gene mutations, production of modifying enzymes that inactivate the active metabolites, low cell wall permeability and efflux related mechanisms (2,9,12,73,102,103). Mycobacteria produce enzymes that degrade or modify defined antibiotics leading to

inactivation (53,94). For example, *Mycobacterium smegmatis (M. smegmatis)* is naturally resistant to RIF, although no mutations have been identified in the *rpoB* gene (72). This suggests that an alternative mechanism plays a role in conferring resistance to RIF. In 1995, it was reported that *M. smegmatis* DSM43756 inactivates RIF by ribosylation, whereby a ribose ring is covalently linked to the RIF molecule (17,42). Gene disruption experiments provided evidence that RIF inactivation via ribosylation was the principal contributor of RIF resistance in *M. smegmatis* (72). However, only limited data exists for the production of degrading and drug modifying enzymes in *M. tuberculosis.* It has been reported previously that bacterial resistance to aminoglycosides can be attributed to enzymatic inactivation of aminoglycosides by phosphotransferases, nucleotidyltransferases and acetyltransferases (18). Acetyltransferase AAC (2')-Ic and the phosphotransferase encoded by *Rv3225c* gene, has been shown to display aminoglycoside modifying activity (25) that resulted in resistance to aminoglycosides in mycobacteria.

In order to design new anti-TB drugs and to develop novel diagnostics it is essential to gain an in depth insight into the mechanisms, other than the classical nsSNPs in known target genes, that confer resistance. This is of particular importance since pathogenic mycobacteria, such as *M. tuberculosis*, are becoming increasingly resistant to many of the first and second line anti-TB drugs. This review gives a broad perspective on the regulation of intracellular anti-TB drug concentration by efflux related mechanisms in mycobacteria.

2. Mechanisms of the mycobacterial intrinsic resistome

The intrinsic resistome is an evolutionary ancient phenotype, and can be defined as the intrinsic resistance of any bacterial species that has not been acquired as a result of recent use of antibiotics (27), and is therefore independent of previous antibiotic selective pressure. Intrinsic resistance is usually the result of reduced permeability of the bacterial envelope and the activity of multidrug efflux pumps (62). This suggests that the main physiological roles of the components of intrinsic resistance is the prevention of influx of toxic components by restricting permeability of the cell or the active transportation of the metabolites or toxic compounds out of the cell.

In mycobacteria, the influx of toxic compounds is significantly restricted by the complex cell wall and lipid bilayer. This waxy lipid-rich cell wall comprising three covalently linked molecules i.e peptidoglycan, arabinogalactan and mycolic acid presents a significant barrier to the penetration of antibiotics (9). The reduction in membrane permeability leads to a decrease in the influx of the drug, thus leading to a decrease in the intracellular drug accumulation (61,66,99).

DNA sequencing has predicted the genome of *M. tuberculosis* strain H37Rv encodes multiple putative efflux proteins, of which the majority have not yet been characterized (2,99). These efflux pump mechanisms probably have a pre-existing physiological role and protect the bacillus against low intracellular levels of toxic molecules and metabolites and maintain cellular homeostasis and physiological balance through transport of the toxins to

the extracellular environment. Recent evidence suggests that mycobacteria extrude multiple drugs (53,94,98) via active efflux systems (55,66,77). However, the efflux of a broad range of structurally unrelated toxic compounds can be considered to be an "accidental and opportunistic" side effect of the transport of unidentified physiological substrates (12,13,71,107). Some efflux pumps are specific for certain antibiotics whilst others extrude promiscuous structurally and functionally unrelated compounds as is the case for Multi Drug Resistance (MDR) efflux pumps (49,53,55). Experimental procedures for the identification of these pumps are limited to laboratory-induced mutants over-expressing efflux pumps. It was suggested by Gagneux *et al.* that clinical isolates have undergone more adaptation than laboratory-induced mutants (29). However, very few studies have been done on clinical isolates and the specific conditions required for the induction of these pumps are not known yet, although it is well recognised that expression of efflux pump genes is tightly regulated (62,71,76). This ensures that efflux pump genes are available when required by the cell to perform their physiological function.

2.1. Multi-Drug Resistance (MDR) efflux pumps

MDR efflux pumps by definition reduce the intracellular concentration of more than one antibiotic to sub-inhibitory levels (53,55) and thereby are thought to promote the emergence of drug resistance. Genes encoding MDR efflux pumps are constitutively expressed in wild type cells (63) and thereby confer a low level of resistance. Current evidence suggests that *M. tuberculosis* contains more than one efflux pump capable of extruding more than one antibiotic, e.g. the Tap efflux pump that extrudes both

aminoglycosides and tetracyclines (TET) (2). MDR efflux pumps and transporters can be over-expressed due to mutations in regulatory regions (62). Alternatively, antibiotics can induce the expression of an MDR efflux pump by interacting with regulatory systems (76), e.g., TET-specific pumps possess regulatory controls that sense the presence of TET which acts as an inducer (33,34). Both of the above mechanisms lead to an increase in the level of drug resistance. Thus, the inactivation or silencing of the efflux pumps (including MDR efflux pumps) could be a possible mechanism of controlling drug resistance. This would render the bacterium more susceptible, allowing lower doses of drugs to be used in the treatment of TB.

MDR transporters are grouped into the following 2 major classes, based on their bioenergetic and structural profiles:

2.1.1. Secondary multi- drug transporters (influx/efflux pumps)

Secondary multi-drug transporters utilize the transmembrane electrochemical gradient of protons or sodium ions to extrude the drugs from the cell (19). They are divided into 4 families; the major facilitator superfamily (MFS), the small multi-drug resistance family (SMR), the resistance nodulation cell division (RND) and multi-drug and toxic compound extrusion (MATE) family (2,49,55). Most RND proteins are either multi-drug or multi-cation transporters (63) and are mostly found in gram negative bacteria. The SMR proteins bind cationic, lipophilic antibiotics and transport them across the membrane in exchange for protons. In *M. tuberculosis, mmr* is an example of an SMR protein that confers resistance to acriflavine, ethidiumbromide and erythromycin (6) (Table 2). The expression of MFS and RND superfamily transporters is known to be subject to regulatory controls

(66,71,97). In some instances the transported substrates acts as the inducer, while in others the transported substrates acts as the repressor by binding to the specific regulatory domains thereby modulating gene expression. An example is the *tap (tub)* gene, encoding a homolog of *tap(for)* in *M. tuberculosis* called *M.tb Tap/ Rv1258c*. The expression of this transporter is dependent on the expression of multi-drug resistant effector gene, *whiB7* (60).

2.1.2. ATP-binding cassette (ABC) type multi-drug transporters

ATP-binding cassette (ABC) type multi-drug transporters utilize the free energy of ATP hydrolysis to pump drugs out of the cell. The ABC transporters occupy about 2.5% of the total genome content of *M. tuberculosis* (12) and can be classified as importers or exporters based on the direction of translocation of their substrate as well as the orientation of receptors and channels (45,46). As their names suggest, importers are involved in the uptake of extracellular molecules while exporters export molecules from the cytoplasm to the external milieu. The ABC importers are composed of 4 domains: 2 membrane spanning domains (MSDs) associated with two cytoplasmic nucleotide domains (NBDs) (45). The NBDs show considerable sequence homology across the spectrum of ABC transporters. Sequence conservation is marked in 5 of the NBDs motifs. Two of the 5 motifs (namely WALKER A and WALKER B motifs) are present in a vast majority of ATP-binding proteins. The remaining 3 motifs characteristically consist of a signature, Histidine loop and Glutamine loop (39,100). Evidence suggest that phosphorylation of the NDB of the ABC transporter *Rv1747*, by serine/threonine kinase *PknF* is a possible mechanism of regulation of this transporter (58).

3. Evidence of efflux mechanisms leading to mycobacterial drug resistance.

3.1. Isoniazid (INH)

There is substantial evidence for efflux related mechanisms in *M. smegmatis,* which is 300 fold more resistant to INH than *M. tuberculosis*. The overexpression of the *M. tuberculosis mmpL7* gene in *M. smegmatis* confers high level INH resistance. With the addition of efflux pump inhibitors (EPI's) Reserpine, CCCP, *ortho*-vanadate, Reserpine and Verapamil in *M. smegmatis* (13), the level of INH resistance decreased. These results suggest that *mmpL7* is involved in the energy-dependent efflux of INH (66). More evidence for efflux related INH resistance comes from analysis of the *iniA* gene in *M. tuberculosis*. It was shown, by gene knock-out experiments, that the *iniA* gene is essential for the activity of an efflux pump that confers resistance to INH and EMB (14) and that *M. tuberculosis* strains lacking the *iniA* gene showed increased susceptibility to INH. The *iniA* deletion also results in an accumulation of intracellular ethidium bromide thereby suggesting that *iniA* plays a role in efflux. This was supported by the observation that on the addition of Reserpine, resistance to both INH and ethidiumbromide were reversed (14). Recent investigation of gene expression differences by qRT-PCR in clinical isolates of *M. tuberculosis*, showed that various MFS efflux pump genes (*efpA*, *PstB, Rv1258c*, *Rv1410c*) and ABC transporters (*Rv1819c* and *Rv2136c*) were overexpressed in the presence of INH (35,43,101). Collectively, these results suggest that INH resistance in *M. tuberculosis* may be attributed to mutations in known genes and could also be influenced or modulated by efflux related mechanisms.

3.2. Rifampicin (RIF)

Although mutations in the *rpoβ* gene have been described in RIF resistant *Mycobacterium avium* (*M. avium)*, many isolates from the *M. avium* and *Mycobacterium intracellulare (M. intracellulare)* group demonstrate a significant level of natural resistance to RIF, possibly as a result of an efficient permeability and exclusion barrier. Low-level RIF resistance in wild-type *M. smegmatis*, *Mycobacterium aurum* (*M. aurum*) and *M. tuberculosis* has been shown to be due to an efflux mechanism that extrudes the drug (69). Despite 95% of RIF resistant clinical *M. tuberculosis* isolates being attributed to mutations in the RRDR, there is evidence to suggest that efflux related RIF resistance mechanisms may play a role in *M. tuberculosis* as RIF has been shown to upregulate the expression of the tap-like pump, *Rv1258c* and other putative efflux pumps *Rv1410c, Rv1819c, Rv2136c* in a clinical isolate of *M. tuberculosis* (35,43,83). In addition, the inorganic phosphate importer *PstB* has been overexpressed in the presence of RIF (35,43,67,78).

3.3. Pyrazinamide (PZA)

PZA resistance in *M. tuberculosis* is primarily attributed to a wide spectrum of mutations in the *pncA* gene that encodes the enzyme pyrazinamidase (PZase) (44,54,73) PZase activates PZA by converting it into active pyrazinoic acid (POA) (104). About 70% of PZA resistant clinical isolates can be attributed to mutations in the *pncA* gene (79,80,86). The *pncA* gene can also be inactivated by the insertion of IS*6110*, thereby conferring the resistance phenotype (50). However, studies by Zhang *et al* confirmed that efflux pumps also play a role in mycobacterial resistance to PZA (87,107). Notably, in non-tuberculous *M. avium* and *M. smegmatis*, natural resistance to PZA is not due to a defective *pncA* gene but is due

to a highly active efflux mechanism that extrudes the active POA from the cell as soon as PZA is converted by PZase (87). The unique susceptibility of *M. tuberculosis* to PZA is due to efficient PZase activity at acidic pH as well as to a defective POA efflux mechanism (107). The definitive components of the POA efflux mechanism remain to be described, although accumulation of radioactive POA in *M. tuberculosis* and its extrusion by non-tuberculous mycobacteria have been demonstrated (87). The efflux pump inhibitor Reserpine has been shown to be an effective inhibitor of the POA efflux pump, increasing the susceptibility of *M. tuberculosis* to PZA by threefold (106).

3.4. Fluoroquinolones (FQs) and Cationic dyes

The FQs target and inactivate DNA gyrase and type II DNA topoisomerase (31,75), which are encoded by *gyrA* and *gyrB* respectively (75). Missense mutations within the Quinolone-Resistance-Determining-Region (QRDR) have been identified and are associated with FQ resistance (31). However, not all FQ resistant clinical *M. tuberculosis* isolates can be explained by mutations in the *gyrA* and *gyrB* genes.

An FQ efflux pump of the MFS superfamily, *LfrA*, was the first efflux pump to be described in *M. smegmatis* (52). LfrA exhibits broad substrate specificity to more hydrophilic FQs. When expressed on a plasmid, *LfrA* mediates low-level resistance to FQ's and other toxic compounds such as ethidium bromide. The disruption of the *lfrA* gene in *M. smegmatis* causes increased sensitivity to ethidium bromide (52) and a minor decrease in the level of Ciprofloxacin (CIP) resistance (89).

Recently, it has been shown that fluoroquinolone resistant *M. tuberculosis* strains with mutations in the *gyrA* and *gyrB* genes were influenced by the efflux pump inhibitors MC207.110 and Reserpine. The levels of resistance for OFL and CIP were reduced between 2- and 6-fold (26). The disruption of the Pst phosphate-specific protein of *M. smegmatis* was also correlated with a decrease in CIP efflux. This resulted in an increased susceptibility to CIP, suggesting an involvement in the efflux of this antimicrobial agent (70). Pasca *et al* showed that the addition of the efflux pump inhibitor Reserpine to CIP resistant *M. tuberculosis* increased the intracellular accumulation of CIP. These results indicate that the Rv2686c-Rv2687c-Rv2688c proteins actively pump out CIP, probably by using ATP hydrolysis as an energy source (65) (Table 2).

3.5 Aminoglycosides and Tetracyclines

Streptomycin (STR), KANA and AMI are some of the known aminoglycosides used for the treatment of MDR-TB. The aminoglycosides target the 30S subunit of the ribosome by binding to the 16sRNA and S12 ribosomal proteins. Mutations in the *rrs* gene (encoding 16sRNA) and the *rpsL* gene (encoding S12 protein) can confer resistance to the aminoglycosides (41,81,96). Recently, mutations in a highly conserved gene , *gidB*, was identified that functions as a rRNA methyltransferase. It was observed that mutations in *gidB* results in low level STR resistance (64). Approximately 65-67% of STR resistant clinical isolates harbour mutations in one of these two genes, however some resistant isolates lack identifiable gene mutations. It has been suggested that the blocking of STR uptake due to membrane impermeability (9) could explain resistance in these cases,

however, efflux related mechanisms leading to aminoglycoside resistance has been recently observed (12,85).

A TAP multidrug efflux pump cloned from *Mycobacterium fortuitum* (*M. fortuitum*) and homologous to that of *M. tuberculosis* has been identified that confers low-level resistance to aminoglycosides and TET when expressed in *M. smegmatis*. Sequence analysis of the *Tap* gene predicts a protein exhibiting similarities to efflux pumps encoded by MFS (2). Another example of efflux related drug resistance to aminoglycosides and TET includes the characterization of the P55 protein isolated from *M. bovis* which was shown to be related to aminoglycoside and tetracycline efflux pumps in mycobacteria (84). Addition of CCCP, Verapamil and Reserpine to the strains expressing P55 decreased the MIC's for both STR and TET. In contrast, the MIC level for both drugs in the control strain did not change. Results therefore indicate that resistance levels as a result of P55 were sensitive to the EPI's and substances that eliminate proton gradient across membranes, suggesting that P55 uses energy from the proton gradient to drive transport of drugs (84). Other examples of aminoglycoside and TET related efflux include the expression of the ABC transporter genes *drrAB* in *M. smegmatis*, which confer resistance to a broad range of clinically relevant antibiotics, including TET, STR and EMB. The addition of Reserpine and Verapamil reversed the resistance phenotype (12).

Recently it has been shown that the transcriptional regulator *whiB7* of *M. tuberculosis* is upregulated in the presence of STR and KANA, although its target gene is yet to be determined (30). The phosphate-transport ATP-binding protein *PstB* was also shown to

extrude STR in *M. smegmatis* (35). For tetracycline specifically, an energy-dependent efflux pump Tet(V) was identified in *M. smegmatis*. The *tet(V)* gene encodes an efflux protein which uses proton motive force to extrude tetracycline from *M. smegmatis*. The Tet(V) protein is not homologous to any known specific TET efflux pump, and remains restricted to *M. smegmatis* and *M. fortuitum* species (22,51).

