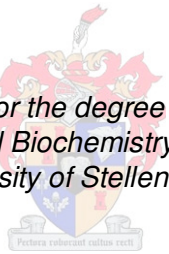
Investigation into Genotypic Diagnostics for *Mycobacterium tuberculosis*

by

Kim Gilberte Pauline Hoek



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Promoters: Prof Robin Mark Warren
Prof Paul David van Helden
Prof Gerhardt Walzl
Faculty of Health Sciences
Department of Biomedical Sciences

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Declaration

By submitting this thesis/dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Abstract

Diagnostic delay is regarded as a major contributor to the continuous rise in tuberculosis (TB) cases and the emergence and transmission of multidrug-resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB). It is therefore essential that more rapid diagnostic methods are developed. Molecular-based assays have the potential for the rapid species-specific diagnosis of TB and associated drug-resistances directly from clinical specimens. We investigated whether high resolution melting analysis (HRM) could enable the rapid diagnosis of TB and associated drug resistance, since the HRM apparatus and reagents are relatively inexpensive and the methodology can easily be implemented in high-incidence, low income regions.

Application of this methodology allowed for the rapid identification of mycobacterial lymphadenitis from fine-needle aspiration biopsy (FNAB) samples in 2 studies. This was done by targeting the region of deletion 9 (RD9), present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the complex. However, the sensitivity of the method was low (51.9% and 46.3%, respectively) when compared to the reference standard (positive cytology and/or positive culture). Despite this limitation our method was able to provide a rapid diagnosis in more than half of the infected patients with a relatively high specificity (94.0% and 83.3%, respectively). We therefore proposed a diagnostic algorithm allowing the early treatment of patients with both HRM and cytology results indicative of mycobacterial disease.

We developed the Fluorometric Assay for Susceptibility Testing of Rifampicin (FAST-Rif) which allowed the rapid diagnosis of MDR-TB by detecting rifampicin (RIF) resistance mutations in the *rpoB* gene with a sensitivity and specificity of 98% and 100%, respectively. The FAST-Rif method was easily adapted to detect ethambutol (EMB) resistance due to mutations in the *embB* gene with a sensitivity and specificity of 94.4% and 98.4% respectively, as compared to DNA sequencing. The FAST-EMB method was a significant improvement over the inaccurate culture-

based method. We identified a strong association between EMB resistance (and pyrazinamide resistance) and MDR-TB and subsequently advised modifications to the current (2008) South African National TB Control Programme draft policy guidelines.

Due to the potential for amplicon release, we adapted the FAST-Rif and FAST-EMB methods to a closed-tube one-step method using the detection of *inhA* promoter mutations conferring isoniazid (INH) resistance as a model. The method (FASTest-inhA) was able to identify *inhA* promoter mutations with a sensitivity and specificity of 100% and 83.3%. These mutations are of particular interest as they confer low level INH resistance and cross-resistance to ethionamide (Eto). Since *inhA* promoter mutations are strongly associated with XDR-TB in the Western and Eastern Cape Provinces of South Africa, data generated by the recently implemented GenoType® MDRTB*Plus* assay may allow individualised treatment regimens to be designed for a patient depending on their INH mutation profile. Our proposed treatment algorithm may be particularly useful in XDR-TB cases, for which only few active drugs remain available.

Since current diagnostic methods all carry advantages and disadvantages, a combination of phenotypic and genotypic-based methodologies may be the best scenario while awaiting superior methods.

Opsomming

Die onvermoë om tuberkulose (TB), multi-weerstandige tuberkulose (MDR-TB) en uiters weerstandige tuberkulose (XDR-TB) vinnig te diagnoseer, is ‘n belangrike oorsaak vir die volgehoue toename en verspreiding daarvan. Dit is noodsaaklik dat diagnostiese toetse wat vinniger resultate oplewer, ontwikkel word. Molukulêre toetsing het die potensiaal om vinnig spesie-spesifieke diagnoses van TB en die weerstandigheid teen TB-medikasie te lewer. Hierdie studie wil vasstel of hoë-resolusie smeltingsanalise (HRS) ‘n vinnige diagnose van TB en die weerstandigheid teen TB-medikasie kan oplewer aangesien die relatiewe lae koste van reagense en apparaat, asook die minimale infrastruktuur en vaardighede wat vir dié toets benodig word, dit uiters geskik maak vir pasiënte in gebiede met ‘n hoë TB-insidensie en lae inkomste.

Die toepassing van die HRS-metode op fynnaald-aspiraatbiopsies in twee afsonderlike studies, het gelei tot die vinnige identifisering van mikrobakteriële-limfadenitis. Dit is bemiddel deur die gebied van delesie 9 (RD9) teenwoordig in *Mycobacterium tuberculosis* en *M. canettii*, maar afwesig in al die ander lede van die kompleks, te teiken. Die sensitiwiteit van die metode was (51.9% en 46.3%, vir die twee studies onderskeidelik) in vergelyking met die verwysingstandaard (positiewe sitologie en/of positiewe kultuur). Ten spyte van dié beperking was ‘n vinnige diagnose in meer as die helfte van geïnfekteerde pasiënte met ‘n redelike hoë spesifisiteit (94.0% en 83.3%, onderskeidelik) moontlik. ‘n Diagnostiese algoritme wat gebaseer is op die resultate van die HRS en sitologie-toetse, is voorgestel om pasiënte vroeër te behandel.

‘n Fluorometriese toets (FAST-Rif) is ontwikkel vir die vinnige diagnose van MDR-TB deur mutasies in die *rpoB*-geen op te spoor met ‘n hoë sensitiwiteit en spesifisiteit (98% en 100%, onderskeidelik). Hierdie mutasies is verantwoordelik vir weerstandigheid teen die antibiotikum rifampicin (FAST-Rif) en word beskou as ‘n vinnige diagnose vir MDR-TB. Die FAST-Rif metode kon maklik aangepas word om mutasies in die *embB*-gene, verantwoordelik vir weerstandigheid teen die antibiotikum ethambutol (EMB), op te spoor. Die FAST-EMB-metode het ‘n sensitiwiteit

en spesifisiteit van 94.4% en 98.4% onderskeidelik getoon in vergelyking met DNS-volgordebepaling. Die FAST-EMB-metode was ‘n betekenisvolle verbetering op die onakkurate kultuurgebaseerde metodes. ‘n Sterk korrelasie tussen EMB-weerstandigheid (en weerstandigheid teen pyrazinamide) en MDR-TB is geïdentifiseer. Vervolgens is veranderinge aan die Suid-Afrikaanse Nasionale TB-beheerprogram se Konsepbeleidsgids (2008) voorgestel.

Om die potensiële vrylating van amplikone te verhoed, is die FAST-Rif en FAST-EMB aangepas tot ‘n enkelstap geslote buissisteem deur gebruik te maak van die opsporing van *inhA*-promotormutasies wat weerstandigheid teen isoniazid (INH) veroorsaak. Die metode het ‘n sensitiwiteit en spesifisiteit van 100% en 83.3% onderskeidelik, getoon. Hierdie mutasies veroorsaak laëvlak weerstandigheid teen INH, maar ook kruisweerstandigheid teen ethionamide (Eto). Aangesien daar ‘n sterk verbintenis tussen *inhA*-promotormutasies en XDR-TB in die Oos- en Wes-Kaapprovinsies van Suid-Afrika is, kan data van die GenoType® MDRTB*Plus*-toets moontlik gebruik word om ‘n meer geïndividualiseerde behandeling te ontwerp afhangende van die pasiënt se INH-mutasieprofiel. Ons behandelingsalgoritme is veral geskik vir XDR-TB-pasiënte vir wie daar weinig aktiewe antibiotika beskikbaar is.

Huidige diagnostiese metodes het almal voor- en nadele, dus bied ‘n kombinasie van fenotipiese en genotipiese metodes moontlik die beste oplossing totdat beter metodes ontwikkel word.

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List of Abbreviations

AFB	- Acid Fast Bacilli
Am	- amikacin
BCG	- *Mycobacterium bovis* bacilli Calmette-Guérin strain
CFP-10	- 10-kDa culture filtrate protein
CFU	- colony forming units
CHER	- Children with HIV Early Antiretroviral therapy trial
Cfz	- clofazimine
Clr	- clarithromycin
Cm	- capreomycin
CPA	- cross-priming amplification
CXR	- Chest X-rays
DABCYL	- 4-(4'-dimethylaminophenylazo) benzoic acid
DNA	- deoxyribonucleic acid
DOTS	- Directly Observed Treatment Short-Course
DST	- drug-susceptibility testing
EMB	- ethambutol
EQA	- external quality assurance
ESAT-6	- early secretory antigenic target 6
Eto	- ethionamide
FAST	- Fluorometric Assay for Susceptibility Testing
FASTest	- Fluorometric Assay for Susceptibility Testing Easy Single-Tube
FNAB	- Fine-needle Aspiration Biopsy
FQ	- fluoroquinolones
HAART	- highly active antiretroviral therapy
HIV	- human immunodeficiency virus

HPLC - High Performance Liquid Chromatography

HRM - High Resolution Melting Analysis

IFN-γ - interferon-gamma

IGRAs - Interferon Gamma Release Assays

INH - isoniazid

IPT - isoniazid preventative therapy

Km - kanamycin

LAM - lipoarabinomannan

LAMP - Loop-mediated isothermal amplification

LED - Light-Emitting Diode

Lfx - levofloxacin

LJ - Löwenstein-Jensen

LTBI - latent tuberculosis infection

MAC - *Mycobacterium avium* complex

MAS-PCR - multiplex allele-specific PCR

MDR-TB - multi-drug resistant tuberculosis

MGIT - Mycobacterial Growth Indicator Tube

MIC - Minimal Inhibitory Concentration

MODS - microscopic-observation drug-susceptibility

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay

NAAT - nucleic acid amplification test

NPV - negative predictive value

NHLS - National Health Laboratory Service

NRA - nitrate reductase assay

NTM - non-tuberculous mycobacteria

OFL - ofloxacin

PANTA - polymyxin B, amphotericin B, Nalidixic acid, trimethoprim and azlocillin

PAS - para-aminosalicylic acid

PCR - polymerase chain reaction

PhaB - phage amplified biological assay

PPV - positive predictive value

PPD - purified protein derivative

PRA - polymerase chain reaction restriction enzyme analysis

PZA - pyrazinamide

QFT-G - Quantiferon®-TB Gold assay

QFT-GIT - Quantiferon®-TB Gold In-Tube assay

RAM - Rapid Analysis of Mycolic Acids

RCA - Rolling Circle Amplification

REMA - Resazurin microtitre assay

RIF - rifampicin

Rfb - rifabutin

SM - streptomycin

SNPs - single nucleotide polymorphisms

SSCP - Single Strand Conformation Polymorphisms analysis

TB - tuberculosis

TLA - thin-layer agar method

T_m - melting temperature

Tr-DNA - transrenal DNA

T-SPOT - T-SPOT®.*TB* assay

TST - Tuberculin Skin Test

WHO - World Health Organisation

XDR-TB - extensively drug-resistant tuberculosis

ZDV - Zidovudine

ZN - Ziehl-Neelsen

General Introduction

Despite the implementation of Directly Observed Treatment Short-course (DOTS) therapy, tuberculosis (TB) remains a high burden disease in many developing countries. It is estimated that between 2000 and 2010, 1 billion people will be newly infected with the aetiological agent *Mycobacterium tuberculosis*, resulting in 200 million TB cases and 35 million deaths.[1]

This increase in TB cases is spurred on by the current human immunodeficiency virus (HIV) epidemic, since 58% of TB cases in South Africa for example are in fact co-infected with HIV.[2] Diagnosis of TB in HIV infected individuals is particularly challenging due to the extra-pulmonary nature of the disease in immunocompromised individuals.

A further concern is that according to the 2008 World Health Organisation (WHO) Global Report on Drug Resistance,[3,4] an estimated 5.3% of all TB cases were in fact classified as multi-drug resistant TB (MDR-TB), which is defined as *M. tuberculosis* resistant *in vitro* to both isoniazid (INH) and rifampicin (RIF). Of these MDR-TB cases, 7% were classified as extensively drug-resistant TB (XDR-TB) cases, defined as MDR-TB with additional resistance to any fluoroquinolone (FQ) and one of the injectable agents.[4] This increase in resistance is largely due to the poor management of the DOTS programme and the inability to rapidly diagnose TB and associated drug resistance. This may lead to increased morbidity and mortality in sufferers, as well as the spread of TB and associated drug resistances to the community.

Currently, TB diagnosis and drug-susceptibility testing are largely based on phenotypic culture methods which result in significant diagnostic delay. Recent advances in phenotypic culture methods have reduced the delay to 2 to 10 days,[5,6,7] however, the culture of viable bacilli requires specialised biosafety facilities and the need for skilled staff. It is therefore essential that improved diagnostics are developed which are both affordable and rapid.

Recently there has been a movement towards the molecular-based assays[8] which have the potential for the rapid species-specific diagnosis of TB and associated drug-resistance. Chapter 1 aims to provide an overview of the current diagnostics (both phenotypic and genotypic-based) available.

Molecular-based assays may drastically reduce turn-around times; however they may be hampered by a high financial cost, the need for downstream processing and the potential for cross-contamination (among the open-tube based assays). An assay which is rapid, sensitive, and specific and does not require downstream processing would be ideal.

The aim of this dissertation is to investigate the potential of high resolution melting (HRM) analysis to improve the diagnosis of TB and associated drug-resistance.

High resolution melting analysis is a relatively new technique based on the concept that specific DNA fragments have specific thermal denaturation profiles which are determined by the nucleotide sequence contained therein.[9] Thus, any change in the nucleotide sequence would alter the thermal denaturation profile, which, in turn, can be detected by measuring the efficiency of binding of a fluorescent dye to the DNA fragment at different temperatures.[10]

We aim to test whether HRM would be able to rapidly identify *M. tuberculosis* DNA from fine-needle aspiration biopsies (FNABs), collected in an inexpensive transport medium, from patients with suspected TB lymphadenitis, the most common manifestation of extra-pulmonary TB in developing countries.[11,12] In Chapter 2 we describe our closed-tube technique targeting the Region of Deletion 9 (RD9), present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the *M. tuberculosis* complex.[13] Although HRM analysis allowed for a rapid and species-specific diagnosis of *M. tuberculosis* lymphadenitis in the majority of patients, we feel that there is potential for further optimisation of the methodology. Chapter 3 investigates whether the sensitivity and specificity of the assay can be improved by circumventing the need for a transport medium bottle and spotting the samples directly onto the simpler FTA® Elute Cards.

In Chapter 4 we investigate the potential of HRM to rapidly diagnose drug-resistance by detecting mutations known to cause rifampicin resistance, an important marker of MDR-TB

(mono-resistance to rifampicin is rare and is mostly accompanied by isoniazid resistance). This "FAST-Rif" ("Fluorometric Assay for Susceptibility Testing of Rifampicin") method overcomes the difficulties associated with detecting nucleotide transversions, since we analyse the thermal denaturation profiles of DNA duplexes formed by annealing DNA fragments (amplified from the rifampicin resistance-determining region (RRDR) of the *rpoB* gene) with (patient sample) and without (wild-type laboratory strain (H37Rv)) nucleotide change (heteroduplex and homoduplex, respectively).

In Chapter 5 we demonstrate that the FAST-Rif method can be easily adapted to detecting resistance towards other antituberculosis drugs. We chose to look at Ethambutol (EMB) as it forms an integral part of first and second-line tuberculosis therapy and current surveillance data regarding the incidence of resistance is inaccurate, suggesting that resistance to EMB is rare.

Both the FAST-Rif and FAST-EMB assays do not address reducing the need for post-amplification processing. This could lead to potential cross-contamination and subsequent misdiagnosis of patients. In Chapter 6 we therefore aim to adapt the FAST method to a single-tube system (FASTest) using the *inhA* promoter gene mutations conferring isoniazid (INH) resistance as a model.

Finally, in Chapters 7 and 8, we aim to demonstrate that data derived from this dissertation, as well as retrospective data analysis, may be used to formulate suggestions as to how to improve treatment policy guidelines, ensuring the appropriate management of patients and preventing the acquisition or spread of other drug-resistance phenotypes.

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Chapter 1

Review: Current Technologies for the Diagnosis of Tuberculosis and Associated Drug-Resistances

1. Introduction

Despite numerous advances in both scientific and medical research, tuberculosis (TB) remains a high burden disease in many countries, with an estimated one third of the world's population currently infected with the aetiological agent, *Mycobacterium tuberculosis*. Failure to rapidly identify TB cases can lead to increased morbidity and mortality in sufferers, as well as the spread of TB to the community.

A further concern is that according to the recent Global Report on Drug Resistance, released by the World Health Organisation (WHO) in 2008[1], an estimated 5.3% of all TB cases were in fact classified as multi-drug resistant TB (MDR-TB), which is defined as *M. tuberculosis* resistant *in vitro* to both isoniazid (INH) and rifampicin (RIF), with or without resistance to other anti-TB drugs. Of these MDR-TB cases, 7% were in fact classified as extensively drug-resistant TB (XDR-TB) cases, which is defined as MDR-TB with additional resistance to any fluoroquinolone (FQ) (e.g. ciprofloxacin, ofloxacin (OFL) or moxifloxacin) and one of the three injectables (e.g. capreomycin (Cm), kanamycin (Km) or amikacin (Am)).[2]

The majority of current diagnostic methodologies are outdated and were implemented several decades ago. They perform inadequately in detecting the presence of *M. tuberculosis* and even worse for drug-susceptibility testing (DST). It is essential that new rapid diagnostics are developed to complement a well-functioning TB Control Program. There are currently a number of diagnostics being developed, however, many of the manufacturers and decision makers originate from first-world, low incidence settings. Diagnostics therefore suited to the TB epidemic, laboratory infrastructure and funding available in these manufacturing countries may not be of use in high burden settings where there are higher proportions of active and latent TB cases.

This chapter aims to review the current diagnostics available, including immunological, bacteriological and genotypic -based methodologies, and to discuss those which show potential for further development.

2. Diagnostic Methodologies

2.1. Clinical investigations

According to the Centres for Disease Control and Prevention, a **clinical** case of TB should have a positive Tuberculin Skin Test (TST) reaction, show signs and symptoms that reflect TB, and be treated with two or more anti-TB antibiotics.[3]

2.1.1 Patient History and Symptom Screening

Clinicians use various "identifiers" to suspect TB in a patient, going so far as to consider their geographical location. Persons from endemic TB areas are subject to high infection pressures and are therefore more likely to be infected. A history of previous TB treatment will also raise the suspicion for TB, as 16% of TB patients are retreatment cases.[1] This could be either due to reactivation of dormant *M. tuberculosis* or due to re-infection.[4] Furthermore, a history of previous TB treatment and non-compliance is a strong indicator of possible drug-resistance. Infection with the human immunodeficiency virus (HIV) also raises suspicion for TB as 58% of TB cases in South Africa for example are in fact co-infected with HIV.[5]

A cough of more than 2 weeks, is one of the most important indicators of active pulmonary TB disease,[6,7] however, there may be no pulmonary involvement in many patients. This is especially true in HIV co-infected individuals and children.[6,7] Using a combination of symptoms (cough, weight loss, fever, haemoptysis and night sweats)[8] may therefore increase the clinical sensitivity of diagnosing active TB in these individuals.

Diagnosis based on signs and symptoms alone, is not necessarily reliable, since certain TB disease symptoms are common to other respiratory ailments (e.g. cough).[8] Therefore additional diagnostic tools are necessary to confirm active TB disease.

2.1.2 Radiological methods

Chest X-rays (CXR) are classically the next step in the diagnostic algorithm for a TB suspect in first-world countries.[9] Typical radiological findings of active TB disease include evidence of cavitation, upper-lobe disease and pulmonary fibrosis.

However, not all TB disease displays radiological changes. Twenty-five to 50% of HIV infected individuals may present with apparently normal CXR[10,11] due to the higher frequency of extra-pulmonary disease in these patients. Nevertheless, many sputum smear-negative TB cases may present with hilar and mediastinal adenopathies[12] or lower-lobe disease[13] which is recognisable on CXR. A recent study in South Africa amongst mine workers who underwent routine annual screening by CXR, showed that the sensitivities and specificities did not differ amongst HIV infected and uninfected individuals.[14]

Despite being able to aid in diagnosing pulmonary disease, CXR remains a non-specific diagnostic for TB. Radiological findings may be difficult to interpret due to alternate manifestations of pulmonary disease such as bacteriological pneumonia. Furthermore, inter- and intra-observer variation is common and may also affect the interpretation of radiological findings.[15] The Chest Radiograph Reading System[15] or the four-point scoring system[16] may improve the sensitivity and specificity of the technique by reducing inter-observer variability. These scoring/reading systems could therefore enhance the accuracy and improve the value of screening TB suspects.

Disadvantages include the restricted availability of CXR in limited resource settings and the need for specialised equipment, high quality film and skilled staff.

In summary, CXR is a useful and cost effective tool for the identification of TB suspects in low incidence settings. Cost may be reduced in high incidence settings if radiography is only used to screen sputum smear-negative TB suspects.[16] However, as this method is non-specific for TB, further diagnostic testing is required to confirm the aetiological agent as *M. tuberculosis.*[17,18]

2.2. Immunological methods

Recently, there has been a steady shift towards immunologically-based diagnostics, which are particularly useful in identifying *M. tuberculosis* infections with a relatively high degree of accuracy. However, infection is not always indicative of TB disease. This is particularly true in high TB disease burden areas, where the majority of persons have been exposed to *M. tuberculosis* at some point in their lifetime. Identifying latent TB infection (LTBI) may however hold some advantages, in that persons at high risk for progression to disease (immunocompromised and high/low age), may be treated with a prophylactic regimen (e.g. isoniazid preventative therapy (IPT)).

2.2.1 Tuberculin Skin Test

The TST, discovered in 1890, is the oldest diagnostic test for TB and remains a crucial surveillance tool in low incidence settings. It involves subcutaneous injection of 0.04 µg (equivalent to 2 tuberculin units) of tuberculin purified protein derivative (PPD, a precipitate of mixed proteins derived from culture filtrate containing multiple (non-specific) mycobacterial antigens), with measurement of the localised immune response (induration) 48 to 72 hours post-injection.

The TST is indicative of mycobacterial infection, but does not discriminate between latent and active disease. Additionally, since PPD is a mycobacterial antigen, it is not specific for *M. tuberculosis*,[19] but for many of the mycobacteria, therefore, it can lead to false positive results in persons vaccinated with the live attenuated *M. bovis* bacilli Calmette-Guérin (BCG) strain and those exposed to some of the environmental or non-tuberculosis mycobacteria (NTM).[20,21] A recent improvement in specificity may be to use the 6-kDa *M. tuberculosis* early secretory antigenic target (ESAT-6).[22] However highly homologous proteins are produced by *M. kansassi*, *M. szulgai* and *M. marinum*.[23-26]

Anergy can occur and is common in immunocompromised individuals due to reduced cell-mediated immunity. This leads to an increase in false negative results and a decrease in the sensitivity of the TST.[20,27] A negative TST result in HIV infected individuals should therefore be interpreted with caution.

The boosting of TST results by BCG vaccination, and anergy in HIV infected individuals, raises the question of what the ideal "cut-off" point of the induration should be to indicate LTBI.[28] Whereas general guidelines regard 10 mm induration as a positive result, there is evidence that cut-offs which are adapted to age, vaccination and HIV status, may improve the sensitivity of the test.[29] McGill university now offers an online tool (http://meakin.mcgill.ca/respepi/homeE.htm) which takes these and other variables (including country of origin, TST induration size, smoking habits and TB contact history) into account to calculate the probability that a TST result is true and the relative risk of progressing to active TB.[30]

As with CXR, inter- and intra-reader variability in determining the size of the induration may also occur. This is further complicated by the need for the patient to return within 48 to 72 hours of administration and thus the sensitivity and success of the test are dependent on whether TB suspects do return within the specified timeframe, and if so, whether the healthcare worker is skilled and available to read the result.

Although the TST is an old technique which is hampered by numerous disadvantages, it remains the method of choice to screen for LTBI. This is particularly useful in developed countries, as high risk individuals would be placed on IPT to limit progression to active disease. This has also proven successful in HIV infected individuals in whom IPT can reduce the risk of developing active TB by 35-76%.[31,32] However, this would not be useful in high incidence settings where it is necessary to discriminate between active and latent disease. A positive TST result would therefore require further investigation, such as microbiological confirmation of active TB disease.

2.2.2 Antibody Detection Kits

M. tuberculosis specific antibody research is a rapidly growing field and many studies have been done which focus on identifying these "biomarkers" of TB disease.[33-36] It is hoped that these biomarkers may provide a much needed point of care diagnostic for active TB disease.[37]

Current research has identified numerous *M. tuberculosis* specific antibodies and host proteins, including those against the novel T cell antigen MTB heparin-binding hemeagglutinin[38] and the CXC chemokine IP-10 (interferon-gamma (IFN-γ)-inducible protein).[39] The use of specific immunity markers such as T cells and cytokine profiles may improve the diagnosis of infection[40] especially in sputum smear-negative individuals.[41,42]

Validation studies of antibody assays in humans have thus far provided inconsistent estimates of sensitivity and specificity and therefore there is not enough evidence to substitute sputum smear microscopy with these assays.[41-44] Further research and development is necessary to improve this methodology.

2.2.3 Interferon Gamma Release Assays

Many studies have been done to determine the validity of Interferon Gamma Release Assays (IGRAs) in TB diagnostics. These tests focus on quantifying the human cellular response to *M. tuberculosis* infection.[37] More specifically, they measure the amount of IFN-γ released from T cells following stimulation with specific *M. tuberculosis* antigens, including the ESAT-6 and the 10-kDa culture filtrate protein (CFP-10). However, these antigens may be present in the BCG vaccine strain and certain NTMs and are therefore not specific to *M. tuberculosis.* [23,24,26,45]

Three commercial IGRAs are currently available, the Quantiferon®-TB Gold assay (QFT-G), the simplified Quantiferon®-TB Gold In-Tube assay (QFT-GIT) (Cellestis Ltd, Carnegie, VIC, Australia) and the T-SPOT®.*TB* assay (T-SPOT) (Oxford Immunotec, Oxford, UK). The QFT-G and QFT-GIT assays analyse whole-blood by enzyme-linked immune-absorbance to detect IFN-

γ, whereas the T-SPOT uses an immunospot technique applied to purified peripheral blood mononuclear cells.[46,47]

The QFT-GIT has a lower average sensitivity (70-77%) than the QFT-G (75-80%) and T-SPOT assays (90-95%).[45,48,49] A recent meta-analysis of IGRAs in various geographical locations pooled the specificities and suggested 99% for the QFT-G, 91 -96% for the QFT-GIT and 93% for the T-SPOT assay.[49,50] Although the specificities of the tests are relatively similar, the T-SPOT test appears to be the most sensitive for the detection of TB infection.[51-53]

As the TST is still regarded as the gold standard in detecting LTBI, studies have been conducted to compare TST to IGRAs. The latter carries numerous advantages over TST including that suspects need only be seen once for testing, the test provides less discomfort (only blood is drawn) and lastly, repeated testing is not affected by "boosting", as is the case for TST. Results of positive IGRAs do however need to be relayed back to the patients, which will necessitate arranging follow-up visits (as is the case with TST analysis). In South Africa, a recent comparison between TST and IGRAs showed a poor correlation between the tests in a high prevalence TB setting amongst HIV infected individuals, with 41%, 28% and 61% of patients positive for TB infection by the TST, QFT-GIT and T-SPOT assays, respectively.[54] In this study, the T-SPOT test proved to once again be the most sensitive assay. However, the TST is the most affordable choice in high incidence, low income countries such as South Africa, where the cost (including laboratory reagent expenses, excluding labour) is only ~R10 per person, whereas the QFN-GIT and T-SPOT cost ~R370 and ~R490 respectively (personal communication, Dr G.F. Black (at a current exchange rate of R7.50:US $1)).

Interferon Gamma Release Assays have a number of drawbacks, including one similar to TST, i.e. there is still much debate regarding the most accurate cut-off point for a positive result. This is especially true for HIV infected individuals who may have a reduced immune response to the antigens. Additionally, IGRAs cannot differentiate between latent and active disease, nor can they determine drug-susceptibility profiles.[48,55-57] The former is especially problematic in high

incidence settings. The reduced sensitivity of the test also implies that cases of active disease may be missed and therefore IGRAs should not be relied on to rule out TB disease.[58]

Since IGRAs are particularly useful in identifying LTBI in low-incidence settings, the test can be used to identify individuals at risk of progression to future disease (children and immunocompromised individuals) and therefore influence initiation of prophylactic therapy in these persons.[48,56,59] Additionally it is thought that IFN-γ circulating levels can be monitored by these tests and may be reflective of effective prophylaxis and treatment, however, this has not been convincingly shown in high incidence settings.[55,60,61]

In summary, IGRAs may provide important data regarding TB infection status in individuals and evidence shows an improved sensitivity over TST, however, diagnosis of active disease is still dependent on microbiological evidence.[62]

2.3. Bacteriological methods

Clinical and immunological diagnostic methods are able to raise the suspicion for TB disease; however, further confirmation by bacteriological or molecular methods is needed to verify active TB disease and determine the drug-susceptibility profiles of the causative organism.

A **laboratory** diagnosis of a TB case is therefore given if *M. tuberculosis* was isolated by culture or amplified by a nucleic acid amplification test (NAAT) from a clinical isolate; or in the case that no culture was available, that Acid Fast Bacilli (AFB) were present in the clinical specimen.[3]

2.3.1 Sputum smear microscopy

Detection of AFB is the oldest diagnostic test for pulmonary TB and was implemented over a century ago. The AFB is usually done by Ziehl-Neelsen (ZN) staining of three sputum samples collected on three separate days.[63] The sputum smears are then scored as 1+, 2+ or 3+

depending on the bacillary count in each microscopic field. This method remains the most widely used and (low) cost-effective diagnostic, which is a key tool in the DOTS strategy, to identify infectious patients with a relatively high speed and acceptable specificity.[64-67] Additionally, sputum conversion (i.e. from positive to less positive or negative) may be a measure of treatment improvement or success.

The estimated current global detection rate of AFB in smear-positive individuals stands at 60%, however this rate is much lower (20-40%) in high incidence settings.[2,68] This low detection rate is due to numerous factors, including that the threshold of detection of the AFB is 10^4 bacilli per millilitre of specimen.[67] Therefore patients with low bacterial loads may be missed by AFB testing, reducing the sensitivity of the test. This is especially likely in HIV positive patients, as they show a lack of Type IV cellular immunity and thus present more often with low bacterial loads.[2,68] Therefore a negative AFB should not be used to exclude TB diagnosis where the clinical suspicion is high and where TB and HIV co-infection is common. Sputum processing followed by concentration, either by centrifugation, filtration or prolonged gravity sedimentation, has been shown to improve sensitivity by as much as 33% over direct microscopy.[65]

The lower sensitivity in high incidence settings may also be due to the elevated number of cases which place an intolerable workload on the laboratories. The reading efficiency of the sputum smears may therefore decrease over time as the technicians become more fatigued. The WHO has acknowledged this and recommends that technicians should be restricted to reading 20 slides per day to prevent fatigue and possible misdiagnosis.[69] The workload may also be reduced by collecting and analysing only two samples (instead of the standard three) in high burden regions if there is a well functioning external quality assurance (EQA) system in place.[70] A further revision to the case definition for sputum smear positive pulmonary TB proposes that only one sample need be positive for AFB, reducing the need to analyse the second sample in these cases.[71] Developments which may improve throughput and reduce cost include apparatus for the bulk staining of AFBs. However, cross-contamination by bacilli may lead to an increase in the number of false positive results. Computational reading of slides may also circumvent technician fatigue and increase throughput. This may not prove as sensitive as highly skilled

staff, but may be an option in settings where the workload is high, where skilled staff is in short supply and where rapid diagnosis outweighs sensitivity.

