Understanding the mechanisms of drug resistance in enhancing rapid molecular detection of drug resistance in *Mycobacterium tuberculosis*

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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

One of the aims of direct observed therapy strategy implemented by the World Health Organization was to prevent the development of drug resistant tuberculosis. However, in recent years a dramatic increase and spread in multidrug resistant tuberculosis has been observed. In this study, a molecular epidemiological approach was used to understand and rapidly detect drug resistance in high incidence tuberculosis communities of the Western Cape, South Africa. Previous studies showed that, drug resistant tuberculosis occurs as a result of spontaneous mutations in particular genes. Using molecular techniques, we developed an algorithm to rapidly detect isoniazid, rifampicin and ethambutol drug resistance in tuberculosis patients from a short term mini culture. Rapid detection of drug resistance is important to prevent future transmission events. In addition, accurate ethambutol resistance testing is of particular importance, since treatment of patients infected with multidrug resistant strains with second line anti-tuberculosis drugs depend on the ethambutol test results. In a comprehensive study, we found that the algorithm performs well when compared to the traditional culture method currently used by the routine laboratories. However, the results showed that more then 90 % of ethambutol resistance is missed by the routine laboratories. This has important implications for the tuberculosis control program, since patients infected with the drug resistant strain may be on inappropriate treatment.

In this study, we found that certain strains have a selective advantage to become drug resistant and transmit and this implies that they are more virulent and fit than other strains. This observation has also been made for strains within the same genotype family. The more transmissible drug resistant strains cause large drug resistant outbreaks.

This study highlights the complexity of the drug resistant epidemic, and confirms that it is a major problem in local communities. Application of molecular methods has provided us with tools to study how resistance might develop. We have demonstrated how we made use of a newly developed method to detect a multidrug resistant outbreak in the study community. The applications of transcriptomics identified several genes that might play a role in isoniazid resistance. Using this data a model was proposed whereby isoniazid resistant strains can compensate for the toxic effect of the drug. Application of comparative genomics by whole genome sequencing will be used to assist us in the further understanding of the mechanisms of drug resistance.

This study also conclude that we should continue in our attempts to develop faster diagnostics for both first and second line drugs and that we must not loose site that all of this research must in the end benefit the patients.

OPSOMMING

Een van die doelstellings van die direkte observasie terapie strategië van die Wêreld Gesondheidsorganisasie is die voorkoming van middelweerstandigheid in tuberkulose. In die laaste paar jaar is daar egter ‘n dramatise toename en verspreiding van middelweerstandige stamme van *Mycobacterium tuberculosis* waargeneem. In hierdie studie is daar van ‘n molekulêre epidemiologiese aanslag gebruik gemaak om die meganismes van middelweerstandigheid verder te verstaan en om tegnieke te ontwikkel om middel weerstandige tuberkulose ten volle te diagnoseer. Deur gebruik te maak van hierdie kennis, is ‘n algoritme ontwikkel om isoniasied, rifampisien en ethambutol weerstandigheid direk van speeksel vas te stel. Akurate toetsing vir ethambutol weerstandbiedendheid is veral noodsaaklik, aangesien verdere behandeling van pasiente wat geinfekteer is met n weerstandige organisme van tweede linie anti-tuberkulose middels afhanklik is. In ‘n omvattende studie is gevind dat die algoritme goed vergelyk met die tradisionele kultuurmetode, wat tans in roetine laboratoriums gebruik word. Bevindings toon dat meer as 90 % van ethambutol weerstandigheid nie deur die roetine laboratoriums opgespoor word nie. Hierdie bevindings het baie belangrike implikasies vir die Tuberkulose Beheer Program, want dit beteken dat pasiente war reeds met middel weerstandige organismes geinfekteer is, dan op verkeerde behandeling is.

Sekere *M. tuberculosis* stamme het‘n selektiewe voordeel om weerstandig te word en dan in die gemeenskap te versprei. Dit impliseer dat hulle meer virulent en fiks is as ander stamme. Hierdie waarnemning is ook gemaak vir sekere stamme binne dieselfde genetipiese stamboom.

Transkripsiestudies het bygedra tot die identifisering van bykomende gene wat ‘n rol mag speel in isoniasied weerstandigheid. Met behulp van hierdie data is ’n model voorstel wat wys hoe ’n isoniasied weerstandige stam kan kompenseer vir die toksiese uitwerking van die anti-

tuberkulose middel. Volledige genoom volgorde bepaling kan gebruik word ten einde verdere meganismes van middel weerstandheid en virulensie verder te kan bestudeer.

Die studie belemtoon die kompleksiteit van die middelweerstandige epidemie en bevestig verder dat dit n ernstige probleem in plaaslike gemeenskappe is. Die gebruik van molekulêre tegnieke het ons in staat gestel om meganismes vir die ontstaan van weerstandigheid te bestudeer. Verder is nuut ontwikkelde metodes gebruik om ’n uitbraak van middel weerstandige tuberkulose binne die studiegemeenskap te identifiseer. Die studie sluit af met n voorstel dat daar deurgaans gewerk moet word aan die ontwikkeling van vinniger diagnostiese metodes om middel weerstandige tuberkulose vir eerste en tweede linie middels te kan identifiseer. Navorsers behoort in ag te neem dat dit uiteindelik die pasiënt is wat by al hierdie stuidies moet baat.

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Chapter 1

Introduction

Tuberculosis (TB) is caused by the bacillus *Mycobacterium tuberculosis* (*M. tuberculosis*) and has been part of the human population since antiquity (11). Soon after the first antibiotic for TB treatment was administered in 1948, the general thought was that the TB epidemic was under control and can be written up in history. However, more then 55 years later, the TB epidemic remains a threat with sub-Saharan Africa having the highest prevalence of TB in the world (1). In the latest TB surveillance data for 2005, South Africa ranked 7th in the world with an prevalence rate for all forms of TB of 600/100 000 (19). The Western Cape Province of South Africa was found to have the highest TB prevalence for 2006 with a TB prevalence rate for all forms of TB of 998/100 000 (8).

Over the past decades, the tuberculosis bacilli became resistant to various anti-TB drugs, making infection control increasingly difficult. Drug resistant TB occurs through selection where in a selective environment (in the presence of a drug), the sensitive bacterial population is killed and the resistant mutant is allowed to grow, and this is called acquired resistance. This form of resistance has resulted in the emergence of multidrug resistant TB (MDR-TB), defined as resistance to Isoniazid (INH) and Rifampicin (RIF), and more recently, extensively drug resistant TB (XDR-TB). XDR-TB is defined as resistance to INH, RIF, plus any fluoroquinolone and at least one of the three injectable second–line drugs (amikacin, kanamycin and capreomycin) (9). According to a weekly report published in 2006 by Center of Disease Control (CDC), it was found that during 2000-2004,

20 % of the TB strains collected world wide were MDR and one tenth of those strains were XDR-TB strains (1). South Africa was listed in the third world report on drug surveillance as one of the hot spots for MDR-TB, where more than 6000 new MDR-TB cases are detected each year (18). However, the extent of XDR-TB in South Africa is not known since second line testing was not performed routinely. Thus, XDR-TB could have been present in South Africa long before the first reports of XDR-TB hit Tugela Ferry (1). Currently there are no new anti-TB drugs available to treat patients. Most of the new promising TB drugs are under development and are still in the preclinical stage. It is predicted that the earliest new drug will be available in 2010 (1). This emerging drug resistant problem is of great concern for the control of TB therefore, there is a need to further understand the mechanisms of drug resistance and to use the knowledge to develop fast and accurate detection methods that can rapidly detect drug resistance.

Routine drug susceptibility testing depends on a positive culture for diagnosis after which a drug susceptibility test is performed which usually takes 3-6 weeks (17). The slow diagnosis and in some cases inaccurate or false negative phenotypic results (15), may be a major contributor to the current drug resistant epidemic. During the last decade molecular methods such as, PCR based technologies and IS*6110* restriction fragment length polymorphism (IS*6110*-RFLP) for example, have increasingly been applied to control drug resistant TB. IS*6110*-RFLP analysis for instance, can be used to distinguish between reactivation and reinfection, while the use of mutational analysis may shed light on primary versus acquired drug resistance.

The combination of IS*6110*-RFLP and drug resistant genotyping can also be used as a powerful tool in the investigation and identification of outbreaks, as well as the transmission dynamics of outbreak

strains. It has also been found that certain strain families are more prominent in certain geographical settings and are often responsible for a large proportion of the disease episodes that occur within that region, making them more transmissible than other strains in the same geographical setting (13,14,16). An example of such a strain family is the Beijing which have been the focus of most MDR-TB outbreaks reported to date (6,7,10).

Molecular biology of TB underwent revolutionary changes over the past decades (4). With the availability of the complete *M. tuberculosis* genome data (3), a new foundation has been laid that will serve as a powerful framework in the understanding of the molecular genetics of drug resistant strains. Also with the application of functional genomic techniques, such as transcriptomics or proteomics, the biology of *M. tuberculosis* can be explored and the response can be measured by using techniques such as microarray analysis, two-dimentional (2-D) gel2-D gel electrophoresis and subsequent quadrupole-time of flight (Q-TOF) analysis. The application of these technologies depends on the type of information that needs to be generated from the data set. The following are examples:

- Comparative genomics: (5)
- Gene regulation: (12)
- Differential gene and protein expression: (2)

The information gained can be used to understand the underlying mechanism that causes *M. tuberculosis* to develop drug resistance. More importantly, it can also be used in future studies for drug discovery, the development of new diagnostic markers, transmissibility and to study virulence.

In this study we **hypothesize** that understanding the mechanisms of drug resistance in *M. tuberculosis* may ultimately lead to the control of drug resistance. The general **aim** of this study is to use molecular techniques to study the molecular mechanisms of drug resistance in *M. tuberculosis* and to apply the knowledge gained to develop new diagnostic methods which can be applied in high incidence communities.

This thesis was divided into the following themes which include:

i. *M. tuberculosis* drug resistance review (Chapter 2)
ii. molecular detection of drug resistance (Chapter 3 and 4)
iii. molecular epidemiology (Chapter 5 and 6) and
iv. differential gene expression (Chapter 7 and 8)

Each chapter is structured according to the journal in which the article was published and if the results in a particular chapter have not been published yet, the specification prescribed for Journal of Clinical Microbiology has been used.

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Chapter 2

Drug resistance in *M. tuberculosis*

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My contribution: Research of data on drug resistance and compiling of data

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ABSTRACT

Anti-tuberculosis drugs are a two-edged sword. While they destroy pathogenic *M. tuberculosis* they also select for drug resistant bacteria against which those drugs are then ineffective. Global surveillance has shown that drug resistant Tuberculosis is widespread and is now a threat to Tuberculosis control programs in many countries. Application of molecular methods during the last decade has greatly changed our understanding of drug resistance in Tuberculosis. Application of molecular epidemiological methods was also central to the description of outbreaks of drug resistance in Tuberculosis. This review describes recommendations for Tuberculosis treatment according to the WHO guidelines, the drug resistance problem in the world, mechanisms of resistance to first line and second line drugs and applications of molecular methods to detect resistance causing gene mutations. It is envisaged that molecular techniques may be important adjuncts to traditional culture based procedures to rapidly screen for drug resistance. Prospective analysis and intervention to prevent transmission may be particularly helpful in areas with ongoing transmission of drug resistant strains as recent mathematical modeling indicate that the burden of MDR-TB cannot be contained in the absence of specific efforts to limit transmission.

1. INTRODUCTION

1.1 *Drug Resistance and Global Surveillance: History*

Shortly after the first anti-tuberculosis (TB) drugs were introduced, streptomycin (STR), para-aminosalicylic acid (PAS), isoniazid (INH)) resistance to these drugs was observed in clinical isolates of *Mycobacterium tuberculosis* (Crofton and Mitchison, 1948). This lead to the need to measure resistance accurately and easily. The Institute Pasteur introduced the critical proportion method in 1961 for drug susceptibility testing in TB and this method became the standard method of use (Espinal, 2003). Studies on drug resistance in various countries in the 1960's showed that developing countries had a much higher incidence of drug resistance than developed countries (Espinal, 2003). By the end of the 1960's rifampicin (RIF) was introduced and with the use of combination therapy, there was a decline in drug resistant and drug susceptible TB in developed countries. This led to a decline in funding and interest in TB control programs. As a result, no concrete monitoring of drug resistance was carried out for the following 20 years (Espinal, 2003). The arrival of HIV/AIDS in the 1980's resulted in an increase in transmission of TB associated with outbreaks of multi-drug-resistant TB (MDR-TB) (Edlin et al., 1992; Fischl et al., 1992) i.e. resistant to INH and RIF. In the early 1990's drug resistance surveillance was resumed in developed countries, but the true incidence remained unclear in the developing world (Cohn et al., 1997).

1.2 *The WHO/IUATLD Global Project on Drug-Resistance Surveillance*

In 1994 the Global Project on Drug-Resistance Surveillance was initiated to monitor the trends of resistance. The first report was published in 1997 and contained data from 35 geographical settings for the period 1994-1996 (World Health Organization, 1997; Pablos-Mendez et al., 1998). The report showed that drug resistance was present globally, and that MDR-TB ranged from 0 % to 14 % in new cases (median: 1.4 %) and 0 % to 54 % in previously treated cases

(median: 13 %). A second report for the period 1996-1999, followed in 2000 and included surveillance data from 58 geographical sites (Espinal, 2003; World Health Organization, 2000). This report confirmed that drug resistant TB was a sufficient problem since MDR-TB ranged from 0-16 % (median: 1 %) among new cases and from 0 % to 48% (median: 9 %) in previously treated cases. The recently published third report has data on 77 geographical sites, collected between 1999 and 2002, representing 20 % of the global total of new smear-positive TB cases (World Health Organization, 2003). Eight countries did not report any MDR-TB amongst new cases, while the highest incidence of MDR-TB amongst new cases occurred in Kazakhstan and Israel (14 %). Significant increases in MDR-TB prevalence were seen in Estonia, Lithuania, Tomsk Oblast (Russian Federation) and Poland and significant decreasing trends in Hong Kong, Thailand and the USA. The highest prevalence of MDR-TB among previously treated cases was reported in Oman (58.3 %, 7/12) and Kazakhstan (56.4 %, 180/319). The annual incidence of MDR-TB in most Western and Central European Countries was estimated to be fewer than 10 cases each. Alarmingly, it is estimated that the annual incidence of MDR-TB for 2 provinces in China (Henan and Hubei) is 1000 and for Kazakhstan and South Africa it is more than 3000. According to the report, the most effective means to prevent the emergence of drug resistance is by implementing the direct observed therapy strategy (DOTS) (World Health Organization, 2003).

1.3 *Current Recommendations for TB Treatment by WHO*

TB persists as a global public health problem and the main focus for the twentieth century is firstly to cure the individual patient and secondly to minimize the transmission of *M. tuberculosis* to other persons (World Health Organization, 2003; Blumberg et al., 2003). The ongoing TB problem has been due to the neglect of TB control by governments, inadequate access and infrastructure, poor patient adherence to medication, poor management of TB control programs, poverty, population growth and migration, and a significant rise in the number of TB cases in HIV infected individuals. Treatment of patients with TB is most successful within a comprehensive framework based upon the following five key components:

- Government commitment
- Case detection by sputum smear microscopy
- Standardized treatment regimen of six to eight months
- A regular, uninterrupted supply of all essential anti-TB drugs
- A standard recording and reporting system

These five key elements are the recommended approach by the World Health Organization (WHO) to TB control and are called the DOTS strategy (Walley, 1997). DOTS are an inexpensive strategy for the detection and treatment of TB. DOTS were implemented as part of an adherence strategy in which patients are observed to swallow each dose of anti-TB medication, until completion of the therapy. Monthly sputum specimens are taken until 2 consecutive specimens are negative. Currently there are four recommended regimens for treating patients with TB infection by drug–susceptible organisms. Each regiment has an initial phase of 2 months intensive phase followed by a choice of several options for the continuation phase of either 4 or 7 months. The recommended regimens together with the number of doses specified by the regimen are described in Table 1.

Table 1. Drug Regimen for Culture-Positive Pulmonary TB Caused by Drug-Susceptible Organisms

Intensive Phase			**Continuation Phase**		
Regimen	**Drugs**	**Doses**	**Regimen**	**Drugs**	**Doses**
1	INH,RIF,PZA, EMB	7 d /wk for 56 doses (8wk) **or** 5 d/wk for 40 doses (8wk)	1	INH/RIF	7d/wk for 126 doses (18 wk) **or** 5d/wk for 90 doses (18 wk)
				INH/RIF	2d/wk for 36 doses (18wk)
				INH/RPT	1 wk for 18 doses (18wk)
2	INH, RIF PZA, EMB	7 d/wk for 14 doses (2wks), then 2 d/wk for 12doses(6wks) **or** 5 d/wk for 10 doses (2wk), then 2 d/wk for 12 doses (6 wk)	2	INH/RIF	2d/ wk for 36 doses
				INH/RPT	1 wk for 18 doses (18wk)
3	INH, RIF PZA, EMB	3d/wk for 24 doses (8wk)	3	INH/RIF	3 wk for 54 doses (18wk)
4	INH, RIF, EMB	7 d/wk for 56 doses (8wk) **or** 5 d/wk for 40 doses (8 wk)	4	INH/RIF	7 d/wk for 217 doses (31 wk) **or** 2d/wk for 62 doses (31 wk)

Legend to Table 1. INH-isoniazid; RIF-rifampicin; RPT-rifapentine; PZA-pyrazinamide;
Note: Streptomycin (STR) efficiency is equal to that of EMB and was use as an interchangeable drug with EMB in the initial phase of treatment. Due to the increase of resistance the drug is rendered less useful. Thus, STR is not recommended to be interchangeable with EMB unless the organism is known to be susceptible to the drug or the patient is from a community in which STR resistance is unlikely. Extracted from Blumberg et al.(Blumberg et al., 2003)

Since the introduction of the DOTS strategy in the early ‘90s by the WHO, considerable progress has been made in global TB control (Sterling et al., 2003). In 1997, the estimated average treatment success rate world wide was almost 80 %. However, less than 25 % of people who are sick with TB are treated through the DOTS strategy (Bastian et al., 2000) . A total of 180 countries (including both developed and undeveloped countries) had adopted and implemented the DOTS strategy by the end of 2002 and 69 % of the global population was living in areas covered by the DOTS strategy (Blumberg et al., 2003). However, even though DOTS programs are in place, treatment success rates are very low in developed countries due to poor management of TB control programs and patient non-compliance (Lienhardt and Ogden, 2004; Bastian et al., 2003) Furthermore, the effectiveness of DOTS is facing new challenges with respect to the spread and increase of MDR-TB and the co-epidemic of TB/HIV (World Health Organization, 2003). WHO and partners have addressed these new challenges and have developed a new strategy called DOTS-Plus for the treatment of MDR-TB and its co-epidemic TB/HIV. The goal of DOTS-plus is to prevent further development and spread of MDR-TB and is a comprehensive management initiative built upon the DOTS strategy (Table 2). It is important to note that DOTS-Plus should only be implemented in areas were the DOTS strategy is in place as there can be no DOTS-plus without an effective DOTS program.

Table 2. DOTS Compared to DOTS-Plus Strategy

DOTS	DOTS-plus
• DOTS prevent emergence of drug resistant TB and MDR-TB • Make primarily use of 1st line drugs that are less expensive	• DOTS-plus design to cure MDR-TB using second line drugs. • Make use of 2nd line drugs that are more toxic and expensive, difficult to treat less effective to administrate and often poorly tolerated DOTS-plus needed in areas where MDR-TB has emerged due to previous inadequate TB control • DOTS-plus only recommended in settings where DOTS strategy is fully in place to prevent against the development of further drug resistance

2. DRUG SUSCEPTIBILITY TESTING

Drug susceptibility testing is carried out on sub-cultured bacteria after the initial positive culture is obtained for diagnosis. It usually takes 3-6 weeks to obtain the initial positive culture with an additional 3 weeks for susceptibility testing (reduced to about 15 days when using the BACTEC system) (Rastogi et al., 1989; Siddiqi et al., 1985; Snider, Jr. et al., 1981; Tarrand and Groschel, 1985). Thus, susceptibility testing is time consuming and costly, and there are numerous problems associated with the standardization of tests and the stability of the drugs in different culture media (Martin-Casabona et al., 1997; Victor et al., 1997). The slow diagnosis of drug resistance may be a major contributor to the transmission of MDR-TB (Victor et al., 2002). The WHO recommended that drug susceptibility testing is done by the proportion method on Löwenstein-Jensen medium, but other media, such as Middlebrook 7H10, 7H11, 7H12 (BACTEC460TB) and other methods, including the absolute concentration and resistance ratio methods, may also be used (World Health Organization, 2001). For the ratio method, serial dilutions are cultured on 2 control media (without the drug) and 2 test media (with two different

drug concentrations). The colonies on the different slants are counted after 21 and 40 days of growth. The proportion of resistant bacilli is calculated by comparing colony counts on drug free and drug containing media. For a resistant isolate the calculated proportion is higher and for a susceptible strain the calculated proportion is lower than the critical proportion (World Health Organization, 2001).

3. MOLECULAR MECHANISMS OF DRUG RESISTANCE

In order to control the drug resistance epidemic it is necessary to gain insight into how *M. tuberculosis* develops drug resistance. This knowledge will help us to understand how to prevent the occurrence of drug resistance as well as identifying genes associated with drug resistance of new drugs. The development of clinical drug resistance in TB is summarized in Figure 1 and is classified as acquired resistance when drug resistant mutants are selected as a result of ineffective treatment or as primary resistance when a patient is infected with a resistant strain. Mutations in the genome of *M. tuberculosis* that can confer resistance to anti-TB drugs occur spontaneously with an estimated frequency of 3.5×10^{-6} for INH and 3.1×10^{-8} for RIF. Because the chromosomal loci responsible for resistance to various drugs are not linked, the risk of a double spontaneous mutation is extremely low: 9×10^{-14} for both INH and RIF (Dooley and Simone, 1994). MDR-TB defined as resistance to at least INH and RIF will thus occur mainly in circumstances where sequential drug resistance follows sustained treatment failure. Treatment can be divided into first line and second line drugs according to the WHO TB treatment regimen and the mechanisms of these will be discussed separately.

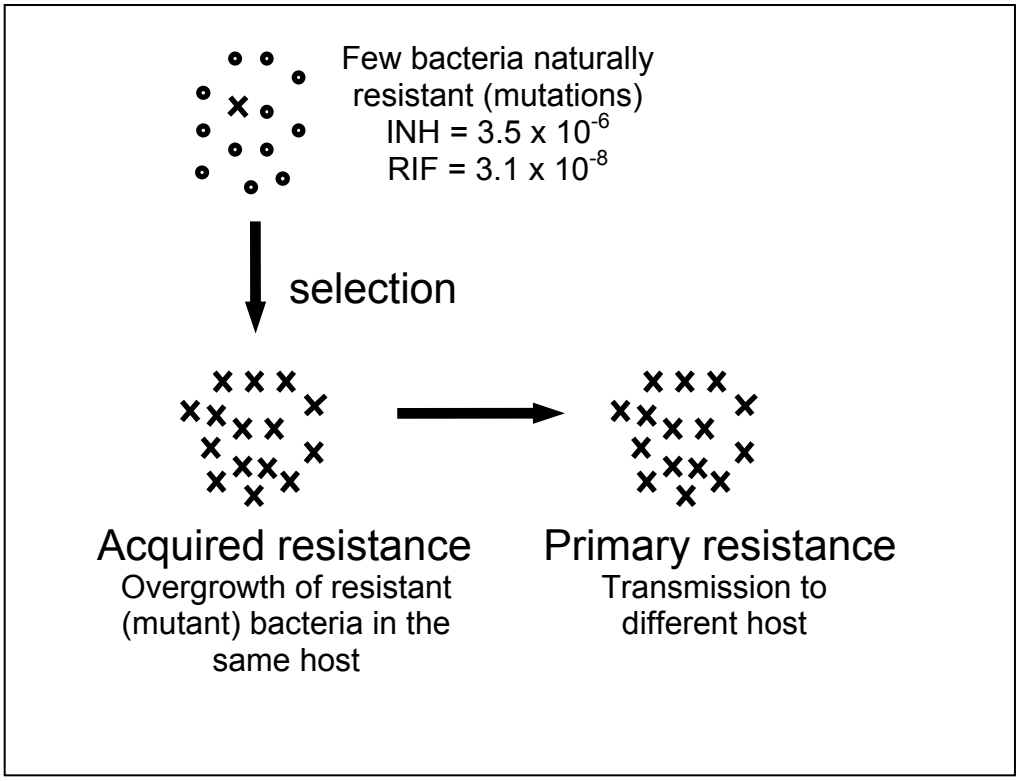

Figure 1. Acquired Resistance Develops due to Natural Selection which is a Function of Ineffective Treatment and Non-Compliance

3.1. FIRST LINE DRUGS

Any drug used in the anti-TB regiment is supposed to have an effective sterilizing activity that is capable of shortening the duration of treatment. Currently, a four-drug regiment is used consisting of INH, RIF, pyrazinamide (PZA) and ethambutol (EMB). Resistance to first line anti-TB drugs has been linked to mutations in at least 10 genes; *katG, inhA, ahpC, kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance, *embB* for EMB resistance, *pncA* for PZA resistance and *rpsL* and *rrs* for STR resistance.

