Evaluation of the Xpert® MTB/RIF assay and its impact on tuberculosis diagnosis and rifampicin resistance screening: Efforts to determine the prevalence of drug resistant TB and *rpo*B gene mutations in the Malawian adult population

Tarsizio Chikaonda

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree

of

Doctor of Philosophy

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DECLARATION

I, Tarsizio Chikaonda, declare that this thesis is the result of my own work, except where otherwise stated. Other sources are acknowledged and a bibliography is appended. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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Tarsizio Chikaonda

20th day of November 2017

DEDICATION

This work is dedicated to my wife Pilira, my children Tarsizious Jnr, Thresfore and Thespesius.

"We cannot win the battle against AIDS if we do not also fight TB. TB is too often a death sentence for people with AIDS. It does not have to be this way"

By

Nelson Mandela

To the memory of my late brother Gregory whose words of encouragement have propelled me to reach this level of study

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS STUDY

Publications

The following manuscripts have been published from this work;

- Brian Barnetta, Runa H. Gokhale, Robert Krysiak, Creto Kanyemba, **Tarsizio Chikaonda**, Mphatso Bokosi, Cornelius Mukuzunga, Friday Saidi, Sam Phiri, Irving F. Hoffman and Mina C. Hosseinipour. **Prevalence of drug resistant TB among outpatients at an HIV/TB clinic in Lilongwe, Malawi** Trans R Soc Trop Med Hyg 2015; 109: 763–768 **(Chapter 3)**

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- **Tarsizio Chikaonda**, Irene Ketseoglou, Nelson Nguluwe, Isaac Thengolose, Felix Nyakwawa, Wendy Stevens, Lesley Scott, Mina Hosseinipour. ***rpo*B gene mutations in rifampicin resistant *Mycobacterium tuberculosis* strains from**

Malawi. National Tuberculosis Conference, Lilongwe, Malawi, 11th to 12th February 2016. Oral presentation.

ABSTRACT

Tuberculosis (TB) remains a global challenge and continues to kill more people than any other bacterial infection. The problem has been complicated with the emergence of multi-drug resistant (MDR) and extensively drug-resistant (XDR) TB. The Xpert MTB/RIF, which detects both *Mycobacterium tuberculosis* (MTB) and resistance to rifampicin (RIF) in a single rapid assay, has greatly improved TB diagnosis and detection of drug resistance.

The main objective of the study was to determine the prevalence of resistance to first line TB drugs among outpatients initiating on treatment in Lilongwe, evaluate performance of Xpert MTB/RIF and to determine the prevalence of mutations responsible for RIF resistance in the *rpo*B gene of MTB strains from Malawi. This was achieved by analysing specimens collected from Bwaila Hospital (Bwaila) and the National TB Reference Laboratory (NTRL), both from Lilongwe district in Malawi.

An observational cohort study was conducted between April 2011 and July 2012 at Bwaila to determine the prevalence of drug-resistant TB among outpatients. The study collected specimens from 702 TB patients of which 420 (59.8%) were HIV co-infected. One (1/349, 0.3%) RIF mono-resistant TB case was detected while isoniazid mono-resistance was detected in 2.9% (10/349) of the newly treated and culture

confirmed cases. The prevalence of MDR-TB was 3.8% (1/26) among previously treated cases and 0.3% (1/349) among new cases. This demonstrates a very low prevalence of drug-resistant TB in the outpatient population.