The specificity of the AFB is also of concern, as it cannot differentiate between *M. tuberculosis* and NTM.[72] This is especially important in HIV infected individuals who may have high rates of opportunistic NTM infections and in regions where TB is not endemic and NTM infection is common.[73]

An additional disadvantage is that up to 30% of patients are unable to produce sputum[37] (this is especially common in children); therefore false-negative results may occur with an associated reduction in sensitivity. Alternate methodologies for improving AFB specimen collection include sputum induction, fiberoptic bronchoscopies and gastric lavage,[37] but implementation of such complex, labour intensive and invasive techniques on large scale is not feasible.

New microbial stains have also been developed to improve the sensitivity and specificity of the AFB test, including the fluorescent auramine-rhodamine stain. This fluorescent technique is more rapid than ZN-staining[74] and increases the sensitivity by almost 10%.[75,76] This increased sensitivity allows for a reduction in the number of samples analysed, thereby decreasing workload and speeding up diagnosis.[77] However, fluorescent microscopes and their mercury vapour lamps are expensive to buy and to maintain. A recent technological advance includes the use of Light-Emitting Diode (LED) Fluorescence Microscopy which reduces the cost of the microscope and bulbs; and does not require a specialised dark room.[78]

2.3.2 Phenotypic culture

Culture of *M. tuberculosis* is regarded as the gold standard for proving a case of TB and it remains the most sensitive method for detecting TB infection in any clinical specimen.[79] Culture is also used (preferentially to sputum smear microscopy) to confirm treatment success[37] as the method requires only 10-100 *M. tuberculosis* organisms to be present in the sample, in contrast to the 10^4 necessary for the AFB stain.[80]

The success of culture is dependent on the quality of the specimen collected and the transport conditions thereof to the laboratory. The specimens then need to be decontaminated to prevent overgrowth of normal flora present in the host prior to incubation in a culture medium.

The solid phase egg-based Löwenstein-Jensen (LJ) slant method was long considered the gold standard culture method in resource limited settings. It requires prolonged incubation of 6 to 8 weeks before a negative result is confirmed and the average time to a positive result exceeds 4 weeks. It is a cost-effective technique, but requires infrastructure and skilled staff. The agar phase method uses either 7H10 or 7H11 Middlebrook media to improve detection time of positive results to less than 4 weeks, but also requires incubation of 6-8 weeks before a result may be classified as negative.

A major drawback of both the solid and agar phase methods is that they are dependent on the growth rate of the bacteria and therefore various improvements have been proposed to speed up the recovery of *M. tuberculosis* from culture, including the use of liquid phase and automated systems.[81-83]

The liquid culture media systems are the most modern and rapid systems and include the use of 7H12 Middlebrook and other media. They are more rapid than the solid and agar phase culturing systems and reduce time to positive results in smear positive samples to less than 2 weeks if combined with genotypic techniques for rapid species identification. Longer incubation times are necessary for sputum smear negative cases. A disadvantage of the more sensitive liquid culture media is that there is a higher rate of bacterial and fungal contamination amongst the samples, including a higher rate of NTM recovery. Thus, additional speciation by genotypic methods to confirm the presence of *M. tuberculosis* is recommended.[84] Cross-contamination is also more common, even in experienced laboratories where 2.5 to 10% of samples may be contaminated by another sample.[85]

Automation can speed up diagnosis, reduce contamination and improve sensitivity. These methods rely on the non-radiometric detection of bacterial growth and include systems such as the MB/BacT (Biomerieux), BACTEC 9000 (Becton Dickinson), ESPII (TREK Diagnostic

Systems, Inc) and Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson). The latter is rapidly becoming the method of choice in high throughput settings and can be done manually or by automation.[86] The MGIT method has a sensitivity of up to 10% higher than that of the traditional solid or agar medium cultures methods.[87-90]

Cultures, in combination with smear microscopy, increase the sensitivity of diagnosing TB and also form the basis of further testing, including DST, mycobacterial speciation and epidemiological investigations; however, it is essential that more rapid methods are developed to prevent the lengthy diagnostic delay.

2.3.3 Phenotypic Drug Susceptibility testing

Phenotypic culturing is considered the most significant determinant of drug-susceptibility as it can define resistance irrespective of the molecular mechanism responsible for resistance. Various methodologies have been reported which vary according to the anti-TB drug being tested and the laboratory resources available in the respective settings. Inconsistent results are a common occurrence[91] and resistance is often underreported, this being especially true for those drugs for which the methods have not yet been standardised.[79]

The gold standard for DST is the indirect-proportion method on agar medium.[92,93] This requires a pure culture of a specimen from a clinical source and the subsequent inoculation thereof onto solid agar media containing specific concentrations of anti-TB drugs. Resistance is then defined by growth on the drug-containing media in comparison to a drug-free control. This indirect method therefore implies that results can be obtained only 4-8 weeks following standard culture. This has many implications including that the patients may have higher morbidity and mortality during the lengthy delay prior to diagnosis and correct therapy, and that there may be transmission of drug-resistant strains of *M. tuberculosis* to close contacts. An additional disadvantage to indirect testing and culture is that expensive reagents and biosafety facilities are needed.[94]

Liquid based cultures may accelerate the process and most of the automated methods such as the BACTEC and MGIT systems have been adapted to do DST. These can reduce time to DST results to 2-4 weeks by the indirect method[95] and are particularly reliable for the first-line drugs INH and RIF.[92,93] However, this commercial liquid culture system is not always available.

Direct susceptibility testing may reduce the diagnostic delay further, in that a pure culture is not needed and turnaround time can be reduced to 1-3 weeks.[95] However, indirect testing is preferred, since the potential for bacterial contamination and the presence of NTMs in direct cultures is higher.

A further limitation of phenotypic DST in high incidence countries is that it is only requested following treatment failure. This extends the diagnostic delay even further. Furthermore, standard culture-based bacteriological DST is not always an accurate indicator of true drug-resistance. This is particularly evident in the case of ethambutol (EMB) resistance as the diagnostic breakpoint (5 to 7.5 µg/ml) is close to the Minimal Inhibitory Concentration (MIC) of EMB and true resistance may therefore be missed (see Chapter 5 and 7).[91]

The ideal improvement to phenotypic DST methods would be to accelerate the growth rate of the bacteria, however in the absence of such a possibility, various modifications of the standard culturing techniques have been proposed.

2.3.4 Microscopic detection of early Mycobacterial growth

i) Thin-layer agar

The thin-layer agar (TLA) method was originally developed to rapidly identify *M. tuberculosis*, since the method has been shown to be able to detect a positive culture in an average of 11.5 days (as compared to the 30.5 days by LJ medium).[96] Additionally the method is able to provide clues regarding speciation, due to the characteristic cording pattern of *M. tuberculosis* which is visible under the microscope.[96] A recent large multi-centre study in South America found that the

TLA was more sensitive in detecting *M. tuberculosis* infection than LJ culture was (92.6% and 84.7% respectively), however they did observe a slight increase in the contamination rates in the TLA method.[96]

This method has been adapted towards DST, by the use of quadrant petri-dishes which are then filled with thin layers of 7H11 media containing the critical concentrations of the drugs being tested. One segment remains drug-free to control for growth. Growth on the drug-containing segments would imply drug resistance. This method has been optimised successfully to enable the detection of RIF, OFL and Km resistance with sensitivities and specificities of 100% for RIF (compared to the BACTEC MGIT 960 method), 100% for OFL and 100% and 98.7% respectively for Km (as compared to the proportion method).[97] The majority of the results were available in a median of 10 days by the indirect method, following a pre-isolation culture step on LJ medium.[97]

A recent study investigated direct inoculation of decontaminated sputum samples onto quadrant plates with each quarter either containing para-nitrobenzoic acid, INH, RIF or being drug-free. As a direct test, the TLA method showed a sensitivity of 91.3% (as compared to 84.7% and 96.7% for the LJ and BACTEC MGIT 960 methods, respectively). The contamination rate (4.1%) and time to detection (10 days) was similar to that of the BACTEC MGIT 960 (2.2% and 7.1 days, respectively), but lower than with direct LJ.[98]

The TLA method does not require sophisticated equipment or specialized CO_2 incubators, only a standard microscope.[99] Additionally, the use of solid agar reduces the risk of generating aerosols, thereby reducing the risk to the laboratory personnel. However, specialised safety conditions are still necessary.

ii) The Microscopic-observation drug-susceptibility assay

The microscopic-observation drug-susceptibility (MODS) assay is similar to the TLA method, however the assay uses liquid culturing media in 24-well plates and analysis is done by inverted microscope.[100] The 7H9 broth contains PANTA (polymyxin B, amphotericin B, Nalidixic acid, trimethoprim and azlocillin) which helps limit the growth of contaminating bacteria and fungi.

Drug-containing and drug-free control wells can then be inoculated with sputum (direct method) or with culture (indirect method). The plates are sealed in Ziplock® bags to prevent aerosol release and contamination.[101] Growth in both the drug-containing and drug-free wells indicates that the isolate is drug-resistant to that specific drug. Speciation is then also done by identifying the cording morphology by microscopy.[101]

The MODS assay performs well on both smear positive and smear negative sputum samples. A recent meta-analysis showed that the sensitivity and specificity for detecting RIF and INH resistance was 96% and 96%; and 92% and 96%, respectively.[94] The average time to results for direct testing was 15-29 days,[94] which is longer than for the TLA methodology. However a turnaround time of 9 days has been reported in an Ethiopian setting.[100]

As with the TLA, the MODS assay does not require specialised equipment, although an inverted microscope is not always available in resource limited settings.[101] Furthermore, the assay must be done in a specialised biosafety laboratory. The test costs an average of US$ 2-3 and does not require great technical skill.[102,103] However, the method does carry a high workload, as plates need to be analysed daily.

An additional disadvantage for both the TLA and MODS assays is that although the cording morphology is unique to the Mycobacteria, the pattern may also be evident in certain NTMs, including *M. kansassi.*[102]

2.3.5 Colorimetric assays

A potential improvement regarding speed and cost of DST in low resource settings is the use of colorimetric assays of which various adaptations have been proposed.

i) Alamar Blue/Resazurin assay

The Alamar Blue assay was first proposed in 1995 for the detection of drug-resistant *M. tuberculosis*.[104] A subsequent study has found that Alamar Blue and Resazurin are in fact the

same compounds, however Resazurin is a non-proprietary agent which is more cost-effective.[105] The blue compound is added to liquid culture medium and is reduced to a pink colour by bacterial metabolism. The addition of anti-TB drugs to the media and subsequent colour change (from blue to pink) therefore indicates the presence of a resistant isolate.

This methodology has been used to detect INH, RIF, streptomycin (SM) and EMB resistance and the results correlated well with the gold standard agar proportion method. Additionally, results were obtainable within 7-14 days by indirect testing.[104]

To enable high-throughput, the Alamar Blue and Resazurin assays have been adapted to microplate formats.[106,107] The Resazurin microtitre assay (REMA) has also been successfully adapted to detect resistance to pyrazinamide (PZA) (for which conventional DST is difficult due to the low pH required for testing)[108] and various second-line anti-TB drugs including OFL, ethionamide (Eto), Km, Cm, and para-aminosalicylic acid (PAS).[109,110]

ii) MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is mostly done as an indirect DST during which the yellow compound is reduced to form purple MTT formazan crystals by metabolically active *M. tuberculosis.* The dissolved crystals may then be analysed by spectrophotometry. The compound is more expensive than the non-proprietary Resazurin and there is the need for an extra solubilisation step.[111]

This assay has been used to detect INH and RIF resistance with similar sensitivities and specificities to the agar proportion method[112] and as with the Alamar Blue and Resazurin assays, it has also been adapted to a higher throughput microplate format for the second line drugs Km, Cm, Eto, PAS, clarithromycin (Clr), clofazimine (Cfz), levofloxacin (Lfx) and rifabutin (Rfb) which correlates well with the 7H11 agar proportion method.[113]

The MTT assay also performs well as a direct method using sputum samples for detecting rifampicin resistance with an average time to results of 2 weeks.[114]

iii) Nitrate reductase assay

M. tuberculosis can reduce nitrate to nitrite by the nitro-reductase enzyme. This inherent ability can be exploited to enable more rapid detection of the bacteria and DST during culture.

The nitrate reductase assay (NRA) involves the addition of potassium or sodium nitrate (1000mg/L) to standard culture medium (solid, agar or liquid) which may also contain critical concentrations of antibiotics (the concentration that inhibits the growth of 99% of cells in antibiotic sensitive strains of *M. tuberculosis*).[115] If *M. tuberculosis* is present in the media and is able to grow successfully, the nitro-reductase enzyme will convert nitrate to nitrite which can be detected by the addition of the Griess reagent (0.2% naphthylenediamine dihydrochloride, 2% sulphanilamide and 5% phosphoric acid) which causes a pink-purple colorimetric reaction in the reduced samples. A colour reaction in a culture containing antibiotic will therefore indicate resistance to that anti-TB drug.[116]

The NRA was initially done as an indirect assay on solid LJ media; however, in an attempt to shorten detection time, the feasibility of the method has been investigated in liquid cultures and by the direct method.[116,117] The average time for the detection of *M. tuberculosis* by the indirect method is 23 days[94] and high correlation is seen with the standard phenotyping methods, especially for the detection of INH and RIF resistance. A recent meta-analysis showed that the pooled sensitivity and specificity for detecting RIF resistance with NRA was 99% and 100% and for detecting INH resistance, 94% and 100% respectively.[94] Sensitivities for SM and EMB resistance are however much lower due to the inherent difficulties associated with standard phenotypic DST for these drugs,[1] therefore they require further analysis to reach the WHO proposed minimum efficiencies.[118] The method has also been investigated in the detection of PZA resistance, which is notoriously difficult to do due to the low pH required by the test.[108] By using nicotinamide resistance as a marker of PZA resistance, the test could be done in a neutral pH and showed a sensitivity and specificity of 91% and 94% respectively.[119]

Although the Colorimetric assays are simple to perform and are particularly useful in resource limited settings, the tests still require biosafety laboratories and may be affected by bacterial contamination and therefore subsequent speciation is still necessary.[79]

2.3.6 Phage Amplification Assays

To address diagnostic delay, the use of mycobacteriophages (which can replicate rapidly) has been investigated. The assay requires infecting mycobacterial cultures with mycobacteriophages (most commonly the mycobacteriophage D29).[120] These phages are then able to replicate within viable mycobacteria, which can be detected either by the phage amplified biological (PhaB) assay, or by a luciferase reporter assay.[120] The former involves plating the phage onto the rapid growing *M. smegmatis* species which then undergoes lysis and characteristic plaque formation as the phage replicates. This will provide a numerical indication as to the number of viable bacteria in the original culture. Alternatively, the luciferase reporter phage assay produces light. Phage assays can also be used in DST by adding anti-TB agents to the cultures. Non-viable bacteria, i.e. those which are drug-susceptible, will not be able to support phage infection and replication in the presence of the antibiotics.[120]

Numerous validation studies have been done and a recent meta-analysis of 19 studies (including 8 commercial assays (FASTPlaque-TB kit, Biotec Laboratories Ltd., Ipswich, UK) and 7 luciferase reporter assays) reported that 11 of the 19 studies showed sensitivities and specificities exceeding 95% for detecting RIF resistance. In addition 13 of the 19 studies showed more than 95% agreement with the reference standards. A higher sensitivity correlated with using culture isolates rather than sputum samples.[120] In addition, in a direct comparison with smear microscopy, the phage-based assay showed a slightly higher diagnostic accuracy in detecting *M. tuberculosis* disease.[121]

In summary, the phage-based diagnosis of *M. tuberculosis* infection and associated drug-resistance is a rapid (<2 days turnaround time on culture isolates),[79] low cost and highly sensitive

method. However, high rates of false positives have been observed[120] and contamination may affect results. Phages are also able to infect and replicate in multiple mycobacterial species, necessitating further speciation by alternative tests.[79]

2.4. Genotypic methods

Most phenotypic diagnostic methodologies are hampered by the slow growth rate of the mycobacteria and also need further speciation to confirm the aetiological agent as *M. tuberculosis*. Therefore it may be wise to focus on genetic markers within and specific to the *M. tuberculosis* genome to accelerate the diagnosis of both TB and anti-TB drug resistance. Genotypic-based diagnoses therefore have a number of potential advantages over phenotypic based methods in that (1) there are more data points available to analyse than with phenotypic-based testing, (2) determination of the phenotype is not dependant on the culturing of the bacteria and (3) the method is more specific and can be performed more rapidly. However, phenotypic-based methodologies by culture remain the gold standard for the diagnosis of *M. tuberculosis* infection and associated drug-resistance.

2.4.1 Hybridisation Assays

Line-probe hybridisation assays are based on the use of the polymerase chain reaction (PCR) to amplify regions of interest in the mycobacterial genome followed by reverse hybridisation to sequence specific probes. These hybridisation assays are often designed as deoxyribonucleic acid (DNA)-strips which can simultaneously detect *M. tuberculosis* complex infection and anti-TB drug resistance.

In 2008, the WHO released a policy statement in which they suggest that the molecular line-probe assays that detect RIF resistance (thereby rapidly identifying MDR-TB patients), are the most advanced novel diagnostic technology currently available.[122] WHO recommended that

these assays (more specifically the GenoType® MTBDR*plus* assay (HAIN Lifescience, GmbH, Nehren, Germany)) be implemented directly on all smear-positive sputum samples to enable rapid detection of MDR-TB.[122,123] However, they acknowledge that mycobacterial culture is still necessary for sputum smear-negative samples and for second-line DST.[122]

Two commercial line-probe assays are currently available. The INNO-LiPA Rif TB assay (Innogenetics, Ghent, Belgium) can detect *M. tuberculosis* complex and RIF resistance with a recent systematic review of the method reporting sensitivities and specificities ranging from 82 to 100 and 92 to 100% respectively.[124] However, this assay is not able to detect additional anti-TB drug resistances.[123]

An improved assay, the GenoType® MTBDR*plus* assay is able to detect the most common mutations in the *rpoB* (responsible for RIF resistance) and the *katG* and *inhA* promoter genes (responsible for resistance to INH).[123] A recent meta-analysis reported the sensitivity and specificity for detecting RIF resistance to be 98.1% and 98.7%, respectively, and slightly lower for detecting INH resistance (84.3% and 99.5%, respectively).[123] The average time to analysis was only 2 days when done directly on sputum positive samples. The assay also performed well in the highly endemic setting of South Africa with the cost being half of that for conventional phenotypic testing.[125]

A possible application of results obtained from the GenoType® MTBDR*plus* assay would be in treatment guidance. Cases which show *inhA* promoter mutations may benefit from the use of high dose INH therapy[125] as these mutations confer low level resistance to INH (as compared to the high levels of resistance encoded by *katG* mutations).

Recently, Hain LifeSciences released the GenoType® MTBDR*sl* assay which is capable of detecting resistance to FQs, Cm/Am/Km and EMB by targeting the *gyrA*, *rrs* and *embB* genes, respectively. A recent study investigated the accuracy of the assay on culture isolates and sputum samples compared to conventional second-line DST and reported combined sensitivities and specificities of 90.2% and 100% for FQ, 83.3% and 100% for Cm, 86.8% and 100% for Am/Km and 59% and 99.1% for EMB resistance, with a turnaround time of only 6 hours.[126]

Hybridisation assays do however have a number of disadvantages, including that these assays focus only on the most prominent single nucleotide polymorphisms (SNPs) and not all the known SNPs that encode anti-TB drug resistance. As yet unknown SNPs will of course also remain undetected. Additionally, these assays require well-trained staff, some dedicated equipment and specialised laboratory infrastructure (including dedicated work stations to prevent cross-contamination). Lastly, but perhaps most importantly, the technique is an open-tube format, with the potential for release of amplicons and therefore an increased risk for cross-contamination. This may have serious consequences, including an increased rate of false-positive results and thus, inappropriate treatment. A possible solution would be to do random repeat assays so as to control for contamination.

2.4.2 DNA chip technology

DNA Biochip technology is similar to that of the hybridisation assays, in that PCR is done and the products are hybridised to a solid phase containing the oligonucleotide probes designed to target regions of interest. Binding of the DNA to the probe produces a fluorescent signal which can be detected by confocal microscopy. These Biochips are able to simultaneously detect the aetiological agent as well as associated drug-resistance.[127,128]

Numerous commercial and in-house DNA Chips have been developed including the Combichip™ Mycobacterial chip (GeneIn, Busan, Korea) and the DNA microarray (LCD array) (Chipron, Berlin, Germany), which can detect resistance to RIF and INH,[129,130] the TB-Biochip oligonucleotide microarray system (Argonne/Engelhardt biochips, USA/Russia) and the high density DNA probe arrays which can detect only RIF resistance;[131,132] and the biological microchip TB-Biochip-2 (Engelhardt Institute, Moscow, Russia) which can detect for FQ resistance.[133]

Sensitivities and specificities vary amongst the different systems, but are similar to those of other NAATs. Reduced sensitivities for detecting resistance are mainly due to undetected mutations,

which occur elsewhere in the genome, or have not yet been identified.[131] Biochips have been used in high incidence settings and the gel-based biochips able to detect INH, RIF and FQ resistance have been certified by the Russian National Regulatory Agency for routine use in clinical applications.[134]

However, the major drawback of DNA chip technology is the exceptionally high cost of the equipment and the difficulty in the interpretation of the data and results.

2.4.3 Multiplex Ligation-dependent Probe Amplification

A recent improvement to the solid phase hybridisation assays is the liquid phase multiplex ligation-dependent probe amplification (MLPA). This has the potential for overcoming defective probes which may be incorporated on the solid-based systems during manufacturing, transport, or incorrect handling. Probes in the liquid system can be prepared in bulk and subsequently validated on well-characterised strains.[135]

The method involves the overnight hybridisation of well-designed probes targeting sequences of interest to denatured DNA and subsequent ligation of the probes. Amplification of these ligated probe sequences is then done by PCR using one set of primers which are complementary to all the pre-designed probes and the resultant amplification products are then visualised by gel electrophoresis (if probes were designed to have different lengths) or by capillary electrophoresis.[135]

A recent study[135] investigated the use of MLPA in *M. tuberculosis* and designed an assay which could provide information on drug resistance (probes targeted 5 codons in *rpoB*, the *inhA* -15 and *katG* -315 promoter codons; and the *embB*306 codon; for RIF, INH and EMB resistance respectively). The assay also included probes which could identify which principal genotypic group the isolate was derived from (*gyrA* codon 95 and *katG* codon 463), the putative virulent strain Haarlem (*ogt* codon 15) and the various Beijing lineages (*mutT2* codon 58, *mutT4* codon 48, *ogt* codon 12 and *ogt* codon 37). Furthermore, speciation was done (*16S* rRNA gene and

IS*6110*) and in the case of *M. tuberculosis* isolates, analysis allowed the differentiation of the "ancient" and modern forms (TbD1).[135] Results were obtainable (in a single tube) within 24 h following short term culture (2 weeks) in Middlebrook 7H9 medium and were 100% specific for all but 2 probes (*mutT4* codon 48 and *embB* codon 306). The method could detect all the isolates harbouring mutations targeted by the probes; however some resistance was not detected, since the assay identified only 71% (20/28) of the RIF resistant isolates and 82% (27/34) of the INH resistant isolates. Results for EMB were not reported. The reduced sensitivity of the assay may be due to the drug-resistance probes chosen. These should be reflective of the most common drug-resistance mutations occurring in the regions analysed. However, the assay allows for the easy substitution of more region-relevant probes. Furthermore, the authors reported preliminary success of the assay applied directly to diluted sputum samples.[135]

2.4.4 Single Strand Conformation Polymorphism analysis

Single Strand Conformation Polymorphism analysis (SSCP) is based on the electrophoretic mobility change that a nucleotide substitution causes in single stranded DNA fragments. The technique involves PCR amplification of the DNA region of interest, subsequent denaturation to form single stranded DNA fragments and then electrophoresis on a polyacrylamide gel. Mobility shifts compared to the wild-type reference sample, indicate the presence of a SNP.[136]

Screening of the *rpoB, katG, inhA* and *ahpC* genes in the genome of *M. tuberculosis* by PCR-SSCP showed a sensitivity of 96 and 87% for the detection of RIF and INH resistance respectively.[137] Specificity in both cases was 100%.[137] Nested-PCR followed by SSCP analysis for RIF susceptibility has been shown to be similar to conventional DST and DNA sequencing.[138] A recent report investigated the use of nested PCR-SSCP in detecting PZA resistance and showed a sensitivity of 86% and specificity of 96% on Chelex purified sputum samples.[139] The reduced sensitivity was due to a number of PZA resistant samples which did not harbour mutations in the target gene, *pncA*.

Since the assay is not able to identify which specific SNP is involved in causing the resistance, the method relies on ensuring that the region of interest targeted contains resistance-causing mutations only and no silent polymorphisms. An additional disadvantage is that the method is labour intensive, requires a high level of technical skill and that results take 24 hours to generate.[136]

2.4.5 High Resolution Melting Analysis

The relatively new technology of High Resolution Melting Analysis (HRM) is similar to SSCP analysis, since it can also screen for mutations. However, in contrast to assessing the electrophoretic mobility of single stranded DNA, it is based on the thermal denaturation profiles of specific DNA fragments which are dependent on the nucleotide sequence contained therein.[140] Thus, any change in the nucleotide sequence would alter the thermal denaturation profile, which, in turn, can be detected by measuring the efficiency of binding of a fluorescent dye to the DNA fragment at different temperatures.[141] Numerous HRM platforms are commercially available, including the Rotorgene-Q (Qiagen, Venlo, Netherlands), LightCycler LC480 (Roche, Basel, Switzerland) and HR-1 (Idaho Technology, Utah, USA) instruments. Several fluorescent dyes have also emerged which are ideal for HRM applications as they are fully saturating and DNA intercalating.

Nucleotide transversions (A:T and G:C) can be difficult to detect directly as they have very little influence on the overall thermal denaturation profile. This can be overcome by analysing DNA heteroduplexes (formed between DNA fragments with and without nucleotide change (heteroduplex and homoduplex, respectively)).[142]

A recent study applied this technology to detect RIF resistance mutations with simultaneous *M. tuberculosis* complex speciation and reported a sensitivity and specificity of 98% and 100%, respectively when applied to purified DNA. No statistical difference was detected in the performance of this "FAST-Rif" ("Fluorometric Assay for Susceptibility Testing of Rifampicin")

method when applied to short-term boiled cultures.[143] However, the FAST-Rif method was hampered by the open-tube format, which could lead to amplicon cross-contamination and the reporting of false positive results. A possible solution would be to "seed" the reaction with wild-type DNA as was done in a similar study in Austria.[144] However, this study used purified DNA from clinical samples, which adds cost and causes a lengthy diagnostic delay. Additionally, the seeding of PCR reactions, could lead to false negative results if the clinical specimens fail to amplify.

HRM for the detection of drug-resistance is further hampered by difficulties associated with multiplexing (i.e. for the detection of multiple gene targets) to detect resistance to more than one drug in a single run; however, this remains a promising technology due to the rapid assay time (~2 hours), high throughput and automation capabilities, and the relatively inexpensive equipment required.

2.4.6 Isothermal amplification methods

i) Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) relies on auto-cycling strand displacement DNA amplification which is done at a uniform temperature by the enzyme *Bst* polymerase. This implies that no expensive PCR equipment is needed, as the technique requires only a standard laboratory water bath or heating block to maintain the uniform temperature. The resulting stem-looped amplicons can be visually detected by the formation of a white magnesium pyrophosphate precipitate.[145] However, this precipitate may be difficult to see. A possible improvement involves the addition of the fluorescent SYBR Green I dye following amplification, however, this would increase the risk of cross-contamination and false-positive results by the need to open the tube.[146]

Recently the addition of calcein (a fluorescent metal indicator) has enabled improved identification of successful amplification while maintaining a closed-tube format. When successful

DNA amplification occurs, pyrophosphate ions ($P_2O_7^{4-}$) are generated which allow the release of manganous ion (Mn^{2+}) which quenches the calcein, thereby emitting a more prominent green fluorescent signal.[146]

The increased specificity of the LAMP technique is due to the incorporation of multiple oligonucleotide primers targeting the same region. The enhanced DNA amplification efficiency ensures amplification of up to 50 times more than by standard PCR.[147]

Numerous LAMP-based assays have been designed to detect *M. tuberculosis,* target areas specific to Mycobacteria include the *gyrB*,[145] *rrs*[148] and *rimM*[146] (encoding 16S rRNA-processing protein) genes. A recent study investigated the use of the highly repetitive IS*6110* Insertion Sequence and reported enhanced sensitivity in both purified and crude samples, detecting essentially all smear-positive specimens and half of smear-negative specimens.[149]

In summary, LAMP is a rapid (<90 min), highly specific and sensitive technique, which requires minimal infrastructure and laboratory skill. However, the technique has only been developed to detect *M. tuberculosis* and currently provide no indication of TB antibiotic susceptibility.

ii) Cross-priming Amplification

An improved methodology for detecting smear negative TB involves adaptation of the LAMP technique to include cross-priming amplification (CPA). Ustar Biotech (Hangzhou, China) has developed a CPA and detection kit contained in a closed plastic device, which includes 6 to 8 cross-linked primers targeting the *gyrB* gene. The CPA occurs with the aid of the *Bst* enzyme at an isothermal temperature (in a waterbath) and the amplified products then hybridise to a lateral flow strip housed within the same plastic device. A recent publication reported sensitivities and specificities of 92.8% and 98.8% compared to the BacT/Alert 3D liquid culture method in purified sputum samples. Furthermore, they found that CPA was more sensitive at detecting sputum smear negative TB than the LAMP assay.[150]

In summary, CPA shows many of the same advantages of the LAMP assay, with improved smear-negative TB detection, however, as with LAMP, the method has not been adapted to

detect drug susceptibility and no internal control system is used. Furthermore, the fairly complicated sputum processing in a biological safety cabinet needs to be simplified to improve turnaround times.[150]

iii) Rolling Circle amplification

Rolling Circle Amplification (RCA) is a relatively novel amplification technique which can significantly improve the detection of nsSNPs by incorporating padlock probes which are complementary to the 5' and 3' ends of the target region.[151] These are then bridged by a genetic linker region, so that when hybridisation to the complementary sequence occurs in the correct orientation, DNA ligase will form a closed circular molecule, which is then exponentially amplified by RCA.[152] Perfect complementarities are necessary for successful amplification, therefore non-specific binding is restricted. Correct probe design is essential for enhanced specificity, with the 5'-probe generally designed to have a higher melting temperature (T_m) and the 3'-probe to have a lower T_m than that used for ligation of the probe.[152] The inclusion of an exonucleolysis step, which removes the ligated padlock probes and template PCR product, ensures that amplification is ligation-dependant. Rolling Circle Amplification, as with LAMP can be done under isothermal conditions using standard laboratory equipment such as a heating block or waterbath.[151] Resultant amplification products may be monitored on real-time PCR instruments following the addition of a fluorescent intercalating dye, with the presence of a signal indicating successful probe binding.[153]

Only one study has been done using RCA for drug-susceptibility testing in *M. tuberculosis*. This study used probes specific to the two most prominent nsSNPs (one in codon 315 of the *katG* and one at position -15 of the *inhA* gene) conferring INH resistance on purified DNA isolates.[153] Although it is believed that over 80% of INH-resistant isolates harbour one of these two mutations,[154] the study showed that only 61% of their INH resistant isolates harboured one of these mutations and furthermore, that 26% of their INH resistant study population had no mutations in the *inhA* and *katG* genes. Sensitivities and specificities were not reported.[153]

While RCA is a rapid and cost-effective amplification technique, it is hampered by the number of nsSNPs that can be detected in a single analysis run. Furthermore, the absence of probe binding and a signal may indicate a false result in the case where amplification failed. Further investigation into including amplification controls and the addition of multiple nsSNP targets may increase the sensitivity of the assay. Validation on crude isolates is also necessary as culture and DNA purification are costly and time-consuming.