3.1.1 *Isoniazid*

3.1.1.1 *KatG*

INH or isonicotinic acid hydrazide, was synthesized in the early 1900's but its anti-TB action was first detected in 1951 (Heym et al., 1999; Slayden and Barry, III, 2000; Rattan et al., 1998). INH enters the cell as a prodrug that is activated by a catalase peroxidase encoded by *katG*. The peroxidase activity of the enzyme is necessary to activate INH to a toxic substance in the bacterial cell (Zhang et al., 1992). This toxic substance subsequently affects intracellular targets such as mycolic acid biosynthesis which are an important component of the cell wall. A lack of mycolic acid synthesis eventually results in loss of cellular integrity and the bacteria die (Barry, III et al., 1998). Middlebrook et al. initially demonstrated that a loss of catalase activity can result in INH resistance (Middlebrook, 1954). Subsequently genetic studies demonstrated that transformation of INH-resistant *Mycobacterium smegmatis* and *M. tuberculosis* strains with a functional *katG* gene restored INH susceptibility and that *katG* deletions give rise to INH resistance (Zhang et al., 1992; Zhang et al., 1993). However, mutations in this gene are more frequent than deletions in clinical isolates and these can lower the activity of the enzyme. Most

mutations are found between codons 138 and 328 with the most commonly observed gene alteration being at codon 315 of the *katG* gene (Slayden and Barry, III, 2000). The Ser315Thr substitution is estimated to occur in 30-60 % of INH resistant isolates (Ramaswamy and Musser, 1998; Musser et al., 1996; Slayden and Barry, III, 2000). The *katG* 463 (CGG-CTG) (Arg-Leu) amino acid substitutions is the most common polymorphism found in the *katG* gene and is not associated with INH resistance.

3.1.1.2. *ahpC*

It has been observed that a loss of *katG* activity due to the S315T amino acid substitution is often accompanied by an increase in expression of an alkyl hydroperoxide reductase (*ahpC*) protein that is capable of detoxifying damaging organic peroxides (Sherman et al., 1996). Five different nucleotide alterations have been identified in the promoter region of the *ahpC* gene, which lead to over expression of *ahpC* and INH resistance (Ramaswamy and Musser, 1998). *AhpC* overexpression exerts a detoxifying effect on organic peroxides within the cell and protects the bacteria against oxidative damage but does not provide protection against INH. *KatG* expression can also be up regulated under conditions of oxidative stress. The correlation between polymorphic sites in the *ahpC* regulatory region with INH resistance in *M. tuberculosis* requires further examination.

3.1.1.3. *inhA*

One of the targets for activated INH is the protein encoded by the *inhA* locus. InhA is an enoyl–acyl carrier protein (ACP) reductase which is proposed to be the primary target for resistance to INH and ethionamide (ETH) (Banerjee et al., 1994). ETH, a second line drug, is a structural analog of INH that is also thought to inhibit mycolic acid biosynthesis and several studies have suggested that low-level INH resistance is correlated with resistance to ETH. Activated INH

binds to the InhA-NADH complex to form a ternary complex that results in inhibition of mycolic acid biosynthesis. Six point mutations associated with INH resistance within the structural *inhA* gene have been identified (Ile16Thr, Ile21Thr, Ile21Val, Ile47Thr, Val78Ala and Ile95Pro) (Ramaswamy and Musser, 1998; Basso and Blanchard, 1998). A Ser94Ala substitution results in a decreased binding affinity of *inhA* for NADH, resulting in mycolic acid synthesis inhibition. Although these mutations in the structural *InhA* gene are associated with INH resistance, it is not frequently reported in clinical isolates. *InhA* promoter mutations are more frequently seen and are present at positions -24(G-T), -16(A-G), or -8(T-G/A) and -15(C-T). These promoter mutations result in over expression of *inhA* leading to low level INH resistance. To date approximately 70-80 % of INH resistance in clinical isolates of *M. tuberculosis* can be attributed to mutations in the *katG* and *inhA* genes (Ramaswamy and Musser, 1998).

3.1.1.4. *kasA*

There seems to be considerable dispute within the literature as to the role of *kasA* as a possible target for INH resistance (Sherman et al., 1996). This gene encodes a β-ketoacyl-ACP synthase involved in the synthesis of mycolic acids. Mutations have been described in this gene that confers low levels of INH resistance. Genotypic analysis of the *kas*A gene reveals 4 different amino acid substitutions involving codon 66 (GAT-AAT), codon 269 (GGT-AGT), codon 312 (GGC-AGC) and codon 413 (TTC-TTA) (Ramaswamy and Musser, 1998; Mdluli et al., 1998). However, similar mutations were also found in INH susceptible isolates (Lee et al., 1999; Piatek et al., 2000). Nevertheless, the possibility of *kasA* constituting an additional resistance mechanism should not be completely excluded.

3.1.1.5 *ndh*

In 1998 another mechanism for INH resistance in *M. smegmatis* was described by Miesel et al. (Miesel et al., 1998). The *ndh* gene encodes NADH dehydrogenase that is bound to the active site of *inhA* to form the ternary complex with activated INH. Structural studies have shown that a reactive form of INH attacks the NAD (H) co-factor and generates a covalent INH-NAD adduct. Mutations in the *ndh* gene, encoding NADH dehydrogenase, cause defects in the enzymatic activity. Thus, defects in the oxidation of NADH to NAD result in NADH accumulation and NAD depletion (Lee et al., 2001). These high levels of NADH can then inhibit the binding of the INH-NAD adduct to the active site of the InhA enzyme (Rozwarski et al., 1998; Miesel et al., 1998). Prominent point mutations in the *ndh* gene at codons 110 and 268 (T110A and R268H) were detected in 9.5% of INH resistant samples. These similar mutations were not detected in the INH susceptible group (Lee et al., 2001).

3.1.2 *Rifampincin*

RIF was fist introduced in 1972 as an anti-TB drug and has excellent sterilizing activity (Rattan et al., 1998; Ramaswamy and Musser, 1998). The action of RIF in combination with PZA has allowed a shortening of routine TB treatment from 1 year to 6 months. RIF in combination with INH forms the backbone of short-course chemotherapy. It is interesting to note that mono resistance to INH is common but mono resistance to RIF is quite rare. It has thus been proposed that resistance to RIF can be used as a surrogate marker for MDR-TB as nearly 90% of RIF resistant strains are also INH resistant (Somoskovi et al., 2001). RIF interferes with transcription by the DNA-dependent RNA polymerase. RNA polymerase is composed of four different subunits (α, β, β$^{'}$ and σ) encoded by *rpoA, rpoB, rpoC* and *rpoD* genes respectively. RIF binds to the β-subunit hindering transcription and thereby killing the organism. Extensive studies on the *rpoB* gene in RIF resistant isolates of *M. tuberculosis* identified a variety of mutations and short

deletions in the gene. A total of 69 single nucleotide changes; 3 insertions, 16 deletion and 38 multiple nucleotide changes have been reported (Herrera et al., 2003). More than 95% of all missense mutations are located in a 51bp core region (rifampin resistance determining region) of the *rpoB* gene between codons 507-533 with the most common changes in codons Ser531Leu, His526Tyr and Asp516Val. These changes occur in more than 70% of RIF resistant isolates (Rattan et al., 1998; Ramaswamy and Musser, 1998; Herrera et al., 2003). Furthermore, the minimal inhibitory concentration (MIC) showed that high level of RIF resistance is associated with mutations in codon 526 and 531, whereas alterations in codon 511,516, 518 and 522 result in low level RIF resistance.

3.1.3. *Pyrazinamide*

PZA, a nicotinamide analog, was first discovered to have anti-TB activity in 1952. PZA targets an enzyme involved in fatty-acid synthesis and is responsible for killing persistent tubercle bacilli in the initial intensive phase of chemotherapy (Somoskovi et al., 2001). However, during the first two days of treatment, PZA has no bactericidal activity against rapidly growing bacilli (Zhang and Mitchison, 2003). PZA on the other hand has effective sterilizing activity and shortens the chemotherapeutic regiment from 12 to 6 months. PZA is a prodrug which is converted to its active form, pyrazinoic acid (POA) by the pyrazinamidase (PZase) encoded by *pncA*. The activity of PZA is highly specific for *M. tuberculosis,* as it has no effect on other mycobacteria. *Mycobacterium bovis* is naturally resistant to PZA due to a unique C-G point mutation in codon 169 of the *pncA* gene. PZA is only active against *M. tuberculosis* at acidic pH where POA accumulates in the cytoplasm due to an ineffective efflux pump. Accumulation of POA results in the lowering of intracellular pH to a level that inactivates a vital fatty acid synthase (Zimhony et al., 2004). Cloning and characterization of the *M. tuberculosis pncA* gene by Scorpio et al. (Scorpio and Zhang, 1996) showed that *pncA* mutations conferred PZA

resistance. Various *pncA* mutations have been identified in more than 70 % of PZA resistant clinical isolates scattered throughout the *pncA* gene but thus far no mutational hot spot has been identified (Scorpio and Zhang, 1996)·(Sreevatsan et al., 1997b; Scorpio et al., 1997). In a study from Peru it was found that 59 % of MDR patients also had M. tuberculosis resistant to PZA(Saravia et al., 2005). PZA susceptibility testing is not done routinely in many countries due to technical difficulties. Thus the extent of PZA resistance globally is largely unknown. A study done by Louw et al (Louw et al., 2006) showed that PZA resistance is common amongst drug-resistant clinical *M. tuberculosis* isolates from South Africa**.** PZA resistance was shown to be strongly associated with MDR-TB and therefore it was concluded that PZA should not be relied upon in managing patients with MDR-TB in this setting. PZA resistant isolates had diverse nucleotide changes scattered throughout the *pncA* gene. Mutations in the *pncA* gene correlate well with phenotypic resistance to PZA. However, PZA resistant isolates without *pncA* mutations were also observed suggesting that another mechanism may be involved in conferring PZA resistance in these isolates. In addition, not all mutations (e.g. $Thr_{114}Met$) were associated with PZA resistance. In summary, the complexity of PZA resistance makes the development of molecular methods for rapid diagnosis difficult.

3.1.4. *Ethambutol*

EMB, a first line drug, is used in combination with other drugs and is specific to the mycobacteria. EMB inhibits an arabinosyl transferase (*embB*) involved in cell wall biosynthesis (Takayama and Kilburn, 1989). Telenti et al. (Telenti et al., 1997) identified 3 genes, designated *emb*CAB, that encode homologous arabinosyl transferase enzymes involved in EMB resistance. Various studies have identified five mutations in codon 306 [(ATG-GTG), (ATG-CTG), (ATG-ATA), (ATG-ATC) and (ATG-ATT)] which result in three different amino acid substitutions (Val, Leu and Ile) in EMB-resistant isolates (Lee et al., 2002; Sreevatsan et al., 1997c;

Mokrousov et al., 2002b; Ramaswamy et al., 2000). These five mutations are associated with 70-90 % of all EMB resistant isolates (Ramaswamy and Musser, 1998). Missense mutations were identified in three additional codons: Phe285leu, Phe330Val and Thr630Ile in EMB resistant isolates. MIC's were generally higher for strains with Met306Leu, Met306Val, Phe330Val and Thr630Ile substitutions than those organisms with Met306Ile substitutions. Mutations outside of codon 306 are present but quite rare. In a study recently done by Johnson *et. al*. (Johnson et al., 2006) it was shown that genotypic analysis identified mutations at codon 306 of the *embB* gene rendering resistance to EMB. However, routine phenotypic analysis failed to identify EMB resistance in 91.4 % of resistant isolates in this setting and confirm the difficulty of EMB phenotypic testing. The inability to accurately detect true EMB resistance by the culture based method has a negative impact on the TB control program. Molecular–based methods offer a rapid diagnosis of EMB resistance and could thereby benefit the management of TB patents within days. However a number of EMB phenotypic resistant isolates (about 30 %) still lack an identified mutation in *embB*. There is therefore a need to fully understand the mechanism of EMB resistance in clinical isolates.

3.1.5. Streptomycin

STR, an aminocyclitol glycoside, is an alternative first line anti-TB drug recommended by the WHO (Cooksey et al., 1996). STR is therefore used in the retreatment of TB cases together with the four drug regimen that includes INH, RIF, PZA and EMB (Brzostek et al., 2004). The effect of STR has been demonstrated to take place at the ribosomal level (Telenti et al., 1993). STR interacts with the 16S rRNA and S12 ribosomal protein (*rrs* and *rpsL)* (Escalante et al., 1998; Finken et al., 1993; Sreevatsan et al., 1996; Abbadi et al., 2001), inducing ribosomal changes, which cause misreading of the mRNA and inhibition of protein synthesis. Although STR is a recommended anti-TB drug, is it less effective against *M. tuberculosis* than INH and RIF. Point

mutations in STR resistant isolates have been reported in *rrs* and *rpsL* genes in 65-67 % of STR resistant isolates (Ramaswamy and Musser, 1998). In the *rrs* gene a C-T transition at positions 491, 512 and 516, and a A-C/T transversion at position 513 were observed in the highly conserved 530 loop. The 530 loop region is part of the aminoacyl–tRNA binding site and is involved in the decoding process (Carter et al., 2000). The C-T transition at codon 491 is not responsible for resistance to STR as it occurs in both STR resistant and susceptible isolates but is strongly associated with the global spread of *M. tuberculosis* with a Western Cape F11 genotype (van Rie et al., 2001; Victor et al., 2001). Other mutations in the 915 loop [903 (C-A/G) and 904 (A-G)] have also been reported to have an association with STR resistance (Carter et al., 2000). Mutations in the *rpsL* gene at codon 43 (AAG-AGG/ACG) (Lys-Arg/Thr) and codon 88 (AAG-AGG/CAG) (Lys-Arg/Gln) are associated with STR resistance. MIC analysis of STR resistant isolates indicate that amino acid replacements in the *rpsL* genes correlate with a high level of resistance, whereas mutations in the *rrs* gene correlate with an intermediate level of resistance (Cooksey et al., 1996) (Meier et al., 1996). In addition, it has been suggested that low levels of STR resistance are also associated with altered cell permeability or rare mutations which lie outside of the *rrs* and *rpsL* genes.

3.2. SECOND LINE DRUGS USED IN TB TREATMENT

According to the WHO the following drugs can be classified as second line drugs: aminoglycosides (kanamycin and amikacin) polypeptides (capreomycin, viomycin and enviomycin), fluoroquinolones (ofloxacin, ciprofloxacin, and gatifloxacin), D-cycloserine and thionamides (ethionamide and prothionamide) (World Health Organization, 2001). Unfortunately, second-line drugs are inherently more toxic and less effective than first-line drugs (World Health Organization, 2001). Second line drugs are mostly used in the treatment of MDR-TB and as a result prolong the total treatment time from 6 to 9 months (Cheng et al., 2004). The current understanding of molecular mechanisms associated with resistance to second line drugs

are summarized in Table 3. The phenotypic methods to detect resistance to second line drugs are less well established and the molecular mechanisms of resistance are also less defined.

3.2.1 *Fluoroquinolones*

Ciproflaxin (CIP) and ofloxacin (OFL) are the two fluoroquinolones (FQs) used as second-line drugs in MDR-TB treatment (World Health Organization, 2001). The quinolones target and inactivate DNA gyrase, a type II DNA topoisomerase (Cynamon and Sklaney, 2003; Ginsburg et al., 2003; Rattan et al., 1998). DNA gyrase is encoded by *gyrA* and *gyrB* (Rattan et al., 1998; Takiff et al., 1994) and introduces negative supercoils in closed circular DNA molecules (Rattan et al., 1998; Ramaswamy and Musser, 1998). The quinolone resistance-determining region (QRDR) is a conserved region in the *gyrA* (320 bp) and *gyrB* (375 bp) genes (Ginsburg et al., 2003) which is the point of interaction of FQ and gyrase (Ginsburg et al., 2003). Missense mutations in codon 90, 91, and 94 of *gyrA* are associated with resistance to FQs (Takiff et al., 1994; Xu et al., 1996). A 16-fold increase in resistance was observed for isolates with a Ala90Val substitution, a 30-fold increase for Asp94Asn or His94Tyr and a 60-fold increase for Asp94Gly (Xu et al., 1996). A polymorphism at *gyrA* codon 95 is not associated with FQ resistance, and is used, with the *katG*463 polymorphism, to classify *M. tuberculosis* into 3 phylogenetic groups (Sreevatsan et al., 1997a).

Table 3. Properties of Resistance to Various Second-line Anti-TB Drugs

Second-line drug	Gene locus	Gene product	Known polymorphism	Most frequently mutated codons associated with resistance	MIC [a] (µg/ml)	Methods for genotypic detection of resistance	Reference
Fluoroquinolones Ofloxacin Cipromycin	*gyr*A	DNA gyrase	*gyrA* 95	*gyr* 90, 91, 94	OFL: 1.0-2.0 CIP: 0.5-4.0	Cloning & expression PCR-SSCP[b] DNA sequencing	(Takiff et al., 1994; Pletz et al., 2004; Rattan et al., 1998; Ginsburg et al., 2003; Cheng et al., 2004)
Aminoglycosides Kanamycin Amikacin	*rrs*	16 S rRNA		*rrs* 1400	KAN: > 200 AMI: > 256	IS*6110*-RFLP PCR-RFLP[c]	(Ramaswamy and Musser, 1998; Takiff et al., 1994; Taniguchi et al., 1997; Suzuki et al., 1998; Ramaswamy et al., 2004; Vannelli et al., 2002)
Ethionamide	*inhA* *ethA* *ethR*	enoyl-ACP reductase Flavin monooxygenase Transcriptional repressor		*inhA*21, 94, 44	≥ 25 ≥200	DNA Sequencing	(Baulard et al., 2000; Morlock et al., 2003; Cynamon and Sklaney, 2003)
D- cycloserine	*alr* *ddl*	D-alanine racemase D-alanine: D-alanine ligase			≥ 300	Cloning & expression DNA Sequencing	(Caceres et al., 1997; Feng and Barletta, 2003)
Viomycin	*rrs*	16S rRNA			20	DNA Sequencing	(Ramaswamy and Musser, 1998; Taniguchi et al., 1997; Suzuki et al., 1995)

Legend to table 3. a) Minimum inhibitory concentration. b) Polymerase chain reaction- single strand conformation polymorphism. c) Polymerase chain reaction-Restriction fragment length polymorphism.

3.2.2. *Aminoglycosides*

Kanamycin (KAN) and Aminokacin (AMI) are aminoglycosides which inhibit protein synthesis and thus cannot be used against dormant *M. tuberculosis*. Aminoglycosides bind to bacterial ribosomes and disturb the elongation of the peptide chain in the bacteria. Mutations in the *rrs* gene encoding for 16s rRNA are associated with resistance to KAN and AMI. Nucleotide changes at positions 1400, 1401 and 1483 of the *rrs* gene have been found to be specifically associated with KAN resistance (Suzuki et al., 1998). An A→G change at codon 1400 in the *rrs* gene showed resistance to KAN of MICs more that 200 μg/ml (Taniguchi et al., 1997; Suzuki et al., 1998).

32.3. *Ethionamide*

Ethionamide (ETH) is an important drug in the treatment of MDR-TB, and is mechanistically and structurally analogous to INH. Like INH, ETH is also thought to be a prodrug that is activated by bacterial metabolism. The activated drug then disrupts cell wall biosynthesis by inhibiting mycolic acid synthesis. Mutations in the promoter of the *inhA* gene are associated with resistance to INH and ETH (Morlock et al., 2003). *EthA* catalyses a two step activation of ETH and gene alterations leading to reduced EthA activity lead to ETH resistance (Engohang-Ndong et al., 2004; Morlock et al., 2003; Vannelli et al., 2002). The expression of *ethA* is under the control of the neighbouring *ethR* gene encoding a repressor. *EthR* negatively regulates the expression of *ethA*, by binding upstream of *ethA* to suppress *ethA* expression (Engohang-Ndong et al., 2004).

3.10 *D-Cycloserine*

D-cycloserine (DCS) is a cyclic analog of D-alanine which is one of the central molecules of the cross linking step of peptidoglycan assembly (Ramaswamy and Musser, 1998; Feng and Barletta, 2003; David, 2001; Caceres et al., 1997). DCS inhibits cell wall synthesis by competing with D-Alanine for the enzymes D-alanyl-D-alanine synthetase (Ddl) and D-alanine racemase (Alr) and also inhibiting the synthesis of these proteins. Over expression of *alr* cause DCS resistance. A G→T transversion in the *alr* promoter may lead to the overexpression of *alr* (Feng and Barletta, 2003; Ramaswamy and Musser, 1998).

3.11 *Peptides*

Viomycin (VIO) and capreomycin (CAP) are basic peptide antibiotics that inhibit prokaryotic protein synthesis and are used as second-line anti-TB drugs. Earlier studies have shown that resistance to VIO in *M. smegmatis* is caused by alterations in the 30S or 50S ribosomal subunits (Taniguchi et al., 1997). Mutations in the *rrs* gene that encodes the 16S rRNA is associated with resistance to VIO and CAP, specifically a G→A or G→T nucleotide change at codon 1473 (Taniguchi et al., 1997).

4. MOLECULAR METHODS TO PREDICT DRUG RESISTANCE

M. tuberculosis is a very slow growing organism and the use of molecular methods for the identification of mutations in resistance-causing genes may offer a means to rapidly screen *M. tuberculosis* isolates for antibiotic resistance. Mutation screening methods are fast and include methods such as DNA sequencing, probe based hybridization methods, PCR-RFLP, single–strand conformation polymorphism (SSCP), heteroduplex analysis (HA), molecular beacons and ARMS-PCR (Victor et al., 2002). The end results for each of these methods are given as a combined photo in Figure 2.

4.1. *Sequencing*

PCR amplification followed by DNA sequencing is the most widely used technique to identify mutations associated with drug resistance in TB (Victor et al., 2002). This technique is costly and require expertise, which make it unpractical for use in routine laboratories, especially in developing countries, where simple, cost effective drug susceptibility testing is needed (Victor et al., 2002).

4.2. *Probe-Based Hybridization Methods*

In these assays, amplified PCR products of genes known to confer drug resistance are hybridized to an allele-specific labeled probe that is complementary to the wild type or mutant sequence of the gene. This can then be visualized by autoradiography, enhanced chemiluminescence, alkaline phosphatase or other detection systems. These methods include the Dot-blot and Line blot essays and the commercially available INNO-LIPA RIF-TB test (Innogenetics, Belgium) (Victor et al., 1999; Mokrousov et al., 2004).