For TB diagnosis, the performance of Xpert MTB/RIF was evaluated retrospectively using 351 specimens (frozen stored pellets) from this cohort. Of 348 sputum pellets with valid Xpert MTB/RIF results, 200 (57%) were from HIV/TB co-infected while 148 (43%) were from HIV uninfected individuals. Among these, 219 (63%) were smear-negative and 129 (37%) were smear-positive. When compared to culture, Xpert MTB/RIF demonstrated a sensitivity of 93.8% (95% CI: 89.4% - 96.8%) and specificity of 97.4% (95% CI: 93.5% - 99.3%) with a positive predictive value of 97.8% (95% CI 94.6% - 99.4%) and negative predictive value of 92.6% (95% CI 87.4% - 96.1%). Within this cohort, Xpert MTB/RIF correctly identified 185 of 186 (98.8%) RIF-sensitive MTB and 2/2 (100%) RIF-resistant MTB when compared with MTBDR*plus* version 2. DNA sequencing resolved the one discrepant result in favour of MTBDR*plus*. In addition, Xpert correctly identified four non-tuberculous mycobacteria (NTM) [*M. avium* (n=1), *M. intracellulare* (n=3)] as negative for MTB.

To determine the prevalence of mutations in the *rpo*B gene, the Rifampicin Resistance Determining Region (codon 507 – 533) was sequenced from specimens from both

Bwaila and the National Tuberculosis Reference Laboratory (NTRL). Specimens that were reported with RIF resistance by Xpert MTB/RIF were eligible for DNA sequencing. A total of 43 specimens were successfully sequenced and mutations were detected in 37/43 specimens. Mutations were frequently detected in codons 526, 531 and 516. Mutations were not detected in 6/43 (14%) specimens. Among the specimens with no mutations, 2/6 (33%) were RIF-sensitive while 4/6 (67%) were RIF-resistant on MTBDR*plus* assay.

Overall, the prevalence of MDR-TB is low among outpatients seeking treatment at Bwaila which emphasises the effectiveness of the National TB programme in Malawi. The use of Xpert MTB/RIF in this population can increase the number of patients starting on appropriate TB treatment earlier and offer a greater opportunity in increasing TB case detection among high risk and vulnerable groups. This technology can reduce the TB burden in the population including people living with HIV. Disease transmission can be reduced with the use of Xpert MTB/RIF because of early treatment initiation and therefore likely to shorten the sputum culture conversion times for patients with RIF-resistant TB. Xpert MTB/RIF is a useful tool due to its diagnostic accuracy although our findings show a lower sensitivity in smear-negative/culture-positive specimens. Further evaluations of Xpert MTB/RIF assay's performance are required in detecting smear-negative/Xpert-positive cases to gauge its diagnostic value over a longer time period.