2.4.7. Molecular Beacons

The real-time based molecular beacon technique is able to detect amplicons as they are synthesised and can distinguish single nucleotide substitutions.[155,156] The beacons consist of hairpin-shaped DNA probes flanked by a fluorescence quencher such as DABCYL (4-(4'-dimethylaminophenylazo) benzoic acid) and a fluorophore (e.g. fluoroscein).[157] When the beacon binds to the complementary DNA target sequence, the probe undergoes a conformational change which releases the fluorophore from the quencher, resulting in a fluorescent signal which can be detected by real-time instrumentation.[158]

Advantages of the method include that the assay can be done in a closed tube system and that the fluorescent output eliminates the need for post-amplification processing and associated amplicon release.[157] Additionally, multiple mutations may be targeted in a single reaction due to the availability of various fluorophores with different emission spectra.[157] Molecular Beacons have also been shown to be useful in smear-negative culture positive sputum samples.[159]

A recent meta-analysis of Molecular Beacon technology for DST showed a pooled sensitivity and specificity of 97 and 100% for RIF resistance and 82 and 100% for INH resistance on clinical isolates, respectively.[160] The reduced sensitivity in detecting INH resistance detection may be due to mutations in other regions of the genome. Most studies focused on mutations in the *katG* and *inhA* genes, which confer the majority of INH resistance in MDR-TB patients.[157]

The Foundation for Innovative New Diagnostics (FIND) have sponsored the development of an automated sputum processing and real-time based molecular beacon assay, the Xpert MTB/RIF Assay (Cepheid Inc., Sunnyvale, California, USA), which can detect *M. tuberculosis* complex and associated RIF resistance directly from sputum sample using ultra-sensitive hemi-nested PCR. A recent validation study reported a detection limit of 4.5 genomes or 131 colony forming units (CFU)/ml (in clinical specimens) per reaction and a turnaround time of less than 2 hours.[161] Additional advantages of the system include the use of independent cartridges, which allow individual runs without the need for processing samples in batches. The system is also fully automated in that sample decontamination, PCR and real-time analysis occurs within the apparatus. An internal amplification control, *Bacillus globigii* is also included in each cartridge. The Xpert MTB/RIF Assay was able to detect *M. tuberculosis* complex with a 100% sensitivity and specificity in smear-positive clinical samples, however, sensitivity was reduced to 71.7% in smear-negative cases. The method showed 100% sensitivity and specificity for detecting 19 of the most common mutations occurring in the *rpoB* gene among culture-positive retreatment cases from Uganda.[161]

Molecular beacons and the automated Xpert MTB/RIF Assay show promise, however, detailed cost analysis and further validation studies in high-incidence settings needs to be done.

2.4.8 Multiplex allele-specific PCR

The multiplex allele-specific PCR (MAS-PCR) includes several oligonucleotide primers in a single PCR reaction which are designed to be allele-specific, implying that they will bind only if the allele targeted is present in the sample. Subsequent analysis of the amplicon banding patterns by gel-electrophoresis will reveal the allele present and the associated drug-susceptibility profile.[162]

This technique has shown to be successful in detecting the most common mutations in the *katG* and *inhA* genes[153] encoding INH resistance and the *rpoB* gene encoding RIF resistance.[162] These studies revealed high specificities; however, sensitivities were low (even less than the

GenoType® MTBDR*plus* hybridisation assay).[162] This may be due to the limited number of mutations targeted, since only the most prominent nsSNPs causing anti-TB drug resistance are targeted.

An additional disadvantage of MAS-PCR is that it is unable to distinguish heterogeneous populations.[162] This is especially important in cases of acquired drug-resistance as these specimens consist of both drug-susceptible and -resistant bacteria. Furthermore, MAS-PCR does not address diagnostic delay as culturing of samples is necessary, including a costly and time-consuming DNA purification step. No studies have yet investigated the accuracy of the technique on crude sputum specimens. Finally, the post-amplification processing by electrophoresis requires the opening of tubes, allowing the potential release of amplicons. This is a particular concern in high-throughput laboratories where cross-contamination may lead to incorrect DST results and patient treatment.

Validation of MAS-PCR directly on crude sputum specimens and the addition of more targets in other genes may improve the assay, however at this point there are more sensitive and specific molecular DST methods available.

2.4.9. Polymerase Chain Reaction Restriction Enzyme Analysis

Speciation of Mycobacteria is important in identifying the causative agent of disease, since treatment may differ for the various species. The gold standard for speciation involves sequencing of 16*S* rRNA, however, this is costly and not possible in many laboratories.[163] Polymerase chain reaction restriction enzyme analysis (PRA) was proposed as a simple and rapid amplification method that could speciate Mycobacteria.[164] The method involves amplification of a 441bp region of the *hsp65* gene and subsequent restriction digestion with the *Bst*EII and *Hae*III enzymes. The digested DNA is then run on standard agarose gels and the resulting banding patterns are compared to species-specific patterns described in published tables[164] or speciated by a web-based algorithm (http://app.chuv.ch/prasite).

Numerous studies have been done to validate PRA. Recent data shows that the technique is able to amplify mycobacterial DNA from 60% of smear-positive specimens that are not infected with *M. tuberculosis* and is able to speciate NTMs in 94% of those successfully amplified.[165] The amplification rate improves with increasing smear positivity, with AFB 3+ slides showing an identification rate of 100%.[166] When compared to bacterial phenotypic identification, PRA shows 86% concordance and is able to differentiate between many species of mycobacteria, including the *M. avium* complex (MAC) which is common in HIV infected individuals.[167]

A recent multicentre analysis of 8 laboratories was done to compare reproducibility of results on boiled cultures of 18 different mycobacterial species.[163] The PRA showed a success rate from 44% to 100% across the laboratories, with accuracy being associated with electrophoresis running conditions, the use of appropriate DNA size markers and the interpretation of the banding patterns.[163]

Despite PRA being relatively cost-effective compared to sequencing of 16*S* rRNA and phenotypic classification, standardisation of the methodology and the equipment used is needed for accurate speciation. Additionally, training is necessary for correct interpretation of results. The PRA method has to our knowledge not been applied to detect drug-resistance, however multiplexing would be problematic due to the various restriction sites and enzyme conditions.

2.4.10. In-house PCR

To reduce the costs incurred by the purchasing of commercial assays, various in-house PCR based assays have been proposed. These vary in technique and post-amplification analysis and include many of the techniques described in this section (Section 2.4).

Recent meta-analyses of in-house PCR for the detection of *M. tuberculosis* in sputum samples found varying results between the reported methods and could therefore not provide pooled sensitivities and specificities.[168,169] However, the use of nested-PCR methods and IS*6110* as a target has been associated with higher diagnostic accuracy.[168]

Although in-house PCR methods are generally more sensitive than the commercial assays in detecting pulmonary TB, they are less specific and the lack of direct comparisons between the various reported "home brew" tests complicate the recommendation of any of these in routine diagnosis of *M. tuberculosis* or associated drug-resistance.[170]

2.4.11. Commercial NAATs

Numerous commercialised NAATs have been developed based on techniques described in this section (Section 2.4), including the Gen-Probe Amplified *M. tuberculosis* Direct test (AMTD) (Gen-Probe Inc., San Diego, California, USA), Roche Amplicor Mtb test (Roche Molecular Systems, Branchburg, New Jersey), BD-ProbeTec Direct (SDA) (Becton Dickinson Diagnostic Systems, Sparks, Maryland, USA) and the GenoType® MTBDR*plus* assay (HAIN Lifescience, GmbH, Nehren, Germany).[171] Many of these have been approved by the American Food and Drug Administration (FDA) for use in sputum-positive specimens. Recently, a second generation AMTD (E-AMTD) was FDA approved for use on smear-negative specimens.[171]

Most of these methods rely on *M. tuberculosis* detection by amplification and analysis of the 16*S* rRNA or IS*6110* regions. Fully automated systems such as the COBAS Amplicor MTB (Roche Diagnostic Systems, Mannheim, Germany) and the Xpert MTB/RIF Assay (Cepheid Inc., Sunnyvale, California, USA) are also available.

As is the case with in-house PCR assays, a recent meta-analysis of commercial assays for both smear-negative and -positive samples, reported significant heterogeneity ($p<0.001$) between commercial NAATs, with sensitivities and specificities ranging from 36 to 100% and 54 to 100%, respectively, compared to culture.[171] This could be due to the various methodologies employed by each commercial test, but the results remained heterogeneous even when stratifying by each type of commercial test.[171] However, the overall accuracy of commercial assays is higher than with in-house assays, due to the high standards of optimisation, numerous validation studies, inclusion of amplification controls and the standardisation of reagents.[172]

The commercial assays have significantly reduced turn-around times, however the cost of the assays and analysis equipment needed, along with the limited number of resistance-causing mutations targeted are their major drawbacks.

2.4.12. Sequencing

Sequencing is considered the gold standard in genotypic analysis, since it can reveal the complete genetic profile of the region targeted. Any NAAT should be validated against sequencing results as well as phenotypic tests to determine diagnostic accuracy. Not only can sequencing be used in species identification (by analysis of 16*S* rRNA)[173] and searching for the presence of known resistance-causing mutations, but it can also be used to screen for novel SNPs which may be associated with drug-resistance.[101]

However, the major drawbacks of sequencing are the cost associated with the test, the technical skill required to operate the expensive equipment and analyse the resultant electrophereograms and the extensive infrastructure needed. This includes the need for separate rooms for DNA extraction, sample preparation, PCR, amplification analysis, purification of PCR products and DNA sequencing.

Whole genome sequencing is gaining popularity in research spheres (such as at the Broad Institute, MA, USA) as it reveals the genetic sequence of an entire genome. It is hoped that future improvements in the technology and drastic reductions in the cost thereof, may make whole genome sequencing more accessible in resource limited countries and that genotypic testing by this methodology will become routine practice.

In summary, PCR-based genotypic diagnostic tests hold a number of disadvantages in that they carry a high financial cost, require technically skilled staff and that there is the potential for cross-contamination among the open-tube based assays, which is especially serious in high-

throughput laboratories. Additionally, a negative result should be interpreted with caution since PCR inhibitors may be present in the sample analysed. Importantly in the case of DST results, not all the relevant resistance-causing mutations have been discovered. Although the specificity of the NAATs remains high, the variable sensitivities should be addressed. The most promising characteristic of NAATs is the drastic reduction in turn-around times, which are a vast improvement over the phenotypic-based assays, however, as NAATs are able to detect non-viable bacteria as well, these assays should be interpreted with caution and empirical treatment undertaken until results can be confirmed by phenotypic and clinical data.

2.5. Others

2.5.1. Urine-based tests

i) Lipoarabinomannan analysis

More recently, the detection of lipoarabinomannan (LAM) antigen in urine samples of TB suspects has been investigated and has led to the development of a commercial ELISA-based assay (Clearview® TB *ELISA*, Inverness Medical Innovations, Bedford, UK).[174] However, preliminary validation studies have revealed variable sensitivities, with a recent study reporting detection rates as low as 8% in sputum positive patients.[174]

ii) Transrenal DNA

Short fragments of free DNA arising from the mycobacterial site of infection transverse the renal barrier and are excreted from the body in urine.[40] These transrenal DNA (Tr-DNA) fragments are detectable by nested-PCR from the soluble portion of urine, using modified extraction and amplification conditions to isolate the low molecular weight fragments.[40]

Previous studies showed promising results amongst HIV infected individuals for detecting *M. tuberculosis* infection,[175] however, later studies have shown varying results regarding diagnostic accuracy.[176,177] These studies however, targeted larger (>500bp) DNA fragments in urine

sediments. Recent data suggests that shorter (150 to 200bp) Tr-DNA can be detected (by using nested PCR targeting the IS*6110* region) from the soluble portion of urine in 79% of culture positive TB patients and is undetectable in unaffected control subjects.[40] Preliminary data also showed that Tr-DNA was no longer detectable in patients who were undergoing effective treatment.[40] The development of a commercial-based Tr-DNA for the detection of *M. tuberculosis* infection is now underway (Xenomics, NY, USA).

A particular advantage of the method is that it is non-invasive and may therefore be particularly useful in the diagnosis of children and in patients from whom sputum samples are difficult to obtain. Further evaluation in HIV-infected individuals is necessary to confirm the increased sensitivity in these patients. Multi-centre validation studies are necessary to evaluate the diagnostic accuracy amongst HIV-infected individuals and those with extra-pulmonary disease.

2.5.2 Rapid Analysis of Mycolic acids

High Performance Liquid Chromatography (HPLC) can be used to do Rapid Analysis of Mycolic Acids (RAM),[178] which quantitates mycolic acids extracted from an unknown organism. These mycolic acids are actively produced during vegetative growth and the resulting HPLC ultraviolet or fluorescent patterns can be analysed by software and compared to a database of known HPLC profiles to identify the organism.

High performance liquid chromatography has been shown to be successful in speciation of mycobacteria isolated from short-term cultured clinical specimens.[179] Additionally the chromatograms can also provide a quantitative measure of the total mycolic acids which show an almost linear relationship with the numbers of CFU/ml. Drug susceptibility testing can therefore be done by analysing peak heights after incubation with anti-TB drugs.[180] A recent study showed an agreement of the RAM method with phenotypic DST of 99.5% for INH, EMB ad PZA and 98.7% for RIF after 72 h of incubation, however, sensitivities and specificities were not reported.[178] As the action of INH is on the mycolic acids of *M. tuberculosis*, results for INH were

more rapid and could be obtained following 30 min of short-term incubation in the Bactec 460 radiometric growth system and subsequent mycolic acid extraction.[178]

Rapid analysis of mycolic acids requires an extensive mycolic acid extraction procedure and is sensitive to bacterial contamination which may affect test results and lead to inaccurate diagnosis. Despite the possibility of automation, the cost of the HPLC equipment discredits this methodology in high incidence, low income areas.[79]

2.5.3. Adenosine Deaminase activity (ADA)

Adenosine deaminase activity is a measurable response (most commonly by the Giusti method)[181] which is activated by monocytes during the local inflammatory response caused by *M. tuberculosis* infection.[182]

A review of the literature indicates that ADA testing is not accurate or useful for the diagnosis of pulmonary TB; however it shows promise for the detection of pleural TB and TB meningitis.[170,183,184]

The determination of the ideal cut-off value for a positive reaction is still under debate and may affect diagnostic accuracy. An additional disadvantage is that ADA can be identified in other pathologic conditions which negatively influences the specificity of the assay.[185,186]

2.5.4. African pouched rats

A recent study investigated the use of trained giant African pouched rats (*Cricetomys gambianus*) to identify positive sputum specimens by olfactory perception of mycobacterial specific volatile compounds).[69] They showed a sensitivity in detecting *M. tuberculosis* infection similar to smear microscopy; however the advantage lay in the rats' resistance to TB infection and their high-throughput capabilities. One rat was able to screen 140 samples in 40 min

compared to the 40 samples a technician would process in a day (WHO recommends 20 samples per day).[69] The trained rats were therefore up to 30 times faster than technicians.

This detection system may have application in high TB incidence regions, however, the effect of HIV-co-infection on sensitivity and specificity warrants further investigation. Additionally, training of rats is a lengthy process and average rat life-span (8 years) and cost of upkeep should be taken into account.

Despite the abundance of clinical, immunological, phenotypic and genotypic methodologies for detecting both M. tuberculosis and associated drug-resistance, there is no test with a high diagnostic accuracy which can be used at the bed-side (point of care). All tests have their advantages and disadvantages, with the possible solution to improved accuracy lying rather in using a combination of methodologies. For this, attention to cost, the need for infrastructure and technical expertise is necessary. Furthermore, the lack of accurate diagnostics for second-line drug-resistance warrants further investigation.

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Chapter 2

Combining Fine-Needle Aspiration Biopsy (FNAB) and High-Resolution Melt Analysis to Reduce Diagnostic Delay in Mycobacterial Lymphadenitis

C. A. Wright[1], K. G. P. Hoek[2], B. J. Marais[3], P. D. van Helden[2], and R. M. Warren[2]

[1]Division of Anatomical Pathology, Department of Pathology, [2]DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, [3]Department of Paediatrics and Child Health, Faculty of Health Sciences, Stellenbosch University, South Africa

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1. Abstract

Tuberculous lymphadenitis is the most common cause of extra-pulmonary tuberculosis (TB) in developing countries. Lymphadenitis caused by non-tuberculous mycobacteria (NTM) requires consideration, particularly in immunocompromised patients and children in developed countries. Fine-Needle Aspiration Biopsy (FNAB) offers a valuable specimen collection technique, but culture confirmation, mycobacterial speciation and drug resistance testing (if indicated) is often unavailable in TB endemic areas and result in unacceptable diagnostic delay.

We evaluated the diagnostic value of high-resolution DNA melting (HRM) analysis in the diagnosis of mycobacterial lymphadenopathy using FNAB and an inexpensive transport medium. Specimens were collected from patients referred to the FNAB Clinic at Tygerberg Hospital (June 2007–May 2008) with clinical mycobacterial lymphadenitis. Cytology, culture, and HRM were performed on all specimens. The reference standard for disease was defined as positive cytology (morphological evidence plus mycobacterial visualisation) and/or a positive culture.

Specimens were collected from 104 patients and mycobacterial disease was confirmed in 53 (51.0%); 52 *Mycobacterium tuberculosis* and 1 NTM. Cytology was positive in 84.9% (45/53) and culture in 66.0% (35/53) of patients. HRM identified 52.8% (28/53) of cases. By using the defined reference standard, we recorded 52.8% sensitivity and 94.1% specificity (positive predictive value 90.3%) with HRM analysis.

HRM analysis allowed rapid and species specific diagnosis of mycobacterial lymphadenitis in the majority of patients, permitting early institution of appropriate therapy. Optimisation of this technique requires further study.

2. Introduction

Resource limited countries carry the brunt of the global tuberculosis (TB) epidemic, particularly those affected by the parallel human immunodeficiency virus (HIV) pandemic.[1] According to the most recent World Health Organisation (WHO) estimates, in 2007, there were 9.3 million incident (newly diagnosed) TB cases of whom 1.4 million (14.8%) were HIV infected. The African region accounted for 79% of HIV-infected TB cases.[1] Although TB incidence rates seem to plateau and/or decline in most regions, absolute numbers continue to rise due to increases in population size. The projected scale of the epidemic and ongoing transmission of drug resistant TB remains alarming.[1] WHO estimated that between 2000 and 2010, 1 billion people will be newly infected with *M. tuberculosis*, resulting in 200 million TB cases and 35 million deaths.[2]

Peripheral lymphadenitis is the most common extra-pulmonary manifestation of TB.[3,4] TB lymphadenitis is also the most common cause of persistent cervical lymphadenopathy in children from TB endemic areas.[5-7] In developed countries where the incidence of TB is low, NTM are frequently responsible for mycobacterial lymphadenitis, particularly in children and HIV-infected immunocompromised adults.[3,8,9] In patients with a peripheral lymph node mass Fine-Needle Aspiration Biopsy (FNAB) is a valuable and underutilised specimen collection technique. This simple and safe procedure allows rapid confirmation of mycobacterial disease using cytomorphology and direct mycobacterial visualisation with either Ziehl-Neelsen (ZN) staining or fluorescence microscopy.

Mycobacterial culture is required for accurate species determination (speciation) and drug susceptibility testing,[10-13] because organisms in the *M. tuberculosis* complex are morphologically indistinguishable. The amount of material harvested during FNAB is minimal, and the needle needs to be rinsed at the bedside in liquid medium to facilitate culture. Although FNAB can be safely performed as an outpatient procedure by well-trained nurses,[14] the need for direct inoculation and unavailability of liquid culture tubes limited decentralisation. Use of an inexpensive transport medium for direct inoculation has been described,[15] which should facilitate

mycobacterial culture from FNAB's performed in rural clinics and hospitals. Direct bedside inoculation at the time of FNAB collection provides an excellent diagnostic yield, but culture results may take up to 6 weeks and requires additional speciation.[10,16]

Performing nucleic acid amplification tests (NAATs) on FNAB specimens may provide a rapid species specific diagnosis and expedite access to appropriate therapy. A recent systematic review demonstrated highly variable results with NAATs to diagnose TB lymphadenitis, reported sensitivities ranged between 2 and 100% (specificities 28–100%).[17] Most NAATs analyse the polymerase chain reaction (PCR) products by gel electrophoresis or other open tube formats, which afford the opportunity for laboratory cross-contamination. These technically challenging techniques will pose problems in countries with limited laboratory resources. High-resolution DNA melting analysis (HRM) is a simple ‘‘closed tube’’ technique that reduces the risk of cross-contamination. Specific PCR amplicons are identified according to their characteristic DNA melting profiles. The amplicons are combined with a saturating dye that fluoresces in the presence of double stranded DNA. It is heated through a range of temperatures while fluorescence is monitored.[18] As the double stranded DNA dissociates (melts) into single strands, the fluorescence decreases. The melting peak of the specific infectious agent is identified. *M. tuberculosis* products melt at 90°C and other members of the *M. tuberculosis* complex at 86°C. This simple technique can be used in routine diagnostic laboratories.[18,19]

The aim of our study was to evaluate the value of PCR-based HRM analysis,[18] to provide a rapid and accurate diagnosis of mycobacterial disease using routinely collected FNAB specimens directly inoculated into liquid transport medium.

3. Materials and Methods

All adults and children referred to the FNAB Clinic at Tygerberg Hospital (June 2007–May 2008) with a superficial lymph node mass suggestive of possible mycobacterial lymphadenitis and in whom written informed consent to participate in the study was obtained, were included.

3.1. Specimen Collection

FNAB was done following standard protocol as an outpatient procedure by a pathologist.[10] The lymph node was stabilised by the aspirator and two needle passes were performed using a 23- or 25-g needle attached to a 10 ml syringe while applying a constant suction of no more than 2 ml. From each aspirate two smears were prepared one fixed with commercial cytology fixative for Papanicolaou staining and the other air dried for the Giemsa and subsequent ZN staining. The residual material in the syringe and needle was collected by withdrawing an aliquot of liquid growth media from the TB transport bottle into the syringe and then expelling the contents back into the bottle. No additional needle passes were performed to collect material for microbiology or PCR. The TB transport bottles were prepared ‘‘in-house’’ in a laminar flow cabinet: 10 ml headspace glass vials containing 1 ml of Middlebrook 7H9 broth (with 0.2% glycerol and 0.05% Tween 80 added), sealed with 20 mm TFE/Sil Septa and 20 mm Aluminium open top seals and autoclaved at 120℃ for 20 min.[15]

3.2. Cytology

Stained smears were evaluated for adequacy and to make a morphological diagnosis. If any specific lesion such as a lymphoepithelial cyst or neoplasia was present, this was reported. Alternatively, we reported ‘‘non-specific reactive lymphadenopathy’’ or ‘‘cytologically consistent

with mycobacterial infection'', based on specific morphological criteria.[20-22] In immunocompetent patients with TB, epithelioid granulomata and epithelioid histiocytes may be identified in a background of reactive lymphocytes and plasma cells (Figure 1). A small amount of amorphous necrosis may also be identified. In immunocompromised patients, the cytological picture is that of abundant necrosis in which neutrophils and cellular debris are prominent (Figure 2). In patients with lymphadenitis due to *M. bovis* BCG or NTM infection, histiocytes with abundant foamy cytoplasm may be present.[8,23,24]

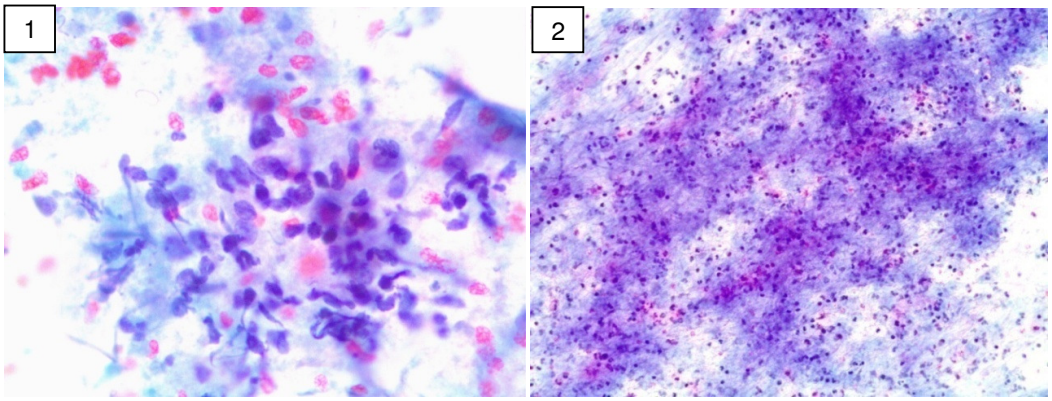


Figure 1. Epithelioid granuloma in an immune competent patient. (Papanicolaou, X400).

Figure 2. Abundant necrosis in which neutrophils and cellular debris are prominent consistent with tuberculous lymphadenitis in an immune compromised patient. (Papanicolaou, X400).

Mycobacteria were visualised using a ZN stain performed on a Giemsa stained slide according to a slightly modified technique (Figure 3). Smears were differentiated in 3% acid alcohol for 1 minute and counterstained with 1% methylene blue for 10 seconds only. In addition, one of the Papanicolaou stained slides was screened using a Zeiss Axiophot microscope with a fluorescent attachment and a wide-band blue excitation filter (450–480 nm); mycobacteria fluoresce as brilliant yellow bacilli, thin and slightly curved with polar enhancement and a uniform length of 2.0–2.7 microns (Figure 4).[11]

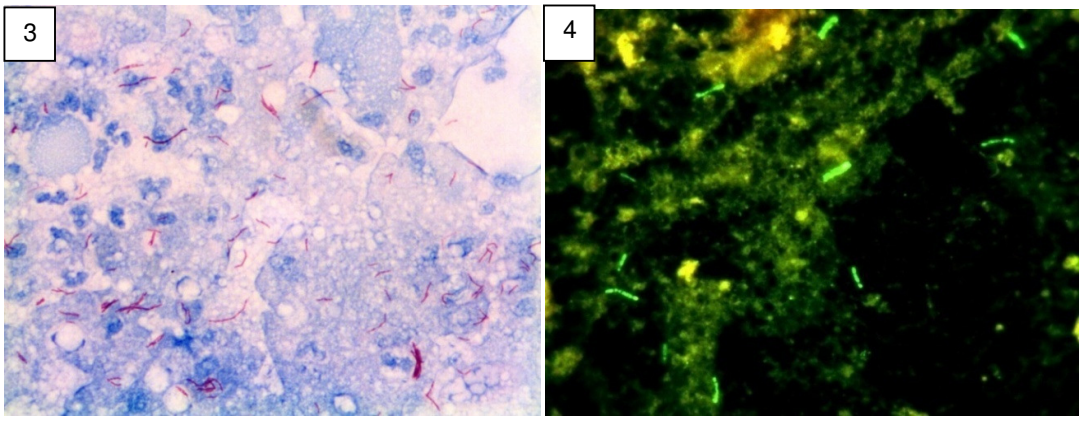


Figure 3. Modified ZN stain in a lymph node aspirate. (Ziehl-Neelsen, X400).

Figure 4. Autofluorescence of mycobacteria in a lymph node aspirate. (Papanicolaou, X1000 with a wide-band blue excitation filter)

3.3. Microbiology

In the laboratory, 0.5 ml of the media was aspirated from the TB transport bottle, inoculated into a separate MGIT tube containing the PANTA (polymyxin B, amphotericin B, Nalidixic acid, trimethoprim and azlocillin) antibiotic mixture and incubated in a BACTEC MGIT 960 machine for 42 days. All positive MGIT tubes were confirmed to contain acid-fast bacilli in the absence of bacterial contamination by ZN staining. Mycobacteria were identified as *M. tuberculosis* or other by standard PCR.[25]

3.4. Extraction of Mycobacterial DNA

The TB transport bottles containing the remaining 0.5 ml media were stored at -20℃ until further analysis. Mycobacteria within a 250 µl aliquot were pelleted by centrifugation at full speed (14 000 rpm) for 10 minutes. The supernatant was discarded and the bacterial pellet was resuspended in 1 ml phosphate buffered saline and re-centrifuged at full speed (14 000 rpm) to remove residual blood. Thereafter, the bacterial pellet was resuspended in 30 µl ddH_2O and heat

inactivated at 100°C for 20 minutes. The lysed bacterial extract was stored on filter paper (FTA® Classic Card Collection, Storage, and Purification system, Whatman, UK). Genomic DNA was eluted from the filter paper cards following manufacturers' instructions (high pH, low pH protocol) and served as the template for subsequent PCR amplification.

3.5. Speciation and HRM analysis

Primers were designed to amplify the Region of Deletion 9 (RD9), present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the *M. tuberculosis* complex. PCR was performed as previously described[19] using an annealing temperature of 62°C and the following primers RD9Fs1 5'-CAA GTT GCC GTT TCG AGC C-3', RD9FR 5'-GCT ACC CTC GAC CAA GTG TT-3' and RD9INT 5'-CAA TGT TTG TTG CGC TGC-3'.[26] Resulting amplification products underwent HRM analysis in a Rotorgene™ 6000 real-time rotary analyser (Corbett Life Science, Australia). The thermal denaturation profiles were measured as previously described.[19] The infectious agent was identified by the Rotorgene™ 6000 software according to the presence of derivative melt peaks located within defined temperature bins. *M. tuberculosis* PCR products melt at 90°C (*M. tuberculosis* could not be differentiated from *M. canettii*, but *M. canettii* is very rarely observed and has not been recorded in the study setting) whereas PCR products of other members of the *M. tuberculosis* complex melted at 86°C (Figure 6).

3.6. Reference Standard for Mycobacterial Disease

The reference standard for the presence of mycobacterial disease[17] was defined as positive cytology (morphology consistent with mycobacterial infection plus direct visualisation of the organism) and/or positive culture with speciation.

3.7. Statistical Analysis

We assessed the diagnostic accuracy of PCR-based HRM analysis compared to cytology, mycobacterial culture and the reference standard as defined using Pearson's Chi square ($\chi 2$) and Fisher's exact tests. All analyses were conducted using Statistica Version 8. Ethics approval was obtained from the Institutional Review Board of Stellenbosch University (N05/03/043).

4. Results

FNAB specimens were collected from 104 patients with possible mycobacterial lymphadenitis in whom complete cytopathology and mycobacterial culture results were available together with PCR-based HRM analysis. The median age was 30 years with a range from 4 months to 62 years. Children less than 13 years of age compromised 21.2% of the study population. There was no significant difference in gender distribution. HIV status was known in 46.1% of patients (48/104) and 75% of these were HIV positive (36/48). There were 23 HIV-positive patients and 6 HIV negative patients with mycobacterial disease as defined by the reference standard above. Table I summarises the demographics and diagnostic outcome of the study population.

Applying the defined reference standard 53 of the 104 patients (51.0 %) were diagnosed with mycobacterial lymphadenitis. Mycobacterial culture was positive in 35/53 (66.0 %) patients; 34 *M. tuberculosis* and 1 NTM. Cytology was positive in 84.9% (45/53) of the cases. Cytology and culture were both positive in 27/53 (50.9%) cases, whereas HRM analysis was positive in 52.8% (28/53) of cases identified using the reference standard. The calculated sensitivity of HRM analysis was 52.8% and the specificity 94.1% (Table 1) with a negative predictive value of 65.8% and a positive predictive value of 90.3%.