4.3. *PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)*

Mutations associated with resistance can be identified by digestion of amplified PCR products with a restriction enzyme that cuts at the specific polymorphic DNA sequence followed by gel electrophoresis. Since not all mutations result in the gain or loss of a restriction site, general use of RFLP to screen for mutations associated with drug resistance
is limited (Victor et al., 2002).

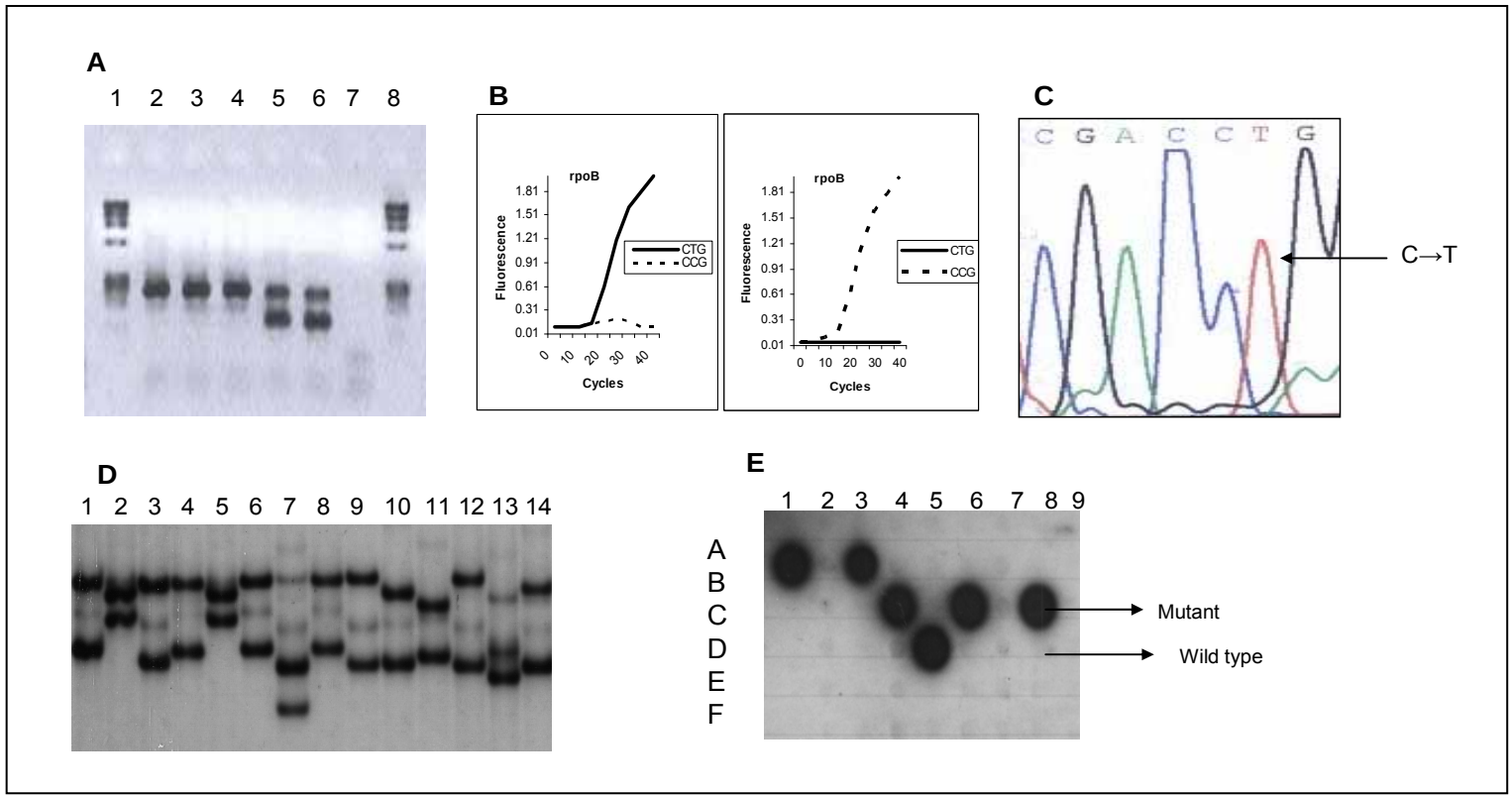

Figure 2: Molecular Methods for Detecting Gene Mutations Associated with Resistance to Anti-TB Drugs

Legend to figure 2. A-E are typical examples of final results obtained by PCR-based mutation screening methods. The DNA template can be pure DNA extracted from a culture, sputum or crude DNA templates prepared from culture or sputum. In all the examples PCR amplification of the DNA is followed by the different mutation detection methods (A-E).
A: ARMS-PCR analysis of *embB* gene. Lane 1 and 8 = Molecular marker and Samples 5 and 6 are mutants. B: Molecular beacon genotyping of the *rpoB* gene showing a T→C mutation. C: Sequence analysis showed a C→T mutation at nt161 in the *pncA* gene D: SSCP analysis with samples having different mutations (visualized as different band mobility shifts) in the of *rpoB* gene. Heteroduplex analysis would give similar band mobility shifts on the gel. E: DOT-BLOT analysis with *rpsL 43* mutant probe showing wild type (lane D8) and mutant (lane C8) controls and mutant (STR resistant) clinical isolates in B1, B3, C4, D5, C6 and C8.

4.4. *Single Stranded Conformation Polymorphism Analysis (SSCP)*

SSCP is a gel based method that can detect short stretches of DNA approximately 175-250bp in size. Small changes in a nucleotide sequence result in differences in secondary structures as well as measurable DNA mobility shifts that are detected on a non-denaturing polyacrylamide gel. To date various studies have applied PCR-SSCP to identify mutational changes associated with drug resistance in *M. tuberculosis* for frontline drugs like, RIF and INH (Kim et al., 2004; Cardoso et al., 2004; Fang et al., 1999; Heym et al., 1995; Pretorius et al., 1995). However, PCR-SSCP analysis has been found to be technically demanding and not sufficiently sensitive. Furthermore SSCP conditions must be carefully evaluated since not all mutations will be detected under the same conditions.

4.5 *Heteroduplex Analysis (HA)*

HA depends on the conformation of duplex DNA when analyzed in native gels. Heteroduplexes are formed when PCR amplification products from known wild type and unknown mutant sequences are heated and re-annealed. The DNA strand will form a mismatched heteroduplex if there is a sequence difference between the strands of the wild type and tested DNA. These heteroduplexes have an altered electrophoretic mobility when compared to homoduplexes, since the mismatches tend to retard the migration of DNA during electrophoresis. There are two types of heterodoplexes. The "bubble" type is formed between DNA fragments with single base differences and the bulge type is formed when there are deletions or insertions present within the two fragments. Recently, temperature mediated HA has been applied to the detection of mutations associated with mutations in *rpoB, katG, rpsL, embB* and *pncA* genes (Mohamed et al., 2004; Cooksey et al., 2002). Neither HA nor the SSCP analysis are 100% sensitive although Rosetti et al. found that HA detected more mutants (Nataraj et al., 1999). However, HA has

certain disadvantages in that it has been found to be insensitive to G-C rich regions and is very time consuming (Nataraj et al., 1999).

4.6. *Molecular Beacons*

Molecular beacons are single-stranded oligonucleotide hybridization probes which can be used as amplicon detector probes in diagnostic assays. A beacon consists of a stem-loop structure in which the stem contains a fluorophore on one arm and a quencher on the other end of the arm. The loop contains the probe which is complementary to the target DNA. If the molecular beacon is free in a solution it will not fluoresce, because the stem places the fluorophore so close to the non-fluorescent quencher that they transiently share electrons, eliminating the ability of the fluorophore to fluoresce. However, in the presence of complementary target DNA the probe undergoes a conformational change that enables them to fluoresce brightly. Different colored fluorophores (different primers) can be used simultaneously to detect multiple targets (each target will give a different color) in the same reaction. Molecular beacons are very specific and can discriminate between single nucleotide substitutions. Thus they are ideally suited for genotyping and have been used in the detection of drug resistance in *M. tuberculosis* (El Hajj et al., 2001; Piatek et al., 2000; Piatek et al., 1998).

4.7. *Amplification Refractory Mutation System (ARMS)-PCR*

ARMS also known as allelic specific PCR (ASPCR) or PCR amplification of specific alleles (PASA) is a well established technique used for the detection of any point mutation or small deletions (Newton et al., 1989). ARMS-PCR, is usually a multiplex reaction where three (or more) primers are used to amplify the same region simultaneously. One of the three primers is specific for the mutant allele and will work with a common primer during amplification. The mismatch is usually located at or near the 3' end of the primer. The third primer will work with

the same common primer to generate an amplified fragment which is larger than the fragment from the mutant allele primer - this serves as an internal control for amplification. Amplification is detected by gel electrophoresis and the genotypic classification is determined by assessing which amplification products are present. An amplification product should always be present in the larger internal control amplified fragment; if this is the case then the absence or presence of the smaller product will indicate the presence or absence of a mutant allele. This technique has successfully been used for the detection of mutations associated with RIF resistance in *M. tuberculosis* (Fan et al., 2003). Figure 2A indicates how the amplified products in the multiplex reaction are distinguished on a gel.

5. APPLICATIONS

One of the major advantages of PCR based methods is the speed by which the result can be obtained (Siddiqi et al., 1985; Snider, Jr. et al., 1981; Tarrand and Groschel, 1985). It is envisaged that molecular techniques may be important adjuncts to traditional culture based procedures to rapidly screen for drug resistance. Prospective analysis and intervention to prevent transmission may be particularly helpful in areas with ongoing transmission of drug resistant strains as reported previously (van Rie et al., 1999). In addition, molecular prediction may also be useful in drug surveillance studies to further improve the confidence limit of the data in these studies if this test is performed on a subset of the samples. Enhanced efforts are necessary to better understand the molecular mechanisms of resistance to second line anti-TB drugs in clinical isolates. However, implementation for both rapid diagnosis and surveillance requires proper quality control guidelines and controls, which is currently not in place yet for molecular prediction of drug resistance in TB. Although molecular methods are more rapid, and can be done directly from a clinical sample there are important limitations when compared to conventional phenotypic methods. These include a lack of sensitivity since not all molecular

mechanisms leading to drug resistance are known, therefore not all resistant isolates will be detected. Molecular methods may also predict resistance genotypes that are expressed at levels that may not clinically be relevant (Victor et al., 2002).

Transmission and Epidemic Drug Resistant Strains

There is much debate about the relative contribution of acquired and primary resistance to the burden of drug resistant TB in different communities. This controversy focuses on whether MDR strains are transmissible or whether the mutations that confer drug resistance also impair the reproductive function of the organism (fitness of the strain). Evidence that MDR strains have the potential to transmission comes from a series of MDR-TB outbreaks that have been reported over the past decade. These have been identified in hospitals (Fischl et al., 1992; Edlin et al., 1992; Bifani et al., 1996; Cooksey et al., 1996), amongst health care workers (Beck-Sague et al., 1992; Pearson et al., 1992; Jereb et al., 1995) and in prisons (Valway et al., 1994) and have focused attention on MDR-TB as a major public health issue. Application of molecular epidemiological methods was central to the identification and description of all these outbreaks.

The most extensive MDR-TB outbreak reported to date occurred in 267 patients from New York, who were infected by Beijing/W genotype (Frieden et al., 1996). This cluster of cases included drug resistant isolates that were resistant to all first-line anti-TB drugs. The authors speculate that the delay in diagnosis and administering appropriate therapy resulted in prolonging infectiousness and placed healthcare workers and other hospital residents (or contacts) at risk of infection for nosocomial infection. This difficult-to-treat strain has subsequently disseminated to other US cities and Paris and the authors showed by using molecular methods, how this initially fully drug susceptible strain clonally expanded to result in a MDR phenotype by sequential acquisition of resistance conferring mutations in several genes

(Bifani et al., 1996). Since then, the drug resistant Beijing/W genotype has been the focus of extensive investigations and Beijing drug resistant and susceptible genotypes have been found to be widely spread throughout the world (Glynn et al., 2002), including in South Africa (van Rie et al., 1999) and Russia (Mokrousov et al., 2002a). Beijing/W genotypes can be identified by their characteristic multi-banded IS*6110* restriction fragment-length polymorphism (RFLP) patterns, a specific spoligotype pattern characterized by the presence of spoligotype spacers 35-43 (Bifani et al., 2002) and resistance conferring gene mutations. Although these data led many to propose that Beijing/W strains behaved differently from other strains, more recent work suggests that MDR outbreaks are not limited to the Beijing/W genotype. Smaller outbreaks involving other MDR-TB genotypes have been reported in other settings such as the Czech Republic, Portugal and Norway (Kubin et al., 1999; Portugal et al., 1999). However, since much of the MDR burden falls in developing countries in which routine surveillance does not usually include molecular fingerprinting, little is known about the characteristics of circulating drug resistant strains in much of the world. It is therefore possible that there are other MDR strains, as widespread as Beijing/W, which have not been recognized and reported as such.

FUTURE

Enhanced efforts are necessary to better understand the molecular mechanisms of resistance in second line anti-TB drugs in clinical isolates. The next generation of molecular methods for the prediction of drug resistance in *M. tuberculosis* will possibly consists of matrix hybridization formats such as DNA oligonucleotide arrays on slides or silicon micron chips (Castellino, 1997; Vernet et al., 2004), particularly if these systems can be fully automated and re-used. This may be particularly useful for mutations in the *rpoB* gene, which can serve as a marker for MDR-TB (Watterson et al., 1998) and also for the multiple loci that are involved in INH resistance (Table

1). Selection of a limited number of target mutations which enable the detection of the majority of drug resistance (van Rie et al., 2001) would be useful in this strategy. It is essential that developments for new techniques must consider the fact that the majority of drug resistant cases occur in resource-poor countries (Raviglione et al., 1995) and therefore the methodologies must not only be cheap but also robust.

There are other rapid methods which do not depend on the detection of mutations to predict drug resistance. One promising method is phage amplification technology in which mycobacteriophages (bacteriophages specific for mycobacteria) are used as an indicator of the presence of viable *M. tuberculosis* in a clinical specimen (Albert et al., 2002; Eltringham et al., 1999; McNerney et al., 2000). The phage assay can also be adapted for the detection of drug resistance.

Application of rapid methods to break chains of ongoing transmission of drug resistant TB will increasingly become important as recent mathematical modeling indicate that the burden of MDR-TB cannot be contained in the absence of specific efforts to limit transmission (Cohen and Murray, 2004; Blower and Chou, 2004). This may include rapid detection of drug resistance by molecular methods.

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Chapter 3

Routine drug susceptibility testing using molecular techniques enhances diagnosis in a high incidence TB community.

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Sample preparation
PCR, Dot-Blot analysis and Sequencing
Data analysis and interpretation
Writing and editing manuscript

ABSTRACT

Sputum samples were collected from tuberculosis (TB) patients in a high TB incidence area in the Western Cape, South Africa. The aim of this study was to evaluate the performance and time to diagnosis of a genotypic drug susceptibility testing (DST) method. During June 2000 and November 2003, a total of 1540 samples were sent for DST to the national health laboratory services, and of those, a phenotypic DST result was obtained for 1373 samples whereas a genotypic DST result was obtained for 1301 of 1540 samples. Performance-based calculations were done on 1244 samples for which both a phenotypic and genotypic result were available. The reproducibility of the genotypic and phenotypic DST methods was 97 % and 95 %, respectively. The sensitivity and specificity of the genotypic DST method was 68 % and 99 % for Isoniazid and 87 % and 99 % for Rifampicin, respectively. Smear gradation was found to influence the performance of the genotypic DST method. The genotypic DST method gave accurate DST results for 75 % of the samples within 20 days (range, 15-25), whereas the phenotypic DST results were only available for 75 % of the samples after 38 days (range, 26-115) ($p<0.001$). This study showed that the genotypic DST could improve TB control by rapid diagnosis of drug resistant tuberculosis, thereby decreasing the risk of transmission.

INTRODUCTION

In resource poor countries, the World Health Organization (WHO) guidelines recommend the diagnosis of tuberculosis (TB) by smear microscopy in all new TB cases and smear microscopy, culture and drug susceptibility testing (DST) in re-treatment cases (18). If a new case fails to convert after 2 to 3 months of first line therapy, culture and DST is requested. Routine phenotypic DST methods are culture based and is initially done to detect isoniazid (INH) and rifampicin (RIF) resistance. If resistance to INH and RIF is found, DST for ethambutol (EMB) is requested. DST usually takes between 3-6 weeks, resulting in long diagnostic delays. These delays are further exacerbated in new cases with primary drug resistance, given that DST is only initiated after 2 to 3 months of first line therapy. Such long delays and the administration of inappropriate therapy during the delay period may lead to the further acquisition of drug resistance, as well as the dissemination of drug resistant strains through transmission. Thus, to improve the outcome and prevent transmission of drug resistant TB, robust and effective alternative diagnostic tests are required that will enable the identification of drug resistant TB (DR-TB) within a few days after sputum collection.

Resistance to first line anti-TB drugs develops through the sequential accumulation of mutations in genes targeted by the different drugs. To date, 11 genes have been linked to resistance to the first line anti-TB drugs (7,15); *katG, inhA* ,*inhA* promoter, *ahpC, kasA* and *ndh* for INH resistance; *rpoB* for RIF resistance *embB* for EMB resistance, *pncA* for pyrazinimide (PZA) resistance and *rpsL* and *rrs* for streptomycin (STR) resistance (8,16). Mutations in specific codons can therefore be used to rapidly detect drug resistance, since drug susceptible samples lack the corresponding gene mutation. In the last few years this concept has led to considerable progress in the development of screening tools for the detection of DR-TB. The first paper on mutation detection for drug resistant TB was published in 1993 (11) and since then, numerous

markers and molecular methods have been described to detect drug resistant gene mutations. These methods include polymerase chain reaction (PCR) or other nucleic acid amplification methods followed by DNA sequencing, probe methods, PCR-restriction fragment length polymorphism (PCR-RFLP), single–strand conformation polymorphism (SSCP), heteroduplex analysis (HA), molecular beacons and ARMS-PCR (3,4,7,9,11,14,17). Application of these molecular tools proved to be rapid and effective in low burden settings.

The aim of this study was to compare the performance (sensitivity, specificity and reproducibility) and time to diagnosis of a molecular genotyping DST method to the "gold standard" in order to detect INH and RIF resistance in high incidence TB communities.

MATERIALS AND METHODS.

Study Setting.

TB cases from the Boland, Overberg, South Cape and Karoo region were treated according to the National Tuberculosis programme (NTP) of South Africa. Sputum samples were collected from suspect TB cases attending 9 primary health care clinics and a TB referral hospital during the period June 2000 and November 2003 and sent to the National Health Laboratory services (NHLS) for TB diagnosis by smear microscopy. Sputum from re-treatment and new cases who failed to convert after 2 months of intensive phase anti-TB treatment was subjected to culture and DST.

Phenotypic DST.

Sputum samples were decontaminated with a mixture of sodium hydroxide, sodium citrate and N-acetyl-L-cysteine and neutralized with sodium phosphate (1). At this point each sample was

divided into two equal parts. Part 1 was sent to Division of Molecular Biology and Human Genetics, University of Stellenbosch for genotypic DST. Part 2 was used at NHLS for routine microscopy, culture and phenotypic DST analysis. Cultures were grown in BACTEC 12B medium (Becton Dickinson, Maryland, USA) with PANTA and growth of *Mycobacteria* was confirmed by Ziehl-Neelsen (ZN) staining. All ZN positive samples were then subjected to a niacin test to confirm the presence of *Mycobacterium tuberculosis* complex. If the culture was ZN negative after the 35 days of incubation, a final result of negative was reported. DST testing was only done on niacin positive cultures using the proportion method on Löwenstein-Jensen medium containing critical concentrations of 0.2 µg for isoniazid (INH) or 30 µg for rifampicin (RIF). Resistance was defined as 1 % or more bacterial growth in comparison with a control in which the tested drug was absent.

Genotypic DST.

Decontaminated sputum samples (part 1) were collected (usually 20-25 samples) weekly from the NHLS for genotypic DST. Each sample was inoculated into 1 ml of BACTEC 12B media, which contained 0.1 ml PANTA–plus (Becton Dickinson, Maryland, USA), and incubated in a 50 ml Falcon tube (Greiner Bio-One, Germany) at 37 °C for 5 days. A negative control containing water was inoculated after every 5th sputum sample in a batch to control for possible cross-contamination. After short term culture, the bacteria were pelleted by centrifugation at 10 000 x g for 20 minutes and the supernatant was aspirated into 150 µl. A crude DNA template was prepared by boiling each sample at 100 °C for 20 minutes. These crude DNA templates were used to PCR amplify chromosomal domains containing mutations associated with INH (*katg315*, *inhA-15* promoter) and RIF (*rpoB531*, *rpoB526*, *rpoB516)* resistance as described previously (13,14). PCR amplification of each batch was performed in 4 separate rooms to minimize amplicon contamination. A water control, DNA from genotypically characterized drug

resistant and susceptible controls, and the negative control prepared during short term culture were incorporated with each PCR amplification reaction (13). Amplicons generated were visualized after electrophoretic fractionation in 1.5 % agarose gel in 1 x TBE buffer and staining with ethidium bromide. Ten microliters of the PCR amplified product was then denatured by the addition of 190 µL of denaturing buffer containing 0.4 N NaOH and 25 mM EDTA and then spotted onto a Hybond-N^+ membrane using a dot-blot apparatus (Bio-Rad). Hybridization was done using ^{32}P labelled oligonucleotide probes which were directed towards mutations in drug resistant genes most frequently found in the local isolates and in other parts of the world as described previously (13,14). Results were scored based on discrimination between genotypically well-characterized controls on the blot. DNA sequencing was done on selected PCR amplified products using the ABI PRISM DNA model 3130xl sequencer (Applied Biosystems, Foster City,CA 94404, USA).

Data Analysis

The statistical program Statistica 7.1 (stasoft, Inc (2005) (www.statsoft.com)) was used to calculate the performance (sensitivity and specificity) of the molecular method at a confidence interval of 95 %. Reproducibility of the phenotyping and the probe method was calculated by comparing follow-up DST results from the same patient, while the accuracy of the genotypic DST method was compared to the results obtained by DNA sequencing. The time for reporting DST results was calculated by subtracting the date the phenotypic or genotypic DST result was available from the date the sputum samples were received. The time for reporting DST for samples that had lost viability during phenotypic DST were calculated from the first time that specific isolates was received for DST and reported lost viability until a second or third sample was requested and the DST results was available. The time for reporting genotypic DST results

was calculated from the day a specific batch of samples was received at the University of Stellenbosch until the genotypic results were available.

RESULTS

Between June 2000 and November 2003, 3038 sputum samples were collected from patients' resident in the study setting and subjected to culture (Fig 1). DST was requested and performed on 1540 of these cultures, of which 1373 (89 %) cultures gave a DST result. The remaining 167 cultures were either contaminated lost viability or were found to be Non-Tuberculosis Mycobacteria (NTM). Phenotypic INH and RIF resistance was identified in 279 (20 %) and 177 (13 %) of the cultures, respectively. Resistance to both INH and RIF (MDR-TB) was identified in 165 (12 %) of the cultures. The reproducibility of the routine phenotypic DST method was 95 % (kappa value 0.8) when the DST results of sequential follow-up samples were compared.

Genotyping was done on all of the samples submitted for culture and DST testing in order to determine the efficiency of mutation detection by the genotyping method. A definitive genotypic DST result was obtained for 1301 of the samples. The remaining 239 failed to produce a product after PCR amplification. Genotypic DST showed that 188 (14 %) of the samples were INH resistant, 155 (12 %) were RIF resistant and 117 (9 %) of the samples were MDR-TB. The reproducibility of the genotyping DST method was 97 % (kappa value 0.9) when sequential follow-up samples were compared. Genotypic DST had an accuracy of 97 % (kappa value 0.9) when compared to DNA sequencing. Phenotypic DST results were available for 1244 (95.6 %) of the PCR positive samples (Table 1).

Fig 1. Flow diagram showing phenotypic and genotypic DST of sputum samples.