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ABBREVIATIONS

AFB – Acid Fast Bacilli

AMK – Amikacin

ART – Anti-Retroviral Treatment

BSL3 – Biosafety Level 3 Laboratory

CAP – Capreomycin

CFU – Colony Forming Units

CFZ - Clofazimine

CIP - Ciprofloxacin

CS – Cycloserine

CSF - Cerebral Spinal fluid

Ct – Cycle Threshold

CXR – Chest X-Ray

dH_2O – Deionised Water

DNA – Deoxynucleic Acid

DOTS - Directly Observed Treatment Short-course

DR-TB – Drug Resistant Tuberculosis

DST - Drug Susceptibility Testing

EHP - Essential Health Package

EMB - Ethambutol

EPTB – Extra-Pulmonary Tuberculosis

ETO - Ethionamide

FDC - Fixed Dose Combination

GC – Genus Control

HAART - Highly Active Antiretroviral Therapy

HIV – Human Immunodeficiency Virus

HIV/TB – HIV and TB coinfection

INH – Isoniazid

IRIS - Immune Reconstitution Inflammatory Syndrome

IUATLD – International Union Against Tuberculosis and Lung Disease

KAN – Kanamycin

LAM - Latin America Mediterranean

LFX – Levofloxacin

LIS - Laboratory Information System

LJ - Lowenstein-Jensen

LOD – Limit of Detection

LPA – Line Probe Assay

LZD - Linezolid

MDR-TB -Multidrug Resistant Tuberculosis

MGIT – Mycobacterium Growth Indicator Tube

mRNA – Messenger RNA

M.tb – Mycobacterium Tuberculosis

MTBC- Mycobacterium Tuberculosis Complex

NAAT – Nucleic Acid Amplification Test

NaOH – Sodium Hydroxide

NPV – Negative Predictive Value

NTM – Non-Tuberculosis Mycobacterium

NTP – National Tuberculosis Programme

NTRL – National Tuberculosis ReferenceLlaboratory

OFX - Ofloxacin

PAS - P-aminosalicylic acid

PCR – Polymerase Chain Reaction

PPE – Personal Protective Equipment

PITC – Provider Initiated Testing and Counselling

PLHIV – People Living with HIV

POC – Point of Care

PPV – Positive Predictive Value

PTB – Pulmonary Tuberculosis

PZA – Pyrazinamide

RFLP – Restriction Fragment Length Polymorphism

RIF – Rifampicin

RNA – Ribonucleic Acid

*rpo*B – RNA Polymerase β Subunit

RRDR – Rifampicin Resistant Determining Region

RR-TB – Rifampicin Resistant Tuberculosis

rt-PCR – Real time PCR

SNP - Single Nucleotide Polymorphism

SR – Sample Reagent

STR – Streptomycin

TB – Tuberculosis

TB-DR – Tuberculosis Drug Resistance

TSR – Treatment Success Rate

UNC – University of North Carolina

WHO – World Health Organisation

XDR-TB – Extensively Drug Resistant Tuberculosis

ZN – Ziehl-Neelson

Thesis Layout

This thesis is divided into 7 chapters: Chapter 1 provides literature review; Chapter 2 provides overview of tuberculosis in Malawi and research objectives; Chapter 3 describes the prevalence of drug resistant tuberculosis among outpatients; Chapter 4 describes the accuracy of Xpert MTB/RIF among presumptive TB outpatients; Chapter 5 describes molecular characterization and distribution patterns of RIF resistant tuberculosis strains from Malawi; Chapter 6 provides conclusions and recommendations from the study findings; Chapter 7 contains appendices.

CHAPTER 1

Literature Review

1.1 Global Overview of Tuberculosis

Tuberculosis (TB) is a chronic, infectious, airborne disease caused by the bacillus *Mycobacterium tuberculosis* (MTB). TB remains one of the leading single-agent, potentially curable infectious diseases and an important public health problem (1) that dates back to 1882 when Robert Koch discovered the causative agent (2, 3). TB was first declared a global emergency by the World Health Organisation (WHO) in 1993, in recognition of the large increase of notified cases worldwide (4). TB continues to be a major cause of morbidity and mortality in the developing world. The global impact of TB is significant with an annual estimate of 10.4 million new (incident) TB cases and over 1.4 million deaths due to TB in 2015 (1). This global disease burden and death caused by TB makes it one of the most dangerous and important diseases in the history of mankind (1).

The majority of the 2015 TB cases (61%) occurred in Asia, India (23%) and China alone accounted for 10% of the total cases. Relative to population, the WHO African Region had the highest rate of cases (26%) and deaths in 2015 (Figure 1) (1). With an exception of a few countries, the overall TB incidence rates are beginning to fall with an average of 1.5% per year (5).