Table1. Demographics and diagnostic outcome

	Numbers	Percentage
Number of cases	104	
Age		
≤ 2 years	6	5.8
≤13 years	22	21.2
> 13 years	77	74
Unknown	5	5
Gender		
Male	48	46.2.
Female	51	49.0
Unknown	5	4.8
HIV status		
Positive	36	34.6
Negative	12	11.5
Unknown	56	53.9
Cytodiagnoses		
Malignancy	12	11.5
Reactive node	43	41.4
Other (e.g. cyst)	4	3.9
Cytology positive mycobacterial infection	45	43.3
Cases with mycobacterial disease		
Culture **and**/**or** cytology positive (Reference Std)	53	100
Cytology positive	45	84.9
Culture positive	35	66.0
Culture positive and cytology positive	27	50.9
PCR positive mycobacterial infection	28	52.8

Concordance between the different diagnostic modalities and HRM analysis was poor, with Kappa values of 0.39 vs. cytology, 0.27 vs. mycobacterial culture, 0.19 vs. both cytology and culture, and 0.45 vs. cytology and/or culture (the defined reference standard). Figure 5 shows the insensitivity of the HRM method as 59% of isolates confirmed to contain *M. tuberculosis* by cytology and culture were not detected by this method.

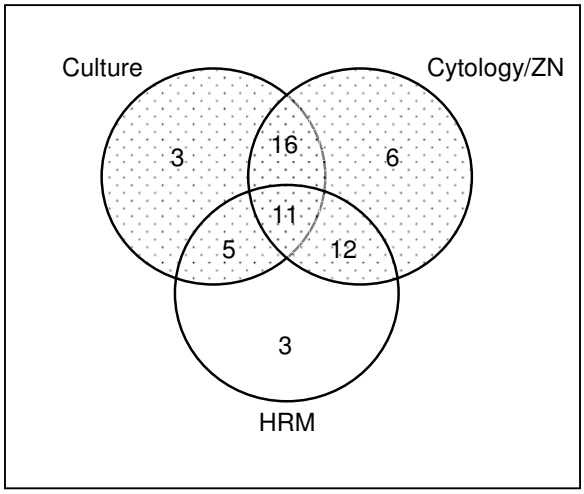
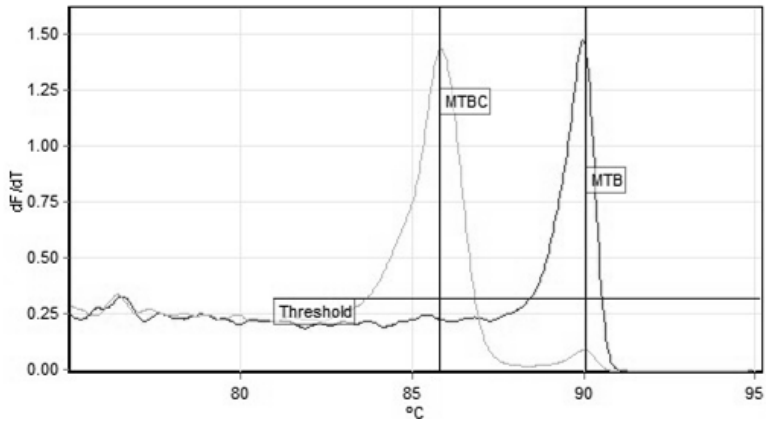


Figure 5. A Venn diagram depicting the number of samples positive for the different diagnostic methodologies. The reference standard for mycobacterial disease (shaded areas) is a positive Culture and/or Cytology/ZN result

To investigate a possible association between mycobacterial load and the result of PCR-based HRM analysis, we tested for any correlation between the time to positivity (TTP, often used as a surrogate of mycobacterial load) and PCR outcome. Among those with positive mycobacterial cultures, no correlation between the time to positivity and PCR-based HRM analysis could be demonstrated; mean TTP in PCR positive cases was 18 days compared with 22 days in PCR negative cases ($p = 0.22$) (Figure 6).



No.	Colour	Name	Genotype	Peak 1
1		M.tuberculosis	MTB	89.98 (MTB)
2		M.bovis	MTB complex	85.85 (MTBC)

Figure 6. Rotorgene software depicting the derivative melt peaks located within defined temperature bins. *M. tuberculosis* products melted at 90°C and *M. tuberculosis* complex at 86°C.

5. Discussion

With global coordination of control efforts TB incidence rates seem to be stabilising and showing signs of decline in all six world regions. However, the TB disease burden in areas plagued by the concurrent HIV pandemic remains at unprecedented levels.[1] Although there is some assistance for the development of strong laboratory networks in developing countries, the existing infrastructure remains poor in most TB endemic areas. South Africa, despite its relatively good infrastructure and health care services, in 2007 reported a national TB incidence of 739.6/100,000 population and an adult TB incidence in the Western Cape Province, a high burden region, of 1005.7/100.000.[27]

In TB endemic areas with ongoing transmission, children constitute a significant percentage of the total caseload, estimated at 15–20%.[28] Immune immaturity and/or compromise, most often due to HIV infection,[29,30] influence the risk to develop extra pulmonary and/or disseminated TB as well as the likelihood of rapid disease progression and TB-related mortality.[31,32] Autopsy studies in Africa[30,33-36] have shown that up to 54% of deaths in HIV-infected adults and 20% in HIV-infected children are due to TB. Reducing this mortality and morbidity necessitates early detection, efficient diagnosis and timely institution of appropriate therapy.

FNAB is a simple, non-invasive specimen collection technique in patients with peripheral lymph node masses.[10] It assists with rapid diagnosis of mycobacterial disease, but also helps to rule out alternative diagnoses that may require urgent treatment such as neoplasia. Diagnostic sensitivity is dependent on the experience of the pathologist and for mycobacterial disease varies from 32 to 78%.[10,37,38] Identification of the organism is essential particularly in immune compromised patients, as other opportunistic infections such as fungal organisms may elicit a similar morphological reaction pattern. Mycobacteria may be visualised using ZN staining, which is a very simple stain but has suboptimal sensitivity (20– 62%).[10,17,39,40] Autofluorescence, using the ability of mycobacteria on Papanicolaou stains smears to fluoresce using a blue excitation filter requires no additional staining and improves sensitivity (65–67%).[10,11] Culture is often regarded as the ‘‘gold standard’’, but is limited by poor sensitivity (reported to be as low as 2–34%)[38,40] and is highly dependent on the culture medium and inoculation technique used. A positive result is delayed by 1–6 weeks and requires additional PCR-based testing for speciation.

Direct NAAT application provides results in 3 to 6 hours and has been evaluated with respiratory and non-respiratory specimens such as FNABs.[17,39-43] Test sensitivity with respiratory specimens is highly dependent on mycobacterial load and use current PCR-based tests are only advised in patients with sputum smear-positive TB. Results with extra-pulmonary disease have been variable but recent studies have shown excellent results. Use of a nested PCR in cervical TB lymphadenitis in Mexican children showed a sensitivity of 96% and a specificity of 93%[46] whereas a study utilising DNA from dried and fixed cytology smears showed a sensitivity of 85%

and specificity of 95% using nested PCR, although reference standards are not necessarily based on bacteriological confirmation of disease.[44]

The majority of these studies use agarose gel electrophoresis to visualise the products, which is not practical in a microbiology laboratory. The open tube nature of the procedure also allows the release of amplicons, which pose a real risk of cross contamination. A recent systematic review evaluating the diagnostic utility of NAATs in TB lymphadenitis identified 36 peer-reviewed publications.[17] The authors found marked variation in populations, test techniques, reference standards, volume of material utilised and quality indicators. Few studies controlled for cross-contamination and inhibitors in clinical samples, thereby increasing the possibility of both false positive and false negative results. The reference standard used most frequently was culture, although the authors regard the sensitivity of culture from FNAB to be about 62%. Using an imperfect reference standard may lead to an underestimation of test performance, and this is a major limitation that is rarely acknowledged.[45]

The current study attempted to address some of the shortcomings noted above. The reference standard used is a combination of positive cytology, defined as cytomorphological features consistent with mycobacterial infection combined with identification of the organism using ZN staining or autofluorescence and/or positive culture. The residual material from the fine-needle aspirates was collected in TB transport medium in a sealed bottle, minimising the possibility of contamination. After washing and concentration of the specimen, the pellets were placed on filter paper for storage and to remove inhibitors present in the specimen. After extraction of DNA, the products were amplified using primers designed to amplify the Region of Deletion 9 (RD9), present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the complex.[19] The amplified products were then identified using high resolution melt analysis, which is a closed tube format that minimises the possibility of cross-contamination.

This technique is rapid and simple, and the equipment required for the HRM such as the Rotorgene 6000™ real-time rotary analyser (Corbett Life Science, Australia) is relatively inexpensive. Up to 72 samples may be processed in a cycle which takes approximately 3 h. The

results of PCR using this technique did not differ significantly from that of cytology or culture when these were assessed independently against the reference standard. The sensitivity is relatively low, but the specificity is high at 94.1% as is the PPV of 90.3%. This enables appropriate therapy to be implemented early with a high degree of confidence in the majority of patients with disease, while continuing with culture in the PCR negative cases.

Limited sample volume may have accounted for the low sensitivity observed. No additional needle passes or aspirates were performed and the material collected was limited to the residual amount left in the needle. Sensitivity may be improved if an additional needle pass is performed to obtain material for PCR. No single diagnostic modality was adequately sensitive to enable it to be used alone. FNAB provides material for cytology, culture and PCR using the transport medium described. This is of particular value in resource limited countries where laboratories tend to be centralised.[1] Cytology slides once prepared are stable at room temperature, as is the transport medium where the mycobacterial organism has been shown to remain viable for up to 7 days at room temperature, enabling both culture and PCR.[14]

Ideally, the PCR technique could be refined, increasing the sensitivity and thereby eliminating the need for culture, which is costly and lengthy. Use of the transport bottle facilitates both culture and PCR analysis, but material may be deposited directly onto filter paper for subsequent PCR analysis. Refined techniques need to remain simple inexpensive and appropriate for use in routine laboratories in countries with limited resources and skills that bear the burden of this devastating and persistent disease.

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Chapter 3

High-Resolution Melt Analysis for the Detection of Mycobacterial Lymphadenitis Using Whatman FTA® *Elute* Cards

K. G. P. Hoek[1], C. A. Wright[2], B. J. Marais[3], P. D. van Helden[1], and R. M. Warren[1]

[1] DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, [2] Division of Anatomical Pathology, Department of Pathology, [3]Department of Paediatrics and Child Health, Faculty of Health Sciences, Stellenbosch University, South Africa

1. Abstract

The increased incidence of tuberculosis (TB) lymphadenitis due to the human immunodeficiency virus (HIV) epidemic and the significant diagnostic delay by routine culturing methods has led to the need for a more rapid diagnostic method. We recently reported a rapid and specific molecular-based method which consisted of high-resolution melting (HRM) analysis of fine needle aspiration biopsies (FNAB) inoculated into transport medium bottles and subsequently spotted onto FTA® Classic Cards (Whatman, UK). In the current study, we aimed to simplify the procedure by circumventing the need for a transport medium bottle and spotting the samples directly onto the simpler FTA® *Elute* Cards.

Fine-Needle Aspiration Biopsy samples were analysed from 65 adults and children with possible mycobacterial lymphadenitis and disease was confirmed in 41 patients (63.1%) according to the reference standard (positive cytology and/or a positive culture). Cytology was positive in 75.6% and culture in 80.5% of patients. HRM identified 46.3% (19/41) TB lymphadenitis samples with a sensitivity and specificity of 46.3% and 83.3% respectively as compared to the reference standard. This was slightly less than by the previously described method.

The use of the simpler more cost-effective FTA® *Elute* Card with the omission of the transport medium bottles did not provide an improvement over the previously described indirect method. However, both methods were able to provide a rapid and specific diagnosis in ~50% of patients, thereby allowing prompt initiation of therapy. The remainder of cases would be diagnosed at a later stage through routine phenotypic culturing methods.

We therefore propose a diagnostic algorithm which takes HRM and cytology results into consideration in that if HRM and cytology are indicative of mycobacterial disease, then treatment is initiated, if HRM and cytology do not correlate or are both negative for disease, then culture results are awaited before treatment initiation.

2. Introduction

The number of lymphadenitis cases is steadily rising due to the increased incidence of extra-pulmonary tuberculosis (TB) spurred on by the human immunodeficiency virus (HIV) pandemic.[1,2] These lymph node masses are most often analysed by Fine-Needle Aspiration Biopsy (FNAB), which is a simple, relatively non-invasive outpatient procedure[3] providing samples for further investigations including cytomorphology and TB culture.[4]

While phenotypic culture of *Mycobacterium tuberculosis* from a FNAB is regarded as the gold standard for diagnosing TB lymphadenitis, the success thereof is dependent on the quality of the specimen collected. Transport of the FNAB to the laboratories is also problematic and the standard liquid inoculation tubes such as the Mycobacterial Growth Indicator Tubes (MGIT, Beckton Dickinson, USA)[3] have limited shelf lives and require specific storage requirements. Furthermore, due to the slow growth rate of mycobacteria, culture methods may take 2 to 6 weeks to generate results, therefore leading to a significant diagnostic delay. Cytomorphology and direct mycobacterial visualisation with either Ziehl-Neelsen (ZN) staining or fluorescence microscopy are more rapid techniques; however they are not considered as a definite diagnosis of *M. tuberculosis* infection and require further speciation.

To overcome these problems, we recently reported the use of an inexpensive transport medium for direct inoculation[5] of FNAB. We subsequently evaluated the diagnostic value of a more rapid molecular-based methodology, high-resolution melting (HRM) analysis in the diagnosis of *M. tuberculosis* lymphadenopathy. As reported[4] initial HRM directly from the transport medium bottles had a very low sensitivity, possibly due to the high dilution of the bacteria within the samples. We therefore concentrated this DNA and then spotted it onto the FTA® Classic Card Collection, Storage, and Purification system (Whatman, UK). Genomic DNA was eluted from the cards using a high pH, low pH protocol (according to manufacturer's instructions). The HRM methodology was able to detect 52.8% (28/53) of TB lymphadenitis cases with a sensitivity and specificity of 52.8% and 94.1%, respectively. The positive predictive value (PPV) was 90.3% and

the negative predictive value (NPV) was 65.8%.[4] Although HRM analysis allowed a rapid and species-specific diagnosis of *M. tuberculosis* lymphadenitis in the majority of patients, we felt that there was a potential for further optimisation of the methodology.

The aim of this study was to investigate the sensitivity and specificity of HRM diagnosis of TB lymphadenitis by the direct spotting of the FNAB samples onto a simpler, more cost-effective FTA® *Elute* Card Collection, Storage, and Purification system (Whatman, UK), which consists of a shortened protocol and circumvents the need for an intermediary transport bottle.

3. Materials and Methods

3.1. Specimen Collection

Fine-Needle Aspiration Biopsy samples were obtained from 83 adults and children with a superficial lymph node mass suggestive of possible mycobacterial lymphadenitis at the FNAB Clinic at Tygerberg Hospital between the time periods May 2009 to January 2010. Informed consent was obtained from all the study participants and ethical approval granted by the University of Stellenbosch, South Africa (N05/03/043).

Fine-Needle Aspiration Biopsy was done following standard protocol by a pathologist.[3] Two needle passes were done and from each aspirate two smears were prepared for microscopic investigations. The residual material in the syringe and needle (from the first pass) was used to inoculate a MGIT tube for routine phenotypic culture by the National Health Laboratory Services (NHLS), and the residual material from the second pass was expressed onto the FTA® *Elute* Card Collection, Storage, and Purification system (Whatman, UK). The cards were then transported to the laboratory in individual plastic pouches and kept at room temperature for a minimum of 24 hours to allow the specimens to dry.

3.2. Phenotypic investigations

The stained smears were investigated for cytomorphological changes by Papanicolaou stain and for the presence of gram negative bacteria by a modified ZN stain as previously described.[4] The slides were not identifiable as having been prepared from the first or second pass.

The residual material from the first pass was cultured in a MGIT tube in a BACTEC 960 machine for 42 days according to the NLHS standard operating procedure. All positive MGIT tubes were confirmed to contain acid-fast bacilli in the absence of bacterial contamination by ZN staining. Mycobacteria were identified as *M. tuberculosis* or other by standard PCR.[6]

3.3. Extraction of Mycobacterial DNA

The FTA® *Elute* Cards were processed according to the manufacturer's instructions. Briefly, a 3mm disk from the centre of the spotted sample was transferred to an Eppendorf tube containing 500µl of ddH_2O. The tubes were pulse vortexed three times and the 500µl ddH_2O was aspirated and substituted with 30µl ddH_2O. After a brief centrifugation (10 seconds) to ensure that the disks were fully submerged, the tubes were incubated at 95°C for 30 min. The samples were then pulse vortexed 60 times and centrifuged so as to separate the matrix from the eluate, which was subsequently transferred to a clean Eppendorf tube and stored at -20°C until further analysis.

3.4. Speciation and HRM analysis

Molecular analysis (PCR and HRM) of the respective FNAB samples was done as previously described,[4] targeting the Region of Deletion 9 (RD9), present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the *M. tuberculosis* complex.[7] Samples which failed to amplify were reanalysed using PCR primers specific to the human beta-actin gene (hu-â-actin F 5'-GAG CTA CGA GCT GCC TGA CG-3' and hu- â-actin R 5'-GTA GTT TAG TGG ATG CCA

CAG GAC T-3'). If amplification failed again, these samples were excluded from further analysis due to the lack of DNA in these samples.

3.5. Reference Standard for Mycobacterial Disease

The reference standard for the presence of mycobacterial disease[8] was defined as positive cytology (morphology consistent with mycobacterial infection plus direct visualisation of the organism either by ZN staining or autoflourescence following Papanicolaou staining)[4] and/or positive culture with speciation.[4]

3.6. Statistical Analysis

We assessed the diagnostic accuracy of PCR-based HRM analysis compared to cytology, mycobacterial culture and the reference standard as defined using Pearson's Chi square ($\chi 2$) and Fisher's exact tests. All analyses were conducted using Statistica Version 8.

4. Results

FNAB specimens were analysed from 65 of 83 patients with possible mycobacterial lymphadenitis for which complete cytopathology and mycobacterial culture results were available together with PCR-based HRM analysis. Nine samples did not deliver any DNA by the extraction technique (confirmed by reanalysis targeting the human beta-actin gene). Three samples were missing culture results and the remaining 6 samples lacked cytology results. These 18 samples were excluded from the statistical analysis. Applying the defined reference standard 41 of the 65

patients (63.1%) were diagnosed with mycobacterial lymphadenitis. This was slightly more than the previously reported 51.0% (Table 1).[4]

Cytology/ZN and HRM was positive in slightly fewer patients than in the previous study (75.6% vs. 84.9% and 46.3% vs. 58.5%, respectively), whereas culture positivity was higher (80.5% vs. 66.0%). Similar results were obtained for samples that were both cytology/ZN and culture positive (56.1% vs. 51.0). More importantly the concordance between the various diagnostic modalities and HRM analysis remained poor, with Kappa values of 0.19 vs. cytology/ZN, 0.26 vs. mycobacterial culture, 0.26 vs. cytology/ZN and culture; and 0.26 vs. cytology/ZN and/or culture (the defined reference standard).

HRM analysis could correctly identify 19 out of 41 TB lymphadenitis samples (defined by the reference standard) with a sensitivity and specificity of 46.3% and 83.3% respectively, with a PPV of 82.6% and a NPV of 47.6% (Figure 1). This was slightly less than by the indirect method.[4] High resolution melt analysis incorrectly identified 4/24 samples as containing *M. tuberculosis* DNA. Further investigation revealed that 3 of these patients had been transferred from community clinics and therefore limited data was available regarding previous TB history (1 patient was HIV positive, 1 was a child and 1 had repeated TB investigations).

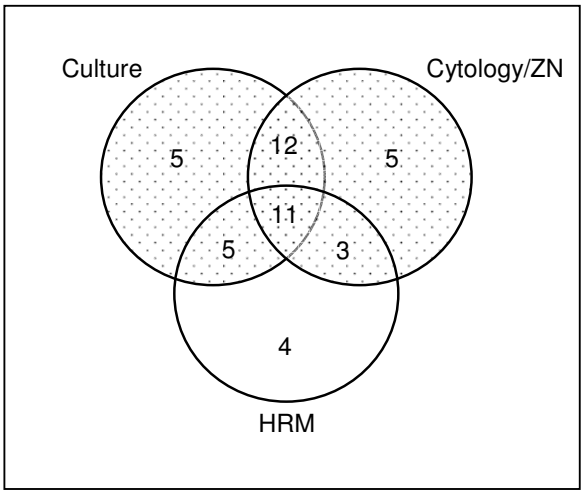

Figure 1. A Venn diagram depicting the number of samples positive for the different diagnostic methodologies. The reference standard for mycobacterial disease (shaded areas) is a positive Culture and/or Cytology/ZN result.

Table1. Comparison of results obtained from spotting specimens from the transport bottles onto the FTA Classic storage system (indirect method)[4] to that of direct specimen spotting onto the FTA *Elute* storage system (direct method).

	Indirect method[4]	**Direct method**
Numbers of cases	104	65
Cases with Mycobacterial disease =		
(Reference standard: Culture **and/or** Cytology or ZN positive)	53 (51.0%)	41 (63.1%)
Cytology or ZN positive	45 (84.9%)	31 (75.6%)
Culture positive	35 (66.0%)	33 (80.5%)
HRM positive	28 (52.8%)	19 (46.3%)
Culture **and** Cytology/ZN positive	27 (51.0%)	23 (56.1%)
Concordance		
HRM vs. Cytology/ZN	0.39	0.19
HRM vs. Culture	0.27	0.26
HRM vs. Culture **and** Cytology/ZN positive	0.19	0.26
HRM vs. Reference Standard	0.45	0.26
Performance HRM vs. Reference Standard		
Sensitivity	52.8%	46.3%
Specificity	94.1%	83.3%
Positive Predictive Value (PPV)	90.3%	82.6%
Negative Predictive Value (NPV)	65.8%	47.6%

5. Discussion

The use of the simpler more cost-effective FTA® *Elute* Card with the omission of a transport medium did not provide an improvement over the previously described indirect method. Furthermore, feedback from the clinicians and nursing staff doing the FNAB revealed that the direct spotting of the sample onto the FTA® *Elute* Card posed a health risk and was difficult to do as the target area of the card is small and the FNAB samples are difficult to expel from the syringes used. Often residual sample remained in the neck of the syringe. The previous method, which involved rinsing the syringe in the transport medium bottles, was able to loosen this residual tissue and also prevented unnecessary exposure of the staff to any infectious material. A further limitation of the methodology is that it is difficult to know how well DNA dissociates from the FTA® *Elute* Card matrix during the extraction procedure and how well PCR inhibitors remain trapped by the same matrix. Furthermore, despite experimenting with various volumes of input DNA in the PCR reaction, we did not investigate if larger volumes (>4µl per 25µl reaction) would significantly improve the sensitivity of the assay. We propose that in future, the samples will be collected in the transport media bottles and that alternative DNA concentration and purification methods be investigated to improve the sensitivity of the assay. We will also investigate the performance of the Xpert MTB/Rif Assay (Cepheid Inc. Sunnyvale, California, USA) on these FNA samples.

The poor performance of the assay may have been due to the failure to annotate which slides were obtained from which pass. Ideally all the diagnostic modalities should be compared to samples obtained from the same pass (i.e. either all from the first or second pass) so as to overcome possible sampling errors/bias. However, despite the low sensitivity (46.3%) of the HRM analysis, 19 of 41 patients were able to have a rapid and specific (83.3%) diagnosis within one day of undergoing a FNAB. This implies that these patients could be started on early anti-tuberculosis treatment, thereby reducing transmission of disease, morbidity and mortality. Although 22 patients were missed by HRM analysis, these patients could be confirmed at a later

stage by phenotypic culture. We therefore propose the following diagnostic algorithm while awaiting improved diagnostic methods: if HRM analysis is positive for the presence of *M. tuberculosis* DNA and cytology is suggestive of Mycobacterial disease, then anti-tuberculosis treatment is started. If HRM analysis is positive, but cytology does not indicate Mycobacterial disease, or if HRM analysis is negative, then await phenotypic culture results before initiating treatment.

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Chapter 4

Fluorometric Assay for Testing Rifampicin Susceptibility of *Mycobacterium tuberculosis* Complex

K. G. P. Hoek, N. C. Gey van Pittius, H. Moolman-Smook, K. Carelse-Tofa, A. Jordaan, G. D. van der Spuy, E. Streicher, T. C. Victor, P. D. van Helden, and R. M. Warren

DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences, Faculty of Health Sciences, Stellenbosch University, South Africa

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1. Abstract

The emergence and transmission of multidrug-resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB) have raised concern about diagnostic delay associated with culture-based drug susceptibility testing methods. The association between rifampicin resistance and MDR-TB or XDR-TB makes it an important genetic marker for genotypic drug susceptibility testing. In this chapter, we describe the analysis of the physical properties of the rifampicin resistance-determining region (RRDR) in the *rpoB* gene by high-resolution thermal melt analysis as a method for detecting rifampicin resistance in *Mycobacterium tuberculosis* complex. The RRDR from the *M. tuberculosis* complex was amplified by PCR from DNA templates extracted from sputum cultures of *M. tuberculosis* or the laboratory strain (H37Rv) in the presence of a fluorescent DNA binding dye. Subsequent mixing of the amplification products from the respective sputum cultures and the laboratory strain and thermocycling allowed the formation of DNA duplexes. The thermal denaturation properties of these DNA duplexes were determined by measuring the derivative of the intensity of fluorescence at different temperatures. Analysis of DNA extracted from 153 sputum cultures showed a sensitivity of 98% and a specificity of 100% for the detection of rifampicin resistance compared to the "gold standard" culture-based phenotyping method. No statistical difference was detected in the performance of the method when applied to crude DNA from 134 boiled cultures. This method, named "FAST-Rif" ("Fluorometric Assay for Susceptibility Testing of Rifampicin"), allowed the rapid, reliable, and easy detection of genotypic rifampicin resistance as a marker for MDR-TB and XDR-TB.

2. Introduction

Drug resistance remains a serious threat to tuberculosis (TB) control worldwide.[1] This is especially true for multidrug-resistant TB (MDR-TB), which is defined as disease caused by *Mycobacterium tuberculosis* that is resistant in vitro to both isoniazid and rifampicin, with or without resistance to other anti-TB drugs. A recent survey estimated that 424,203 MDR-TB cases were diagnosed worldwide among new and retreatment cases in 2004.[2] In 2004, a study of samples submitted to selected national reference laboratories worldwide estimated that in the 4 years between 2000 and 2004, 20% of the samples tested represented MDR-TB cases and that 10% of these were extensively drug-resistant TB (XDR-TB) cases (defined at that time as resistant to three of six classes of second-line drugs).[3] In October 2006, the World Health Organization redefined XDR-TB as being MDR-TB with additional resistance to any fluoroquinolone (e.g., ciprofloxacin, ofloxacin, or moxifloxacin) and to at least one of three injectable second-line anti-TB drugs used in treatment (capreomycin, kanamycin, or amikacin).[4]

In order to combat the threat of drug resistance, it is essential that new rapid diagnostics are developed to complement a well-functioning TB control program. Recent advances in phenotypic drug susceptibility testing include the use of mycobacterial growth indicators[5,6] and phage-based assays.[7] Although these methods are able to report phenotypic resistance in 2 to 10 days, the culture of viable bacilli poses a health risk to laboratory personnel and thereby requires high levels of biosafety.

To overcome these limitations and to improve the speed of detection of drug resistance, numerous PCR-based methods have been described.[8] However, the number of different non-synonymous single nucleotide polymorphisms (nsSNPs) conferring resistance remains a major challenge to the successful development of genotypic drug susceptibility testing methods. Pragmatically, this has been partially circumvented by developing assays which analyse the most prominent nsSNPs, with some reduction in sensitivity and specificity due to this. However, many of these methods are hampered by the need for downstream processing to enable the detection

of nsSNPs within the PCR-amplified domain (e.g., hybridisation to immobilised oligonucleotides,[9] microarray,[10] dot blot hybridisation,[11] denaturing high-performance liquid chromatography,[12] and DNA sequencing[13,14]). The complexity of these methods and the need for multiple steps to perform them greatly increase the risk of cross-contamination and thereby misdiagnosis. An assay which is rapid, sensitive, and specific and does not require downstream processing, thereby minimising cross-contamination, would be ideal.

A study by Williams *et.al.*,[15] showed that heteroduplexes could be used to determine rifampicin susceptibility by analysing conformational changes created by nsSNPs in the DNA fragments. We proposed that the analysis of thermal denaturation profiles of heteroduplexes could be used to enhance the detection of nsSNPs conferring resistance in *M. tuberculosis*. In theory, the thermal denaturation profile of a specific DNA fragment is dependent on the nucleotide sequence of that fragment.[16] Thus, any change in the nucleotide sequence would alter the thermal denaturation profile, which in turn could be detected by measuring the efficiency of binding of a fluorescent dye to the DNA fragment at different temperatures.[17]

However, nucleotide transversions (A:T and G:C) remain difficult to detect, since they have very little influence on the overall thermal denaturation profile. To circumvent this limitation, we proposed that the efficiency of detecting transversions and transitions could be enhanced by analysing the thermal denaturation profiles of DNA duplexes formed by annealing DNA fragments with and without nucleotide change (heteroduplex and homoduplex, respectively).

In this study, we tested these concepts by analysing the unique thermal denaturation properties of the rifampicin resistance- determining region (RRDR) of the *rpoB* gene to develop a method for the detection of rifampicin resistance. Mono-resistance to rifampicin is rare and is mostly accompanied by isoniazid resistance. Therefore, the rifampicin resistance profile could be used as a marker for suspected MDR-TB and XDR-TB cases.

3. Materials and Methods

3.1. Preparation of pure DNA templates

M. tuberculosis was cultured from sputa (obtained from TB patients) on Löwenstein-Jensen medium, and genomic DNA was extracted as previously described.[18]

3.2. Preparation of crude DNA templates

Decontaminated sputum specimens were cultured at 37°C in BACTEC 12B medium (Becton Dickinson) for 7 days in the BACTEC 460 system, and the bacteria were pelleted by centrifugation, resuspended in 100 µl BACTEC 12B medium, and boiled to generate a crude-DNA template.[19]

3.3. Drug susceptibility testing

Drug susceptibility testing was done by the National Health Laboratory Service, using the indirect proportion method on Middlebrook medium containing critical concentrations of 0.2 µg/ml isoniazid and 30 µg/ml rifampicin. Following DNA sequencing and high-resolution thermal melt analysis, specimens with discrepant phenotypic results were subjected to rifampicin drug susceptibility testing in mycobacterial growth indicator tubes (Becton Dickinson) according to the manufacturer's instructions.

3.4. DNA sequencing of the RRDR of the *rpoB* gene

The DNA sequence of the RRDR of the *rpoB* gene of each sputum culture (pure and crude DNA) was determined as previously described.[20] The genotypes and phenotypes of the respective

isolates were determined prior to high-resolution thermal melt analysis, and these results were blinded to the high-resolution thermal melt operator.