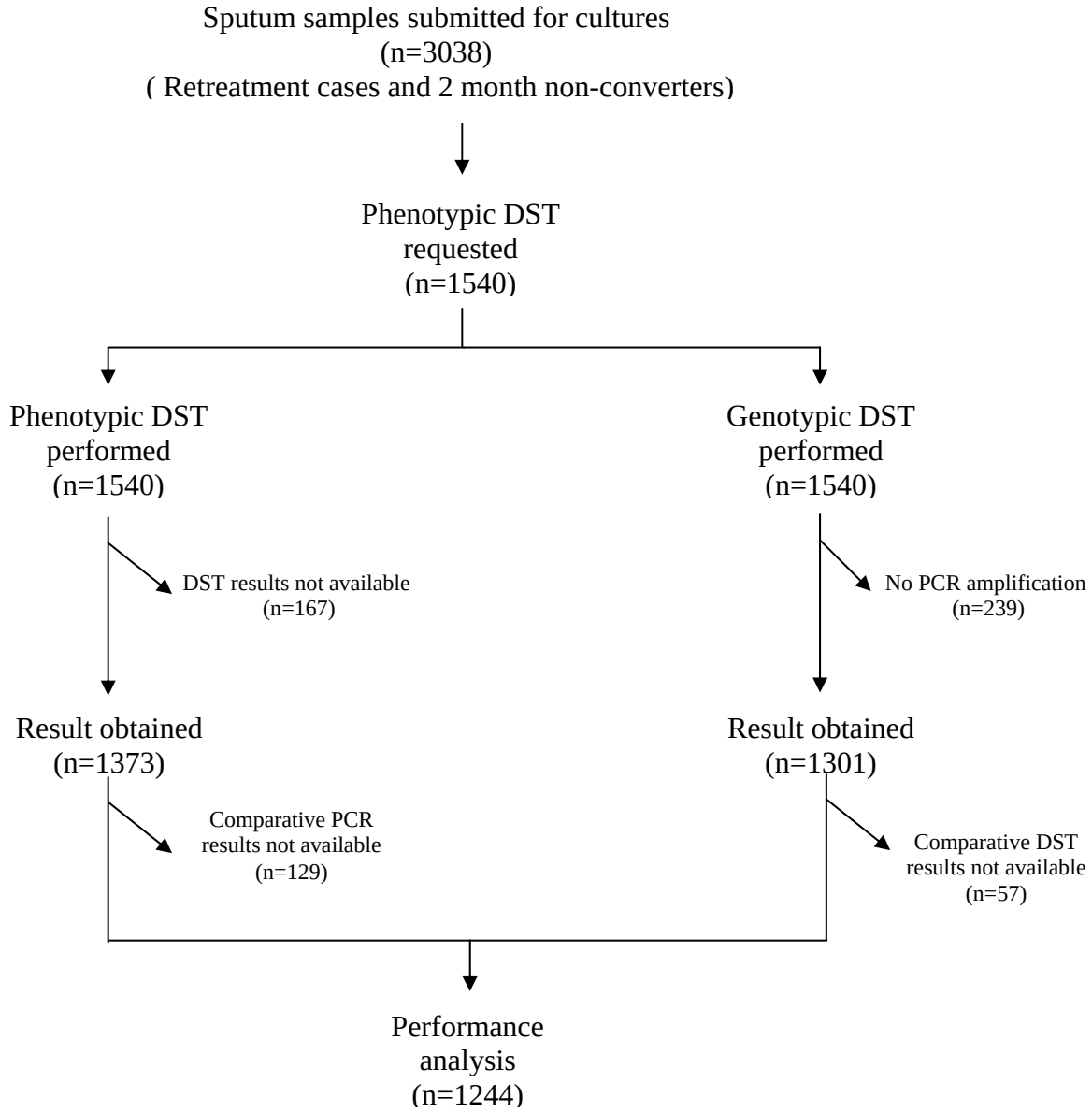

Table 1: Comparative analysis of phenotypic and genotypic DST.

	n=1244	
	Phenotype	Genotype
INH^S	1000 (80 %)	1065 (86 %)
INH^R	244 (20 %)	28 *inhA* prom (2 %) 151 *katG* (12 %)
RIF^S	1095 (88 %)	1102 (89 %)
RIF^R	149 (12 %)	142 (11 %)
MDR	141 (11 %)	108 (9 %)

Legend to Table 1: S-susceptible; R-resistant

Performance was calculated on 1244 samples for which both a phenotypic and genotypic DST results was available. Performance calculations comparing the genotyping DST method to the gold standard phenotypic DST method showed a sensitivity and specificity for INH resistance of 68 % and 99 %, respectively. The positive and negative predictive values were 92 % and 93 %, respectively. The sensitivity and specificity for genotypic RIF resistant testing was 87 % and 99 % respectively, while the positive and negative predictive values were 92 % and 98 % respectively. The sensitivity and specificity for genotypic MDR testing were 72 % and 99 %, respectively, while the positive and negative predictive values were 94 % and 97 % respectively.

To determine whether smear gradation strongly influenced the predictive value of the genotyping method, PCR amplification ability was compared to the sample bacterial load. A positive correlation between the bacterial load and amplification was observed, as 6 % (37/311) of the high (smear 2+ and 3+) and 12 % (51/881) of the low (smear 1+) bacterial load samples were not amplifiable. Smear results were not available for 181 sputum samples.

To determine whether the genotypic DST method could shorten the interval for diagnosing drug resistance, the time to a positive DST result, between the phenotypic and genotypic methods, was compared. Genotype DST results were available for 75 % of the samples within 20 days

(range, 15-25), whereas 75 % of the phenotype result were only available after 38 days (range, 26-115) (Fig 2). This difference was statistically significant according to the Wilcoxon matched pair test ($p<0.001$).

Thirty-four (20 %) of the samples that failed to give a phenotypic DST result were PCR amplifiable. These samples either lost viability or were contaminated and subsequent samples were requested for DST. In these cases the mean delay was 133 days (range 50-1403 days) before a phenotypic DST result was available. In contrast the genotypic DST results were available within 27 days (range, 18-30).

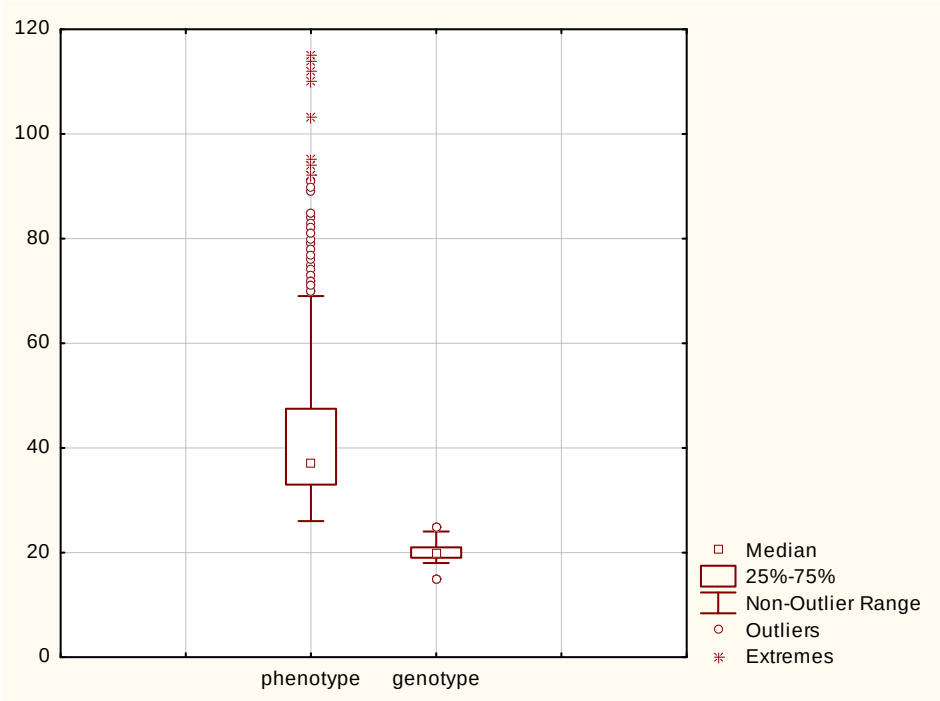

Fig 2. Time to a positive DST result using either phenotypic or genotypic DST. The mean diagnostic delay for the phenotypic method was 38days (range 26 to 115), while the mean diagnostic delay for the genotypic method was 20 days (range 15 to 25).

DISCUSSION

It is well known that DST for some drugs is difficult due to technical reasons and these results are not always accurate (5,6). In addition, it may take up to 6 weeks to get a phenotypic DST result and during this time many transmission events may take place. Alternative methods need to be evaluated to improve the speed of diagnosis of DR-TB and especially MDR-TB. Molecular techniques have been applied widely to detect mutations associated with specific drugs to overcome these problems.

This is a comprehensive study to evaluate the performance (sensitivity and specificity) of a genotypic method to detect drug resistance in a high TB incidence area. In this study, we have concentrated on identifying the most frequent mutations associated with resistance to INH and RIF (9,11). We found that our genotypic DST method compared favourably against the culture method for INH and RIF. However, it was noted that the sensitivity for INH was low since many of the resistance carrying gene mutations could not be identified. This observation is not unique since the molecular mechanisms for INH resistance are not fully understood and 25-30 % of phenotypic INH resistance associated mutations are still unaccounted for (12). An important finding was that 75 % of the genotypic results from all samples tested were available in a significantly shorter time interval in comparison to conventional culture based phenotyping methods. Such a time saving may have important implications for the control of DR-TB as it may reduce the chance for further transmission events of DR-TB. Furthermore, the genotypic DST method was able to detect drug resistance in samples which lost viability, circumventing the need to request follow-up sputum and thus reducing the overall cost and resources as it will not be necessary to tract patients in rural areas.

The performance of any new molecular method will depend on the efficiency of the sample preparation procedure. Amplification of drug resistant genes directly from sputum samples

without the need for culture remains a major problem. For many genotypic methods PCR analysis directly from sputum samples with very low bacterial load is extremely difficult. This may be the most important reason why molecular testing has not yet been introduced for routine testing on a large scale. We found that the establishment of short term mini-cultures from decontaminated sputum samples and the use of a specific Hot star *Taq* polymerase (Qiagen) gave the most consistent results. The implementation of DNA extraction protocols such as the nuclisens magnetic extraction kit (Biomérieux, Netherlands) may significantly improve detection of drug resistance from scanty positive sputum samples.

In the local community, the majority of drug resistance is due to transmission of a previously drug resistant strain (10). Recently, a bleak picture has emerged for TB control programs with the discovery of extensively drug resistant TB (XDR-TB) strains, raising concerns of a future epidemic of virtually untreatable TB (2). If any genotypic method could rapidly (despite low sensitivity) detect the majority of drug resistant strains in a high incidence community, the use of such a technique will be an advantage for the control of DR-TB. Therefore, it is urgent that molecular methods are developed and evaluated with the aim of rapid detection of MDR- and XDR-TB.

From the lessons learned in this study, the following recommendations can be made i) molecular methods can aid in the rapid detection of DR-TB. This is particularly useful for the detection of RIF resistance which is a marker for MDR-TB and XDR-TB, ii) implementation of any new test must be done in close collaboration with clinicians and the TB control program, iii) a positive genotype result, for a known drug resistant gene causing mutation, can be regarded as true resistance, iv) if possible, discrepant results between genotype and phenotype testing should be subjected to DNA sequence analysis, v) resistance genotyping must initially test for RIF

resistance followed by testing for other drugs (including genotypic DST for second line drugs to identify XDR-TB). We conclude that this study showed that a molecular method to rapidly detect drug resistance can add considerable value to the control of DR-TB in a high incidence area

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Chapter 4

ETHAMBUTOL RESISTANCE TESTING BY MUTATION DETECTION.

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My contribution to this project:
Planning of project
Sample preparation
PCR, Dot-Blot analysis and Sequencing
Design of new probes
Design of multiplex PCR
Data analysis and interpretation
Writing of manuscript

Abstract

Objective: To identify chromosomal mutations that confers resistance to ethambutol (EMB) in *M. tuberculosis.*

Design: Drug resistant (n=235) and drug susceptible (n=117) *M. tuberculosis* isolates collected from the Western Cape in South Africa were subjected to *emb*B gene analysis and the results were compared to phenotypic EMB testing.

Results: Genotypic analysis identified mutations at codon 306 of the *emb*B gene in 20 % (n=47/235) of the resistant isolates in comparison to only 1.7 % (n=4/235) which were phenotypically resistant to EMB by the agar diffusion method. No gene mutations were detected in susceptible isolates. Phenotypic re-testing in BACTEC demonstrated that the 47 genotypically resistant isolates were phenotypically resistant to EMB. This implies that 91.4 % (n=43/47) of EMB resistance had been phenotypically missed by routine laboratory procedures. EMB resistance was closely linked to MDR as 87.2 % (n=41/47) of the EMB resistant isolates was resistant to both Isoniazid (INH) and Rifampicin (RIF). A newly developed one-step Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) method correctly detected the EMB resistant genotype.

Conclusion: Implementation of more accurate diagnosis of EMB resistance may enhance patient management in South Africa since the standardized treatment of MDR-TB with second line drugs is currently dependent on the outcome of the EMB resistance test.

INTRODUCTION

Ethambutol (EMB), a first-line anti-tuberculosis (TB) drug, was introduced in 1961 and recommended for use in combination with isoniazid (INH), rifampin (RIF), streptomycin (STR), and pyrazinamide (PZA) [1]. Association studies implicate arabinosyltransferases as the targets for EMB, which are involved in the biosynthesis of the cell wall components arabinogalactan (AG) and lipoarabinomannan (LAM). EMB action on the tubercle bacilli is bacteriostatic due to the inhibitory effect of the drug in the transfer of AG into the cell wall. This process leads to the accumulation of mycolic acid [2]. As a result, the synthesis of sugars necessary for cell wall construction, is prevented, and thereby cell death follows.

In 1997 Telenti and colleagues [3] used EMB resistant isolates to identify the *emb*CAB gene locus involved in the biosynthesis of the mycobacterial cell wall. The *M. tuberculosis emb*CAB locus is a 10kb operon that encodes for arabinosyltransferases in three contiguous genes, namely, *emb*C, *emb*A and *emb*B, that have a 60 % similarity to each other [3]. Sreevatson [4] confirmed the finding that certain mutations in the *emb*CAB operon of *M. tuberculosis* were exclusively associated with EMB-resistant clinical isolates [3;4]. In particular, amino acid replacements at position 306 of *emb*B were represented in approximately 90 % of EMB-resistant isolates, but not EMB-susceptible isolates [5-7]. Five distinct structural mutations in the *emb*B gene have been described, with amino acid replacements of the wild-type Met306 with Ileu, Leu or Val, in EMB resistant isolates [8]. This finding suggested *emb*B as one of the targets of EMB. Mutations outside the 306 codon have been found but are less frequent [7]. EMB susceptible isolates lack the corresponding gene mutations [4;7].

A close association with gene mutations and phenotypic culture-based EMB susceptibility testing will only be evident if the phenotypic test is highly sensitive and specific. This is

currently not the case in routine diagnostics laboratories in many countries. Phenotypic EMB resistance detection in cultures differs widely and lacks uniformity amongst different laboratories [6;9;10]. In a quality assurance study done during 1997 between 16 supranational reference laboratories within the World Health Organization (WHO) network, a vast lack in uniformity amongst phenotypic EMB testing was observed [9]. The National Health Laboratory Services (NHLS) in Cape Town is a high-throughput routine diagnostic laboratory and receives on average 550 samples per day for TB testing. Drug susceptibility testing, which includes EMB, is carried out on a subset of these samples by means of the agar proportional method [11;12]. As from March 2003, drug susceptible testing is only done for INH, RIF and EMB. The treatment regimen of MDR-TB cases with second line anti-TB drugs is then based on the outcome of the EMB test as recommended by the national TB control program [12].

In this study a molecular approach was used to screen for gene mutations considered to be associated with EMB resistance. Clinical isolates found to be resistant (any drug) by routine culture drug susceptibility testing, as well as fully drug susceptible isolates were used in this investigation. The aim of this study was to correlate mutational analysis at codon 306 of the *embB* gene to EMB culture susceptibility testing of *M. tuberculosis* isolates in a routine laboratory surrounding a high incidence area.

STUDY POPULATION AND METHODS

Isolates Two sets of *M. tuberculosis* isolates were collected between November 2000 and June 2003 from 72 rural clinics in the Boland/Overberg and Southern Cape/Karoo region. in the Western Cape, South Africa. The first set of 235 drug resistant (resistant against any drug) *M.*

tuberculosis isolates (in BACTEC 12B media) represent one isolate per patient. The second set consisted of 117 fully drug susceptible isolates over the same time period from the same region.

Culture drug susceptibility testing Isolates (n=352) were initially tested for EMB resistance in the routine diagnostic laboratory (NHLS) by the agar diffusion method on Middlebrook 7H11 slants (supplemented with Tween 80 (Merck, Darmstead, Germany) and 100ml Oleic Albumin dextrose Catalase (Biolab diagnostics, Gauteng, South Africa)) at a critical EMB concentration of 7.5µg/ml. Results of sensitivity testing were read at 21 days.

Mutational analysis The *embB* gene was amplified using crude DNA templates and visualized on a 1.5 % agarose gel in 1 x TBE buffer with ethidium bromide using a previously described method [13]. Five microliter of PCR template was used for PCR dot-blot hybridization as described previously [13]. Wild type and mutant-specific oligonucleotides, for the *emb*B 306 codon (Table 1), were used for the detection of mutations associated with EMB resistance. Dot-blot analysis was carried out as described previously [13].

Table 1 Probes used for dot-blot hybridisation

Probe	Sequence	Tm	Reference
Emb306wt	C CTG GGC atg GCC CGA GTC G	70°C	Victor *et al.* (1999)
$Emb306_{ATA}$	C CTG GGC AT“a” GCC CGA GTC G	68°C	Current
$Emb306_{ATC}$	C CTG GGC AT“c” GCC CGA GTC G	70°C	Current
$Emb\ 306_{ATT}$	C CTG GGC AT“t” GCC CGA GTC G	68°C	Current
$Emb\ 306_{GTG}$	C CTG GGC “g”TG GCC CGA GTC G	72°C	Current
$Emb\ 306_{CTG}$	C CTG GGC “c”TG GCC CGA GTC G	72°C	Current

Legend to Table 1: atg, wild type probe; AT“a”, AT“c”, AT“t”,”g”TG and “c”TG, mutant probes.

Handling of discrepant results Phenotypic re-testing of EMB was done using the BACTEC method at a prescribed critical concentration of 2.5µg/ml in the Department of Medical Biochemistry, University of Stellenbosch. Each of the isolates was checked weekly, and the susceptibility results were recorded between 5-12 days. Discrepant results between phenotyping and genotyping were further evaluated by DNA sequencing on selected samples with an ABI PRISM DNA sequencer (model 377, Perkin Elmer, Foster City, US).

Performance of Phenotypic and Genotypic test After investigation of all discrepancies, the corrected EMB resistance pattern for each of the isolates was determined. The corrected EMB resistant pattern was then used as the “gold standard” to evaluate and compare the performance of both the phenotype and genotype EMB resistant testing as was done previously [10;14].

Statistics: The difference between phenotypic and genotypic results was calculated using the statistical program GraphPad Prism version 4.03.

Amplification refractory mutation system (ARMS)-PCR. An ARMS multiplex PCR method has been developed to detect EMB associated gene mutations in a one-step PCR reaction. The primer sets was Emb151 5' CGG CAT GCG CCG GCT GAT TC '3, Emb151wt 5' ACG GCT ACA TCC TGG GCA TG '3 and Emb131 5' TCC ACA GAC TGG GT CGC TG '3 at a Tm of 66°C. For the Hot Star PCR reaction (Qiagen), 5µl crude DNA lysate was added to a PCR mix (100µl) containing a 2.25mM $MgCl_2$, 250µM of deoxynucleotide triphosphates, 20µl Q Buffer, 2.5 units Taq, 10µL Emb 131 reverse primer, 6µl Emb151 forward primer and 6µl Emb151wt wild type primer. DNA amplification of 40 cycles was done as fellows: 95°C for 15min, 95 °C for 1min, 66 °C for 1min, 72 °C for 1min, and a final extension of 72 °C for 7min. PCR products were analyzed by electrophoresis on a 1.5% agarose gel and stained with ethidium bromide.

RESULTS

Phenotypic drug resistance pattern by routine surveillance. On routine investigation of the 235 resistant isolates, only 1.7% (4/235) was found to be resistant to EMB using a critical concentration of 7.5µg/ml on Middlebrook 7H11 slants (Table 2). Additional BACTEC culture analysis confirmed that these four isolates (R120, R179, R212 and R222) were resistant to EMB. They were also resistant to other first and second line anti-TB drugs.

Table 2: Phenotypic and genotyping of all isolates with a mutant *emb*B306 allele.

Isolate	Resistant Pattern	*embB*306	Corrected Phenotype
R 162	INH	ATA	INH, EMB
R 163	INH	ATA	INH, EMB
R 327	INH	ATA	INH, EMB
R 7	INH,RIF	GTG*	INH,RIF, EMB
R 12	INH,RIF	GTG*	INH,RIF, EMB
R 39	INH,RIF	ATA*	INH,RIF, EMB
R 132	INH,RIF	ATA*	INH,RIF, EMB
R 178	INH,RIF	ATA	INH,RIF, EMB
R 269	INH,RIF	GTG*	INH,RIF, EMB
R 373	INH,RIF	ATA	INH,RIF, EMB
R 296	INH,RIF,ETH	ATA	INH,RIF,ETH, EMB
R 1	INH,RIF,STR	GTG	INH,RIF,STR, EMB
R 10	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 14	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 17	INH,RIF,STR	GTG	INH,RIF,STR, EMB
R 24	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 41	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 44	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 56	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 58	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 60	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 63	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 65	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 73	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 102	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 145	INH,RIF,STR	GTG	INH,RIF,STR, EMB
R 221	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 223	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 235	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 364	INH,RIF,STR	GTG	INH,RIF,STR, EMB
R 391	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 432	INH,RIF,STR	ATC	INH,RIF,STR, EMB
R 439	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 451	INH,RIF,STR	ATA	INH,RIF,STR, EMB
R 543	INH,RIF,STR	ATC*	INH,RIF,STR, EMB
R 565	INH,RIF,STR	ATA*	INH,RIF,STR, EMB
R 590	INH,RIF,STR	GTG*	INH,RIF,STR, EMB

R 619	INH,RIF,STR	ATC*	INH,RIF,STR, EMB
R 120	INH,RIF,STR,**EMB**	wt*	INH,RIF,STR,EMB
R 40	INH,RIF,STR,ETH	ATC*	INH,RIF,STR,ETH, EMB
R 118	INH,RIF,STR,ETH	GTG	INH,RIF,STR,ETH, EMB
R 394	INH,RIF,STR,ETH	ATC	INH,RIF,STR,ETH, EMB
R 212	INH,RIF,STR,ETH,**EMB**	ATA	INH,RIF,STR,ETH,EMB
R 222	INH,RIF,STR,ETH,**EMB**	ATC	INH,RIF,STR,ETH,EMB
R 257	INH,STR	GTG*	INH,STR, EMB
R 479	INH,STR	ATA	INH,STR, EMB
R 179	INH,STR,ETH,**EMB**	GTG	INH,STR,ETH,EMB

Legend to Table 2: INH, Isoniazid; RIF, Rifampicin; STR, Streptomycin; EMB, Ethambutol; ETH, Ethionamide; *, Sequence; W, wild type, R, resistant, S,Susceptible; MIC, Minimal inhibitory concentration

Genotypic resistant Pattern. Mutations could only be detected in three of the four phenotypically EMB resistant isolates which have the following *embB*306 gene mutations: isolate R179 Met306Val (ATG→GTG), R212 Met306Ile (ATG→ATA) and R222 Met306Ile (ATG→ATC). No mutations in the *embB* gene could be detected in isolate R120, even after DNA sequencing of the entire gene. Mutations at codon *embB*306 were present in 47 of 235 isolates (20 %) phenotypically classified as susceptible to EMB after initial routine screening (Table 2). The difference between phenotypic and genotypic results was statistically significant ($p<0.0001$). The following *embB*306 gene mutations were associated with these 47 isolates: ATG-ATA (n=21), ATG-ATC (n=15) and (ATG-GTG) (n=11) respectively. No mutations were observed by dot-blot or sequence analysis at codon 306 in the 117 fully EMB susceptible isolates from the same geographical area collected during the same time period.

