MOLECULAR CHARACTERIZATION AND DRUG SUSCEPTIBILITY OF ISOLATES FROM MDR-TB PATIENTS IN THE EASTERN CAPE AND NORTH WEST PROVINCES OF SOUTH AFRICA

by

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DECLARATION

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SUMMARY

South Africa is among the countries facing rising numbers of *Mycobacterium tuberculosis* (Mtb) drug resistant strains. In 2000, DOTS-Plus strategy was introduced nationally to combat drug resistant tuberculosis (TB). This necessitated the introduction of drug susceptibility testing for second-line drugs (SLDs) in order to detect and treat cases in a timely and effective manner. However, this was only routinely implemented following the description of extensively drug resistant (XDR-TB, defined as MDR-TB plus resistance to a fluoroquinolone and a second-line injectable, in South Africa in 2006.

The impact of implementing a standardized MDR-TB therapy policy in South Africa on individual treatment outcomes and acquisition of additional drug resistance has not been widely documented. Improved knowledge of factors that lead to acquisition of second-line drug resistance will help better predict who is most at risk of drug resistance and contribute to the development of new tools and strategies to combat MDR-TB. To fill this gap, we sought to determine the prevalence of SLD resistance among MDR-TB patients in the DOTS-Plus cohort and its impact on treatment outcomes for these patients in two provinces in South Africa; Eastern Cape (EC) and North West (NW) province.

The results show that treatment success was strongly influenced by the setting where the patients were treated. Default and death accounted for 58.1% (193/333) of all unfavourable outcomes in provinces. The EC province had the lowest (13.4%, 51/381) cure rate and the highest default rate of 38.3%; compared to a default rate of 6.39% in NW.

This study also describes the resistance patterns against second line drugs among newly diagnosed MDR-TB patients in the NW and EC province using Genotype MTBDR*sl* assay (version 1) and targeted sequencing of genes known to confer resistance, and how these patients acquired resistance during treatment. These finding have important implications for infection control, because undiagnosed highly resistant strains could have been transmitted to contacts during treatment. The concordance between Genotype MTBDR*sl* and sequencing was 82% for all *gyrA* gene and 67% for the *rrs* gene. Resistance to all drugs (including ethambutol) tested at baseline was 15.8% (47/298) and resistance to both ofloxacin and kanamycin was 1.3% (4/298). Heteroresistance associated with the *gyrA* and *embB* gene was also observed.

Furthermore, the study discusses the implementation of the DOTS-Plus policy with regards to whether it significantly contributed to the emergence of XDR-TB in individual patients. Implications for implementation of standardized MDR-TB treatment in the absence of knowledge of baseline resistance are also discussed.

Analysis of 48 MDR-TB patients, with initial and last available isolates, showed that 45,8% gained resistance to second line drugs during treatment which suggests that the combination of in-hospital treatment with a standardized MDR-TB treatment regimen increased the risk to the patient gaining XDR during treatment.

This thesis has contributed to our understanding of drug resistance in TB, and implications of implementing standardized MDR-TB treatment in South Africa. We propose an algorithm for rapidly diagnosing patients that are at risk of extensively drug resistant tuberculosis (XDR-TB) using a combination of the methods endorsed by the World Health Organization (WHO).

OPSOMMING

Suid-Afrika is ‘n land met stygende getalle middelweerstandige stamme van *Mycobacterium tuberculosis*. In 2000 is die DOTS-plus strategie oor die hele land ingestel om middelweerstandige tuberkulose (TB) te beveg. Dit het die bekendstelling van middelvatbaarheidstoetsing vir tweedeliniemiddels genoodsaak om gevalle tydig en effektief op te spoor en te behandel. Dit is egter eers in 2006, na die beskrywing van uitgebreide middelweerstandige (XDR)-TB, omskryf as MDR-TB plus weerstand teen fluorokinoloon en ’n tweedelinie inspuitbare middel as roetine geïmplementeer in Suid-Afrika.

Die impak van die implementering van 'n gestandaardiseerde MDR-TB-terapiebeleid op individuele behandelingsuitkomste en die opdoen van addisionele middelweerstandigheid in SA is nie goed gedokumenteer nie. Verbeterde kennis van die faktore wat tot die opdoen van tweedeliniemiddelweerstandigheid lei, sal lei tot beter voorspellings oor wie die hoogste risiko loop vir middelweerstandigheid, en ook bydra tot die ontwikkeling van nuwe middels en strategieë om MDR-TB te beveg. Ten einde hierdie gaping te vul, het ons probeer vasstel wat die voorkomssyfer van tweedeliniemiddelweerstandigheid onder MDR-TB-pasiënte in die DOTS-plus-studiegroep is en die uitwerking daarvan op behandelingsuitkomste vir hierdie pasiënte in twee provinsies in Suid-Afrika, naamlik die Oos-Kaap en Noordwes.

Die resultate toon dat behandelingsukses sterk beïnvloed word deur die plek waar die pasiënte behandel is. 58.1% (193/333) van alle ongunstige uitkomste in die provinsies is te wyte aan versuiming van behandeling en sterfte. Die Oos-Kaap het die laagste genesingskoers (13.4%, 51/381) en die hoogste versuimingskoers (38.3%) gehad, in vergelyking met 'n versuimingskoers van 6.39% in Noordwes.

Hierdie studie beskryf ook die weerstandspatrone teen tweedeliniemiddels onder nuut-gediagnoseerde MDR-TB-pasiënte in Noordwes en die Oos-Kaap met genotipe-MTBDR*sl*-toetsing (weergawe 1) en geteikende DNS volgordebapaling van gene bekend vir die oordra van weerstandigheid, en hoe hierdie pasiënte weerstand gedurende die behandeling opgebou het. Hierdie bevindinge het belangrike implikasies vir infeksiebeheer omdat ongediagnoseerde, hoogs weerstandige stamme na kontakte gedurende behandeling oorgedra kan word. Die ooreenstemming tussen die genotipe-MTBDR*sl* en DNS volgorde was 82% vir al die *gyrA*-gene en 67% vir die *rrs*-geen. Weerstandigheid teen alle middels (insluitende etambutol) wat op aanvangsvlak getoets is, was 15.8% (47/298) en weerstandigheid teen sowel ofloksasien as kanamisien was 1.3% (4/298).

Heteroweerstandigheid wat met sowel die *gyrA*-geen as die *embB*-geen geassosieer word, is ook waargeneem.

Die studie bespreek verder die implementering van die DOTS-plus beleid en of dit betekenisvol aanleiding gee tot die verskyning van XDR-TB in individuele pasiënte. Die implikasies vir die implementering van gestandaardiseerde MDR-TB-behandeling met gebrek aan enige kennis oor aanvangsweerstandigheid, word ook bespreek.

Die ontleding van 48 MDR-TB pasiënte, met ’n aanvanklike en ’n laaste kultuur toon dat 45,8% weerstand teen tweedeliniemiddels gedurende behandeling opgebou het. Dit dui daarop dat die kombinasie van behandeling in 'n hospitaal met ’n gestandaardiseerde behandelingsplan die risiko vir die pasiënt verhoog om XDR gedurende behandeling op te doen.

Hierdie tesis dra by tot ons kennis oor en begrip van middelweerstandigheid in TB en die implikasies van die implementering van gestandaardiseerde MDR-TB-behandeling in Suid-Afrika. Ons doen 'n algoritme aan die hand om pasiënte wat gevaar loop om uitgebreide middelweerstandige tuberkulose (XDR-TB) op te doen, vinnig te diagnoseer met 'n kombinasie van metodes wat deur die Wêreld-gesondheidsorganisasie (WGO) goedgekeur is.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

In 2014, an estimated 9.6 million people developed tuberculosis (TB) and 1.5 million died from the disease, 400,000 of whom were HIV-positive.[1] The global TB rate has been falling by 1.5% per year – far slower than the 10% yearly declines needed to end TB within twenty years. The African Region had 28% of the world's TB cases, higher than the previously published estimate for 2012, accounting for 74% of people who are HIV positive. The global increase in drug resistance, particularly multidrug resistant TB (MDR-TB), is very concerning due to limited treatment options and much higher risk of death compared to drug susceptible TB. Only 50% of the MDR-TB patients who receive treatment survive. Of the estimated 480 000 new cases of MDR-TB in 2014, a quarter (123 000) were reported as diagnosed. India, the Russian Federation and South Africa (SA) accounted for almost half of the total reported cases. Worldwide, 58% of previously treated patients and 12% of new cases were tested for drug resistance, up from 17% and 8.5% respectively in 2013. This improvement is partly due to the adoption of rapid molecular tests and improved reporting from laboratories.[2] MDR-TB is defined as TB that is resistant to isoniazid (INH) and rifampicin (RMP]), the two backbone drugs used in treat drug susceptible TB. According to the WHO Global Tuberculosis Report, the proportion of new cases with MDR-TB was 3.3%, and 20% of previously treated cases in 2014, percentages that have changed little in recent years.[1] On average, an estimated 9.7% of patients with MDR-TB had extensively drug resistant TB (XDR-TB) defined as MDR-TB with additional resistance to any fluoroquinolone (FQ) and to at least one of three injectable second-line TB drugs (capreomycin [CAP], kanamycin [KAN] or amikacin [AM]).

In 2016, the National Institute for Communicable Diseases (NICD) estimated MDR prevalence of 2.1% in new cases and 4.6% in retreatment cases with an overall, MDR-TB estimate of 2.8% shifting from 2.9% in the previous survey 2001-02. [3] Mpumalanga province showed the highest overall MDR estimate at 5.1%; notably higher than four other provinces: Eastern Cape 2.1%; Limpopo 1.6% North West 2.6% and Northern Cape 1.7%. The prevalence of any isoniazid resistance (9.3%) was higher than that of any rifampicin resistance (4.6%). The isoniazid mono-resistance levels were similar in new cases at 5.5% while in previously treated cases it was 6.5%. The TB-HIV co-infection rate was 63.2% nationally and highest in Mpumalanga 76.8%, followed by Gauteng 74.6%. The lowest rates were in the Western Cape and Northern Cape at 47.4% and 51.7% respectively. [3] Approximately 10.5% of MDR cases

in South Africa have extensively drug-resistant tuberculosis (XDR-TB). [4-5] Studies have shown that the high mortality of XDR-TB patients in SA (41%-68%) is likely to be associated with a high level of HIV co-infection in TB patients. [1-2 5, 6]

In 2013, *Mycobacterium tuberculosis* infections with even more complete resistance were identified. These isolates showed *in-vitro* resistance to all first and second–line drugs tested (13 drugs) and were subsequently termed totally drug resistant (TDR) or extremely drug resistant (XXDR) TB strains.[7-8] The patients infected with XDR and XXDR-TB have no reasonable treatment options and therefore may increase the risk of disease transmission among community contacts. There is evidence in South Arica that virtually untreatable strains of TB have become established and are circulating in the Eastern Cape Province and KwaZulu-Natal province of South Africa.[9]

In KwaZulu-Natal, Max O'Donnell et al. presented outcomes for 114 adults treated for XDR-TB at the provincial drug-resistant TB referral hospital in KwaZulu-Natal.[10] All cases started treatment between December 2006 and October 2007 and outcomes were ascertained from routine hospital records. Most were treated with a standardised regimen consisting of capreomycin (CAP), pyrazinamide (PZA), and p-aminosalicylic acid (PAS), ethionamide (ETO), ethambutol (ETH) and cycloserine (Cs) or terizidone (Trd). There was no laboratory data regarding drug susceptibility over and above the routine diagnostic tests used to identify the XDR-TB. Although culture remains the gold standard for TB and MDR/XDR-TB diagnosis, its complex requirements of laboratory infrastructure, equipment and personnel, as well as biosafety considerations and relatively long turnaround times, limit its potential for rapid diagnosis, especially in resource-limited settings. [11]

Molecular techniques have provided new ways to study distribution of the mutation frequency and patterns and evolutionary genetics of the pathogen, which are all essential for effective control and prevention of TB. The Genotype MTBDR*sl* assay is the only rapid commercial test endorsed by the WHO for the detection of resistance to the main second-line drugs (SLD).[11] However, as this assay is limited to key commonly described mutations known to cause antibiotic resistance to second line drugs and do not allow the detection of new mutations, Direct Sequencing is used an alternative approach that allows for comprehensive detection of mutations associated with resistance to second-line anti-TB drugs.[12] Furthermore, to gain an insight into the epidemiology of *Mycobacterium tuberculosis* isolates, several molecular tying methods are used e.g spacer oligotyping (Spoligotyping), *IS6110*-restriction fragment length polymorphisms (RFLP), and mycobacterial interspersed repetitive units-variable-number of tandem DNA repeats (MIRU-VNTR).[13] Spoligotyping relies on identifying polymorphisms in

the spacer units in the direct repeat (DR) region of the genome. This region contains multiple, conserved 36 base pairs (bp) DRs that have distinctive, individual spacer sequences that range from 34-41 bp in length spread between each DR. Spoligotyping has been used for rapid identification of laboratory error and contamination, and also establishing whether a strain is identical to or different from other strains found within a study community. [14-15] When this principle is applied to serial isolates collected from a single patient, it is possible to relate the genotype of the infecting strain to the genotype of a strain from a prior episode of disease.[15] Strains with shared genotypes are thought to represent ongoing transmission, while strains with unique genotypes are thought to represent reactivation.[12]

Spoligotyping provides some important advantages over other genotyping techniques. These are simplicity, rapidity, high reproducibility and stability of the results, with the latter being expressed in a simple digital pattern, readily named and databased. However, spoligotyping has relatively low discriminatory capacity for strains without a copy of IS6110 and also for those with a low copy number of less than six, which makes it necessary to use secondary fingerprinting methods to prove clonality between isolates. [16] Spoligotyping in combination with MIRU-VNTR has been used to replace RFLP-*IS6110*. [12]

In Max O'Donnell's study only one in five MDR-TB cases (22%) had a successful outcome (cure or completion) - 42% died, 17% defaulted and 19% failed treatment. Of the 42 cases with culture conversion during treatment, one in six showed culture reversion, suggesting the emergence of additional resistance during treatment because of lack of drug susceptibility testing for second line drugs to identify XDR-TB.[10] Similarly, Pietersen and colleagues prospectively followed 107 patients from three provinces in South Africa who had been diagnosed with XDR-TB between August 2002 and February 2008.[6] All were treated empirically as inpatients with a median of eight drugs. Genotypic testing of a subset of patients showed resistance to at least eight drugs. Fifty-six patients died in the hospital, six transferred out of the region, and 45 patients were discharged. Of those discharged into the community, 19 had failed treatment and a third of those were smear positive at discharge. DNA fingerprinting showed that, in one instance, an XDR-TB patient who had failed treatment and was subsequently discharged, infected his brother who later died. [17] Together these studies clearly provide evidence that outcomes with standardised or empiric treatment regimens for XDR-TB are poor.

1.2 RATIONALE OF THE STUDY

Resistance to anti-TB drugs is a major challenge for TB control programs. This situation poses a serious problem for low income countries, especially those with a high prevalence of HIV. Another problem is that the MDR-TB treatment is lengthy, more difficult and the disease is costly to cure. Also, patients that are severely immunosuppressed by HIV infection have a lower response rate and higher fatality rate than HIV negative cases for MDR-TB treatment. The emergence of drug-resistant tuberculosis (DR-TB) is often attributed to the failure to implement proper TB control programs and to correctly manage TB cases.[5] The WHO reports that 218,231 RR-TB cases were from South Africa.[18] Molecular epidemiologic studies agree that the majority of new DR-TB cases SA are due to transmission of already resistant strains, rather than acquisition.[19] In contrast, XDR-TB appears to be acquired due to ineffective treatment of MDR-TB in some provinces and transmitted in others.[20-22]

The Directly Observed Treatment (DOTS), short-course strategy was formulated by the World Health Assembly in 1991 and adopted by the South African National TB Control Programme in June 1996. DOTS comprises of five elements (i) fully supervised treatment with a standardized short-course regimen; (ii) case detection, with special attention to the use of sputum microscopy; (iii) reliable drug provision; (iv) effective monitoring of TB control programmes; and (v) government commitment to TB control. [23-24] Based on the Directly Observed Therapy (DOT) strategy, DOTS-Plus was designed to manage MDR-TB using second-line drugs. The DOTS-Plus (DP) strategy in SA consisted of the following: treatment at dedicated MDR-TB referral facilities, specialised teams overseeing all aspects of MDR-TB management at the referral centres, a standardised treatment regimen, regular monitoring of patients during treatment, extensive documentation, and ambulatory treatment after discharge, and patient follow-up for 5 years after treatment completion. In 2001, the South African national policy on MDR-TB recommended a standardised regimen of four to six months of kanamycin, pyrazinamide, ethambutol, ofloxacin and ethionamide during the initial intensive phase. This phase was followed by 12-18 months of ethambutol, ofloxacin and ethionamide in the continuation phase. Cycloserine was used as an ethambutol replacement when resistance to ethambutol was detected. The treatment guidelines were then revised during 2012 to recommend moxifloxacin (Mfx) instead of ofloxacin (Ofx). Referrals to MDR-TB centres were based on either proven MDR-TB following sputum culture and susceptibility tests or clinical suspicion after failure of therapy. BACTEC 460 TB system (Beckton Dickinson, Sparks, MD, USA) was used as the gold standard, for both diagnosis and drug susceptibility testing (DST) of MDR-TB.

In 2008 the GenoType MTBDR*plus* assay (MTBDR), which detects resistance to both INH and RIF within 48 hours, was endorsed by the WHO for the diagnosis of pulmonary TB (PTB).[21] The assay was implemented as a replacement for culture for the rapid detection of *M. tuberculosis* and drug resistance directly from sputum. The standard DST method for second line drugs was culture based—either conventional agar proportion or liquid. In 2011 the WHO endorsed Xpert MTB/RIF assay was introduced as the primary method for rapid diagnosis of TB, replacing smear microscopy. Prior to this, the diagnostic tests available included the acid fast bacilli (AFB) smear (which miss half of active cases, especially in HIV-coinfected patients), a chest X-ray (CXR) (which performs sub-optimally in HIV co-infected individuals) and mycobacterial culture and culture-based drug susceptibility tests for both first and second-line TB drugs. Culture was the gold standard for the diagnosis of MDR-TB, but the turnaround time is undesirably long (up to six weeks). The test was only performed on a subset of patients with high clinical suspicion for acquired resistance, and the resulting delays have an adverse impact on infection control.

The development of efficient laboratory strategies for rapid and reliable antimicrobial susceptibility testing of *M. tuberculosis* is important for proper management of patients, particularly those with multidrug resistant tuberculosis. Traditional determination of drug resistance is by, three different growth based laboratory methods

The success of the standardized regimen implemented by DOTS-Plus is dependent on how much additional resistance is present in MDR-TB patients at the baseline, for both the majority and potential minority strains.[22] If many patients have baseline resistance to drugs in the standardized regimen, then starting them on DOTS-Plus puts them on a weakened regimen. The impact of implementing the standardized MDR-TB therapy policy in South Africa on individual treatment outcomes and acquisition of additional drug resistance has not been evaluated. Improved knowledge of factors that lead to acquisition of second-line drug resistance will help better predict who is most at risk of drug resistance and contribute to the development of new tools and strategies to combat MDR-TB. To fill this knowledge gap, this thesis aimed to determine the prevalence of SLD resistance among MDR-TB patients in the DOTS-Plus cohort that were receiving treatment at dedicated facilities and its impact on treatment outcomes for these patients.

1.3 OVERALL HYPOTHESIS

A sub-optimal implementation of the DOTS-Plus in-hospital and standardized MDR-TB treatment regimen has led to emergence of extensively drug-resistant tuberculosis (XDR-TB).

Certain *M. tuberculosis* strain lineages are more likely to acquire drug resistance and are associated with poor treatment outcomes.

1.4 AIMS OF THE STUDY

To 1) study the prevailing and evolving genetic characteristics (genetic fingerprints, mutations) and drug susceptibility profiles of sequential MDR-TB isolates infecting patients in two provinces in South Africa and 2) correlate these genetic characteristics with clinical outcomes associated with strain-specific *M. tuberculosis* infections.

1.4.1 Primary Objectives

The primary objectives of this study are to characterize consecutive serial isolates from MDR-TB patients residing in two South African provinces and receiving hospitalized standardized MDRT-TB treatment. Our aims are:

- To screen all MDR-TB isolates collected from a cohort of patients in the Eastern Cape and North-West Province for resistance to second-line anti-TB drugs (SLDs) and to identify their respective resistance profiles.
- To describe changes over time in secondary drug resistance among MDR-TB patients.
- To describe the prevalence of SLD resistance among MDR-TB patients in the DP cohort and its impact on clinically defined treatment outcomes.
- To assess impact of implementing a standardized MDR-TB treatment regimen in two South African provinces.
- To describe the prevalence of SLD resistance among MDR-TB patients in the DP cohort and its impact on treatment outcomes.

1.4.2 Secondary Objectives

These relate to clinical outcome and patient-related issues:

- To determine if MDR-TB isolates with certain genotypes are more likely to acquire resistance to SLDs which are associated with poor outcomes (death, treatment failure, time to sputum negativity).
- To determine whether certain MDR-TB genotypes irrespective of DST are linked to poorer clinical outcomes.
- To determine the correlation between fingerprinting, drug susceptibility patterns, mutations and clinical outcome of a cohort of MDR-TB patients.

- To investigate the occurrence of re-infections and mixed infections in a cohort of 264 MDR-TB patients.

1.4.3 This thesis is divided into the following chapters:

I. Review: Phenotypic and genotypic techniques for the detection of extensively drug resistant tuberculosis (XDR-TB) (Chapter 2). I was responsible for the conceptual development and the collation of the literature and writing of the review. This chapter is prepared for submission to PloS One.

II. Provincial differences in treatment outcomes of multidrug resistant tuberculosis patients under DOTS-Plus programme: A retrospective cohort study in two provinces of South Africa (Chapter 3). Clinical data was I was responsible for the conceptual design of the study, writing, statistical analysis with assistance from Sidney Atwood. This chapter is prepared for submission to PloS One.

III. Detection of second-line drug resistance in *Mycobacterium tuberculosis* isolates Genotype MTBDR*sl* (version 1) assay and DNA sequencing (Chapter 4). Patient contact, sample and clinical data collection was done by the research team of the South African Medical Research Council. I was responsible the DNA extraction, Drug Susceptibility Testing of 2nd line drugs and analysis. DNA samples were sent to the University of Stellenbosch Sequencing Lab for sequencing, with laboratory advice and assistance from Dr Lizma Streicher.

IV. Emergence of additional drug resistance during standardized MDR-TB treatment (Chapter 5). The gene mutation data was sourced from chapter 4, analysis and writing was my resposnsibility. I was responsible for most of the Spoligotyping experiment with laboratory advice and assistance from Dr Lizma Streicher. This chapter is prepared for submission to the International Journal of Tuberculosis and Lung Disease.

V. Characteristics and treatment outcomes of patients with multidrug resistant tuberculosis in South Africa (Chapter 6). Clinical data collection was done by the research team of the South African Medical Research Council. I was responsible for writing and analysis with assistance from Biostatistics Department of the South African Medical Research Council.

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CHAPTER 2

REVIEW: PHENOTYPIC AND GENOTYPIC TECHNIQUES FOR THE DETECTION OF EXTENSIVELY DRUG RESISTANT TUBERCULOSIS (XDR-TB)

2.1 ABSTRACT

Background: Tuberculosis remains a global problem due to several reasons including inadequate treatment programmes, the HIV epidemic, increasing economic deprivation, lack of laboratory capacity and the emergence of drug resistant TB (DR-TB). Early and accurate diagnosis of drug resistance in TB is important so that effective treatment is provided as soon as possible to ensure rapid cure. In this review, we discuss the mechanisms of second line drug resistance, strategies for determining drug resistance and implementation of molecular TB diagnostic tests.

Objectives: To propose an algorithm for implementation of one or a combination of the methods endorsed by WHO for rapid identification of second-line resistance and thereby XDR-TB in a high TB and HIV burden country.

Results: The accuracy of sequencing by pyrosequencing is comparable to that of Sanger sequencing. Despite the simplicity, low cost, and relative rapidity of pyrosequencing, results still have to be confirmed by a phenotypical culture-based method for correct management of XDR-TB.

Conclusions: More than one method is needed because phenotypic methods do not satisfy the requirement of rapid results and genotyping is a better alternative for rapid detection of XDR-TB. We therefore recommend use of rapid molecular methods, pyrosequencing, for DST of XDR-TB.

2.2 INTRODUCTION

Tuberculosis remains a serious global health challenge as a result of several reasons including inadequate treatment programmes, the HIV epidemic, increasing economic deprivation and the emergence of drug resistant TB (DR-TB). DR-TB develops either due to infection with a resistant strain (transmitted), or as a result of inadequate treatment (acquired), for example when a patient receives a weak treatment regimen, is given poor quality drugs or has malabsorption of medications.[1-3] Non-compliance has been cited as the main contributor to the burden of drug resistance TB. From a patient perspective, lack of money to pay for transportation, long distance from clinics, negative attitudes from the healthcare providers discourages the patients from treatment adherence. [4-5] Lack of information, stigma, side-effects of drugs and poor integration of TB-HIV services plays its share in poor treatment adherence. [6] Poor knowledge about TB and the efficacy of treatment beginning of their treatment also has a share. [7]

Years of issues with care delivery has led to the emergence of virtually untreatable forms of TB, now termed extensively drug-resistant tuberculosis (XDR-TB), defined as resistance to at least isoniazid (INH) and rifampicin (RIF) also known as multidrug-resistance (MDR-TB), and in addition, to any fluoroquinolones (FQs) (such as ofloxacin or moxifloxacin) and to at least one of three injectable second-line drugs (amikacin (AM), capreomycin (CAP) or kanamycin (KAN)).[8-9] This review will discuss the mechanisms of second-line drug resistance, strategies for determining drug resistance and implementation of molecular TB diagnostic tests. The following section is focused on epidemiology of XDR-TB, diagnostic and treatment challenges of XDR-TB.