3.5. PCR amplification of the RRDR

DNA templates (pure or crude) extracted from different sputum cultures, the laboratory strain (H37Rv, having a rifampicin-susceptible genotype), different members of the *M. tuberculosis* complex (*M. canettii*, *M. tuberculosis*, *M. africanum*, *M. microti*, *M. pinnipedii*, *M. caprae*, and *M. bovis* [Mycobacterial Reference Laboratory, Institut Pasteur, France]), or different species of non-tuberculous mycobacteria (*M. alvei*, *M. arupense*, *M. avium*, *M. chelonae*, *M. elephantis*, *M. fortuitium*, *M. goodii*, *M. intracellulare*, *M. interjectum*, *M. kansasii*, *M. kumamotonense*, *M. marinum*, *M. montefiorense*, *M. monacense*, *M. moriokaense*, *M. paraffinicum*, *M. peregrinum*, *M. porcinum*, *M. rhodesiae*, *and M. terrae* [speciated by 16S rRNA DNA sequencing][21]) were subjected to PCR amplification (Figure 1, step 1) in a reaction mixture containing 25 ng DNA template, 5 µl Q-Buffer, 2.5 µl 10X buffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM deoxynucleotide triphosphates, 1 µl of each primer (50 pmol/ µl) (rpoB Forward, 5'-CGC CGC GAT CAA GGA GTT C-3'; rpoB Reverse short, 5'-GCC CGG CAC GCT CAT GT-3'), 1 µl (1/100 dilution) Syto 9 fluorescent dye (Molecular Probes), and 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) and made up to 25 µl with distilled water. Amplification was initiated by incubation at 95°C for 15 min, followed by 35 to 45 cycles at 94°C for 45 s, 64°C for 45 s, and 72°C for 45 s. After the last cycle, the samples were incubated at 72°C for 10 min. Amplification was confirmed by high-resolution melt analysis (see below). To minimise laboratory cross-contamination, the preparation of the PCR mixes, the addition of the DNA, and the PCR amplification were conducted in physically separated rooms. Negative controls (water) were included to detect reagent contamination.

3.6. DNA duplex formation

After amplification, a 12.5 µl aliquot of the PCR product containing the amplified RRDR from each sputum culture was mixed with a 12.5 µl aliquot of the amplified RRDR from the laboratory strain H37Rv (Figure 1, step 2). Each mixture was subjected to a second round of cycling without adding additional PCR components (95°C for 1 min, followed by 10 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, followed by incubation at 72°C for 5 min). This procedure generated DNA duplexes between the respective RRDR amplification products from the sputum culture and the standard laboratory strain.

3.7. High-resolution thermal melt analysis

The DNA duplexes were subjected to high-resolution thermal melt analysis in a Rotorgene 6000 real-time analyser (Corbett, Australia) (Figure 1, step 3). The thermal denaturation profile was measured over the temperature range from 80°C to 95°C, and fluorometric readings were taken every 0.1°C. Rotorgene software was used to calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT), thereby generating a plot where the derivative peak(s) represents the T_m value of the DNA duplexes. Rifampicin drug susceptibility was assigned by the software according to the presence of a derivative peak(s) located within a defined temperature bin(s) (width, 1.5°C). A derivative plot with a single derivative peak (homoduplex) was classified as drug susceptible, while a derivative plot with two derivative peaks (homo- and heteroduplexes) was classified as drug resistant.

3.8. Statistical analysis

The statistical software program Statistica 7.1 was used to calculate the sensitivity and specificity of PCR amplification or genotypic drug susceptibility testing at a confidence interval (CI) of 95%.

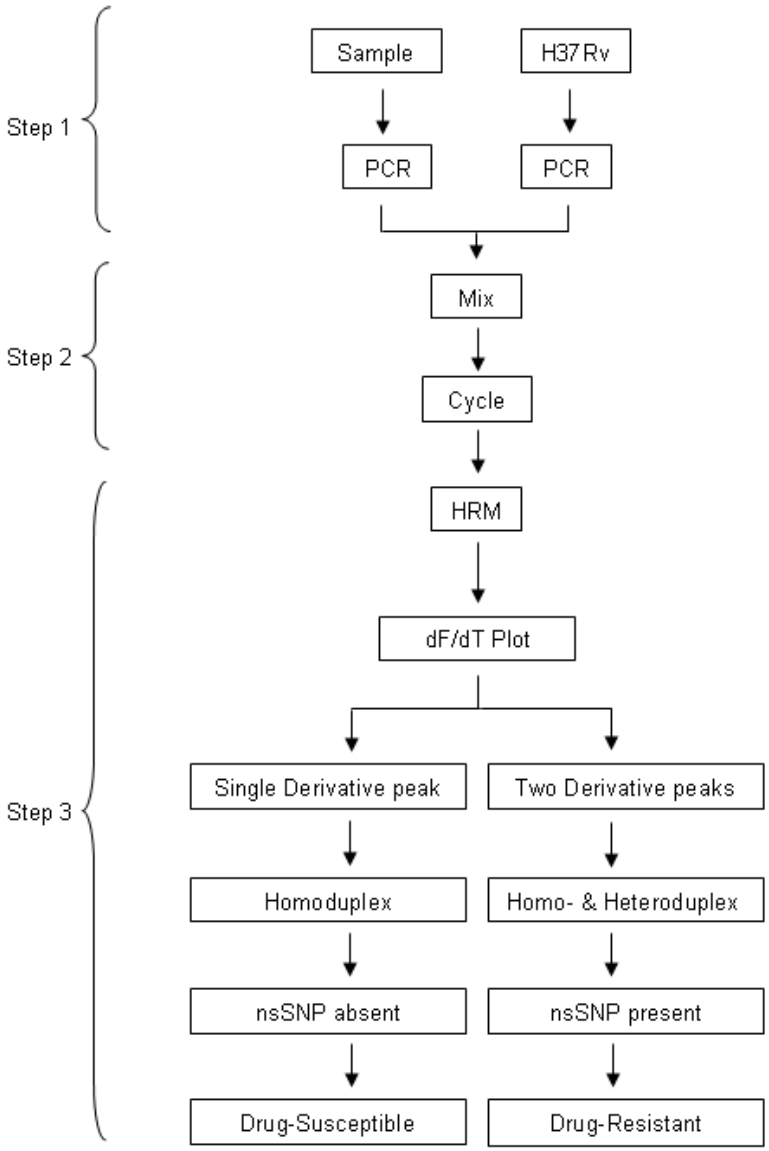

Figure. 1. Flow diagram of the high-resolution thermal melt genotypic drug susceptibility testing methodology. **In step 1**, DNA extracted from the laboratory strain (H37Rv) or the respective sputum cultures was subjected to PCR amplification of the RRDR of the *M. tuberculosis* complex *rpoB* gene. **In step 2**, RRDR DNA duplexes were formed between the amplified RRDR of the laboratory strain and the amplified RRDR of the respective sputum cultures by mixing of the PCR products and subsequent thermocycling. **In step 3**, DNA duplexes were subjected to high-resolution thermal melt analysis (HRM). Genotypic drug susceptibility was scored according to the shape of the dF/dT plot and the presence of derivative peaks in defined temperature bins.

4. Results

To determine the specificity of the primers used for the PCR amplification of the *M. tuberculosis* complex RRDR, DNA templates from 7 different members of the *M. tuberculosis* complex and 20 different species of non-tuberculous mycobacteria were amplified. The specificity of *M. tuberculosis* complex RRDR amplification was 100% compared to the 16S rRNA DNA sequence data.

To test whether the thermal denaturation properties of the RRDR could be used to detect nsSNPs conferring rifampicin resistance, DNA duplexes (formed between the RRDRs amplified from DNA extracted from phenotypically and genotypically well-characterised sputum cultures [n = 34] and the laboratory strain [H37Rv], respectively) were subjected to high-resolution thermal melt analysis in the presence of a saturating concentration of the fluorescent dye. Figure 2 shows the dF/dT values of DNA duplexes. Rifampicin-susceptible isolates were characterised by the presence of a single derivative peak, representing homoduplexes (Figure 2A), while rifampicin resistant isolates were characterised by the presence of two distinct derivative peaks at defined temperatures, representing homo- and heteroduplexes, respectively (Figure 2B). This demonstrated that the high-resolution thermal melt method could be used to identify nsSNPs conferring rifampicin resistance.

To determine the performance of this method, purified DNA from 80 phenotypically rifampicin-susceptible and 73 phenotypically rifampicin-resistant sputum cultures was analysed (the operator was blinded to the genotypic and phenotypic rifampicin resistance data). The method showed a sensitivity of 98% (95% CI, 94% to 101%) and a specificity of 100% (95% CI, 100% to 100%) for scoring the presence of nsSNPs encoding rifampicin resistance in relation to the “gold standard” phenotypic drug susceptibility testing method (see Appendix 1). The positive predictive value was 100%, and the negative predictive value was 97%.

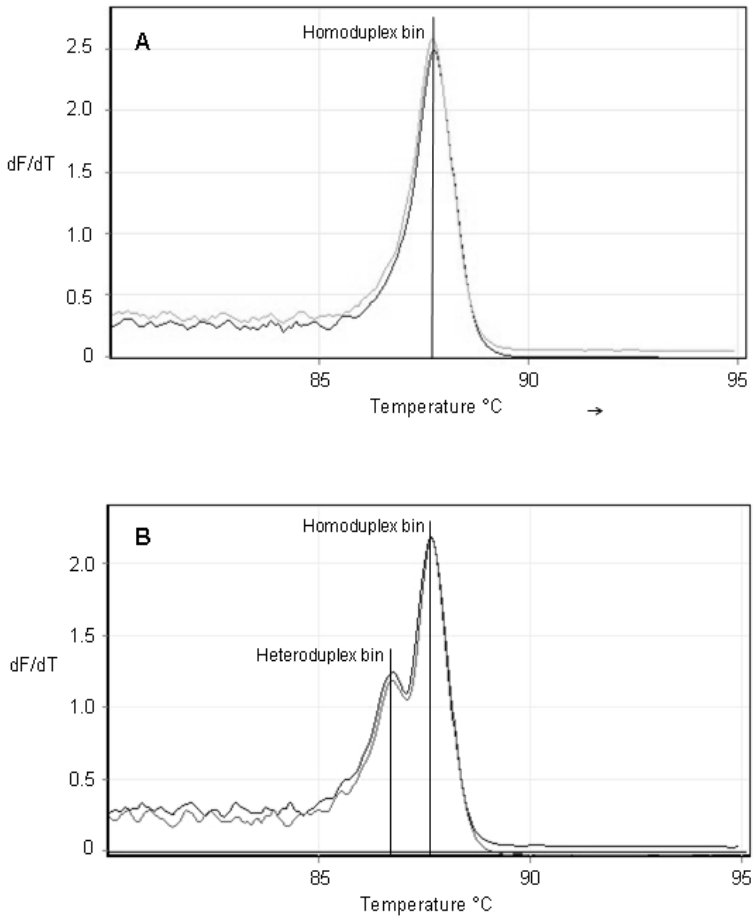

Figure. 2. dF/dT values of the DNA duplexes. (A) Isolates were scored as susceptible to rifampicin based on the presence of a single derivative peak within a defined temperature bin (homoduplex). (B) Isolates were scored as resistant to rifampicin based on the presence of two derivative peaks in defined temperature bins (homo- and heteroduplex).

To determine whether the high-resolution thermal denaturation genotyping technique could be used to analyse crude DNA templates, 44 phenotypically rifampicin-susceptible and 90 phenotypically rifampicin-resistant boiled sputum cultures were analysed. Performance analysis showed the technique had a sensitivity of 94% (95% CI, 90% to 99%) and a specificity of 96% (95% CI, 90% to 101%) (see Appendix 2). The positive predictive value was 98%, and the negative predictive value was 88%.

Using the thermal denaturation genotyping technique, a total of 14 different nsSNPs were detected at six codons in the RRDR and an insertion between codons 514 and 515 (data not shown).

Discussion

In this chapter, we report the development of a novel PCR-based fluorometric assay for testing of susceptibility to rifampicin, called FAST-Rif. This method allowed for the rapid detection of 15 different mutational events in the RRDR of the *rpoB* gene conferring rifampicin resistance in *M. tuberculosis* complex. The advantage of this method over previously described methods[9-14] was the simplicity of the method, the broad spectrum of nsSNPs detected by each analysis, and the high sensitivity and specificity achieved.

However, the method did not provide information about which nsSNP conferred resistance. This should not be seen as a limitation, since the primary objective for routine diagnostics would only be to determine the presence or absence of an nsSNP conferring resistance, thereby enabling the correct treatment to be administered to the patient. The sensitivity of this technique was not confounded by the presence of synonymous single nucleotide polymorphisms, since these have not been identified in the RRDR.[22] We acknowledge that our method was not able to identify nsSNPs conferring rifampicin resistance which fell outside of the RRDR. This implies that approximately 5% of cases with rifampicin resistance will be not be detected by the described method. Similarly, most of the previously described genotypic drug susceptibility testing methods[9-14] would not be able to detect nsSNPs outside of the RRDR.

In this study, the FAST-Rif method was based on two independent PCRs, which ensured the optimal non-competitive amplification of both the clinical and reference templates. Attempts to convert the method to a single-tube format were hampered by the competitive nature of the PCR. We envisage that this method could be adapted to a single-tube format if the two PCRs were

separated by a septum. After PCR amplification, the two PCRs could be mixed by inverting the tube, thereby avoiding the need to open the reaction tube. This would have further advantages, since it would simplify the method while avoiding the risk of cross-contamination following aerosolisation of amplicons. Furthermore, the single-tube (South African provisional patent 2007/06915, see Appendix 3) format would allow for high throughput and automation.

We acknowledge that the quality and amount of the input DNA remain a bottleneck for the applicability of all PCR based genotypic drug susceptibility testing methods. However, in this study we showed that the FAST-Rif method could efficiently amplify "crude DNA", thereby enabling a diagnosis to be made within a matter of 3 to 4 h following short-term culture. This diagnostic interval could be shortened further if the RRDR could be efficiently and routinely amplified from *M. tuberculosis* DNA purified from sputum.

Application of this methodology in the routine screening of clinical isolates would enable the rapid diagnosis of rifampicin resistance[23], thereby increasing the suspicion index for both MDR-TB and XDR-TB. This information would permit the attending clinician to request drug susceptibility testing for additional anti-TB drugs, thereby ensuring the appropriate management of the TB case and preventing the acquisition of other drug resistance phenotypes. Furthermore, the rapid diagnosis of rifampicin resistance would assist in limiting the transmission of drug-resistant TB to close contacts.

Given the affordability of the described method (cost not exceeding that of a standard PCR), as well as the ease of use, it should be possible to implement this methodology to improve case detection in most settings, thereby enhancing the overall control of the drug-resistant-TB epidemic. The versatility of this method implies that it could be adapted to detect nsSNPs conferring resistance to other anti-TB drugs.

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Chapter 5

Adaptation of the Fluorometric Assay for Susceptibility Testing to detect Ethambutol Resistance

1. Abstract

Ethambutol (EMB) forms an integral part of first and second-line tuberculosis therapy due to the inaccuracy/lack of surveillance data, suggesting that resistance to EMB is regarded rare. Drug-susceptibility testing (DST) for EMB is inaccurate and may lead to underestimation of true resistance frequencies, which may have a negative effect on the TB control program. Improved methodologies involve molecular-based testing, focusing on the *embB* gene conferring EMB resistance. We describe a fluorometric assay based on the formation of DNA duplexes and the use of High Resolution Melting analysis to rapidly detect EMB resistance in cohorts of isoniazid mono-resistant, rifampicin mono-resistant and multi-drug resistant (MDR-TB) tuberculosis isolates.

The method was optimised on a subset of 98 well-characterised (DNA sequencing) EMB susceptible and resistant samples. The region flanking codon 306 of the *embB* gene from the *Mycobacterium tuberculosis* complex was amplified by PCR from crude cultures of *M. tuberculosis* or the laboratory strain (H37Rv) in the presence of a fluorescent DNA binding dye. Subsequent mixing of the amplification products from the respective sputum cultures and the laboratory strain and thermocycling allowed the formation of DNA duplexes. The thermal denaturation properties of these DNA duplexes were determined by measuring the derivative of the intensity of fluorescence at different temperatures. Analysis of the 97 amplifiable well-characterised isolates showed a sensitivity and specificity of 94.4% and 98.4% respectively, compared to DNA sequencing, with a positive predictive value (PPV) of 97.1% and a negative predictive value (NPV) of 96.8% respectively.

We applied the method to a cohort of crude samples and found that EMB resistance was not associated with rifampicin mono-resistance, since only 4.3% of 47 isolates harboured mutations in the *embB*306 region. Initial screening of 86 isoniazid mono-resistant isolates revealed that 8.1% were resistant to ethambutol, however, subsequent sequencing of these ethambutol

resistant isolates showed the presence of mutations in the *rpoB* gene, implying additional resistance to rifampicin (i.e. MDR-TB).

Analysis of 228 MDR-TB isolates showed a strong association of EMB resistance with MDR-TB, since 57.5% of the isolates harboured mutations in the *embB*306 region, conferring EMB resistance. In contrast, only 9.2% were phenotypically resistant by routine culture on solid media.

The method proved rapid and reliable for the detection of EMB resistance and data could be used to help reformulate the South African Tuberculosis Program guidelines, thereby ensuring the appropriate management of patients and preventing the acquisition or spread of other drug-resistance phenotypes.

2. Introduction

Ethambutol (EMB) is a first-line anti-tuberculosis drug which targets arabinosyltransferase (encoded by the *embB* gene) involved in cell wall biosynthesis.[1] Inhibition of this enzyme by the drug results in the failure to synthesise the cell wall, an accumulation of mycolic acids in the bacteria and subsequent cell death.[2] Ethambutol forms an integral part of the Directly Observed Treatment Short-Course (DOTS) strategy and its use is continued in second-line therapy (the DOTS Plus strategy) due to the shortage of alternative second-line drugs and surveillance data (or lack thereof), which suggests that resistance to EMB is rare.[3] However, DST for EMB is inaccurate,[4] as the diagnostic breakpoint (5 to 7.5 µg/ml) is close to the Minimal Inhibitory Concentration (MIC) of EMB and true resistance may therefore be missed.[5]

Despite numerous publications arguing for[6-10] and against[2,11,12] mutations in codon 306 of the *embB* gene causing EMB resistance, a recent allelic exchange experiment in EMB susceptible and resistant strains showed that *embB* codon 306 mutations do increase the EMB MIC to cause

low level EMB resistance (MIC < 16 µg/ml).[5] These *embB* codon 306 mutations may interact with mutations in other genes to generate high-level EMB resistance (MIC = 16 to 40 µg/ml).[5]

A recent study done in Cape Town, South Africa, showed that routine phenotypic analysis by the National Health Laboratory Services (NHLS) failed to identify 91% of EMB resistant isolates using the indirect proportion method on Middlebrook solid medium compared to the liquid culture medium-based method, confirming the inaccuracy of EMB phenotypic testing.[4] This inaccuracy was further confirmed by genotypic sequencing of codon 306 of the *embB* gene which harbours 70-90% of the EMB resistance-causing mutations.[13]

The difficulties associated with phenotypic EMB susceptibility testing may have inadvertently had a negative effect on the TB control program. This hypothesis is further strengthened by evidence showing a strong association of EMB resistance with that of isoniazid and rifampicin resistance (multi-drug resistant (MDR-TB)).[4] Therefore failure to recognise EMB resistance may be implicated in the development of MDR-TB and extensively drug-resistant (XDR-TB) tuberculosis.[14] New draft policy guidelines (2008) on the treatment of MDR-TB (formulated by the South African National TB Control Programme) suggest that drug-susceptibility testing (DST) for EMB determines the treatment regimen for MDR/XDR-TB. Therefore it is essential that improved DST methods are implemented for the accurate detection of EMB resistance.

We describe an adaptation of the Fluorometric Assay for Susceptibility Testing of Rifampicin resistance (FAST-RIF)[15] to detect EMB resistance (FAST-EMB), based on the analysis of the thermal denaturation profiles of DNA duplexes spanning codon 306 of the *embB* gene of *Mycobacterium tuberculosis*.

3. Materials and Methods

3.1. Preparation of DNA templates

M. tuberculosis cultured from 98 sputum cultures was used to optimise the FAST-EMB method. The *embB* gene for each specimen had been previously sequenced,[4] demonstrating mutations at codon 306 in 37 specimens (EMB resistant) and wild type sequences in 61 specimens (EMB sensitive).

Crude DNA extracted from 47 rifampicin mono-resistant samples, 86 isoniazid mono-resistant samples and 228 MDR-TB patients was used to determine the incidence of EMB resistance amongst these sub-groups.

Briefly, each sputum specimen was cultured at 37℃ in BACTEC 12B medium (Becton Dickinson) for 7 days in the BACTEC 460 system, and the bacteria were pelleted by centrifugation, resuspended in 100 µl BACTEC 12B medium, and boiled to generate a crude-DNA template.[16]

3.2. Drug susceptibility testing

Drug susceptibility testing was done by the National Health Laboratory Service, using the indirect proportion method on Middlebrook medium containing critical concentrations of 5µg/ml EMB.

The phenotypes and genotypes of the respective isolates were determined prior to High Resolution Melting (HRM) analysis, and these results were blinded to the HRM analysis operator (K.H).

3.3. PCR amplification and HRM analysis of the *embB gene*

Crude DNA templates obtained from different sputum cultures and the laboratory strain (H37Rv, pan susceptible) were subjected to PCR amplification as previously described.[15] Briefly, each reaction mixture consisted of 2µl crude DNA template, 5 µl Q-Buffer, 2.5 µl 10X buffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM deoxynucleotide triphosphates, 1 µl of each primer (50 pmol/ µl) (*embB* 2F 5'- CAT GTC ATC GGC GCG AAT-3', *embB* R new 5'-GCG CCA GCA GGT TGT AAT ACC-3'), 1 µl (1/100 dilution) Syto 9 fluorescent dye (Molecular Probes), and 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) and was made up to 25 µl with distilled water. Amplification was initiated by incubation at 95℃ for 15 min, followed by 45 cycles at 94℃ for 45 s, 62℃ for 45 s, and 72℃ for 45 s. After the last cycle, the samples were incubated at 72℃ for 2 min.

Amplification products of the sputum cultures and the laboratory wild-type strain were mixed (1:1) and allowed to form DNA duplexes as previously described.[15] Resultant homo- and heteroduplexes were subjected to HRM analysis in the Rotorgene 6000 real-time analyser (Corbett, Australia). The thermal denaturation profiles were measured over the temperature range from 80℃ to 95℃, and fluorometric readings were taken every 0.1℃. The presence of absence of mutations within the amplified domain was assigned by the software according to the presence of a derivative peak(s) located within a defined temperature bin(s). A derivative plot with a single derivative peak (homoduplex) was classified as EMB susceptible, while a derivative plot with two derivative peaks (homo- and heteroduplexes) was classified as resistant to EMB.

3.4. Statistical analysis

The statistical software program Statistica 7.1 was used to calculate the sensitivity and specificity of the FAST-EMB method as compared to DNA sequencing of the *embB* gene region of interest at a confidence interval (CI) of 95%.

4. Results

The methodology was optimised on a cohort of 98 genetically well-characterised crude DNA samples. HRM analysis showed the presence of DNA homo- and/or heteroduplexes. EMB-susceptible isolates were identified by the presence of a single derivative peak (T_m = 86.2°C), representing homoduplexes (Figure 1A), while EMB resistant isolates were identified by the presence of two distinct derivative peaks at defined temperatures (T_m = 86.2°C and 84.4°C), representing homo- and heteroduplexes, respectively (Figure 1B). The FAST-EMB method could correctly identify all 34 of 36 (94.4%) amplifiable isolates harbouring one of the three most common *embB* codon 306 mutations (GTG, ATC or ATA) and 60 of 61 (98.4%) wildtype (ATG) isolates. The discrepant results were re-submitted for DNA sequencing to confirm their *embB*306 sequences. The two samples which were incorrectly scored by HRM analysis as EMB susceptible did in fact harbour *embB* codon 306 (GTG) mutations. The sample incorrectly scored as resistant could not be confirmed by re-sequencing as the working sample was depleted and subsequent efforts at re-culturing were unsuccessful due to a loss of viability. One isolate also repeatedly failed to amplify (using various primer sets) following re-culture; this could be due to the presence of inhibitors. The method showed a sensitivity and specificity of 94.4% and 98.4% respectively as compared to DNA sequencing, with a positive predictive value (PPV) of 97.1% and a negative predictive value (NPV) of 96.8% respectively (excluding the amplification failure).

High resolution melting analysis of 47 rifampicin mono-resistant and 86 isoniazid mono-resistant isolates (DST according to the NHLS) showed an incidence of concurrent EMB resistance of 4.3% and 8.14%, respectively. The 7 isoniazid mono-resistant isolates which showed mutations in the *embB* gene underwent sequencing of the *rpoB* gene and were found to harbour mutations conferring resistance to rifampicin, confirming the association with MDR-TB.

High resolution melting analysis of 228 crude DNA extracts from MDR-TB cultures from the Western Cape showed that 131 (57.5%) harboured mutations in the *embB* gene region analysed, suggesting resistance to EMB. In contrast, only 9.2% (13/141 tested by routine DST) were

phenotypically resistant by routine culture on solid media. Sequencing of a random selection of 20 of these isolates showed correlation in 19/20 samples. The single discrepant result was due to a rare mutation in codon 328 of the *embB* gene which fell outside of the region targeted by this assay. These results confirm that EMB resistance is strongly associated with MDR-TB.

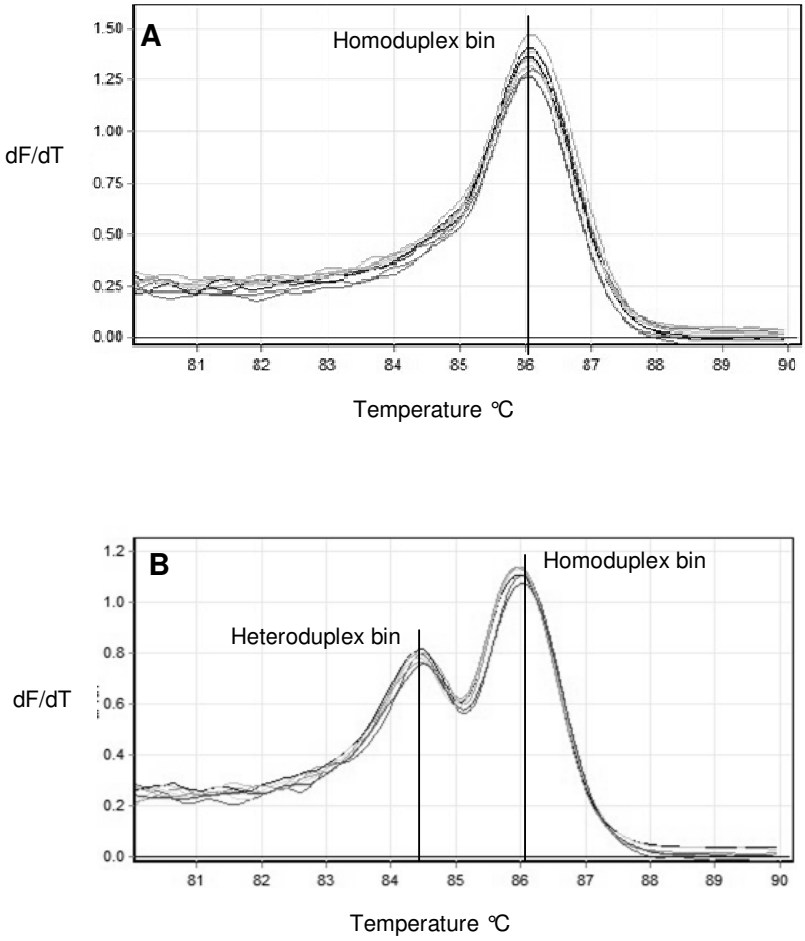

Figure 1. dF/dT values of the DNA duplexes. (A) Isolates were scored as susceptible to EMB based on the presence of a single derivative peak within a defined temperature bin (homoduplex). (B) Isolates were scored as resistant to EMB based on the presence of two derivative peaks in defined temperature bins (homo- and heteroduplex).

Discussion

Despite evidence for the association of *embB* mutations and ethambutol resistance,[5] the clinical significance of these mutations remains controversial.[2,11,12] A recent publication documenting the evolution of drug resistance showed positive selection for EMB resistance.[17] These isolates harboured a mutation in the *embB* gene at codon 306. Together this suggests that the concentration of EMB used in the current treatment regimen is sufficient to select for low level EMB resistant strains and that this treatment with the standard dose would be of little benefit in patients with strains harbouring the *embB*306 mutations. Thus it is essential that genetic based DST tools are developed to rapidly classify isolates of *M. tuberculosis* as resistant or susceptible to EMB.

We describe the successful adaptation of the FAST-Rif method[15] to allow for the reliable detection of EMB resistance by changing only the primers used to amplify the target region in the *embB* gene. The method was highly sensitive and specific (94.4% and 98.4%, respectively), compared to DNA sequencing and also detected resistance missed by routine DST.

Analysis of 47 rifampicin mono-resistant isolates revealed a weak association with EMB resistance. However, 8.1% of the 86 isoniazid mono-resistant isolates harboured mutations in the *embB* gene. This was of particular concern as these patients would not be considered as MDR-TB cases and would therefore receive an incorrect (first-line) treatment regimen in which only 2 effective drugs are prescribed (rifampicin and pyrazinamide). However, subsequent sequencing of the *rpoB* gene in these samples revealed mutations in this gene which confer resistance to rifampicin. These isolates were therefore incorrectly classified as isoniazid mono-resistant by phenotypic drug-susceptibility testing, implying that these MDR-TB patients are unintentionally being treated during first-line therapy with only one effective drug (pyrazinamide) which is not used in the continuation phase of treatment. This may therefore lead to the more rapid evolution of pre-XDR and XDR-TB in these patients.

Analysis of 228 MDR-TB isolates collected in early 2008 indicated that 57% of the isolates were in fact resistant to EMB, thereby confirming the strong association of EMB resistance in MDR-TB.[4] Most importantly, routine phenotypic DST failed to identify EMB resistance in 85.4% of these MDR-TB isolates. This finding is similar to the previously reported 90% resistance missed in the same setting.[4]

The strong association of EMB resistance with MDR-TB and the inaccuracy of routine phenotypic DST have potentially serious implications for the South African Tuberculosis Program's treatment guidelines. We believe that our data should be used to help reformulate current draft guidelines and ensure the appropriate management of patients. This is especially important as the current draft policy guidelines (South African National TB Control Programme) suggest that DST for EMB determines the treatment regimen for MDR/XDR-TB. If phenotypically susceptible, the guidelines suggest that EMB should be included in the regimen as the 5th drug. If resistant, EMB should be replaced with terizidone or cycloserine as the 5th drug. We propose that in the absence of genotypic-based DST for EMB, it should be assumed that all MDR-TB cases are resistant to EMB and that these patients should be treated with terizidone or cycloserine as well as receive 4 other effective anti-tuberculosis drugs. This is in line with the WHO guidelines[18] which state that EMB may be included in the regimen, but not counted.

The FAST-EMB shares similar advantages to that of the FAST-Rif method, as it is a simple, cost effective method that allows for the rapid identification of EMB resistance (3 to 4 h following short-term culture). The method was not able to provide information on which nsSNP conferred resistance and failed to detect resistance due to mutations occurring in regions outside of the *embB* codons analysed. However, the method was a considerable improvement over phenotypic-based DST. Unfortunately the methodology is based on two independent PCRs and required the opening of tubes to generate DNA duplexes, thus there is a potential for cross-contamination. It would therefore be useful to convert the method to a single-tube format.

Given the affordability and superior accuracy of the described FAST-EMB method, it would be useful in the routine diagnosis of EMB drug-resistance and should be considered for

implementation into the TB control program so as to ensure the appropriate management of the TB case and to preventing the acquisition or spread of further drug-resistance phenotypes. Furthermore, we recommend that the FAST-EMB be used on all isolates with rifampicin resistant results (a marker of MDR-TB) by the GenoType® MTBDR*plus* assay (HAIN Lifescience, GmbH, Nehren, Germany) due to the high rates of undetected EMB resistance amongst MDR-TB isolates.