Investigation of discrepant results. Forty four isolates showed potential false –negative EMB testing by routine screening. Retesting of the culture-based false negative samples by additional DNA sequence analysis (n=12) confirmed the initial dot blot results (Table 2). Phenotypic re-testing by the radiometric BACTEC method confirmed that these isolates were resistant to EMB at the critical concentration of 2.5 µg/ml. The results from both phenotypic and genotypic re-testing were then used to establish the corrected EMB resistant phenotype for each isolate. Collectively these results strongly suggest that the additional 43 isolates with *emb*B306 gene mutations are indeed resistant to EMB and that 91.4 % of EMB resistance was not detected during the initial screening for EMB resistance in the routine laboratory.

Performance of genotypic EMB resistant testing. Comparative analysis showed that both phenotype (BACTEC) and genotype method have a high specificity (100 %) and sensitivity (97 %). However, the specificity and sensitivity of the proportional method was 100 % and 8.3 % respectively. Thus the original EMB resistant screening by the phenotypic method was extremely low (8.3 %) in comparison to the sensitivity (97 %) of the genotypic and BACTEC phenotype methods.

EMB resistance is strongly associated with MDR-TB. Further analyses of the drug resistance of the isolates indicated that none of the isolates are EMB mono-resistant. An interesting observation is that EMB resistance was predominantly (87 %) associated with MDR isolates; 11 % of isolates were associated with other multiple drug resistant combinations.

ARMS-PCR. An ARMS PCR-based method was develop to improve rapid detection of EMB resistance. After PCR amplification a 260 bp DNA fragment for EMB resistant isolates and a double band of 260 bp and 150 bp respectively for EMB susceptible isolates was generated (Fig.1). Isolates (n=50) screened in a blind study using the ARMS–PCR method corresponded 100 % to dot-blot analysis done on the same subset of isolates.

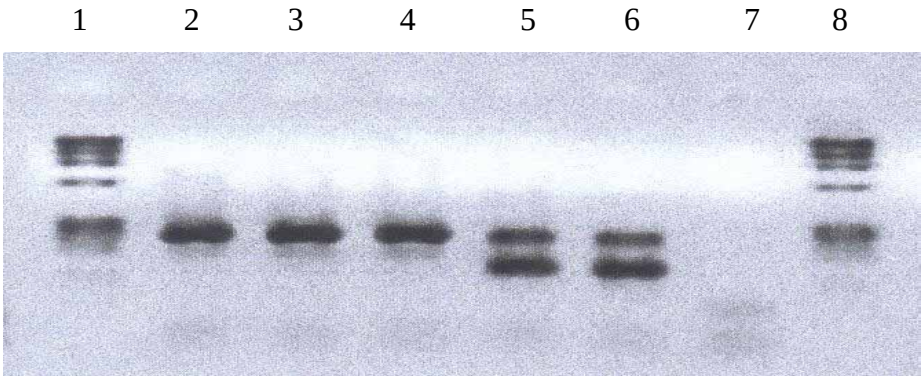

Fig. 1. ARMS PCR amplification of EMB. Lane: 2-4 EMB resistant isolates lane, 5-6 EMB susceptible isolates, lane: 1 and 8: DNA size marker IX (Roche) and lane 7: blank

DISCUSSION

The control of drug resistant TB relies upon the incorporation of drug susceptibility testing as part of national TB control programs. Since 1996, drug-susceptibility testing in South Africa has only been done on cultures obtained from retreatment cases and new patients with treatment failure. This routine drug-susceptibility testing is done by the NHLS, Cape Town, using the agar proportion method on Middlebrook slants containing critical concentrations of 0.2 µg/ml INH or 30 µg/ml RIF. Additional drug-susceptibility testing was done for cultures found to be resistant

to INH and/or RIF. In 2002, standardized treatment of MDR-TB was introduced as part of the national TB control program and as from March 2003 drug susceptibility testing became limited to INH, RIF, and EMB [12]. Ethambutol is used during the continuation phase of the standard retreatment regiment in South Africa as it is a valuable bacteriostatic agent for preventing the emergence of resistance to other TB drugs [12]. All retreatment and treatment failure cases are initially treated with a combination of INH, RIF, EMB, STR and PZA until the drug susceptibility test for INH, RIF and EMB are available. Such testing takes on average two months (range 30-98 days). If EMB resistance is identified, EMB is replaced with Cycloserine in the second line treatment regimen. Therefore the outcome of EMB drug susceptibility testing is critical in deciding the most appropriate treatment regimen. It is well known that testing of susceptibility to INH and RIF is more reliable than testing susceptibility to EMB, STR and PZA[15-17]. This study supports previous reports which have raised concern over false negative EMB susceptibility results [6;10]. In this study, the routine laboratory method for EMB resistance testing came under investigation and we observed that there are significant limitations associated with the sensitivity of the currently used proportion method. Our findings, together with those of others [6;10] suggest that the culture based method and critical drug concentration used for EMB testing needs to be critically re-examined. The reason for the low sensitivity observed in this study may include: - 1) The use of LJ slants instead of plates may cause false susceptible results, as the colonies formed on the LJ slant will have the same color as the media making resistant detection difficult; 2) stability of the EMB in the test medium is important as EMB shows reduced activity in culture media as the drug is partially inactivated during media preparation [1]; 3) the reading period after inoculation is vital as false susceptible results can be obtained if the reading time after inoculation is incorrect for that specific phenotypic test used[18] ; 4) the addition of Tween 80 can have an effect on susceptibility testing [1]. However, this is

unlikely since the phenotypic EMB results in BACTEC correlate with the genotypic findings in the study.

In contrast to the indirect proportion method using solid media, we showed that the phenotypic BACTEC method (gold standard) correlated directly with the genotyping data. The specificity and sensitivity of the genotyping method was 100 % and 97 % respectively when compared to the BACTEC method. The primary limitations of the BACTEC culture based technique is that the results are available only after ± 30 days which further delays the implementation of appropriate therapy.

The strong correlation between the BACTEC phenotype and the genotype methods implies that in this setting, mutations detected via PCR would be the appropriate method for rapid EMB resistance testing. As mutations conferring EMB resistance occur predominantly at one base in a single codon. We have developed a simple and robust multiplex PCR-ARMS method to rapidly identify isolates which are susceptible to EMB. This PCR amplification method generates two products; an internal control to confirm that the sample is amplifiable and a specific PCR product to demonstrate the presence of a "wild type" sequence at codon 306 in the *emb*B gene. The inability to amplify the latter product strongly suggests a mutation at codon 306 and this implies resistance to EMB (provided the internal amplification control generates a specific product). Our results show that the method works extremely well, and has now been applied to directly amplify DNA from sputum specimens (data not shown). Further research is necessary to determine the cost-effectiveness of this technique. The method has certain limitations: 1) emerging EMB resistance will not be detected as the presence of the EMB sensitive strain's "wild type" sequence will generate an amplification product, 2) isolates resistant to EMB via a mutation outside of the *emb*B gene will not be detected. However, such mutations account for less than 10% of EMB resistance [19]. Despite these limitations, our results suggested that patients

infected with *M. tuberculosis* resistant to EMB will benefit from rapid diagnosis. The inability to accurately determine EMB susceptibility has consequences for both the patient and control program. Prolonged time delay between diagnosis of TB and the initiation of appropriate therapy may allow for the amplification of additional resistance markers. This in turn may prolong treatment, thereby placing unnecessary strain on the patient, increasing the risk of default and exacerbating the overall cost of treatment. Most importantly, diagnostic delay has the potential to increase the risk of transmission. The development of rapid molecular based drug susceptibility testing methods offers a rapid diagnosis and thereby could direct appropriate patient management within days of TB diagnosis. This is particularly important for the TB control program given the close (direct) association of EMB resistance with MDR-TB.

CONCLUSION

This study shows that there is a need to re-evaluate the routine phenotype susceptibility testing of EMB and suggests that genotypic detection of EMB resistance would do more to enhance the management of MDR-TB cases.

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Chapter 5

A clone of the drug resistant Beijing strain family of *Mycobacterium tuberculosis* has a higher propensity to transmit and cause drug-resistant TB

Will be submitted to Journal of Medical Microbiology as a note (1000 words)

My contribution to this project:
Planning of project
Primer design, PCR, Mutational analysis
Sequencing
Identification of outbreak strain
Data analysis and interpretation
Writing of manuscript

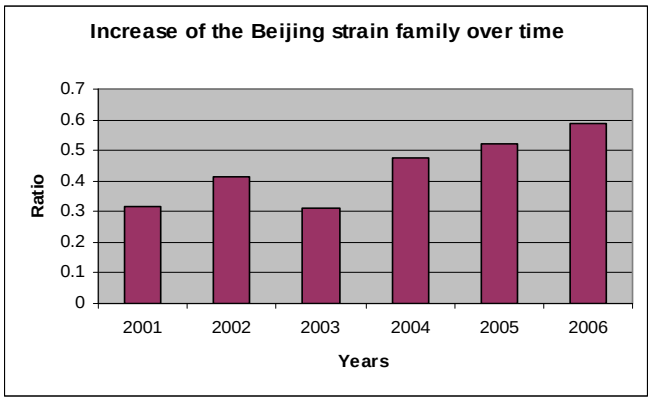
The increase in drug resistant tuberculosis (TB) and especially multi-drug resistant tuberculosis (MDR-TB) is of concern. South Africa was listed in the third World Report on Drug Surveillance as one of the hot spots for MDR-TB, where more than 6000 new MDR-TB cases are detected each year (8). Recently, a bleaker picture has emerged with the discovery of extensively drug resistant tuberculosis (XDR-TB), an emerging threat for patients and control programs, since XDR-TB strains have developed resistance to first line and most second line drugs.

Molecular methods such as strain genotyping (1,4) and drug resistance genotyping, have become valuable tools with which to study the epidemiology of drug resistant TB. These methods are also central to the identification and control of TB and MDR-TB in various areas around the world (7). The Beijing strain family is widely spread around the world and has been involved in many

outbreaks. It has been hypothesize that the Beijing family may have an enhanced propensity to transmit and cause disease (2,5,6).

From February 2001 to December 2006, *M. tuberculosis* isolates from 1200 re-treatment cases from the Boland- Overberg-South Cape Karoo regions (BOKS) in the Western Cape South Africa were found to be drug resistant. Molecular analysis of these isolates (first isolate per case) showed that 67 % of drug resistance in this region was driven by 4 strain families; Beijing (31 %), followed by X1 (22 %), LAM3 (10 %) and the S family (4 %). Fig 1a shows that the proportion of drug resistant TB due to the Beijing strain family (n=375) increased steadily over time in relation to other drug resistant strain families (n=825). This suggests that the Beijing strain families may have enhanced fitness compared to the non-Beijing strain families. Additional, IS*6110*-RFLP analysis (1) of the Beijing strain family identified several drug resistant clones, of which cluster 220 is the largest (166/375; 45 %). After 2001, cluster 220 increased steadily (except for 2003 when sampling was low for an unknown reason) relative to other Beijing drug resistant strain clusters (Fig. 1b). These observations suggest that the transmission characteristics of *M. tuberculosis* strains are not conserved within strain families but rather that individual sub-lineages have unique transmission characteristics. The majority (63 %) of Beijing cluster 220 was phenotypically and genotypically resistant to INH and RIF (therefore MDR) and 36 % were resistant to INH only.

A



B

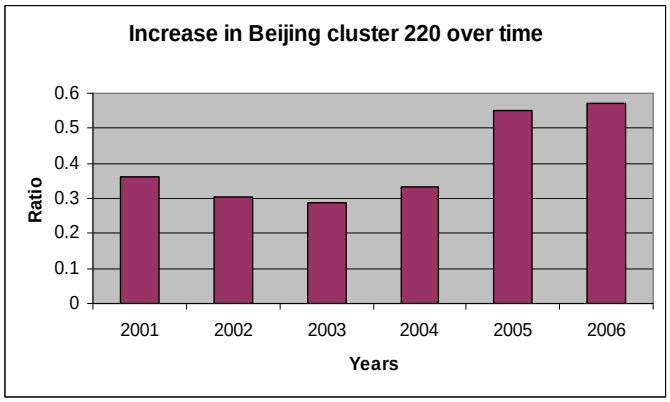


Fig. **1a)** Ratio of the Beijing drug resistant genotype in comparison to non-Beijing resistant genotypes.
1b) Ratio of Beijing cluster 220 in comparison to other Beijing genotypes

Thus far, no Beijing cluster 220 drug susceptible isolate has been identified in the region. More then 90 % (n=151/166) of Beijing cluster 220 isolates have the *inhA*-15 (C-T) promoter mutation (conferring INH resistance) and 80 % had an identical *rpoB* 531_{TTG} mutation (conferring RIF resistance) while 13 % of the RIF resistant isolates had other mutations (Table 1). These results imply that cluster 220 was initially fully drug susceptible, and then in a step-wise manner became resistant to INH followed by gaining additional resistance to RIF. The over-representation of Beijing cluster 220 in the whole geographical region strongly suggests sequential acquisition and amplification of resistance followed by recent spread in the community. It is possible that the *inhA* promoter mutation may have given cluster 220 a selective advantage to develop additional resistance and spread in the community. Geographical mapping indicated that the Beijing cluster 220 drug resistant genotype is widespread in the BOKS region and was involved in a recent MDR outbreak in a high school in Cape Town (3). The result raises concern for the spread of drug resistant strains in vulnerable populations. Greater vigilance is required to contain the drug resistant TB epidemic in high prevalence settings. The emergence of this Beijing sub-lineage that shows

increased transmissibility may have important implications for the tuberculosis Control Program and it is important that early diagnosis and contact tracing should be a top priority in order to curb the spread of this transmissible drug resistant strain.

Table1. Beijing Cluster 220 Phenotype and Genotype

	Phenotype			Genotype			
	INH mono	RIF	MDR	*inhA*-15 prom	*rpoB*531ttg	other rpoB mut	wt
n=166	61 (36 %)	105 (64 %)	104 (63 %)	151 (91 %)	84 (80 %)	14 (13%)	7 (6 %)

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Chapter 6

An Outbreak of drug resistant tuberculosis caused by a Beijing strain in the Western Cape, South Africa

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My contribution to this project:

Planning of project
Sample preparation, PCR, Mutational analysis and Sequencing
Geographical mapping and identification of outbreak strain
Data analysis and interpretation
Writing and editing of manuscript

SUMMARY

During October 2005, 4 children in a school in Cape Town were identified with MDR-TB. Genetic analysis confirmed that these isolates belonged to a single cluster (Beijing cluster 220) and all harboured a -15 *inhA*$_{C\text{-}T}$ promoter mutation demonstrating transmission. Genetic analysis of isolates cultured from patients from the Boland–Overberg-South Cape-Karoo and Cape Town regions showed that 28 % (58/209) of patients infected with a Beijing strain had the cluster 220 genotypes and all harboured the same -15 *inhA*$_{C\text{-}T}$ promoter mutation. The presence of these transmissible MDR-TB strains may pose a threat to the community and rigorous infection control measures are needed to ensure the safety of those exposed.

INTRODUCTION

During 2004, over 44 000 people were diagnosed with TB in the Western Cape Province of South Africa, of which 450-600 cases had multidrug resistant (MDR) TB [1]. The TB epidemic in this area is primarily driven by four strain families with the Beijing strain family predominating amongst drug resistant strains [2]. In this study we have used molecular methods to investigate the geographical distribution of a strain identified in a recent MDR-TB outbreak amongst children at a high school in Cape Town, South-Africa.

MATERIALS and METHODS

During October 2005, 4 adolescents from a school in Cape Town were identified by the TB control program with MDR-TB after not responding to first-line anti-TB treatment. DNA from the first culture from each case was extracted and typed by the IS*6110*–restriction fragment length polymorphism (RFLP) [3] method, spoligotyping [4] and DNA sequencing [5].

In addition, we used a novel multiplex PCR method to rapidly detect the outbreak strain in a one step reaction, as describe below. Primers were designed according to the IS*6110* junction (point of insertion: 954.19) Fig1a, unique to the Beijing cluster 220 strain type identified in the 4 school cases. Each PCR reaction contained 1 µl DNA template, 5 µl Q-Buffer, 2.5 µl 10 x Buffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM dNTP's, 0.5 µl of each primer (50 pmol/µl) (ESAT-6-for 5' cag cag cag tgg aat ttc gc 3", ESAT-6-rev 5' tcc cag tga cgt tgc ctt c 3, IS*6110*-xhoIfor 5' ttc aac cat cgc cgc ctc tac 3' and 954.19-rev 5' aat cgt tga tgc tgg cgc tat gaa cc 3') and 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) made up to 25 µl with H_20. Amplification was initiated by incubation at 95 °C for 15 minutes, followed by 35 cycles at 94 °C for 1 minute, 62 °C for 1 minute, and 72 °C for 1 minute. After the last cycle, the samples were incubated at

72°C for 10 minutes. PCR amplification products were electrophoretically fractionated in 1.5 % agarose in 1 x TBE pH 8·3 at 6V/cm for 4 hours, and visualized by staining with ethidium bromide. A 270 bp PCR product indicated the presence of *M. tuberculosis*, while the 160 bp PCR product indicated the presence of the strain (Beijing cluster 220) (Fig.1). This PCR method is able to identify all 220 cluster isolates, and although it may also pick up 322 and 219, these occur very infrequently in our population.

This PCR method was used to retrospectively screen archived Beijing isolates (n = 209 drug resistant, 100 drug susceptible Beijing genotypes), collected over a 4 year period between February 2001 and May 2004 from the Boland–Overberg-South Cape-Karoo (BOKS) [2] regions and from an epidemiological field site in metropolitan Cape Town [6] in the Western Cape Province South Africa. The set of samples included both new and re-treatment cases of infected Beijing isolates. This study was approved by the ethics committee (ethics approval 2002/C118) of Stellenbosch University, Tygerberg, South Africa and was done on request of the Provincial Health Department as part of an outbreak investigation and therefore individual written consent was not obtained from the parents of the children.

RESULTS and DISCUSSION

Spoligotyping showed that the 4 adolescents from one school were infected with Beijing strains. IS*6110*-RFLP typing confirmed that these isolates all had identical IS*6110* hybridization bands (fig 1a) and belonged to the Beijing cluster 220. DNA sequencing showed that all 4 isolates had a -15 *inhA*$_{C\text{-}T}$ promoter mutation (conferring isoniazid resistance) and *rpoB*531$_{TCG\text{-}TTG}$ gene mutation (conferring rifampicin resistance).

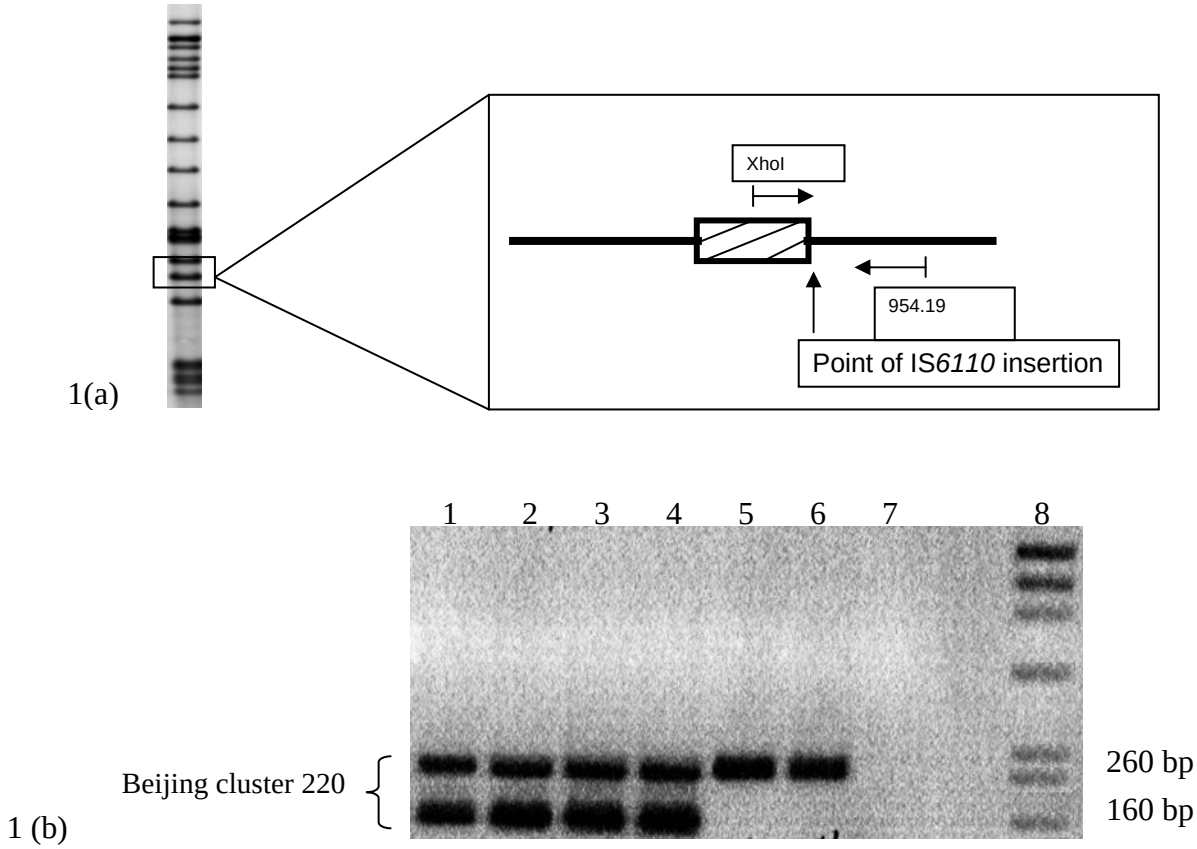

Figure 1: A. A representative IS*6110*-RFLP fingerprint of Beijing drug resistant cluster 220 genotype. **B.** PCR amplification of the Beijing cluster 220 genotype (lanes 1-4), *M. tuberculosis* Beijing cluster 208 (lane 5), H37Rv control (lane 6) and molecular marker (lane 8)

These results confirmed that the isolates were identical and reflected recent transmission. The newly developed PCR-based method correctly identified the Beijing cluster 220 genotype (fig 1b) from the 4 school children. Using this method, we found that 28% (58/209) of the archived *M. tuberculosis* Beijing drug resistant isolates had the Beijing cluster 220 genotype. All INH resistant Beijing cluster 220 genotypes from the BOKS and Cape Town regions had the same -15 $inhA_{C\text{-}T}$ promoter mutation. Forty-four percent (25/58) of these isolates also had *rpoB* gene mutations (rpoB531 $_{TCG\text{-}TTG}$, n=23; rpoB526 $_{TCG\text{-}TAC}$, n=2) associated with rifampicin resistance and are thus MDR. A total of 100 drug susceptible isolates was screened for the -15 $inhA_{C\text{-}T}$ promoter mutation and to date, no Beijing cluster 220 drug susceptible isolates, harbouring that specific mutation, has been identified. Geographical mapping indicated that the Beijing cluster

220 drug resistant genotype is widespread in the Western Cape (fig 2). Most of the cases were found in the Worcester (n=18), Mosselbaai (n=11), George (n=9), Cape Town (n=10) and other areas (n=10) respectively (fig 2). Thirteen paediatric cases were also shown to be infected with the Beijing cluster 220 drug resistant genotype in a study conducted from March 2003 through August 2005 at Tygerberg Children's Hospital, in the Western Cape Province (personal communication, Dr. Simon Schaaf). The results demonstrate that all 4 school children were infected with a Beijing cluster 220 MDR genotype which appears to be widely distributed in the Western Cape. Although a susceptible counterpart of Beijing cluster 220 has not been found, it is assumed that this strain was originally fully drug susceptible and acquired drug resistance by sequential selection (initially isoniazid, followed by rifampicin) and that the resistant progeny were then disseminated by transmission. The dominance of this Beijing drug resistant cluster 220 genotype suggests that it may be more fit than other drug resistant clusters from the same Beijing strain family which were less frequently observed in local communities.