4. Efflux pump genes not implicated in drug resistance

Antibiotic resistance characteristics have facilitated the identification of many efflux proteins. However, *efpA* was discovered fortuitously during the screening of genes for novel *M. tuberculosis* membrane proteins (24). EfpA is predicted to be highly related to members of the QacA transporter family (Qac TF), which is also known as the drug resistance transporter family. Thus far, *efpA* has not been implicated in drug resistance, although all other members of the QacA TF mediate resistance to antibiotics. EfpA was detected in all drug-susceptible and drug-resistant *M. tuberculosis* isolates examined (24).

5. Concluding remarks

With the identification of chromosomal mutations in drug resistant pathogens, it was thought that the war against drug resistance in mycobacteria can potentially be won. However, the organisms have adapted their ancient mechanisms of toxin removal opportunistically to aid in fighting the battle against antibiotics. The opportunistic intrinsic resistance and the ability to evolve higher levels of resistance involve a complex network of non-classical antibiotic resistance genes and mechanisms. Therefore, the identification and

characterization of the genes and mechanisms forming these networks will contribute to the accurate prediction of the emergence of antibiotic resistance. In addition, it will help to define new potential drug targets against intrinsically resistant bacterial pathogens.

Clinical use of compounds such as efflux pump inhibitors may serve to decrease intrinsic resistance in pathogens and lower the frequency of the emerging resistant mycobacterial strains. Therefore, it would be beneficial to inhibit efflux pump activity of a specific gene and thereby improve the clinical efficacy of various anti-TB drugs that are substrates of such efflux pumps.

No new TB drugs have been developed and implemented into the TB treatment regime for 40 years. Therefore, it is imperative to understand drug resistance mechanisms, specifically efflux related drug resistance, so as to aid in improving existing drug treatments, identifying new potential drug targets and to define new drug development strategies. A better understanding of these mechanisms will aid in the rapid detection and prevention of transmission of drug resistant *M. tuberculosis* strains (82) and can lead to more efficient anti-TB treatment.

This review will be submitted to an international journal for potential publication.

Table 1: Genes associated with resistance to various anti-TB drugs

Drugs	Chemical properties of drug	Discovery date	Drug mode of action	Gene	Target enzyme	References
INH*	Hydrophilic	1952	Inhibits cell wall synthesis	*KatG*	catalase peroxidase	(47,73,75,105)
				InhA	fatty acid enoyl acyl carrier protein reductase A	
				ahpC	alkyl hydroperoxidase reductase	
				kasA	B-ketoacyl-ACP synthase	
				Ndh	NADH dehydrogenase	
RIF*	Hydrophobic	1966	Inhibits RNA synthesis	*rpoB*	B-subunit of RNA polymerase	(73,75,91,105)
STR***	Hydrophilic	1944	Inhibits translation	*rpsL*	16S rRNA ribosomal subunits	(64,73,105)
				Rrs		
				gidB		
EMB*	Hydrophilic	1961	Inhibits cell wall synthesis	*embCAB*	arabinosyl transferase	(73,105)
PZA*	Hydrophilic	1952	Disrupts plasmamembrane and energy metabolism	*pncA*	Pyrazinamidase,	(79,105)
				IS*6110* insertion		
FQ**	Hydrophobic	1963	Introduces negative supercoils in DNA molecules	*gyrA*	DNA gyrase	(16,31,32,105)
				gyrB		
AMINO-GLYCOSIDES**	Hydrophilic	1957	Inhibits translation	*rrs, rpsL*	16S rRNA ribosomal subunits	(3,48,56,88,92,105)
PEPTIDES**	Hydrophilic	1957		*Rrs*		
				tlyA		
ETH**	Hydrophilic	1956	Disrupts cell wall biosythesis	*InhA*	fatty acid enoyl acyl carrier protein reductase A	(7,59,105)
				ethA	flavin monooxygenase	
				ethR	Transcriptional repressor	

Legend to Table 1. INH-Isoniazid; RIF-Rifampicin; STR-Streptomycin; EMB-Ethambutol; PZA-Pyrazinamide; FQ-Fluoroquinolones; VIO-Viomycin; CAP-Capreomycin; ETH-Ethionamide

*First-line drug for the treatment of TB; **Second line drug for the treatment of MDR-TB; ***Alternative first-line drug for retreatment TB cases

Note: The FQ for the treatment of MDR-TB consist of Moxifloxacin; Aminoglycosides consist of Kanamycin and Amikacin; Peptides consist of Capreomycin and Viomycin

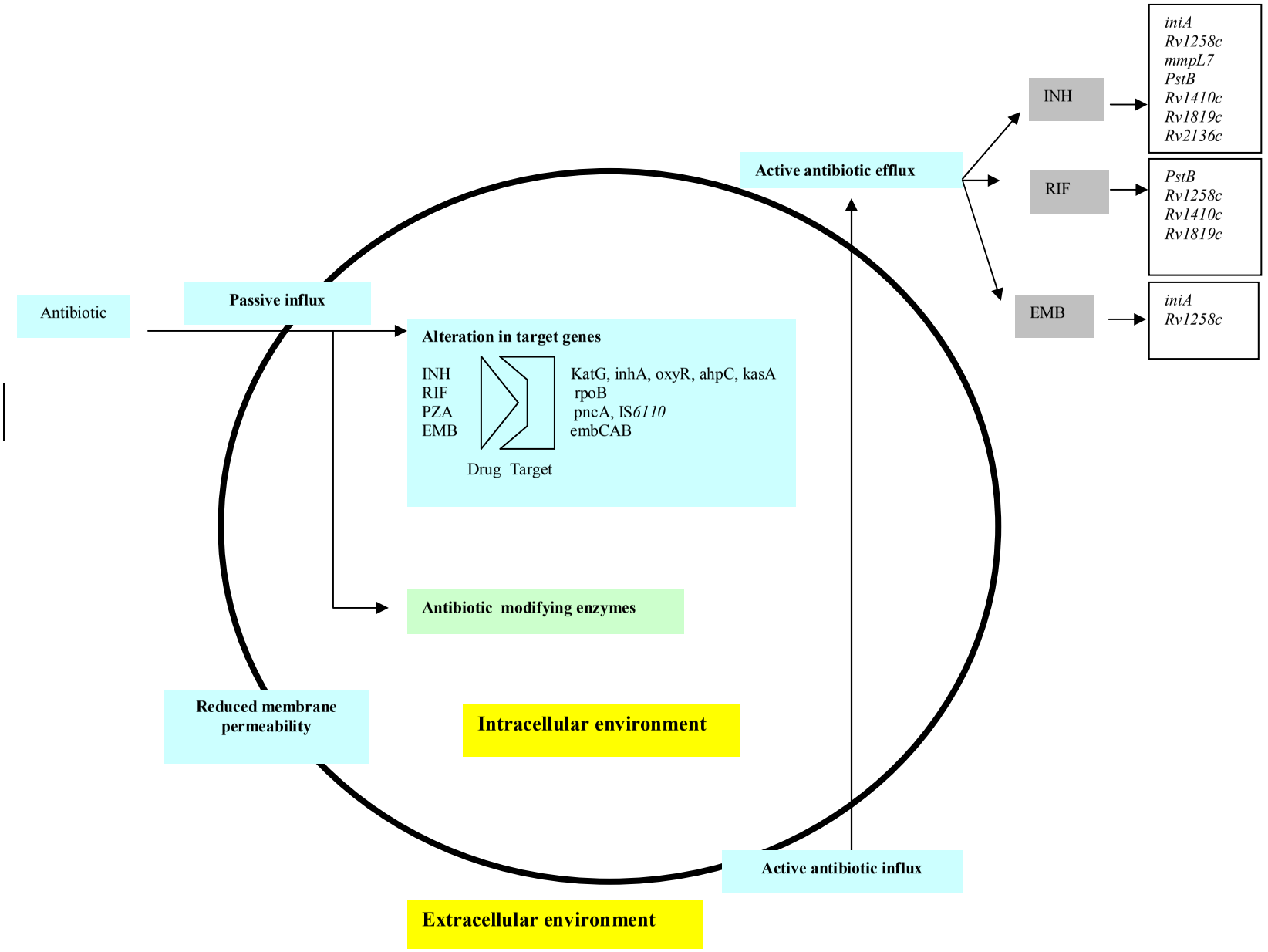

Figure 1: Illustration of the mechanisms conferring resistance to first-line anti-Tb drugs.

Table 2: Reported putative efflux pump genes and transporters that may play a role in drug resistance in mycobacteria

Gene	Possible Drugs extruded	Transporter	Function	Protein Product	Reference
PstB	INH, RIF, EMB, CIP	ABC	Active import of inorganic phosphate and export of drugs	Phosphate-transport ATP-binding protein	(5,8,15,78)
Rv2686c	CIP	ABC	Active transport of drugs	Integral membrane ABC transporter	(8,15,65)
Rv2687c	CIP	ABC	Export of highly hydrophobic drugs	Antibiotic transport integral protein	(8,15,65)
Rv2688c	CIP	ABC	Export of toxic compounds	Antibiotic-transport ATP-binding protein	(8,15,65)
Rv1747	INH	ABC	Transport of drug across the membrane.	conserved transmembrane ATP-binding protein	(8,15)
drrA	TET, STREP, EMB	ABC	Export of antibiotic in the cell wall.	ATP-binding protein drrA	(8,12,15)
drrB	TET, STREP, EMB	ABC	Export of antibiotic in the cell wall.	ATP-binding protein drrB	(8,12,15)
drrC	TET, STREP, EMB	ABC	Export of antibiotic in the cell wall.	ATP-binding protein drrC	(8,12,15)
Rv1348	Multiple Drugs	ABC	Active export/translocation of drugs across the membrane.	Probable drugs-transport transmembrane ATP-binding protein	(15)
Rv1456c	Undetermined	ABC	Active export of antibiotic across the membrane	Integral membrane protein	(15)
Rv1463	Undetermined	ABC	Active transport and energy coupling across the membrane.	Probable conserved ATP-binding protein	(15)
Rv1258c	INH, RIF, EMB, OFL	MFS	Export of drugs	Conserved membrane protein	(15,20,43,83)
Rv2994	Undetermined	MFS	Efflux of drugs	Conserved membrane protein	(15,20,51)
Rv1877	TET, KANA, Erythromycin	MFS	Efflux of drugs	Conserved membrane protein	(15,20,51)
Rv1634	Undetermined	MFS	Efflux of sugars and drugs	Drug efflux membrane protein	(15,19,20,51)
efpA	Possibly INH	MFS	Export of drugs	Integral membrane efflux protein	(15,24,51)
Rv2333c	Tetracycline	MFS	Efflux of drugs	conserved integral membrane transport protein	(15,20)
Rv2459c	Drugs	MFS	Transport of substrates	conserved integral membrane transport protein	(15,20)
Rv3239c	Sugar or drugs	MFS	could be involved in efflux	conserved transmembrane transport protein	(15,20)
Rv3728	Sugar or drugs	MFS	Involved in efflux	conserved two-domain membrane protein	(15,20)
mmpL7	INH	RND	Export of antibiotic	transmembrane transport protein	(15,23,66,68)
emrB	Undetermined	SMR	Export of multiple drugs	Integral membrane efflux protein	(15,21)
whiB7	RIF	regulatory protein	Transcriptional regulation	transcriptional regulatory protein and effector gene	(15,83)
Rv2989	Undetermined	transcriptional regulator	transcriptional mechanism	transcriptional regulatory protein	(15)
iniA	INH, EMB	Membrane protein	Drug transport	INH inductible protein iniA	(4,14,15)
iniB	INH	Membrane protein	Drug transport	INH inductible protein iniB	(4,14,15)
iniC	INH	Membrane protein	Transcriptional mechanism	INH inductible protein iniC	(4,14,15)
Rv1002c	Undetermined	Membrane protein	Unknown function	Integral membrane protein	(15)
Rv3806c	Undetermined	Membrane protein	Unknown Function	Integral membrane protein	(15)
Rv3679	Undetermined	ATPase	Extrusion of anions	Probable anion transporter	(15)

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CHAPTER 3

Frequency and implications of pyrazinamide resistance in managing previously treated tuberculosis patients.

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- Planning of project
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SUMMARY

Objective: To determine the extent of Pyrazinamide (PZA) resistance in isolates from previously treated patients from the Western Cape, South Africa.

Design: Drug-resistant, resistant to one or more drugs other than PZA (PZA resistance is not routinely determined) (n=127), and drug-susceptible (n=47) clinical isolates of *M. tuberculosis* from previously treated patients from the Western Cape, South Africa, were phenotypically (BACTEC MGIT 960) and genotypically (*pncA* gene sequencing) analysed for PZA resistance.

Results: MGIT analysis found that 68 of the 127 drug-resistant isolates were PZA resistant. Nearly all (63/68) PZA resistant isolates had diverse nucleotide changes scattered throughout the *pncA* gene and 5 PZA resistant isolates had no *pncA* mutations. Forty-six of the forty-seven phenotypically susceptible isolates were susceptible to PZA, while one isolate was PZA mono-resistant (OR = 53.0, 95% CI = 7.1-396.5). A *pncA* polymorphism ($Thr_{114}Met$) not conferring PZA resistance was also identified. PZA resistance was strongly associated with multidrug-resistant TB (MDR-TB).

Conclusion: An alarmingly high proportion of South African drug-resistant *M. tuberculosis* isolates are PZA resistant indicating that PZA should not be relied upon in managing patients with MDR-TB in the Western Cape, South Africa. A method for the rapid detection of PZA resistance would be beneficial in managing patients with suspected drug resistance.

Keywords: Pyrazinamide, *pncA*, tuberculosis

INTRODUCTION

Pyrazinamide (PZA) is an integral component of drug regimens for both drug sensitive and drug resistant TB. Because it is effective in the acidic microenvironment produced during acute inflammation, it is believed to play a unique role in killing a subpopulation of semi-dormant TB bacilli that are not easily killed by other antibiotics.[1;2] The addition of PZA to standard chemotherapeutic regimes for TB allowed the duration of therapy to be shortened to 6 months and has been credited with the success of current DOTS strategy.[3] Since PZA resistance is believed to be rare, the drug is also an important element in second line regimens used to treat MDR-TB for which rifampicin (RMP, R) and isoniazid (INH, H) are ineffective.

PZA is a prodrug that is converted intracellularly to the active moiety, pyrazinoic acid (POA), by the mycobacterial enzyme pyrazinamidase (PZase).[1;2] POA accumulates within *M.tuberculosis* bacilli when the extracellular pH is acidic.[4-6] This accumulation of POA lowers the intracellular pH which is thought to lead to the inactivation of a vital target enzyme such as fatty acid synthase I (*fasI*).[3]

Multiple studies have demonstrated that mutations in *pncA*, the gene that encodes for PZase, are a major mechanism of resistance to PZA.[7] Previously identified mutations include missense mutations that cause amino acid substitutions, nucleotide insertions or deletions, and mutations in the putative promoter region of the *pncA* gene that alter gene expression. Although some researchers have found that most PZA resistant TB isolates have *pncA* mutations, others have reported that a substantial proportion of resistant strains lack these mutations and have proposed additional mechanisms of resistance including the non-uptake of the pro-drug.[7]

As PZA is only active at low pH (pH 5.5), susceptibility testing for PZA is complicated by the poor growth and subsequent death of mycobacteria at low pH in the test medium.[7;8] For these technical reasons, PZA susceptibility testing is not done routinely in many countries and the extent of PZA resistance globally is largely unknown.

Over the past decade, the prevalence of MDR-TB has increased in parts of South Africa. While a study conducted in the early 1990s in the Western Cape identified MDR-TB amongst 1% of previously untreated cases,[9] MDR-TB cases increased to 2% during 2001 to 2002.[10]

In this study, we describe the phenotypic and genotypic identification of PZA resistance in clinical isolates of *M. tuberculosis* from the Boland/Overberg and South Cape/Karoo (BOKS) regions of South Africa.