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Chapter 6

Single-tube Detection of Drug-resistance in *Mycobacterium tuberculosis* – Detection of *inhA* Promoter Mutations conferring Isoniazid Resistance

1. Abstract

Diagnostic delay is an important contributing factor to the increase in multi-drug resistant tuberculosis (MDR-TB) cases. Genotypic-based assays may drastically reduce turn-around times; however they are hampered by a high financial cost and the potential for cross-contamination (among the open-tube based assays). We describe a single, closed-tube fluorometric method for the detection of *inhA* promoter mutations and associated isoniazid and ethionamide resistance.

Thermal denaturation profiles of homo- and heteroduplexes formed between DNA from a reference sequence (wild type) and a clinical sample were analysed by high-resolution melting analysis. Briefly, the clinical isolates were PCR amplified and the resultant amplicons then annealed to a modified complementary single stranded DNA oligonucleotide allowing extension of the amplicon and the incorporation of the adaptor sequence. Primers specific to the adaptor sequence and the original 3' sequence ensured a final PCR product that reflected amplification of both the wild type sequence and the sequence present in the clinical isolate.

The method was able to identify *inhA* promoter mutations from crude DNA obtained from the sputum cultures of 77 patients with INH resistant TB. The sensitivity and specificity was 100% and 83.3%, respectively compared to DNA sequencing analysis. The positive predictive value (PPV) was 87.2% and the negative predictive value (NPV), 100%. The specificity of the assay was improved to 93.5% by visual analysis of the derivative plots by experienced and inexperienced operators. Sensitivity remained high (98.4%) and the pooled PPV and NPV was 94.7% and 98.0%, respectively. Visual scoring was more specific than automatic scoring due to limitations with the Rotorgene software.

Rapid identification of *inhA* promoter mutations would help to guide treatment regarding the inclusion of ethionamide and the use of high dose isoniazid therapy.

2. Introduction

The global incidence of multi-drug resistant tuberculosis (MDR-TB) cases is steadily increasing[1,2] despite the implementation of Directly Observed Treatment Short-course (DOTS) therapy in most countries. This increase is largely attributed to poor management of the DOTS programme and the inability to rapidly diagnose drug resistance. Currently, drug-susceptibility testing is largely based on culture which is hampered by lengthy turn-around times, expensive equipment, high reagent costs and the need for skilled staff and extensive biosafety facilities. It is therefore essential that improved diagnostics are developed which are both affordable and rapid.

Recent advances in molecular line-probe assays has led the World Health Organisation (WHO) to release a policy statement[3] in which they recommend that these assays (more specifically the GenoType® MTBDR*plus* assay (HAIN Lifescience, Germany)) be implemented directly on all smear-positive sputum samples to enable rapid (<2 days) detection of MDR-TB.[3,4] This assay targets the most common mutations in the *rpoB* gene (responsible for rifampicin (RIF) resistance) and the *katG* and *inhA* promoter genes (responsible for resistance to isoniazid (INH)).[4] A recent meta-analysis demonstrated that the application of this test reduces diagnostic delay to less than 48 hours,[4] with high sensitivity and specificity to identify RIF resistance which is an important marker of MDR- and extensively drug-resistant (XDR)-TB. Specificity was excellent for INH as well, although sensitivity estimates were modest and variable, since several genes are involved in conferring INH resistance and probes for these genes are not included in the current test. This may lead to false-negative results in cases where resistance is due to mutations elsewhere in the genome, or where they are situated outside the probe area of the assay. More importantly, the GenoType® MTBDR*plus* assay is an open-tube assay which allows for the potential release of amplicons, thereby leading to a possible increased rate of false-positive results and subsequent inappropriate treatment. This is a particular concern in high-throughput laboratories and this requires specialised infrastructure with highly skilled staff.

Current efforts in the development of a closed-tube assay include the automated real-time based molecular beacon Xpert MTB/RIF Assay[5] (Cepheid Inc., Sunnyvale, California, USA). This method is capable of detecting multiple targets in the genome in less than 24 hours; however, it is hampered by the high cost of the analysis equipment.

We recently reported a fluorescence-based assay, the Fluorometric Assay for Susceptibility Testing to detect RIF (FAST-Rif)[6] and ethambutol (FAST-EMB) resistance which rely on the analysis of the thermal denaturation profiles of amplification products of specific DNA fragments/targets.[6] In this assay the thermal denaturation profile was dependent on the nucleotide sequence of the DNA fragment,[7] therefore any changes in the nucleotide sequence would alter this profile, which, in turn, could be detected by measuring the efficiency of binding of a fluorescent dye to the DNA fragment at different temperatures.[8] To enable the efficient detection of A:T and G:C nucleotide transversions, the FAST-Rif and FAST-EMB techniques relied on the generation of DNA homo- and heteroduplexes (formed between amplification products generated from a wild type reference strain and the clinical sample). However, this step required 2 independent PCR reactions with post-amplification mixing of the PCR products. This open-tube system, as is the case with the GenoType® MTBDR*plus* assay, could lead to potential cross-contamination. Furthermore, the amplification of the reference strain in the absence of amplification of the clinical isolate may lead to the overrepresentation of false-susceptible results.

A possible solution to these limitations would be to seed the PCR reaction with the wild type reference strain prior to amplification.[9] Homo- and heteroduplexes would then be generated as amplification proceeds. This approach has recently been followed using purified DNA from clinical samples,[9] however, this purification step adds cost to the assay and increases diagnostic delay. Furthermore, the seeding of PCR reactions does not overcome the possibility of false-susceptible results in cases where a clinical sample may fail to amplify due to insufficient DNA.

In this study we developed a single, closed-tube fluorometric method which aimed to overcome the need for post-amplification processing, thereby preventing the release of amplicons. Figure 1 illustrates the method of simultaneous amplification of both the wild type and clinical DNA target

templates. We present a proof of concept for this FAST easy single-tube method using the *inhA* gene promoter target as a model and have termed the method FASTest-inhA. The *inhA* gene encodes the enoyl-acyl carrier protein reductase which is responsible for low-level resistance to INH, however the resistance-causing mutations are more frequently located in the upstream promoter region.[10] Although INH resistance may be due to mutations in a number of genes, over 80% of these are present in either the *katG* gene or the *inhA* gene promoter region.[11]

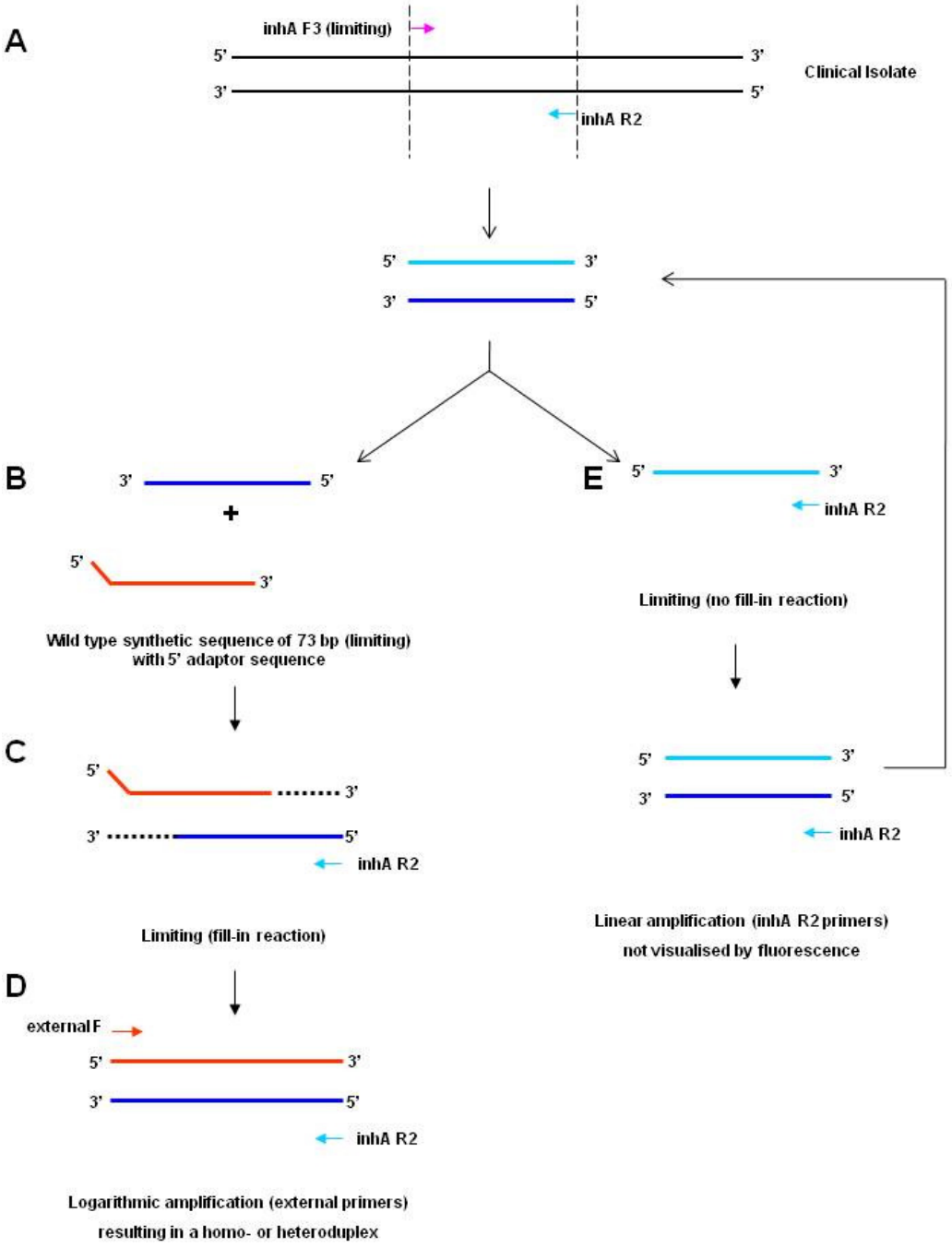

Figure 1. Proposed FASTest-inhA method. **(A)** During the initial amplification step, primers complementary to a specific target sequence in the genome of the clinical isolate are used for amplification (primers inhA F3 and inhA R2, see Table 1). **(B)** The 3' to 5' amplification product can now anneal to the complementary single stranded wild type DNA oligonucleotide (with a unique 5'-adaptor sequence) allowing **(C)** extension of the amplicon and the incorporation of the adaptor sequence. **(D)** The extended amplicons are amplified with primers complementary to the adaptor sequence (primer external F, see Table 1) as well as primers complementary to

the original 3' sequence (primer inhA R2, see Table 1). The resulting PCR products will reflect amplification of both the wild type sequence and the sequence present in the clinical isolate. These products have the potential to form either homo- or heteroduplexes depending on the sequence of the DNA in the clinical isolate. **(E)** The 5' to 3' amplification product cannot anneal to the single stranded wild type DNA oligonucleotide and will only undergo linear amplification which should be undetectable by high resolution melt (HRM) analysis.

3. Materials and Methods

3.1. Preparation of pure DNA templates

Mycobacterium tuberculosis from 11 sputum samples (obtained from patients with MDR-TB and XDR-TB) was cultured on Löwenstein-Jensen medium and genomic DNA was extracted as previously described.[12] This purified DNA was used to optimise the FASTest-inhA method.

3.2. Preparation of crude DNA templates

Decontaminated sputum specimens from 77 randomly selected patients with INH-mono resistant, MDR-TB and XDR-TB were cultured at 37℃ in BACTEC 12B medium (Becton Dickinson) for 7 days in the BACTEC 460 system, and the bacteria were pelleted by centrifugation, resuspended in 100 µl BACTEC 12B medium, and boiled to generate a crude-DNA template as previously described.[13] The growth index of the cultures was not measured.

3.3. Drug-susceptibility testing

Drug-susceptibility testing was done by the National Health Laboratory Service (NHLS) as previously described using the indirect proportion method on Middlebrook medium containing

critical concentrations of 0.2 µg/ml isoniazid (INH). All isolates analysed were resistant to INH (mutations either in the *inhA* gene promoter or the *katG* gene (used as negative controls)).

3.4. DNA sequencing of the *inhA* gene promoter region

The DNA sequence of the *inhA* gene promoter region of each sputum culture was determined by PCR amplification in a reaction mixture containing 2 µl crude DNA template, 5 µl Q-Buffer, 2.5 µl 10X buffer, 2 µl 25 mM $MgCl_2$, 4 µl 10 mM deoxynucleotide triphosphates, 1 µl of each primer (50 pmol/µl) (inhA P5 and inhA P3, Table 1) and 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) and made up to 25 µl with distilled water. Amplification was initiated by incubation at 95℃ for 15 min, followed by 45 cycles each at 94℃ for 45 s, 60℃ for 45 s, and 72℃ for 45 s. Samples were then incubated at 72℃ for 10 min to ensure complete product extension and were sent for subsequent sequencing analysis. The genotypes remained blinded to the high resolution melt (HRM) operator. The 11 pure DNA templates consisted of 8 samples with mutations at positions either -15 or -17 of the *inhA* promoter region and 3 wild type specimens (INH resistance due to mutations in the *katG* gene). The crude dataset consisted of 41 samples harbouring mutations at positions -8, -15 and -17 of the *inhA* gene promoter region and 36 specimens having a wild type sequence (INH resistance due to mutations in the *katG* gene).

3.5. Asymmetric PCR amplification of the *inhA* gene promoter region

DNA templates (pure or crude) extracted from different sputum cultures were subjected to PCR amplification in a reaction mixture containing 50 ng purified or 2 µl crude DNA template, 5 µl Q-Buffer, 2.5 µl 10X buffer, 3 µl 25 mM $MgCl_2$, 4 µl 10 mM deoxynucleotide triphosphates, 1 µl of each primer (external F, inhA F3 and inhA R2), 1 µl of the synthesised wild type sequence, 1 µl (1/100 dilution) Syto 9 fluorescent dye (Molecular Probes), and 0.125 µl HotStarTaq DNA polymerase (Qiagen, Germany) and made up to 25 µl with distilled water. The working

concentrations used and the sequences of the oligonucleotide primers and the synthesised wild type oligonucleotide are shown in Table 1.

Amplification was initiated by incubation at 95℃ for 15 min, followed by 2 cycles each at 94℃ for 45 s, 67℃ - 64℃ (decreasing by 1℃ every 2 cycles) for 45 s, and 72℃ for 45 s. After the 8 touchdown cycles, the samples underwent a further 35 cycles at 94℃ for 45 s, 62℃ for 45 s, and 72℃ for 45 s. Samples were then incubated at 72℃ for 10 min to ensure complete product extension. Homo- and heteroduplexes were formed during amplification, which was subsequently confirmed by HRM analysis (see below).

To minimise laboratory cross-contamination, the preparation of the PCR mixes, the addition of the DNA, and the PCR amplification were conducted in physically separated rooms. Negative controls (water) were included to detect reagent contamination.

Table 1. Oligonucleotide sequences and concentrations used

Primer Name	**pmol/µl**	**5' to 3' Sequence**
wild type	0.5	5'-ACATCCTACACACGCTCTCATGTCTCGTGGACATACC GATTTCGGCCCGGCCGCGGCGAGACGATAGGTTGTC-3'
inhA F3	7.5	5'-CTCGTGGACATACCGATTTCG-3'
inhA R2	50	5'-CTGAACGGGATACGAATGGG-3'
external F	50	5'-ACATCCTACACACGCTCTCATGTC-3'
inhA P5 *	50	5'-CGCAGCCAGGGCCTCGCTG-3'
inhA P3 *	50	5'-CTCCGGTAACCAGGACTGA-3'

*primers used for DNA sequencing

3.6. High-resolution melting analysis

The DNA duplexes were subjected to HRM analysis in a Rotorgene 6000 real-time analyser (Qiagen, Germany). The thermal denaturation profiles were measured over the temperature range of 80°C to 90°C, and fluorometric readings were taken every 0.1°C (ramp time was 2 seconds per 0.1°C). The Rotorgene software was used to calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT), thereby generating a plot where the derivative peak(s) represents the T_m value of the DNA duplexes. Clinical samples were classified as either wild type or mutant *inhA* gene promoter sequences by the software according to the presence of a derivative peak(s) located within a defined temperature bin(s). A derivative plot with a single derivative peak at a T_m of 86.64°C (homoduplex, bin width 1°C) was classified as wild type for the *inhA* gene promoter region, while a derivative plot with two derivative peaks at T_m's of 86.64°C and 85.5°C (homo- and heteroduplexes, bin width 1°C and 0.5°C respectively) was classified as harbouring a mutation in this region.

The HRM derivative plots of the 77 consecutive samples of the dataset were also scored visually by 3 blinded operators (one experienced and two inexperienced).

3.7. Statistical analysis

The statistical software program Statistica 7.1 was used to calculate the sensitivity and specificity of PCR amplification for detecting the presence of *inhA* gene promoter mutations at a confidence interval (CI) of 95% compared to DNA sequencing analysis.

4. Results

The methodology was optimised on a cohort of 11 genetically well-characterised purified DNA samples obtained from sputum cultures from patients with MDR-TB and XDR-TB. In all instances the HRM analysis was able to differentiate between homo- and heteroduplexes (Figure 2). Mutations at position -15 and -17 of the *inhA* gene promoter region were correctly identified in 100% (n=8) of the isolates which harboured an *inhA* promoter mutation. All the wild type specimens were correctly identified by HRM analysis.

To determine whether the single-tube HRM method could be used to analyse crude DNA templates, decontaminated sputum specimens from 77 patients with INH-mono resistant TB, MDR-TB or XDR-TB were analysed. Rotorgene software correctly identified 100% (n=41) of the isolates containing an *inhA* promoter mutation. However, this software incorrectly scored 6 of the 36 wild type samples as harbouring a mutation leading to an overall specificity of 83.3% (95% CI, 70% to 96.7%) compared to DNA sequencing analysis. The sensitivity of the assay was 100% (95% CI, 100% to 100%), with a positive predictive value (PPV) of 87.2% and a negative predictive value (NPV) of 100%. The performance of the automatic scoring could not be improved further due to software limitations. Figure 3 shows the typical derivative plots generated, including correctly scored mutation harbouring isolates; and correctly and incorrectly scored wild type isolates. Isolates harbouring a mutation in the *inhA* gene promoter showed a homoduplex peak with a lower T_m than wild type isolates which had a slightly higher T_m.

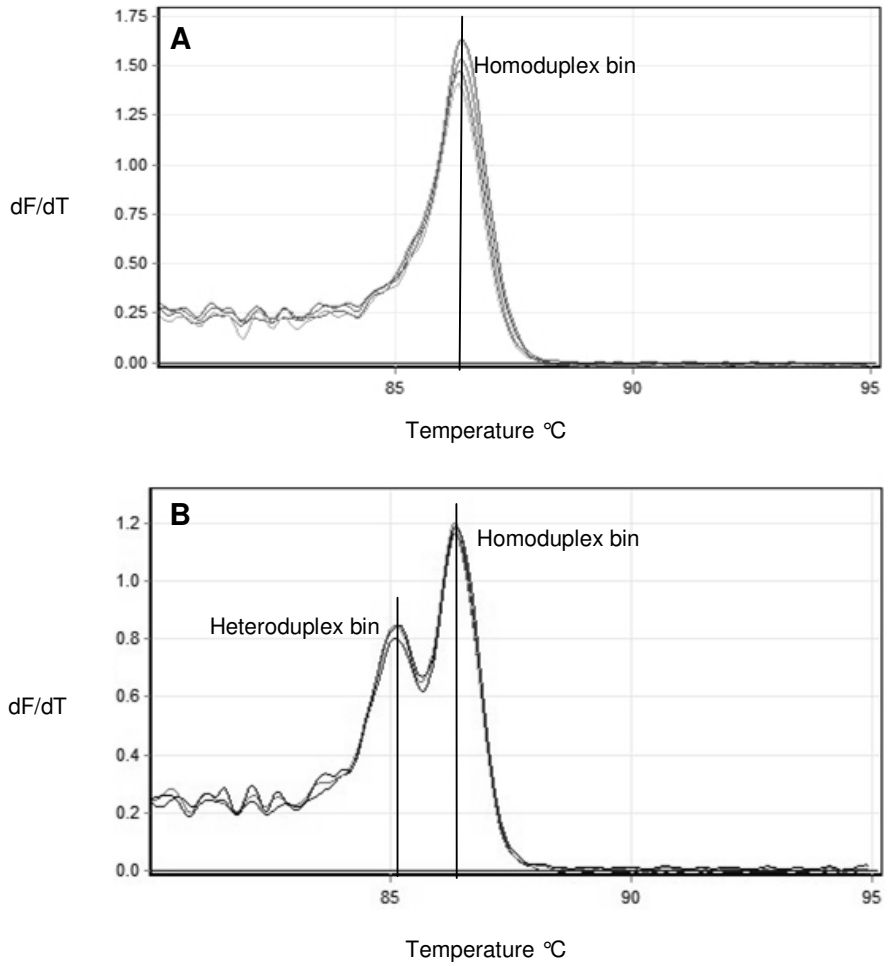

Figure 2. dF/dT values of the DNA duplexes from purified DNA. **(A)** Isolates were scored as wild type for *inhA* gene promoter mutations based on the presence of a single derivative peak within a defined temperature bin (homoduplex, width 1°C). **(B)** Isolates were scored as harbouring a mutation in the *inhA* promoter region based on the presence of two derivative peaks in defined temperature bins (homo- and heteroduplex, width 1°C and 0.5°C respectively).

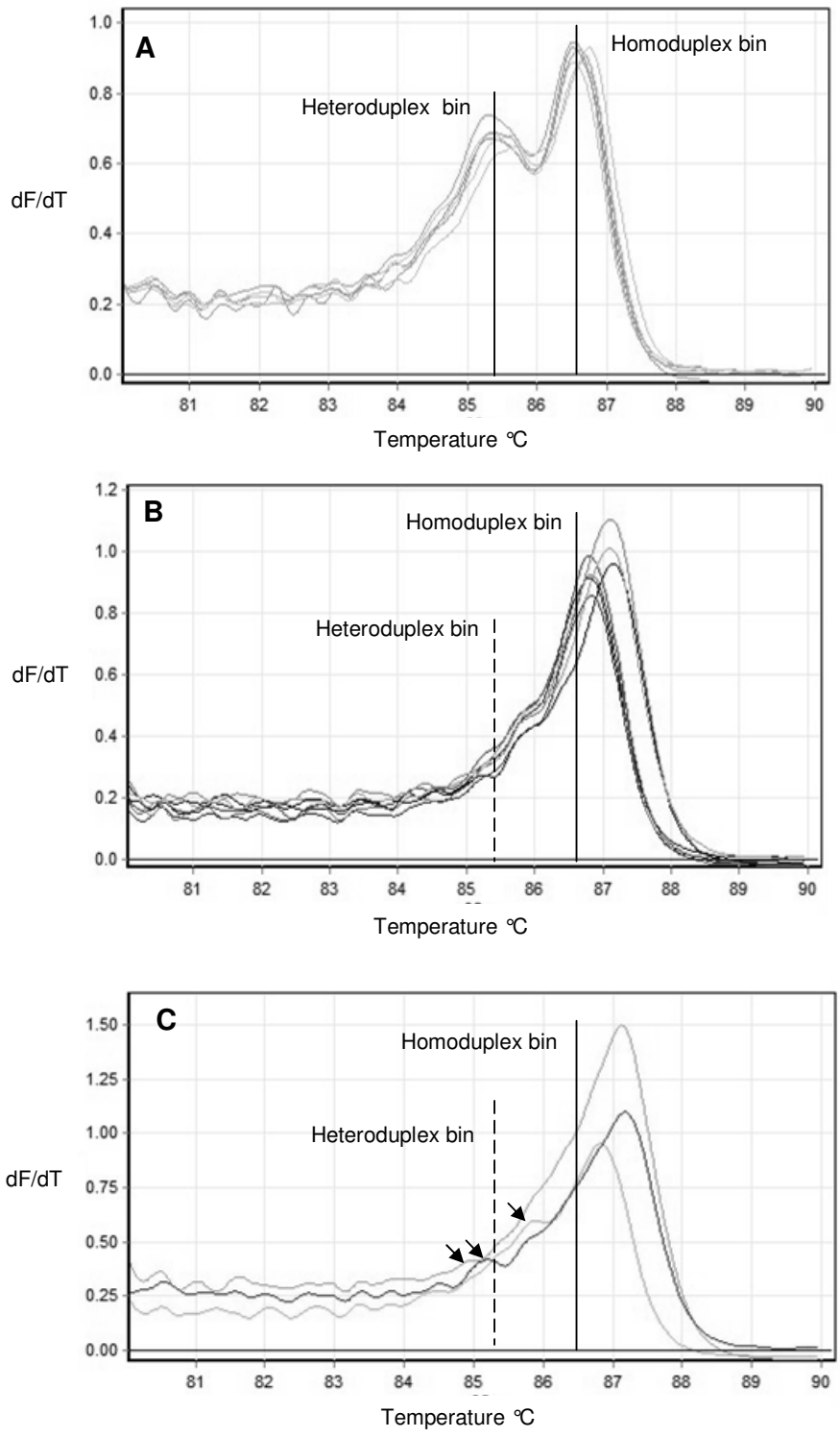

Figure 3. dF/dT values of the DNA duplexes from crude DNA. **(A)** Isolates were correctly scored as harbouring a mutation in the *inhA* gene promoter region based on the presence of two derivative

peaks in defined temperature bins (homo- and heteroduplex, width 1°C and 0.5°C respectively). **(B)** Derivative peaks shifted to the right, but were correctly scored as wild type using the same defined temperature bins (homoduplex). **(C)** Isolates were incorrectly scored as harbouring a mutation due to the presence of a small peak in the defined heteroduplex temperature bin (arrows).

To determine whether the scoring performance could be improved, 77 HRM derivative plots (including the 6 scored incorrectly by the software) were visually scored by one experienced and two inexperienced operators (following a short 5 min training session). The experienced and inexperienced operators showed a pooled sensitivity and specificity of 98.4 % (range: 97.6% to 100%) and 93.5% (range: 86.1% to 100%) for detecting mutations in the *inhA* promoter gene compared to DNA sequencing. The average PPV was 94.7% and the NPV was 98.0%, respectively. The inter-reader agreement was high, with a Fleisch Kappa score of 0.88 (95% CI: 0.79 to 0.95). Visual scoring was thus more specific than automatic scoring by the Rotorgene software.

5. Discussion

We report the successful development of a single closed-tube fluorometric assay for the detection of mutations (and associated drug-resistance) using purified or crude *M. tuberculosis* DNA templates. This FASTest-inhA method was able to identify mutations occurring at positions -8, -15 and -17 of the *inhA* gene promoter. However, the current Rotorgene software was unable to distinguish between background noise and signal in a small proportion (7.8%) of the wild type sequence samples.

This could be directly ascribed to the presence of impurities in the crude DNA templates (a known limitation of HRM methodologies)[14] which resulted in derivative curves which showed

inter-sample variation according to peak height and peak T_m. Adding a DNA purification step would largely resolve the above limitations, however, this additional step would increase the cost of the assay and contribute to diagnostic delay. A possible solution would be to introduce synthesised oligonucleotide sequences in each PCR reaction to act as calibrators allowing the normalisation of derivative curves.[15] Custom software can then be developed to align the calibrators of each sample in an analysis run, thereby aligning the peaks and minimising T_m variation.[15] The resultant homo- and heteroduplex bins can then be narrowed so as to avoid the scoring of aberrant peaks or overlapping samples. Introducing additional oligonucleotides into a PCR reaction may however reduce amplification efficiency;[15] therefore it would be more advantageous to find an alternative solution. We propose that the manufacturers design an additional software application which will normalise all the derivative plots of a run according the homoduplex peak, to a pre-defined T_m. Aligning this homoduplex peak would allow the heteroduplex peak to align in samples harbouring a mutation, while keeping wild type samples with the smaller false peak aligned at a lower peak height and different T_m. Once again, narrowing analysis bins should be able to differentiate the samples more accurately (see Figure 4).

The FASTest-inhA assay was a significant improvement over the FAST-Rif[6] and FAST-EMB methods in that we included an amplification control, which ensured that amplification failures could be identified (only clinical samples containing *M. tuberculosis* DNA will be amplified). Furthermore, the closed tube system reduced the time to results (<2hours following short term culture) and the risk of amplicon release. However, in its present format, the assay may not be as advantageous as the GenoType® MTBDR*plus* assay which provides a more powerful readout, including speciation and multiple drug-resistance targets. However, the FASTest-inhA method does overcome inter-reader variation and lays the foundation for a considerably more affordable and safer closed-tube assay, which could be adapted to multiple targets. The assay also has the potential for large scale automation, which should be particularly attractive in low-income, high TB incidence settings.

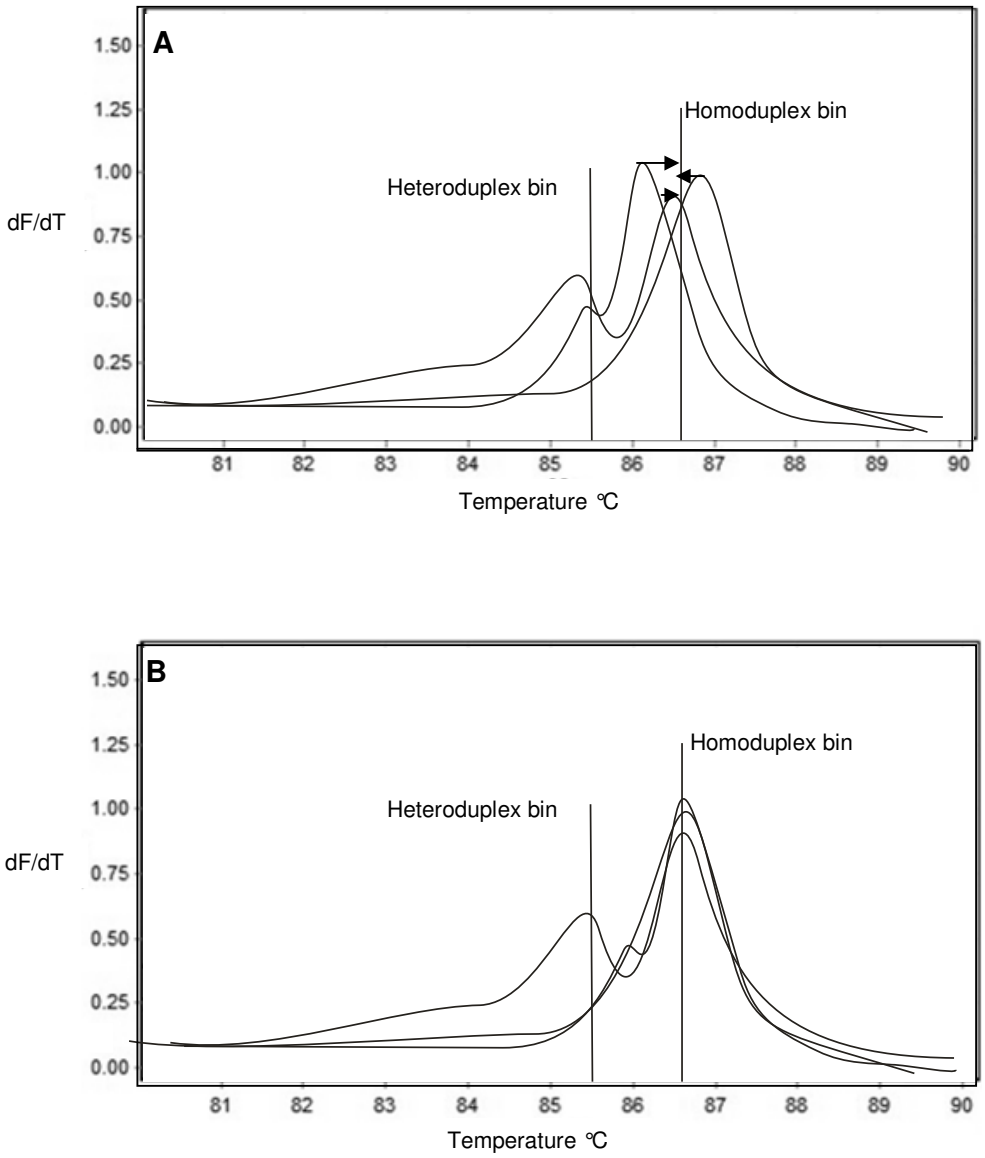

Figure 4. Illustration of proposed normalisation of thermal denaturation peaks. **(A)** Typical output of 3 isolates (1 with a mutation and 2 without). The peaks are not aligned. **(B)** Expected output following normalisation to a pre-defined T_m for the homoduplex peak (in this case 86.7℃). The wild-type isolate with the small first peak will now be scored correctly as the bins can be narrowed to exclude background noise.