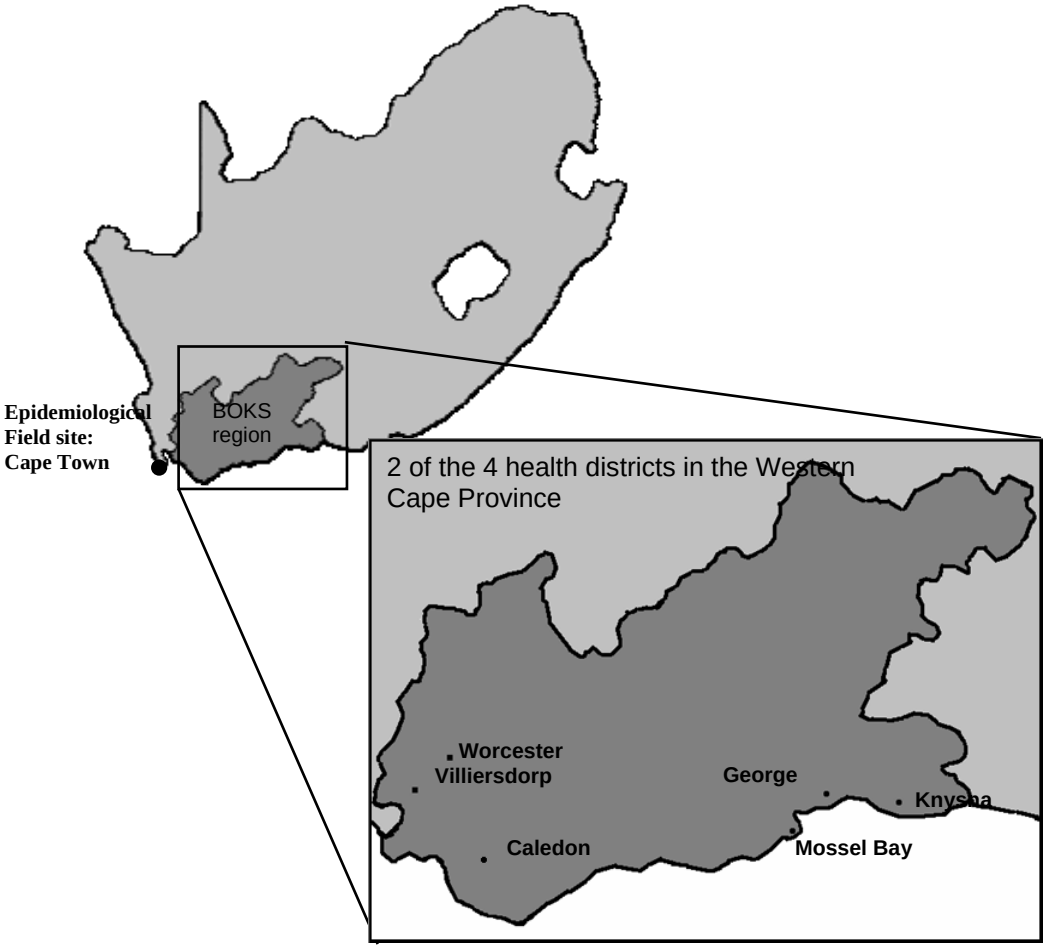

Fig 2. Clinic location where the cases presented with disease were used to construct a geographical map of Beijing cluster 220 genotypes. The ratio of Beijing cluster 220 in relation with the total number of Beijing strains found in each location is indicated below. Cases with cluster 220 were distributed in large towns as follows: Worcester (n=18/45), Mossel Bay (n=11/18), George (n=9/25), Cape Town (n=10/71), Knysna (n=3/13), Villiersdorp (n=2/5), Caledon (n=2/2) and other areas (n=3/30

Application of molecular methods such as were used in this study, have been central to the description and control of other MDR-TB outbreaks.[7] Previous culture-based drug surveillance studies from the same region have failed to identify drug resistant outbreaks and suggested that drug resistance is largely acquired.[8;9] The occurrence of this outbreak in a setting in which a direct observed therapy strategy (DOTS) programme was in place, testifies to the need to dedicate more resources to intensify the basic DOTS strategy as well as extend them to include

specific activities directed at the diagnosis and treatment of MDR-TB (DOTS Plus). The presence of these transmissible drug resistant strains poses a threat to healthcare workers and others in the community and suggests that rigorous infection control measures are needed to ensure the safety of those exposed to infectious sources with highly transmissible MDR-TB strains.

ACKNOWLEDGEMENTS

We thank the South African National Research Foundation (grant Gun 2054278) and DST/NRF Centre of Excellence in Biomedical Tuberculosis Research), the Welcome Trust (grant Ref. 072402/Z03/Z), the NIH (grant R21 A155800-01), the IAEA (grant SAF6008) and the Andrew W Mellon scholarship. The authors would also like to thank TB in the 21st Century Consortium, an international network supported by the Norwegian Research Council and the Centre for prevention of global Infectious, University of Oslo.

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Chapter 7

Gene expression patterns of a susceptible and resistant *Mycobacterium tuberculosis* Beijing strain after exposure to non- lethal concentrations of INH

My contribution to this project: Planning of project
Strain culture, Growth curve, RNA isolation, cDNA synthesis, QRT-PCR, Primer Design, DNA sequencing
Data analysis, Gene expression, microarray, proposed INH mechanism
Writing and editing of manuscript

ABSTRACT

Currently, isoniazid still forms an important part of the first line anti-tuberculosis therapy, but the molecular mechanisms conferring isoniazid resistance remains poorly defined. Currently there are 5 genes known to be associated with isoniazid resistance an recently an additional 11 genes was identified by microarray analysis that might play a role in isoniazid resistance. The aim in this study was to quantify the expression level of genes, induced by INH, in a susceptible and resistant *M. tuberculosis strain* Beijing strain family. Two Beijing cluster 208 isogenic strains were selected from an existing strain bank and treated with sublethal concentrations of isoniazid. Quantitative real-time PCR analysis was performed on the 11 genes that were induced upon INH treatment. After 5 hrs of exposure, most of the isoniazid induced genes in both the resistant and susceptible strain showed a low level of response to INH treatment except for *accD6, acpM, efpA* and *Rv1772.* After 24 hrs of treatment most of the INH induced genes was found to be significantly regulated. Using this data we proposed a mechanism (based on the QRT-PCR results whereby drug resistant strains can compensate for the toxic effect of the drug. In addition we conclude that resistance in tuberculosis may be a combination of mutations in certain genes and their regulatory domains which regulate the intracellular concentration of the drug.

INTRODUCTION

Soon after Isoniazid (INH) was introduced in 1952, INH resistant *Mycobacterium tuberculosis* (*M. tuberculosis*) strains were noted (2,4,13,14,18). Currently, INH still forms an important part of the first line anti-tuberculosis (anti-TB) therapy, but the molecular mechanisms of INH action and resistance remains poorly defined (11,12). It is proposed that INH enters the bacterium as a prodrug that is activated by catalase–peroxidase (CP), a product of the *katG* gene (7). Reduced or loss of CP activity due to gene mutations, or a whole *katG* gene deletion, is one of the primary mechanisms conferring resistance to INH (8). In addition, a further 4 genes (*inhA*, *kasA, ndh* and *ahpC*) are associated with INH resistance (1,9). However, mutations in these 5 genes are absent in 25-30 % of INH resistant clinical isolates collected from various geographical regions (15,16). These results imply that other genes or mechanisms are involved in INH resistance (10).

Studies using microarray technology have demonstrated additional genes that are induced in the bacterium after exposed to INH (3,5,17,18). Some of these genes encode for Type II fatty acid synthase (FAS-II) enzymes, which are important for mycolic acid biosynthesis. Other genes that are affected by INH and that mediate the toxic effect of the drug include *acpM, kasA*, *kasB*, *fbpC; fadE, efpA, iniA, iniB, ahpC, accD6, Rv1592 and Rv1772*. However, even though they fall within the INH pathway, none of these genes could thus far be directly linked to INH resistance by gene associated mutation.

The aim in this study was to quantify the expression level of genes, induced by INH, in a susceptible and resistant *M. tuberculosis strain* Beijing strain family.

MATERIALS AND METHODS

Strain selection

Between February 2001 to December 2006, phenotypic drug resistant Beijing isolates (n=370) were selected from an existing strain bank containing, phenotypic, genotypic and demographical information, at the University of Stellenbosch. A minimum inhibitory concentration (MIC) of 0.1 µg/ml of INH was considered susceptible and 1 µg/ml of INH was considered as moderately resistant whereas, 4 µg/ml of INH was considered highly resistant (6). Primers were designed for PCR amplification and DNA sequence analysis was done on all samples to identify mutations in the 5 genes (*katG, inhA, kasA, ndh* and *ahpC*) known to be associated with INH resistance (Tables 1 and 2). After screening of the 5 genes, no gene associated mutation could be identified in 30 % (118/370) of the Beijing drug resistant samples. IS*6110*-restriction fragment length polymorphism (IS*6110*-RFLP) analysis showed that the Beijing cluster 208 was the predominant cluster amongst these 118 samples. A subset of these 118 samples (phenotypically resistant, but no INH gene associated mutations) was then selected to screen for mutations in an additional 15 genes (as well as genes for which the promoters are known) whose gene expression was known to be influenced by INH exposure (18) (Tables 1 and 2). When no mutations could be identified after screening the additional 15 genes and their promoters, representatives of genetically isogenic (identical spoligotype, IS*6110* RFLP pattern and DNA vs DNA microarray analysis (DNA vs DNA microarray data not shown)) (Fig 1 and 2) Beijing cluster 208 strains was selected from those isolates. These strains included a Beijing fully susceptible (K636) and a INH mono resistant (R55) strain. A laboratory strain, H_{37}Rv was used as a control.

Table 1. Genes analyzed to detect nucleotide sequence variations

Gene	Product and Function
**KatG*	catalase-peroxidase
**inhA*	Enoyl ACP reductase
**inhA promoter*	
**kasA*	β-Ketoacyl ACP synthase
**ahpC*	Alkylhydroperoxidase
**ndh*	NADH dehydrogenase
•*furA*	Ferric uptake regulator
•*mabA*	3-Ketoacyl reductase
•*Rv0340*	Unknown
•*iniB*	INH-inducible gene
•*iniA*	INH-inducible gene
•*iniC*	INH-inducible gene
•*srmR homolog*	regulatory gene
•*fabD*	Malonyl-CoA ACP transacylase
•*accD6*	Acetyl-CoA carboxy;ase
•*fbpC*	trehalose dimycolyl transferase
•*fadE24*	Fatty acyl-CoA dehydrogenase
•*nhoA*	Acrylamine *N*-acetyl transferase
•*efpA*	Efflux protein
•*Rv1592c*	Unknown
•*Rv1772*	unknown

* The 5 genes known to be involved in INH resistance (*katG, inhA, kasA*, *ndh* and *ahpC*) that were used to screen for mutations associated with INH resistance; • Fifteen genes known to be induced after exposure to INH (18).

Table 2. PCR primers and conditions used to amplify INH gene associated mutations

Genes region	Forward primers	Reverse Primers	Size (bp)	Tm (°C)	Reference
katG	5' CAGAAACCACCACCGGAGCC '3	5' GCTGGTGATCGCGTCCTTAC '3	945	59	Current
	5' TGGCCGCGGCGGTCGACATT '3	5' TCGGGGTCGTTGACCTCCCA '3	804	66	Current
	5' CCGACGATGCTGGCCACTGA '3	5' GACCTCGACAAGCGCCCGCA'3	997	64	Current
inhA prom	5' CGCAGCCAGGGCCTCGCTG '3	5' CTCCGGTAACCAGGACTGA '3	246	60	Current
inhA	5' CACGTTACGCTCGTGGACAT '3	5' GGGTTCATGATCGGCAGGAG '3	1300	64	Current
kas operon	5' CTGAAAGCATCGACAGCAATG '3	5' GCA ACG CAA TCA CGT GTC TA	500	59	Current
kasA	5' GTTCAGGCGAGGCTTGAG 3'	5' GCGATGTCGTGCTTCAGTAA '3	1293	50	(10)
fabD	5' AAAAACATAGCTTACAGGCCCG '3	5' GTTGTGTACAAATCGAACTGACG '3	1071	59	(10)
accD6	5' GACAGGAGACCTGCGATGAC '3	5' GGCAGAACAATCCGACCA '3	655	59	(10)
	5' GGTTGGTCGGATTGTTC '3	5' CCTCGCGCGTGCGATTCTG '3	842	55	(10)
ahpC prom	5' CGACTG GCTCATATCGAGAA '3	5' ATGAGAGCGGTGAGCTGGTA '3	411	49	(10)
ahpC	5' GCTGATTGTCCGAGAGCATCG '3	5' GGTCGCGTAGGCAGTGCCCC '3	701	60	(10)
	5' CCGATGAGAGCGGTGAGCTG '3	5' ACCACTGCTTTGCCGCCACC '3	236	66	(10)
furA	5' GCGATCGGGTCCTAGCAG '3	5' TTCATATGACCCACGACGG '3	641	61	(10)
ndh	5' ATCACCACCGCCGCTGAAGC 3'	5' GTTCGGGTACCCGGGAATG '3	1134	65	(10)
	5' CATTCCCGGGTACCCGAAC '3	5' GTCGACCGTTTTGGCGTTGG '3	535	65	(10)
mabA	5' CACGTTACGCTCGTGGACAT	5' AATCCGTTTGCCGTCCAGCA '3	500	60	(10)
Rv0340	5' ATGCGTCGTATGCTTGG '3	5' CCAAACACCTATCGGGATC '3	850	54	(10)
iniBAC prom	5' GAAATACCCGACACGACCAG '3	5' CCACCGATGAGATTTGGTG '3	450	59	Current
iniB	5' ATAAGTTCCGGACCGGCG '3	5' CGACAGATGAGGCATAGCAG '3	1053	56	(10)
iniB-iniA	5' TTGAACGGCGCTGCTATG '3	5' GTGCTGATGTCATCGACGG '3	1070	60	(10)
iniA	5' CAACCGCAGCGGTTGACAT '3	5' CCGCATGCCGATAATCATT 3'	1079	61	(10)
iniA-iniC	5' GGAATCGAAACCGCTGCG '3	5' CCAGCCCACCGATCTGTTTGA '3	1090	55	(10)
iniC	5' TCCTGTTGCGCACCCTGAAC '3	5' AACATGTTCCACCCGGTGGC '3	1040	65	(10)
srmR homolog	5' GCCAGTACCGGATCGACG '3	5'GTGTCGCGGACATCCTGG'3	819	63	(10)
	5' CCAGGATGTCCGCGACACC '3	5'TGTCCGGGTGCGAGCAAC'3	835	67	(10)
fbpC	5' GCATGGGTCTCTCCTCTG '3	5' GCTGATACCAGTCGGTGTAG '3	635	55	(10)
	5' GCCAATCCAGTTTCTACACCG '3	5' ATGTTCCACACATCGGC '3	732	55	(10)
fadE24	5' GCTATCAGATGCCTGGCG '3	5' GTGGGATCGAATAGTGGCTG '3	890	59	(10)
nhoA	5' CTAGTGGCGCGAGCAGAC '3	5' CCGACGTCGACGAGATAGC '3	927	61	(10)
	5' GCTATCTCGTCGACGTCG '3	5' GCATTCTACGTCTACGCCG '3	662	59	(10)
efpA prom	5' GGGGCTATGAAGAAGTGCTG '3	5' GCTCTGAAGCGGTCTCCTC '3	500	59	Current
efpA	5'ACCTCCCGCGATCATCG-3'	5' CGTCGAGCTTCATCCGTTC '3	810	59	(10)
Rv1592 prom	5' AAAGAATTCGCGCTCTCAA '3	5' GTGGGAAGTAGAACGGATCG '3	450	59	Current
Rv1592c	5' AACTCGGCGTACCCAACC '3	5' ACACGCTCGGAATTCAAGG '3	804	55	(10)
	5' GAGCGTGTCGGGTTGTCC '3	5' CGAGGTTGTGTGCCAGGTC '3	834	55	(10)
Rv 1772 prom	5' CCGAAGAGCGATGAGGATAC '3	5' TCATGTTTCCGATTGAGGTG '3	500	59	Current
Rv1772	5' CGGGTGTTTCTCAACGAC '3	5' GGACTGGACTCGCTGATTG '3	633	55	(10)

***** All PCR reactions were run for 40 cycles and proceeded by a denaturation step at 95 °C for 15 min and included a final extension step at 72 °C for 10 min

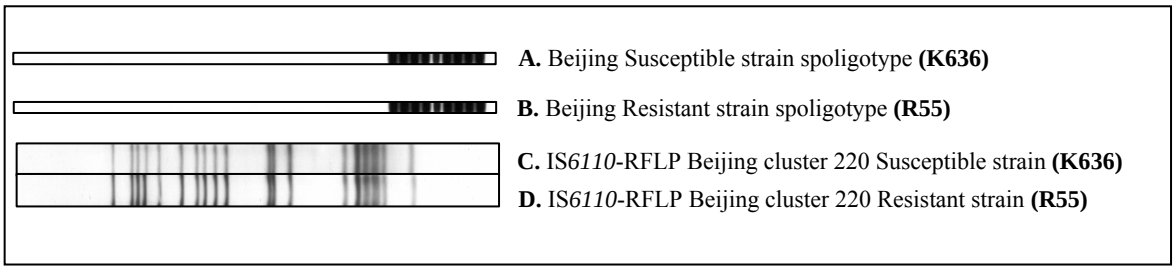

Fig 1. Spoligotype patterns and IS*6110*-RFLP profiles of strains investigated. **A)** Beijing K636 susceptible spoligotype, **B)** Beijing R55 resistant spoligotype **C)** RFLP-IS*6110* profile of K636 Beijing cluster 208 susceptible strain **D)** RFLP-IS*6110* profile of R55 Beijing cluster 208 resistant strain.

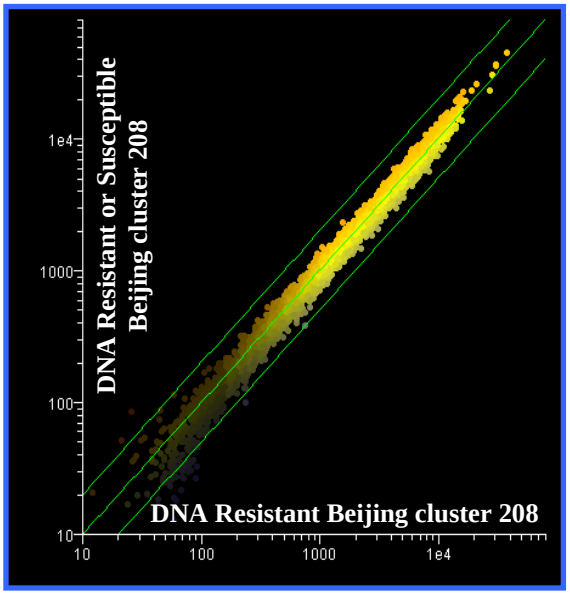

Fig 2. DNA vs DNA Microarray analysis: **2)** DNA Microarary analysis was performed on genomic DNA obtained from the (drug susceptible strain (K636) vs the INH mono drug resistant strain (R55). No differences were identified, indicating that these two strains were isogenic (data not shown).

Culture and drug treatment

The *M. tuberculosis* $H_{37}Rv$, Beijing susceptible (K636) and resistant (R55) strains were grown on Löwenstein–Jensen (LJ) slants at 37 °C for three weeks with continuous aeration. One LJ was used to start an initial culture for subsequent RNA analysis. Ten milliliters 7H9 Middlebrook medium (Becton, Dickinson, Sparks, MD 21152, USA) (supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween 80 and 10 % ADC) in vented screw-cap, tissue culture flasks (Greiner Bio-One, Maybach street, Germany) was incubated with colonies from the LJ slant at 37 °C (without shaking). After 10 days, cultures were sub-cultured into 20 ml 7H9 Middlebrook media (supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween 80 and 10 % ADC) and after 10-14 days of growth (0.7-0.8 OD_{600}), 1 ml aliquots were taken and stored at -80 °C.

Two biological replicate experiments were performed (which included 2 biological and 2 technical replica experiments for each strain) in which a starter culture was set up by diluting an inoculum of the initial culture 1:100 into 80 ml fresh 7H9 media and incubating at 37 °C without shaking until mid log phase (0.6-0.8 OD_{600}). At this point the liquid culture was divided into two portions; INH was added to 40 ml of the culture, while the remaining 40 ml was untreated and served as the control. Drug treatment was done by adding filtered-sterilized stock solution of INH in water (INH was prepared as per manufacture's instructions) (Becton Dickinson, Maryland 21152, USA) to achieve a final concentration of 0.1 µg/ml. Cultures were harvested by centrifugation after completion of the drug treatment periods of 5 hrs and 24 hrs. Similarly, the control liquid cultures that were not exposed to INH were harvested at 5 hrs and 24 hrs.

RNA isolation

One hundred milliliter of a 5M guanidinium thiocyanate (GITC) (Sigma-Aldrich, St. Louis, USA) solution (5 M GTC, 0.5% sodium *N*-lauryl sarcosine, 0.1 M ß-mercaptoethanol, 12.5ml of a 1M sodium citrate pH 7.0 and 1% Tween 80 made up to 500 ml with RNase free water and filter sterilized) was added to each 2 x 20 ml culture. Bacteria were harvested by centrifugation (20 min. 3000 x g, 19 °C) and the top aqueous layer was discarded. The pellet was resuspended in 1.5 ml Trizol (Life Technologes, Gaithersburg, MD). The trizol suspension was then transferred to a FAST Prep tube (Bio 101, Vista CA) and mechanically disrupted in a Ribolyzer (reciprocal shaker: Hybaid) for 45 sec at 6.5W for 2 cycles and cooled on ice for 1 minute between pulses. The samples were centrifuged at 13000 r.p.m. for 1 min in a microfuge and the trizol solution that is above the bed of beads was collected into a 2 ml phase lock gel tube (Eppendorf, Hamburg 22331, Germany) tube containing 300 µ1 of chloroform isoamyl alcohol (24:1 v/v) (Sigma-Aldrich,St. Louis, MO, USA). The tube was inverted several times before the aqueous layer was recovered by centrifugation (5 min, 13 000 r.p.m). The aqueous layer was transferred to a clean 1.5 ml eppendorf tube and the RNA was precipitated with the addition of an equal volume of isopropanol (Merck, Darmstadt, Germany). This was followed by incubation at -20 °C for 16 hrs. The crude RNA fraction was then pelleted (30 min, 13 000 r.p.m, 4°C) and washed with 1 ml 70 % ethanol (Merck, Darmstadt, Germany), air dried and re-dissolved to a final volume of 70 µl. Crude nucleic acid concentration was determined spectrophotometrically. Remaining traces of DNA were digested by treating 30 µg RNA with 6 units DNase I (Ambion) at 37 °C for 30 min. Followed by DNase I inactivation at 70°C for 10 min. The quality and concentration of the purified total RNA was analysed by gel electrophoresis, as well as by analysis using the Agilent 2100 Bio Analyzer (Agilent Technologies).