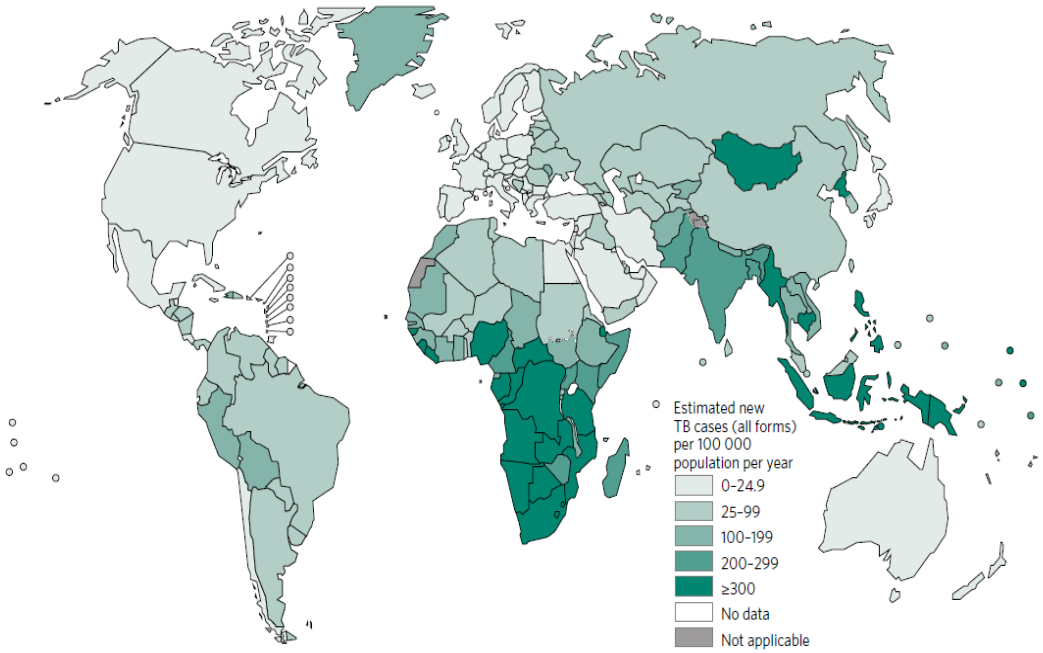

Figure 1: Estimated TB incidence rates for 2015 (1).

However, the global decline in the TB incidence rate is below the first milestone of the End TB Strategy which is aimed at reaching an annual global reduction of 4 – 5% in the TB incidence rate and a 10% reduction in the global case fatality ratio by 2020. To achieve these milestones, it requires a substantial acceleration in the current rate of progress in reducing the TB disease burden (1).

The emergence of drug resistance to currently available anti-TB drugs guarantees that TB will remain a significant public health concern in the coming century. Multi-drug-

resistant TB (MDR-TB) has emerged as a major public health concern in several countries and is a serious threat to global TB control (1). Globally, it is estimated that 3.9% of new cases and 21% of previously treated cases have MDR-TB/RR-TB and that 9.5% of these have extensively drug resistant TB (XDR-TB) (1). In 2015, MDR-TB cases accounted for 83% of all MDR-TB/RR-TB cases worldwide, with varying proportions by country and region. Of the estimated 580,000 incident cases, there were approximately 250,000 deaths due to MDR-TB/RR-TB (1).

The emergence of XDR-TB strains, defined as MDR-TB strains resistant to any fluoroquinolone and at least one of the three injectable second-line drugs i.e. Kanamycin (KAN), Capreomycin (CAP) or Amikacin (AMK) has resulted in poorer treatment outcomes with high mortality especially in TB/HIV co-infected individuals (6, 7, 8, 9). By the end of 2015, about 117 WHO member states detected XDR-TB cases. About 7,579 of the XDR-TB cases were detected by 74 countries, of which 1,100 were reported in Africa (1).

1.2 Pathogenesis of Tuberculosis

TB mainly affects the lung parenchyma or the tracheobronchial tree (pulmonary TB; PTB) but can also affect other sites such as tissues or organs outside of the lungs (extra-pulmonary TB; EPTB) e.g. Pleura, lymph nodes, joints and bones, meninges etc (10, 11). TB is transmitted from person to person through air by droplet nuclei which are produced when persons with PTB or laryngeal tuberculosis sneeze, cough, speak or sing. Additionally, droplet nuclei can be generated during bronchoscopy, sputum induction, aerosol treatments and through manipulation of lesions or processing of secretions or tissues (12). The droplet nuclei are carried down the bronchial tree upon inhalation and implant in the alveoli or respiratory bronchioles in the lungs. Depending on bacterial virulence and inherent microbicidal ability of the alveolar macrophage, the ingested tubercle bacilli can either establish an infection in the lung (active TB) or not (latent TB) (12).