In addition, we acknowledge that our method was not able to identify INH resistance which was due to mutations in genes other than the *inhA* promoter region, however, *inhA* promoter mutations are of particular interest as these confer low level INH resistance, therefore cases which harbour these mutations may benefit from the use of high/standard dose INH therapy.[16] Recent data from the Western Cape Province of South Africa showed that 53.6% of INH mono-resistant clinical samples and 38.2% of MDR-TB samples harboured mutations in the *inhA* gene or promoter region.[17] These mutations were also strongly associated (70%) with XDR-TB in a cohort of 85 XDR-TB patients.[16] This implies that these patients have low level INH resistance and may benefit from high dose INH therapy.[16] This is of particular clinical importance in patients resistant to most or all of the available antibiotics. Additionally, *inhA* promoter mutations have been implicated in cross-resistance to the second-line anti-tuberculosis drug ethionamide, which forms part of the treatment regimen for MDR-TB.[16] Thus genotypic drug-susceptibility testing (including the GenoType® MTBDR*plus* assay) for INH resistance through mutations in the *inhA* gene may be extremely beneficial in tailoring MDR-TB and XDR-TB treatment regimens.[16]

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Chapter 7

Resistance to pyrazinamide and ethambutol compromises MDR/XDR-TB treatment

K. G. P. Hoek,[1] H. S. Schaaf,[2] N. C. Gey van Pittius,[1] P. D. van Helden,[1] and R. M. Warren[1]

[1] *DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences,* [2] *Department of Paediatrics and Child Health, Faculty of Health Sciences, Stellenbosch University, South Africa*

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1. Abstract

The increase in multidrug-resistant tuberculosis (MDR-TB), defined as *Mycobacterium tuberculosis* resistant *in vitro* to at least isoniazid (INH) and rifampicin (RIF), is a global concern. It is estimated that 511 000 MDR-TB cases occur globally each year. The World Health Organisation (WHO) consequently released an emergency update on their management guidelines, recommending that treatment of MDR-TB should include at least 4 effective drugs, and that standardised treatment regimens should be based on resistance patterns for each country/region. Most importantly, treatment regimens should not depend on the results of drug susceptibility testing (DST) for ethambutol (EMB) or pyrazinamide (PZA). In response, the South African Department of Health prepared a draft drug-resistant TB treatment policy in which PZA remains one of the 4 effective drugs, while EMB should be replaced with terizidone or cycloserine, if there is resistance to EMB (disregarding inaccurate DST). In South Africa, there is a high frequency of undetected EMB and PZA resistance and their association with MDR-TB. Therefore, we recommend that the WHO guidelines in which 4 other effective drugs are used to treat MDR-TB, be followed more closely. EMB and PZA can be included if they are not counted as one of the 4 effective drugs. However, this does not address the root cause of the amplification of resistance in undiagnosed MDR-TB patients in South Africa, which can only be achieved by the implementation of rapid DST methods in all TB cases before initiating therapy. This protocol would curb the amplification of resistance and the evolution of XDR-TB.

2. Forum

The number of MDR-TB cases has steadily increased despite the widespread implementation of the directly observed treatment short-course (DOTS) and DOTS-plus strategies.[1,2] A survey in South Africa estimates that 1.8% of new TB cases and 6.7% of retreatment TB cases are MDR-TB,[1] equating to approximately 14 000 MDR-TB cases each year.[1] The manner in which the WHO DOTS strategy has been implemented in South Africa to control TB might have inadvertently lead to the amplification of resistance in MDR-TB cases.[3-5] Consequently, it is important to review the epidemiology of drug resistance in the country and make informed evidence-based suggestions on improving the current treatment strategy.

The WHO DOTS-plus treatment guidelines for treating MDR-TB focus on the use of various bactericidal or bacteriostatic anti-tuberculosis drugs with a proven efficacy against *M. tuberculosis*. They are classed as either first-line (normally used to treat new and drug-susceptible TB cases) or second-line (normally used to treat MDR-TB or extensively drug-resistant TB (XDR-TB)). EMB and PZA, two first-line drugs, are often used in combination with various second-line drugs to treat MDR/XDR-TB. Their inclusion in the latter treatment regimen was based on the absence of alternative second-line drugs and surveillance data (or lack thereof), which suggests that resistance to EMB and PZA is rare.[6] However, DST for both EMB and PZA is inaccurate.[7,8] The National Health Laboratory Service (NHLS) in Cape Town missed 90% of EMB resistance using the indirect proportion method on Middlebrook solid medium compared with a liquid culture medium.[7] Of the EMB-resistant isolates, 87% were also resistant to INH and RIF. These results were confirmed by DNA sequencing which identified mutations in the *embB* gene which conferred resistance to EMB in all the above isolates.[7,9] In 2008, we showed in a cohort of 228 MDR-TB isolates from the Western Cape that 131 (57.5%) harboured mutations in the *embB* gene, suggesting that they were resistant to EMB (see Chapter 5). Only 9.4% were phenotypically resistant by routine culture on solid media. In a drug resistance surveillance study in children in the Western Cape from March 2007 through February 2009, 12

out of 24 (50%) with confirmed MDR-TB were phenotypically resistant to EMB, confirming the high rate of EMB resistance in adult MDR-TB cases (HSS, personal communication).

DST for PZA is not routinely performed in South Africa owing to the complexity of the culture conditions (low pH medium is required, which negatively affects the growth and viability of *M. tuberculosis*).[10] To address the largely unknown frequency of resistance to PZA, a recent study (using the non-radiometric BACTEC mycobacterial growth indicator tube (MGIT) 960 method) showed that 53.5% of drug-resistant isolates (various resistance patterns) from the Western Cape were resistant to PZA. This finding was confirmed by DNA sequencing of the *pncA* gene, which encodes for the mechanism of resistance.[8,11] This study importantly showed a highly significant association between MDR-TB and PZA resistance ($p<0.001$)[8] that was confirmed in isolates collected as part of a national drug-resistance survey where 52.1% of MDR-TB isolates showed additional resistance to PZA.[12]

The association between EMB and PZA resistance and MDR-TB in South Africa (a setting where MDR-TB is primarily transmitted)[13] stems from the manner in which the DOTS strategy has been implemented since 1996. New TB cases are routinely diagnosed by sputum smear microscopy or culture without DST. In the absence of routine DST, it is assumed that all new TB cases are drug-susceptible until treatment failure or relapse occurs. Therefore, according to the current 2004 South African treatment guidelines (South African National Tuberculosis Control Programme Practical Guidelines 2004), a new TB patient with undiagnosed MDR-TB will be treated with 4 first-line drugs (INH, RIF, PZA and EMB) during the intensive phase of therapy (the first 2 months), which implies that the treatment regimen will contain only 2 effective drugs: EMB and PZA. Since PZA is a poor companion drug to prevent the acquisition of resistance, it is highly probable that resistance to EMB and/or PZA will follow, as is evident by the above data. If patients fail to show sputum conversion after 5 months of treatment, they would be regarded as a treatment failure and be shifted to the category II regimen (i.e. South African current retreatment guidelines) with the addition of streptomycin (SM) in the first 2 months of retreatment. DST will then be requested (which may take months),[14] during which time resistance to any remaining effective drugs may develop (i.e. EMB, PZA and/or SM). Consequently, the MDR-TB epidemic

will become largely associated with EMB, PZA and SM resistance. In addition, during the diagnostic delay period, transmission to close contacts may occur, thereby perpetuating the MDR-TB epidemic.

According to the South African National Tuberculosis Control Programme Practical Guidelines 2004, a patient will only be placed on the standardised treatment for MDR-TB after DST results become available. This could be at least 6 to 7 months after initial therapy started. The patient will be placed on the current standardised MDR-TB treatment regimen which includes a fluoroquinolone (usually ofloxacin or ciprofloxacin), amikacin (Am) or kanamycin (Km), ethionamide (for which the strain may be resistant if *inhA* promoter region mutation is the cause of INH resistance), PZA and EMB (replaced with cycloserine or terizidone if resistant to EMB). Resistance to PZA,[8] EMB[7] and ethionamide[15] is commonly associated with MDR-TB and, if not detected, patients with MDR-TB may only receive 2 effective drugs (a fluoroquinolone and Am or Km) of which Am or Km is not used during the 12 to18-month continuation phase of therapy. This situation could lead to unintentional monotherapy during the continuation phase, with possible acquisition of resistance to the fluoroquinolones, leading to pre-XDR-TB (one resistance mutation away from XDR-TB).[13] Therefore, many MDR/XDR-TB cases are presently being inadvertently under-treated, which may strongly influence treatment outcome. This scenario may be repeated as treatment regimens are adjusted, leading to the eventual evolution of XDR-TB.[4]

To address the difficulties associated with DST for EMB and PZA, the WHO in 2008 released an emergency update on guidelines for treating drug-resistant TB in which they acknowledged that MDR-TB treatment regimens should not be dependent on the results of DST for EMB or PZA.[16] They recommended that treatment of MDR/XDR-TB should include at least 4 drugs with certain or almost certain effectiveness. Treatment regimens can be individualised or standardised if resistance patterns for the country/region are known. EMB could be included in a regimen provided that it is not counted as one of the effective 4 drugs, and that PZA may be used for the entire treatment if deemed effective (based on DST) but must also not be counted as one of the 4 effective drugs. Ciprofloxacin is no longer recommended for treatment of TB.[16]

New draft policy guidelines (2008) are being formulated by the South African National TB Control Programme based on the WHO recommendations. These guidelines aim to address diagnostic delay as well as treatment of MDR/XDR-TB. They suggest that DST should be done on patients failing to show clinical or bacteriological improvement at 2 months of treatment or at 3 months in cases where the intensive phase was extended (as opposed to 5 months in the old guidelines). In contrast to the WHO, these guidelines recommend that DST for EMB determines the treatment regimen for MDR/XDR-TB. If susceptible, EMB should be included in the regimen as the 5th drug (this disregards the inaccuracy of EMB DST). If resistant, EMB should be replaced with terizidone or cycloserine as the 5th drug. These guidelines also recommend the inclusion of PZA as one of the 4 effective drugs but acknowledge that, in the absence of DST, it should be assumed that all isolates are resistant. Given the strong evidence of high levels of resistance to PZA in South Africa associated with MDR-TB, we suggest that, in the absence of DST for PZA, this drug should not be counted as one of the 4 effective drugs, but may be included in the regimen.

In light of the WHO recommendations and the high levels of undetected resistance to EMB and PZA in South Africa, it is essential that the revised guidelines for MDR-TB treatment are implemented in South Africa together with improved routine DST. In the interim, it should be assumed that all MDR-TB cases are resistant to EMB and PZA and, although treatment with these drugs can be continued, they should not be counted as one of the 4 effective drugs, to prevent the emergence of additional resistance and the possible evolution of XDR-TB.

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Chapter 8

Mycobacterial pharmacogenetics of *inhA* promoter mutations: A gateway to the emergence of XDR-TB in South Africa?

B. Müller,[1] E. M. Streicher,[1] K. G. P. Hoek,[1] M. Tait,[1] A. Trollip,[2] M. E. Bosman,[3] G. J. Coetzee,[4] E. M. Chabula-Nxiweni,[5] E. Hoosain,[5] N. C. Gey van Pittius,[1] T. C. Victor,[1] P. D. van Helden,[1] R. M. Warren[1]

[1] *DST/NRF Centre of Excellence for Biomedical Tuberculosis Research/MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Department of Biomedical Sciences;* [2] *Biotec Laboratories SA (Pty) Ltd, Cape Town, South Africa;* [3] *National Health Laboratory Service, Greenpoint, Cape Town, South Africa;* [4] *National TB Reference Laboratory, National Institute for Communicable Diseases, National Health Laboratory Service, Johannesburg, South Africa;* [5] *Nelson Mandela Bay Municipality, Port Elizabeth, South Africa*

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1. Abstract

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

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

DESIGN: Analysis of the frequency distribution of isoniazid resistance conferring mutations in a population sample of drug-resistant strains of *M. tuberculosis*.

RESULTS: In the Western Cape and Eastern Cape Provinces, respectively, the percentage of strains exhibiting *inhA* promoter mutations increased significantly from 47.8% and 70.7% in multidrug-resistant (MDR) strains to 85.5% and 91.9% in XDR strains. Data from the Western Cape revealed that significantly more XDR strains showed mutations in the *inhA* promoter than in *katG* (85.5% vs. 60.9%; $p<0.001$), whilst the respective proportions were equal for INH resistant non-MDR strains (~30%).

CONCLUSIONS: *InhA* promoter mutations are strongly associated with XDR tuberculosis in South Africa. We suggest that this is due to the dual resistance to ethionamide and (low-dose) isoniazid conferred by *inhA* promoter mutations. The use of molecular probe assays such as the GenoType® MTBDR*plus* assay, which allow for the detection of *inhA* promoter mutations, could enable adjustment of the treatment regimens depending on the pharmacogenetic properties of the mutations detected.

2. Introduction

Multidrug-resistant tuberculosis (MDR-TB), defined by resistance to at least isoniazid (INH) and rifampicin (RIF), threatens TB control programs in many parts of the world.[1] Amplification of drug resistance in MDR-TB can lead to virtually untreatable extensively drug-resistant tuberculosis (XDR-TB), defined as MDR-TB plus additional resistance to a fluoroquinolone and one of the injectable second-line drugs kanamycin (Km), amikacin (Am) or capreomycin (Cm).[2-5]

In South Africa, all new cases of TB are treated with a combination of four anti-TB drugs [INH, RIF, pyrazinamide (PZA) and ethambutol (EMB)] in the absence of drug susceptibility testing (DST).[6,7] Streptomycin is added to this regimen for retreatment cases while DST is being performed. If resistance to INH and RIF is detected, the treatment regimen is adjusted to include second-line anti-TB drugs. Since 2002 the treatment of MDR-TB has been largely standardised and includes a fluoroquinolone [mostly ofloxacin (OFL)], an aminoglycoside (Am or Km), PZA, EMB or cycloserine, and ethionamide (Eto).[6,8] This regimen assumes that resistance to EMB and PZA is rare. However, there is mounting microbiological evidence to suggest a strong association between MDR-TB and EMB and PZA resistance.[6,9,10] Furthermore, several molecular epidemiological studies have suggested that MDR-TB is largely transmitted.[11-15] This questions the validity of the current treatment guidelines[6] and calls for the development of rapid diagnostics for DST.[16]

Cases of drug-resistant TB emerge due to ineffective treatment, patient non-adherence to therapy, or the transmission of drug-resistant strains of *M. tuberculosis*.[2-4] On a molecular level, drug resistance in *M. tuberculosis* develops through spontaneous mutations in target genes followed by the natural selection of these resistant bacteria upon exposure to anti-TB drugs.[3,4] Various drug resistance causing mutations in *M. tuberculosis* have been characterised to date.[3,17,18] It is well documented that mutations in the *inhA* promoter confer low-level resistance to INH and cross-resistance to Eto, while mutations in *kat*G at codon 315 exclusively confer high-level resistance to INH.[18-21] Similarly, mutations in the *rpoB* gene account for >95% of RIF

resistance.[22] Thus, molecular assays identifying specific drug resistance causing mutations may be used to accelerate DST, which is considered critical to prevent amplification and transmission of drug resistance.[16] In this respect, the WHO endorsed GenoType® MTBDR*plus* assay has been implemented in several settings to assist culture based DST; it detects most of the mutations conferring RIF resistance as well as the principal mutations in the *katG* gene and *inhA* promoter which confer INH resistance.[16]

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

3. Materials and Methods

3.1 Data from the Western Cape Province

Strains of *M. tuberculosis* used for this study were selected from a large collection of drug-resistant strains, isolated from altogether 4524 TB patients mostly between 2001 and 2009. Between 2001 and 2007, drug-resistant isolates from patients visiting health facilities in two of the four health districts in the Western Cape Province were collected. From 2008, drug-resistant isolates from patients visiting health facilities in all of the health districts were collected. Routine DST was done by the National Health Laboratory Service (NHLS) and included testing for INH, RIF and EMB resistance. In 2007, routine DST was revised to include testing for OFL, Am and Eto resistance. Mutations in the *inhA* promoter and *katG* were identified by dot-blot techniques or partial gene sequencing as explained elsewhere.[23] For the analysis of the frequency of mutations in the *inhA* promoter and *katG* in INH resistant strains (Table 1), only sequencing data was

considered. Isolates from our collection of strains that were included in this analysis were INH resistant and were tested for mutations in either the *inhA* promoter or the *katG* gene or both. Multiple isolates from the same patient were included only if they showed different mutations. The convenience sample for this analysis included isolates obtained from 527 patients. For the analysis of the mutation patterns in different drug resistance classes (Table 2, Figure 1), all available data including results from dot-blot analyses was considered. Isolates that were included in this analysis were tested for mutations in both the *inhA* promoter and *katG.* A convenience sample was selected for INH resistant non-MDR (INH mono-resistant and poly-resistant) and MDR strains. However, the majority of XDR-TB strains were analysed. Only one *M. tuberculosis* isolate per patient was considered and the analysed sample contained isolates from 459 patients.

This study was approved by the Stellenbosch University Ethics Committee.

3.2 Data from the Eastern Cape Province

During the period from July 2008 to January 2009 a convenience sample of isolates from 263 patients corresponding to approximately one third of all MDR-TB isolates cultured from patients visiting heath facilities in the Eastern Cape Province was collected. Routine DST was done by the NHLS, Eastern Cape. Mutations in the *inhA* promoter were identified by DNA sequencing.[23]

3.3 Statistical analyses

Statistical analyses were performed in Stata/IC v10.1. Associations between distinct drug resistance groups and the occurrence of mutations in *katG* or the *inhA* promoter were determined by the Pearson's chi-squared test. The difference in the proportions of XDR strains with mutations in *katG* or the *inhA* promoter was determined by the two-sample z-test of proportions. A level of $\alpha<0.05$ was considered statistically significant.

4. Results

4.1 Western Cape Province

Mutations identified by partial sequencing of the *katG* gene and the *inhA* promoter are listed in Table 1. *KatG* mutations at codon 315 and *inhA* promoter mutations at nucleotide position -15 constituted the majority (32.2% and 58.2%, respectively) of the observed nucleotide changes (Table 1). Of all mutations identified, the MTBDR*plus* assay could potentially detect alterations in *katG* codon 315 and in the *inhA* promoter at nucleotide positions -8, -15 and -17, either specifically through a mutation probe or through the absence of a hybridization signal for one of the wild-type probes. Consequently, in theory, the MTBDR*plus* assay might detect up to 96.4% of the mutation events herein identified by sequencing (Table 1).

To investigate any potential associations between sequence changes in *katG* or the *inhA* promoter and XDR-TB, we calculated the proportion of strains with mutations in these genetic regions for different drug resistance classes (INH resistant non-MDR strains, MDR strains and XDR strains; Table 2, Figure 1). The percentage of strains with an *inhA* promoter mutation increased significantly from 30.1% in INH resistant non-MDR strains to 58.5% in MDR strains and 85.5% in XDR strains (overall 2.8-fold increase, X^2=74.6, p<0.001; Table 2, Figure 1). Although the percentage of strains with a *katG* mutation also increased, the rate of increase was lower (2.1-fold, X^2=28.7, p<0.001) and attributable to a strain cluster, which also harboured an *inhA* promoter mutation (data not shown). Significantly more XDR strains showed mutations in the *inhA* promoter than in *katG* (85.5% vs. 60.9%, p<0.001), whilst the respective proportions were equal for INH resistant non-MDR strains (~30%; Table 2, Figure 1).

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

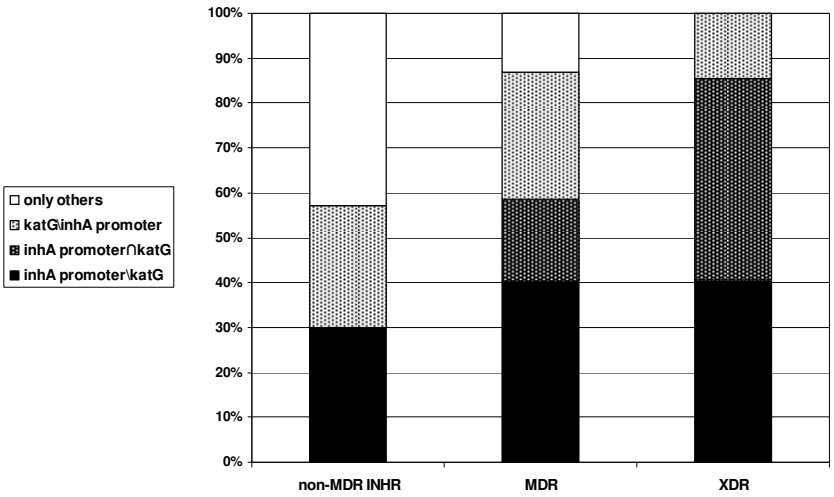

Figure 1. Patterns of point mutations in the *inhA* promoter and *katG* by drug resistance class in strains of *M. tuberculosis* in the Western Cape Province.

Key: The MDR group includes XDR group

only others: Strains with no mutations detected in *katG* or the *inhA* promoter

*katG**inhA* promoter: Strains with mutations detected in *katG* but not in the *inhA* promoter

inhA promoter∩*katG*: Strains with mutations detected in the *inhA* promoter and in *katG*

inhA promoter*katG*: Strains with mutations detected in the *inhA* promoter but not in *katG*

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

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

Position: Amino-acid positions are indicated for transcribed regions (*katG*) and base-pair positions are indicated for untranscribed regions (*inhA* promoter)

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

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

Sum: Number of INH resistant strains tested for a specific mutation for the indicated gene at the indicated position

% Present: Among the INH resistant strains tested, percentage of strains showing a mutation for the indicated gene at the indicated position

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

% Cum. Mutations: Cumulative percentage of % Mutations

Total: Total number of detected mutation events

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

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

MDR group includes XDR group

inhA promoter*katG*: Strains with mutations detected in the *inhA* promoter but not in *katG*

inhA promoter∩*katG*: Strains with mutations detected in the *inhA* promoter and in *katG*

*katG**inhA* promoter: Strains with mutations detected in *katG* but not in the *inhA* promoter

only others: Strains with no mutations detected in *katG* or the *inhA* promoter

inhA promoter: Strains with mutations detected in the *inhA* promoter

katG: Strains with mutations detected in *katG*

Total: Total number of strains tested

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

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

CI: 95% confidence intervals

* One-sided, 97.5% confidence interval

4.2 Eastern Cape Province

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

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

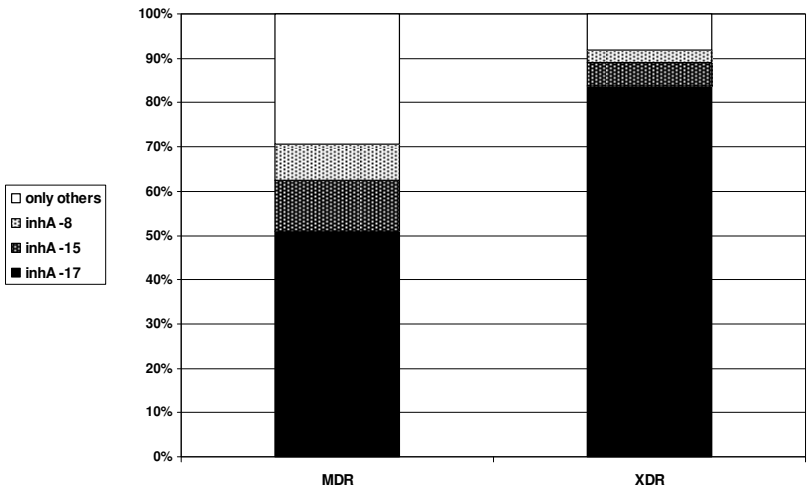

Figure 2. Frequency of point mutations in MDR and XDR strains of *M. tuberculosis* in the Eastern Cape Province

Key: MDR group includes XDR group
only others: Strains with no mutations detected in *katG* or the *inhA* promoter
*katG**inhA* promoter: Strains with mutations detected in *katG* but not in the *inhA* promoter
inhA promoter∩*katG*: Strains with mutations detected in the *inhA* promoter and in *katG*
inhA promoter*katG*: Strains with mutations detected in the *inhA* promoter but not in *katG*

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

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

MDR group includes XDR group

Position: Nucleotide positions are indicated

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

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

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

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

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

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

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

CI: 95% confidence intervals

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

5. Discussion

Our results show a significant association between *inhA* promoter mutations and XDR-TB in two South African Provinces (Tables 2 and 3, Figures 1 and 2). This may suggest a selective advantage of strains harbouring an *inhA* promoter mutation to become XDR strains, within the current treatment regimen. At least two distinct processes may explain the observed accumulation of XDR-TB strains with an *inhA* promoter mutation. First, *inhA* promoter mutations may have evolved during exposure to INH during first-line treatment. MDR strains evolving from such strains also would have demonstrated cross resistance to Eto. If Eto resistance remained undetected there was an increased probability for these strains to acquire resistance to second-line drugs due to the treatment regimen having one less effective drug. Second, a significant proportion of XDR-TB strains initially might have been susceptible to Eto; however, continuous exposure to this drug could have lead to the acquisition of *inhA* promoter mutations and Eto resistance.[3,4,17-21]

The strong association between the presence of *inhA* promoter mutations and XDR-TB may also apply to other regions in South Africa and other parts of the world. In a recent study in Portugal, 98.0% of 50 MDR strains tested showed resistance to Eto and 91.4% of 58 MDR strains tested showed an *inhA* promoter mutation.[24] A retrospective study on XDR-TB isolates from four provinces of South Africa showed that 30 of the 41 strains tested (73.2%), were resistant to Eto.[25] These findings may again be explained by the scenario described above. Future studies should investigate to what extend the observed association between *inhA* promoter mutations and XDR-TB also applies to other settings.

Our population study has some limitations. Mutation analyses of *katG* and the *inhA* promoter of isolates from the Western Cape Province was done on a convenience sample of INH resistant non-MDR and MDR strains. However, the majority of the XDR strains from this setting have been analysed and no bias is evident for this group of strains. We know that the Beijing lineage of

strains was overrepresented in our sample of the INH resistant non-MDR and MDR strains. In the Western Cape Province, drug-resistant Beijing strains are strongly associated with *inhA* promoter mutations [[11,14], unpublished results]. Therefore, our analyses overestimated the proportion of strains with an *inhA* promoter mutation in INH resistant non-MDR and in MDR strains. Moreover, XDR strains were overrepresented in our sample of MDR strains of the Western Cape Province. Therefore, the increase in the proportion of strains with an *inhA* promoter mutation from non-MDR INH resistant strains to XDR strains is likely to be even more pronounced and our analyses have been conservative with regards to this observation.

Our results may have been influenced by the considerable contribution of transmission of drug-resistant strains to MDR-TB, in South Africa.[11-15] Therefore, the percentage composition of distinct drug resistance mutations in different drug resistance groups may fluctuate if significant changes in the transmission rates of strains occur that are associated with specific mutations. It is also conceivable that the observed association between *inhA* promoter mutations and XDR-TB is not due to a selective advantage of these mutations, but is caused by their coincidental presence in a few highly transmitted drug-resistant strains that are particularly successful for other reasons. An alternative hypothesis for a potential selective advantage of *inhA* promoter mutations in the evolution of XDR-TB may be decreased fitness cost of these mutations compared to mutations in *katG*. Our analyses could have been distorted due to the partial sequencing only of *katG* in this study. However, other mutations in *katG* than those described here are infrequently detected in INH resistant clinical isolates[3,18] (Table 1) and their influence on our analyses is therefore likely to be minor or absent.

Our study suggests that in principle, the MTBDR*plus* assay could detect 96.4% of all mutation events identified by partial sequencing of the *katG* gene and the i*nhA* promoter of an extensive sample of INH resistant strains of *M. tuberculosis* from the Western Cape Province (Table 1). However, for this setting, our results clearly showed that mutations in genes other than *katG* or the *inhA* promoter accounted for more than 40% and 12% of all cases of INH resistant non-MDR and MDR cases, respectively. This illustrates that culture based phenotypic DST should not be

replaced but rather assisted by molecular drug resistance typing techniques in order to achieve the highest possible sensitivity for the detection of drug-resistant isolates. Otherwise, this will inevitably lead to the selection and spread of INH resistant strains with properties that remain undetected by the molecular identification methods currently in use.

The MTBDR*plus* assay has been implemented for routine DST in several countries including some provinces in South Africa. With the introduction of the assay to assist culture based DST, information on the presence of the most important drug resistance mutations in isolates of *M. tuberculosis* from TB patients becomes readily available to clinicians. This may allow adjustment of the treatment regimens depending on the pharmacogenetic properties of the mutations detected.[26]

For TB patients infected with strains harbouring an *inhA* promoter mutation, Eto must not be considered an effective drug of the treatment regimen. However, because the *inhA* promoter mutation only confers low-level resistance to INH, high-dose or standard dose INH may be included in the treatment regimen of such cases if the infecting strains do not also exhibit any additional high-level INH resistance mutations.[26] In many settings, the most frequently observed high-level INH resistance mutations are found in codon 315 of *katG*,[3] which are detectable by the MTBDR*plus* assay.[16] The information that high-dose INH may be included in treatment regimens is particularly valuable in XDR-TB cases, for which only few active drugs remain available. In this respect, our observation that approximately 40% of the MDR and XDR strains from the Western Cape Province exhibit a mutation in the *inhA* promoter but not in *katG*, is of special importance.

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

Genetic region exhibiting a drug resistance mutation			Drug resistance class	
rpoB (RIF high level resistance)	*katG* (INH high level resistance)	*inhA* promoter (INH low level resistance)		
negative	negative	negative	non-MDR	Use standard first line drugs.
negative	negative	positive	non-MDR INH resistant	Do not consider INH and ETH as effective drugs for treatment. Use second line drugs other than ETH to replace INH in the drug regimen. High-dose INH may be considered for treatment.
negative	positive	negative	non-MDR INH resistant	Do not consider INH or high-dose INH as an effective drug for treatment. Replace INH by an effective second line drug for treatment.
negative	positive	positive	non-MDR INH resistant	Do not consider INH or high-dose INH and ETH as effective drugs for treatment. Use second line drugs other than ETH to replace INH in the treatment regimen.
positive	negative	negative	non-MDR RIF resistant	Do not consider RIF as an effective drug for treatment. Replace RIF by an effective second line drug for treatment.
positive	negative	positive	MDR	Use second line drugs for treatment. Do not consider ETH, EMB and PZA as effective drugs for treatment. High-dose INH may be considered for treatment.
positive	positive	negative	MDR	Use second line drugs for treatment. Do not consider high-dose INH, EMB and PZA as effective drugs for treatment.
positive	positive	positive	MDR	Use second line drugs for treatment. Do not consider high-dose INH, ETH, EMB and PZA as effective drugs for treatment.