Globally, 4 044 patients with XDR-TB were enrolled in treatment in 2014 (an increase from 3 284 in 2013).[10] Although the reporting of data has improved since 2008, XDR-TB prevalence is still believed to be underestimated because most countries have limited ability to perform drug susceptibility testing (DST) for second-line drugs. Because many cases of XDR-TB are never diagnosed, let alone properly treated, they remain high risk for on-going propagation of the epidemic. Most cases of MDR-TB and XDR-TB in South Africa have been detected in KwaZulu-Natal, the Western Cape, and Eastern Cape Provinces. In 2009, the respective figures of patients diagnosed were 1,773; 2,078; 1,858 for MDR-TB and 254; 72 and 123 for XDR-TB. [11] In South Africa, the number of people with TB initiating treatment decreased from 406 082 to 332 170 between 2009 and 2013. However, during the same period, the number of patients who initiated treatment for MDR-TB more than doubled, from 4 143 to 10 179, with a 45% treatment success rate.[11] The treatment success rate of XDR-TB

was between 15% and 20%.[7] Reasons for the poor treatment outcomes are many, including poor linkage and retention to care, poor tracing mechanisms and limited access to new agents for example bedaquiline and linezolid. The recent discovery of a totally drug-resistant TB in the Eastern Cape raises the concern that untreatable strains will soon spread more widely. This new strain has shown resistance to at least 10 anti-TB drugs currently in use in the public health sector.[12]

In order to respond to the dual epidemics of HIV and TB, SA developed an integrated National Strategic Plan (NSP) for HIV, STIs and TB (2012 - 2016).[13] One of the goals of the NSP was to reduce the number of new TB infections and deaths from TB by half by 2016. Among the strategies to achieve these goals are: 1) Intensified Case Finding (ICF); 2) TB infection control; 3) workplace health policies on TB and HIV; 4) provision of IPT; 5) prevention of MDRTB; and 6) reduction of TB-related stigma. In addition, the strategic plan prioritizes testing and screening for HIV and TB, improved contract tracing, early diagnosis and rapid enrolment into treatment and integration of HIV and TB care in the healthcare system.[13] To achieve these targets, in 2011 the National Tuberculosis Programme (NTP) recommended ambulatory over hospital-based treatment for MDR-TB cases in order to achieve rapid diagnosis and treatment initiation.[14]

Definitive diagnosis of MDR-TB and XDR-TB requires that *M. tuberculosis* be isolated and identified, and drug-susceptibility testing (DST) completed.[15] Using conventional methodologies, including growth detection, identification of *M. tuberculosis* and DST for first and second-line drugs, may take eight to twelve weeks. Because delays in diagnosis and treatment can lead to poor individual outcomes and increased spread of disease, the WHO and partners have proposed a global XDR-TB response plan, calling for implementation of rapid methods to screen patients at risk of rifampicin resistant-TB.[16] Rapid tests can provide results within days (even without culture, i.e. directly on specimens) and thus enable prompt and more appropriate treatment, decrease morbidity and mortality and interrupt transmission. Treatment of MDR-TB is difficult due to delayed diagnosis and, once identified, ineffective drugs that are more costly, toxic and lengthy than drug susceptible therapies. MDR-TB treatment regimens ideally include five drugs and lasts for 18-24 months. In South Africa, fluoroquinolones and injectable aminoglycosides are the two backbone drugs used in MDR-TB treatment. From 2010, ethambutol was replaced by cycloserine (Cs) or terizidone (Trd).[17]

Since 2008, much attention has been given to rapid techniques for second-line drug susceptibility. Currently available rapid tests to detect XDR-TB are Line Probe Assay, Pyrosequencing, Whole Genome Sequencing, and Reverse Line Hybridization Assay (see Table 2.).[18-22] The expense and technical requirements involved in these PCR tests puts them

out of reach of most patients in TB-endemic countries. The high risk of transmission of TB makes cost-effective and rapid detection crucial to control the spread of infection.[23] The WHO has endorsed commercial rapid liquid TB culture methods.[24] and two molecular assays[25] for simultaneous detection of both *M. tuberculosis* and resistance to isoniazid (INH) and or rifampicin (RIF). Although several drugs are mentioned per class, only one of them needs to be tested because of cross-resistance between members of that class.[26] In 2009 the WHO endorsed the use of Genotype MTBDR*sl* assay for the detection of resistance to second-line anti-tuberculosis drugs to rule out XDR-TB, but it cannot be used to define XDR-TB for surveillance purposes. This assay significantly improves diagnostic yield while simultaneously decreasing diagnostic delay for reporting second-line DST.[25] Limitations of Genotype MTBDR*sl* are that there is incomplete cross-resistance between the second-line injectables, and the assay does not allow for specific resistance to individual second-line injectables to be determined.[27]

It was therefore imperative to develop, improve and evaluate diagnostic methods to rapidly identify second-line resistance and thereby XDR-TB. This review will focus on the most promising currently available diagnostic techniques and will highlight strengths and weaknesses of the different assays. An algorithm will be proposed for the implementation of one or a combination of the methods endorsed by WHO into a routine program of a high TB and HIV burdened country and how healthcare professionals can adapt to use and interpret the results.

The emergence of XDR-TB continues to threaten national TB programmes around the world. Drug resistance most often develops when first- or second-line TB drugs are misused or mismanaged (when patients do not take the full course of treatment or doctors prescribe the wrong dosage, duration or drugs for treatment) and thereby become ineffective and also due to person to person transmission. The XDR *M. tuberculosis* strains are more likely than the non–XDR MDR strains to be clustered, suggesting that transmission plays a critical role in the new incidence of XDR-TB.[26] In contrast, findings by Ioerger et al. suggest that XDR drug resistance in the Beijing strains in the Western Cape is not spreading clonally, but continues to be acquired independently in different strains.[28] XDR-TB infection is particularly problematic for individuals with HIV or other conditions weakening the immune system. Patients who are coinfected with HIV have varying degrees of intestinal absorption of TB drugs[29,30] and of treatment failure with standard regimens,[31, 32] both of which potentially increase the risk of acquiring or amplifying TB drug resistance.[33] Treating XDR-TB successfully is difficult, because of the lengthy treatment duration of 18–30 months, and the difficulty in tolerating side effects and toxicities of second-line medications.[28]

In 2005, large numbers of patients with MDR-TB and XDR-TB were identified at a rural hospital in Tugela Ferry, KwaZulu-Natal. Systematic surveillance undertaken at the hospital between January 2005 and March 2006 revealed that, of 542 patients with positive sputum TB culture results, 221 (41%) had MDR-TB and 53 (10%) had TB caused by *M. tuberculosis* strains with resistance to all six drugs tested (isoniazid, rifampicin, ethambutol, streptomycin, ciprofloxacin, and kanamycin).[12] The mortality rate among patients with XDR-TB was 98% most of whom were co-infected with HIV, with a median survival time of 16 days from the time of collection of diagnostic sputum samples.[34] By year-end 2008, XDR-TB cases diagnosed in Tugela Ferry had increased to 463, though the mortality rate had fallen to 82% and most patients had a median survival time of 28.5 days after sputum collection.[35] Available data from different studies shows that treatment duration is longer and outcomes are generally poorer for XDR-TB, compared with patients with MDR-TB. However, an observational cohort study by Sotgiu et al. showed that XDR-TB can be successfully treated in up to 65% of patients, with better outcomes for those not coinfected with HIV. [36] According to a review study by Jacobson et al. the proportion of patients who experience favourable outcomes is in the range of 18% to 67%, and the percentage of patients who received a later-generation fluoroquinolone was significantly associated with the proportion with favourable outcomes. [37] These studies also suggest that patients receiving later-generation fluoroquinolones have a 40% increase in favourable outcomes, compared with patients not receiving later-generation fluoroquinolones. [37]

2.3 MECHANISMS OF SECOND-LINE DRUG RESISTANCE

2.3.1 Fluoroquinolones

The most common mechanisms by which bacteria acquire resistance to fluoroquinolones is by spontaneous mutations in chromosomal genes that alter the structure of gyrase or topoisomerase IV or both. Resistance mutations occur in the DNA gyrase gene encoding two short discrete segments termed the quinolone resistance-determining regions (QRDR) of *GyrA* subunit (QRDR-A) and less frequently in *GyrB* (QRDR-B), respectively.[33] Specific amino acid substitutions and the number of resistance mutations in the QRDR lead to the development of different levels of fluoroquinolone resistance. Individual mutations in *gyrA* may confer low-level resistance (MIC < 2 mg/L),[34] while high-level (MIC 6.0, 8.0 and 10.0 mg/L) resistance to fluoroquinolones usually requires multiple mutations in *gyrA*, or concurrent mutations in *gyrA* and *gyrB.*[22] Asp94Gly substitutions in the *gyrA* gene are associated with

high level OFX resistance [35] rendering the DNA gyrase protein conformation more difficult for fluoroquinolones to bind to, resulting in higher MIC values.

From the earlier studies, DNA sequencing of *gyrA* showed that certain strains possessed a naturally occurring polymorphism at codon 95 (Ser95Thr), which did not have a significant impact on fluoroquinolone susceptibility as it occurs in both fluoroquinolone-susceptible and fluoroquinolone-resistant strains. Instead, mutation at codon 95 of *gyrA* gene serves as an evolutionary marker for classification of *Mycobacterium tuberculosis* strains into Principal Genetic Groups (PGG).[25,26] Group 1 has the allele combination *katG* codon 463 CTG (Leu) and *gyrA* codon 95 ACC (Thr); group 2 has *katG* 463 CGG (Arg) and *gyrA* codon 95 ACC (Thr), and group 3 organisms have *katG* 463 CGG (Arg) and *gyrA* codon 95 AGC (Ser).[36]

Studies have also shown that mutations in the QRDR region of *gyrA* account for 42–100% [37-39, 35] of fluoroquinolone resistance, suggesting that there could be other alternative mechanisms of resistance like efflux pumps, which export toxins out of the cell thereby reducing the intracellular concentration resulting in low level resistance.[40] Several mycobacterial efflux pumps associated with FQs resistance have been described. These efflux pumps include the pumps of the Major Facilitator Superfamily (MFS) family (lfrA, Rv1634 and Rv1258c) and ATP Binding Cassette (ABC) transporters (DrrAB, PstB and Rv2686c-2687c-2688c). [41] In addition to the two efflux pumps mentioned above, numerous efflux determinants of fluoroquinolone resistance in mycobacteria have been described. [42-43] A further explanation for the absence of a correlation between FQ resistance and *gyrA* mutation is the occurrence of FQ heteroresistance. Some studies reported between 22 and 31% of patients being infected with both wild-type and QRDR mutant *M. tuberculosis* strains.[36,33,44] A systematic review study by Avalos et al. showed A90V as the most frequent, followed by D94G mutation within *gyrA* gene in South Africa (Table 1).[45]

Table 1: The cumulative frequencies of *gyrA* point mutations in FQ resistant isolates in South Africa

Mutation	No. of resistant isolates examined	No. of susceptible isolates examined	No. of resistant isolates with mutations	No. of susceptible isolates with mutations	Frequency of mutation among resistant isolates	Frequency of mutation among susceptible isolates
A90V	280	258	65	0	0.33	0
D94G	280	258	92	0	0.23	0
D94A	280	258	30	0	0.11	0
D94N	280	258	27	0	0.10	0
S91P	280	258	15	0	0.05	0
D94Y	280	258	2	0	0.01	0
G88C	275	250	3	0	0.01	0

Source Avalos E, Catanzaro D, Catanzaro A, Ganiats T, Brodine S, Alcaraz J, et al. Frequency and Geographic Distribution of gyrA and gyrB Mutations Associated with Fluoroquinolone Resistance in Clinical *Mycobacterium tuberculosis* Isolates: A Systematic Review. PLoS One. 2015; 10.

Fluoroquinolone resistance due to *gyrB* mutations was thought to be rare, however, clinical isolates resistant to FQs with *gyrB* mutations and wild type (WT) *gyrA* loci have been reported in several studies.[43-45] *GyrB* mutations at amino acid positions Arg-485, Asp-495, Asn-510, Thr-511, Arg-516 Asn-533, Asn-538 and Ala-54 have been reported.[44-46,48-50]

Although ofloxacin is commonly used because of its relatively lower cost, moxifloxacin (a later-generation fluoroquinolone) is more effective than ofloxacin for patients with MDR-TB, even with ofloxacin-resistant strains.[51] Moxifloxacin is a synthetic, broad spectrum 8-methoxy fluoroquinolone antibacterial agent which has shown high bioavailability, a good curative effect, lower MICs and minimal adverse effects.[41, 51-52] This has led WHO to recommend substituting moxifloxacin when there is resistance to early generation fluoroquinolones like ofloxacin and ciprofloxacin.[53] It targets the mycobacterial topoisomerase II DNA gyrase and blocks the movement of replication forks and transcription complexes. It is active against strains with low levels of resistance (MIC, 0.5 µg/ml) and reduces the mortality associated with strains with intermediate resistance (MIC, 2 µg/ml). [51] However, it is inactive, against strains with high levels of resistance (MIC, >2 µg/ml). [51]

2.3.2 Aminoglycosides

Kanamycin and amikacin are injectable aminoglycosides used in the treatment of multidrug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB). These drugs are more toxic, less effective and more costly than the standard anti-TB regimen. They are considered as a reserve therapy and are used when patients are intolerant to first-line drugs or cannot take first line drugs.[53]

Aminoglycosides inhibit protein synthesis by binding to the 16S rRNA in the 30S ribosomal subunit.[54] Resistance develops through mutation in the *rrs* gene at position A1401T and G1484T, thereby altering the binding between the aminoglycoside and the 16srRNA (~1,400-bp) with high-level resistance MICs of ≥80 µg/ml.[47-57] High level kanamycin resistant strains are also cross resistant to other ribosome binding antibiotics including capreomycin and amikacin.[58] In contrast, low-level KAN-resistant (5 µg/ml < MIC < 80 µg/ml) strains generally exhibit resistance to KAN only. More recently, studies have shown that mutations in the *eis* promoter are associated with KAN resistance. Zaunbrecher found that up to 80% of strains showing low-level KAN resistance harboured mutations in the -10 and -35 promoter region of the aminoglycoside acetyltransferase gene *eis.*[56] The KAN resistance conferred by *eis* promoter mutations is due to the significant increase in *eis* transcript levels and corresponding increase in the levels of an enzyme that acetylates and inactivates KAN.[56]

2.3.3 Ethionamide

Ethionamide is a derivative of isonicotinic acid structurally similar to isoniazid. It is also a pro-drug requiring activation by a monooxygenase encoded by the *ethA* gene. It interferes with the mycolic acid synthesis by forming an adduct with NAD that inhibits the enoyl-ACP reductase enzyme.[59] *ethA* expression is regulated by the transcriptional repressor *EthR*.[60] Resistance to ethionamide occurs because of mutations in *etaA*/*ethA*, *ethR* and also mutations in *inhA* promotor, which cause resistance to both isoniazid and ethionamide.[61-62] Consequently, cross-resistance to these two antibiotics has been observed in clinical isolates.[63] Strains with low-level resistance to INH frequently display low-level ethionamide resistance, whereas high-level INH-resistant strains typically remain ethionamide susceptible.[64] In the case of low-level resistance to INH, patients may benefit from high doses of isoniazid instead of ethionamide.[65]

2.3.4 Pyrazinamide

Pyrazinamide is an analogue of nicotinamide and its introduction into the regimen allowed a reduction in the duration of treatment to six months.[59] It has the characteristic of inhibiting semi-dormant bacilli residing in acidic environments such as those found in the TB lesions.[66] Pyrazinamide is also a pro-drug that needs to be converted to its active form, pyrazinoic acid, by the enzyme pyrazinamidase/nicotinamidase coded by the *pncA* gene.[67] The proposed mechanism of action of pyrazinamide involves conversion of pyrazinamide to pyrazinoic acid, which disrupts the bacterial membrane energetics inhibiting membrane transport.[68] Pyrazinamide would enter the bacterial cell by passive diffusion and after conversion to pyrazinoic acid it is excreted by a weak efflux pump. Under acid conditions, the protonated

pyrazinoic acid would be reabsorbed into the cell and accumulated inside, due to an inefficient efflux pump, resulting in cellular damage.[69] One study has also found that pyrazinoic acid and its n-propyl ester can inhibit the fatty acid synthase type I in replicating *M. tuberculosis* bacilli.[70]

Mutations in the gene *pncA* remain as the most common finding in pyrazinamide resistant strains. These mutations are scattered throughout the gene, however most occur in a 561-bp region in the open reading frame or in an 82-bp region of its putative promoter.[71] The study review by Whitfield et al. reported that SNPs are distributed throughout the entire *pncA* gene, with more than 600 unique polymorphisms observed in approximately 400 positions in *pncA* (including the upstream flanking region).[72] Shi et al. confirmed that the ribosomal protein S1 (RpsA), encoded by the *rpsA* gene, was a target of POA which might be associated with PZA resistance in clinical *M. tuberculosis* isolates. However, based on the current evidence, the contribution of mutations in *rpsA* to pyrazinamide resistance remains limited. [73-75]

2.4 DRUG SUSCEPTIBILITY TESTING

In general, there are two different strategies for determining drug resistance; the phenotypic and genotypic/molecular methods. The phenotypic susceptibility testing is based on the determination of growth or inhibition of growth in the presence of antibiotics, whereas molecular methods detect gene mutations that are known to be associated with resistance to certain antibiotics. Drug susceptibility testing (DST) of *M. tuberculosis is* generally carried out after a culture is isolated from a clinical specimen. This takes four to six weeks, first to isolate a culture and then to perform drug susceptibility testing (indirect DST). Specimens for culture methods have to be decontaminated prior to being cultured to prevent overgrowth by other micro-organisms. All decontamination methods are to some extent also harmful to mycobacteria, and the culture is therefore not 100% sensitive.[76] Phenotypic tests are also greatly affected by the inoculum size as well as the viability of the strains.

2.4.1 Phenotypic second-line drug susceptibility testing

Second-line DST for certain drugs has not been standardized throughout the world, due to technical difficulties related to in vitro drug instability, drug loss caused by protein binding, heat inactivation, filter sterilization, incomplete dissolution and/or varying drug potency.[77] Laboratory technique, medium pH, incubation temperature and incubation time also influence DST results.[77] In addition, the drug critical concentration defining resistance is often very close to the minimal inhibitory concentration (MIC) required to achieve antimycobacterial

activity, increasing the probability of misclassification of susceptibility or resistance, and leading to poor reproducibility of DST results.[48] For *M. tuberculosis,* the antimicrobial susceptibility testing breakpoint (also known as the "critical concentration") is defined as "the lowest concentration of drug that will inhibit 95% of wild strains of *M. tuberculosis* that have never been exposed to drugs, while at the same time not inhibiting clinical strains of *M. tuberculosis* that are considered to be resistant (e.g. from patients who are not responding to therapy)".[78]

Internationally, few laboratories have the required capacity and expertise to reliably test for all classes of available anti-TB drugs. These laboratories are largely limited to resource-rich settings. Newer phenotypic techniques aim for a more rapid detection of growth by using the metabolic activities of growing bacteria.[79]. These techniques, namely automated liquid culture systems, are difficult to implement in the countries where they are most needed owing to high cost, technical complexity and lack of appropriately trained laboratory staff. As a result, conventional culture and DST methods using egg-based or agar-based media are still the most widely used in resource-limited settings, leading to long diagnostic delays. Even in sophisticated and well-resourced environments, wide variations in second-line DST systems and methods have been reported, reflecting the difficulties in securing reproducibility and optimizing the clinical relevance of DST results. [15]

Rapid liquid culture-based techniques have been established that can detect growth-dependent changes such as oxygen consumption (Mycobacteria Growth Indicator Tube [MGIT] (Becton-Dickinson, Sparks, MD) and VersaTREK (Trek Diagnostic systems, West Lake, OH).[80] Furthermore, a more rapid detection of growth can also be achieved by microscopic observation of liquid cultures in tissue-culture plates (microscopic-observation drug-susceptibility [MODS] assay). Since mycobacteriophages are able to only replicate in living cells, phage-based tests have also been developed for speeding up DST. [81]

There are several drugs used to treat MDR-TB for which drug susceptibility testing (DST) is desired. The comparisons of different detection methods (both phenotypic and molecular) for XDR-TB are discussed in Table 2. Below is a list of WHO approved phenotypic tests for identification of drug resistance.

a) *BACTEC MGIT 960 system:* The diagnostic method endorsed by the World Health Organization (WHO) as a gold standard for the diagnosis of XDR-TB is the automated liquid systems (BACTEC MGIT 960 system). The BACTEC MGIT 960 system is an automated continuously monitoring system, based on the detection of bacterial growth in

drug-containing media which is compared to a drug-free control tube.[82] A modified version of the conventional modified agar proportion method (MOP) has been developed for this system, in order to test the susceptibility of *M. tuberculosis* to the second-line drugs.[78] Although it is still technically demanding, it significantly shortens turnaround times for DSTs compared to conventional methods on solid media. The average reporting time for the DST results ranges from five to seven days for the BACTEC MGIT 960 system and two to four weeks using the conventional Löwenstein-Jensen medium.[83] However**,** currently no commercially available kit exists.[84] This makes it more error-prone as the working solutions of each drug should be prepared by the users and could lead to procedural inaccuracies**.** Other drawbacks of this test include i) it is labour-intensive with regards to the handling of vials and maintenance of the instrument, and (ii) there is a potential risk of cross-contamination of the cultures. Automated liquid systems for second-line DST are more prone to contamination and the manipulation of large volumes of infectious material requires appropriate and adequate biosafety measures.[85]

b) *Agar Proportion Method:* The proportion method consists of calculating the proportion of resistant bacilli present in an isolate. Two appropriate bacillary dilutions, one high and one low, are inoculated on a drug containing and drug free medium, in order to provide numerable colonies on both agar media reference.[84]The ratio of the number of colonies obtained on the drug containing medium to the number of colonies obtained on the drug free medium indicates the proportion of resistant bacilli present in the strain.[86] The isolate is resistant if more than 1% of the number of colonies on the control agar growth is visible on the drug agar plate. This method is time consuming, requiring up to two months for the results to be obtained. This may lead to substantial delays in the identification and treatment of MDR-TB patients, and may also contribute to MDR-TB transmission in communities.

c) *Nitrate Reductase Assay (NRA):* The NRA, also known as the Griess method, is a simple technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite. By incorporating 1 mg/ml potassium nitrate (KNO_3) in a Lowenstein–Jensen medium, the reduction of nitrate can be detected using the Griess reagent, which produces a coloured reaction.[87] In the presence of an antibiotic at the critical concentration, development of a red–pink colour in the medium represents resistance. Susceptible strains lose the capacity to reduce nitrate in the presence of the antibiotic, thus no colour is produced. The use of nitrate reduction as an indicator of growth, before colonies can be seen macroscopically, reduces the turnaround time to achieve results compared to conventional methods. The WHO recommends that the NRA be used as a direct test on smear-positive sputum

specimens or as an indirect test on *M. tuberculosis* isolates grown from a conventional solid culture.[88] Data also shows that the sensitivity of NRA to second-line drugs is 100% and the turnaround time is 10 to 14 days.[89] NRA is simple, cost-effective and the use of solid media minimises cross-contamination and biohazard risks. It requires less sophisticated equipment and is a method of choice in resource limited countries.

2.4.2 Molecular Methods

These methods search for the genetic determinants of resistance rather than the resistance phenotype. In general, they have two basic steps: a molecular nucleic acid amplification step such as PCR to amplify sections of the *M. tuberculosis* genome known to be altered in resistant strains, and a second step of assessing the amplified products for specific mutations.[90]

Current molecular methods for detection of genetic mutations associated with second-line resistance include second-generation LPAs, pyrosequencing and targeted sequencing which offer an alternative to the conventional methods. However, only a few mutations conferring resistance have been described for most second-line drugs and testing is technically demanding and expensive.

a) Line Probe Assay: The GenoType MTBDR*sl* (version 1) that was released in 2009, allows the simultaneous detection of the *M. tuberculosis* complex and resistance to fluoroquinolones, aminoglycosides or ethambutol on TB bacteria grown in culture from a patient specimen, therefore allowing the rapid detection of XDR-TB [91] and can be used in combination with the MTBDR*plus* test to identify XDR-TB. The major advantage of Genotype MTBDR*sl* assay is that the test can also be directly applied to smear-positive sputum samples, giving rapid drug susceptibility results without the need for culture. It detects the presence of mutations in genes that cause drug resistance to fluoroquinolones, or second-line injectable drugs, or both. However, it does not report whether there is resistance to individual drugs within these categories (ofloxacin and levofloxacin in the case of the fluoroquinolones; amikacin, kanamycin, and capreomycin in the case of second-line injectable drugs) but detects the presence of mutations in genes that cause drug resistance for drug classes (ie FQs, second line injectable drugs (SLIDs) or both). [92]

Findings from several studies have shown that Genotype MTBDR*sl* assay has the poorest sensitivity for kanamycin resistance.[93-94] In line with this observation, Huang et al showed that the low sensitivity of Genotype MTBDR*sl* assay was due to the presence of specific mutations not detected by the test, located in the *eis* gene promoter region.[95] These mutations are

responsible for more than 80% of low-level KAN resistant cases,[96] making this genetic region a valuable molecular marker of KAN resistance. The disadvantages of LPA's are that they are labour intensive and require highly trained personnel and dedicated laboratory space. Another limitation is that only a limited number of mutations can be detected with a single test. Thus, in cases where the mutations are outside of the targeted regions, the wild-type banding patterns appear, leading to false negative (susceptible) results.