STUDY POPULATION AND METHODS

Study setting

The study was conducted between September 2000 and December 2002 in 72 primary health care clinics in the BOKS region of the Western Cape Province, South Africa. The incidence of new smear positive TB cases in this province in 2002 was 571/100 000 population.[11] TB cases were diagnosed by passive case finding and treated according to National TB Control Programme guidelines. Following DOTS recommendations, smear positive cases were treated for 2 months on a 4-drug Fixed Combination preparation (INH, RMP, PZA, ethambutol [EMB, E]). If patients remained smear positive after 2 months, sputum culture and drug susceptibility testing (DST) were performed and the patient was clinically reassessed. Routine DST was performed by the National Health Laboratory Services in Green Point by the indirect proportion method on Middlebrook 7H11 medium, and was therefore only available for previously treated patients. All drug-resistant patients were treated with individualized chemotherapy based on DST results and as specified by the patient's physician. This study was approved by the ethics committee of Stellenbosch University, Tygerberg, South Africa.

Isolates and DNA

A total of 127 drug-resistant (resistant to one or more drugs other than PZA) *M. tuberculosis* isolates were analysed in this study. These isolates were randomly selected from previously treated patients attending clinics in the BOKS regions during the period from 22 February 2001 to 1 December 2002. Genotyping showed that these drug-resistant isolates represent various highly prevalent *M. tuberculosis* drug-resistant genotypes from this region which include Beijing/W, F11, F28, LCC and unique genotypes which have been previously described in this region.[12] Strain families were defined according to IS*6110* restriction fragment length polymorphism analysis, with the dice coefficient and a similarity index of ≥65%. Spoligotyping confirmed

the presence of *M. tuberculosis* in all of the isolates analysed.[12;13] Fourty-seven randomly selected drug-susceptible isolates, representing the same strain families as the drug-resistant isolates, collected from previously treated patients during the period from 17 January 2002 to 1 December 2002 from the BOKS region were also available for this study.

PZA susceptibility testing

Susceptibility to PZA was tested by using the non-radiometric BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 method according to the BACTEC manual. The test medium is a modified Middlebrook 7H9 broth which supports the growth and detection of mycobacteria at a reduced pH of 5.9. The recommended critical concentration of 100μg/ml PZA was used in the BACTEC MGIT 960 test to discriminate between PZA susceptible and PZA resistant isolates. Four PZA resistant isolates from a supranational reference laboratory in South Africa were included as additional controls.

PCR amplification of the *pncA* gene

Using the genome sequence of the *M. tuberculosis* H37Rv reference strain (http://genolist.pasteur.fr/TubercuList) and DNAMAN version 4.1 software (Lynnon Biosoft, Vaudreuil-Dorion, Quebec, Canada), primers were designed to amplify a 615bp product which included the *pncA* coding region as well as flanking regions. The *pncA* F primer 5` AGTCGCCCGAACGTATGGTG 3` was designed to anneal –25bp upstream of the start codon (ATG) and the *pncA* R primer 5` CAACAGTTCATCCCGGTTCG 3`annealed 29 bp downstream of the stop codon. Purified mycobacterial DNA (2 μl) was added to PCR reagents which include 2x Q solution, 1x PCR buffer, 2mM $MgCl_2$, 0.4mM dNTPs, 50 μM of each primer and 1.25U Hot Star Taq polymerase (Qiagen, San Diego, CA, USA). The PCR

reactions were performed under the following thermocycling conditions: 15 minutes (min) denaturation at 95 ^{o}C followed by 35 amplification cycles (each cycle: 94 ^{o}C for 1 min, 62 ^{o}C for 1 min, 1 min extension at 72 ^{o}C) and the final elongation step of 10 min at 72 ^{o}C in a thermal cycler (Perkin Elmer Gene Amp PCR system 2400, Perkin Elmer, Cape Town, South Africa). Successful PCR amplification was confirmed by gel electrophoresis (100 Volts, 1 hour) on a 2% agarose gel after staining with ethidium bromide.

Sequencing of the *pncA* gene

PCR products were cleaned with exonuclease I / shrimp alkaline phosphatase (*Exo* I and SAP-IT) according to the instructions of the manufacturer (Amersham, Vienna, Austria). Typically, 2µl of ExoSAP-IT (containing Exonuclease I and Shrimp alkaline phosphatase) was added to 5µl PCR product. The reaction was mixed and incubated at 37^{o}C for 15 min in a thermal cycler (PTC-100 Programmable Thermal Controller, MJ Research Inc, Ramsey, MN, USA). The enzymes were inactivated by heating to 80^{o}C for 15 min and thereafter 7µl distilled water was added before DNA sequencing. The diluted products were sequenced (ABI PRISM DNA Sequencer model 377, Perkin Elmer) in both directions with the *pncA* F and *pncA* R primers, respectively.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism, Version 4.03. Differences between variables were expressed as odds ratio (OR) with 95% confidence intervals (95% CI).

RESULTS

PZA resistance among drug-resistant clinical *M. tuberculosis* isolates from South Africa

BACTEC/MGIT analyses showed that a total of 68 of the 127 (53.5%) drug-resistant isolates from previously treated patients were phenotypically resistant to PZA at a critical concentration of 100μg/ml PZA. Forty-six of the forty-seven (98%) isolates that were phenotypically susceptible to other drugs were found to be susceptible to PZA. However, one isolate (isolate S19) which was phenotypically susceptible to other drugs, was PZA resistant, suggesting PZA mono-resistance (OR = 53.0, 95% CI = 7.1-396.5) (Table).

Correlation of mutations in the *pncA* gene with phenotypic resistance to PZA

Of the 68 (93%) PZA-resistant isolates, 63 had nucleotide changes that include non-synonymous mutations, insertions and deletions in the *pncA* gene that resulted in a change of the amino acid sequence of the PZase protein (Table). These mutations were diverse and scattered along the gene. The Figure shows the positions of the 28 different mutations found in the *pncA* gene, and illustrates the high diversity of mutations in the gene and the absence of a common hotspot for mutation. Five of the 68 (7%) PZA-resistant isolates did not have any *pncA* gene mutations. Re-testing these 5 isolates confirmed the PZA resistant phenotype. This suggests that another mechanism might be involved in conferring PZA resistance in these isolates. From these results it is evident that mutations in the *pncA* gene are common in the drug-resistant isolates from previously treated patients and that the *pncA* gene mutations correlate well with PZA resistance.

Some *pncA* mutations are not associated with resistance to PZA

A non-synonymous nucleotide change (C→T at nt341, $Thr_{114}Met$) in the *pncA* gene was identified in 10 of the 47 phenotypically susceptible isolates and was not found in any of the drug-resistant isolates. This nucleotide change did not confer the PZA resistance phenotype and can therefore be regarded as a polymorphism.

PZA resistance is associated with MDR-TB.

A total 62/68 (91%) drug resistant isolates that were phenotypically resistant to PZA were also resistant to INH and RMP ($p<0.001$). Therefore, it can be concluded that PZA resistance was strongly associated with MDR-TB.

Table: Genotypic analysis of the *pncA* gene in the PZA-resistant isolates

Family/ R numbers	Resistance pattern	Nucleotide substitution (position)	Amino acid substitution
LCC			
24	INH, RMP, SM	Insertion of GG (389)	Frameshift
40	INH, RMP, SM, ETH		
56	INH, RMP, SM		
58	INH, RMP, SM		
63	INH, RMP, SM		
221	INH, RMP, SM		
432	INH, RMP, SM		
543	INH, RMP, SM		
619	INH, RMP, SM, KM		
67	INH, RMP, SM	Deletion of T (172)	Frameshift
166	INH, SM		
181	INH, SM		
212	INH, RMP, EMB, SM, ETH, TH		
223	INH, RMP, SM		
309	INH, RMP, SM		
439	INH, RMP, SM		
451	INH, RMP, SM		
495	INH, RMP, SM		
498	INH, SM		
577	INH, RMP, SM		
599	INH, RMP, SM		
615	INH, SM	A→C (535)	$Ser_{179}Arg$
357	INH, RMP, SM	G→C (554)	$Ser_{185}Thr$
364	INH, RMP, SM	T→C (476)	$Leu_{159}Pro$
478	INH, RMP, SM		
365	INH, RMP	G→C (145)	$Asp_{49}His$
118	INH, RMP, SM, ETH	G→A (415)	$Val_{139}Met$
607	INH, RMP, SM		
32	INH, RMP, SM	T→C (476)	$Leu_{159}Pro$
597	INH, RMP, SM		
32	INH, RMP, SM	G→C (554)	$Ser_{185}Thr$
41	INH, RMP, SM, TH	T→G (202)	$Trp_{68}Gly$
44	INH, RMP, SM		
47	INH, RMP, SM		
323	INH, RMP, SM		
381	INH, RMP, SM		
479	INH, SM		
565	INH, RMP, SM		
41	INH, RMP, SM, TH	G→T (543)	$Glu_{181}Asp$
Beijing			
374	INH, RMP	G→T (25)	$Val_{9}Leu$
391	INH, RMP, SM, TH	T→C (40)	$Cys_{14}His$
394	INH, RMP, SM, ETH, KM		
283	INH, RMP	T→G (269)	$Ile_{90}Ser$
405	INH, RMP, SM	C→G (309)	$Tyr_{103}STOP$
431	INH, RMP, SM		
462	INH, RMP, SM	G→A (319)	$Glu_{107}Lys$
328	INH, RMP	G→C (554)	$Ser_{185}Thr$
431	INH, RMP, SM		
134*	INH, RMP	No mutation	
214*	INH, RMP		
F11			
18	INH, RMP, OFX	G→C (136)	$Ala_{46}Pro$
269	INH, RMP	T→C (202)	$Trp_{68}Arg$
398	INH, RMP		
224	INH, RMP	G→A (215)	$Cys_{72}Tyr$
269	INH, RMP	G→C (554)	$Ser_{185}Thr$
103	INH, RMP		
Unique			
82	INH, RMP, SM	C→T (161)	$Pro_{54}Leu$
228	INH, RMP	G→T (109)	$Glu_{37}STOP$
351	INH, SM		
371	INH, RMP, SM		
371	INH, RMP, SM	G→C (554)	$Ser_{185}Thr$
202	INH, RMP, ETH, TH, KM, OFX	C→T (169)	$His_{57}Tyr$
455	INH, RMP		
455	INH, RMP	G→C (554)	$Ser_{185}Thr$
50	INH, RMP	C→T (211)	$His_{71}Tyr$
75	INH, RMP	A→C (340)	$Thr_{114}Pro$
75	INH, RMP	A→C (535)	$Ser_{179}Arg$
270	INH, RMP, SM	T→G (94)	$Leu_{35}Arg$
124	INH, RMP, SM	C→G (346)	$Leu_{116}Val$
230	INH, RMP, SM	A→G (407)	$Asp_{136}Gly$
120	INH, RMP, SM, EMB	T→C (202)	$Trp_{68}Arg$
108*	INH, RMP	No mutation	
219*	INH, RMP		
463*	INH, RMP, SM		
S19	PZA†		

* PZA-resistant isolates with no *pncA* mutations

† PZA-monoresistant isolate

PZA= pyrazinamide; INH= isoniazid; RMP= rifampicin; SM= streptomycin; ETH= ethionamide; KM= kanamycin; TH= thioacetone; OFX= ofloxacin; EMB= ethambutol

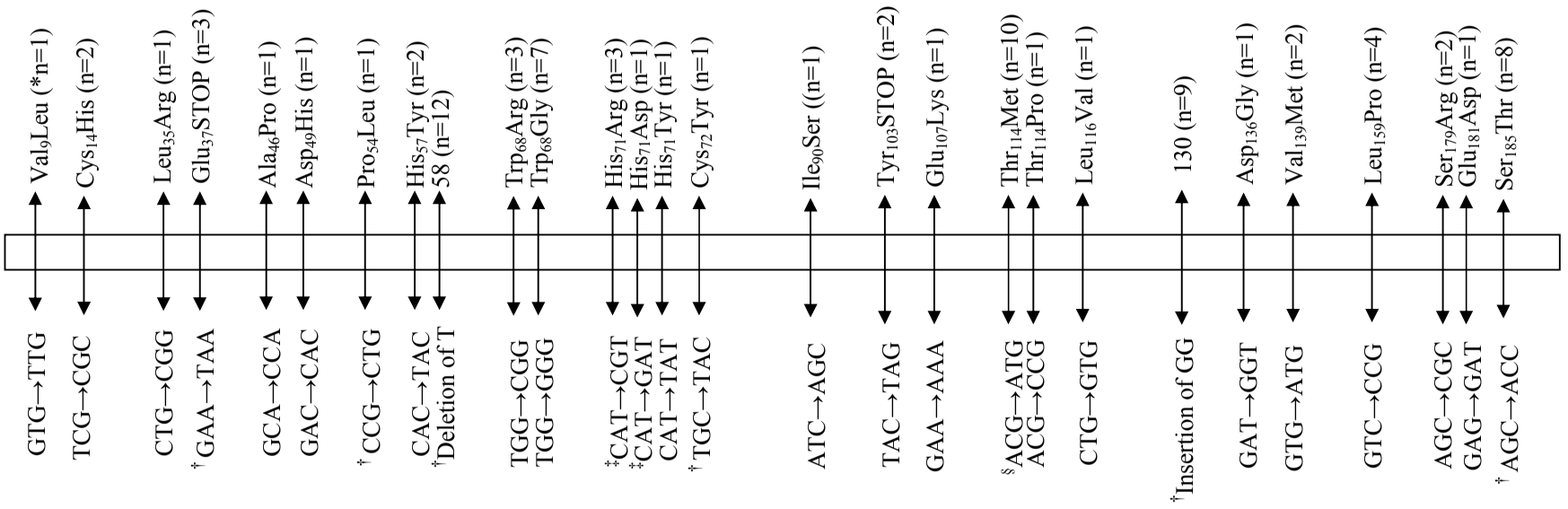

Figure: Representation of the various mutations and the polymorphism found in the 561 bp *pncA* gene of *M. tuberculosis*. The nucleotide change is shown below the schematic, and the amino acid change is shown with the associated codon in the *pncA* gene is depicted above. * Number of isolates with specific mutation. † Newly identified mutations in the study. ‡Mutations found in PZA-resistant isolates from the supranational laboratory. § Polymorphism found in fully sensitive isolate. bp= base pairs; PZA= pyrazinamide

DISCUSSION

Here we show that PZA resistance is common in drug-resistant clinical *M .tuberculosis* isolates from previously treated patients from the defined region and that there is a strong association ($p<0.001$) between mutations in the *pncA* gene and resistance to PZA. The mutations were highly diverse and scattered along the *pncA* gene. This diversity will make it difficult to develop a molecular based method, for rapid identification of PZA resistance other than sequencing the entire gene. Of the 28 (79%) previously described *pncA* gene mutations, 22 were found to confer PZA resistance, as in other studies.[7;8] In addition to previously described mutations in the *pncA* gene, we describe a further 6 mutations which confer PZA resistance. These mutations include a deletion of T at nt172, an insertion of GG at nt389, C→T at nt161 ($Pro_{54}Leu$), G→A at nt215 ($Cys_{72}Tyr$), G→T at nt109 ($Gly_{37}STOP$), G→C at nt554 ($Ser_{185}Thr$). We also describe a polymorphism, C→T at nt341 ($Thr_{114}Met$) that is not associated with PZA resistance. Although *pncA* gene mutations are a major mechanism of PZA resistance, isolates without *pncA* mutations were also found to be PZA-resistant. This suggests the existence of a second mechanism involved in conferring PZA resistance. Zhang *et al*. provided direct evidence that efflux pumps play a role in mycobacterial resistance to PZA.[6;14]

In this study, PZA resistance was strongly associated with MDR-TB. Patients can be infected by a PZA resistant *M. tuberculosis* strain due to 2 mechanisms. One of these may involve antibiotic selection and thus may reflect the manner in which these patients were treated prior to drug susceptibility testing. Currently, patients presenting for the first time are treated for 2 months with a standardized intensive phase regimen (HRZE), followed by 4 month continuation phase (HR). Failure to respond will usually lead to DST. Therefore, if the strain is already resistant to INH and RMP, PZA drug-resistance could develop as result of the

failure of EMB to protect PZA or in the presence of INH, RMP and EMB resistance as the result of monotherapy. Patients presenting for retreatment will usually receive the World Health Organixation (WHO) recommended retreatment regimen of 2 months intensive phase treatment of HRZE + streptomycin (SM), 1 month of HRZE and 5 months of HRE, and DST will be requested at diagnosis. Again, resistance to INH and RMP, while awaiting the DST results, is dependent upon SM and EMB. Both SM and EMB are only moderately effective agents in this respect[15]. Should EMB resistance already exist, only SM will stand between PZA and the development of resistance. The other mechanism is via transmission of a PZA resistant *M. tuberculosis* strain, where a previous study has shown that transmission is a significant force driving drug resistant TB in this region[12]. The rapid detection of these resistant isolates would, of course, greatly assist the more effective management of these drug resistant patients and contribute to the prevention of further spread of these life-threatening bacilli. Scorpio *et al*. attempted to rapidly identify PZA resistant *M. tuberculosis* strains by direct sequencing of PCR products and PCR single-stranded conformational polymorphism (PCR-SSCP)[16]. However, the observation that PZA resistance is caused by multiple mutations in the *pncA* gene [16] makes the development of a molecular test for their rapid identification difficult.