Tuberculosis disease manifests differently depending on several factors including both host and micro-related characteristics and interactions (10). Prior to the HIV epidemic, most TB infections (approximately 85%) were limited to the lungs and the remainder involved non-pulmonary or both pulmonary and non-pulmonary sites (13). However, in individuals with HIV infection, about 38% have PTB, 30% have

EPTB and 32% have both PTB and EPTB (14). Signs and symptoms of PTB include cough for at least two weeks, night sweats, chills, fever, haemoptysis (coughing up blood), fatigue and weight loss. Signs and symptoms of EPTB depend on the site of disease (10, 12, 15).

1.3 Global TB/HIV Co-infection

HIV infection is the strongest risk factor for TB (16). TB/HIV co-infection form a deadly combination because each enhances progression of the other (17, 18). HIV increases the risk of activating latent TB infection (LTBI) due to a reduction in the body's immune defences (19, 20, 21, 22). The risk of developing TB disease is 20 - 30 times greater in HIV infected individuals that in those not infected with HIV (5). The clinical presentation and laboratory diagnosis of active TB is complicated in HIV infected individuals (23). HIV-infected patients with active pulmonary TB (PTB) often have low bacillary loads (24), absence of Acid Fast Bacilli (AFB) on sputum smear microscopy (negative sputum smear results), atypical chest radiographic abnormalities and an increased prevalence of EPTB (13). In advanced stages of HIV infection, signs and symptoms of EPTB are often masked (3). This can lead to misdiagnosis or delays in diagnosis using conventional TB diagnostic methods, resulting in a higher morbidity and mortality (17, 18).

Globally, among the 10.4 million new TB cases in 2015, 1.2 million (11%) were co-infected with HIV. The majority of TB/HIV co-infections were from the WHO African Region with over 50% being from parts of Southern Africa (Figure 2). In Sub-Saharan Africa, up to 71% of TB cases are HIV associated (1).

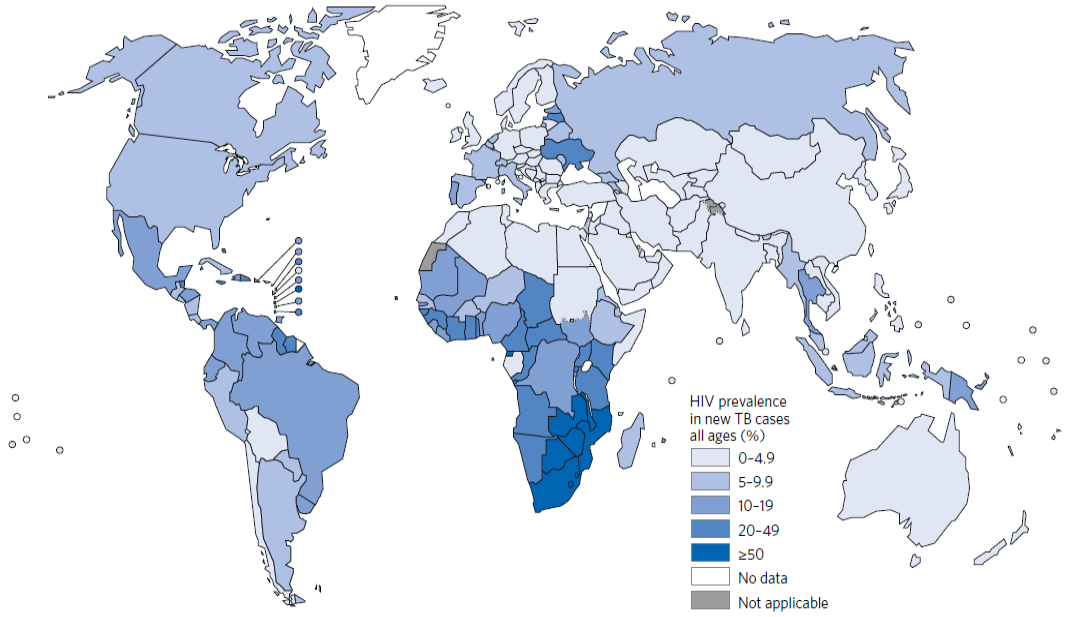

Figure 2: Diagram depicting the percentage of new tuberculosis cases with HIV infection in 2015 (1).