To improve the overall performance of Genotype MTBDR*sl*, and in particular its sensitivity for KAN resistance, a new version of the assay (i.e. Genotype MTBDR*sl* v2.0) has recently been developed.[97]

Genotype MTBDR*sl* v2.0 includes two new target genetic regions: the *eis* promoter region −10 to −14 (in addition to *rrs*) and the *gyrB* quinolone resistance-determining region (QRDR) (in addition to *gyrA*) for the detection of mutations at codons 536 to 541. The target region for *emb* (*embB*) has been removed. Genotype MTBDR*sl* v2.0 thus includes a total of 27 probes for the detection of resistance exclusively to second-line drugs. The inclusion of probes for the detection of mutations in the *eis* promoter region increased the overall sensitivity of Genotype MTBDR*sl* v2.0 for the detection of SLID resistance to 86.4% (95% CI, 79.9% to 91.0%) and the sensitivity for the detection of kanamycin resistance to 96% for both the isolates and the clinical specimens, which is higher than the pooled sensitivity values for Genotype MTBDR*sl* v1.0 (66.9%; 95% CI, 44.1% to 83.8).[98]

The inclusion of probes for the detection of mutations in the *eis* promoter region in the Genotype MTBDR*sl* v2.0 test has resulted in a higher sensitivity for detection of kanamycin resistance for both direct and indirect testing (96% and 95.4%, respectively) than that seen with the original version of the assay. The test sensitivities for detection of FLQ resistance remained unchanged (93% and 83.6% for direct and indirect testing, respectively). Moreover, Genotype MTBDR*sl* v2.0 showed better performance characteristics than v1.0 for the detection of XDR-TB, with high specificity and sensitivities of 81.8% and 80.4% for direct and indirect testing, respectively. Genotype MTBDR*sl* v2.0 thus represents a reliable test for the rapid detection of resistance to second-line drugs and a useful screening tool to guide the initiation of appropriate MDR-TB treatment.[99]

b) *DNA sequencing*: This method is based on DNA sequencing technology using the chain-termination method. While DNA sequencing of variable genomic regions offers a rapid and accurate identification of mycobacteria, this method is not yet practical for use outside

academic settings and is too expensive for most resource-poor countries. Streicher et al. have demonstrated that targeted DNA sequencing is also suitable for clinical trials. [100]

c) *Pyrosequencing:* Pyrosequencing (PSQ) is a semi-automated sequencing method based on real-time monitoring of DNA synthesis, optimized to analyse short DNA sequences. It is based on the quantitative detection of released pyrophosphate during DNA synthesis. In a cascade of enzymatic reactions, light is generated at intensities proportional to the numbers of incorporated nucleotides.[101] PSQ has a short turnaround time and is less hands-on and easier to handle than conventional sequencing method.[102] The entire procedure, from extracting DNA to reporting results, can be accomplished within six hours using a protocol that can be easily integrated into the workflow of diagnostic or public health laboratories. Although this method is easier to perform and less expensive than traditional sequencing, the shorter sequences are not as discriminating. It cannot be practically applied when mutations are spread widely over a gene, as is the case in *pncA.* It is therefore difficult to use for pyrazinamide resistance detection.[103] By using automated sequencers and well-trained staff, sequencing assays can be completed and identification results can be reported within one to three days. However, the installation, maintenance and running of automated DNA sequencing on a daily basis are currently too expensive and labour-intensive for use in resource poor settings.[104]

d) *Targeted Next-Generation Sequencing (NGS):* In contrast to Pyrosequencing, NGS allows interrogation of many genes with sequencing of much longer segments in a relatively short time, but requires sophisticated software to handle huge data sets. NGS has vastly improved nucleic acid sequencing with respect to amount, speed and cost of data obtained.[105] Unlike conventional sequencing techniques, it is based on the immobilization of the DNA samples onto a solid support, cyclic sequencing reactions using automated fluidics devices and detection of molecular events by imaging.[106] In contrast to most Sanger sequencers, the Ion Torrent does not utilize (expensive and maintenance-requiring) lasers that typically require modified fluorescence-based, light-sensitive chemistries and it has a much smaller overall footprint. The current NGS platforms need higher DNA concentrations (>50 ng), necessitating growth from cultures for testing. Colman et al have found out that the use of “single molecule-overlapping reads” (SMOR) analysis with next generation DNA sequencing for determination of ultra-rare target alleles subpopulations at (≥0.1%) in complex mixtures provides increased sensitivity over standard DNA sequencing.[105,107] Moreover, it leads to lower error bias and can be used directly on patient samples. It can be applied to low levels of degraded DNA, which is often the case with clinical samples. This approach could lead to early detection and monitoring

of antibiotic resistant subpopulations, which, if left undetected, could lead to selection of the resistant population and ultimately, treatment failure.[108-109]

e) *Reverse Line Hybridization Assay:* The reverse line blot hybridization (RLBH) assay is a probe-based method where multiple oligonucleotide probes carrying the mutant sequence and wild-type sequence are immobilized on nitrocellulose strips and hybridized with biotin-labelled PCR products. The membrane can be stripped and reused up to seven times without compromising the results. A study by Ajban et al. showed sensitivities for detection of resistance to FQs and second-line aminoglycosides to 95.34% and 94.8%, respectively.[104]

   The RLBH assay has a turnaround time of three days when performed directly from a smear-positive clinical specimen. Compared to PCR-based sequencing and DNA microarray techniques, RLBH can be performed without expensive devices.[104] However, the RLBH assay is time-consuming and technically complex, limiting its usefulness in the point-of-care setting.

2.4.3 Quality control and standardization of methods for the genetic and phenotypic tests

In 2007, the WHO issued policy guidance on the use of liquid TB culture, DST and rapid species identification in low-resource settings.[110] The WHO policy recommends phased implementation of these systems as part of a country-specific, comprehensive plan for laboratory capacity strengthening and addresses key issues including biosafety, customer support, staff training, maintenance of infrastructure and equipment, specimen transport and the reporting of results. The WHO has established a structured, systematic process to rapidly review the evidence base for new TB diagnostics, ensuring that new tools meet the required performance standards. All laboratories should be accredited by a recognized national/international accrediting organization and should participate in internal and external quality assurance/quality control activities in conjunction with a reference laboratory. These programs will assess the reproducibility and the inter-laboratory variability of the methods used and adherence to standardized testing procedures.[110] All laboratories should have a document control system in operation that will detect and correct significant clerical or analytic errors that could affect patient management.

Table 2: Comparison of different detection methods for XDR-TB

	Conventional Methods			Molecular methods			
Performance criteria	**BACTEC MGIT 960 system**	**Proportion method**	**Nitrate Reductase Assay**	**Line Probe Assay**	**Sanger DNA sequencing**	**Pyrosequencing**	**Reverse Line Hybridization Assay**
Turnaround-time	8 days	28days or 42days	10-14	6-8 hours	2 days	1-2hrs	3 days
Sensitivity (Fluoroquinolones)	90%	90.2%	100%	[C]22% (EMB low)	100%	87%	95.3%
Laboratory infrastructure required	Yes	No	No	Yes Separate procedure rooms	Yes	Yes	Yes
Training	Extensive	Moderate	Moderate	Moderate	Moderate	Moderate	Moderate
Material costs	High	Low	Very low	High costs	High tech equipment, High maintenance, High reagent cost	Moderate cost Or Inexpensive	Low
Commercial kit for second-line drugs	Yes	No	No	Yes	No	No	No
Biohazard risk	High	High	Less	High	Less	Less	Less

Source: [C] Olga Ignatyeva, Irina Kontsevaya, Alexander Kovalyov, Yanina Balabanova, Vladislav Nikolayevskyy, Kadri Toit, Anda Dragan, Daniela Maxim, Svetlana Mironova, Tiina Kummik, Ionela Muntean, Ekaterina Koshkarova and Francis Drobniewskiaff. Detection of Resistance to Second-Line Antituberculosis Drugs by Use of the Genotype MTBDR*sl* Assay: a Multicenter Evaluation and Feasibility Study, J. *Clin. Microbiol.* May 2012. 50(5):1593-1597(46)

2.5 IMPLEMENTATION OF NEW MOLECULAR TB DIAGNOSTIC TESTS FOR SECOND-LINE DRUG SUSCEPTIBILITY TESTING: CLINIC AND LAB READINESS

The implementation of rapid new TB diagnostic techniques is resource-demanding and difficult. Many of the tools becoming available require sophisticated, expensive laboratory biosafety infrastructure that must be established before these tools can be used. One of the challenges that must be addressed before the implementation of a new test is to determine how quality assurance will be provided. The South African policy recommends that all laboratories that perform drug susceptibility testing (DST) have internal quality assurance measures in place and participate in external proficiency testing programmes.[(111)] In this section, we discuss implementation and lessons learnt, specifically focusing on lab readiness and clinic challenges.

2.5.1 Technical Complexity

While it may be easy to perform the test, it is often more challenging for trainees to become proficient in properly reading the molecular results and interpreting the results correctly for the clinicians. There is cross-resistance between the second-line injectables and the assay does

not allow for specific resistance to individual second-line injectables to be determined. Additionally, confusion with LPA often arises if a wild-type band is missing but a corresponding mutation band is not present. The disappearance of a wild-type band and the mutant band in the same gene may indicate a mixed population which makes results difficult to interpret. Within these mixed populations, *M. avium* is the most common and clinically relevant non-tuberculous mycobacteria (NTM) species, especially among HIV-infected persons. One can either repeat this test or perform speciation tests to exclude the possibility of contamination with NTMs. Recently, LPAs have been developed for detection and identification of NTMs and these are now commercially available. However, rapid speciation facilities, either on-site or through a local reference laboratory, are not readily available in many resource-limited settings.[112]

However, phenotypic methods also have their own challenges. A complex internal control system has to be established to ensure drug efficiency, that the proper concentration of the drug is used and the quality of the media itself. In general, standardization of these techniques is difficult, because the efficiency of the drugs alters in different media and with different methods. [113] Other general laboratory requirements for implementation of a new diagnostic test for TB are summarized in Table 3.

2.5.2 Operational and logistical issues

Some molecular techniques have complex requirements, and therefore it is important to clearly map the processes involved in specimen collection and transportation from clinics, delivery of specimens to the laboratory and reporting results back to the clinic. Staff training in safety procedures and quality control should be considered before beginning implementation. Training at management levels, retention of trained personnel, enhancement of quality-assurance systems, enhancement of results-reporting mechanisms and supply procurement are some of the laboratory aspects that should be considered. In general, laboratories need to ensure sustainable systems for long-term provision of instrument maintenance and the skill set required for routine service.

Table 3: Laboratory requirements for the implementation of new TB diagnostic test

Activities	Comments
Cooperation and coordination between facilities	-
Long term contract with the supplier	To ensure uninterrupted supply of stock
Proper infrastructure	To ensure uninterrupted electricity supply, availability of equipment
Technical assistance	To assist troubleshooting related to unusual results and contamination
Cost of products	-

2.6. ALGORITHM FOR IMPLEMENTATION OF DRUG SUSCEPTIBILITY TECHNIQUES FOR SECOND-LINE DRUGS IN A HIGH TB BURDEN COUNTRY

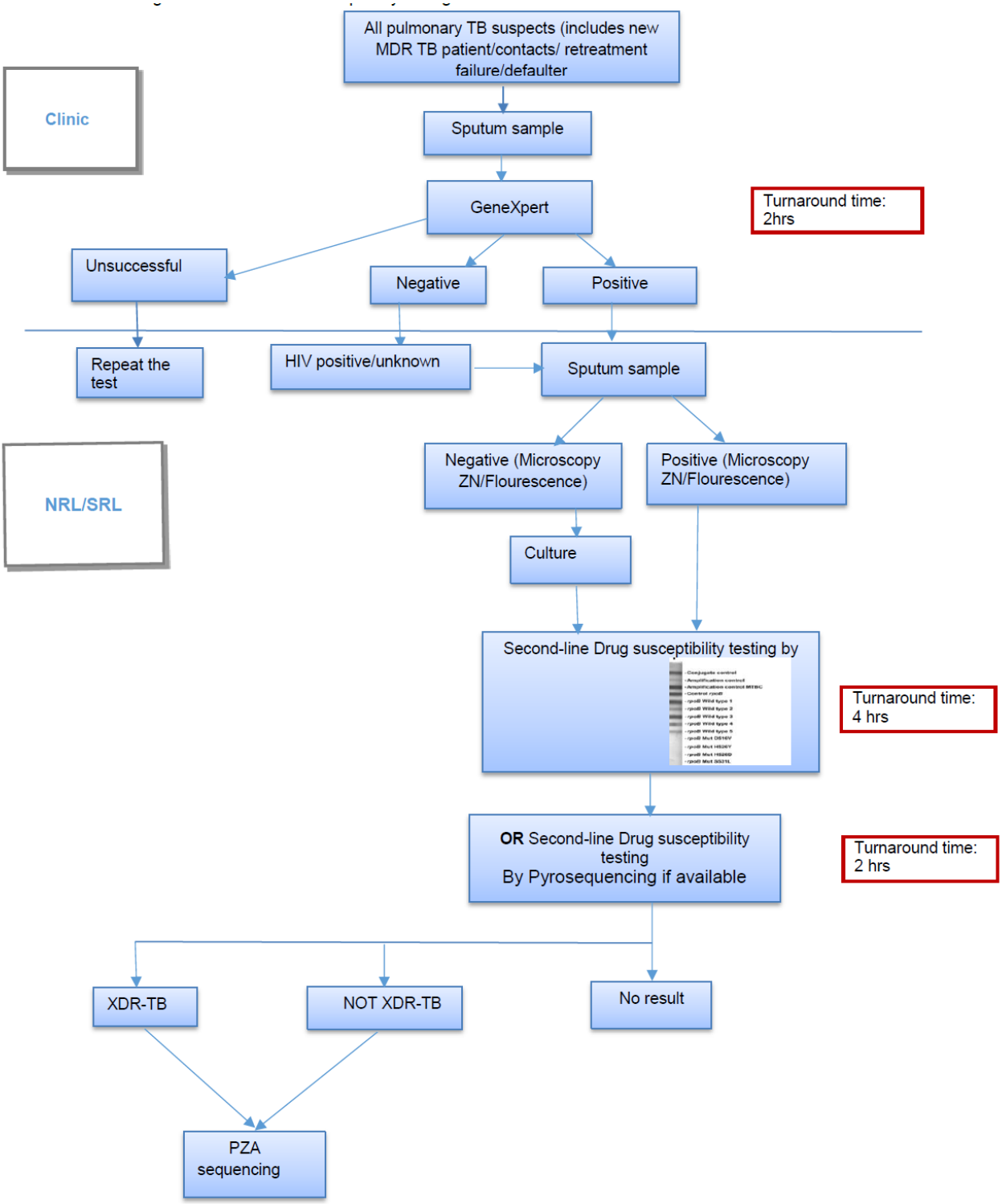
Algorithms should be designed to use existing laboratory services so that specimens can be referred to the appropriate level for tests that are not available at the peripheral level laboratories. Such referrals are particularly important when patients are suspected of having drug-resistant TB, HIV associated TB, when the patients are children or when they have extrapulmonary disease. Several rapid methods [(114)] have been introduced to detect XDR-TB, but in spite of all these developments there is still a need to confirm the DST results for XDR-TB using a liquid based conventional method. Of note is LPA which is limited in the number of loci examined and the lack of capacity to differentiate silent mutations from those that effect drug efficacy, leading to false positive results. [(115)] Although sequencing *M. tuberculosis* from sputum from suspected XDR-TB patients suggests it has a role in the management of TB,[(116)] data analysis remains a bottleneck, requiring specialist expertise not readily available in clinical laboratories. Thus, to obtain the best performance of laboratory tests it is very important to link techniques that complement each other in an algorithm for detection of XDR-TB. South African policy guidelines recommend GeneXpert (GXP) and LPA for early triaging and treatment initiation for patients with DR-TB. Second-line DST of strains, identified as RR/MDR-TB using GXP, is also recommended in order to diagnose or rule out XDR-TB. In specific instances (i.e. when screening contacts of known XDR-TB patients), a second-line DST should be requested together with first-line DST.

Below is the proposed algorithm for detecting patients that are at risk of XDR-TB for example treatment failure or default, HIV-infected cases or contacts of drug-resistant cases using a combination of molecular techniques with rapid potential for detection of XDR-TB patients that

may be missed by MGIT 960. In order to rapidly detect second-line drug resistance, the Genotype MTBDR*sl* assay and pyrosequencing have been proposed in this algorithm.[100] Pyrosequencing is based on the quantitative detection of released pyrophosphate during DNA synthesis,[92] while the Genotype MTBDR*sl* v2.0 assay simultaneously detects resistance aminoglycosides/cyclic peptides and fluoroquinolones through detection of mutations in the relevant genes.[11]

Below is the proposed algorithm for detecting patients that are at risk of XDR-TB for example treatment failure or default, HIV-infected cases or contacts of drug-resistant cases using a combination of molecular techniques with rapid potential for detection of XDR-TB patients that may be missed by MGIT 960. In order to rapidly detect second-line drug resistance, the Genotype MTBDR*sl* assay and pyrosequencing have been proposed in this algorithm

Figure 1: A proposed algorithm to link the molecular line probe assay with solid culture- and liquid culture-based growth detection and susceptibility testing of XDR-TB



2.7 CONCLUSION

Early and accurate diagnosis of drug resistance in TB is important so that effective treatment is provided as soon as possible to ensure rapid cure. To initiate effective treatment, it is important to rapidly obtain the results of DST for second-line drugs and put a stop to the transmission of drug-resistant TB.

The high mortality rate and risk of nosocomial transmission of XDR-TB have stimulated intensive interest in the development and use of rapid DSTs.[117] Significant progress has been made in the development of rapid and accurate detection of drug-resistant TB, most notably molecular based methods. These technologies have brought great improvement in the detection and drug susceptibility testing of drug-resistant *M.tuberculosis* and have enhanced the identification of the disease in HIV-positive individuals. However, it is important to realize that their diagnostic performance vary greatly in terms of turnaround time, cost and complexity. Other factors to be considered are appropriateness to the setting, including initial and recurrent costs and sustainability.

Case detection of DR-TB in SA has increased to 218 231[1] following a nationwide roll-out of Xpert MTB/RIF in 2011. However, this may have limited impact on on-going transmission of the disease unless the majority of cases are rapidly initiated on effective treatment. Second-line DST for AMK and OFX is carried out routinely for all RIF-resistant samples using culture and Genotype MTBDR*sl*. The disadvantage of this is that culture results only become available four weeks after initiation of MDR treatment and should be actively followed up, as detection of further resistance represents XDR or pre-XDR-TB and necessitates modification of treatment. [118]. Concerns over the sensitivity of the Genotype MTBDRs/ v1, particularly for resistance to the injectables, have delayed implementation in the routine diagnostic algorithm due to the fact that it is more sensitive to smear positive sputum and cultivated samples. To address this limitation, the Genotype MTBDR*sl* second version is now available. It is more sensitive and can therefore also be performed using smear-negative pulmonary samples.

Discordance between GXP, LPA and/or phenotypic test results is not uncommon, and may cause confusion and delay treatment initiation. This highlights the importance of sending a second sample for confirmation of positive GXP results, and ideally a third sample to be taken at treatment initiation for culture and DST to provide further confirmation in cases of initial discordance. In patients with discordant results, correlation with clinical findings is also crucial and specialist consultation is necessary to ensure that patients receive the most appropriate

treatment without adversely affecting their chance of cure, but simultaneously limiting exposure to unnecessary and toxic drugs.[118]

Table 3 shows that most rapid methods require adequate laboratories that developing countries do not have access to. In practice, more than one method is needed because phenotypic methods do not satisfy the requirement of rapid results and genotyping is a better alternative for rapid detection of XDR-TB. We therefore recommend use of rapid molecular methods for example PSQ for DST of XDR-TB.

The accuracy of sequencing by PSQ is comparable to that of Sanger sequencing, and the availability of sequence information enables users to study the association of drug MICs with each mutation which makes it a useful tool for predicting the level of drug resistance. [67] In a study conducted by Catanzaro, PSQ produced more interpretable results than Genotype MTBDR*sl* for DST against the second-line drugs AMK, KAN, and CAP. [108] This strategy may decrease the infectious period, reduce treatment failure and may decrease defaults while reducing costs. [72] Despite the simplicity, low cost and relative rapidity of pyrosequencing, the methodology requires intensive staff training and more expensive equipment. However, for correct management of XDR-TB patients, results still have to be confirmed by a phenotypical culture-based method, given the limited number of mutations evaluated by current genotypic tests and incomplete understanding of the mechanisms of resistance to second-line drugs.

On that basis, PSQ should be considered as a supplemental method for rapid detection of XDR-TB. Its ability to can detect all known and unknown mutations may help guide treatment decisions before the availability of phenotypic DST results.

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CHAPTER 3

PROVINCIAL DIFFERENCES IN TREATMENT OUTCOMES OF MULTIDRUG RESISTANT TUBERCULOSIS PATIENTS UNDER DOTS-PLUS PROGRAMME: A RETROSPECTIVE COHORT STUDY IN TWO PROVINCES OF SOUTH AFRICA

3.1 ABSTRACT

Background: DOTS strategy was implemented in South Africa in 1996. However, the number of TB cases continued to rise. Expansion of the DOTS strategy in SA followed rapidly in the following years and by the end of 2003; there was complete coverage in all nine provinces. The importance of addressing drug resistant TB was further amplified by more recent reports on totally drug-resistant tuberculosis.

Objectives: To evaluate MDR treatment outcomes in two different provinces (Eastern Cape (EC) and North West (NW) province) for the period 2000-2004 and also identify risk factors associated with unfavourable treatment outcomes of MDR-TB.

Methods: This study included all laboratory confirmed MDR-TB patients from two rural provinces chosen on the basis of their different socioeconomic circumstances and provincial TB programs.

Results: The treatment success rate of tuberculosis patients on MDR-TB treatment was low (40%). A higher number of female than male MDR-TB cases were observed (330 vs 225 cases, respectively) in these two provinces. Treatment success was strongly influenced by the setting where the patients were treated. The EC province had the lowest (13.4%) cure rate and the highest default rate of 38.3%. Mortality was significantly associated with HIV-coinfection in both provinces.

Conclusions: The absence of routine second-line drug susceptibility testing and the treatment of MDR-TB with substandard treatment regimen could have led to suboptimal diagnosis and poor treatment outcomes. This shows how regional differences in executing the TB Control Programme can adversely affect treatment outcomes, and therefore need constant, strict monitoring and evaluation of these programmes.

3.2 INTRODUCTION

The World Health Organisation (WHO) reported an estimated 130 000 TB cases in 1995 in South Africa (SA) representing one of the highest annual TB incidences (311 per 100 000 population) in the world. [1] The TB incidence did however vary dramatically by both geographical region and population group. South Africa adopted the Directly Observed Short-Course (DOTS) strategy in 1996 after tuberculosis (TB) was declared a national emergency by the WHO, with the aim of improving the TB cure rate to at least 80% and improving the TB microscopy services. However, the number of TB cases continued to rise.[2] Expansion of the DOTS strategy in SA followed rapidly in the following years and by the end of 2003, there was complete coverage in all nine provinces.

Recognising that multidrug-resistant tuberculosis (MDR-TB) poses a considerable risk to public health, the WHO and partners launched "DOTS-Plus for MDR-TB" to manage MDR-TB in 1999.[3] MDR-TB is defined as a specific form of TB that is resistant to two most effective first-line anti-tuberculosis drugs and accounted for at least 480 000 incident cases and 210 000 attributed deaths globally in 2013.[4] In 2000, the SA National TB Control Programme (TBCP) implemented the DOTS-Plus programmes which included a standardised approach to MDR-TB management through dedicated provincial MDR-TB centres. Despite the implementation of this strategy, SA still has the highest burden of morbidity and mortality due to TB, partly due to human immunodeficiency virus (HIV) co-infection. In SA, as many as 70% of patients with TB are co-infected with HIV.[5] It is estimated that of all cases of HIV associated TB that occur worldwide annually, 30% are in SA.[6]

The prevalence of extensively drug-resistant (XDR) TB, which is MDR with additional resistance to both quinolones and second-line injectable agents,[4] is also on the rise. The highest rates of MDR and XDR-TB in the country were noted for the Western Cape, Eastern Cape and KwaZulu-Natal provinces with treatment success rates below 50% for MDR-TB and considerably poorer outcomes for XDR-TB.[7]

There are also a significant number of patients dying with undetected TB.[9] A post-mortem study conducted in one hospital in KwaZulu-Natal (KZN) showed that TB rates are much higher than estimated.[8] Furthermore and even more alarming, was the observation that of those patients who were not receiving TB treatment, 42% were culture positive.[9] In 2013, 26 023 laboratory-confirmed RR-TB cases were reported in South Africa with only 10 663 (41%) reported to have been initiated on second-line anti-tuberculosis treatment.[10]

Statistics from KZN, WC and EC show that they have the highest burden of DR-TB cases in South Africa. For example, the number of MDR-TB cases diagnosed in KZN, EC and WC in 2014 was 6 630, 2 205 and 2 072 respectively[10] and the number of XDR-TB cases diagnosed were 147, 240 and 175 respectively.[11] In 2000, the treatment of patients with MDR-TB with standardized second-line regimens became a national policy and was implemented through the creation of a network of dedicated provincial MDR-TB referral centres.[12] Despite the implementation of dedicated referral centres and a standardised MDR-TB treatment regimen, the number of laboratory-confirmed cases of MDR-TB in South Africa increased from 2,000 cases in 2005 to 7 350 cases in 2007.[13]

The importance of addressing drug resistant TB was further amplified by more recent reports on totally drug-resistant tuberculosis.[14] Previous reports of MDR-TB have shown poor outcomes in HIV-positive patients, with significant mortality in the first few weeks after initiation. Other identified risk factors for unfavourable treatment outcomes include alcohol abuse, treatment interruption and being male.[14-17] In a report by the MRC, rapid time to treatment initiation of MDR-TB treatment in EC had no significant effect on the treatment outcomes[18] which meant there could be other risk factors.

In this study we aimed to evaluate MDR treatment outcomes in two different provinces (Eastern Cape (EC) and North West (NW) province) for the period 2000-2004 and also identify risk factors associated with unfavourable treatment outcomes of MDR-TB.

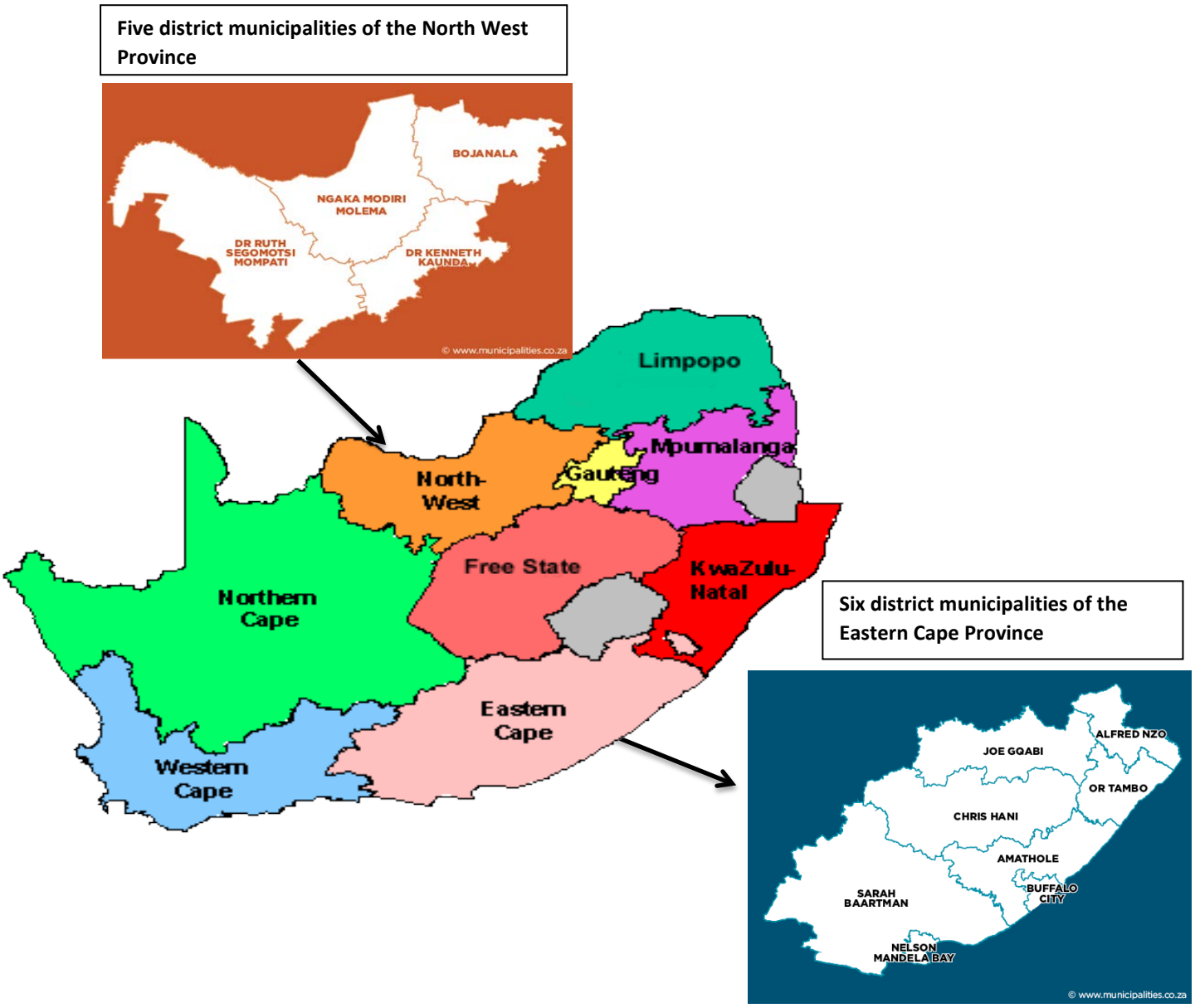
3.3 MATERIALS AND METHODS

3.3.1 Study setting

This study included all laboratory confirmed MDR-TB patients from two rural provinces chosen on the basis of their differences in socioeconomic circumstances, epidemiologic profile TB and provincial TB programs (see Figure 2 for the location of both provinces). The Eastern Cape is the second largest province in the country with a population of approximately 6.9 million people and is characterized by poor socioeconomic conditions. [18]) As of 2013, 755 610 individuals were living with HIV in the Eastern Cape Province. [19] In contrast, the North West province is a third smallest province with a population size of 3.7 million [19] and is a centre of mining activity. The North West had nearly half a million (496,000) HIV positive in 2014.[20] The number of MDR-TB cases diagnosed in EC and NW in 2010 was 1782 and 158 respectively and the number of XDR-TB cases 320 and 14 respectively.[21]

During the study period, the standardised approach to DOTS-Plus in SA comprised of treatment at dedicated MDR-TB referral facilities/centres. MDR-TB referral centres were regarded as centres of expertise and therefore were responsible for treatment of MDR tuberculosis patients, even after discharge. Second-line drugs were only available through these centres, but the bed numbers were limited. Some patients would wait as long as six months to get a bed to initiate treatment. Time from diagnosis to treatment initiation of more than 180 days was common in MDR-TB patients from EC. Patients were admitted for at least the first four months of therapy or preferably until sputum conversion, defined as two consecutive negative cultures, at least 30 days apart. Before patient discharge, arrangements were made with designated clinics to ensure adherence monitoring and to obtain regular feedback on patient progress. In addition, MDR-TB drugs were supplied to the designated clinics referral hospitals treating MDR-TB on a patient name basis. Time from diagnosis (defined as date of the positive culture result at the initial outpatient evaluation) to treatment initiation was recorded. Patients were put on ambulatory treatment after discharge, provided that directly observed treatment was ensured. Final treatment outcomes were recorded at the referral hospital. Patients were followed-up for two years after treatment completion. Six-monthly visits were required to assess symptoms and signs of recurrence.