WHO guidelines for the management of a patient with apparent MDR-TB suggest two approaches depending upon whether the DST results are available[17]. Should they not be available following the failure of the recommended retreatment regimen, a "third line" regimen is recommended. This should include at least 3 drugs never used: kanamycin, ethionamide and ofloxacin and pyrazinamide. If the DST results are available, an individualized regimen can be constructed, but, again the use of PZA is recommended. PZA has a limited role in preventing the emergence of drug resistance.[18] However, it has been

proven in clinical trials that regimens containing PZA have a lower bacteriological relapse rate than regimens without PZA.[19]

An interesting finding in Quebec, Canada is that many clinical isolates are PZA mono-resistant and they all share the same *pncA* mutation profile.[20] In a study from Peru 47 of 80 (59%) MDR patients also had *M. tuberculosis* resistant to PZA.[21] Our results indicate a similar level of PZA resistance amongst patients with MDR isolates. PZA resistance may thus be much more common than previously suspected and this confirms the dictum that it is unwise to place any reliance upon a drug to which the patient has previously been exposed when managing treatment failures. Further surveillance studies are needed to estimate the prevalence of PZA resistance in *M. tuberculosis* strains in South Africa.

CONCLUSION

This study highlights the presence of additional PZA resistance amongst patients with MDR-TB in the Western Cape Province of South Africa. PZA susceptibility testing of *M. tuberculosis* isolates is not routinely done in this region and our findings indicate that PZA should not be relied upon in the management of patients with MDR-TB in the Western Cape. A methodology for the rapid detection of PZA resistance would be of considerable clinical benefit in managing patients with suspected drug resistance.

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CHAPTER 4

Rv2629 191A/C nucleotide change is not associated with Rifampicin resistance

My contribution:

Literature search

Sample selection

PCR amplification, Sequencing

Data analysis

Writing and editing of manuscript

ABSTRACT

A non-synonymous single nucleotide polymorphism (nsSNP) at position 191A/C in the *Mycobacterium tuberculosis* gene *Rv2629* was recently identified by proteomics and sequencing and was reported to be significantly associated with Rifampicin (RIF) resistance. It was further claimed that this nsSNP could serve as a potential marker for the rapid diagnostics of multi-drug resistant tuberculosis. To test this hypothesis we quantified the frequency of this nsSNP in drug susceptible and drug resistant clinical isolates from different evolutionary lineages of *M. tuberculosis*. In contrast to the results reported elsewhere, our results showed that 191A/C *Rv2629* was significantly associated with *M. tuberculosis* isolates from the Beijing evolutionary lineage ($p<0.0001$), irrespective of their RIF phenotype. Accordingly, we conclude that 191A/C *Rv2629* is a polymorphism associated with the *M. tuberculosis* Beijing evolutionary lineage and does not confer RIF resistance. Thus, the 191A/C *Rv2629* nsSNP cannot be used as a genetic marker of multi-drug resistance.

TEXT

In 1972 Rifampicin (RIF) was included in the treatment regimen as a first-line anti-tuberculosis (anti-TB) drug due to it's exceptional sterilizing activity. Together with Isoniazid (INH), Ethambutol (EMB) and Pyrazinamide (PZA), this drug forms an integral part of the current intensive and continuation phases of short-course treatment (1). Resistance to RIF is considered to be a reliable surrogate marker for multi-drug resistant TB (MDR-TB) (2). Therefore, identifying and understanding RIF resistance is of great importance and has led to the development of methods for the rapid identification of RIF resistance (2-4). These methods are based on the observation that ninety-five percent of RIF resistance can be attributed to mutations in the Rifampicin Resistance Determining Region (RRDR) of the *Mycobacterium tuberculosis rpoβ* gene (5). This suggests that other mechanisms or genes may be responsible for conferring RIF resistance in the remaining 5% of cases. In an attempt to discover such mechanisms, Wang *et al* used a proteomic approach to identify over-expressed proteins specifically associated with RIF-resistance (6). Sequencing of the gene *Rv2629,* encoding one of these over-expressed proteins, identified a non-synonymous nucleotide polymorphism (nsSNP) at codon 191A/C. The authors demonstrated that this nsSNP was significantly associated with RIF resistance in *M. tuberculosis* (6) and thereby concluded that this nsSNP was a potential biomarker for the rapid detection of RIF resistance in *M. tuberculosis.*

To test this hypothesis, 90 RIF-resistant and 67 RIF-susceptible *M. tuberculosis* clinical isolates were randomly selected for analysis from a longitudinal collection of well

characterized clinical isolates. Each isolate was genotyped by spoligotyping (7) and grouped into major evolutionary lineages according to the presence of specific spoligotype signatures (8). The selected isolates were representative of prevalent *M. tuberculosis* evolutionary lineages, including the Beijing (28%), F11 (12%), F28 (5%), and LCC (26%) lineages which together constitute 71% of drug resistant isolates cultured from cases in the Western Cape, South Africa (9). A 695bp fragment of the *Rv2629* gene was amplified with primers that were previously described (6). PCR reactions were performed under the following thermocycling conditions: 15 minutes (min) denaturation at 95^{o}C followed by 40 amplification cycles (each cycle: 94^{o}C for 1 min, 62^{o}C for 1 min, 1 min extension at 72^{o}C) and an elongation step of 10 min at 72^{o}C. PCR products were cleaned with Exonuclease I / Shrimp Alkaline Phosphatase (*Exo* I and SAP-IT) according to the manufacturer's instructions (Amersham, Vienna, Austria). The products were sequenced using an ABI PRISM DNA Sequencer model 377, Perkin Elmer. Statistical analysis was done using GraphPad Prism, Version 5.00.

Sequencing analysis of the *Rv2629* gene showed that the 191A/C nsSNP was present in 48% (n = 43/90) of RIF-resistant isolates. This nsSNP was also present in 64% (n = 43/67) of RIF-susceptible isolates, thereby suggesting that the *Rv2629* nsSNP was a polymorphism and was not associated with RIF-resistance.

Grouping of isolates according to their spoligotype signatures showed that the *Rv2629* nsSNP was significantly associated with *M. tuberculosis* isolates of the Beijing

evolutionary lineage ($p<0.0001$). This association occurred irrespective of RIF-resistance phenotype (Table1). Accordingly, our results do not support the hypothesis put forward by Wang *et al* that the 191A/C nsSNP was a marker for RIF resistance in *M. tuberculosis*. Our results confirm a recent report which indicated that this nsSNP was not associated with RIF resistance, but was significantly associated with the Beijing lineage (10). Thus we conclude that the 191 A/C nsSNP is a marker identifying isolates from the Beijing evolutionary lineage. Furthermore, this nsSNP can not be used in a genetic test to diagnose RIF resistance. Therefore, the rapid diagnosis of RIF resistance is still restricted to analysing SNP's in the RRDR region of the *rpoB* gene.

Since the early 1990's, *M. tuberculosis* isolates from the Beijing evolutionary lineage have become the centre of research in many settings. Isolates from this lineage are thought to have evolved unique properties, including the ability to evade the protective immunity conferred by BCG, as well as the ability to acquire and transmit drug-resistance (11-14). However, the functional role of this nsSNP in pathogenesis remains to be determined.

Table1: Characteristics of RIF-susceptible and RIF-resistant *M. tuberculosis* isolates

	RIF-resistant (n=90)		RIF-susceptible (n=67)	
	WT Rv2629	191A/C *Rv2629*	WT Rv2629	191A/C *Rv2629*
Beijing	0	43 (47.78%)	0	43 (64.18%)
Non-Beijing	47 (52.2%)	0	24 (35.82%)	0

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CHAPTER 5

Evidence that the level of Rifampicin resistance in *Mycobacterium tuberculosis* evolves through a multi-step mechanism.

My contribution:

Planning of project
Literature search
Culturing of *M. tuberculosis* clinical isolates
Rifampicin MIC determination by BACTEC
Determining the effect of Verapamil/ Reserpine on MIC's
Involved in proposing of RIF resistance model
Writing and editing of manuscript

ABSTRACT

Drug resistance in *Mycobacterium tuberculosis* has been shown to evolve through spontaneous mutation and subsequent antibiotic selection. Resistance to rifampicin (RIF) is associated with non-synonymous single nucleotide polymorphisms (nsSNPs) in the *rpoB* gene. Different nsSNPs have been shown to influence the RIF minimum inhibitory concentration (MIC) presumably as a result of their varying effects on the structure of the β-subunit of RNA polymerase (RpoB). In this study, genetically closely related clinical isolates with identical IS*6110* and spoligotype genotypes and identical mutations conferring resistance to isoniazid, rifampicin, ethambutol and pyrazinamide were selected for RIF MIC testing. BACTEC analysis showed that their MICs varied widely despite identical nsSNPs conferring resistance. The same phenomenon was observed for genetically unrelated RIF resistant clinical isolates. Susceptibility to RIF could be partially restored by inhibition of efflux pumps with either Reserpine or Verapamil (p= 0.0000; 95% CI). This provides the first evidence that efflux pumps may modulate the level of RIF- resistance in clinical isolates of *M. tuberculosis* over and above the level of resistance conferred by *rpoB* mutations. Accordingly, a model was constructed proposing that the evolution of RIF resistance levels is a multi-step process whereby an initial nsSNP confers low level resistance, while subsequent mutations and/or gene regulation effects confers varying levels of RIF resistance. We conclude that the evolution of strains with varying levels of RIF resistance is more complex than the "one mutation – one gene" scenario.

INTRODUCTION

Rifampicin (RIF) is one of the most important antibiotics used to treat tuberculosis as it has strong sterilizing activity against rapidly growing extra- and intra-cellular *Mycobacterium tuberculosis* bacilli. RIF has been shown to diffuse freely through the cell wall of the organism (12) and to inhibit the early steps of gene transcription by binding to the β-subunit of RNA polymerase (RpoB) encoded by the *rpoB* gene (4). Resistance to RIF develops by a process of spontaneous mutation and antibiotic selection, particularly during periods of poor adherence or monotherapy. Luria-Delbrück fluctuation tests show that mutations conferring RIF resistance appears spontaneously at a rate of 10^{-9} to 10^{-8} per cell division (5,8). The non-synonymous single nucleotide polymorphisms (nsSNPs) conferring RIF resistance occur exclusively in the *rpoB* gene, with 95% occurring within an 81 bp region known as the RIF resistance-determining region (RRDR) (16). Each nsSNP alters the structure of RpoB, thereby altering the dissociation constant between the mutated RpoB ($RpoB_{mut}$) and RIF in such a manner that this directly influences the RIF minimum inhibitory concentration (MIC) (3,4). Accordingly a direct relationship between specific nsSNPs in the *rpoB* gene and varying MICs could be expected (8).

This study aims to challenge the dogma that a simple relationship exists between nsSNP in the *rpoB* gene and resistance to RIF. We provide evidence to demonstrate that the development of RIF resistance is a multi-step process which ultimately defines the level of resistance.

METHODS

Drug resistant *M. tuberculosis* bacilli were cultured from sputum samples collected from patients attending health-care clinics in the Western Cape, South Africa (15) and genotypically characterised by IS*6110* DNA fingerprinting (17) and spoligotyping (11) using internationally standardised methods. The nsSNPs conferring resistance to isoniazid, rifampicin, ethambutol and pyrazinamide were determined by DNA sequencing of the *katG*, *inhA* promoter, *rpoB*, *embB* and *pncA* genes, respectively. *M. tuberculosis* isolates representing recent MDR-TB outbreaks (defined as having identical IS*6110* DNA fingerprints, spoligotypes and nsSNP's conferring resistance to isoniazid, rifampicin, ethambutol and pyrazinamide) as well as genetically unrelated drug resistant clinical isolates (resistant isolates representative of the same and other evolutionary lineages, showing the *rpoB* Ser531Leu mutation, but different other resistance conferring mutations) were selected. In addition, susceptible clinical isolates (RIF susceptible isolates from the same evolutionary lineages as the MDR-TB outbreak isolates and showing the same IS*6110* DNA fingerprints) were selected (9,14,18).

The MIC for RIF for each of the selected isolates was determined by inoculating a 100 µl aliquot of a mid-log phase culture into enriched BACTEC 12B medium (Becton Dickinson, USA) containing between 2 to 200µg/ml RIF for the analysis of RIF resistant isolates and between 0.0002 to 2µg/ml RIF for the analysis of RIF susceptible isolates in increments of 10. All cultures were incubated at 37°C and the growth index (GI) for each isolate (at each RIF concentration) was measured daily for 9 consecutive days and compared to the GI of

the corresponding isolate grown (diluted 1/100) in the absence of RIF. H37Rv (ATCC 35828) was included as a RIF susceptible control. The MIC for each isolate was defined as the lowest concentration of RIF at which there was a complete absence of growth. All experiments were done in duplicate in which <5% variation was observed and each experiment was repeated on at least two separate occasions.

To determine whether the efflux pump inhibitors reserpine or verapamil had affected *M. tuberculosis* growth in the absence of rifampicin, the selected isolates were cultured in the presence of varying concentrations of reserpine (10 to 100 µg/ml) or verapamil (10 to 500 µg/ml) in enriched BACTEC 12B medium at 37°C (supplementary data). The GI for each isolate was measured daily over a period of 9 days and compared to the growth index of the corresponding isolate grown in the absence of reserpine or verapamil.

To determine whether reserpine or verapamil could restore RIF susceptibility, the drug resistant isolates were cultured at 37°C in enriched BACTEC 12B medium containing either reserpine (80 µg/ml) or verapamil (50 µg/ml) together with varying concentrations of RIF (2 to 150 µg/ml). These selected isolates represent MDR-TB outbreak strains with identical resistance conferring mutations. RIF susceptible isolates were cultured at 37°C in enriched BACTEC 12B medium containing either (30 µg/ml) or verapamil (10 µg/ml) together with 0.002µg/ml RIF. The GI of both the drug resistant and drug susceptible isolates measured daily over a period of 9 days and compared to the growth index of the corresponding isolate grown in the absence of reserpine or verapamil but in the presence of varying concentrations of RIF.

The % susceptibility restored was calculated at day 5 for the isolates by using the following formula: $$\frac{((GI_{RIF} - GI_{EPI+RIF}) - (GI_{CONTROL} - GI_{EPI}))}{GI_{RIF}}$$

where GI_{RIF} is the growth index of the isolate grown in the presence of only RIF; $GI_{EPI+RIF}$ is the growth index of the isolate grown in the presence of both the EPI and RIF; $GI_{CONTROL}$ is the growth index of the isolate grown without any treatment and GI_{EPI} is the growth index of the isolate grown in the presence of only optimal concentrations of EPI.

Statistical analysis to determine the effect of the various treatments over time was done using STATISTICA VERSION 7.

RESULTS

The MICs for RIF were found to be highly variable among isolates representing recent MDR-TB outbreaks (clonal expansion) and ranged between 10 and 170 µg/ml RIF in BACTEC 12B media (Table 1). This variation was largely independent of the *M. tuberculosis* genotype and the investigated two resistance conferring nsSNP's (Ser531Leu, Asp516Val) in the *rpoB* gene (Table 1). This variation in the MIC's for RIF was also observed in other drug-resistant isolates (Table 2). Together these results suggest that a single *rpoB* mutation alone cannot explain the variation in the MIC's for RIF in drug resistant clinical isolates.