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

6. Acknowledgements

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General Conclusion

Despite the abundance of clinical, immunological and genotypic-based methodologies for detecting both *Mycobacterium tuberculosis* and associated drug-resistance, phenotypic culture still remains the gold standard. However, this method is dependent on the growth rate of *M. tuberculosis,* which is notoriously slow (doubling rate of 16 hours in optimal laboratory conditions).[1] Diagnostic delay is regarded as a major contributor to the continuous rise in tuberculosis cases and the associated emergence and transmission of multidrug-resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB). It is therefore essential that new and more rapid diagnostic methods are developed.

Genotypic-based assays show the most promise as they circumvent the need for culture and have the potential to provide a species-specific result within hours of obtaining a sample. Numerous methodologies have been described including hybridisation-based assays,[2,3] DNA Chip technologies,[4,5] multiplex ligation-dependent probe amplification,[6] single strand conformation polymorphism analysis,[7,8] isothermal amplification methods,[9-11] molecular beacons,[12,13] multiplex allele-specific PCR,[11,14] PCR restriction enzyme analysis,[15,16] and various other in-house[17] and commercial-based methodologies. Although DNA sequencing is considered the gold standard in genotypic-based assays, the cost of the equipment and reagents is too high for routine implementation in low-income countries. This is also the case with many of the other methodologies listed above, some of which are further hampered by the need for post-amplification processing. An open-tube format allows for the potential release of amplicons and the possibility of cross-contamination and reporting of false positives.

We therefore investigated the potential of high resolution melting analysis (HRM) to improve the rapid diagnosis of TB and associated drug resistance. The HRM apparatus and reagents are

relatively inexpensive and the methodology can easily be implemented in high-incidence, low income regions.

We firstly demonstrated that HRM was capable of rapidly identifying mycobacterial lymphadenitis from fine-needle aspiration biopsy (FNAB) samples. These FNABs were rinsed in an inexpensive transport medium and subsequently spotted onto FTA® Classic Cards (Whatman, UK) for later purification. The HRM method delivered a species-specific result by targeting the region of deletion 9 (RD9), present in *M. tuberculosis* and *M. canettii*, but absent from all other members of the complex[18] (human infection with *M. canettii* is rare). The method showed a sensitivity of 51.9% and a specificity of 94.0% as compared to the reference standard (positive cytology and/or positive culture with speciation). To simplify the method, we circumvented the need for the transport medium and spotted the samples directly onto the simpler FTA® *Elute* Cards. However, the procedure posed an increased risk to the health practitioners doing the FNAB, due to the potential for aerosolisation of any infectious material. Furthermore the samples often remained in the luer of the syringe and were not capable of being fully expelled onto the cards (using the previous method it was possible to remove this trapped sample by rinsing in the transport medium). The method delivered a slightly lower sensitivity and specificity (46.3% and 83.3%, respectively) than by rinsing in the transport medium. Unfortunately, none of the diagnostic modalities (HRM, cytology or culture) were sufficiently sensitive which may be due to the paucibacillary nature of mycobacterial lymphadenitis.

Despite the low sensitivities of both methods, HRM was able to rapidly identify more than half (study 1 = 57.4%, study 2 = 56.1%) of the patients with mycobacterial lymphadenitis with a relatively high specificity. Early treatment could therefore be initiated in these patients with a high degree of confidence. We have suggested a diagnostic algorithm which takes HRM and cytology results into consideration. 1) If the HRM analysis is positive for the presence of *M. tuberculosis* DNA and cytology is suggestive of mycobacterial disease, anti-TB treatment is initiated. 2) If HRM analysis is positive, but cytology does not indicate mycobacterial disease, or if HRM analysis is negative, then await phenotypic culture results before initiating treatment. Future studies are planned to improve this algorithm as well as to test alternate genotypic methods

including the GenoType® MDRTB*Plus* (Hain Lifesciences, Germany) and the GeneXpert MTB/RIF Assays (Cepheid Inc., California, USA) which are capable of rapidly identifying MDR-TB. We will reinvestigate storing the sample in a transport medium as it allows for dislodging any trapped sample as well as subsequent analysis by culture.

High resolution melting was also used to identify mutations within target regions. We developed the Fluorometric Assay for Susceptibility Testing of Rifampicin (FAST-Rif) which was capable of rapidly identifying MDR-TB by detecting *M. tuberculosis* complex-specific rifampicin (RIF) resistance mutations. The method involved PCR amplification of the rifampicin resistance-determining region (RRDR) of the *rpoB* gene from short-term cultures (clinical isolates) and from a wild type laboratory strain (H37Rv) in the presence of a fluorescent DNA binding dye. These amplification products were then mixed to form DNA duplexes which were then analysed by HRM. The method had a sensitivity of 98% and a specificity of 100% for the detection of RIF resistance compared to the gold standard culture-based phenotyping method. The advantage of this method over previously described methods[19-24] was its simplicity, the broad spectrum of non-synonymous single nucleotide polymorphisms (nsSNPs) detected by each analysis (15 different mutational events in the RRDR of the *rpoB* gene), and the high sensitivity and specificity achieved.

The HRM method proved versatile in that we were able to adapt it to rapidly identify ethambutol (EMB) resistance by detecting mutations in the *embB* gene. Ethambutol is an integral part of first-line therapy and its use is continued in second-line therapy due to surveillance data suggesting that EMB resistance is rare.[25] However, drug-susceptibility testing (DST) for EMB is inaccurate[26] and the recent evidence demonstrating the clinical significance of mutations in the *embB* gene[27] suggests that genotypic-based diagnosis of EMB resistance may be more accurate. Our FAST-EMB method showed a sensitivity and specificity of 94.4% and 98.4% respectively for detecting mutations in the *embB* gene as compared to DNA sequencing. We found that although EMB resistance was not associated with RIF mono-resistance or isoniazid (INH) mono-resistance, there was a strong association with MDR-TB, since 57.5% of the isolates (n=228) harboured

mutations in the *embB* 306 region, conferring EMB resistance. In contrast, only 9.2% were phenotypically resistant by routine culture.

Based on the strong association of EMB resistance and MDR-TB identified by this study and other studies,[26,27] as well as the strong association of pyrazinamide (PZA) resistance and MDR-TB,[25,28] we believe that the continued use of EMB and PZA based on the results of phenotypic DST will/has lead to the rapid evolution of MDR and XDR-TB in these patients. The World Health Organisation (WHO) has acknowledged that MDR-TB treatment regimens should not be dependent on the results of DST for EMB or PZA and that at least 4 drugs with certain or almost certain effectiveness are used (EMB and PZA are not counted).[29] In contrast to the WHO, the new South African National TB Control Programme draft policy guidelines (2008) advise that DST for EMB determines the treatment regimen for MDR/XDR-TB. If susceptible, EMB should be included in the regimen as the 5th drug. If resistant, EMB should be replaced with terizidone or cycloserine as the 5th drug (this disregards the inaccuracy of DST). These guidelines also recommend the inclusion of PZA as one of the 4 effective drugs but acknowledge that, in the absence of DST, it should be assumed that all isolates are resistant.

In light of the WHO recommendations and the high levels of undetected resistance to EMB and PZA in South Africa we propose that in the absence of an accurate DST method for both EMB and PZA, that it should be assumed that all MDR-TB cases are resistant to both anti-TB drugs and that although treatment with these drugs can be continued, they should not be counted as one of the 4 effective drugs. Adopting this notion may prevent the emergence of additional resistance and the possible evolution of XDR-TB. Furthermore, given the affordability and superior accuracy of the described FAST-EMB method over routine DST, it would be useful in the routine diagnosis of EMB drug-resistance and should be considered for implementation into the TB control program so as to ensure the appropriate management of the TB case and to prevent the acquisition or spread of further drug-resistance phenotypes.

The FAST-Rif and FAST-EMB methods were rapid, cost effective, highly sensitive and specific and, in the case of EMB, a considerable improvement over phenotypic-based DST. We

acknowledge that these methods require post-amplification processing (to mix amplification products) with the potential for cross-contamination and the possible reporting of false positive results. This is especially worrying in high throughput laboratories. Efforts to convert the method to a single-tube format were hampered by the competitive nature of the PCR. We therefore designed a tube with a septum (South African provisional patent 2007/06915, see Appendix 3) which would allow simultaneous and non-competitive amplification of the two PCRs. After PCR amplification, the two PCRs could be mixed by inverting the tube, thereby avoiding the need to open the reaction tube. We are in the process of approaching manufacturers to develop a prototype so as to provide a proof of concept. An alternative solution would be to seed the PCR reaction with the wild type reference strain prior to amplification.[30] However, the seeding of the PCR reaction, as well as the use of a tube with a septum, will not overcome the possibility of false-susceptible results in cases where a clinical sample may fail to amplify due to insufficient DNA.

We were however able to convert the FAST method to a closed-tube one-step method (FASTest-inhA) using the detection of *inhA* promoter mutations conferring INH and associated ethionamide (Eto) resistance as a model. Briefly, in a single closed tube, clinical isolates were PCR amplified and as amplicons were generated, one of the strands (3' to 5') annealed to a modified complementary single stranded DNA oligonucleotide (5' to 3') allowing extension of this amplicon and the incorporation of an adaptor sequence. Primers specific to the adaptor sequence and the original 3' sequence ensured a final PCR product that reflected amplification of both the wild type sequence and the sequence present in the clinical isolate. The method was able to identify *inhA* promoter mutations with a sensitivity and specificity of 100% and 83.3%, respectively compared to DNA sequencing analysis. The specificity of the assay was improved to 93.5% by visual analysis of the derivative plots by experienced and inexperienced operators. Visual scoring was more specific than automatic scoring due to limitations with the Rotorgene software, which was unable to distinguish between background noise and signal in a small proportion (7.8%) of the wild type sequence samples. This could potentially be improved by adding a DNA purification step; however, this would increase the cost of the assay and contribute further to diagnostic

delay. Alternatively, synthesised oligonucleotide sequences could be introduced in each PCR reaction to act as calibrators, thereby allowing the normalisation of derivative curves to these calibrators.[31] However, the addition of these oligonucleotides into the PCR reaction may reduce amplification efficiency.[31] We therefore proposed the design of an add-on software application which would normalise all the derivative plots of a run according the homoduplex peak, to a pre-defined T_m. Aligning this homoduplex peak would allow the heteroduplex peak to align in samples harbouring a mutation, while keeping wild type samples with the smaller false peak aligned at a lower peak height and different T_m. This would then allow the samples to be scored more accurately.

The FASTest-inhA assay was a significant improvement over the FAST-Rif[18] and FAST-EMB methods in that it included an amplification control, which ensured that amplification failures could be identified (only clinical samples containing *M. tuberculosis* DNA will be amplified). Furthermore, the closed-tube system reduced the time to results (<2hours following short term culture) and the risk of amplicon release. This diagnostic interval could be shortened further if our targets could be efficiently and routinely amplified directly from sputum. The method may lay the foundation for a more affordable and safer closed-tube assay, which could be adapted to multiple targets. The assay also has the potential for large scale automation, which should be particularly attractive in low-income, high TB incidence settings.

Although our methods were not able to identify the particular mutations involved in resistance, this should not be seen as a limitation, since the primary objective for routine diagnostics would only be to determine the presence or absence of an nsSNP conferring resistance, thereby enabling the correct treatment to be administered to the patient. We specifically targeted regions within the *rpoB, embB* and *inhA* promoter genes in which no known synonymous SNPs have been identified. We also acknowledge that our method was not able to detect nsSNPs conferring RIF, EMB and INH resistance which fell outside of the areas we targeted; however, in the case of RIF and EMB we would miss only 5%[32] and 10-30%[33] of resistance, respectively. Similarly, the majority of the genotypic-based drug susceptibility testing methods listed above would also not be able to detect nsSNPs outside of these regions.

Furthermore, although over 80% of the mutations conferring INH resistance occur in either the *katG* or *inhA* promoter gene,[33] *inhA* promoter mutations are of particular interest as these confer low level INH resistance, therefore cases which harbour these mutations may benefit from the use of high dose INH therapy.[34] These mutations have also been implicated in cross-resistance to the second-line anti-tuberculosis drug Eto, which forms part of the second-line treatment regimen for MDR-TB.[34]

We provided data showing that in the Western and Eastern Cape Provinces of South Africa, *inhA* promoter mutations are strongly associated with XDR-TB, which we believe may be due to the dual resistance to Eto and (low-dose) INH conferred by these mutations. We also suggest that since 40% of INH resistant (non-MDR-TB) and 12 % of MDR-TB cases do not harbour a mutation in either the *katG* or *inhA* promoter genes in our setting, that culture-based DST is still necessary, but could be complemented by genotypic-based studies in order to achieve the highest possible sensitivity for the detection of drug-resistant isolates. The GenoType® MTBDR*plus* assay has been implemented for routine DST in several countries including some provinces in South Africa. The data generated by this assay may provide clinicians the opportunity to adjust treatment regimens for a patient depending on the pharmacogenetic properties of the mutations detected.[34] We subsequently proposed guidelines for the individualised treatment of TB patients based on the drug resistance mutation patterns detected by the MTBDR*plus* assay. Ethionamide should not be considered an effective drug in TB patients infected with strains harbouring an *inhA* promoter mutation, however high-dose INH may be included in the treatment regimen of such cases if the infecting strains do not also exhibit any additional high-level INH resistance mutations (most commonly occurring in the *katG* gene).[34] This treatment algorithm may be particularly useful in XDR-TB cases, for which only few active drugs remain available.

In conclusion, we have shown that our methods are simple and affordable and they can easily be adapted to provide species-specific diagnosis of *M. tuberculosis* infection and various associated drug-resistances. Furthermore, such technology can be applied to advise individualised treatment regimens. However, the lack of diagnostics for second-line drug resistance is worrying

and our method proved difficult to adapt towards these drugs. We believe this may be due to a number of reasons, including that the genes and mutations conferring second-line drug resistance are less well defined (and in some cases still unknown) and that in our setting, XDR-TB is more often acquired, implying that patients often have a mixed population of both wild-type and mutation carrying strains in their clinical specimens.[35] Finally a universal problem of all the genotypic-based assays which target DNA is that they are able to detect non-viable bacteria. We therefore advise that while awaiting the ideal diagnostic, improved accuracy may lie in using a combination of both phenotypic and genotypic-based methodologies.

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Appendix 1

Table 1: Comparison of the results of the rifampicin drug-susceptibility testing methods followed, including the gold standard phenotyping method, DNA sequencing and the FAST-RIF analysis, on purified DNA specimens.

Sample	Rif Phenotype (Corrected)[1]	rpoB codon mutated	FAST-RIF
1	Sensitive		Sensitive
2	Resistant	531	Resistant
3	Resistant	531	Resistant
4	Sensitive		Sensitive
5	Sensitive		Sensitive
6	Sensitive		Sensitive
7	Sensitive		Sensitive
8	Sensitive		Sensitive
9	Sensitive		Sensitive
10	Sensitive		Sensitive
11	Sensitive		Sensitive
12	Sensitive		Sensitive
13	Resistant	531	Resistant
14	Sensitive		Sensitive
15	Resistant	531	Resistant
16	Sensitive		Sensitive
17	Sensitive		Sensitive
18	Sensitive		Sensitive
19	Resistant	531	Resistant
20	Resistant	526	Resistant
21	Resistant	531	Resistant
22	Sensitive		Sensitive
23	Sensitive		Sensitive
24	Sensitive		Sensitive
25	Sensitive		Sensitive
26	Sensitive		Sensitive
27	Resistant	531	Resistant
28	Resistant	531	Resistant
29	Resistant	531	Resistant
30	Resistant	531	Resistant
31	Resistant	531	Resistant
32	Sensitive		Sensitive
33	Sensitive		Sensitive
34	Sensitive		Sensitive
35	Sensitive		Sensitive
36	Sensitive		Sensitive
37	Resistant	531	Resistant
38	Resistant	531	Resistant
39	Resistant	wt [2]	Sensitive
40	Sensitive		Sensitive
41	Sensitive		Sensitive
42	Resistant	531	Resistant
43	Sensitive		Sensitive
44	Sensitive	wt	Sensitive
45	Resistant	531	Resistant
46	Sensitive		Sensitive
47	Resistant	526	Resistant

48	Sensitive		Sensitive
49	Resistant	526	Resistant
50	Resistant	531	Resistant
51	Sensitive		Sensitive
52	Resistant	526	Resistant
53	Resistant	531	Resistant
54	Sensitive		Sensitive
55	Resistant	531	Resistant
56	Resistant	531	Resistant
57	Resistant	531	Resistant
58	Sensitive		Sensitive
59	Sensitive		Sensitive
60	Resistant	531	Resistant
61	Resistant	531	Resistant
62	Resistant	531	Resistant
63	Sensitive		Sensitive
64	Resistant	531	Resistant
65	Resistant	533	Resistant
66	Resistant	ins	Resistant
67	Resistant	531	Resistant
68	Resistant	531	Resistant
69	Sensitive		Sensitive
70	Sensitive		Sensitive
71	Resistant	531	Resistant
72	Resistant	wt [2]	Sensitive
73	Resistant	531	Resistant
74	Sensitive		Sensitive
75	Resistant	531	Resistant
76	Resistant	531	Resistant
77	Resistant	531	Resistant
78	Resistant	531	Resistant
79	Resistant	531	Resistant
80	Sensitive		Sensitive
81	Sensitive		Sensitive
82	Resistant	516	Resistant
83	Sensitive		Sensitive
84	Sensitive		Sensitive
85	Resistant	516	Resistant
86	Resistant	531	Resistant
87	Sensitive		Sensitive
88	Resistant	531	Resistant
89	Resistant	531	Resistant
90	Sensitive		Sensitive
91	Resistant	531	Resistant
92	Resistant	531	Resistant
93	Resistant	516 & 526	Resistant
94	Sensitive		Sensitive
95	Resistant	531	Resistant
96	Sensitive		Sensitive
97	Sensitive		Sensitive
98	Resistant	531	Resistant
99	Sensitive		Sensitive
100	Resistant	526	Resistant
101	Sensitive		Sensitive

102	Sensitive		Sensitive
103	Sensitive		Sensitive
104	Sensitive		Sensitive
105	Resistant	531	Resistant
106	Resistant	531	Resistant
107	Resistant	531	Resistant
108	Resistant	531	Resistant
109	Resistant	533 & 511	Resistant
110	Sensitive		Sensitive
111	Resistant	531	Resistant
112	Sensitive		Sensitive
113	Sensitive		Sensitive
114	Resistant	531	Resistant
115	Sensitive		Sensitive
116	Resistant	531	Resistant
117	Resistant	531	Resistant
118	Resistant	531	Resistant
119	Sensitive		Sensitive
120	Resistant	531	Resistant
121	Resistant	531	Resistant
122	Resistant	531	Resistant
123	Resistant	531	Resistant
124	Sensitive		Sensitive
125	Sensitive		Sensitive
126	Resistant	531	Resistant
127	Sensitive		Sensitive
128	Sensitive		Sensitive
129	Sensitive		Sensitive
130	Resistant	531	Resistant
131	Resistant	531	Resistant
132	Sensitive		Sensitive
133	Sensitive		Sensitive
134	Sensitive		Sensitive
135	Resistant	531	Resistant
136	Resistant	531	Resistant
137	Resistant	526	Resistant
138	Sensitive		Sensitive
139	Resistant	531	Resistant
140	Resistant	531	Resistant
141	Resistant	wt [3]	Resistant
142	Resistant	531	Resistant
143	Sensitive		Sensitive
144	Resistant	531	Resistant
145	Sensitive		Sensitive
146	Sensitive		Sensitive
147	Resistant	531	Resistant
148	Sensitive		Sensitive
149	Sensitive	wt	Sensitive
150	Resistant	531	Resistant
151	Sensitive		Sensitive
152	Sensitive		Sensitive
153	Resistant	531	Resistant

Appendix 2

Table 1: Comparison of the results of the rifampicin drug-susceptibility testing methods followed including the gold standard phenotyping method, DNA sequencing and the FAST-RIF analysis, on crude DNA specimens.

Sample	Rif Phenotype (Corrected)[1]	rpoB codon mutated	FAST-RIF
1	Resistant	531	Resistant
2	Resistant	531	Resistant
3	Sensitive		Sensitive
4	Resistant	526	Resistant
5	Sensitive		Sensitive
6	Sensitive		Sensitive
7	Sensitive		Sensitive
8	Sensitive		Sensitive
9	Resistant	533	Resistant
10	Sensitive	511 [4]	Resistant
11	Resistant	531	Resistant
12	Resistant	516	Resistant
13	Resistant	516	Resistant
14	Resistant	wt [2]	Sensitive
15	Resistant	533	Resistant
16	Sensitive		Sensitive
17	Sensitive		Sensitive
18	Resistant	526 [5]	Sensitive
19	Resistant	513	Resistant
20	Sensitive		Sensitive
21	Resistant	533	Resistant
22	Resistant	531	Resistant
23	Sensitive		Sensitive
24	Sensitive		Sensitive
25	Resistant	516	Resistant
26	Resistant	531	Resistant
27	Resistant	531	Resistant
28	Resistant	513	Resistant
29	Resistant	516	Resistant
30	Resistant	516	Resistant
31	Resistant	531	Resistant
32	Resistant	531	Resistant
33	Resistant	531	Resistant
34	Sensitive		Sensitive
35	Resistant	531	Resistant
36	Resistant	531	Resistant
37	Resistant	531	Resistant
38	Resistant	531	Resistant
39	Resistant	531 [5]	Sensitive
40	Sensitive		Sensitive
41	Sensitive		Sensitive
42	Resistant	531	Resistant
43	Resistant	531	Resistant
44	Resistant	516	Resistant
45	Resistant	516	Resistant
46	Resistant	531	Resistant
47	Resistant	wt [2]	Sensitive

48	Resistant	516	Resistant
49	Resistant	531	Resistant
50	Resistant	526	Resistant
51	Resistant	526	Resistant
52	Resistant	516 & 511	Resistant
53	Resistant	526	Resistant
54	Resistant	531	Resistant
55	Sensitive		Sensitive
56	Sensitive		Sensitive
57	Resistant	526	Resistant
58	Resistant		Resistant
59	Sensitive		Sensitive
60	Resistant	531	Resistant
61	Resistant	531	Resistant
62	Resistant	531	Resistant
63	Sensitive		Sensitive
64	Sensitive		Sensitive
65	Resistant	531	Resistant
66	Sensitive		Sensitive
67	Resistant	531	Resistant
68	Resistant	531	Resistant
69	Sensitive		Sensitive
70	Resistant	531	Resistant
71	Sensitive		Sensitive
72	Resistant	531	Resistant
73	Resistant	531	Resistant
74	Sensitive		Sensitive
75	Resistant	531	Resistant
76	Sensitive		Sensitive
77	Sensitive		Sensitive
78	Resistant	531	Resistant
79	Sensitive	wt	Sensitive
80	Resistant	531	Resistant
81	Resistant	531	Resistant
82	Resistant	531	Resistant
83	Sensitive		Sensitive
84	Sensitive	wt	Sensitive
85	Sensitive		Sensitive
86	Sensitive		Sensitive
87	Sensitive		Sensitive
88	Resistant	531	Resistant
89	Resistant	516	Resistant
90	Resistant	531	Resistant
91	Resistant	531	Resistant
92	Resistant	526	Resistant
93	Resistant	531	Resistant
94	Resistant	531	Resistant
95	Sensitive		Sensitive
96	Resistant	531	Resistant
97	Sensitive		Sensitive
98	Resistant	531	Resistant
99	Resistant	531	Resistant
100	Resistant	526	Resistant
101	Sensitive	wt	Sensitive

102	Resistant	526	Resistant
103	Resistant	531	Resistant
104	Resistant	531	Resistant
105	Resistant	531	Resistant
106	Resistant	531	Resistant
107	Sensitive		Sensitive
108	Resistant	531	Resistant
109	Sensitive		Sensitive
110	Sensitive		Sensitive
111	Sensitive	511 [4]	Sensitive
112	Sensitive	531 [3]	Resistant
113	Resistant	531	Resistant
114	Sensitive		Sensitive
115	Resistant	516	Resistant
116	Resistant	531	Resistant
117	Resistant	531	Resistant
118	Sensitive		Sensitive
119	Resistant	531	Resistant
120	Resistant	531	Resistant
121	Resistant	533	Resistant
122	Resistant	531	Resistant
123	Resistant	531	Resistant
124	Resistant	wt [2]	Sensitive
125	Sensitive		Sensitive
126	Resistant	wt [2]	Sensitive
127	Sensitive		Sensitive
128	Resistant	531	Resistant
129	Resistant	531	Resistant
130	Resistant	531	Resistant
131	Resistant	531	Resistant
132	Sensitive		Sensitive
133	Resistant	531	Resistant
134	Resistant	531	Resistant

[1] Corrected phenotype result following reanalysis of any discrepant results
[2] The mutation conferring rifampicin resistance lies outside of the RRDR
[3] The culture became contaminated and no further tests could be performed
[4] Correct result – may implicate that mutations in the codon 511 do not confer Rifampicin resistance
[5] DNA quality may have affected results

REPUBLIC OF SOUTH AFRICA

REPUBLIEK VAN SUID AFRIKA

PATENTS ACT, 1978

CERTIFICATE

In accordance with section 44 (1) of the Patents Act, No. 57 of 1978, it is hereby certified that:

1.SOUTH AFRICAN MEDICAL RESEARCH COUNCIL
1.UNIVERSITY OF STELLENBOSCH

Has been granted a patent in respect of an invention described and claimed in complete specification deposited at the Patent Office under the number

2008/09666

A copy of the complete specification is annexed, together with the relevant Form P2.

In testimony thereof, the seal of the Patent Office has been affixed at Pretoria with effect from the **25** day of **November 2009**

.....
Registrar of Patents

FORM P.2

REPUBLIC OF SOUTH AFRICA — REGISTER OF PATENTS — PATENTS ACT, 1978

OFFICIAL APPLICATION NO.	LODGING DATE: PROVISIONAL	ACCEPTANCE DATE
22 ZA 2008/09665		18-09-2009
INTERNATIONAL CLASSIFICATION	LODGING DATE: COMPLETE	GRANTED DATE
B01L, C12M, B01F	12 NOVEMBER 2008	25-11-2009

FULL NAME(S) OF APPLICANT(S)/PATENTEE(S)

71

1. SOUTH AFRICAN MEDICAL RESEARCH COUNCIL
2. UNIVERSITY OF STELLENBOSCH

APPLICANTS SUBSTITUTED:	DATE REGISTERED
71	

ASSIGNEE(S)	DATE REGISTERED
71	

FULL NAME(S) OF INVENTOR(S)

72

1. WARREN, ROBIN MARK
2. HOEK, JEM GILBERTE PAULINE
3. GEY VAN PITTIUS, NIGRAAG CLAUDIUS
4. VAN HELDEN, PAUL DAVID
5. VICTOR, THOMAS CALLDO

PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
N.B. Use International Abbreviation for country (See Schedule 4)	33 ZA	31 2007/06915	32 17 August 2007

TITLE OF INVENTION

54

"LABORATORY MIXING DEVICE"

ADDRESS OF APPLICANT(S)/PATENTEE(S)

1. Francie van Zyl Avenue, Parow Valley, Parow, Republic of South Africa
2. Matieland, Stellenbosch, Republic of South Africa

ADDRESS FOR SERVICE	A & A REF:
74 ADAMS & ADAMS, Pretoria	P6875ZA00 GSK

PATENT OF ADDITION TO NO.	DATE OF ANY CHANGE
61	
FRESH APPLICATION BASED ON	DATE OF ANY CHANGE

A&A P202

A & A Ref No: P687SZA01 GSK

FORM P7

ADAMS & ADAMS
PATENT ATTORNEYS
PRETORIA

REPUBLIC OF SOUTH AFRICA
Patents Act, 1978

COMPLETE SPECIFICATION
(Section 30 (1) - Regulation 28)

21	01	OFFICIAL APPLICATION NO	22	LODGING DATE
				12 NOVEMBER 2008

51	INTERNATIONAL CLASSIFICATION

B01L, C12M, B01F

71	FULL NAME(S) OF APPLICANT(S)

1. SOUTH AFRICAN MEDICAL RESEARCH COUNCIL
2. UNIVERSITY OF STELLENBOSCH

72	FULL NAME(S) OF INVENTOR(S)

1. WARREN, ROBIN MARK
2. HOEK, KIM GILBERTE PAULINE
3. GET VAN PI TIUS, NICOLAAS CLAUDIUS
4. VAN HELDEN, PAUL DAVID
5. VICTOR, THOMAS CALLDO

54	TITLE OF INVENTION

"LABORATORY MIXING DEVICE"

SPOOR & FISHER

FORM P.1

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT
AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) – Regulation 22)

REGISTRAR OF PATENTS, DESIGNS, TRADE MARKS AND COPYRIGHT
SPOOR P
BILLED
17 Aug 07

The granting of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate

OFFICIAL APPLICATION NO.

21	01	2007/06912

S & F REFERENCE: PA/14505/P/1

FULL NAME(S) OF APPLICANT(S)

71	1. SOUTH AFRICAN MEDICAL RESEARCH COUNCIL 2. UNIVERSITY OF STELLENBOSCH

ADDRESS(ES) OF APPLICANT(S)

	1. FRANCIE VAN ZIJL DRIVE, PAROW, 7505, WESTERN CAPE, SOUTH AFRICA 2. VICTORIA STREET, STELLENBOSCH, 7600, WESTERN CAPE, SOUTH AFRICA

TITLE OF INVENTION

54	METHOD FOR DETECTING DNA SEQUENCE POLYMORPHISMS

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2. THE EARLIEST PRIORITY CLAIM IS:

COUNTRY: NIL	NUMBER: NIL	DATE: NIL

~~THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.~~

~~21~~	~~01~~	

~~THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND IS BASED ON APPLICATION NO.~~

~~21~~	~~01~~	

THIS APPLICATION IS ACCOMPANIED BY:

- ☒ 1. A single copy of a provisional specification of 13 pages.
- ☒ 2. Drawings of 11 sheets.
- ☐ 3. Publication particulars and abstract (Form P.8 in duplicate).
- ☐ 4. A copy of figure of the drawings (if any) for the abstract.
- ☐ 5. Assignment of invention.
- ☐ 6. Certified priority document.
- ☐ 7. Translation of the priority document.
- ☐ 8. Assignment of priority rights.
- ☐ 9. A copy of the Form P.2 and the specification of S.A. Patent Application No .
- ☐ 10. Declaration and power of attorney on Form P.3.
- ☐ 11. Request for ante-dating on Form P.4.
- ☐ 12. Request for classification on Form P.9.
- ☒ 13. Form P.2 in duplicate.
- ☐ 14. Other.

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17 AUG 07
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74	ADDRESS FOR SERVICE: SPOOR & FISHER

Dated: 17 August 2007

J.C. McKnight

SPOOR & FISHER
PATENT ATTORNEYS FOR THE APPLICANT(S)

RECEIVED

REGISTRAR OF PATENTS

DOOR & FISHER

FORM P.9

PATENTS ACT, 1978

PROVISIONAL SPECIFICATION

(Section 30(1) – Regulation 27)

OFFICIAL APPLICATION NO.		LODGING DATE	
21	01	22	17 AUGUST 2007

FULL NAMES OF APPLICANTS

71	SOUTH AFRICAN MEDICAL RESEARCH COUNCIL UNIVERSITY OF STELLENBOSCH

FULL NAMES OF INVENTORS

72	HOEK, KIM VAN PITTIUS, NICOLAAS CLAUDIUS GEY VAN HELDEN, PAUL DAVID WARREN, ROBIN MARK VICTOR, TOMMIE

TITLE OF INVENTION

54	METHOD FOR DETECTING DNA SEQUENCE POLYMORPHISMS