Figure 2: Map of South Africa showing the location of the North West and Eastern Cape Province in relation to other provinces



3.3.2 Study population

Patients were diagnosed with MDR-TB at primary health care facilities. Adult men and women, aged 18 years and older, who were referred for treatment at a participating MDR-TB treatment centre between 2000 and 2004, with culture confirmed MDR-TB, were eligible for enrolment in the study. Since 2000, most of the MDR-TB treatment centres in South Africa have been using standardised programmatic management of MDR-TB (DOTS-Plus).[22] Standardised therapy was initiated after obtaining drug susceptibility test results.

3.3.3 Treatment regimen

The standardised treatment regimen for MDR-TB consisted of a four-month intensive phase in which five drugs (kanamycin, pyrazinamide, ofloxacin, ethionamide and either cycloserine or ethambutol) were given daily, followed by a 12-18 month continuation phase in which three drugs (ofloxacin, ethionamide and either ethambutol or cycloserine) were given daily. The continuation phase could be shortened on the basis of clinical judgement provided that 12 months of treatment was given after sputum conversion, defined as two consecutive negative cultures, at least 30 days apart. Daily doses were administered in accordance with published guidelines.[21] Patients were seen at regular intervals during the intensive phase and at least monthly during the continuation phase.

3.3.4 Patient cohort

Between 2000 and 2004, 2 079 MDR-TB patients from nine provinces were enrolled in the cohort study conducted by the MRC and followed up for two years.[7] Of 2 079 patients, only 1 676 were eligible for a two-year follow-up, 403 were not eligible for follow up, of these 367 died and 36 was transferred. During this two year follow-up study, monthly follow-up sputum samples were collected, cultured (three to nine cultures were collected from each patient) and stored for future analysis.

All enrolled subjects had 1) no previous history of treatment for MDR-TB (i.e. no history of treatment with second-line drugs) and 2) had demographic and clinical data (HIV, weight, chest X-ray results, TB history etc.) available in an electronic database. Patients with terminal illnesses were not included in the study. Patients with extra-pulmonary TB were also excluded because they are less likely to have a culture positive sputum sample for the required laboratory tests.

Following provincial policies, HIV counselling and testing were performed where feasible on all patients at the start and completion of MDR-TB treatment, and at the completion of the 24 month follow-up period.

3.3.5 Data collection

Data verification was conducted by the research team which reviewed the charts for all treatment failures and a 10% sample of all other clinical records for accuracy of clinical outcome data (death, failure, default, transferred, treatment completed, cured). Discrepancies were rare, but were resolved through discussion and consensus with the final decision made by the coordinating investigator if necessary.

3.3.6 Data Management and Analysis

The standard WHO definitions for treatment outcome in MDR-TB were used. The outcome was categorized as favourable in cases of cure and if treatment was completed, and as unfavourable in cases of failure, death and default. Classification of treatment outcomes was based on smear and culture evaluation, treating clinicians identified the outcome by marking the corresponding category on the medical record form. Data on DST to first-line drugs -SIRE (site of disease, date of processing sputum, date sputum received, adverse effects, treatment start date, treatment end date and treatment outcome) was electronically captured on Epidata. Patients who were classified as having been transferred out of the program were excluded in the analysis as they did not have monthly sputum culture data.

Demographic and clinical treatment characteristics were analysed and differences between the two provinces were assessed separately by province using the chi-square test. To determine the risk factors for unfavourable outcomes, a univariate logistic regression analysis was conducted in the pooled data from both provinces. Table 4 presents the descriptive statistics for the variables used in the study. Variables which had a P value of <0.05 in univariate analysis were then included in a multivariate logistic regression analysis. Adequacy of the final model was assessed by the Pearson goodness-of-fit test. Kaplan-Meier analysis was used to determine risk factors associated with outcomes in time-to-event-based analyses. Survival was calculated from date of diagnosis of MDR-TB to the date of death. Data were analysed using STATA version 11.0 for Windows (STATA Corporation, College Station, Texas). To examine multivariate factors associated with death, a risks regression was conducted to compute cause-specific relative hazards using the Cox proportional hazards model.

3.3.7 Ethical Statement

Ethical approval to conduct the study was obtained from the ethics committee of the South African Medical Research Committee and the Provincial Ethics Committee in each province.

3.4 RESULTS

3.4.1 Comparison of clinical characteristics

Of the 1 676 patients that were eligible for a two-year follow up in the cohort between 2000 and 2004, 556 were patients from the EC (381) and NW (175) province and were included in the study. Of 556 MDR-TB patients studied, 329 (59.2%) were women and 226 (40.6%) were men. The median age was 37 years (ranging from 18 to 75 years). More than 70% of patients in both provinces were classified with bilateral cavitary disease. Overall, >90% had been treated for drug sensitive TB previously and approximately 40% were HIV-positive. Patients enrolled in both provinces differed in terms of their baseline characteristics; these included gender distribution, culture and smear positivity, BMI distribution, cavitary status and HIV status (Table 4).

EC had more male MDR-TB patients (46.2%) compared to NW, and were more likely to have bilateral disease (Table 4). Time from diagnosis to treatment initiation of more than 180 days was common in MDR-TB patients from NW (91.9%) compared to the patients from EC (71.6%) (Table 4). Other differences seen were that patients from NW were more likely to be HIV positive (46.0% vs. 39.5%) and underweight (73.3% vs. 72.0%), however these differences were not statistically significant.

3.4.2 Treatment outcomes

Overall, 223 (40%) of 556 patients had favourable treatment outcomes at the end of therapy, 157 (28.2%) defaulted, 131 (23.6%) were classified as treatment failures and 36 (6.5%) died during treatment (Table 5). Of the 131 patients who defaulted, 44 (33.6%) died within the first three months of starting treatment and were more likely to have presented late for treatment with a median of 40 days to start MDR-TB treatment after diagnosis.

In the Eastern Cape province, a favourable outcome was achieved in 101/381 (26.5%) cases, consisting of those cured 51/381 (13.4%) and treatment completed 50/381 (13.1%). Unfavourable outcomes consisted of treatment failure (5.35%), default (38.3%) and death (28.9%). In the North West province a favourable outcome was achieved in 122/175 (69.7%) cases, consisting of those cured (46.9%) and treatment completed (22.9%). Unfavourable outcomes included treatment failure (9.1%), and default (6.3%) and death (12.0%). Nine patients transferred out; 2.8% from the North West and 1.1% from the Eastern Cape province.

The difference between treatment outcomes of the two provinces was statistically significant with a P- value of less than 0.05.

Table 4: Clinical and Demographic characteristics of MDR-TB patients from the Eastern Cape (EC) and North West (NW) province

Characteristics	EC province n=381 (%)	NW province n=175 (%)	chi2	P- value
Gender				
Female	204 (53.5)	125 (71.4)	15.63	0.000
Male	176 (46.2)	50 (28.6)		
Age				
<50	336 (89.6)	147 (84.0)	3.50	0.061
≥50	39 (10.4)	2□ (16.0)		
***Culture**				
Negative	24 (6.5)	39 (26.9)	40.42	0.000
Positive	346 (93.5)	106 (73.1)		
Smear				
Negative	65 (19.9)	46 (31.9)	7.98	0.005
Positive	261 (80.1)	98 (68.1)		
BMI				
≤20kg/m^2 (Underweight)	116 (72.0)	85 (73.3)	0.05	0.821
>20kg/m^2)	45 (28.0)	31 (26.7)		
HIV Status				
Negative	196 (60.5)	88 (54.0)	1.89	0.169
Positive	128 (39.5)	75 (46.0)		
TB history				
No	36 (9.7)	9 (5.6)	2.51	0.113
Yes	335 (90.3)	153 (94.4)		
Cavitary status				
Bilateral	255 (89.2)	120 (79.5)	7.62	0.006
Unilateral	31 (10.8)	31 (20.5)		
Time to treatment				
>180 days	267 (71.6)	147 (91.9)	26.59	0.000
≤180 days	106 (28.4)	13 (8.1)		

** Patients were diagnosed with MDR-TB at primary health care facilities. Upon treatment initiation, another sputum specimen for culture was collected, sent to SAMRC laboratory but some were culture negative.*

Table 5: Treatment outcomes in the study cohort of 556 MDR-TB patients by province

Treatment outcomes	Provinces		
	Eastern Cape n (%)	North West n (%)	Overall
Cured	51 (13.4)	82 (46.9)	133 (23.9)
Completed	50 (13.1)	40 (22.9)	90 (16.2)
Died	20 (28.9)	16 (12.0)	36 (6.5)
Defaulted	146 (38.3)	11 (6.3)	157 (28.2)
Failure	110 (5.35)	21 (9.1)	131 (23.6)
Transferred out	4 (1.1)	5 (2.8)	9 (1.6)
TOTAL	**381**	**175**	**556**

3.4.3 Risk factors

Risk factors associated with unfavourable outcomes on univiariate analysis were HIV coinfection [OR-1.68; 95% CI 1.15-2.45], time to treatment of less than 180 days [OR-26.1; CI 9.44-72.1], age less than 50 years [OR-2.00; CI 1.18-3.58] and Eastern Cape province [OR 6.68; CI 4.41-10.1] (Table 6). The only risk factor that was protective against poor outcomes was being female [OR-0.64]. When we adjusted for these factors in the multivariate analysis, we found that HIV infection, being female and in the EC province were the only significant risk factors of unfavourable outcomes, although with wide confidence estimates (Table 6).

The probability of surviving for 19 months (600 days) was 60% in the EC and 69% in the NW province (Fig 3). The survival rate among HIV-ve patients at 20 months of treatment in both provinces was higher than HIV+ve patients (Fig 4). The overall survival rates in this study were found to be 55% and 93% in EC and NW, respectively. A lower survival rate was observed in males from the EC as compared to NW. In this study, surprisingly, patients who started treatment >180 days after diagnosis had better survival (Fig 5). The survival rate at the end of continuation phase (24 months/730 days) in patients less than 50 years of age was 40% and was 80% in patients of more than 50 years of age (Fig 6).

In the multivariate analysis, patients with HIV infection and bilateral disease remained the only significant risk factors of unsuccessful outcomes in the EC (Table 6). After stratifying on Province, those patients in the EC that had bilateral disease and were HIV positive were those with worse treatment outcomes, potentially reflecting sicker patients in that Province (Table 7; Figure 4). Using a Cox proportional hazard analysis HIV coinfection [hazard ratio=2.45 (1.39-

4.32)], time to treatment: less than 180 days [hazard ratio = 110.10 (27.8-435.10)] and being underweight [hazard ratio = 1.90 (0.97-3.75)] stayed associated with poor treatment outcomes in addition to being from the Eastern Cape.

Table 6: Univariate and multivariate analysis of demographic and clinical characteristics on unfavourable outcome, EC and NW province, 2000-2004 (n=556)

Variable	Univariate analysis			Multivariate analysis		
	Odds ratio	**p-value**	**95% Conf. Interval**	**Odds ratio**	**p-value**	**95% Conf. Interval**
HIV +ve	**1.68**	**0.01**	1.15-2.45	**2.01**	**0.04**	1.02-3.93
Bilateral Disease on CXR	1.68	0.06	0.98-2.90	**8.17**	**0.01**	1.80-37.10
Previous TB treatment	1.06	0.85	0.57-1.99	2.08	0.06	0.97-4.49
Time to treatment (<180 days)	**26.1**	**0.00**	9.44-72.10	0.72	0.34	0.36-1.42
BMI (>20kg/m^2)	1.62	0.08	0.94-2.79	0.90	0.82	0.35-2.29
Gender (Female)	**0.64**	**0.01**	0.45-0.91	**7.79**	**0.00**	3.94-15.4
Age ≤50	**2.00**	**0.01**	1.18-3.38	0.71	0.46	0.29-1.75
Province (EC)	**6.68**	**0.00**	4.41-10.10	**8.17**	**0.01**	1.80-37.10

** Bold text shows identified variables with significant p-values less than 0.05*

Table 7: Multivariable analysis of demographic and clinical characteristics on unfavourable outcome by province, EC and NW province, 2000-2004 (n=556)

	Eastern Cape Province			North West Province		
Variable	**Odds ratio**	**p-value**	**95% Conf. Interval**	**Odds ratio**	**p-value**	**95% Conf. Interval**
Age ≤50yrs	1.14	0.79	0.45-2.87	0.38	0.15	0.10- 2.88
Bilateral Disease on CXR	**3.43**	**0.01**	1.46-5.87	0.59	0.24	0.24-1.41
HIV +ve	**2.77**	**0.00**	1.44-5.35	1.33	0.47	0.61- 2.88

*****_Bold text shows identified variables with significant p-values less than 0.05_

Table 8: Hazard ratio for risk factors for treatment outcomes

Variable	Hazard ratio	p-value	95% Conf. Interval
HIV +ve	**2.45**	**0.00**	1.39-4.32
Bilateral Disease on CXR	0.73	0.43	0.34-1.59
BMI (underweight = <20kg/m^2)	**1.90**	0.06	0.97-3.75
Gender (Female)	0.74	0.28	0.42-1.28
Age ≤50	0.73	0.46	0.33-1.66
Time to treatment (<180 days)	**110.10**	**0.00**	27.8-435.10
Prov (EC)	**3.37**	**0.00**	1.88-7.40

* Bold text shows identified variables with significant p-values less than 0.05

Figure 3: Time to death during MDR-TB treatment by province

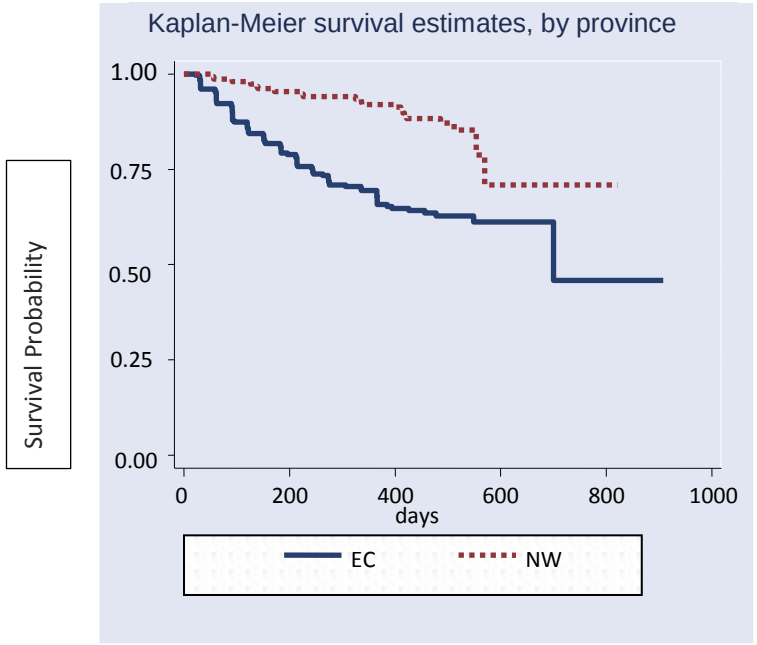


Figure 4: Time to death during MDR-TB treatment by HIV status

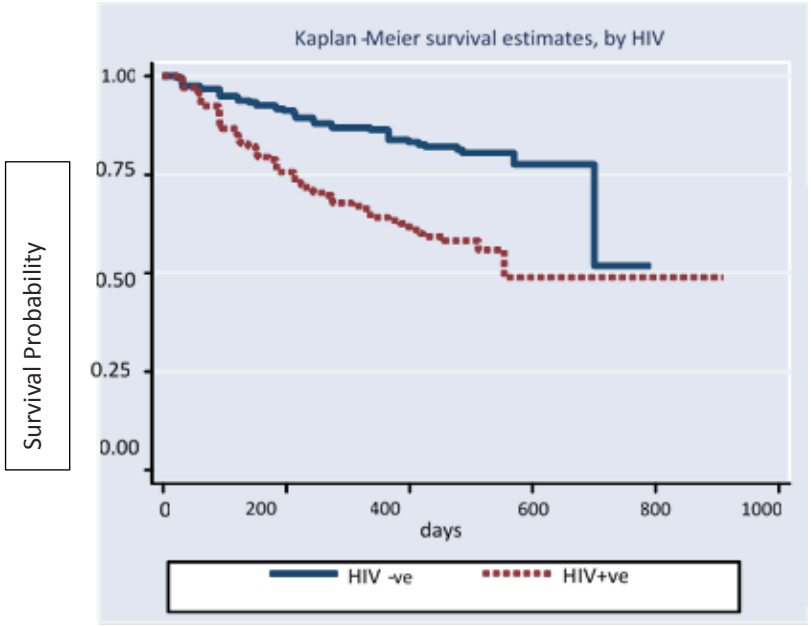
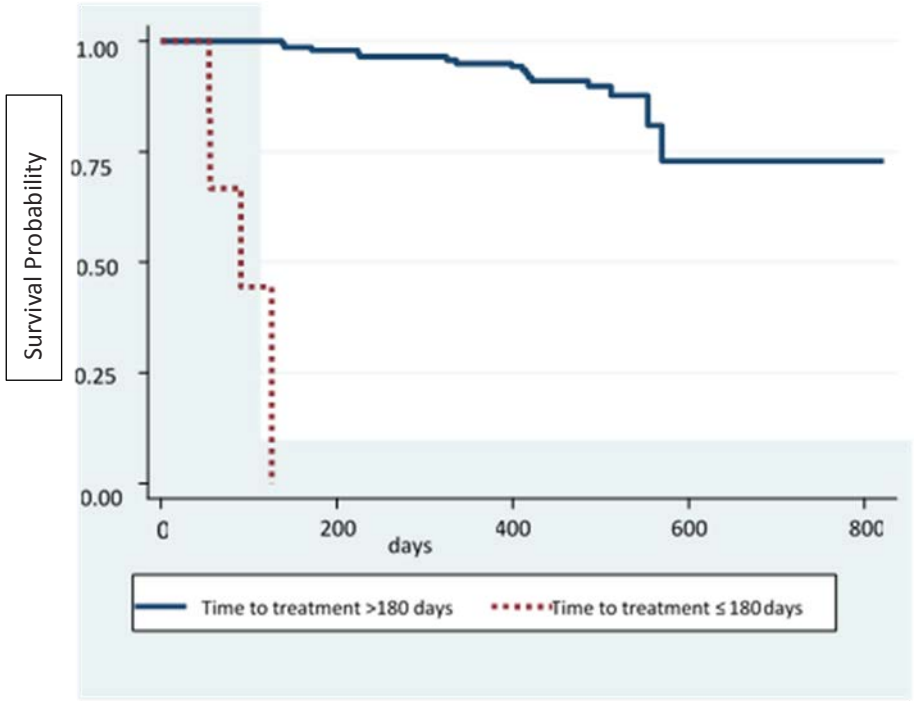


Figure 5: Time to death during MDR-TB treatment by time to treatment after MDR diagnosis (a) >2 years (b) <200 day

a) Kaplan-Meier survival estimates by time to treatment



b) Kaplan-Meier survival estimates by time to treatment

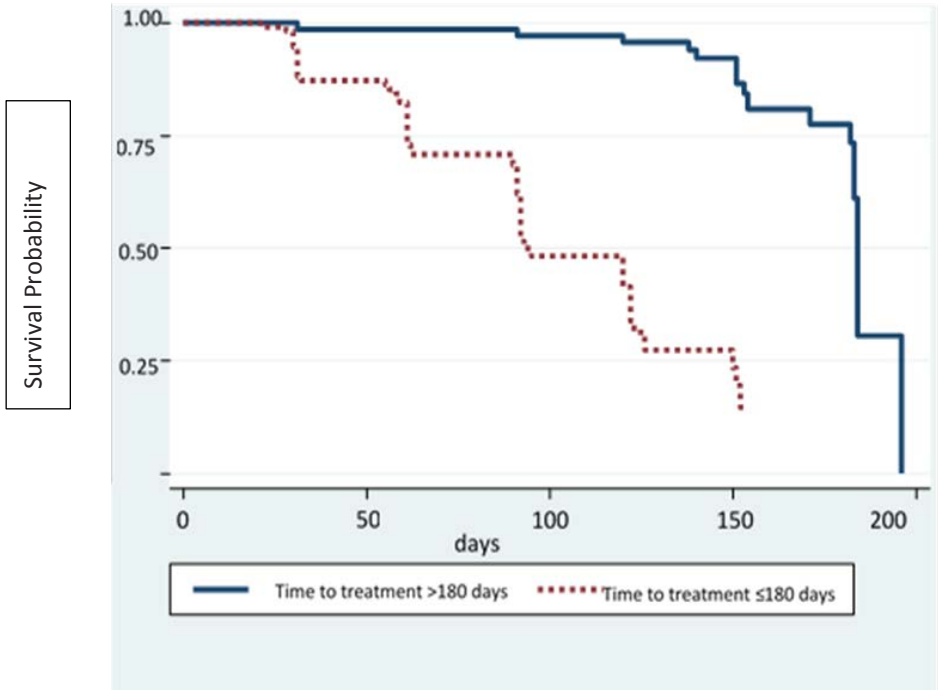
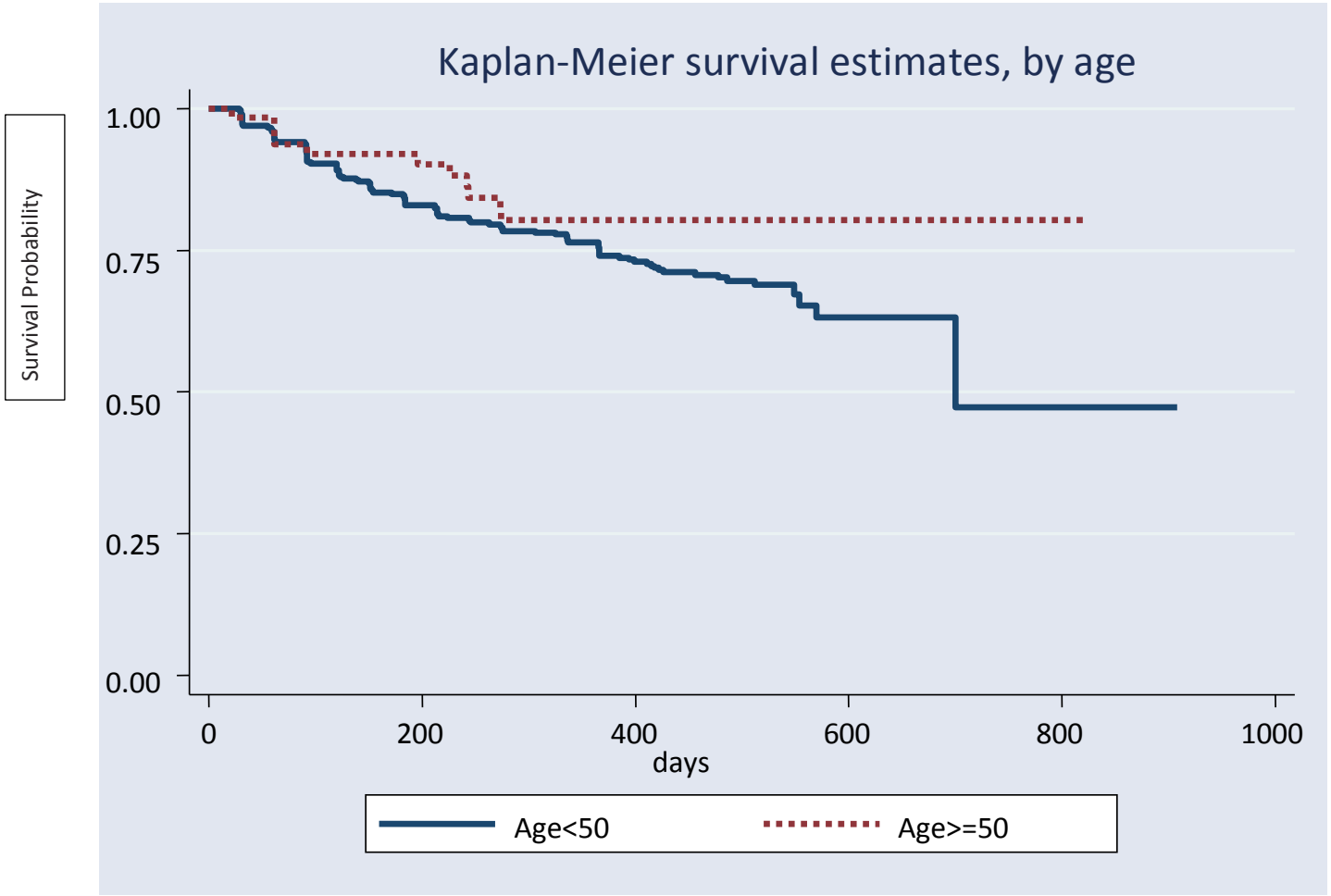


Figure 6: Time to death during MDR-TB treatment by age



3.5 DISCUSSION

Only 223/556 (40%) of patients who started standardized MDR treatment had favourable outcomes in this cohort originating from two rural provinces in SA. Treatment success was strongly influenced by the setting where the patients were treated. Default and death accounted for 58.1% of all unfavourable outcomes.