Table 1: Characteristics of MDR-TB outbreak *M. tuberculosis* isolates

	Isolate	Evolutionary lineage	Spoligotype	IS*6110* cluster type	*rpoB* mutation	Other resistance causing genes	RIF MIC (ug/ml)	% susceptibility restored at 2µg/ml RIF	
								80µg/ml Reserpine	50µg/ml Verapamil
MDR-TB outbreak isolates	R279	Beijing	2	220	TTG (Ser531Leu)	inhA-15prom emb306 GTG (Met306Val) pncA103 TAG (Tyr103Ter)	150	77.29	70.17
	R179						140	69.83	84.63
	R750						90	55.08	76.69
	R405						90	63.13	77.5
	R257						30	80.8	84.8
	R451	Low Copy Clade	115	DRF150	TTG (Ser531Leu)	*katG*315 ACA (Ser315Thr) *emb*306 ATA (Met306Ile) *pncA* Del58	170	67.58	70.34
	R543						160	67.20	71.03
	R439						60	52.67	60.49
	AT970	Atypical Beijing	2	464	GTC (Asp516Val)	*katG*315 ACC (Ser315Asn) inhA-17prom	80	71.56	84.56
	AT720						40	68.25	73.21
	AT787						20	75.10	72.42
	AT881						20	70.18	76.95
	AT802						20	71.23	84.25
	AT776						10	71.46	86.27
	AT695						10	62.45	75.12

Table 2: Characteristics of genetically unrelated drug resistant *M. tuberculosis* isolates.

	Isolate	Evolutionary lineage	Spoligotype	IS*6110* cluster	*rpoB* mutation	RIF MIC (ug/ml)
Drug-resistant isolates	R283	Beijing	2	220	TTG (Ser531Leu)	90
	R778					90
	R326					90
	R676					10
	R328					5
	R122					5
	R610					5
	R1040	F11	77	1027	TTG (Ser531Leu)	120
	R1333					120
	R285					100
	R251					70
	R74	F28	82	11061	TTG (Ser531Leu)	110
	R749		81			110
	R1087		82			80
	R1219		81			30
	R58	Low Copy Clade	115	338	TTG (Ser531Leu)	30
	R212					80
	R223					80
	R432					120
	R440					120
	R779					40
	R1114					70
	R1134					30
	R32	Low Copy Clade	118	338	TTG (Ser531Leu)	80
	R160					40
	R607					70
	R733					80
	R794					30
	R1098					80
	R1161					70

In order to determine whether efflux pumps contributed to the regulation of MICs for RIF, selected isolates from each of the MDR-TB outbreaks were cultured in the presence of varying concentrations of reserpine or verapamil. This study showed that 90% of the growth index of each isolate was retained at a concentration of 80 µg/ml reserpine or 50 µg/ml verapamil (in the absence of RIF), thereby confirming that these compounds at these concentrations were not toxic (Figure 1). Susceptibility to RIF was mostly restored when the respective MDR-TB isolates were cultured in the presence of 2 µg/ml RIF in combination with either 80 µg/ml reserpine or 50 µg/ml verapamil (Figure 1). The combined effect of RIF and reserpine and also RIF and verapamil was statistically significantly different ($p=0.0000$; 95% CI) in comparison to the individual drug treatments. The percentage susceptibility restored for MDR-TB outbreak isolates varied from 62.45 to 77.29% for reserpine and 70.17 to 86.25% for verapamil (Table1, Figure 1). This suggests that inhibition of mycobacterial efflux pumps by either reserpine or verapamil allowed for intracellular accumulation of RIF, thereby promoting the binding of RIF to $RpoB_{mut}$ and the subsequent inhibition of transcription. Regulation of susceptibility towards RIF could not be demonstrated for drug susceptible isolates (Table 3), thereby suggesting that efflux pumps targeted by reserpine or verapamil do not significantly regulate the intracellular concentration of RIF in susceptible isolates.

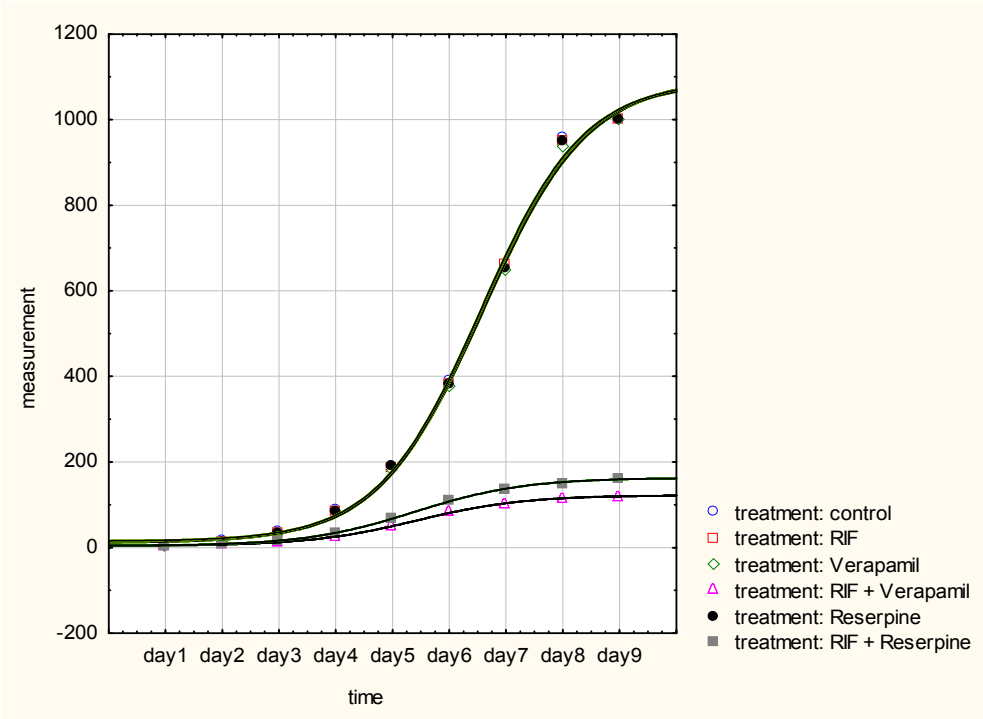


Figure 1: The cumulative effect of treatment over time for all the closely related RIF resistant isolates.

Legend to Figure 1: The combined effect of RIF and reserpine and also RIF and verapamil is statistically significantly different (p=0.0000; 95% CI) in comparison to the individual drug treatments. The % susceptibility restored for genetically closely related strains varied from 62.45 -77.29% for reserpine and 70.17 to 86.25% for verapamil (Table1).

Table 3: Characteristics of RIF susceptible isolates

	Isolate	Spoligotype	IS*6110* cluster type	RIF MIC (ug/ml)	% susceptibility restored at 0.002µg/ml RIF	
					30µg/ml Reserpine	10µg/ml Verapamil
RIF susceptble clones	R284			0.02	4.05	3.97
	R336	2	220	0.02	4.88	3.50
	R333			0.02	3.35	3.26
	R166			0.2	2.15	1.96
	R196	115	DRF150	0.2	1.78	2.10
	R181			0.--2	3.05	1.73

DISCUSSION

In this study we demonstrate that the MIC for RIF varies among drug resistant clinical isolates which have different genetic backgrounds and identical nsSNPs in their *rpoB* genes. This was also observed amongst isolates representing different MDR-TB outbreaks in different geographical regions in South Africa where both the genetic background and *rpoB* mutation are identical (highly similar) (9,14,18). These findings are consistent with the recent report that *in vitro* selected RIF resistant mutants from two different genetic backgrounds, Beijing and non-Beijing, had varying RIF MICs for identical nsSNPs in the *rpoB* gene (8). Thus, in contrast to previous studies which have suggested a direct relationship between a specific *rpoB* mutation and the level of RIF resistance, we conclude that the level of resistance to RIF is determined not only by alterations in the structure of the β-subunit of RNA polymerase but also by other mechanisms that do not involve *rpoB*. Our results challenge the dogma that nsSNP's within the *rpoB* gene are the only mechanism conferring RIF resistance and modulating/regulating RIF resistance levels. Accordingly, we propose the following multi-step pathway for the evolution of the level of RIF resistance. In Figure 2, we define the dissociation constant (Kd^S) between the "wild type" RpoB ($RpoB_{wt}$) and RIF in a pan-susceptible *M. tuberculosis* isolates as *x* nM. Following Piddock *et al* we assume that RIF reaches $RpoB_{wt}$ by passive diffusion and that efflux inhibitors do not significantly influence the intracellular RIF concentration, as demonstrated in this study. Therefore it is likely that the rate of RIF influx into the *M. tuberculosis* bacillus equals the rate of RIF efflux and thereby the intracellular RIF concentration is in equilibrium with the extracellular RIF concentration.

Given that the binding affinity of RIF for RpoB is dependent on the structure of the RpoB protein (3,4) it can be assumed that spontaneous mutation in the RRDR region will influence the dissociation constant (Kd^R) between the "mutant" $RpoB_{mut}$ and RIF, such that $Kd^R > Kd^S$. The magnitude of Kd^R could vary significantly (Figure 2, Step 1). Considering the scenario, $Kd^{R(LOW)}$, such isolates would display a MIC of *ax* nM through passive diffusion of a low concentration of RIF, while $Kd^{R(HIGH)}$ isolates would display a MIC of *bx* nM (where b > a) through passive diffusion of a high concentration of RIF. However, because isolates with identical nsSNPs demonstrate varying MIC's we predicted that $Kd^{R(LOW)}$ isolates would demonstrate a high MIC for RIF if either secondary mutations and/or regulation of gene expression resulted in the active efflux of RIF from the cell (Figure 2 Step 2). Thus, a high concentration of RIF would be required to compensate for active efflux in order to achieve an intracellular concentration which allowed maximal inhibition of RpoB. Conversely, $Kd^{R(HIGH)}$ isolates would display a low MIC for RIF if either secondary mutations and/or regulation of gene expression resulted in the active accumulation of RIF within the cell (Figure 2 Step 2).

This study supports the above hypothesis by demonstrating that efflux pump inhibitors are able to significantly restore RIF susceptibility to the critical concentration of 2 μg/ml. This suggests that nsSNPs in the *rpoB* gene in general confer a low level of RIF resistance. When combined with other mutations or expression changes which regulate the intracellular RIF concentration, however, the combined changes confer a high level of RIF resistance.

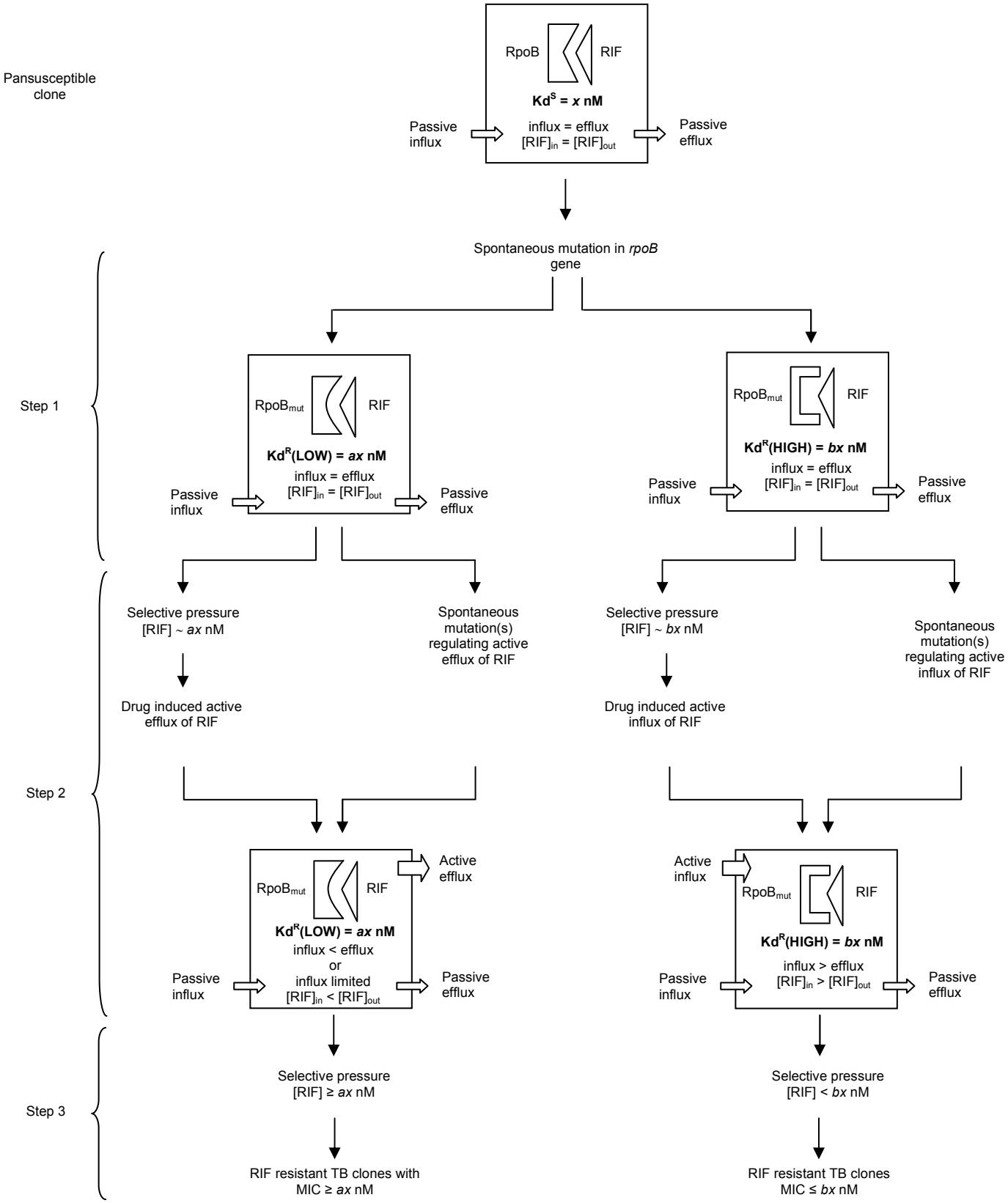
Our results together with previous results (2,7) suggest that efflux pump inhibitors may have an important role in treatment regimens targeted at drug resistant tuberculosis. This is particularly relevant in the context of the limited number of drugs available to treat drug resistant tuberculosis and their associated side effects. The restoration of susceptibility to perhaps the most important anti-tuberculosis drug (rifampicin) would greatly benefit the treatment of MDR-TB and XDR-TB cases. An added benefit would be if efflux pump inhibitors could restore susceptibility to more than one drug. This notion is supported by a recent study which showed that the level of fluoroquinolone resistance could be significantly reduced in *M. tuberculosis* isolates in the presence of the efflux pump inhibitors reserpine or MC207.110 (6).

The introduction to the TB treatment regimen of compounds that restore drug susceptibility is an attractive alternative to aid in the control of drug resistance. However, this study was done *in vitro*, and there is a need to assess the efficacy of the potential drugs *in vivo* (although they have been registered for use in humans in treatment of cardiac diseases). It has been shown that efflux pump inhibitors such as verapamil and reserpine enhanced the killing of intracellular drug-resistant *M. tuberculosis* bacilli by non-killing macrophages (1). Literature suggests that for a drug to be active in patients an antibiotic should have Cmax/MIC>10, thus an MIC of 5 ug/ml (Table 2) might not be low enough to reflect effective patient treatment from a pharmokinetic point of view (10). A clinical study by Prakash *et al* showed that in patients with TB, pre-treatment with Verapamil, which is also a blocker of human P-glycoprotein and cytochrome P450 3A4, increased the serum levels of RIF (13). This suggests that P-glycoprotein and cytochrome P450 play important roles

in the absorption of RIF in TB patients. We acknowledge that these efflux pump inhibitors have limitations, and encourage the development of novel drugs which modulate the intracellular drug concentrations.

We conclude that the evolution of the level of RIF resistance is more complex than the "one mutation – one gene – one MIC" scenario. Our study shows that the value of the MIC is directly linked to the cumulative effect of different evolutionary events. This implies that the bacteriostatic/bacteriocidal efficacy of current drugs may be enhanced by the development of compounds which modulate the intracellular drug concentration.