Quantitative Real-time -PCR (*QRT-PCR*)

cDNA was synthesized from 1µg of purified RNA according to the manufacture's instructions (Quantitect® Reverse Transcriptase kit (Southern Cross Biotechnologies)). Primers for each gene of interest (Table 3) were designed using Primer software 3 version 0.2 (Whitehead Scientific). Light cycler Master mix was prepared by adding 1 µl of a 1:10 dilution of the cDNA, 1 µl of each primer pair (10 µM) (Table 3), 2 µl Light Cycler Faststart DNA Masterplus SYBR green Mix (Roche Diagnostics, Indianapolis,USA 0001) and RNase free water to a final volume of 20 µl. QRT-PCR was performed using a Lightcycler 2.0 system (Roche Diagnostics). A four step PCR parameter protocol was used: (i) denaturation program (95°C for 10min), amplification and quantification program repeated for 40 cycles (95 °C for 10 s, 59 °C for 10 s, 72 °C for 6s), (ii) melting curve program (95 °C for 0 s, 65 °C for 15 s, 95 °C with a heating rate of 0.1 °C/s) and a cooling down program of 40 °C for 30 s. The quantity of cDNA in each reaction was determined from the exponential phase of amplification from the cycle threshold (Ct). The transcriptional level of each candidate gene was quantified by using the delta-delta Ct calculation in which the relative abundance of mRNA of the target gene was normalized relative to the levels of the reference RNA transcripts (16S rRNA and *ftsZ*) and the control samples. Data analyses were done according to the delta-delta Ct equation $R=2^{-(\Delta Ct\ sample-\Delta Ct\ control)}$ and no efficiency correction are performed.

Table 3. Primers used in QRT-PCR

Gene	Forward primer	Reverse Primer	Size (bp)	Tm (°C)	Reference
kasA	5'CCGACCCTGAACTACGAGAC'3	5'AACCCGAACGAGTTGTTGAC'3	100	60	Current
ahpC	5'TCAGCAAGCTCAATGACGAG'3	5'ATCGGGAAGGGTAACGTTTT'3	120	60	Current
iniA	5'GTCATGATTGGCATGCTGTC'3	5'AGCAACCGGTTTTGTTTGTC'3	120	60	Current
iniB	5'GCTAGCCAGATCGGTGTCTC'3	5'GCGTTGGAAGCCAACACT'3	125	60	Current
efpA	5'TAGGTTTCATCCCGTTCGTC'3	5'CACGGTGCATGAAAAATGAG'3	115	60	Current
Rv1592	5'TCCGTTCTACTTCCCACCTG'3	5'TACATGTTCGTGGTCCGGTA'3	120	60	Current
Rv1772	5'TGGGTTCAACAGGAGGTAGC'3	5'CCGAAGAGCGATGAGGATAC'3	120	60	Current
fbpC	5'ATATCTGCAGGTGCCATCC'3	5'ATGTCCCAGCCGTTGTAGTC'3	120	60	Current
fadE	5'AGCCAGCGACAACGACTATT'3	5'TTCACATACGGGACGACGTA'3	120	60	Current
acpM	5' AGGACAAGTACGGCGTCAAG'3	5' GGGGTTCTCCGACTCAATCCT '3	120	60	Current

RESULTS

QRT-PCR analysis was done on 11 genes previously shown to be up regulated when *M. tuberculosis* was exposed to 1 µg/ml of INH (18) (Table 4). QRT-PCR analysis showed the occurrence of significant changes (where a 5 fold increase/decrease in expression was defined as significant) on a transcriptional level of the selected genes in both resistant and susceptible strains after exposure to 0.1 µg/ml of INH for 5 hrs and 24 hrs (Table 5), respectively. After 5hrs of exposure, most of the INH induced genes in both the resistant and susceptible strain showed a low level of response except for *accD6, acpM, efpA* and *Rv1772* that showed significant differences in expression between the susceptible and resistant strain. In comparison to the untreated INH control, it is interesting to note that *accD6*, *acpM, Rv1772* and *efpA* were upregulated in the drug resistant strain (R55). In comparison, the transcript levels of *accD6* and *acpM* were significantly up-regulated in the drug susceptible strain (K636). A delayed response after 24 hrs of INH treatment was observed in which the following genes in the resistant strain (R55) was found to be significantly up-regulated in *ahpC, kasA, iniA, iniB* whereas *fbpC* was found to be down regulated. In the susceptible strain (K636) *iniA, iniB* and *fbpC* found to be significantly up-regulated after 24 hrs while a down regulation was observed in the *acpM* gene.

Table 4. Gene differentially regulated after 1µg/ml of INH treatment (18).

ORF	Gene	Product
Rv3140	*fadE23*	probable acyl-coa dehydrogenase
Rv2846c	*efpA*	possible integral membrane efflux protein
Rv2428	*ahpC*	alkyl hydroperoxide reductase c protein ahpc
Rv2247	*accD6*	acetyl/propionyl-coa carboxylase
Rv2245	*kasA*	beta-ketoacyl-ACP synthase
Rv2244	*acpM*	Acyl carrier protein
Rv1772	*Rv1772*	hypothetical protein
Rv1592c	*Rv1592c*	conserved hypothetical protein
Rv0342	*iniA*	isoniazid inductible gene protein iniA
Rv0341	*iniB*	isoniazid inductible gene protein iniB
Rv0129c	*fbpC*	Trehalose dimycolyl transferae

Table 5. QRT-PCR gene expression profile of INH induced genes

Genes	QRT-PCR			
	Fold change 5hrs		Fold change 24 hrs	
	#K636	$R 55	#K636	$R 55
ahpC	2.3	2.5	2.7	10.77**
kasA	2.7	2.8	1.3	23.3**
accD6	11*	6.7*	15.6**	5*
acpM	52*	4.9*	35.4**	5.6*
iniA	-2.7	1.5	2.3	10.2**
iniB	-2.9	1.6	4.4**	25.7**
Rv1592	3.8	1.6	4.4	2.5
Rv1772	1.7	6.2	2.9	7.7**
fbpC	2.1	1.1	13.4**	-7♦♦
fadE	1.1	-1.1	-1.1	2.1
efpA	2.6	12.5*	3.0	16.7**

Legend 5: $Resistant strain (R55); #susceptible strain (K636); * genes that are upregulated in comparison to control after 5hrs; ** Significant up regulation of genes after 24hrs of treatment; ♦♦ significant down regulated after 24hrs of INH.

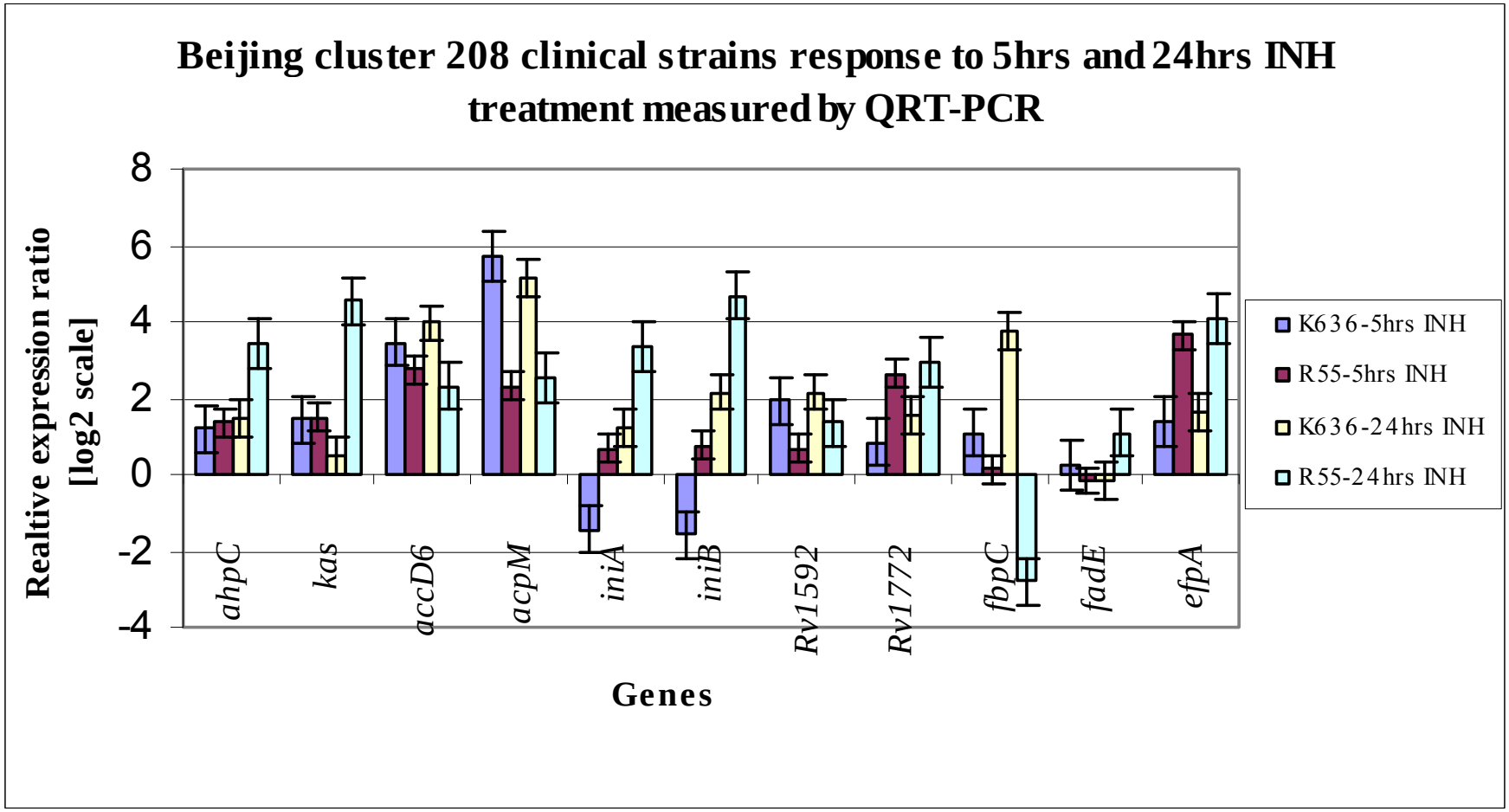

Fig 4. Response of INH induced genes in a Beijing cluster 208 resistant (R55) and susceptible (K636) strain as measured by QRT-PCR analysis. Ratio of the cDNA copies detected in each sample relative to the time zero control by QRT-PCR at 5hrs and 24hrs

Discussion

This is the first report to describe gene expression in well characterized isogenic Beijing strains after exposure to low concentrations of INH which have a bacteriostatic effect on cell growth in pan susceptible strains. Previous reports on INH induced gene expression were based on studies performed on the laboratory strain $H_{37}Rv$ or other resistant clinical isolates after exposure to lethal concentrations of INH (3,5,6,17,18). These studies indicated that a number of genes were regulated after induction with INH. It was suggested that differences in observed gene expression levels could have resulted from either the different strains analyzed or the concentration of INH used. The genes identified are biochemically relevant to the drug's mode of action, but their functional role in acquiring INH resistance in clinical isolates remains unclear. It is also not clear which modulators are involved in the regulation of these genes in certain strains, thereby conferring a resistant phenotype. It is specifically important to know which genes are regulated by low levels INH in resistant clinical isolates since it may provide novel insights into the mechanisms of resistance in clinical isolates where the classical mutations conferring resistance are absent.

Genes that was differentially regulated included acyl-coA dehydrogenase (*fadE)* and Trehalose dimycolyl transferase *(fbpC).* Both of these genes play a role in mycolic acid biosynthesis which form an important part of the cell envelope and inhibition may lead to cell death. *FadE* is a negatively controlled gene that binds to transcription factors that regulate the expression of fatty acid biosynthesis. *FbpC* is an export protein that has a catalytic effect for the maturation of the mycolic acids in the cell wall. *FbpC* was found to be upreglated in the susceptible strain and down regulated in the resistant strain. Other genes identified include a efflux protein (*efpA*) that play an important role in drug tolerance as well as 2 isoniazid inducible gene called *iniA* and *iniB.* The*" ini"*

genes encode transmembrane proteins that act through pump like mechanisms to actively pump the drug out of the cell, resulting in increase resistance to INH. The *kasA* genomic region consists of 5 genes in an operon (*fabD, acpM*, *kasA, kasB* and *accd6*). All of these encode for enzymes that is involved in the fatty acid biosynthesis pathway. It has previously been shown that these genes are differentially regulated in *M. tuberculosis* in the presence of activated INH. *Rv 1592* and *Rv1772* are conserved hypothetical proteins of unknown function. It is likely that these hypothetical proteins may play important roles in the cellular homeostatis of the cell and further studies are required to define their role in *M. tuberculosis*. Thus, the possibility exists that the expression of these genes activates certain pathways, which could be linked to INH resistance.

In this study, we selected a list of candidate genes which have been previously described to be regulated when H_{37}Rv was exposed to 1 μg/ml of INH (18). These include genes from part of the FASII complex, which are important in the pathway for the synthesis of mycolic acids. However, the precise mechanism of action of some of the genes remains unresolved. According to our QRT-PCR results (Table 5, Figure 4 and 5) and current opinion, it is suggested that in a susceptible strain, INH enters the bacterial cell as a prodrug and is activated by catalase peroxidase produced by KatG. Activated INH then binds to the NADH in the pocket of the enoyl-acyl carrier reductase (InhA), by forming a covalent complex between the KasA-AcpM proteins. As a result of this activated INH, mature mycolates are not produced. This accumulation of mycolates is then sensed by the KasA-AcpM resulting in an increase in the expression of AcpM (Fig 5). A increase in the expression of fbpC, one of several exported proteins, was observed. We proposed that fbpC is upregulated due to the accumulation of mycolic acid. As a result can no mature mycolates be transferred to the cell wall, and thus rendering the bacterium susceptible to INH which then result in cell death.

According to our QRT-PCR results (Table 5, Figure 4) we propose (Figure 5) that in a resistant strain (in the absence of any gene mutation), INH enters the cell and is activated by catalase peroxidase. KatG on its own is not sufficient to detoxify H_2O_2, and this imbalance is sensed by the organism resulting in an increase in the synthesis of AhpC. This delayed AhpC response assists with the detoxification of the reactive oxygen species from the bacterial cell, as well as the possible inactivation of INH. This activated INH is then sensed by the *efpA* gene resulting in an up-regulation of expression and as a result INH is actively pumped out of the cell. A delayed increase in expression of *iniA* and *iniB* genes was also observed, suggesting that these proteins may assist in the action of EfpA to detoxify the cell. Down regulation of fbpC was observed implying that mature mycolates was formed and exported to the cell wall assisting in the efflux of INH from the cell. A increase in *accD6* and Rv1772 activity was also observed, however, the role of these proteins in the INH metabolic pathway remains unclear.

We therefore conclude that drug resistance in tuberculosis may be a combination of mutations in certain genes and their regulatory domains which regulate the intracellular concentration of the drug. However, these mutations remain to be determined. The application of whole genome sequencing of these two strains could provide novel insights into the mechanisms used by this strain to evade the toxic effect of INH.

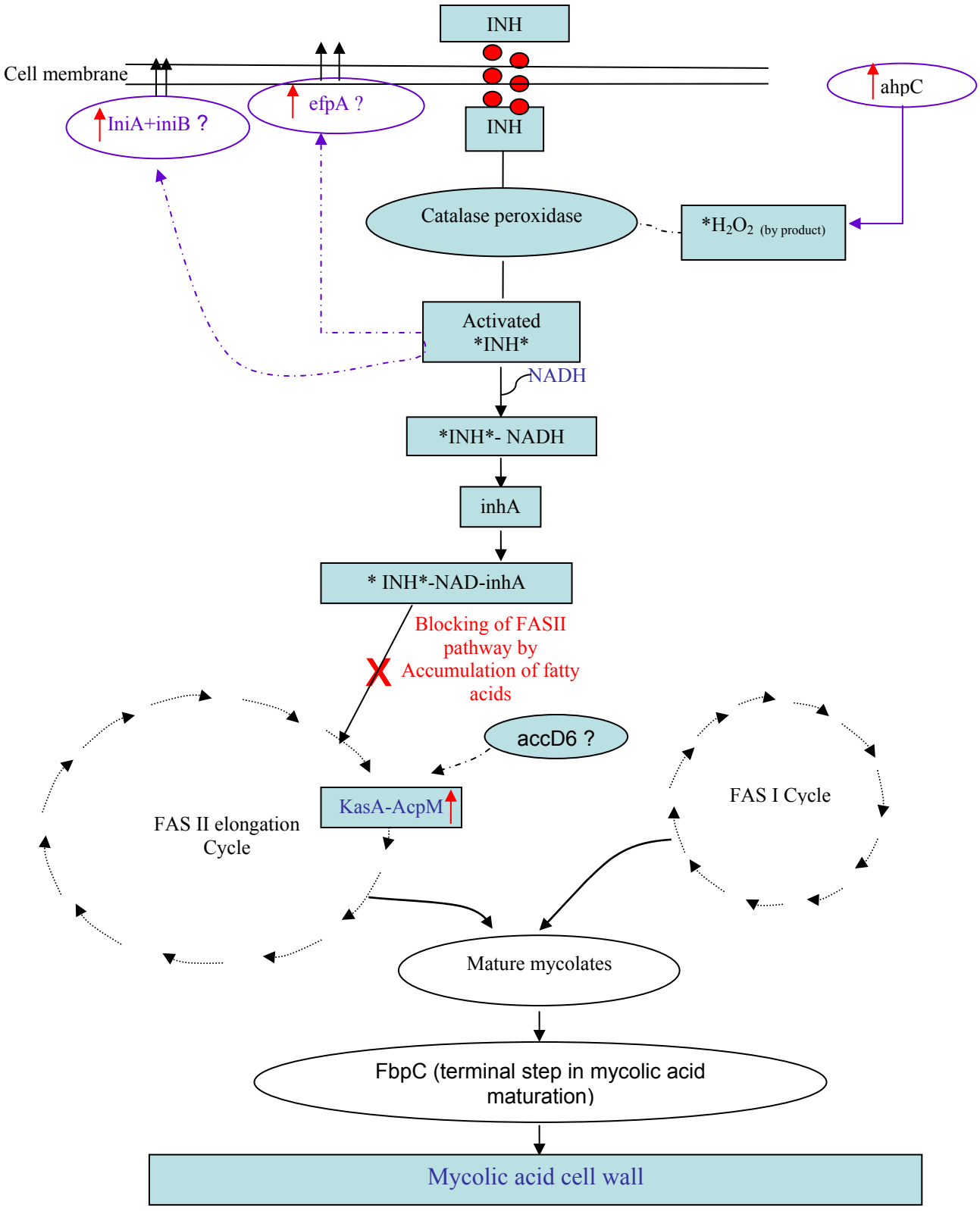

Fig 5. There hypothesize mechanism of INH action in susceptible and resistant strain with respect to results obtained by QRT-PCR

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Chapter 8

Protein expression profile of a susceptible and resistant *Mycobacterium tuberculosis* Beijing lineage after low level INH treatment

My contribution to this project: Planning of project
Strain culture and growth, Protein isolation, 2-D gel electrophoresis Data analysis,
Writing and editing of manuscript

ABSTRACT

Isoniazid is a front line anti-tuberculosis drug. However, about 26 % of isoniazid resistant Beijing clone 208 phenotype lack a known gene associated mutation. Thus, the aim of this study was to make use of proteomics in order to identify any protein that might play a role in isoniazid resistance. A Beijing cluster 208 fully drug susceptible and a isoniazid mono resistant (with no gene associated mutations) was selected from a existing strain bank for protein analysis. Both strains were grown to mid log phase after which it was exposed to 0.1 µg/ml of isoniazid. Cultures were harvested upon completion of drug treatment of 5 hrs and 24 hrs. Differentially expressed protein spots were excised from the gel and used for subsequent Q-TOF analysis. Protein differences identified between Beijing resistant and susceptible *M. tuberculosis* strains irrespective of drug treatment included; major membrane protein, Rv3418c (GroES), and Rv3875 (Esat6). Expression differences observed after isoniazid treatment for the Beijing cluster 208 strain included two hypothetical proteins; Rv3678 and Rv2626c as well as a Rv1980c and Rv2428. Proteins identified were stress induced proteins, thus we hypothesize that the response observed in this study might not be due to the toxic effect of the drug, but rather reflect an isoniazid induced stress response.

INTRODUCTION

The increase in the incidence of drug resistance has stimulated interest in understanding the molecular mechanisms of drug resistant tuberculosis. Completion of the *Mycobacterium tuberculosis* (*M. tuberculosis*) whole genome sequence (8) has enabled the annotation of 3998 genes, which serves as a frame-work to understand how their encoded proteins could aid in the understanding of the development of drug resistance (23). Conventional proteomics relies on high-resolution two-dimensional (2D) gel electrophoresis, mass spectrometry (MS) and database searching for protein identification. MS analysis entails the ionization of unknown proteins using matrix assisted laser adsorption ionization (Maldi) (1). Following ionization, the mass of the peptides is determined using, for example, quadrupole-time of flight (Q-TOF) analysis. These peptide masses can then be compared to a database to identify the protein.

Researchers are currently striving to obtain a more complete understanding of the tuberculosis (TB) proteome and how it is influenced by current anti-TB drugs. Bacteria develop resistance to an antibiotic through spontaneous mutation and antibiotic selection. In the selective environment (in the presence of a drug), the sensitive bacterial population is killed and the resistant mutants are allowed to grow. This type of resistance is called acquired resistance and is defined by mutations in specific genes (16).

Isoniazid (INH) is a first line anti-TB drug and has been in use since 1952. The manner in which INH kills *M. tuberculosis* remains unclear. However, it has been hypothesized that *M. tuberculosis* develops resistance to INH through mutations in genes (*katG*, *inhA*, *ndh*, *ahpC* and *kasA*) or their promoters. These mutations result in blocking the INH from binding to its targets (18,21). However,

mutations in these genes account for only about 70 % of INH resistance (9,16). Thus, approximately 30 % of clinical isolates are phenotypically resistant to INH and do not have mutations in these genes (9,16). This implies that other, as yet unknown mechanisms (or genes), are involved in INH resistance.

Molecular epidemiological studies have suggested that *M. tuberculosis* strains with the Beijing genotype have a higher propensity to acquire drug resistance (12). In this study setting the Beijing strain family is the most prevalent (31 %) drug resistant strain family (Chapter 5) and a quarter of these strains have an INH resistant phenotype in the absence of classical mutations. Thus the aim of this study is to determine what effect a bacteriostatic concentration of INH has on the proteome in order to identify proteins which may be up-regulated and thereby confer a INH resistant phenotype.

MATERIALS AND METHODS:

Setting up initial culture and M. tuberculosis growth rates

An H_{37}Rv, *M. tuberculosis* Beijing cluster 208 fully susceptible (K636) and INH mono-resistant strain (R55) were selected from an existing strain bank at the University of Stellenbosch. An initial culture was set up by scraping colonies from a Lowenstein-Jensen (LJ) slant, and then inoculated into 10 ml 7H9 Middlebrook medium (supplemented with 0.2 % (v/v) glycerol, 0.1 % Tween 80 and 10 % albumin-dextrose-catalase (ADC)) in vented screw-cap, tissue culture flasks (Greiner Bio-One, Maybach street, Germany). The culture was incubated without shaking at 37 °C. After 10 days the cultures were sub-cultured into 20 ml supplemented 7H9 Middlebrook media and after a further 10-14 days of growth (0.7-0.8 OD_{600}) at 37 °C, 1ml aliquots were stored at -20 °C.

Drug treatment

A starter culture was set up by inoculating 0.8 ml freezer stock into 80 ml fresh supplemented 7H9 media (1:100) and incubating at 37 °C (without no shaking) until mid log phase (0.6-0.8 OD_{600}). Thereafter, the culture was divided into two; 40 ml of the culture served as control and the remaining 40 ml was incubated in the presence of INH (dissolved in H_2O) (Becton Dickinson, Maryland 21152, USA) at a final concentration of 0.1 µg/ml at 37 °C for 24 hrs. After incubation at 37 °C, the *M. tuberculosis* bacilli from each culture (cultured in the absence or presence of INH) were harvested by centrifugation (3000 *g*, 20 min).