The EC province had the lower (13.4%) cure rate and the higher default rate of 38.3%. The low cure rate may be due to poor drug compliance caused by adverse drug effects during treatment such as vomiting, nausea, constipation, joint pain etc. Unfortunately, adverse drug effects and comorbidities were not properly reported or recorded on patient files. Besides adverse drug effects, high death rate (28.9%) and also patients who perhaps defaulted for lack of money for transportation to the MDR centres and discontinuing treatment after feeling a bit better could have contributed in the low cure rate that was observed in the province. The poor outcomes in patients with multidrug-resistant tuberculosis could also have been due to highly resistant strains already circulating in the EC. [15]

Age has been identified as an important risk factor for death in tuberculosis patients. In this study, an age of less than 50 years was significantly associated with unfavourable outcomes in univariate analysis. However, this association was no longer significant after adjusting for other factors, including HIV status. Patients less than 50 years are a sexually active group, 41% of patients in this group were HIV positive. However, in other studies, higher death rates have been noted in the elderly patients.[24-25] The observation of a high proportion of HIV positive patients from NW than EC (46.0% vs. 39.5%) is supported by data from a national HIV prevalence study in 2002 among MDR-TB patients in the two provinces which revealed an HIV prevalence of 66.0%- 30.5% in NW and EC respectively.[28]

Findings from this study are comparable with the proportion of successfully treated patients reported in other Green Light Committee (GLC) supported DOTS-Plus projects.[21] A higher number of female than male MDR-TB cases were observed (330 vs. 225 cases, respectively) in these two provinces, which supports earlier data from a surveillance study conducted in South Africa in 2002 of a total of 81 794 TB patients with known sex (95% of all patients) and showed that female TB cases had a 1.2 times higher odds ratio of harbouring MDR-TB strains than male TB cases.[27]

Mortality was associated significantly with HIV-coinfection in both provinces. The presence of bilateral disease was a significant risk factor only in the NW province. A proportion of

confirmed MDR-TB patients continued with the same first line regimen at their centres while waiting for hospitals beds. Antiretrovirals (ARVs) were only available towards the end of the study in both provinces, 2004 in EC and 2003 in NW. In the EC, patients that were HIV positive were transferred to different hospitals or clinics from the MDR centre where they were treated for MDR-TB but were not accredited for ARVs at the time. In the NW province the cure rate was 46.9% and the default rate was 6.3%. The better success rate in NW could be attributed to differences in provincial TB control programs. Patients in the NW province had a better transport system which was arranged from the referral clinics to the MDR centres for their follow-up check-ups.

The limitations of the study included firstly, being a retrospective analysis. Secondly, the CD4 count was not routinely recorded by clinicians in both provinces making this data unavailable for analysis and limiting our description of the cohort in relation to severity of HIV infection and treatment outcomes. Our current finding of 39.5% (EC) and 46.0% (NW) may underrepresent the HIV positive population and may suggest that those patients with an HIV unknown status were early in their HIV disease and therefore more clinically similar to HIV negative MDR-TB patients. Inclusion of time to treatment in survival analysis may have resulted in bias as patients included in the study may be a survival cohort.

The study revealed a consistent association between HIV co-infection and being younger as the most important risk factor for poor outcomes. Sixty patients started treatment after >180 days but did not show a poor outcome compared to patients who received timely treatment. potentially reflecting a survival cohort effect in that those who survived that long had a better chance of surviving overall. These results could also have been influenced by continuing with a first line treatment regimen while waiting for a bed. Further research should evaluate the clinical characteristics of patients who were started on MDR-TB start treatment immediately after diagnosis in these two provinces. It is likely that patients who benefited from early treatment could be the patients that appeared very ill.

The treatment success rate of tuberculosis patients on MDR-TB treatment was low (40%). The key factors to successfully combat this epidemic include earlier diagnosis of MDR-TB, earlier initiation of appropriate second-line TB treatment, and more aggressive HIV testing and ART initiation. The absence of routine second-line drug susceptibility testing and the treatment of MDR-TB with substandard treatment regimen could have led to suboptimal diagnosis and treatment outcomes. To improve treatment outcome of tuberculosis patients in the two provinces, we recommend earlier diagnosis, rapid detection of MDR-TB, initiation, management and monitoring of MDR-TB treatment close to their homes at community health

facilities (CHCs) and PHCs in order to reduce waiting time before patients start treatment. This will address transport issues which make it difficult to access treatment sites and patients will not have to wait for a bed to become available at a referral facility.

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CHAPTER 4

DETECTION OF SECOND-LINE DRUG RESISTANCE IN MDR-TB ISOLATES BY GENOTYPE MTBDR*sl* ASSAY (VERSION 1) AND DIRECT SEQUENCING

4.1 ABSTRACT

Background: Rapid identification of drug resistance and initiation of appropriate treatment is key in order to break the chain of transmission in the communities. The availability of rapid molecular tests for the detection of extensively drug-resistant TB (XDR-TB) is critical in areas with high rates of multidrug-resistant TB (MDR-TB) and XDR-TB.

Objectives: To retrospectively determine resistance patterns against second line drugs among newly diagnosed MDR-TB patients in the North West (NW) and Eastern Cape (EC) province and how they have acquired resistance during treatment.

Methods: The cultures used in this study were collected from two prospective observational studies 1) the DOTS-Plus study and 2) Preserving the Effective TB Treatment Study (PETTS). A total of 306 (206 EC and 100 NW) serial isolates from MDR-TB patients with both initial and last isolates available were examined. Drug resistance to second line drugs was detected by GenoType MTBDR*sl* assay (version 1) and direct sequencing.

Results: Sequencing confirmed that 10 patients had baseline isolates that were ofloxacin sensitive but acquired resistance during treatment. In this study, mutation D94G was the frequently observed mutation [37.0% (27/73)], followed by D94Y [19.2% (14/73)], D94H [15.1% (11/73)], D94A [9.6% (7/73)] and A90V [5.5% (4/73)]. A total of 2/298 (0.7%) of isolates analyzed were detected as heteroresistant. We observed 21% acquisition of resistance to second line drugs during treatment.

Conclusions: The accuracy of the GenoType MTBDR*sl* assay for the detection of FLQ was comparable to that of sequencing. However, the test was less accurate for the detection of aminoglycosides/CAP. To overcome this, we recommend MTBDR*sl* v2.0 which is more sensitive than MTBDR*sl* v1 and is able to detect most common mutations involved in resistance to flouroquinolones and aminoglycosides/CAP. There is evidence of acquisition of resistance to second line drugs during treatment.

4.2 INTRODUCTION

Efficient tuberculosis (TB) control is based on early diagnosis, followed by rapid identification of drug resistance and initiation of appropriate treatment in order to break the chain of transmission in the communities. Notable advances in TB diagnostics have been made in recent years with the development of new tools for the detection and rapid identification of patients with multidrug-resistant tuberculosis (MDR-TB) caused by *M. tuberculosis* (MTB) strains that are resistant to isoniazid (INH) and rifampicin (RIF) and extensively drug resistant tuberculosis (XDR-TB) which is defined as MDR-TB with additional resistance to any fluoroquinolone (FLQ) and to at least one of three injectable second-line drugs, (kanamycin (KAN), amikacin (AMK), and/or capreomycin (CAP).[1]

Globally, an estimated 480 000 people developed MDR-TB in 2014 and an estimated 190 000 died from MDR-TB. According to the World Health Organization (WHO) 2015 Report, XDR-TB had been reported by 105 countries. On average, 9.7% of people with MDR-TB have XDR-TB. Levels and trends of drug resistance to second-line drug resistance vary by country with resistance to at least one second line drug ranging, from 33% in Thailand to 62% in Latvia. Resistance to second-line injectable drugs ranges from 2% in the Philippines to 47% in Latvia, while fluoroquinolone resistance prevalence ranges from 7% in the Philippines to 32% in South Korea.[2] Countries with higher poverty rates tend to have a higher burden of drug resistance due to lack of resources and access to the most up to date treatments.

Routine drug susceptibility testing (DST) for all TB patients prior to starting their TB treatment would be ideal for monitoring the amplification of resistance and also to ensure that the most appropriate treatment is determined. However, because of lack of access to routine DST services in most countries, inconsistent diagnoses frequently lead to inadequate treatment, increased mortality and inaccurate determination of the true burden of the disease. In 2013, the WHO published a report of the Expert Group Meeting (held on 21st March, 2012) that was held to review the evidence on Genotype MTBDR*sl* (Hain Lifescience Nehren, Germany) for policy recommendation.[3] It approved the use of line probe assays for MDR-TB diagnosis, but not as a complete replacement for conventional culture and DST, and has yet to recommend the use of molecular diagnostics for XDR-TB.[3]

In South Africa (SA) second-line DST for amikacin and ofloxacin is carried out routinely since 2014, for all RIF-resistant samples using phenotypic culture-based methods to determine the growth of MTB bacilli in the presence of individual drugs. These results only become available weeks or months following initiation of MDR treatment and should be actively followed up as detection of further resistance represents XDR or pre-XDR-TB and necessitates modification

of treatment. The GenoType MTBDR*s*l version 1 rapidly detects mutations conferring resistance to second-line drugs (specifically fluoroquinolones, ethambutol (EMB) and the injectable agents). The probes contained in the assay do not detect all mutations in these genes but are targeted to the most commonly occurring mutations. Fluoroquinolone resistant isolates are reported to carry mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* gene,[4] and that a small number have mutations in the *gyrB* gene[5-6] It was previously postulated that efflux pump mechanisms account for FQ resistance in isolates with wild-type *gyrAB* genes.[7] Resistance to second-line injectable drugs is detected by targeting the commonly known 1401 and 1484 mutations in the *rrs* gene[8] and ethambutol (EMB) by targeting the *emb* 306 mutation in the *emb* gene.[9]

Evaluation of the performance of this test suggested that the sensitivity for detecting fluoroquinolone resistance is between 75.6% and 90.6%, and the sensitivity for detecting kanamycin resistance is between 77% and 100%.[10-11] In contrast, the sensitivity for detecting ethambutol resistance is only 64.2%.[11] This low sensitivity of the test, particularly for resistance to the injectables, has delayed implementation in the routine diagnostic algorithm and the test is undergoing further evaluation. This assay has diminished accuracy in smear-negative specimens,[12] meaning that culture isolates still need to be awaited to rule-in resistance. Conventional culture and phenotypic DST capacity is still needed to detect XDR-TB.[13]

To improve the overall performance of Genotype MTBDR*sl*, and in particular its sensitivity for KAN resistance, a new version of the assay (i.e. Genotype MTBDR*sl* v2.0) has recently been developed. Genotype MTBDR*sl* v2.0 includes two new target genetic regions: the *eis* promoter region −10 to −14 (in addition to *rrs*) and the *gyrB* quinolone resistance-determining region (QRDR) (in addition to *gyrA*) for the detection of mutations at codons 536 to 541. The target region for EMB (*embB*) has been removed. Genotype MTBDR*sl* v2.0 thus includes a total of 27 probes for the detection of resistance exclusively to second-line drugs.

Automated liquid cultures systems have significantly reduced the turnaround times for drug susceptibility testing (DST) compared to use of solid media. However, it takes more than 10 days for the detection of drug resistance using automated liquid cultures systems. For example, the BACTEC MGIT 960 and BACTEC 460TB need 13.3 days and 10.6 days on average to report the drug resistance results, respectively.[14] Liquid systems are also more prone to contamination potentially resulting in misdiagnosis and consequently to a patient receiving an incorrect treatment regimen.[15-16] Furthermore, identification of *M. tuberculosis* in liquid systems is greatly affected by loss of mycobacterium viability on long-term storage

of samples. A rapid, reliable and accurate test is therefore necessary to avoid clinical deterioration, improve patient management and prevent further transmissions.[17]

Genotype MTBDR*sl* assay (Hain Lifescience GmbH, Nehren, Germany), presents a more rapid alternative for diagnosis of XDR-TB. It was developed using PCR-based amplification and a reverse blotting assay that employs specific probes linked to nitrocellulose strips to detect resistance to second-line drugs and ethambutol.

Susceptibility testing for second-line drugs (fluoroquinolones and injectables) was not routinely performed on MDR-TB specimens during the study period unless specially requested by the attending clinician. Prevalence of primary drug resistance also serves as an epidemiological indicator to assess the success of the TB control programme. The aim of this study was to retrospectively determine resistance patterns against second-line drugs among newly diagnosed MDR-TB patients in the NW and EC province and how they have acquired resistance during treatment.

4.3 STUDY SETTING

MDR-TB isolates were obtained from the SAMRC culture collection. These cultures were collected from two prospective observational studies: 1) the DOTS-Plus study and 2) Preserving the Effective TB Treatment Study (PETTS) which were conducted in five provinces in South Africa.

In both studies, sputum specimens were collected from the patients at the specialised MDR-TB hospitals and sent to the SAMRC in Pretoria where the specimens were cultured for mycobacteria at the start of MDR-TB treatment and thereafter on a monthly basis until the end of their MDR-TB treatment episode. Three to nine cultures were collected from each patient.

For the purpose of this study only two provinces (EC and NW) were studied and therefore 306 (206 EC and 100 NW) serial isolates from patients with both initial and last isolates available were examined (Fig 8). The reason for studying these two provinces is that both provinces are understudied in terms of molecular genotyping of *M. tuberculosis* isolates.

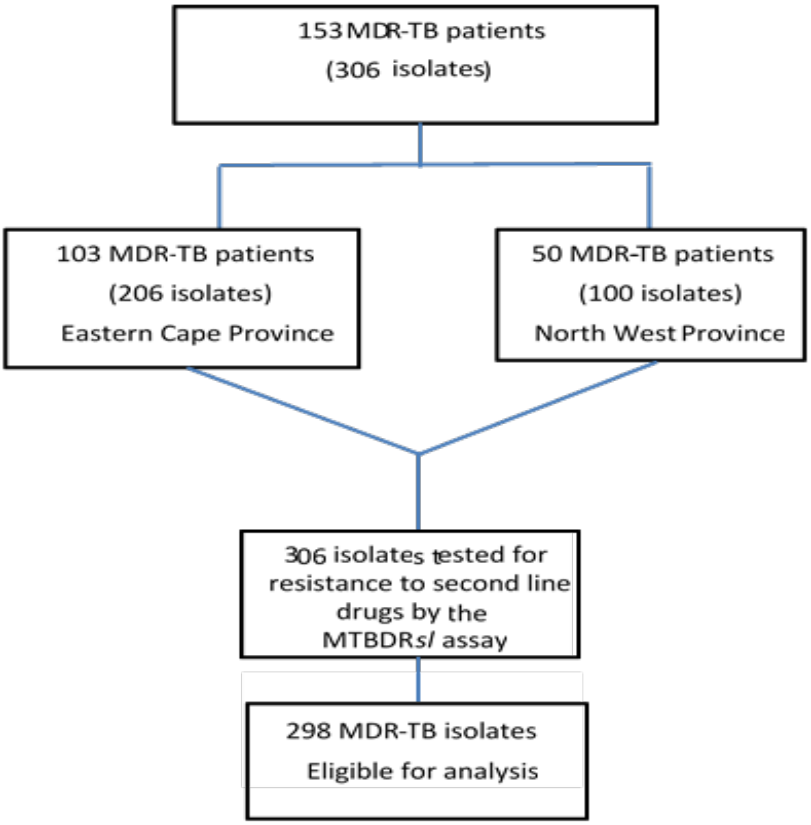
4.4 DESIGN

This was a retrospective descriptive, laboratory based study of tuberculosis isolates from the new MDR-TB patients who presented for treatment at participating MDR-TB treatment centres located in the two provinces (EC and NW) of South Africa between 2000 and 2005.

4.5 SUBJECTS

A total of 306 (206 EC and 100 NW) *M.tuberculosis* isolates collected from MDR-TB patients were included in this study (Fig 7). All enrolled subjects had no previous history of treatment for MDR-TB (in other words no history of treatment with second-line drugs exist). Informed consent was obtained from each study participant before enrolment in the study. Ethical clearance for the collection and use of sputum specimens and usage of patient data followed by the informed consent of each study participant was obtained from the MRC Ethics committee and Provincial Research Committee in each province. Strict patient confidentiality was maintained throughout the entire study.

Figure 7: MDR-*M. tuberculosis* isolates included in the study



4.6 MATERIAL & METHODS

Isolates used in this study were collected from patients clinically and laboratory diagnosed as MDR-TB. Detection and characterization of mutations associated with resistance to fluoroquinolones (FLQ), aminoglycosides/cyclic peptide (AG/CP), and ethambutol (EMB) in the *gyrA*, *rrs* and *embB* genes, respectively, was achieved using Genotype MTBDR*sl* assay (version 1). Targeted DNA sequencing of *gyrA* and *rrs* gene was performed to confirm the results of Genotype MTBDR*sl* assay.

4.6.1 DNA Extraction

M. tuberculosis isolates were grown on Lowenstein-Jensen medium slopes at 37°C for three to four weeks. DNA from mycobacterial cells was extracted using a boiling technique. Briefly, a 1 µl loopful of cells was suspended in 200 µl of TE buffer pH8.0 (10mM Tris-Cl, 1mM EDTA) and heat-killed by incubation at 95°C for 15-20min. The supernatant containing the extracted DNA was collected by centrifugation at 12000 rpm for 7min.

4.6.2 Drug susceptibility testing to second-line drugs

The Genotype MTBDR*sl* assay was performed according to the instructions provided by the manufacturer (Hain Lifescience GmbH, Nehren, Germany).[12] The Genotype MTBDR*sl* assay involves DNA extraction from culture isolates or AFB smear-positive respiratory specimens, followed by PCR amplification and reverse hybridization. It provides a visual reading of test results on nitrocellulose strips.

The Genotype MTBDR*sl* strip contains 22 probes, including two amplification and hybridization controls to verify the test procedures. Besides an *M. tuberculosis* specific probe, PCR control bands for all targeted regions (*gyrA*, *rrs*, and *embB*) are present. For the detection of FLQ resistance, three *gyrA* wild-type probes (WT1, WT2, and WT3) encompass the region of the gene encoding amino acids 85 to 97. Six probes (*gyrA* MUT1 A90V, *gyrA* MUT2 S91P, *gyrA* MUT3A D94A, *gyrA* MUT3B D94N/Y, *gyrA* MUT3C D94G, *gyrA* MUT3D D94H) specifically target the most common mutations. For the detection of AMK-KAN-CAP resistance, two probes cover the wild-type region of *rrs*, while two others (*rrs* MUT1 and MUT2) are designed to assess nucleotide exchanges A1401G and G1484T. For the detection of EMB resistance, *embB* wild-type probe WT1 targets *embB* codon 306, while the *embB* MUT1A and MUT1B probes were designed to bind to nucleotide exchanges ATG/ATA (M306I) and ATG/GTG (M306V).

Results for the Genotype MTBDR*sl* assay are interpreted based on the hybridization (presence of sharp visible band) to the respective probes coated onto the strips. A test is valid and interpretable when Conjugate Controls (CC), Amplification Control (AC) and *M. tuberculosis* complex (TUB) bands are visible along with *gyrA* Locus control (LC), *rrs* LC, *emb* LC. Absence of any one of the bands make the test invalid/indeterminate for the respective target. In addition, presence of a wild type sequence along with the corresponding mutant probe indicates the sample carrying heteroresistant strain.

For a given isolate, if all of the wild-type (WT) probes showed positive staining and the mutant probes produced no staining, the isolate was considered susceptible to the three drugs. In contrast, an isolate was considered resistant if at least one WT probe was absent or if any mutant probe was present.

4.6.3 DNA Sequencing

Targeted DNA sequencing was used to detect second-line drug resistance and confirm the results of Genotype MTBDR*sl* assay. DNA was extracted using a boiling method as well as DNA and flanking sequences (amplification product codons 18 to 132). The sequences of samples were then submitted for PCR, clean-up and sequencing using an ABI 3130XL genetic analyser at the Central Analytic Facility of Stellenbosch University. The chromatograms were analyzed using Chromas software. Mutations conferring ofloxacin resistance were determined by DNA sequencing of the quinolone resistance-determining region (QRDR) of the *gyrA* gene. The primers used for PCR amplification of the gyrase gene were: *gyrA* forward primer 5'–TGA CAT CGA GCA GGA GAT GC–3' and *gyrA* reverse primer: 5'–GGG CTT CGG TGT ACC TCA TC–3'. Mutations conferring to KAN/AMK/CAP resistance were determined by DNA sequencing of the region encompassing nucleotide 1401 of the *rrs* gene (amplification product nucleotides 1339 to 1528). The *rrs* gene (1400-1500 region) was amplified from each isolate using primers: *rrs*290 forward 5'–TGCTAC AATGGCCGGTACAA-3' and *rrs*290 reverse 5'-CTTCCGGTACGGCTACCTTG-3'. We chose to not include the *emb* gene for sequencing as EMB was not considered one of the effective four drugs even though it was included in the regimen.

4.7 RESULTS

A total of 306 MDR *M.tuberculosis* strains isolated from patients with MDR-TB in the EC and NW province of South Africa were tested for resistance to the second-line antituberculosis drugs AMK/KAN/CAP, OFX and EMB. Readable Genotype MTBDR*sl* assay results were obtained for 298 MDR-TB isolates. Representative DNA patterns obtained with Genotype MTBDR*sl* are shown in Figure 8. Susceptibility pattern results obtained by Genotype MTBDR*sl* are presented in Table 9.

Figure 8: Example of DNA patterns obtained by testing MDR-TB isolates using GenoType MTBDR*sl* (v1.0) assay. The positions of the oligonucleotides and control probes are shown on the right side of the strip. From right to left are susceptibility patterns of different isolates; Lane 1, example of isolate OFXr KAN/CAPr EMBr pattern; Lane 2, OFXs KAN/CAPs EMBs; Lane 3, OFXs KAN/CAPs EMBs; Lane 4, OFXr KAN/CAPsEMBr, (heteroresistant); Lane 5, OFXr KAN/CAPsEMBr (heteroresistant); Lane 6, OFXsKAN/CAPsEMBr

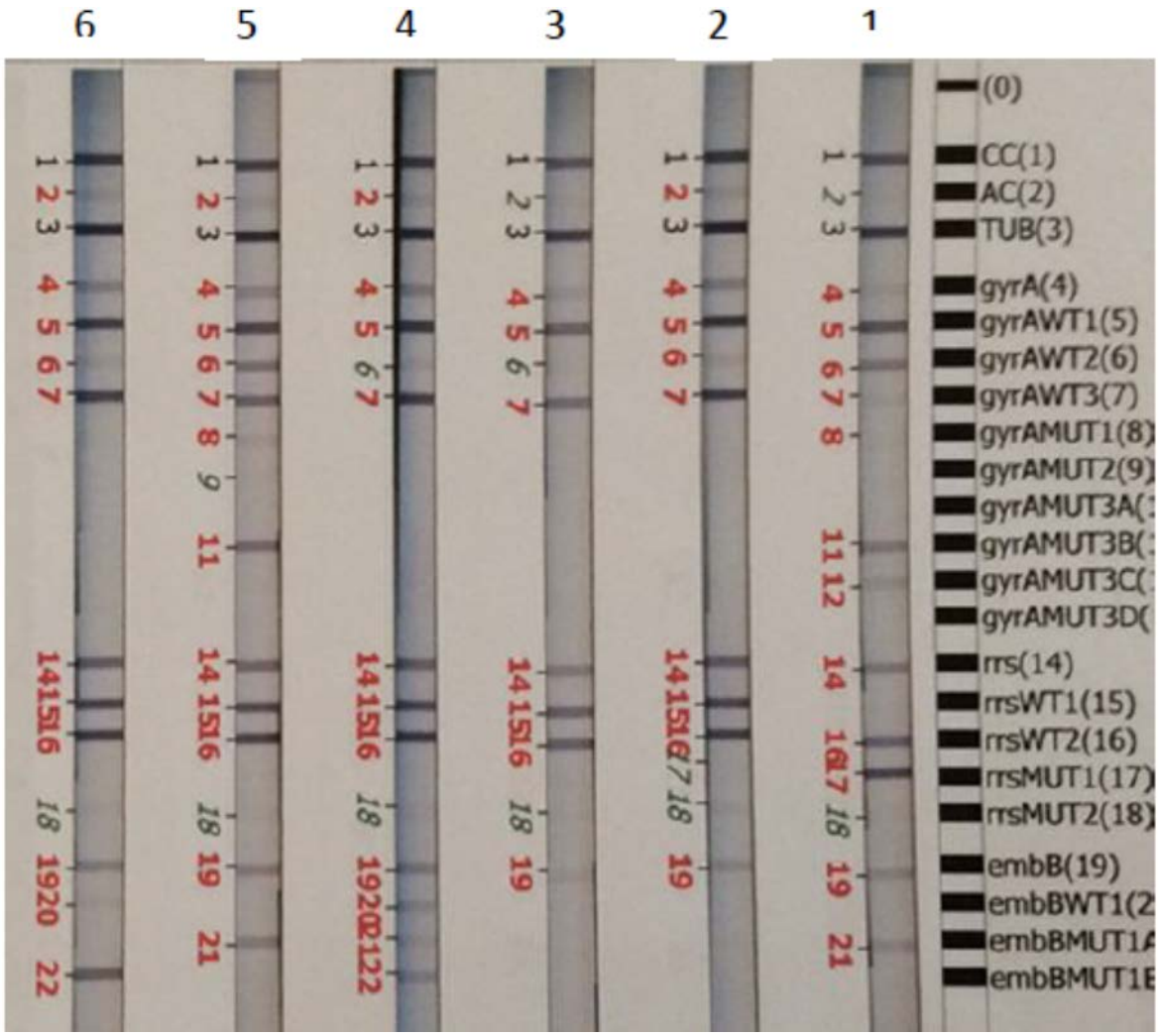


Table 9: Resistance of the 298 MDR *M. tuberculosis* strains to second-line anti-tuberculosis drugs

DRUG RESISTANCE PATTERN	NO OF STRAINS	%
Susceptible to second-line drugs	**93**	**31.0**
OFX only	5	1.7
AMK/KAN/CAP only	3	1.0
EMB only	83	27.9
AMK/KAN/CAP+EMB	44	14.8
OFX+EMB	19	6.4
OFX+AMK/KAN/CAP	4	1.3
OFX+AMK/KAN/CAP+EMB	47	15.8

OFX=Ofloxacin; KAN=Kanamycin; AMK= Amikacin; CAP=Capreomycin; EMB=Ethambutol

Of all the isolates tested 97.4% (298/306) were found valid to all the three targets (*gyr*A (FQ), *rrs* (AG/CP) and *embB* (EMB), respectively) by Genotype MTBDR*sl* assay, and the remaining 2.6% (8/306) were found invalid/indeterminate. Two of 298 isolates had both the wild-type, mutant OFX and EMB bands, suggesting heteroresistance.[18-19] Heteroresistance is the phenomenon of simultaneous occurrence of drug resistant and drug sensitive organisms in the same sample.[4,19] In all eight isolates with invalid results, the *rrs* gene control band was absent. Similar results were obtained after repeating the assay and hence these isolates were excluded from the analysis. The results discussed below are of isolates with valid results.