Figure 2: Proposed model for the evolution of the level of RIF resistance in *M. tuberculosis*



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SUPPLEMENTARY DATA (ADDITIONAL FIGURES)

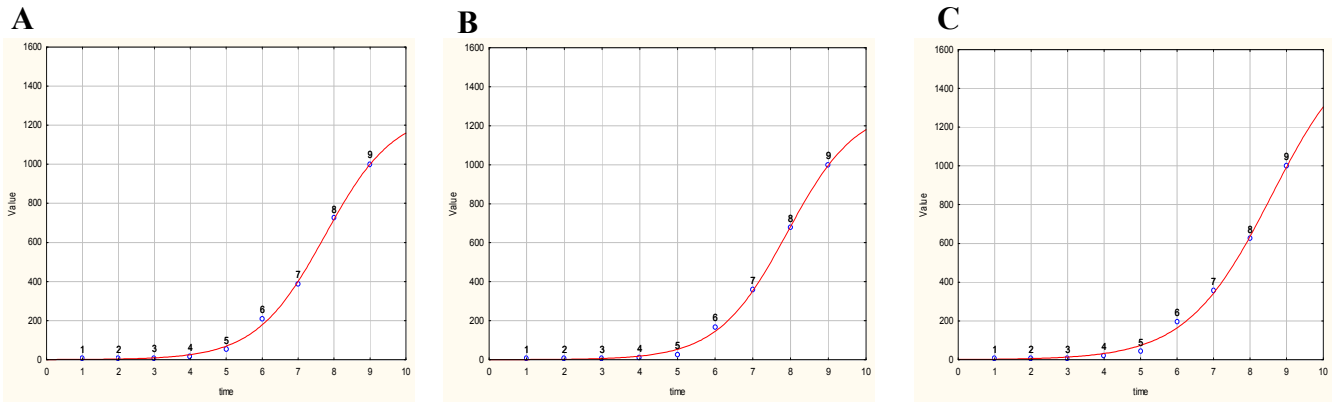

Figure 3: The effect of treatment over time for RIF Resistant isolates to determine the optimum concentration of the efflux pump inhibitor; **A)** RIF resistant isolates without any drug exposure serving as control; **B)** RIF resistant isolate exposed to 50µg/ml Verapamil; **C)** RIF resistant isolate exposed to 80µg/ml Reserpine.

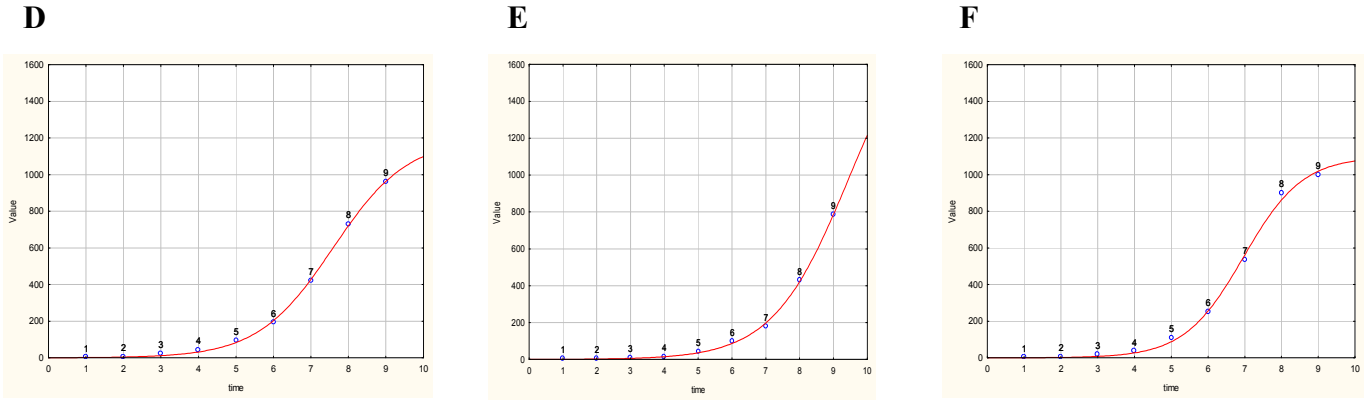

Figure 4: The effect of treatment over time for RIF susceptible isolates to determine the optimum concentration of efflux pump inhibitor; **A)** RIF susceptible isolates without any drug exposure serving as control; **B)** RIF susceptible isolate exposed to 10µg/ml Verapamil; **C)** RIF resistant isolate exposed to 30µg/ml Reserpine.

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CHAPTER 6

Rifampicin induces differential expression of putative transporter genes in *Mycobacterium tuberculosis*

My contribution to this project:

Planning of project

Bioinformatics and literature searches

Selection of putitative candidate efflux genes

Strain selection and culture

RNA extraction and assessment of RNA quality

cDNA synthesis

Primer design, QRT-PCR

Data analysis

Writing and editing of manuscript

ABSTRACT

Central dogma suggests that a single mutation in a specific gene causes a certain level of drug resistance and that this resistance cannot be reversed. We have provided evidence in Chapter 5 that various levels of Rifampicin (RIF) resistance were observed in clinical isolates of *Mycobacterium tuberculosis* with genetically identical backgrounds. In addition, treatment of these isolates with efflux pump inhibitors significantly restored RIF susceptibility. This study is an expansion of these previous findings. Thirty putative efflux genes were identified by using bioinformatics and literature searches for subsequent QRT-PCR assessment. Twenty-eight efflux genes were differentially expressed in response to RIF in the LCC cluster DRF150 strains. Ten genes were up-regulated and 2 genes were down-regulated, irrespective of their level of RIF resistance in the LCC DRF150 strains. The remaining 16 genes were uniquely differentially expressed in isolates with different levels of RIF resistance. Gene expression in Beijing cluster 220 isolates showed differential expression in 21 genes in response to RIF, of which 4 genes were up-regulated and 1 gene was down-regulated in both strains. The remaining 16 genes showed expression levels unique to the isolates with high and low RIF resistance levels. The results suggest that a lineage specific global response is firstly launched, followed by a subsequent unique response that enables the specific strains to cope with or evade the toxic effect of RIF. The results provide the first evidence that putative efflux pumps may contribute to variation in the level RIF-resistance in clinical isolates of *M. tuberculosis*.

INTRODUCTION

Administering of effective drugs is one of the major factors that influence the control of infectious diseases. However, due to the emergence of drug resistance in *Mycobacterium tuberculosis* (*M. tuberculosis)*, some anti-tuberculosis (anti-TB) drugs have become ineffective. Drug resistance in *M. tuberculosis* has been primarily associated with mutations in defined genes (17). However, in a certain percentage of phenotypically resistant clinical isolates of *M. tuberculosis,* classical mutations known to confer resistance were not identified (17). Thus, it was hypothesised that in such cases the *M. tuberculosis* had adapted certain intrinsic mechanisms to evade the toxic and bactericidal effect of the anti-TB drug. A number of recent studies have provided experimental evidence to show that efflux pumps controlling the intracellular concentration of the anti-TB drug may be candidates contributing to drug resistance in mycobacteria (16,27-29). In support of this notion we have demonstrated that the addition of efflux pump inhibitors reserpine and verapamil largely reversed the rifampicin (RIF) resistance phenotype, despite the presence of a mutation in the defined region of the *rpoB* gene (CHAPTER 5). These efflux pump inhibitors generally target ATP Binding Cassette (ABC) transporters.

The completion of the whole genome DNA sequence of several clinical and laboratory strains of *M. tuberculosis* ((http://genolist.pasteur.fr/TubercuList), (http://www.broad.mit.edu)) has enabled the identification of various genes which when annotated show similarity to transporters in other bacterial species (8,10). Transporters are classified based on the direction of drug translocation (i.e. import or export) and their energy source (i.e. ATP hydrolysis or proton motive force (pmf)) (5,10). To date,

expression studies have investigated the role of transporters in drug resistance. These expression studies provide limited understanding of the efflux related drug resistance. An example is the increased transcription of the tap-like protein from the Major Facilitator Superfamily (MFS) (2), encoded by the *Rv1258c* gene of *M. tuberculosis,* upon exposure to RIF and INH in a multi-drug resistant isolate (27). The exact role and contribution of these transporters to drug resistance remains ill-defined and still needs to be assessed. More importantly, the contribution of these putative efflux genes to the level of drug resistance has not been investigated yet.

In this study we aim to test the hypothesis that changes in expression of efflux pump gene(s) regulate the level of RIF resistance over and above the level of resistance conferred by classical *rpoB* mutations clinical isolates of *M. tuberculosis*.

MATERIALS AND METHODS

Bioinformatics and literature searches

The genome sequence of the *M. tuberculosis* H37Rv reference strain and whole genome sequences ((http://genolist.pasteur.fr/TubercuList), (http://www.broad.mit.edu)) of clinical strains were searched to identify various types of transporters predicted by bioinformatic analysis (Figure 1). In addition, a literature search was done to identify genes which showed increased levels of expression in the presence of an anti-TB drug (3,5,10,21,22,25) (Table 1).

Selection and growth of *M. tuberculosis* clinical strains

Four clinical isolates from 2 genetically distinct evolutionary lineages (Beijing lineage cluster 220 and Low Copy Clade (LCC) lineage cluster DRF150), with identical mutations in the *rpoB* gene, lineage specific IS*6110* RFLP and spoligotype fingerprint were selected based on their levels of RIF resistance (as described in Chapter 5). Resistance levels were 30 and 140µg/ml RIF for Beijing cluster 220 isolates, and 60, 170µg/ml RIF for the isolates in LCC cluster DRF150, respectively (Table 2).

Isolates were grown on Lowenstein-Jensen (LJ) solid medium for 3-4 weeks with continuous aeration. Colonies were scraped from the LJ slants and incubated in 5ml Middlebrook 7H9 medium (Becton Dickinson, Sparks, MD 21152, USA) supplemented with 0.2% (v/v) glycerol, 0.1% Tween 80 and 10% ADC in filtered screw cap tissue culture flasks (Greiner Bio-one, Maybachstreet, Germany). After 7-10 days these primary sub-cultures were inspected for contamination by Ziehl-Neelsen gram staining and culture on

blood agar plates. The primary sub-cultures were then sub-cultured in 10ml supplemented Middlebrook 7H9 liquid medium and incubated at 37^{o}C. After 10-14 days of growth (OD_{600}= 0.7-0.8), the secondary sub-cultures were re-inspected for contamination and a 50% glycerol stock was prepared and stored at -80^{o}C.

All subsequent experiments were set up as 2 biological (to assess biological measurements for two independent experiments done on different days) and 2 technical replicates (repeated measures of one biological sample on the same day) for each isolate. Each experimental culture was set up by aliquoting 800µl of the secondary sub-culture in 80ml supplemented 7H9 medium (1:100 dilution) and incubated at 37^{o}C until mid-log phase (OD_{600}= 0.7-0.8). The 80ml culture was then divided into 2 x 40ml cultures. RIF was added to one 40ml at a concentration corresponding to ½ the minimum inhibitory concentration (MIC) of the respective isolate. No anti-TB drugs were added to the other 40ml culture (control). Both cultures were then incubated at 37^{o}C for a further 24 hours.

Table 2: Characteristics of the clinical isolates selected

Isolate	Family	Spoligotype	Cluster	IS6110 RFLP	RIF MIC (ug/ml)
R257[a]	Beijing	2	220		30
R179[b]					140
R439[c]	LCC	115	DRF150		60
R451[d]					170

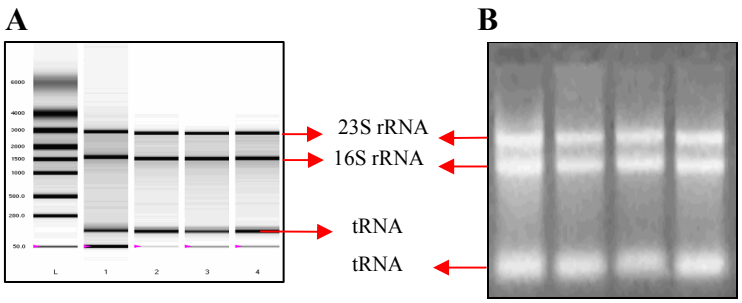
Legend to Table 2: [a]Beijing RIF-resistant isolate with low RIF resistance levels; [b]Beijing RIF resistant isolate with high RIF resistance levels; [c]LCC RIF resistant isolate with low RIF resistance levels; [d]LCC RIF resistant isolate with high RIF resistance levels

RNA extractions and purification

After the 24hour incubation, 200 ml of Guanidine-thiocyanate (GITC) (Sigma-Aldrich, St. Louis, USA) solution (5M GTC, 0.5% sodium *N*- lauroyl sarcosine, 0.1M β-mercaptoethanol, 12.5ml of 1M sodium citrate pH7.0 and 1% Tween 80 made up to 500ml with RNase free water) was added to each 40 ml culture. Cells were harvested by centrifugation (3000 x g, 15 min, 20^{o}C) and the supernatant was discarded. The pelleted cells were then resuspended in 1ml of TRIzol (Life Technologies, Gaithersburg, MD). The suspension was transferred to 2ml screw capped tube containing silica beads (IEPSA Medical diagnostics, South Africa) and ribolyzed (reciprocal shaker, Hybaid) at 6 W for 20 seconds. Thereafter the tube was cooled on ice for 1 minute between pulses. The ribolysis was repeated 3 times. The samples were centrifuged at 13000rpm for 1min and the TRIZOL solution above the beads was transferred to a 2ml phase lock gel tube (Eppendorf, Hamburg 22331, Germany) containing 300µl chloroform/isoamyl alcohol (24:1) (Sigma-Aldrich, St. Louis, Germany). The tube was inverted several times and then centrifuged at 13000rpm for 10min. The top aqueous layer was transferred to a new 1.5ml tube and the crude RNA was precipitated with the addition of an equal volume of isopropanol (Merck, Darmstadt, Germany). The samples was then incubated at -20^{o}C overnight. The crude RNA were collected by centrifugation at 12000 x g, 30 min at 4^{o}C and the pellet was washed with 70% ethanol (Merck, Darmstadt, Germany). The crude RNA was then collected by centrifugation at 12000 x g, for 10 min at 4^{o}C, the 70% ethanol was aspirated and the RNA pellet was air-dried and dissolved in 70µl RNase-free water.

Contaminating chromosomal DNA was digested by typically adding 4µl DNase and 4µl DNase buffer (Whitehead Scientific) to 15µl extracted RNA, followed by incubation at 37^{o}C for 30 minutes. The DNase treated RNA was made up to a final volume of 200µl with RNase-free water. An equal volume of Phenol:Chloroform (4:1) (Sigma-Aldrich, St. Louis, Germany) was added to the diluted RNA, gently mixed and left on ice for 10 min. The tube was centrifuged at top speed for 10 min at room temperature. The top aqueous layer was transferred to a new tube, 0.1 volumes of RNase-free sodium acetate pH 5.2 and 2.5 volumes of 100% RNase-free Ethanol (Merck, Darmstadt, Germany) were added. The tube was the incubated at -20^{o}C overnight. The RNA was collected by centrifugation at 12000 x g, 30 min at 4^{o}C , washed with RNase-free 70% ethanol (Merck, Darmstadt, Germany), re-collected by centrifugation at 12000 x g, for 10 min at 4^{o}C and the ethanol was aspirated off. The purified RNA was air-dried and re-dissolved in 70µl RNase-free water. The quantity and quality of the RNA extracted from each culture was determined by measuring the A_{260}/A_{280} ratio spectrometrically, electrophoretic mobility in non-denaturing 1% agarose gels stained (Figure 2A) and virtual gel electrophoresis on the Experion Software version 2.01 (Bio-rad) (Figure 2B). The RNA preparations were only considered acceptable for subsequent analysis if the presence of the dominant 16S and 23S rRNA species appeared as fairly sharp bands, the A_{260}/A_{280} ratio was between 1.8 and 2.1 and no high molecular weight DNA was detected.

Figure 2



Legend to Figure 2: Visualization of RNA on different systems. A) Assessment of RNA quality by a virtual gel (Experion software -BIORAD); **B)** A typical result obtained on a 1% agarose gel

Primer design for the candidate putative efflux pump genes

PCR primers (Table 3) for reverse transcription and quantitative amplification of the putative efflux pump RNA transcripts were designed using Primer software 3 version 0.2 (Whitehead Scientific), and the whole genome sequence of the *M. tuberculosis* H37Rv reference strain (http://genolist.pasteur.fr/TubercuList).