Although there has been a global decline in the number of TB deaths between 2000 and 2015 (Figure 3), TB remains one of the top 10 causes of death worldwide. In 2015, TB was responsible for 400,000 deaths in HIV infected people. Most of these deaths

occurred in low and medium-income countries, with 75% of them occurring in the WHO African Region (1). The number of TB deaths is unacceptably large considering access to health care and provision of the right treatment to infected individuals (10, 5). The disease is preventable and largely curable if detected early and treated effectively (25).

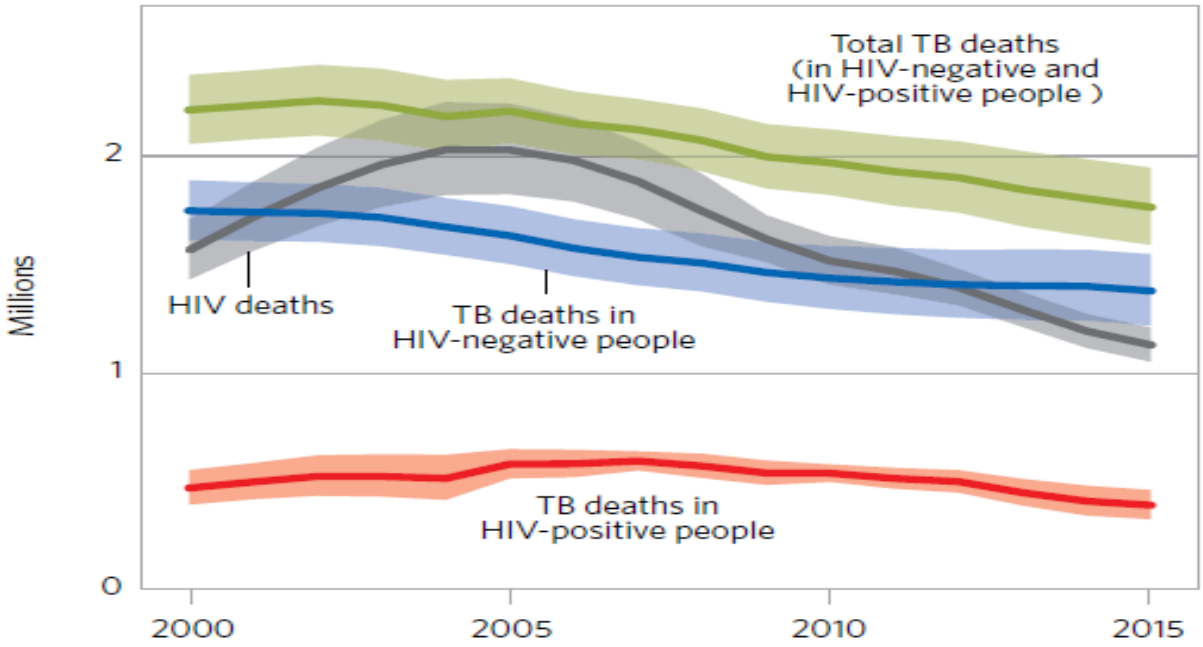

Figure 3: A graphical representation of the global trends of deaths among both HIV-negative and HIV-positive TB patient from 2000 to 2015 (1)

TB treatment in HIV co-infected patients is often challenging due to interactions of drugs used in treating both diseases, their side effects and the risk of developing TB-associated immune reconstitution inflammatory syndrome (IRIS) (26, 27). Furthermore, the mortality risk increases with drug-resistant TB (DR-TB) infections in TB-HIV co-infected patients (28, 29, 30). HIV infection may lead to acquisition of rifamycin resistance due to malabsorption of anti-TB drugs. MDR-TB strains present a major challenge to effective treatment of this infection especially in people living with HIV (PLHIV) (31). MDR-TB appears not to cause infection or disease more readily than drug-susceptible TB in HIV-infected persons (32).