Fluoroquinolone resistance: Table 10 summarizes drug susceptibility patterns of FQ-resistant and FQ-sensitive isolates with valid results. Of the total 298 isolates analysed, 223 (75.5 %) showed wild-type patterns in the *gyrA* gene and the remaining 75 (25%) isolates had mutations detected by the Genotype MTBDR*sl* assay in the *gyrA* gene. One (1.3%) ofloxacin-resistant isolate was detected as heteroresistant, indicating the coexistence of mutant and wild-type strains.

Among the 75/298 (24.5%) OFX-resistant isolates, the *gyrA* MUT 3C/D94G was the most prevalent mutation occurring in 27/75 (36%) isolates followed by the *gyrA* MUT 3B band/D94Y (14/75; 18.7%), *gyrA* MUT 3D/D94H (11/75) is a rare mutation which has been detected only in silico and hence thought to be undetectable in vitro; 14.7%), *gyr*A MUT 3A/ D94A (7/75; 9.3%), and *gyrA* MUT 1/A90V mutation (4/75; 5.3%) (see Table 10).

In 10 of 75 isolates (13.7%) both *gyrA* MUT 3C/D94G and *gyrA* MUT 3B band/D94Y (2/10); *gyrA* MUT 3C/D94G and *gyrA* MUT 3D/D94H (4/10); *gyrA* MUT 3A/ D94A and *gyrA* MUT 1/A90V (4/10) were present (see Table 10).

Table 10: Genotype MTBDR*s*/ test results for the detection of FLQ, AMK-CAP, and EMB resistance in 298 smear-positive MDR-TB isolates with valid results

Resistance and Genotype MTBDR*s*/ pattern (*gyrA*, *rrs*, *embB*)*b*	Resistance and Genotype MTBDR*s*/ pattern (*gyrA*, *rrs*, *embB*)*a*	Gene region of mutations detected	Genotype MTBDR*s*/ result : (No. (%) of strains)
FLQ			
gyrA ΔWT3, MUT 3C	FLQr	D94G	27 (36)
gyrA ΔWT3, MUT 3B	FLQr	D94N/D94Y	14 (18.7)
gyrA WT, MUT 3B	FLQr (heteroresistant)	D94N/D94Y	1 (1.3)
gyrA ΔWT3, MUT 3D	FLQr	D94H	11 (14.7)
gyrA ΔWT3, MUT 3A	FLQr	D94A	7 (9.3)
gyrA ΔWT1, MUT 1	FLQr	A90V	4 (5.3)
gyrA ΔWT1, ΔWT 3, MUT 3A, MUT 3B, MUT3C, MUT 3D, *gyrA* MUT 1	FLQr	D94A, D94N/D94Y, D94G, D94H, A90V	10 (13.3)
gyrA ΔWT3	FLQr	D94X	1 (1.3)
gyrA WT	FLQs	None	223 (75.5)
AMK-KAN-CAP			
rrs ΔWT1, MUT1	AMK/KAN/CAPr	A1401G	70 (23,5)
rrs ΔWT2, MUT2	AMK/KAN/CAP r	G1484T	20 (6.7)
rrs ΔWT1, ΔWT2	AMK/KAN/CAP r	-	8 (2.7)
rrs WT	AMK/KAN/CAPs	None	200 (67.1)
EMB			
embB ΔWT1, MUT1A	EMB^r	M306I	118 (61.4)
embB ΔWT1, MUT1B	EMB^r	M306V	56 (18.8)
embB WT1, MUT1A, MUT1B	EMB^r (heteroresistant)	M306I, M306V	1 (1.3)
embB ΔWT1, MUT1A, MUT1B	EMB^r	M306I, M306V	7 (2.3)
embB ΔWT1, ΔWT2	EMB^r	-	10 (3.4)
embB WT	EMB^s	None	106 (35.6)

- a = r, resistant; s, susceptible.
- b = WT, wild-type pattern with all respective bands; ΔWT, lack of hybridization with the indicated wild-type probe (WT1, WT2, or WT3)

Aminoglycosides/cyclic peptide resistance: Table 10 summarizes drug susceptibility patterns of AMK/KAN/CAP-resistant and AMK/KAN/CAP-sensitive isolates with valid results. Of 298 isolates with valid results, 98 (32.8%) had mutations in the *rrs* gene (see Table 9). Forty eight isolates had acquired resistance to ethambutol. Of the 98 AMK/KAN/CAP resistant isolates, the *rrs* MUT 1/A1401G was the most prevalent mutation occurring in 70/98 (71.4%) isolates. The remaining 20/98 (20.4%) had *rrs* MUT 2/A1484T mutation which has been previously been reported in South Africa by Said et al.[20] while 8/98 (8.2%) were resistant by absence of wild-type bands at positions 1401 and 1402 (WT1) and position 1484 (WT2) in the *rrs* gene with no developing mutations band.

Ethambutol resistance: Table 10 summarizes drug susceptibility patterns of EMB-resistant and EMB-sensitive isolates included in the study. Of 192/298 (64.4%) ethambutol resistant isolates 118 (61.5%) had mutations in the *embB* MUT 1A/M3061. The second most prevalent mutation of the 192 EMB resistant isolates was *embB* MUT 1B/M306V (56/192; 29.1%), followed by isolates with both *embB* MUT 1A/M3061 and *embB* MUT 1B/M306V (8/192; 4.2%) of which one was heteroresistant. The remaining isolates (10/192; 5.2%) were resistant by absence of wild-type patterns with no developing mutations band.

The 298 isolates with valid results included two isolates that were not paired, in other words only the baseline or final isolate was available. One hundred and forty eight (148) patients had DST results for both the baseline and final TB culture positive isolate available. In 50 of 148 patients (34.0%) the DST pattern of the last available isolate was different from that of the patient's initial isolate. Of these, 31 (70.0%) patients had last available isolates that became resistant to an additional drug indicating that the infecting strain gained a mutation conferring resistance during treatment. Eighteen patients had last available isolates that had lost resistance, in other words the final isolate became sensitive after initially being resistant to one or more of the drugs tested. One patient had a final available isolate that had both gained and lost resistance in one of the drugs. Table 11 shows the number of reversions for each of the second-line drugs tested.

Sequencing for detection of Ofloxacin and Kanamycin resistance: Our panel included 104 isolates that were further tested by DNA sequencing to confirm the results of the Genotype MTBDR*sl*. A total of 34/104 (32.7%) and 15/104 (14.4%) isolates were available for sequence analysis associated with mutations in *gyrA* and *rrs* gene respectively, thus 70/104 (67.3%) could not be included because of a bad sequence data either due to an insufficient or poor quality template. Since ethambutol is not considered an active drug in MDR-TB, we

did not sequence the *embB* gene for ethambutol resistance in this study, although it was added to the MDR-TB regimen during the period of the study.

Table 11: QRDR mutations identified in patients whose initial and last isolates (34) had different DST profile

*Patient	DST pattern by Hain assay			No. of months between the initial and final isolates	Mutations in *gyrA* by Direct Sequencing		
	OFX	KAN	EMB		Change in nucleotide	Amino Acid Change	Type of Resistance
1	S R	S S	R R	35	AGC-ACC GAC-AAC; AGC-ACC	S95T D94N; S95T	Gained resistance
2	S S	S R	R R	3	GAC-AAC; AGC-ACC GAC-GGC; AGC-ACC	D94N; S95T D94G; S95T	Gained resistance
3	S R	S S	R S	1	AGC-ACC AGC-ACC	S95T S95T	-
4	S R	S R	S R	9	AGC-ACC AGC-ACC	S95T S95T	-
5	S S	S S	R S	2	AGC-ACC GAC-AGC; AGC-ACC	S95T D94S; S95T	No resistance gained
6	S R	S S	S R	1	AGC-ACC GAC-AGC; AGC-ACC	S95T D94S; S95T	Gained resistance
7	S R	R R	S S	28	AGC-ACC GAC-GCC; AGC-ACC	S95T D94A; S95T	Gained resistance
8	S R	S R	S S	28	AGC-ACC GAC-AGC; AGC-ACC	S95T D94S; S95T	Gained resistance
9	S R	R R	R R	8	AGC-ACC AGC-ACC	S95T S95T	-
10	S R	R R	R R	7	AGC-ACC GAC-GGC; AGC-ACC	S95T D94G; S95T	Gained resistance
11	S R	R R	R R	8	AGC-ACC AGC-ACC	S95T S95T	-
13	R R	S R	S S	17	AGC-ACC AGC-ACC	S95T S95T	-
14	S R	S S	R R	11	AGC-ACC GAC-AGC; AGC-ACC	S95T D94S; S95T	Gained resistance
15	R R	S R	R R	4	GAC-GCC; AGC-ACC GAC-GCC; AGC-ACC	D94A; S95T D94A; S95T	-
16	S R	S R	R R	11	AGC-ACC GAC-AGC; AGC-ACC	S95T D94S; S95T	Gained resistance
17	S R	R R	R R	15	AGC-ACC GAC-GGC; AGC-ACC	S95T D94G;S95T	Gained resistance
18	S R	S S	R R	10	AGC-ACC GAC-GGC; AGC-ACC	S95T D94G;S95T	Gained resistance

**For each patient, the first row is the results of the initial isolate; whereas second row are results of the final isolate*

Analysis of gyrA gene for mutations in Ofloxacin resistant isolates: Comparison between the Genotype MTBDR*sl* assay and the DNA sequencing results of the *gyrA* gene showed discordant results in 6/34 (17.6%) isolates. Of these, four of the 34 isolates resistant by Genotype MTBDR*sl* assay showed wild-type sequence, while two isolates that were sensitive by GenoType MDRTB*sl* assay were resistant by sequencing method. The remaining 28 isolates (82.4%) were correctly identified as OFX resistant by Genotype MTBDR*sl* assay.

The 28 fluoroquinolone-resistant as identified by DNA sequencing displayed various mutations in the *GyrA* QRDR, corresponding either to mutations frequently reported in the literature (mutations in codons 90 and 94) or more rarely encountered mutations (codons 88 and 80) (Table 11).

Analysis of rrs gene for mutations in Kanamycin resistant isolates: Fifteen isolates were available for analysis of mutations in the *rrs* gene. Analysis of the DNA sequencing chromatograms confirmed the presence of an A1401G mutation in the *rrs* gene in 10/15 (67%) patient isolates (not shown). Of these, five of isolates identified as susceptible by Genotype MTBDR*sl* assay had a mutation by sequencing method.

4.8 DISCUSSION

Currently, there are different molecular tests that identify MDR and XDR *M. tuberculosis* and these are mainly DNA sequencing and line probe assays. The Genotype MTBDR*sl* assay is a rapid line probe assay for the detection of resistance to fluoroquinolones, ethambutol and aminoglycocydes in *M. tuberculosis*. In the present study, we assessed the capacity of the Genotype MTBDR*sl* test to detect mutations linked to resistance to OFX, AMK/KAN/CAP and ethambutol and compared them to mutations on genes conferring resistance to OFX and KAN as detected by sequencing.

This retrospective study showed a high prevalence (68.8%) of resistance to second-line drugs for MDR-TB and that of XDR-TB (51/298; 17.1%) as detected by Genotype MTBDR*sl*. The overall rate for reporting a valid test was 97.4% (298/306) and the rate for indeterminate result was 2.6% (8/306). Resistance to all drugs (including ethambutol) tested was 15.8% (47/298) and resistance to both OFX and KAN was 4 (298)1.3%. These findings confirm the high rates reported by Tracy Dalton and colleagues in South Africa that resistance to any second-line drugs was 44.7% and XDR-TB was 10.6%.[21]

A total of 6/34 (17.6%) isolates showed discrepancies of results in the *gyrA* gene between the Genotype MTBDR*sl* and sequencing. Four of the 34 isolates resistant by Genotype MTBDR*sl* assay showed widtype pattern by sequencing, while two isolates that were sensitive by Genotype MDRTB*sl* were resistant by sequencing. Among the *gyrA* targeted mutants in the Genotype MTBDR*sl* assay D94G was the frequently observed mutation [37.0% (27/73)], followed by D94Y [19.2% (14/73)], D94H [15.1% (11/73)], D94A [9.6% (7/73)] and A90V [5.5% (4/73)]. Isolates with the D94Y (19.2%) mutation had both the *gyrA* WT3 deletion + MUT3B hybridization and *gyrA* WT3 + MUT3B hybridization using the Genotype MTBDR*sl* test as observed, as described by Kiet et al.[11]

Furthermore, (2/298) 0.7% isolates analysed were detected as heteroresistant. The heteroresistance was in association to *gyrA and embB* gene, whereas previous studies have reported heteroresistance mostly in relation to *rrs* gene.[11] Heteroresistance might also result from specific treatment regimens, host fitness, differently mutated strains or hypermutable alleles of the DNA repair genes.[4,22] High rates of heteroresistance to FLQ-resistant isolates have been reported before in Germany (21.9%),[16] Russia (16.6%),[4] and Vietnam (21.4%);[20] however, the rates were low in our study (7.9%) and in France (4.2%).[23]

The concordance between Genotype MTBDR*sl* and sequencing was 82% for all *gyrA* gene and 67% for the *rrs* gene. Sequencing confirmed that 10 patients had baseline isolates that were ofloxacin sensitive but acquired resistance during treatment. These patients never received flouroquinolone therapy for TB prior to the initiation of this study. Exposure to OFX and other FQ for the treatment of other bacterial diseases could have contributed to acquiring resistance or they were infected with a MDRTB strain already resistant to FQ ie pre-XDR-TB .[24-25] This finding is concerning because it results in a poor treatment outcome for example early relapse and also because resistance to one fluoroquinolone often means cross resistance to others.[26] There are also reports of OFX-resistant strains of *M. tuberculosis* or the developing of OFX resistance during treatment.[27] This has been attributed to use of fluoroquinolones in patients who have unsuspected tuberculosis. There are reports of the acquisition of fluoroquinolone resistance by Mtb after as little as 23 and 13 days of fluoroquinolone treatment.[28-29] On that basis, the rapid diagnosis of TB drug resistance is recommended to avoid the spread of resistance to second-line drugs.

Mutation in A1401G is related to high level resistance to second-line aminoglycosides.[30-31] In this study, A1401G [71.4% (70/98)] mutation was observed using Genotype MTBDR*sl* assay, followed by A1484T [20.4% (20/98)]. Eight of 98 (8.2%) were resistant by absence of wild-

type bands with no developing mutation bands which could be due to silent mutations in this region, which do not result in an amino acid exchange.

The study had certain limitations. The resistance rate for fluoroquinolones and injectable drugs may have been underestimated because of the unavailability of phenotypic testing. The absence of sequencing data due to poor DNA yield on a certain proportion of samples with follow up isolates means that we could have underestimated resistance to second-line drugs that develops overtime during treatment. When using the boiling technique to isolate DNA, proteins are not removed in samples and this could have inhibited the sequencing reaction. The other reason for the poor DNA yield is likely the long storage time of the culture samples on solid media.

The accuracy of the GenoType MTBDR*sl* assay for the detection of FLQ was comparable to that of sequencing. However, the test was less accurate for the detection of aminoglycosides/CAP. To overcome this we recommend MTBDR*sl* v2.0 which is more sensitive than MTBDR*sl* v1 and is able to detect most common mutations involved in resistance to flouroquinolones and aminoglycosides/CAP. There is evidence of acquisition of resistance to second line drugs during treatment.

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CHAPTER 5

EMERGENCE OF ADDITIONAL DRUG RESISTANCE DURING STANDARDIZED MDR-TB TREATMENT

5.1 ABSTRACT

Background: In order to address the increasing incidence of MDR-TB, the South African National TB Program recommended the implementation of to a standardized treatment regimen in 2000, consisting of 4-month intensive phase with 5 drugs pyrazinamide, ethambutol, ethionamide, ofloxacin, and either amikacin or kanamycin followed by a 12- to 18-month continuation phase without AMK/KAN. This policy advocated that MDR-TB treatment should be conducted in hospital settings until culture conversion.

Objective: To assess the impact of implementing a standardized MDR-TB treatment regimen in two South African provinces, the Eastern Cape (EC) and North West (NW) by retrospectively quantifying the acquisition of drug resistance to second-line drugs in serial cultures.

Methods: A total of 556 MDR-TB patients from the Eastern Cape and North West provinces were enrolled in the study. We investigated whether DST pattern, HIV infection status, and treatment outcomes were associated with the acquisition of drug resistance. Spoligotyping was used to compare the genotypes of the MDR-TB isolates obtained at the time of treatment initiation with last available isolate**.**

Results: Seventy-five percent (n=106) of patients included in the study had poor treatment outcomes (died, defaulted, failed treatment). Of 556 MDR-TB patients, 106 (64 females and 42 males) had DST and spoligotyping results. Of these 48 had an initial and last available isolate, and of these 22 (45.8%) had genetic DST results that differed. In 11/22 (50%) patients the spoligotype patterns changed suggesting reinfection. In 9/22 (41%) of these patients the reinfecting strain had a resistance profile that showed additional resistance relative to the initial isolate (amplification of resistance). In 23 of the 26 (88.5%) remaining patients, the spoligotype pattern of these serial isolates remained constant, suggesting persistent disease. The Beijing and Latin American Mediterranean (LAM) families were found to be more frequent in 48 MDR-TB patients studied, with 29/48 (60.4%) and 7/48 (14.6%) respectively. Out of 29 patients with strains belonging to Beijing family, 24/29 (82.8%) had unfouvarable outcomes.

There was no statistically significant difference in the amplification of resistance between patients who were HIV positive and those who were HIV negative (p-value = 0.250). Acquisition of OFX resistance was more common in patients with poor treatment outcomes (failed defaulted or died) compared to patients with good treatment outcomes (cured, completed treatment) (54% vs. 13%, p-value =0.03879).

Conclusions: Amplification of resistance to second line drugs was observed in 45.8% of MDR-TB patients. This implies that the implementation of standardized MDR-TB treatment in the absence of knowledge of the degree of resistance in circulating MDR-TB strains increases the risk of amplification of resistance during treatment. Furthermore, institutionalized MDR-TB treatment increases the risk of nosocomial reinfection with strains that are more resistant than the initial infecting strains.

5.2 INTRODUCTION

The first multidrug resistant-tuberculosis (MDR-TB) case, defined as resistance to isoniazid (INH) and rifampicin (RIF), was diagnosed in South Africa in mid-1980s. [(1)] A drug resistance survey in the Western Cape Province conducted by the South African Medical Research Council (SA MRC) from July 1992 to May 1993, found 1.1% of new and 4% of retreatment TB patients had MDR-TB.[(2)] Subsequent to this, another survey was conducted in Mpumalanga Province, showing 2% MDR-TB among new patients and 8% among patients previously treated for TB.[(3)] In 2001 the National Drug Resistance Survey, performed by the SAMRC and sponsored by the World Health Organization (WHO), was conducted to estimate the burden of drug resistant TB in all nine South African provinces. The survey found that 1.6% of newly diagnosed and 6.6% of previously treated TB patients had MDR-TB. Drug resistance among newly treated cases for INH, RIF, ethambutol (EMB) and streptomycin (SM) were 2.6%, 0.2%, 0.0% and 1.8% respectively. In addition, the survey reported that resistance to EMB was rare. Resistance to pyrazinamide (PZA) and second-line drugs were not tested.[(4)] One-fourth of all MDR-TB isolates were found to be resistant to all four first-line drugs against which they were tested, but second line drug (SLD)susceptibility testing was not performed, so estimates of the percentage of extensively drug resistant tuberculosis (XDR-TB) isolates are not available.

In response to these findings, in 2002 the South African National TB Program recommended changes to treatment guidelines, from an individualized to a standardized treatment regimen for MDR-TB as proposed by the WHO.[(5)] This included a four- to six-month hospital-based intensive phase treatment consisting of PZA, EMB, ethionamide (ETH), ofloxacin (OFX) and

either amikacin (AMK) or kanamycin (KAN) followed by a 12- to 18-month outpatient continuation of the regimen omitting the injectable (AMK or KAN). The continuation phase could be shortened on the basis of clinical judgement provided that 12 months of treatment was given after sputum conversion (negative culture). Hospital-based intensive phase treatment was recommended to ensure adherence and to monitor for adverse events. On discharge, patients received clinic-based care with directly observed therapy-short course (DOTS) workers monitoring treatment adherence at home. Susceptibility testing for second-line drugs (fluoroquinolones and injectables) was not routinely performed on MDR-TB specimens unless specially requested by the attending clinician. These tests were done by the National TB Reference Laboratory or the National Health Laboratory Services (NHLS). Thus, in the majority of cases, second-line drug sensitivity testing (SLDST) results were not known at treatment initiation. Accordingly, sputum culture conversion from positive to negative for MDR-TB was the most important indicator of efficacy of treatment.

The impact of implementing a standardized MDR-TB therapy policy in South Africa on treatment outcomes and acquisition of additional drug resistance has not been widely evaluated. Numerous reports also suggest that by not testing for additional first- or second-line resistance prior to treatment initiation, and hence not adjusting therapy, increased the risk of the developing additional drug resistance.[5-6]

To measure the genetic relationship between different *M. tuberculosis strains* the spoligotyping technique was used and the results were correlated with drug-susceptibility test results and treatment outcomes. In this study we aimed to assess the impact of implementing a standardized MDR-TB treatment regimen in two South African provinces, the Eastern Cape (EC) and North West (NW) by; 1) retrospectively quantifying the acquired drug resistance to second-line drugs in serial cultures; 2) assessing treatment outcomes and sputum culture conversion; 3) differentiating persistent disease from reinfection. We investigated whether the implementation of this policy significantly contributed to the emergence of extensively drug-resistant tuberculosis (XDR-TB), defined as MDR-TB plus resistance to a fluoroquinolone and a second-line injectable, in individual patients.[7]

5.3 MATERIAL AND METHODS

5.3.1 Study population and isolates

For this study, we retrospectively analysed results of patients prospectively enrolled in the study titled “DOTS-Plus for MDR-TB in South Africa: Systematic Evaluation of Standardized

Treatment Regimen Applied Under Tuberculosis Control Program Conditions" conducted by the SAMRC between July 2000 and December 2004. A total of 1066 MDR-TB patients were enrolled to investigate the clinical and cost effectiveness of the standardised MDR-TB treatment regimen under the TB control programme conditions in South Africa. To meet the inclusion criteria, patients were 18 years and older with confirmed MDR-TB presenting at MDR-TB referral centres in the nine provinces and regarded as eligible for standardised MDR-TB treatment according to the National Tuberculosis Programme (NTP) policy. Patients were excluded if they had a previous history of treatment for MDR-TB (i.e. received second-line drugs).

From this cohort, we selected all patients from the Eastern Cape and North West Provinces, in order to compare the effect of implementation of this standardized MDR-TB treatment regimen in two different provincial settings. To monitor treatment response, monthly serial samples were sent to the MRC Supranational Reference Laboratory for mycobacterial culture and INH and RIF DST using BACTEC 460 radiometric method. All cultures were subsequently stored at 7^0C on Löwenstein–Jensen (L-J) medium and BACTEC vials for future studies. We included in the analysis culture-positive patients in whom both the initial and final available isolates were available. The initial isolate is defined as the first available isolate for a patient and the final isolate is defined as the last available isolate for the same patient. Ethical approval for the study was received from the MRC and the respective provinces.

5.3.2 Sample processing, DNA isolation, and second-line drug susceptibility testing

Patients were diagnosed with MDR-TB at primary health care facilities. As part of routine diagnosis, sputum samples were processed by the NHLS. Upon treatment initiation, sputum specimens were sent to the MRC laboratory in Pretoria where they were cultured for mycobacteria and thereafter on a monthly basis until the end of their MDR-TB treatment episode. The first sputum specimen received by the MRC from each patient represented an initial isolate used in this study. All sputum samples collected from high risk patients (presence of TB-HIV co-infection, first-line treatment failure, TB relapse or contact with a MDR-TB strain, suspected primary drug resistance) were subjected to culture and DST for INH and RIF using the BACTEC 460 radiometric method (Becton Dickinson Diagnostic System, Sparks, MD). As a backup, LJ slants were inoculated with 0.1ml processed specimen and incubated at 37°C for up to eight weeks or until positive. All positive cultures were stored at 7^oC for future use and long term storage resulted in loss of viability.

Specimens that were culture positive on both LJ and BACTEC 460 were used for second-line drug susceptibility testing using Genotype MTBDR*sl* assay (Hain Lifescience GmbH, Germany). DNA from mycobacterial cells was extracted using a boiling technique by scraping one or two scoops of cells from LJ slants. The cells were suspended in 200 µl of TE buffer pH8.0 (10 mM Tris-CI, 1 mM EDTA), and heat-killed by incubation at 95°C for 15 to 20 minutes. Thereafter, the extract was centrifuged at 12000 rpm for 7 minutes and the supernatant containing the extracted DNA was collected by aspiration.

From BACTEC 460 vial, 1.0ml of the culture was removed with a tuberculin needle and syringe and placed in a 2.5ml micro-centrifuge tube. The tube was then closed and centrifuged in a micro-centrifuge for 15 minutes at 13,000 r.p.ms. After centrifugation the supernatant was removed and the sediment re-suspended in 300µl molecular grade water. This suspension was then boiled at 96^{o}C in a water bath for 15 minutes to lyse and inactivate the bacilli. After boiling, the suspension was then placed in a sonicating water bath for a further 15 minutes. 5µl of this supernatant was used for the amplification reaction.

Spoligotyping was done according to the internationally standardised protocol to detect the presence or absence of 43 variable spacers in the direct repeat region of *Mycobacterium tuberculosis* genome.[8] This genotyping method was used to compare the genotypes of the MDR-TB isolates obtained at the time of treatment initiation with the last available isolates**.** Reinfection was defined as an active TB case whose initial and follow-up isolates showed significant differences in spoligotype-defined shared type (ST).[9] Persistent disease was defined as active TB in a patient whose initial and follow-up isolates had the same ST.