Quantitative Real-Time PCR (QRT-PCR)

cDNA was synthesized from 1 µg of highly purified RNA according to the manufacturer's instructions (QuantiTect Reverse Transcriptase kit (Southern Cross Biotechnologies)). Thereafter a 20µl RT-PCR reaction was set up by adding 10µl of 2x Quantitect SYBR Green PCR Master Mix (QuantiTect SYBR Green PCR Master Mix (Qiagen)), 1µl forward and reverse primer (50µM) of each candidate efflux gene (Table 3), 6µl of RNase-free water and 2µl of diluted cDNA (1:10). QRT-PCR was done using a Lightcycler 2.0 system (Roche Diagnostics). A four-step PCR parameter protocol was used: (i) Activation program

(95^{o}C for 15 min); (ii) PCR Program repeated for 50 cycles (95^{o}C for 3s, 60^{o}C for 5s, 72^{o}C for 20s); (iii) melting curve program (95^{o}C for 0s, 60^{o}C for 0s, 95^{o}C with a heating rate of 0.2^{o}C/s) and a cooling down program of 38^{o}C for 10s. Each QRT-PCR experiment was done on duplicate biological samples that were each assayed in duplicate. The quantity of a specific cDNA in each reaction was determined from the exponential phase of amplification from the cycle threshold (Ct).

STATISTICS

Both *16S rRNA* (15) and *Rv1437* (27) were included as reference genes, but *16S rRNA* was used for normalization of RNA levels, because in the experiments *16S rRNA* expression levels were the most stable. The level of gene transcription of each individual gene was quantified by the delta-delta Ct calculation in which the relative abundance of the target gene was normalized relative to the levels of the reference RNA transcripts (*16S rRNA*). Data analyses were done according to the delta-delta CT equation $R=2^{-(\Delta CT\ sample\ -\Delta CT\ control)}$ (20). Only experiments with a standard deviation of <0.5 were included for analysis. Significant fold changes were identified based on The Relative Expression Software Tool - 384 (REST-384©) that assigns significance with a significance level of 5%.

RESULTS

Bioinformatic and literature searches identified 30 putative transporter genes in the genome of *M. tuberculosis* which have been suggested to be involved in drug export/import and metabolism (Table 1). QRT-PCR was done to determine the expression levels of these genes in response to RIF in clinical isolates of *M. tuberculosis* representative of two different evolutionary lineages and different levels of resistance to RIF. Significant differences in the level of expression of most of these genes were identified between isolates cultured in the absence or presence of RIF (at a concentration equivalent to ½ RIF MIC).

Analysis of the LCC isolates showed that 28 genes were differentially expressed in response to RIF (Table 4). No significant gene expression changes were observed for 2 genes (*efpA, Rv2687c*). Ten genes (*Rv1463, Rv2994, Rv3806c, mmpL7, drrABC, emrB, iniA, iniC)* were up-regulated (highlighted yellow) and 2 genes (PstB and *Rv1002c*) were down-regulated (highlighted green) in both strains, irrespective of their level of RIF resistance. An additional 10 genes (highlighted grey) were differentially expressed in the isolate with low level RIF resistance (R439), while a further 6 genes (highlighted pink) were up-regulated in the isolate with high level RIF resistance (R451).

Similarly, the Beijing strains with different levels of RIF resistance showed differential expression of 21 of the selected genes in response to RIF (Table 4). NO significant gene expression changes were observed for 9 genes. Four genes (*whiB7, Rv1747, Rv3239c, Rv1634)* were up-regulated (highlighted blue) and one gene (*Rv1002c*) was down-regulated (highlighted red) in both strains. The remaining 16 genes showed different levels of expression between the

isolates with high or low RIF resistance levels. The low level RIF resistant isolate (R257) showed differential expression of an additional 8 genes (highlighted dark grey), while the high level RIF resistant strain (R179) showed differential expression of an additional 10 genes (highlighted dark green).

Comparison of gene expression in the isolates from the different lineages showed that only one gene *(Rv1002c)* was down- regulated in both the LCC and Beijing strains. The *efpA* gene was the only gene that was not differential expressed in any of the isolates tested.

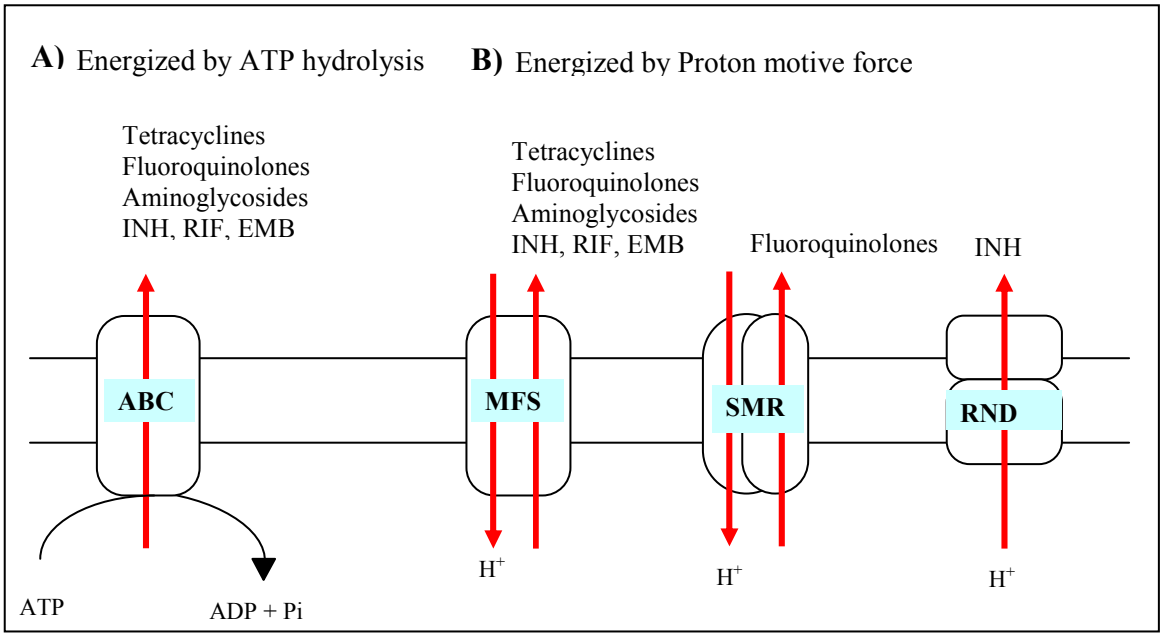


Figure 1: Known transporters associated with drug export in *M. tuberculosis*

Legend to Figure 1: ABC– ATP Binding Cassette (21,26); MFS – Major Facilitator Superfamily (19,24); SMR- Small Multi drug resistance family (11); RND- Resistance Nodulation Cell Division family (22); INH-isoniazid, RIF-rifampicin; EMB-ethambutol

Table 1: Selection of candidate efflux genes based on bioinformatics and literature searches

Effux Gene	Possible Drugs extruded	Transporter	Function	Protein Product	Reference
PstB	INH, RIF, EMB, CIP	ABC	Active import of inorganic phosphate and export of drugs	Phosphate-transport ATP-binding protein	(1,4,5,26)
Rv2686c	CIP	ABC	Active transport of drugs	Integral membrane ABC transporter	(1,5,21)
Rv2687c	CIP	ABC	Export of highly hydrophobic drugs	Antibiotic transport integral protein	(1,5,21)
Rv2688c	CIP	ABC	Export of toxic compounds	Antibiotic-transport ATP-binding protein	(1,5,21)
Rv1747	INH	ABC	Transport of drug across the membrane.	conserved transmembrane ATP-binding protein	(1,5)
drrA	TET, STREP, EMB	ABC	Export of antibiotic in the cell wall.	ATP-binding protein drrA	(1,5,6)
drrB	TET, STREP, EMB	ABC	Export of antibiotic in the cell wall.	ATP-binding protein drrB	(1,5,6)
drrC	TET, STREP, EMB	ABC	Export of antibiotic in the cell wall.	ATP-binding protein drrC	(1,5,6)
Rv1348	Multiple Drugs	ABC	Active export/translocation of drugs across the membrane.	Probable drugs-transport transmembrane ATP-binding protein	(1)
Rv1456c	Undetermined	ABC	Active export of antibiotic across the membrane	Integral membrane protein	(1)
Rv1463	Undetermined	ABC	Active transport and energy coupling across the membrane.	Probable conserved ATP-binding protein	(1)
Rv1258c	INH, RIF, EMB, OFL	MFS	Export of drugs	Conserved membrane protein	(1,10,16,27)
Rv2994	Undetermined	MFS	Efflux of drugs	Conserved membrane protein	(10,18)
Rv1877	TET, KANA, Erythromycin	MFS	Efflux of drugs	Conserved membrane protein	(10,18)
Rv1634	Undetermined	MFS	Efflux of sugars and drugs	Drug efflux membrane protein	(9,10,18)
efpA	Possibly INH	MFS	Export of drugs	Integral membrane efflux protein	(1,14,18)
Rv2333c	Tetracycline	MFS	Efflux of drugs	conserved integral membrane transport protein	(10)
Rv2459c	Drugs	MFS	Transport of substrates	conserved integral membrane transport protein	(10)
Rv3239c	Sugar or drugs	MFS	could be involved in efflux	conserved transmembrane transport protein	(10)
Rv3728	Sugar or drugs	MFS	Involved in efflux	conserved two-domain membrane protein	(10)
emrB	Undetermined	MFS	Export of multiple drugs	Integral membrane efflux protein	(11)
mmpL7	INH	RND	Export of antibiotic	transmembrane transport protein	(1,13,22,23)
whiB7	RIF	regulatory protein	Transcriptional regulation	transcriptional regulatory protein and effector gene	(1,27)
Rv2989	Undetermined	transcriptional regulator	transcriptional mechanism	transcriptional regulatory protein	(1)
iniA	INH, EMB	Membrane protein	Drug transport	INH inductible protein iniA	(1,3,7)
iniB	INH	Membrane protein	Drug transport	INH inductible protein iniB	.(1,3,7)
iniC	INH	Membrane protein	Transcriptional mechanism	INH inductible protein iniC	(1,3,7)
Rv1002c	Undetermined	Membrane protein	Unknown function	Integral membrane protein	(1)
Rv3806c	Undetermined	Membrane protein	Unknown Function	Integral membrane protein	(1)
Rv3679	Undetermined	ATPase	Extrusion of anions	Probable anion transporter	(1)

Legend to Table 1: INH- Isoniazid; RIF-Rifampicin; EMB-Ethambutol; CIP-Ciprofloxacin; TET-Tetracycline; STREP-Streptomycin; OFL-Ofloxacin; KANA-Kanamycin; ABC-ATP-Binding Casssette; MFS-Major Facilitator Superfamily; RND-Resistance Nodulation Cell Division Family; SMR-Small Multi-drug resistance Family

Table 3: Primers used for QRT-PCR analysis

Gene	Primer sequence	Size (bp)	Tm (°C)	Reference
Rv1348	Forward:5`GTT CTT GGG TAC CAC GTT CG 3` Reverse:5`GTG GTC GAA CAC CAC AGT TG 3`	184	60	Current
Rv1456c	Forward:5`ATA TGC ATT CGT CGC TGT TG 3` Reverse: 5`GGG TAC CCC GGT GAA GTA TT 3`	170	59	Current
Rv1463	Forward:5` AGA ACT GCT CAA GCC CAA GA 3` Reverse: 5` ACG TAT TCC GGG TGG ATG TA 3`	174	60	Current
Rv1002c	Forward:5` CAT TTC TGG TGA TGG GCA TT 3` Reverse:5` CCA GGT TCC AGG TCT GTT GT 3`	186	60	Current
Rv3679	Forward:5`AAG AAC AAG CTG CCG GTC TA 3` Reverse:5` GGC AGC GCT TCT AAC AGA GT 3`	200	60	Current
Rv3806c	Forward:5` GTG TCG TCG GCG TAT TTG AT 3` Reverse:5` CGC AGA TAG GTG CTG GTG TA 3`	200	60	Current
efpA	Forward:5` TAG GTT TCA TCC CGT TCG TG 3` Reverse:5` TGA CCA GGT TGG GGA AGT AG 3`	177	60	Current
Rv1258c	Forward:5` GGT ATG CCG TGT TGG CTA TC 3` Reverse:5` CCG CGT CTG TAT CAC GTA GTT 3`	188	60	Current
PstB	Forward:5’ GTT CCC GAT GTC AAT CAT GG 3’ Reverse:5’ ACC ACC AGA GAG TCG AAA CG 3’	166	60	Current
whiB7	Forward:5’ CAG ACA AAG ATT GCC GGT TT 3’ Reverse:5’ TCG AGC CTT GGT CGA ATA TC 3’	194	60	Current
mmpL7	Forward:5’ TGA AAT ACG GAA GCC TGG TC 3’ Reverse:5’ GAG GTA AGA GGC CAG CAC AC 3’	197	60	Current
drrA	Forward:5’ ACG ACC ATG GTG GAC ATC TT 3’ Reverse:5’ AAC ACC AGG TTC TGC TCA CC 3’	170	60	Current
drrB	Forward:5’ CTG AGC TTG CCC ATT TTG AT 3’ Reverse:5’ TCA CCT GTG AGG CTG TCT TG 3’	166	60	Current
drrC	Forward:5’ AAC CGG TTG CTA ACT CGA TG 3’ Reverse:5’ CAG CGG AAC AAT GCT GTA GA 3’	153	60	Current
Rv2686c	Forward:5’ ACG ACA TTC GAG GAC CCT AC 3’ Reverse:5’ ACG ATG ATG CTG GTC AAC AA 3’	190	60	Current

Table 3: Primers used for QRT-PCR analysis (continued).