Whole cell lysate protein isolation

Mycobacterial pellets were washed twice with 1 ml Phosphate buffer saline (pH7.4) containing 1 % (v/v) Tween-20 and centrifuged (13 000 rpm, 10 min). An equal volume (pellet volume) of silica beads (Bio 101,Vista,USA) and 200-500 µl lysis buffer (0·3 % (w/v) SDS, 200mM DTT, 50 mM Tris/HCl pH 7.0, 1 mM phenylmethanesulfonyl (Sigma-Aldrich, Germany) and complete protease inhibitor cocktail (Roche Molecular Biochemicals)) were added to the pellet and mixed by vortexing for 60 seconds. The cell suspension was then incubated at 80 °C for 20 min to inactivate the bacilli, after which it was rybolized (Bio 101, Vista, USA) for 45 seconds at 6.5 W for 2 cycles, with cooling on ice between pulses. The samples were incubated at 100 °C for 10 min after which they were centrifuged (13 000 rpm, 10 min) and the supernatant transferred to a clean 1.5 ml eppendorf tube. Protein concentration were determined according to the Bradford Assay (5).

2D gel electrophoresis.

2D gel electrophoresis was performed as described previously by Betts et al (2), with minor changes. Briefly; approximately 50 µg of total protein was resuspended in rehydration buffer (8M urea, 2 % CHAPS, 10mM DTT, 2 % Pharmalyte and a trace of bromophenol blue) and made, up to a final volume of 200 µl, applied to a pH 4-7 isoelectrical focusing (IEF) strip for 20 hrs. IEF was performed under the following conditions; 100 V for 2 h, 300 V for 2 hrs, 1000 V for 1 hr and 3500 V for 20 hrs. After IEF focusing, strips were stored at -80 °C. Second dimension electrophoresis was done by placing the equilibrated IEF strips onto a 12 % PAGE (http://www.expasy.ch/ch2d/protocols/) and sealing it with agarose (Bio-Rad Laboratories, Hercules, CA 94547). Gels were stained with silver stain compatible with mass spectrometry (http://www.expasy.ch/ch2d/protocols/) (4). Gels were scanned and recorded using a GS-800 Calibrated Densitometer (Biorad) and quantitative spot detection and matching was visually. When protein spot differences were noted, gels were rerun, stained with a silver stain compatible with MS (4) and the spots of interest were excised from the gel.

Scoring of differentially expressed protein spots

Spot differentiation was visually recorded at least three times in three separate experiments. Spot differences for the quadruplicate gels were scored as follows : a protein spot was graded from + to +++, where + indicated the presence of a spot, ++ and +++ indicated an increase in spot intensity. The absence of a protein spot was indicated by a “-“.

Sample preparation for Q-TOF analysis

The excised gel spots were placed in 50 µl of double distilled MilliQ H_2O and sent to Imperial College in London where they were analyzed by Q-TOF analysis. Peptide sequence data generated at Imperial College was then searched against the non-redundant protein MASCOT database (http://www.matrixscience.com) to identify the protein of interest.

RESULTS

Fig 1 shows the electrophoretic fractionation of whole cell lysate (WCL) proteins in a 2D silver-stained gel (pI range of pH 4-7 with a molecular weight of 10-66 kDa) that reveals approximately 300 distinct protein spots.

Comparison of protein expression between the susceptible and resistant strain (irrespective of drug treatment) showed 7 differentially expressed protein spots (Table. 1, Fig 1 and 2A). Only 5 of the 7 protein spots could be structurally and functionally identified by Q-TOF analysis. Three of the 5 protein spots had peptides that matched the annotated genome sequence; the remaining 2 protein spots were isoforms. The major membrane protein (MMP) showed increased expression in the susceptible strain relative to the resistant strain. The protein spot identified as ESAT-6 was found to be more dominant in the susceptible strain compared to the resistant strain. Different isoforms of the GroES protein were identified and variation in spots between the different isoforms was observed. For example, in one protein spot, the protein GroES was found in abundance in the resistant strain

(+++) compared to the susceptible strain (+), while in another protein spot with the same matched peptides (GroES), the protein was found in abundance in the susceptible strain (++) compared to the resistant strain (+).

Comparison between the INH untreated control and 24 hrs INH treated cultures revealed 7 differentially expressed proteins (Table 2, Fig 1 and 2B) of which 5 protein spots could be structurally and functionally identified by Q-TOF analysis in both susceptible and resistant cultures. Four of the 5 protein spots had peptide sequence that matched the annotated genome sequence; the remaining protein spot was an isoform (Table 2). The identified and the relative expression levels for each protein are shown in Table 2. Two of the protein spots encoded for hypothetical proteins (Rv3678 and Rv2626c) while the other three remaining protein spots encoded for ORF with known functions: i) different isoforms of alkyl-hydroperoxide reductase (Rv2428, AhpC) and ii) an immunogenic protein (Rv1980c, Mpt64)). In both resistant and susceptible strains an increase in the expression of the AhpC protein was observed. The Mpt64 protein was found to be down regulated in both resistant and susceptible strains.

Table. 1 Protein differences observed between Beijing Resistant (R55) and Beijing Susceptible (K636) *M. tuberculosis* isolates irrespective of drug treatment

Spot	ORF	Protein	Function	Matched Peptide	MW (kDa)	pI	Resistant	Susceptible
8	No. ID						+	-
9	Unknown Rv number	MMP	Major membrane protein, belongs to alpha-crystallin HspS, Family of small stress induce proteins	(R)SEFAYGSFVR(T) (R)TVSLPVGADEDDIKATYDK(G)	19	5.9	+	+++
10	Unknown Rv number	MMP	Major membrane protein, belongs to alpha-crystallin HspS, Family of small stress induce proteins	(R)SEFAYGSFVR(T) (R)DGQLTIKAER(T)	19	5	+	++
11	No. ID						+	+++
12	Rv3418c	groES	Chaperonin-10	R)DVLAVVSK(-) (K)EKPQEGTVVAVGPGR(W) (K)RIPLDVSEGDTVIYSK(Y)	10	4.6	+++	+
13	Rv3418c	groES	Chaperonin-10	(R)DVLAVVSK(-) (K)EKPQEGTVVAVGPGR(W)	10	4.6	+	++
14	Rv3875	Esat6	Early secretory Ag target, important for virulence	(-)TEQQWNFAGIEAAASAIQGNVTSIHSLTK(L)	6	4.5	+	++

*Relative expression levels was graded from a “+” to a “+++”. Protein spots absent are indicated by “-“.

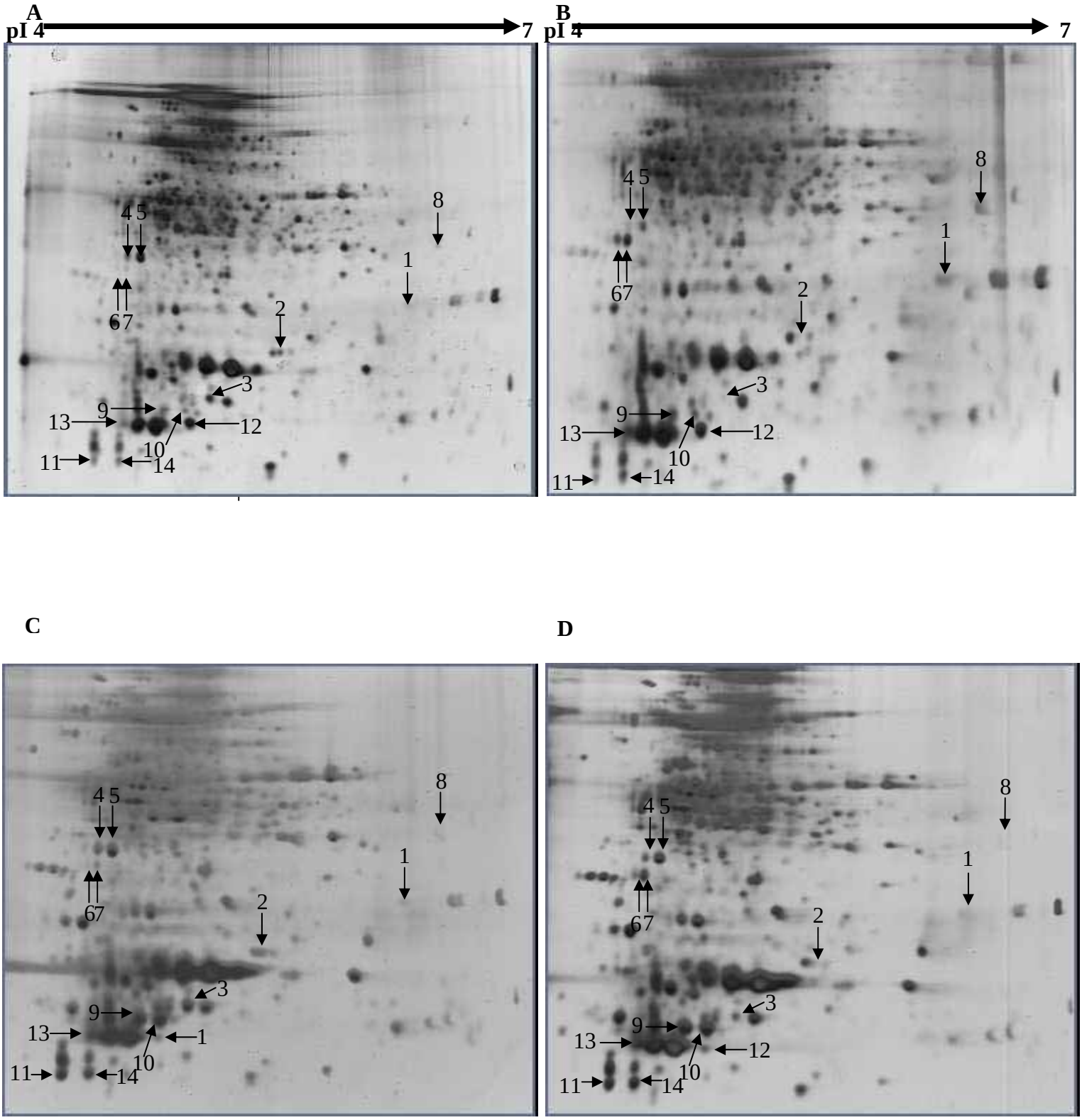
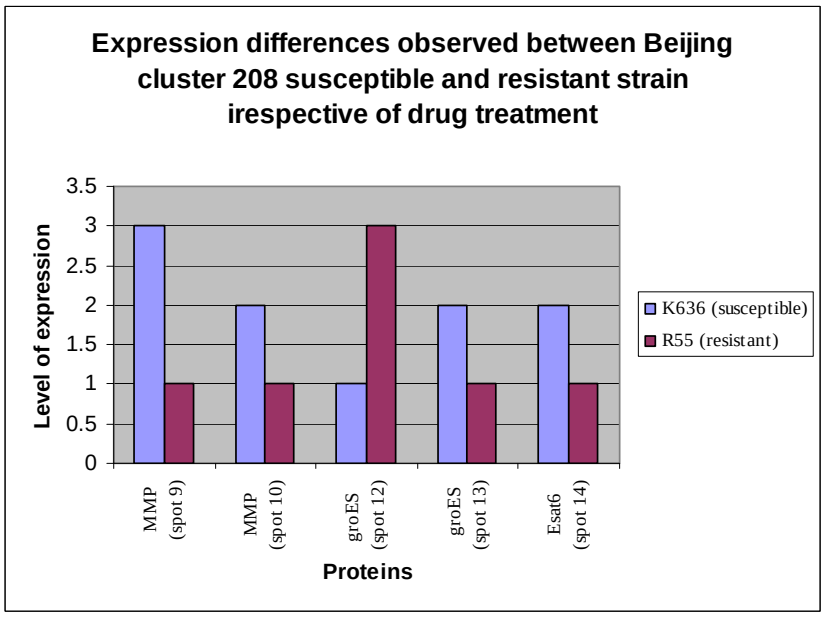


Fig 1. Two-dimensional electrophoresis patterns of *M. tuberculosis* Beijing Cluster 208: A) INH mono resistant isolate (no INH exposure) B) INH mono resistant isolates (treated with 0.1µg/ml INH for 24 hrs) (C) Susceptible isolate control (no INH exposure); D) Susceptible isolate (treated with 0.1µg/ml INH for 24 hrs)

A



B

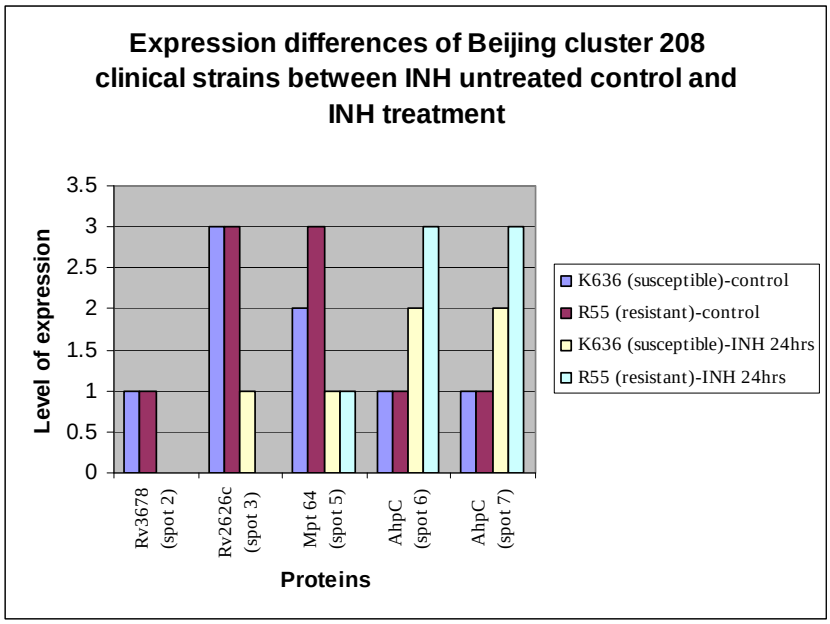


Fig 2. Protein expression differences between Beijing cluster 208 Clinical strains. **A)** Expression differences observed between Beijing INH mono resistant and drug susceptible clinical strains irrespective of drug treatment. **B)** Expression differences of Beijing clinical strain between INH untreated control and INH drug treatment.

Table. 2 Protein differences between Beijing cluster 208 control (no drug added) and 24hrs INH (0.1µg/ml) treatment for both susceptible (K636) and resistant (R55) isolates

							Resistant (R55)		Susceptible (K636)	
Spot	**ORF**	**Protein**	**Function**	**Matched Peptide**	**MW (kDa)**	**pI**	**Control**	**24hrs**	**Control**	**24hrs**
1	No. ID						+	+++	+	+
2	Rv3678	Hypothetical protein	unknown	(R)TGNLVYTAGQLPLEAGKLVR(T) (R)LGQLGVTLPQVAAPLAAYVPAVR(T) (K)VVGFVASAPGFHGQPSVINGASDLLAEVFGDSGAHAR(S)	15	5.1	+	-	+	-
3	Rv2626c	Hypothetical protein	unknown	(R)HLPEHAIVQFVK(A) (K)GLAAGLDPNTATAGELAR(D)			+++	-	+++	+
4	No. ID						++	+	++	+
5	Rv1980c	Mpt 64	Immunogenic protein	(R)GTQAVVLK(V) (K)FLSAATSSTPR(E) (K)SLENYIAQTR(D) (R)DKFLSAATSSTPR(E) (K)VYQNAGGTHPTTTYK(A)	24	4.8	+++	+	++	+
6	Rv2428	AhpC	Alkyl hyroperoxidaseC	(K)DFTFVCPTEIAAFSK(L)	21	4.5	+	+++	+	++
7	RV2428	AhpC	Alkyl hyroperoxidaseC	(R)VVFFWPK(D) (R)ELSQAAGVLNADGVADR(V) (K)DFTFVCPTEIAAFSK(L) (K)PLLTIGDQFPAYQLTALIGGDLSK(V) (K)PLLTIGDQFPAYQLTALIGGDLSKVDAK(Q)	21	4.5	+	+++	+	++

*Relative expression levels were graded from a “+” to a “+++”. Protein spots absent are indicated by “-“

DISCUSSION

In this study, the proteome of a drug resistant and drug susceptible Beijing strain that is dominant in the local community was analyzed to identify differences in expressed proteins after isoniazid exposure. Similar studies but with different experimental approaches, were performed previously (3,11,14,15,19,20). These studies differ with respect to strain type (H_{37}Rv or a clinical strain other than Beijing), growth conditions (early log phase or late log phase cultures or cultures grown under O_2 limitation) and the growth medium used (Sautons medium). Most of the proteins identified in these studies encoded for proteins of either known functional or hypothetical proteins.

When the proteome of the INH induced strains was analyzed, no differences in protein spot intensity could be seen for any of the 5 known proteins (except for AhpC) involved in INH resistance or the additional proteins encoded by the 11 genes thought to be involved in the INH pathway. Previous reports using a early log phase H_{37}Rv culture exposed to 0.1 µg/ml INH showed that 3 proteins (AhpC, KasA and the AcpM) were up-regulated in response to INH (14). The absence of induced proteins thought to be up-regulated by INH could be explained by: i) the translational response of INH in this study was measured by analyzing WCL proteins and a more selective group of INH induced proteins might have been observed if the proteins were fractionated prior to analysis and ii) INH induced proteins responsible for conferring INH resistance fall outside of the pI window of 4-7 with a molecular weight below 30 kDa, iii) high molecular weight proteins may not enter the gel and iv) low levels of INH might not significantly induce a translational response .

Comparison of the Beijing resistant strain to the susceptible strain revealed different isoforms of the MMP. The MMP forms part of the α crystalline (Acr) homolog and consists of a family of small stress-induced proteins (HspS). Acr is a *M. tuberculosis* membrane virulence factor that was found to be more dominant in the susceptible Beijing strain in comparison to the Beijing resistant strain. The Esat 6 protein which plays an important role in virulence was also found to be unregulated in the susceptible strain compared to the resistant strain. These observations are in concordance with the central dogma in which it is believed that susceptible strains are more virulent than drug resistant strains. GroES is a molecular chaperone that cooperates with GroEL during protein folding. These two chaperonins are constitutively express and enhance synthesis of these proteins occur when the bacterium are put under stress. Different isoforms as well as different levels of expression of the same isoform was observed for this protein. We proposed that this phenomenon can be due to the different isoforms present during different stages of protein folding (10).

One of the proteins induced by INH treatment was AhpC, a member of a large family of peroxidases, which is responsible for the antioxidant defense in bacteria (13). Up-regulation of this protein enables the bacterium to evade the toxic effect of INH and in this manner ensures to the bacterium's intracellular survival. In mycobacteria, AhpC not only detoxifies H_2O_2 but also affords protection to cells against oxidative stress exerted by the host immune response (6,7,17). An immunogenic protein Mpt64 was also identified and found to be down regulated. Mpt64 induces strong delayed type hypersensitivity response. Up-regulation of AhpC and down-regulation of Mpt64 protein after INH treatment was consistent with previous reports (14,22). Different isoforms/mobility variants of the AhpC protein and that of the MMP protein could be due to posttranslational modifications.

We hypothesise that the response observed in this study might not be due to the toxic effect that INH has on the bacterium, but rather reflects an INH induced stress response. This observation is based on the fact that the protein spots were up-regulated in both the resistant and susceptible Beijing strains, irrespective of drug treatment.

Based on these observations, we conclude that by using this strategy we were unable to identify new proteins conferring INH resistance as bacteriostatic concentrations of INH does not induce many changes within the proteome of *M. tuberculosis* Beijing cluster 208.

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Chapter 9

Conclusion

Despite the implementation of the direct observed therapy strategy (DOTS) by the World Health Organization, TB still remains a public health problem in many countries. In South Africa, most of the patients infected with *M. tuberculosis* live in poverty under conditions that promote the spread of this infectious disease. Patients diagnosed with an *M. tuberculosis* infection are immediately treated with a 4 anti-TB drug regimen which includes; INH, RIF, EMB and PZA. Drug susceptibility testing (DST) is only done on patients who have previously been treated for TB and on patients who do not respond to two months of therapy. Accurate DST is essential to ensure that the patient receives the most appropriate treatment.

Application of new molecular methods during the past two decades has significantly enhanced our knowledge of TB. Mutations in genomic targets of the drugs have been identified as the major mechanism of drug resistance. In the second theme of this thesis, we have made use of molecular techniques to rapidly identify patients infected with drug resistant strains. We found that more then 90 % of EMB resistance has been missed by the culture procedure used in the routine TB laboratory and that 87 % of these patients were MDR. These results indicate that the inability of culture based techniques to accurately detect EMB resistance could significantly influence the treatment of such MDR cases. Currently, EMB is used as a second-line drug if the *M. tuberculosis* strain is sensitive to EMB. Failure to detect EMB resistance will result in inappropriate therapy which may result in prolonged treatment.

We have successfully detected resistance to INH, RIF and EMB from a 5 day mini culture by using molecular methods. In a comprehensive study, we found that genotypic results from all the samples tested were available in a significantly shorter time interval than the routine culture method. Furthermore, we were able to detect drug resistant–TB in samples which had lost viability (DST by culture cannot be done on these samples) circumventing the need to request follow-up sputum from patients in rural clinics. We proposed a protocol which can assist the control program for fast and accurate detection of drug resistance. In order to stop the spread of drug resistant strains, rapid tests must be introduced into routine laboratories. The TB control program has been informed about our findings and is currently in the progress to further evaluate molecular testing on a larger scale in South Africa.

Molecular epidemiological studies have demonstrated the world wide occurrence of the Beijing genotype, thereby suggesting that this genotype has evolved unique properties. This genotype is currently one of the most well studied *M. tuberculosis* strain families globally. In the third theme of this study, we showed that more than 60 % of drug resistant TB is due to ongoing transmission of an already drug resistant strain. We also observed that strains with the Beijing genotype are the most dominant (successful) drug resistant strains in our study community. The result shows that the proportion of cases infected with strains with the Beijing genotype has increased over time. Furthermore, we found that a specific drug resistant Beijing strain (cluster 220) has a higher propensity to transmit and cause disease as compared to other drug resistant Beijing strains. The high frequency of the Beijing cluster 220 strain raises concern for the spread of drug resistant TB in vulnerable populations. This enhances the possibility of outbreaks of MDR TB, such as were

identified in a high school in Cape Town, South Africa. We recommend that greater vigilance is required to contain this drug resistant Beijing lineage (and other outbreak drug resistant strains) and that molecular detection should be central in the control program for the detection of these outbreak strains. This can be achieved by the development and implementation of rapid diagnostics, ensuring treatment adherence and intensify the screening of contacts.

Many studies, including our study, have identified a subset of INH resistant clinical isolates without known drug resistance causing gene mutations. By using a transcriptional and translational approached (theme 4), we found several genes that are differentially expressed/translated between clinical isolates with different INH resistance phenotypes.

In this study, we proposed a mechanism by which genes induced by INH (*efpA, acpM, iniA and iniB*) in a resistant strain, with no gene associated mutation, might be able to compensate for the toxic effect of the drug. The results also suggest that the susceptible bacterium sense a different response where the final execution step is the inhibition of mycolic acid.

When the transcriptomics and proteomics result was compared, no overlap (except for the identification of the *ahpC* protein) was found between the two techniques. The level of a given mRNA is not positively correlated with the expression of the associated protein due to the occurrence of post transcriptional and post translation modification that might take place as well as the turn around time of mRNA into protein. QRT-PCR was used to validate the expression levels of the previously INH induced genes.

We come to the conclusion that treatment of drug resistant TB patients with standard concentrations of INH may be sub-optimal as the bacteria have other mechanisms of coping with low concentrations of INH. This must be particularly important in patients which are not compliant to anti-TB treatment. The genome of *M. tuberculosis* encodes several efflux putative proteins. Little is known about the role of these proteins in the regulation of drug resistance in *M. tuberculosis.* It is also possible that subtle changes in regulons that regulate gene expression may play a role in drug resistance. In order to fully understand the complete mechanism of resistance to INH, we propose that future studies should include whole genome sequence analysis.

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