5.3.3 Statistical analysis

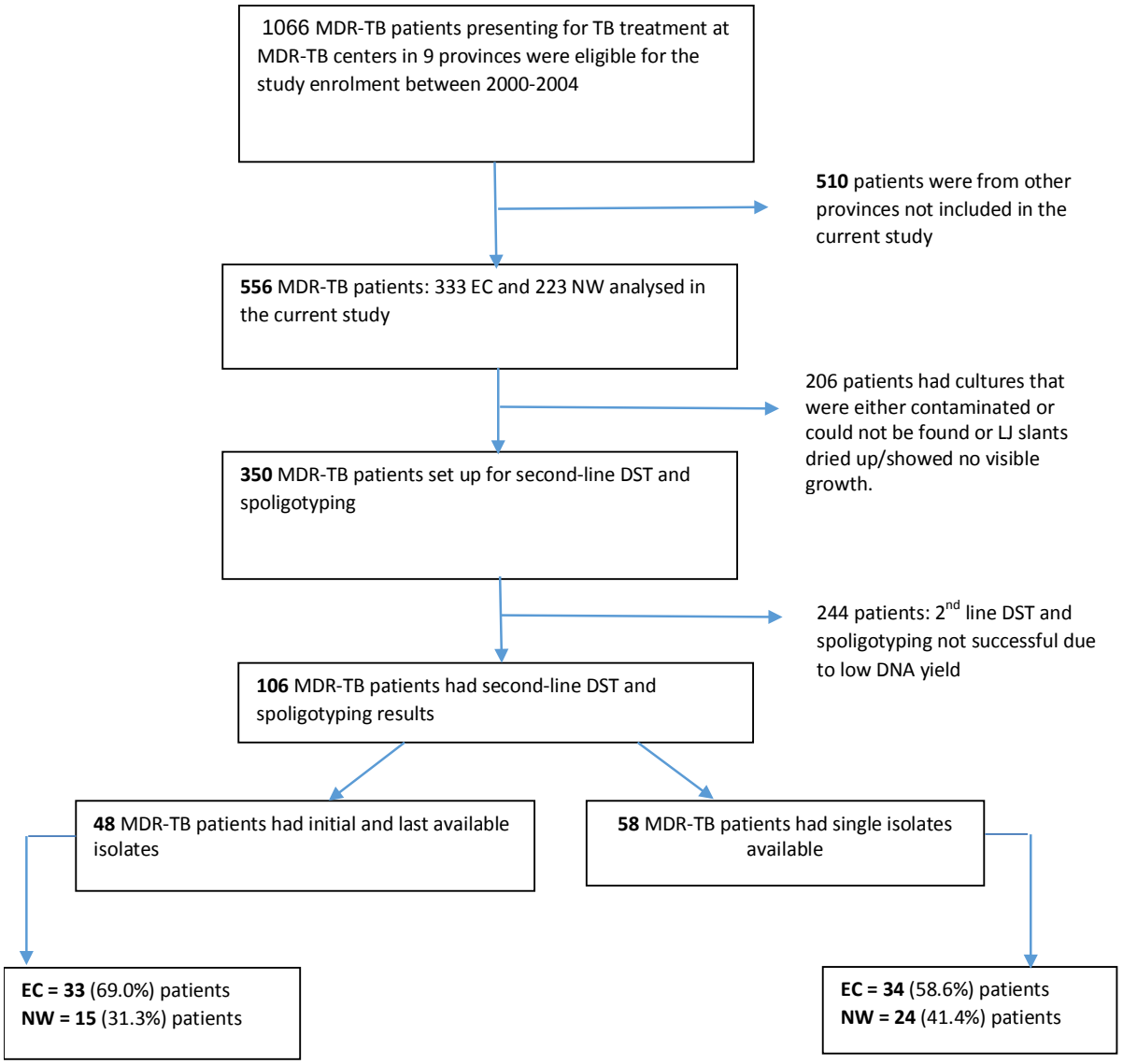
The statistical software STATA version 12.0 (StataCorp, USA) was used to analyse the data. The data is expressed in numbers and percentages. The χ^2 test was used to compare the differences between patients included and excluded in the study. $P<0.05$ was considered to indicate a statistically significant difference between two groups.

5.4 RESULTS

Among 1066 MDR-TB patients eligible for the study in nine South African provinces from 2000 to 2004, 556 (52.2%) were from EC and NW province (Figure 9). Stored cultures were available from 350 (62.9%) MDR-TB patients, however, the yield of DNA was insufficient from 244 (69.7%) of these cultures and were excluded from subsequent analysis. Among the

remaining 106 MDR-TB patients that had both second-line drug susceptibility testing (SLDST) and spoligotyping results, 48 (33 EC, 15 NW) had an initial and last isolate available while 58 (34 EC, 24 NW) had only a single isolate available.

Figure 9: The study selection process



Out of 106 patients included in the study, there were 64 female and 42 male patients (Table 12). The age of patients ranged from 18 years to 65 years. A high proportion of these patients, 64 (60%) were HIV-positive. Twenty-six percent of patients included in the study had successful outcomes (cure or treatment completion). Thirty percent were defaulters, 35.9%

died, and 6.6% failed treatment. Sixty-three percent of patients were from the Eastern Cape and 37% from the North West province.

There were no statistically significant differences observed between patients included in the study versus those excluded in terms of age (p=0.513), provincial origin (p=0.439), HIV status (p=0.889) and treatment outcomes (p=0.239) except for there were more females in the excluded population (p=0.031) (Table 12).

Table 12: Clinical characteristics of MDR-TB patients included in the study

Characteristics	Included Patients N=106 (%)	Excluded Patients N=450 (%)	Total N=556 (%)	p-value
Age				0.513
18-24	19 (18.10)	51 (11.5)	70 (12.75)	
25-34	26 (24.8)	127 (28.6)	153 (27.9)	
35-44	33 (31.4)	159 (35.8)	192 (35.0)	
45-54	19 (18.1)	78 (17.6)	97 (17.67)	
55-64	6 (5.7)	24 (5.4)	30 (5.46)	
≥65	2 (1.9)	5 (1.13)	7 (1.28)	
Gender				0.031
Female	64 (60.0)	320 (71.0)	384 (69.0)	
Male	42 (40.0)	130 (29.0)	172 (31.0)	
Provincial Origin				0.439
EC	67 (63.0)	266 (59.0)	333 (59.9)	
NW	39 (37.0)	184 (41.0)	223 (40.1)	
HIV status				0.889
HIV-positive	42 (40.0)	175 (36.0)	217 (39.0)	
HIV negative	64 (60.0)	275 (61.1)	339 (61.0)	
Treatment outcome:				0.239
Successful Outcome (cure or treatment completion)	27 (25.5)	139 (30.9)	166 (29.9)	
Default	32 (30.2)	126 (28.0)	158 (28.4)	
Death	38 (35.9)	119 (26.4.)	157 (28.2)	
Treatment Failure	7 (6.6)	41 (9.1)	48 (8.6)	
Unknown treatment outcomes	2 (1.89)	25 (5.6)	27 (4.9)	
***Conversion category**:				0.000
Early-conversion (2-6weeks)	37 (28.2))	94 (71.8)	131 (100)	
Mid-conversion (7-18weeks)	25 (17.9)	115 (82.1)	140 (100)	
Late conversion (≥18weeks)	5 (45.5)	6 (54.6)	11 (100)	
Never-conversion	12 (17.2)	166 (93.3)	178 (100)	
Baseline smear status:				0.002
Negative	60 (56.6)	170 (37.8)	230 (41.4)	
Positive	36 (34.0)	216 (48.0)	252 (45.3)	
No data available	10 (9.4)	64 (14.2)	74 (13.3)	

* Information missing for 27 cases among patients included in the study and 69 cases among patients not included in the study

Genotypic analysis of the isolates from the 48 patients with initial and last available isolates showed that 26 (54.2%) patients had an initial isolate with mutations identical to the final isolate (Table 13). In 23 of the 26 (88.5%) patients, the spoligotype pattern of these serial isolates remained constant, suggesting persistent disease, while in the remaining three (11.5%) patients the spoligotype pattern changed during the course of disease, suggesting reinfection with a strain with the same resistance conferring mutations (Table 14).

Table 13: Drug resistance characteristics and spoligopatterns of 48 patients with initial and final isolate

	DST Identical (n=26)	**DST Different (n=22)**	**TOTAL**
Spoligotype Pattern Identical	23 (88.5%)	11 (50.0%)	**34 (71.0%)**
Spoligotype Pattern Different	3 (11.5%)	11 (50.0%)	**14 (29.0%)**
TOTAL	**26 (54.0%)**	**22 (46.0%)**	**48 (100%)**

Drug-susceptibility testing showed that 3/26 (6.3%) of these patients had XDR-TB at baseline, while 9/26 (34.6%) had pre-XDR-TB. Six out of 26 (15%) patients had resistance to OFX and 3/26 (12%) had resistance to KAN/CAP. Out of the 26 patients, 19 showed culture conversion at three months later while the remaining seven patients did not achieve sputum culture conversion (Table 14). Five (67.9%) patients were cured, six (14.3%) died, 10 (17.9%) defaulted, 5 (19%) completed treatment and none failed treatment.

Twenty-two (46.0%) of 48 patients who had initial and final isolates had genetic DST results that differed (Table 15). Eleven (50.0%) of these 22 patients had an initial isolate with a spoligotype pattern that differed from the final, suggesting reinfection while another eleven (50%) patients had initial isolates with a spoligotype identical to the final isolate, suggesting acquisition of resistance. Taking in account the low discriminatory power of spoligotyping, it is likely that some additional reinfection might have been missed. Of the 11 cases where reinfection was observed, nine were reinfected with a strain harbouring additional resistance conferring mutations, while two were reinfected with a strain harbouring fewer mutations (Table 15)**.** A higher proportion of reinfection cases were observed in the Eastern Cape province 8/11 (72.7%) than in the North West province 3/11 (27.3%).

The Beijing and Latin American Mediterranean (LAM) families were found to be more frequent in 48 MDR-TB patients studied, with 29/48 (60.4%) and 7/48 (14.6%) respectively (Table 14 &15). Other spoligotypes i.e (LAM), S family, T, X, Haarlem (H), and East- African Indian (EAI)

were observed in one or two patients. The Beijing family was more common 21/29 (72%) in cases form the Eastern Cape. Beijing genotype was also the predominant genotype among patients that had gained resistance 14/22 (63.6%). Of these, 28.6% were reinfected with a strain harbouring additional resistance conferring mutations. Out of 29 patients with strains belonging to Beijing family, 24/29 (82.8%) had unfavourable outcomes, 13 (44.8%) of patients died during TB treatment, 10 (34.5%) defaulted and 1 (3.4%) failed treatment.

The proportion of isolates that developed resistance during treatment ranged from 79.0% for OFX, 10.5% for KAN/CAP and 10.5% for EMB (Table 15). Of the 17 patients who gained OFX resistance, seven (41.2%) also gained resistance to KAN/CAP or EMB. In 33.3% of isolates, resistance to OFX was gained during the intensive phase of treatment (Table 15).

Table 14: Rate and amplification of drug resistance among 26 MDR-TB patients who did not gain additional resistance i) =Initial isolate ii)=Final isolate

Isolate No.	Relevant Characteristics							Drug resistance pattern		
-	HIV status	Treatment outcome	Time to gain resistance in months	*Time to Initial Culture conversion(weeks)	Time to Final outcome (wks)	Province	Genotype	OFX	KAN/CAP	EMB
1075 (i)	positive	defaulted	9	Never converted	49	EC	LAM4	S	S	R
(ii)	positive	defaulted				EC	LAM4	S	S	R
1117(i)	negative	died	1	Never converted	29	EC	Beijing	R	R	R
(ii)	negative	died				EC	Beijing	R	R	R
1207 (i)	negative	cure	2	4	71	EC	Beijing	S	S	S
(ii)	negative	cure				EC	Indo-Oceanic	S	S	R
1345 (i)	negative	died	1	24	33	EC	Beijing	R	R	R
(ii)	negative	died				EC	Beijing	R	R	R
1370 (i)	positive	died	3	Never converted	5	EC	Beijing	R	S	R
(ii)	positive	died				EC	Beijing	R	S	R
1455 (i)	positive	defaulted	6	Never converted	39	EC	Beijing	R	R	R
(ii)	positive	defaulted				EC	Beijing	R	R	R
160 (i)	positive	cure	1	13	70	NW	T1	S	S	R
(ii)	positive	cure				NW	T1	S	S	R
164 (i)	positive	died	11	3	13	NW	Beijing	R	S	R
(ii)	positive	died				NW	Beijing	R	S	R
1678 (i)	positive	cure	1	13	71	NW	X3	S	S	S
(ii)	positive	cure				NW	X3	S	S	S
1724 (i)	positive	cure	1	unknown	72	NW	LAM4	S	S	S
(ii)	positive	cure				NW	LAM4	S	S	S
1823 (i)	positive	completed	10	28	73	EC	MANU2	R	S	R
(ii)	positive	completed				EC	LAM4	R	S	R
1884 (i)	negative	defaulted	2	28	73	EC	Beijing	S	S	R
(ii)	negative	defaulted				EC	Beijing	S	S	R
2138 (i)	negative	died	3	unknown	unknown	EC	Beijing	S	S	R
(ii)	negative	died				EC	Beijing	S	S	R
2353 (i)	negative	defaulted	1	Never converted	17	EC	Beijing	S	S	S
(ii)	negative	defaulted				EC	Beijing	S	S	S
2438 (i)	negative	defaulted	3	13	70	EC	X3	S	S	S
(ii)	negative	defaulted				EC	X3	S	S	S
2974 (i)	positive	defaulted	3	unknown	70	EC	Beijing	S	S	S
(ii)	positive	defaulted				EC	Beijing	S	S	S
530 (i)	negative	completed	20	8	70	NW	LAM9	S	S	R
(ii)	negative	completed				NW	LAM9	S	S	R
568 (i)	positive	completed	2	10	65	EC	Beijing	S	S	S
(ii)	positive	completed				EC	Beijing	S	S	S
621 (i)	negative	defaulted	9	Never converted	69	EC	H3	S	R	S
(ii)	negative	defaulted				EC	Beijing	S	R	S
643 (i)	positive	defaulted	1	9	59	NW	Beijing	S	S	S
(ii)	positive	defaulted				NW	Beijing	S	S	S
668 (i)	negative	cure	9	10	65	EC	Beijing	S	R	R
(ii)	negative	cure				EC	Beijing	S	R	R
713 (i)	negative	defaulted	4	Never converted	91	EC	Beijing	R	S	R
(ii)	negative	defaulted				EC	Beijing	R	S	R
742 (i)	negative	defaulted	1	9	38	NW	LAM4	S	S	S
(ii)	negative	defaulted				NW	LAM4	S	S	S
990 (i)	negative	died	4	11	59	NW	Euro-American	S	R	S
(ii)	negative	died				NW	Euro-American	S	R	S
795(ii)	positive	completed	3	12	72	NW	H1	S	S	R
(ii)	positive	completed				NW	H1	S	S	R
793(ii)	positive	completed	5	24	70	NW	LAM11_ZWE	S	S	S
(ii)	positive	completed				NW	LAM11_ZWE	S	S	S

Table 15: Rate and amplification of drug resistance among 22 MDR-TB patients who gained resistance/sensitivity to second-line drugs during

Isolate no.	Relevant Characteristics							Drug resistance pattern			Cohort
-	HIV status	Treatment outcome	Time to gain resistance in months	*Time to Initial Culture conversion(weeks)	Time to Final outcome (wks)	Province	Genotype	OFX	KAN/CAP	EMB	Persistent/Reinfection
97 (i)	Negative	Died	8	Never converted	34	EC	Beijing	S	S	R	
(ii)	Negative	Died	-	-		EC	Beijing	R	R	R	Persistent 1
113 (i)	Not known	Died	3	Never converted	56	EC	Beijing	S	S	R	
(ii)	Not known	Died	-	-		EC	Beijing	S	R	R	Persistent 2
143 (i)	Negative	Cured	7	19	73	NW	H1	S	S	R	
(ii)	Negative	Cured	-	-		NW	H1	S	S	S	Persistent 3
187 (i)	Negative	Died	9	Never converted	50	EC	Indo-Oceanic	S	S	S	
(ii)	Negative	Died	-	--		EC	Beijing	R	R	R	Reinfection A
352 (i)	Negative	Completed	3	Not known	71	EC	H3	S	S	S	
(ii)	Negative	Completed	-			EC	H1	R	R	R	Reinfection B
544 (i)	Positive	Completed	29	Not known	Not known	NW	X3	S	S	R	
(ii)	Positive	Completed	-			NW	XE	R	S	R	Reinfection C
717 (i)	Negative	Died	22	Never converted	92	EC	Beijing	S	R	R	
(ii)	Negative	Died	-			EC	Beijing	R	R	R	Persistent 4
756 (i)	Positive	Died	6	Never converted	24	EC	Beijing	S	R	R	
(ii)	Positive	Died	-	-		EC	Beijing	R	R	R	Persistent 5
780 (i)	Positive	Failure	27	Never converted	144	EC	Beijing	S	S	S	
(ii)	Positive	Failure	-	-		EC	Beijing	R	R	S	Persistent 6
913 (i)	Positive	Defaulted	16	Never converted	70	EC	S	R	R	R	
(ii)	Positive	Defaulted	-	-		EC	LAM3	R	R	S	Reinfection D
1031 (i)	Positive	Defaulted	8	Never converted	39	EC	Euro-American	R	R	R	
(ii)	Positive	Defaulted	-	-		EC	Beijing	S	S	S	Reinfection E
1207 (i)	Negative	Cured	2	16	71	EC	Beijing	S	S	S	
(ii)	Negative	Cured	--	-		EC	Indo-Oceanic	S	S	R	Reinfection F
1971 (i)	Positive	Died	1	11	62	EC	Beijing	S	S	R	
(ii)	Positive	Died	-	-		EC	Beijing	R	S	R	Persistent 7
2040 (i)	Positive	Died	11	54	65	EC	LAM3	S	S	R	
(ii)	Positive	Died	-	-		EC	T1	R	R	R	Reinfection G
2072 (i)	Negative	Completed	8	24	103	EC	Beijing	S	S	R	
(ii)	Negative	Completed	-	-		EC	X3	R	S	R	Reinfection H
2371 (i)	Negative	Defaulted	1	18	69	EC	Beijing	S	R	R	
(ii)	Negative	Defaulted	-	-		EC	Beijing	R	S	R	Persistent 8
2468 (i)	Negative	Cured	1	17	70	NW	X2	S	S	S	
(ii)	Negative	Cured	-	-		NW	X2	S	S	R	Persistent 9
788 (i)	Not known	Not known	13	Not known	Not known	EC	S	S	R	R	
(ii)	Not known	Not known	-	-		EC	LAM3	R	R	R	Reinfection I
809 (i)	Negative	Died	15	20	30	EC	Beijing	S	R	R	
(ii)	Negative	Died	-	-		EC	X3	R	R	R	Persistent 10
864 (i)	Positive	Died	10	56	72	EC	X3	S	S	R	
(ii)	Positive	Died	-	-		EC	X3	R	S	R	Persistent 11
912 (i)	Positive	Died	2	1	21	NW	LAM4	S	S	R	
(ii)	Positive	Died	-	-		NW	Beijing	S	R	R	Reinfection J
975 (i)	Positive	Defaulted	6	65	65	NW	LAM3	S	S	S	
(ii)	Positive	Defaulted	-	-		NW	Beijing	R	S	R	Reinfection K

The median initial sputum culture conversion time was 19 weeks in patients who gained resistance to one or more additional drugs versus 18 weeks among those who did not gain resistance (p-value =0.001). Those who did not convert were more likely to die or had defaulted treatment for multidrug resistant TB.

We next investigated whether DST pattern, HIV infection status and treatment outcomes were associated with the acquisition of drug resistance (Table 15). Six (40.0%) of 15 isolates that had gained OFX resistance were from patients that were HIV-negative, whereas nine (60.0%) of 15 were from HIV-positive patients. There was no statistically significant difference in the amplification of resistance between patients who were HIV positive and those who were HIV negative (p-value = 0.250). Amplification of OFX resistance was more common in patients with poor treatment outcomes (failed defaulted or died) compared to patients with good treatment outcomes (cured, completed treatment) (54% vs. 13%, p-value =0.03879).

5.5 DISCUSSION AND CONCLUSION

In this study, we investigated whether the implementation of an in-hospital and standardized MDR-TB treatment regimen contributed to patients gaining additional resistance during treatment. We assessed this policy in two South African provinces, the Eastern Cape (EC) and the North West (NW), by retrospectively quantifying the extent of additional resistance to second-line drugs that was gained during standardized MDR-TB treatment.

Our analysis of 48 MDR-TB patients, with initial and last available isolates, showed that 45.8% gained resistance to second-line drugs during treatment. These results are extremely concerning as they suggest that these hospitalized patients being treated with a standardized MDR-TB treatment regimen were at significant risk of acquiring additional resistance or becoming infected with *M. tuberculosis* strains that had more resistance than their baseline infecting strain. Alarmingly, 17% of MDR-TB patients gained resistance to a level of XDR-TB during treatment. These findings are in agreement with previous reports which have shown that additional resistance gained during treatment increases the risk of poor outcomes including death [11-12] and the emergence of XDR-TB. [13] A clear association between resistance to OFX and poor treatment outcome in MDR-TB patients have been shown in several studies.[14-16] In the current study, resistance to OFX was frequently gained (31.3%) of which 10.4% was gained during the intensive phase of treatment.

This study showed that two mechanisms were responsible for resistance to be gained during treatment. Firstly, by spoligotyping, we found in 23% of patients the genotypes of the initial

and final isolates differed significantly, suggesting nosocomial reinfection. This implies that patients are susceptible to reinfection during treatment [17] following exposure to strains which harbour less or more resistance causing mutations. Using only spoligotyping could have underestimated the proportion of reinfection, as showed in the studies performed in the Netherlands.[18] To obtain a better estimation of the reinfection proportion, secondary genotyping methods such as the IS*6110*-restriction fragment length polymorphism (IS*6110*-RFLP) and mycobacterial interspersed repetitive units-variable number of DNA tandem repeats (MIRU VNTR) have been proposed. While the two methods have been highly discriminatory, one drawback is a large amount of sample required to obtain sufficient amounts of DNA. In this study we could not grow the bacteria as the stored culture was not viable. This resulted in low DNA yield as observed during DNA Sequencing.

Reinfection with a strain which has additional mutations can be explained by the fact that the reinfecting strains are at a selective advantage when exposed to the standardized treatment regimen when compared to the initial strain. However, this does not hold true for situations where the reinfection strain has less resistance causing mutations unless there are additional mutations which were not tested for in this study. Additionally, this could also reflect the difference in the level of fitness between the two strains present in these patients.[19-21]

The fact that nosocomial transmission occurs implies that patients remained infectious despite starting the MDR-TB standardized treatment. To stop transmission of MDR-TB, studies have shown that patients need to be initiated promptly on effective treatment [22] and greater infection control methods need to be implemented. This may be largely overcome with the implementation of community based treatment for MDR-TB as is currently recommended by the SA-National Tuberculosis Programme (NTP) guidelines. Secondly, this study showed that in 22/106 (21%) additional mutations conferring resistance were acquired during treatment implying that additional resistance could have developed as a result of treatment with an inappropriate regimen for an extended period of time.

We acknowledge that this study has limitations. Only patients with initial and follow up positive cultures, not those with one culture available, were included in the study. This study could have underestimated or overestimated the proportion of patients with acquired drug resistance to second-line drugs even though it is administered to all new patients with MDR-TB. However, this is unlikely as the study could not identify any difference between the included and excluded group.

Also, we did not investigate all other resistance markers for gaining resistance. As a result of the non-viability of cultures for most patients, we used Genotype MTBDR*sl* assay to detect second-line drug resistance. Genotype MTBDR*sl* assay cannot be used to "rule out" the presence of underlying resistant populations which may mean that the frequency of amplification of resistance may be higher than estimated. Lastly, the use of spoligotyping, which has a lower level of discrimination, may be insufficient to identify reinfection in genetically related strains leading to an under-estimate of reinfection. Another limitation of this study is that we did not further differentiate Beijing isolates into typical and "atypical" isolates and therefore could associate certain genotypes with specific drug resistance patterns.

In conclusion, the high levels of amplification of drug resistance to second-line drugs observed in this study generally reflect the implementation of a weak standard regimen and the requirement of patients to remain cohorted in hospital for much of the initial treatment. Lack of early DST may have resulted in the use of an ineffective treatment regimen, thereby leading to acquired drug resistance. In view of the increasing MDR-TB, there is a need for rapid comprehensive DST for early detection of drug resistance and strengthened regimens in order to provide patients with improved second-line treatment options, improve treatment success rates and reduce rates of treatment failure and mortality rates.

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CHAPTER 6
CHARACTERISTICS AND TREATMENT OUTCOMES OF PATIENTS WITH MULTI-DRUG RESISTANT TUBERCULOSIS IN SOUTH AFRICA

6.1 ABSTRACT

Background: This retrospective cohort study reports on rates of drug resistance and treatment outcomes for MDR-TB patients notified between 2000 and 2004 and examines factors associated with death/failure.

Objectives: To describe the clinical characteristics and prevalence of second line drug (SLD) resistance among MDR-TB patients in the DOTS-Plus (DP) cohort and its impact on clinically defined treatment outcomes.

Methods: This retrospective study included all MDR-TB patients registered in the DP cohort between 2001 and 2004. Frequencies and cross-tabulations were used to describe the variables and chi square (x^2) for associations. Significant variables (<0.001) were entered into a logistic regression analysis

Results: Among the 2212 drug resistant patients 2079 were new MDR-TB patients included in the analysis. Of the 2079 patients, 1290 (62.0%) were male, and 436 (21.1%) were HIV positive. The treatment success rate was 51.3%, followed by 20.4% default rate, 17.7% death rate and 8.9% failure rate. Mortality rate was significantly high (94.8%) among patients who have been treated for drug susceptibile TB before compared to new MDR-TB patients (5.2%). In the multivariate analysis patients with low weight, resistance to pyrazinamide and ofloxacin remained the only statistically significant factors associated with a lower chance of having favourable outcomes. Compared with first line drugs, second line drug resistance (kanamycin and ofloxacin, cycloserine was higher in patients with unfavourable outcomes. Logistic-regression analysis for factors associated with death/failure yielded odds ratios that were significant for weight <50kg, resistance to PZA and OFX.

Conclusions: High default (20.4%) and death rate (17.7%) indicate urgent need for early identification and referral of patients with the history of treatment of TB, shorter and effective regimen in order improve patient outcomes and reduce ongoing MDR-TB transmission within the community.