Gene	Primer sequence	Size (bp)	Tm (ºC)	Reference
Rv2687c	Forward:5' CTA CAG GTG CGG CAG AAG TT 3' Reverse:5' GAC GAA GAA GAA CCC GAT GA 3'	150	60	Current
Rv2688c	Forward:5' ACA GTC CCA CCG AAC TGA AG 3' Reverse:5' ATG AAT GGT CTC GAC GTG GT 3'	164	60	Current
Rv1747	Forward:5' TCT GGA GCT GTT CGT TGA TG 3' Reverse:5' ACC CAG GAC ATC TGG TCA AG 3'	192	60	Current
iniA	Foward:5' AAG ATG ATC CAG CGT CTG CT 3' Reverse:5'TTG ACC TGG CTC AGG ATA CC 3'	173	60	Current
iniB	Forward:5' GCT AGC CAG ATC GGT GTC TC 3' Reverse:5' CGA CAG ATG AGG CAT AGC AG 3'	171	60	Current
iniC	Forward:5' CAA CGA CAT TGA ACG ACG AC 3' Reverse:5' GAA CGG ATC GTT GAG TGG AT 3'	179	60	Current
emrB	Forward:5' TTC GAC TAC ATG GGC CTC TT 3' Reverse:5' TAT GAG CGG ATG TTC TGT GC 3'	183	60	Current
Rv1634	Forward:5' CCA CCA ACG AGT TTC TGA CA 3' Reverse:5' ACC CCA TCA GAT ACG ACG AG 3'	174	60	Current
Rv1877	Forward:5' AAT CGC TGT ACC TGG TCG TC 3' Reverse:5' CGG TCC AGG AAG TTT ACG AA 3'	190	60	Current
Rv2333c	Forward:5' TGA TCT TTC TCG ACG CAC TG 3' Reverse:5' CAG CGT GAA CAA CGA AAC AC 3'	200	60	Current
Rv2459	Forward:5'TGG ACG TCA ACA TCG TCA AT 3' Reverse:5'GTG ACC CCG AAC ACA AAA CT 3'	178	60	Current
Rv2989	Forward:5' GAA AGC GTG CAG GTA TAT CG 3' Reverse:5' ACA CCG CCT TTG GCA ATA C 3'	190	60	Current
Rv2994	Forward:5' CTA TCT CAC GCG GGT CTG TT 3' Reverse:5' ACA GGA AGA CAC CGA TCC AC 3	187	60	Current
Rv3239c	Forward:5' CGG ACG CTG ACC CTA TTA GA 3' Reverse:5' ACA TGC AGT CGA CCG TTG TA 3'	200	60	Current
Rv3728	Forward:5' GAT GGC ATC GGA AAA AGT GT 3' Reverse:5' CAC CAG CTC CAT GAT TTG TG 3'	154	60	Current

Table 4: Differential gene expression profile of putative efflux genes

Fold change after 24hrs exposure to ½ RIF MIC

Genes	LCC cluster DRF150		Beijing Cluster 220	
	[a]R439	[b]R451	[c]R257	[d]R179
whiB7	-2.08#	2.89*	5.72*	6.80*
Rv1456c	1.35	111.43*	1.07	-4.08#
Rv1463	2.07*	62.90*	-1.39	-1.81
Rv1747	2.34*	1.17	2.16*	7.92*
Rv2994	2.31*	2.60*	1.90	-2.45#
Rv1877	-3.28#	2.07*	-1.29	-1.36
Rv2333c	-4.79#	1.71	-1.25	-2.22#
Rv2459c	-6.80#	1.41	2.63*	-2.92#
Rv3806c	2.93*	113.77*	-1.71	-1.79
Rv1258c	-3.82#	-1.42	2.02*	1.10
efpA	-1.61	-1.90	1.34	1.33
PstB	-3.88#	-2.64#	-2.27#	11.43*
Rv1002c	-2.48#	-2.48#	-2.76#	-8.43#
Rv1348	-1.21	3.12*	-1.38	-1.10
Rv3679	1.11	152.75*	1.04	3.93*
mmpL7	3.81*	2.05*	2.93*	-1.13
Rv2686c	2.34*	1.62	-1.40	1.10
Rv2687c	1.28	-1.93	-2.15#	-1.14
Rv2688c	10.85*	-1.49	2.29*	1.32
drrA	2.45*	2.64*	-1.83	-1.64
drrB	7.16*	4.36*	1.03	3.48*
drrC	3.52*	2.11*	-1.39	1.29
emrB	4.53*	2.45*	1.85	10.09*
Rv3728	-8.25#	-1.10	1.90	-22.86#
Rv2989	-6.68#	1.66	1.55	-2.01
Rv3239c	-1.62	2.23	20.32*	4.32*
iniA	6.32*	2.52*	1.92	-1.55
iniB	1.28	7.89*	8.93*	-10.85#
iniC	18.06*	3.56*	-62.69#	1.21
Rv1634	7.29*	-1.61	89.26*	11.47*

Legend to Table 4: [a]LCC cluster DRF150 RIF resistant strain (RIF MIC=60µg/ml); [b] LCC cluster DRF150 RIF resistant strain (RIF MIC=170µg/ml); [c]Resistant strain of Beijing cluster 220 (RIF MIC=30µg/ml); [d]Resistant strain of Beijing cluster 220 (RIF MIC=140µg/ml); *significantly upregulated; #significantly down regulated

DISCUSSION

Efflux pumps are typically membrane proteins that can remove toxic compounds from the cell by active transport. Thus such pumps may play an important role in the development of resistance to commonly used drugs. This can be affected by either an increase in *de novo* synthesis of the pump itself, the activation of a pre-existing efflux pump through mutation or by drug pump interaction. This is supported by the observation that RIF could induce a change in expression of the efflux gene (*Rv1258c)* (27). Our results confirm that most of the genes putatively associated with drug resistance through either regulation of influx or efflux were differentially expressed in response to RIF. The differential expression of the respective efflux genes was largely lineage/isolate specific with the exception of *Rv1002c* which was down regulated in both the isolates from the LCC and Beijing lineages. In this study, expression of the *efpA* gene was not affected by the exposure to RIF. It has been shown previously that *efpA* was up-regulated upon exposure to INH (14). However, this efflux gene could not be implicated in conferring drug resistance.

We propose that the extrusion of RIF from the different strains by numerous putative efflux pumps may regulate the intracellular concentrations of RIF resulting in different levels of RIF resistance. We also suggest that this complex network of differentially expressed genes may reflect a response to the toxic nature of RIF at concentrations above the critical concentration used in standard drug sensitivity tests. Under these conditions RIF may act as an inducer that stimulates expression of the various efflux pumps. Alternatively, the hydrophobic nature of the RIF molecule may in itself stimulate the activity of the efflux

pumps, as has been shown for aminoglycosides that induce/stimulate specific efflux pumps (12). However, the latter effect was not addressed in this study.

Our initial hypothesis suggested that isolates with identical genetic backgrounds would respond similarly to the same anti-TB drug pressure. This is in part reflected in our results as a number of genes are either up-regulated or down-regulated in a lineage dependent manner. We propose that the differential expression of genes seen in isolates from the same lineage reflects either differences in the genetic makeup of the closely related strains or that the different RIF concentrations used (1/2 MIC) would have had different toxic/killing effects. Analysis of the kill curves used to determine the MIC values showed that bacterial growth was inhibited by approximately 18-20% at ½ the RIF MIC (sub-inhibitory concentrations) for each individual isolate (Chapter 5). Thus we cannot exclude the fact that our observations also include a RIF killing effect. This may be reflected by the apparent down regulation of certain genes as RIF is a transcription inhibitor. We acknowledge this limitation and suggest that exposure to a therapeutic dose may alleviate this confounding factor.

From the results in this study we propose the following: In a rifampicin sensitive strain, the addition of RIF will primarily affect the functional activity of rpoB and consequently inhibiting transcription. Under such conditions it will be difficult to asses the toxic effect of RIF (Figure 5a). With the addition of RIF to a rifampicin resistant strain, the mutation in the *rpoB* gene prevents the binding of RIF to rpoB and thus transcription will proceed. Increasing the concentration of RIF will have one of two effects: promote interaction between RIF and rpoB leading to inhibition of transcription and cell death or provide a

toxic effect to which the cell will react by changing gene expression that will influence expression of numerous toxic response genes including efflux pumps (Figure 5b). It is currently unknown whether metabolism of RIF occurs under such conditions to produce metabolites which in turn have a toxic effect (Figure 5b). It should be noted that only a limited number of efflux pump genes can be stimulated, due to the energy requirements of the cell. Depletion of the intracellular ATP pool, would lead to cell death.

From this study we conclude that the level of drug resistance in *M. tuberculosis* is attributed to a combination of mutations in target genes as well as a toxic response which regulates the intracellular concentration of the drug.

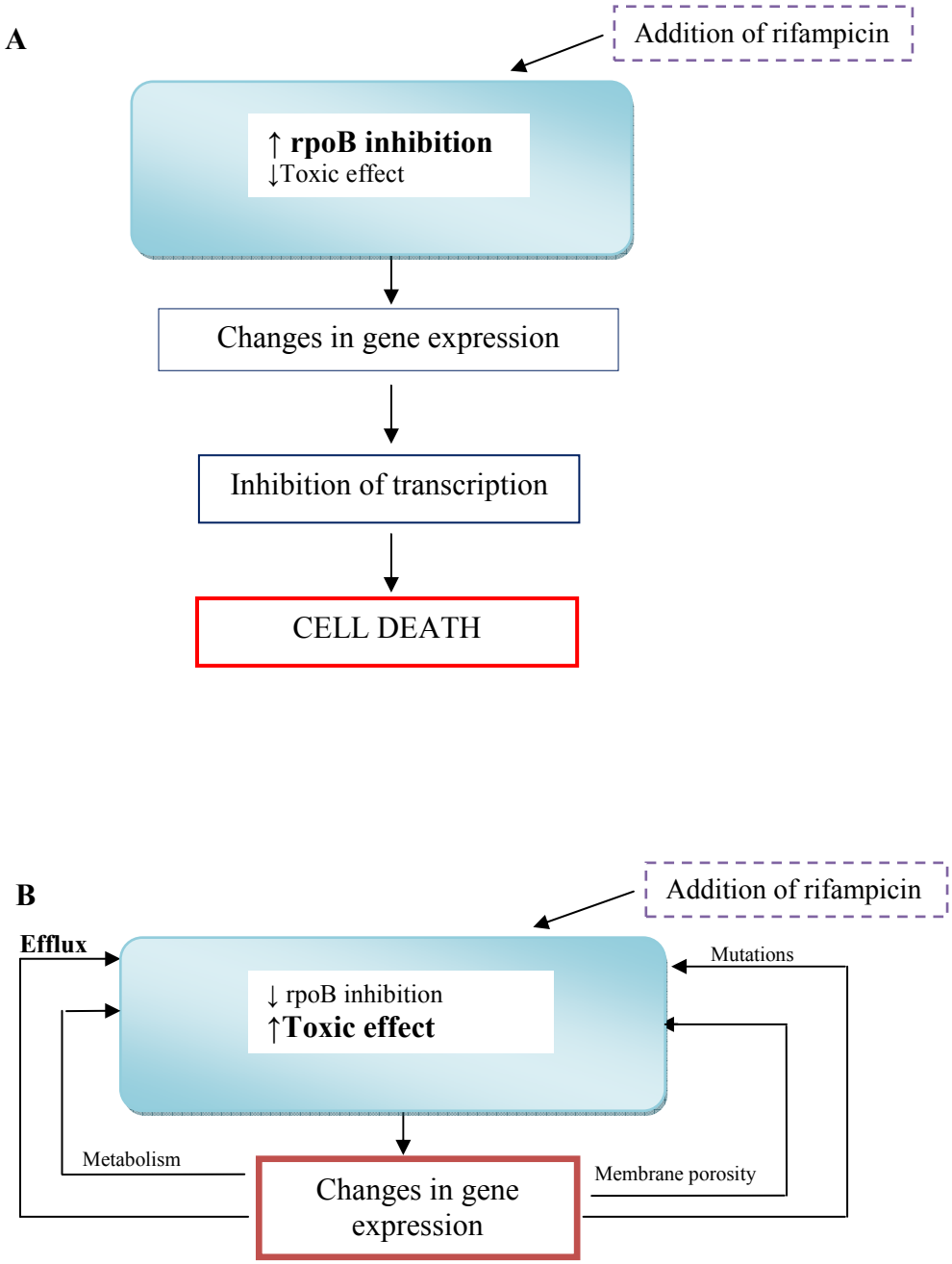


Figure 5: The effect of the addition of rifampicin. A) The effect of the addition of RIF to a RIF sensitive strain; B) The effect of the addition of RIF to a RIF resistant strain.

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CHAPTER 7

CONCLUSION

Drug resistant tuberculosis (TB) has emerged globally despite stringent control strategies. This suggests that *Mycobacterium tuberculosis* has adapted and evolved mechanisms to cope with or evade the mode of action of various anti-TB drugs. Rapid diagnosis of patients infected with a drug resistant strain is thus of great importance in order to prevent the transmission of these difficult to treat strains.

Phenotypic diagnosis of drug resistance remains a lengthy and technically challenging process and is not routinely done in many countries with a high burden of drug resistant TB. In South Africa, initial drug susceptibility testing (DST) is only done for isoniazid (INH) and rifampicin (RIF) on high risk patients. If it is a multi-drug resistant TB (MDR-TB) strain (resistant to INH and RIF), then only resistance to ethambutol (EMB) is tested and the patient is treated accordingly with second-line anti-TB drugs. No additional DST is done on these cultures. Pyrazinamide (PZA) is included in the initial intensive phase treatment regimen for TB, together with INH, RIF and EMB. However, due to the technical difficulty in PZA phenotypic drug susceptibility testing, PZA resistance testing is not routinely done in South Africa. Therefore, the prevalence of PZA resistance in TB patients in South Africa is largely unknown. Alarmingly, this study showed that more than 50% of previously treated TB patients in the Western Cape of South Africa, are phenotypically and genotypically resistant

to PZA. The correlation of phenotypic to genotypic resistance (mutations in the *pncA* gene) was 93%. Although these mutations can be used as a marker to identify PZA resistance, they are scattered and highly diverse in the *pncA* gene (without mutation clustering), thereby making it difficult to rapidly identify PZA resistance by genotypic methods other than gene sequencing. We also observed that 91% of the PZA resistance was associated with MDR-TB. Accurate PZA testing is essential to ensure that patients receive appropriate treatment. The failure to detect PZA resistance will result in inappropriate therapy which may lead to prolonged treatment. This is a potential crisis, due to the fact that PZA is carried through to the next phase when treating patients infected with extreme drug resistant TB (XDR-TB) strains. Therefore, this study showed that PZA should not be relied upon in managing patients with MDR-and XDR-TB in South Africa.

RIF resistance has been suggested as a marker to identify MDR-TB and a number of molecular methods have been employed to rapidly genotype RIF resistance. Recently, an additional nucleotide change (*Rv2629* 191 A/C), other than the known *rpoB* mutations, was identified and shown to be associated with RIF resistance. However, in contrast to what was reported, we have shown that this nucleotide change was not associated with resistance to RIF but was a polymorphism significantly associated with the Beijing genotype. Therefore, this nucleotide change can not be used as a marker for the rapid identification of RIF resistance, but it can however be useful in identifying clinical isolates from the Beijing genotype, which has a greater propensity to spread and cause disease than other strain families.

It is well known that mutations in the *rpoB* gene confer RIF resistance. It has also been accepted that certain mutations confer a certain level of RIF resistance. We have shown by using phenotypic methods that the level of RIF resistance varied in closely related clinical isolates with identical mutations conferring resistance to RIF, INH, EMB and PZA. This observation holds true for different evolutionary lineages, including the Low Copy Clade (LCC) and Beijing, which were reported in drug resistant outbreaks in South Africa. We therefore conclude that a specific mutation in the resistance conferring *rpoB* gene alone can not explain the variation in the level of RIF resistance in genetically closely related clinical isolates. By further investigating this phenomenon, we have shown that the addition of compounds that inhibit the activity of efflux pumps (such as verapamil and reserpine), together with RIF, significantly reversed the resistance phenotype of the strains to become RIF susceptible. For this reason, we proposed a model suggesting that the initial mutation in the *rpoB* gene results in low/moderate resistance and that other unknown mechanisms (eg. efflux pumps, regulators or other genes) are responsible for the bacterium becoming hyper-resistant to RIF. The addition of compounds that inhibit efflux activity has opened a new avenue for reviewing existing drugs in combination with anti-TB drugs for the treatment of this disease. Apart from normal susceptible TB, this could potentially be promising therapy for the treatment of MDR-and XDR-TB.

To further investigate efflux pumps as targets for novel treatment regimens, putative efflux genes were identified by bioinformatics and literature searches. Assessment of expression of these putative efflux genes by QRT-PCR showed significant differential expression of numerous efflux genes in response to RIF for the investigated lineages. In the LCC lineage, a

number of the same genes were differentially expressed in both isolates with high and the low level of RIF resistance. The same observation was made for the isolates in the Beijing lineage. This suggests that a global response is firstly launched to evade the toxic effect of RIF. However, isolate specific gene expression changes were also observed in the isolates from the different evolutionary lineages, indicating a unique response. This suggests that each isolate has adapted to make unique changes in efflux gene expression, in combination with the global response, to evade the toxic effect of RIF. Our model suggests that in a RIF resistant strain, with an initial defined *rpoB* gene mutation, RIF has a toxic effect on the cellular functions that will result in changes in gene expression. These changes will then stimulate the activity of other mechanisms such as the activity of efflux pumps, that will ultimately regulate the intracellular drug accumulation and subsequent level of RIF resistance.

Initial whole genome sequence of the same low and high level rifampicin resistant clinical isolates that were used in this study indicate that only a few SNP's and indels are different between these two sets of strains. Since there are only a few differences it will not be difficult to assess functionality of potential candidates by point mutagenesis and knock in/out of genes. This will lead to the unravelling of the mechanisms leading to the development of RIF hyper-resistance in well characterized clinical isolates. However, comparative sequence analysis of more isolates is essential to get a full picture of the evolution of drug resistance. New targets identified from comparative sequence data will be useful for future drug design.

Future studies will include the assessment of the effect on efflux pump inhibitors on *in vitro* laboratory-generated mutants, as it has been reported that the level of RIF resistance also differs in these isolates with identical mutations.

Other Publication

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