6.2 INTRODUCTION

In 2014 it was estimated that there were 480 000 of multi drug-resistant tuberculosis (MDR-TB) cases globally.[1] MDR-TB is defined as tuberculosis strains with resistance towards the two most potent anti-tuberculosis drugs, isoniazid and rifampicin. Globally MDR-TB is typified by < 50% successful treatment outcomes, and high death rates.[2] South Africa ranks second among the highest burden MDR-TB countries,[3] and has been treating MDR-TB patients since 2000, with equally dismal treatment outcomes.[4-5] In 2005 strains of MDR-TB with resistance to two groups of second-line drugs (SLDs): kanamycin (KAN) or amikacin (AMK) and ofloxcacin (OFX) were described[6], and termed extensively drug-resistant TB (XDR-TB). At that time concerns about the impact of XDR-TB on global TB control targets emerged as patients with this form of disease were characterised by exceptionally high death rates. [7]

XDR-TB, as for MDR-TB, is a laboratory diagnosis and requires that *Mycobacterium tuberculosis* (Mtb) to be cultivated from sputa or other clinical specimens, whereupon sensitivities against SLDs are done by growing the organisms in presence of the drugs in the BACTEC MGIT 960 system. As Mtb grows slowly in vitro, the results of drug susceptibility testing (DST) can take up to 12 weeks to be available, during which period the patient may deteriorate due to sub-optimal treatment and during which time additional drug resistance can be acquired. It is believed that resistance against any of the two classes of SLDs will have an adverse impact on treatment outcomes, but the relative contribution of resistance as compared to other clinical signs of the patient is unknown. Despite having SLD-DST available, performing these assays remain challenging, most important are the difficulties in standardization of the methodology. Furthermore, very little is known about the correlation between bacteriologically demonstrated drug-resistance, either by phenotypic or genotypic methods,[8] with clinical resistance, where clinical resistance is defined as failure or death. Such information would be useful not only to estimate the extent of the problem in this high-risk population, but also for reviewing the tuberculosis control program and for developing the necessary steps for preventing the spread of MDR-TB in the country. The use of SLD-DST to guide individualized regimens has been highlighted more recently with reports on patients with drug-resistance to all anti-TB drugs[9-10] and the need to be cautionary in defining different classes of SLD resistance in the absence of data on the correlation with clinical response to treatment.[2]

In 2000, South Africa implemented standardized programmatic management of MDR-TB in a study known as DOTS-Plus (DP).[11] In this protocol, 2079 MDR-TB patients with newly culture-confirmed disease, from all nine provinces, were enrolled from 2000-2004. Only isoniazid (INH) and rifampicin (RIF) susceptibility were done as background resistance of the

SLDs AMK/KAN and the fluoroquinolones (OFX) were believed to be low due to limited used and availability prior to 2000. It was however recognised that some patients would have primary or acquired resistance to SLD, and that these patients may require individualized regimens. Therefore, any patient that was still culture-positive at any stage during months 6 to 9 of treatment was eligible for having SLD susceptibility testing done on a culture, and adjustment of regimen, depending on the clinician's decision.

The aim of this study was to 1) describe the prevalence of SLD resistance among MDR-TB patients in the DP cohort 2) its impact on clinically defined treatment outcomes and identify risk factors for death and failure.

6.3 MATERIAL & METHODS

6.3.1 Patient population

New MDR-TB patients were prospectively enrolled from 2000-2004 into the DOTS-Plus (DP) cohort [5] for standardized treatment. Full description of the cohort and treatment outcomes were as previously described by Farley et al.[5] All nine provinces in the country participated in DP, and the provincial MDR-TB hospitals had the option to submit monthly sputum specimens to the South African Medical Research Council (SAMRC) for culture and biobanking. A SLD treatment regimen was used, consisting of a 4 month hospital-based intensive phase of pyrazinamide (PZA), ethambutol (EMB) or cycloserine/terizidone (Cs/Trd) (if resistant to ethambutol), ethionamide (ETO), ciprofloxacin/ofloxacin (CFX/OFX), and amikacin/kanamycin (AMK/KAN). This was followed by an additional 12 to 18 months, outpatient based continuation phase depending on culture conversion, in which the injectable agent and PZA were omitted. Susceptibility testing for SLDs was not routinely performed on MDR-TB specimens during the study period unless specially requested by the attending clinician. Case Report Forms (CRFs) were prospectively completed by a clinician or nurse. The patient data collected included demographic data, baseline treatment and clinical information, as well as monthly treatment and clinical data. HIV testing was offered to all patients upon start of MDR-TB treatment, but antiretroviral (ART) therapy has only been available to MDR-TB patients since late 2004. Standardized treatment outcome and culture conversion definitions were used.[12] Under DP patient demographic, baseline clinical, treatment regimen, response to treatment and drug sensitivity (phenotypic or genotypic) data were collected.

6.3.2 Bacteriology and Second-line Drug Susceptibility Testing

The MDR-TB provincial hospitals had the option to send monthly sputum specimens to the SAMRC TB Laboratory in Pretoria for culturing, biobanking, and SL DST when deemed necessary. This however resulted in only selected sputa from patients' being submitted to SAMRC, which limited the applicability of any SLD resistance findings across the cohort. Upon receiving sputum specimens at the SAMRC's laboratory, specimens were cultured in duplicate on Lowenstein-Jensen (LJ) slants and stored at 7°C. For patients not converting at months 6-9, duplicate slants were sent from the SAMRC to the National Health Laboratory Service (NHLS) for SLD susceptibility testing on MGIT. Drugs tested were kanamycin (5 mg/l), amikacin (4 µg/mL), ofloxacin (2.0 µg/ml), pyrazinamide (100 µg/ml), cycloserine (5 mg/l) and ethionamide (5.0 µg/ml).

6.3.3 Data Management

Data were captured in an Epi-Data, and exported to SAS. Frequencies and cross-tabulations were used to describe the variables and chi square (x^2) for associations. Significant variables (<0.001) were entered into a logistic regression analysis

A favourable outcome was defined as a treatment outcome of cure or completed and unfavourable outcome a treatment outcome of death, default or failure. Patients who transferred out of the MDR Facility and patients who defaulted during treatment were excluded from data analysis.

Descriptive analysis: continuous data were summarised using means standard deviances, and 95% CI as appropriate. The students' t-test was used to compare the two groups in univariate analysis when the assumption of normality was met, else nonparametric test such as the Mann-Whitney rank sum test statistic was used. Categorical variables were tabulated and Pearsons chi-square test statistic or Fishers' exact test statistic was used to test for statistical significance.

6.4 ETHICS

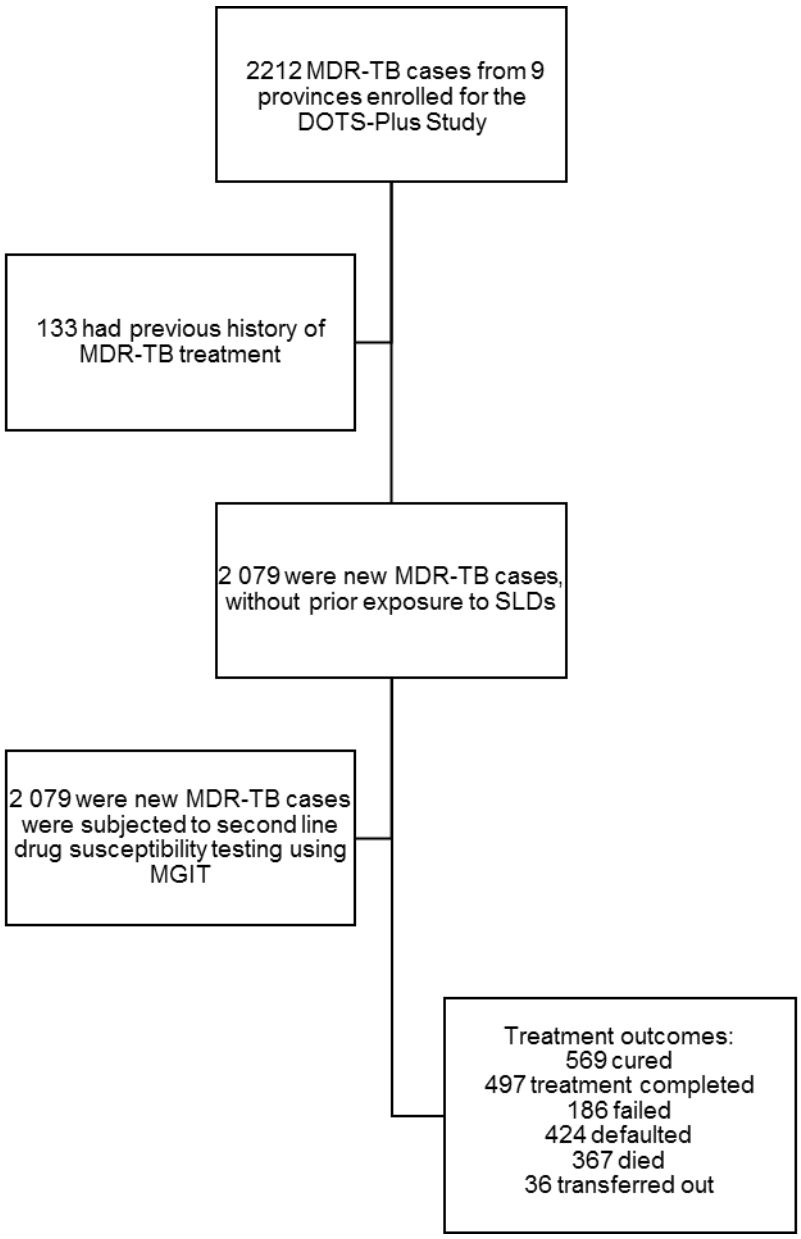
The DOTS-Plus study was approved by the Ethics Committee of the South African Medical Research Council (SAMRC) and research committees of the nine provinces, and patients gave informed consent. It also subsequently received review and approval from the Johns Hopkins University Bloomberg School of Public Health Investigational Review Board.

6.5 RESULTS

Baseline characteristics

Between 2000-2004, a total of 2212 DR-TB patients were enrolled into the DOTS-Plus cohort. Among the registered cases, 133 (6.0%) were found to have been previously treated for MDR-TB. The remaining 2 079 were new MDR-TB cases from all 9 different provinces of South Africa. These cases were included in the analysis of this study, see (Figure 1).

Figure 10: Schematic description of patients included in the study



The cohort of 2079 patients consisted of mostly males (62.0%). The majority (55.7%) of patients had bilateral cavity disease and the human immunodeficiency virus (HIV) status was known for 60.8%, among which 34.5% of those screened were HIV-positive. Half of the 2079 patients (1066, 51.3%) were successfully treated, 36 (1.7%) were transferred out, 186 (8.9%) failed treatment, the default rate was 424 (20.4%) and 378 (17.7%) of patients died. Table 16 shows the baseline demographic and clinical characteristics of patients included in the study. There were no statistically significant differences observed in terms of gender (p=0.058), age (p=0.061), cavitary status (p=0.322) (Table 16). One hundred and seventy nine 9.5%, patients had not been treated before for drug susceptible tuberculosis prior to initiation of MDR-TB treatment (Table 16).

Drug Resistance Pattern

At onset of treatment EMB susceptibility was known for 2079 of patients, of which 659 (31.7%) were resistant. For 436 (20.9%) of patients phenotypic KAN susceptibility results were known, among which 65 (15.0%) were resistant. For 326 (15.7%) of patients OFX susceptibilty (phenotypic) were known, among which 47 (14.4%) were resistant. A total of 309 (14.9%) of patients in the cohort had susceptibility results for both OFX and KAN, amongst which 30 (9.7%) met the criteria for XDR-TB. The most common second-line resistance observed was ethionamide, followed by KAN, Cs and OFX.

Table 16. Demographics and clinical characteristics of patients, N=2079

Characteristic	Cured n (%)	Completed n (%)	Failed n (%)	Defaulted n (%)	Died n (%)	Transfer Out n (%)	Total	P-value
Gender								0.058
Male	357 (62.8)	325 (65.4)	101 (54.3)	266 (62.9)	215 (58.7)	26 (72.2)	1290	
Female	211 (37.1)	172 (34.6)	85 (45.7)	157 (37.1)	151 (41.3)	10 (27.8)	786	
Unknown	1 (0.05)	0 (0.0)	0 (0.0)	1 (0.05)	1 (0.05)	0 (0.0)	3	
Age (years)								0.061
18-24	93 (16.6)	63 (12.7)	31 (16.9)	51 (12.1)	45 (12.3)	3 (8.3)	286	
25-34	174 (31.1)	155 (31.2)	50 (27.3)	147 (35.0)	120 (32.9)	13 (36.1)	655	
35-44	148 (26.4)	150 (30.2)	61 (33.3)	143 (34.0)	133 (36.4)	10 (27.8)	645	
45-54	101 (18.0)	98 (19.7)	25 (13.7)	53 (12.6)	49 (13.4)	7 (19.4)	333	
55-64	35 (6.3)	28 (5.6)	14 (7.7)	23 (5.5)	14 (3.8)	3 (8.3)	117	
>65	9 (16.1)	5 (1.0)	2 (1.1)	3 (0.7)	4 (1.1)	0 (0.0)	23	
*Any co-morbidity								0.000
Unknown	231 (41.5)	295 (62.1)	107 (58.5)	170 (43.0)	243 (68.6)	21 (61.8)	1067	
Yes	42 (7.5)	17 (3.6)	7 (3.8)	7 (1.8)	8 (2.3)	3 (8.8)	94	
No	284 (51.1)	163 (34.3)	69 (37.7)	218 (55.2)	103 (29.1)	10 (29.4)	847	
Cavitary status								0.322
Unilateral (yes)	67 (11.8)	59 (11.9)	15 (8.1)	34 (6.8)	33 (6.6)	1 (2.8)	209	
Bilateral (yes)	318 (55.9)	264 (53.1)	113 (60.8)	240 (48.3)	203 (40.8)	21 (58.3)	1159	
Unknown	184 (32.3)	174 (35.0)	58 (31.1)	150 (30.2)	131 (26.4)	14 (39.1)	711	
HIV status at diagnosis								0.000

Characteristic	Cured n (%)	Completed n (%)	Failed n (%)	Defaulted n (%)	Died n (%)	Transfer Out n (%)	Total	P-value
Positive	118 (23.5)	120 (28.2)	27 (18.2)	97 (26.1)	157 (49.7)	10 (34.5)	436	
Negative	245 (48.8)	205 (48.1)	82 (55.4)	182 (48.9)	101 (32.1)	13 (44.8)	828	
Unknown	139 (27.7)	101 (23.7)	39 (26.4)	93 (25.0)	58 (18.4)	6 (20.7)	529	
Weight								0.000
>65kg	53 (10.3)	54 (13.3)	5 (3.1)	27 (7.6)	12 (3.9)	6 (17.6)	157	
>=50-65kg	249 (48.3)	207 (51.1)	68 (40.5)	160 (44.9)	100 (32.9)	17 (50.0)	801	
<50kg	213 (41.4)	144 (35.6)	95 (56.5)	169 (47.5)	192 (63.2)	11 (32.4)	824	
Unknown								
Patient classification								0.001
New MDR, never treated for TB before	51 (9.1)	48 (9.7)	22 (11.8)	55 (13.1)	19 (5.8)	2 (5.6)	197	
New MDR, treated for TB	518 (91.0)	449 (90.3)	164 (88.2)	369 (87.0)	348 (94.8)	34 (94.4)	1882	
Drug Resistance								0.000
Rifampicin	569 (100)	497 (100)	186 (100)	424 (100)	367 (100)	36 (100)	2079	
Isoniazid	569 (100)	497 (100)	186 (100)	424 (100)	367 (100)	36 (100)	2079	
Ethambutol	223/569 (39.2)	159/497 (32.1)	82/186 (44.1)	89/242 (21.1)	97/367 (26.4)	9/36 (25.0)	659	
Pyrazinamide	9/10 (90.0)	3/ (12) (25.0)	25/34 (73.5)	16/25 (64.0)	26/42 (62.0)	1/1 (100)	80	
Ethionamide	52/185 (28.1)	26/62 (42.0)	35/72 (49.0)	21/53 (39.6)	27/66 (41.0)	0 (0.0)	161	
Kanamycin	12/187 (6.4)	5/61 (8.2)	21/72 (3)	12/52 (29.2)	15/62 (24.2)	0/2 (0.0)	65	
Ofloxacin	6/124 (4.8)	1/47 (2.1)	13/58 (22.4)	11/38 (28.9)	16/57 (28.1)	0/2 (0.0)	47	
Cycloserine	25/142 (17.6)	13/36 (36.1)	9/20 (45)	5/19 (26.3)	5/16 (31.3)	0 (0.0)	57	

Factors associated with favourable outcomes

In the multivariate analysis (see Table 17), patients with a lower weight, resistance to pyrazinamide and OFX remained the only statistically significant factors with less chance of having favourable outcomes (defined as a treatment outcome of cure or completed).

Table 17. Multivariate analysis of demographic and clinical characteristics on favourable outcomes (n=2079)

Variable	Multivariate analysis		
	Odds ratio	p-value	95% Conf. Interval
Weight <50kg	**0.58**	**0.00**	0.46-0 .74
COMORB	0.99	0.99	0.90-1.10
HIV +ve	1.52	0.04	1.01-1.33
New MDR, with no previous TB treatment	1.10	0.55	0.81-1.50
Resistance to ethambutol	1.03	0.64	0.91-1.17
Resistance to kanamycin	0.80	0.23	0.56-1.15
Resistance to ethionamide	0.58	0.03	0.36-0.93
Resistance to pyrazinamide	**0.16**	**0.00**	0.08-0.32
Resistance to ofloxacin	**0.56**	**0.00**	0.41-0.75
Resistance to cycloserine	**2.22**	**0.00**	1.37-3.63

Values highlighted bold are significant risk factors

Risk factors for death or failure

In a univariate analysis we compared variable cured/completed and died/ failed. Except gender and classification of MDR-TB, all other variables included in the study were significantly different between completed/cure and death/failure.

In multivariate logistic regression analysis, weight <50kg (OR, 0.38; 95% CI, 0.27-0.53, P<0.001), resistance to PZA (OR, 0.14; 95% CI, 0.07-0.31, P<0.001), resistance to OFX (OR, 0.58; 95% CI, 0.41-0.83, P<0.001) showed risk for death or failure, as presented in Table 18.

Table 18. Multivariate analysis of factors associated with death/failure

Variable	Multivariate analysis		
	Odds ratio	p-value	95% Conf. Interval
Weight <50kg	0.38	**0.00**	0.27-0.53
Gender	0.77	0.21	0.51-1.14
COMORB	1.09	0.23	0.94-1.27
HIV +ve	1.16	0.08	0.98-1.36
Resistance to ethambutol	0.99	0.89	0.84-1.17
Resistance to kanamycin	0.91	0.67	0.59-1.39
Resistance to ethionamide	0.47	0.01	0.27-0.82
Resistance to pyrazinamide	0.14	**0.00**	0.07- 0.31
Resistance to ofloxacin	0.58	**0.00**	0.41-0.83
Resistance to cycloserine	1.98	0.02	1.12-3.49

Values highlighted bold are significant risk factors

6.6 DISCUSSION

MDR-TB is more difficult to treat and successful outcome rate is low compared to drug susceptible TB. Accurate and rapid diagnosis of MDR-TB is important not only to put patients on effective treatment but also importantly to prevent further transmission of resistant strains. In addition, understanding the risk factors responsible for unfavourable treatment outcomes (defined as death, failure or default) among MDR-TB patients is necessary to improve the treatment outcomes.

Half of the 2079 patients (1066, 51.3%) were successfully treated, 36 (1.7%) were transferred out, 186 (8.9%) failed treatment, the default rate was 424 (20.4%) and 367(17.7%) of patients died. The Eastern Cape province had the highest number (34.4%) of default cases followed by the Western Cape province (20.5%); which is in agreement with other studies.[16-17] High default rate observed in this study is consistent with previous reports by Farley et. al on outcomes of DR-TB patients in eight provinces between 2000 and 2004, and a study by Brust et. al among DR-TB patients in KwaZulu-Natal (2000-2003), which both found a 21% default rate.[5,18]

The proportion of isolates with resistance to EMB amongst new patients was 31.7% higher than the drug resistance survey conducted in South Africa from 2001 to 2002 (0.8%).[19] Although phenotypic DST of *M. tuberculosis* is considered as the gold standard, liquid culture based system, including MGIT 960 system has been reported to yield poor reproducible DST

results which may result in some misclassification.[20] This is due to a narrow range between the critical breakpoint for EMB resistance and the MIC for susceptible strains. The low frequency of resistance to Cs could be related to the difficulties in testing.

All of the 2079 cases had primary MDR which is indicative of ongoing transmission of MDR-TB. Factors which allow for ongoing MDR-TB transmission, such as high rates of failure or default of MDR-TB treatment, may have created the ideal environment for MDR-TB dissemination. Given the high rates of treatment failure in our study, consideration should be given to either individualize treatment regimens or increasing the number of medications in the standardized regimen. This could be also be due to diagnostic delay or delay in the initiation of treatment. Delay in starting appropriate MDR treatment after diagnosis could be another factor for advanced disease (56% bilateral disease) observed in this study. The 56% of bilateral disease observed in this study is not too different from other findings.[21]

Of note, was the high mortality (18.2%) with almost 60% of them being HIV positive. Our findings are consistent with other reports which shows high mortality among HIV coinfected MDR-TB patients.[22-24] Efforts to reduce mortality must focus on earlier diagnosis and early initiation of second-line TB and antiretroviral therapy. Our study took place before the availability of antiretroviral therapy in the public sector in South Africa.

A multivariate analysis of demographic and clinical characteristics with effect on favourable outcomes HIV status, new MDR-TB with no previous TB history, resistance to EMB and Cs had protective effect against favourable outcomes (OR=1.52, OR=1.10, 0R=1.03, OR=2.22 respectively) but this was not statistically significant, with the exception of Cs.

Logistic-regression analysis for factors associated with death/failure yielded odds ratios that were significant for weight <50kg, resistance to PZA and OFX, which complements other studies.[25] In this study gender was a higher risk 7 more times for death or failure, but this was not significant.

Our study has limitations, predominantly those associated with multi-site large scale operational research projects conducted under routine programmatic settings. Firstly, we attempted to control completeness of CRFs, uniformity across the sites/provinces and accuracy of data collection during frequent site visits, calculated culture conversion and assigned treatment outcomes ourselves. Secondly, the MDR-TB provincial hospitals had the option to send monthly sputum specimens to the SAMRC TB Laboratory in Pretoria for culturing and bio-banking, and SLDST when deemed necessary. This however resulted in that

only for selected patients sputa were submitted to the SAMRC, which limited the applicability of any SLD resistance findings across the cohort.

Treatment with first-line drugs before microbiological investigation may have resulted in increased drug resistance drug-resistant organisms. Since the treatment options are so limited for M/XDR-TB, strains that are resistant to all existing anti-TB medications are likely to emerge. Timely identification and referral of patients with the history of treatment failure for culture and DST need to be strengthened in order improve patient outcomes and reduce ongoing MDR-TB transmission within the community. Thus, as recommended by WHO and South African national tuberculosis management guidelines, rapid identification molecular methods and a shorter, cheaper treatment regimen for MDR-TB is essential for earlier treatment initiation and better outcomes.[26-28]

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CHAPTER 7

CONCLUSIONS AND FUTURE WORK

7.1 CONCLUSIONS AND RECOMMENDATIONS

Drug resistance has compromised the success of treatment of TB and MDR-TB and has caused high morbidity and mortality particularly among HIV coinfected patients. This necessitated the introduction of rapid drug susceptibility testing (DST) for second-line drugs (SLDs) in order to detect, initiate effective anti-TB treatment and prevent ongoing transmission of M/XDR-TB. Currently available rapid tests to detect second line drug resistance require expensive laboratory biosafety infrastructure that developing countries do not have access to. They are expensive and require appropriately trained laboratory staff.

From a global perspective, many laboratories rely on conventional phenotypic drug susceptibility testing which is even more problematic. The standardization of second line drug susceptibility testing (SLDST) is difficult, the technique has a long turnaround time with 3-4 weeks and some drugs often show discordant results (particularly ethambutol).[1] Until recently, phenotypic automated liquid system has been used as the gold standard for susceptibility testing of second line drugs.[2]

The use of Genotype MTBDR*sl* for rapid detection of second line drug resistance has recently been endorsed by a WHO. [3] However only a limited number of mutations can be detected with a single test. Thus, in cases where the mutations are outside of the targeted regions, the wild-type banding patterns appear, leading to false negative (susceptible) results.[4] Pyrosequencing (PSQ) has the potential to overcome such problems.This study therefore proposes an algorithm that uses PSQ for DST of XDR-TB.

This study also describes the prevalence of SLD resistance among MDR-TB patients in the DOTS-Plus cohort and its impact on treatment outcomes for these patients in two provinces in South Africa; Eastern Cape (EC) and North West (NW) province. Improved knowledge of factors that lead to acquisition of second-line drug resistance will help better predict who is most at risk of drug resistance and contribute to the development of new tools and strategies to combat MDR-TB. In this study treatment success was strongly influenced by the setting where the patients were treated. HIV co-infection and being younger was identified as an important risk factor for poor outcomes. The shortcomings of the CD4 count not routinely recorded by clinicians in both provinces and limiting our description of the cohort in relation to severity of HIV infection and treatment outcomes was highlighted.

In this study, the resistance patterns against SLDs among newly diagnosed MDR-TB patients in both provinces using Genotype MTBDR*sl* assay (v 1.0) and how these patients acquired resistance during treatment is discussed. The concordance between MTBDR*sl* and sequencing for *gyrA* and *rrs* gene was not as high as reported in other studies.[5-6] The resistance rate may have been underestimated due to unavailability of phenotypic testing. We recommend MTBDR*sl* v2.0 which is more sensitive than MTBDR*sl* v1 and is able to detect most common mutations involved in resistance to flouroquinolones and aminoglycosides/CAP. We also encountered low rates of heteroresistance in association with *gyrA* and *embB* gene, as reported previously. [7-8] Our findings also show that 45.8% gained resistance to SLDs during treatment. Our results are worrying as they suggest that these hospitalized patients being treated with a standardized MDR-TB treatment regimen were at significant risk of acquiring additional resistance or becoming infected with *M. tuberculosis* strains that had more resistance than their baseline infecting strain.

The high levels of amplification of drug resistance to second-line drugs shown in this study generally reflect the implementation of a weak standard regimen and the requirement of patients to remain hospitalized for much of the initial treatment. These findings should be taken into account when formulating the national policy on treatment regimens for MDR-TB. In view of high SLD resistance, we recommend rapid identification molecular methods and a shorter, cheaper treatment regimen for MDR-TB and implementation of community based treatment for MDR-TB as is currently recommended by WHO and national guidelines.[9-10]

7.2 FUTURE WORK

This thesis has contributed to our understanding of drug resistance in TB, and implications of implementing standardized MDR-TB treatment in South Africa. The baseline information of the drug resistant strains of TB that are circulating in the country is required in order to better understand TB disease. The epidemiological database of drug-resistant TB allows for ongoing studies comparing drug resistance patterns across the entire country and the region. Such increased knowledge would help in monitoring trends in the prevalence of drug resistance and treatment outcomes which are essential for TB control over time.

Our analysis of time from diagnosis to treatment initiation showed that patients had started treatment after >180 days did not show a poor outcome compared to patients who received timely treatment especially in the EC. This potentially reflects a survival cohort effect in that those who survived that long had a better chance of survival. Further research should evaluate

clinical characteristics of patients who were started on MDR-TB treatment start immediately after diagnosis in these two provinces.

The importance of reinfection in nosocomial transmission has previously been described by Andrews et al.[11] In our study spoligotyping could have underestimated the proportion of reinfection. Secondary genotyping methods with better discriminatory power such as the IS*6110*-restriction fragment length polymorphism (IS*6110*-RFLP) and mycobacterial interspersed repetitive units-variable number of DNA tandem repeats (MIRU VNTR) will give better estimation of the proportion of reinfection.

These finding also have important implications for infection control, because undiagnosed highly resistant strains could have been transmitted to contacts during treatment. Further research must be conducted to find the most efficient infection control measures to reduce transmission of MDR-TB.

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