1.4 Drug development and TB Chemotherapy

When TB infection cannot be sufficiently contained by host immunity, antibiotics are used as exogenous agents to fight the disease. Treatment for TB dates back to 1944 with the discovery of P-aminosalicylic acid (PAS) and streptomycin (STR) (33, 34). The first use of multi-drug therapy against TB was initiated after 1950 drug efficacy studies showed that a combination of PAS and STR was more effective than when either of the two was used alone (35, 36). Treatment efficacy greatly improved when Isoniazid (INH) was added to the treatment regimen in 1952. In some cases, the cure rate reached 100% although the treatment duration remained relatively long (18 to 24 months) (36). The duration of treatment reduced to 18 months when PAS was

substituted with Ethambutol (EMB) in 1960. The introduction of rifampicin (RIF) in the early 1970s and pyrazinamide (PZA) in 1980 further reduced the duration of treatment to 9 and 6 months respectively (35).

In general, the current first line treatment drugs for TB include RIF, INH, EMB, PZA, and STR (Table 1). The treatment duration using these drugs is six (6) months. Treatment for DR-TB is complex, usually requiring two or more years of therapy, with drugs that are less potent and more toxic than the drugs used to treat drug-susceptible TB (5).

Second-line drugs (Table 2) include varying combinations of AMK, CAP, KAN, Ciprofloxacin (CIP) and Ofloxacin (OFX) based on their effectiveness and safety. Linezolid (LZD) and clofazimine (CFZ) are now recommended by the WHO as core-medicines in treating MDR-TB. Additionally, the WHO has recommended treating all rifampicin resistant TB (RR-TB) cases with a MDR-TB regimen, regardless of their susceptibility to INH (1). For all patients with pulmonary MDR-TB/RR-TB (with an exception of pregnant women), a standardised shorter MDR-TB regime of 9 – 12 months is now recommended if there is no resistance to second-line drugs (1). The

mode of action of each of these anti-TB drugs is well understood as described in Tables 1 and 2. Their relevance indicate the significance of their bacterial targets (35).

1.5 Development of Drug Resistance

Global TB control is hampered by the emergence and spread of DR-TB strains (37). The Phenomenon of drug resistance in TB was first described during the initial human trial of TB treatment in 1948 mainly due to the use of STR as a monotherapy (38). After deciphering the full genome sequence of MTB in 1998 (39), the knowledge of the mechanisms of resistance to anti-TB drugs has increased.

1.5.1 Intrinsic Drug Resistance

The mycobacterium cell envelope, which is comprised of a capsule-like polysaccharide coat and a thick hydrophobic cell wall which is rich in long-chain fatty acids such as C_{60} and C_{90} (40), offers an intrinsic resistance mechanism. Drug uptake is minimised due to multi-drug efflux pumps (41) while the highly hydrophobic multi-layer cell envelope provides an effective permeability barrier (42). Presence of arabinogalactan coupled with the unusual length of the fatty acids also contributes to its poor permeability (39, 43).

1.5.2 Acquired Drug Resistance

Development of drug resistance in MTB is through genetic mutations and not by the acquisition of new DNA (2) as is the case with other bacteria. Confirmation of the causality between drug resistance and mutations has been made for only a subset of genes (44). Drug resistance in MTB is mainly acquired by spontaneous chromosomal mutations at low frequency of mycobacterial replication, producing a selection of resistant strains (37). Such mutations affect either the bacterial enzyme that converts a prodrug into an active drug or the drug target itself (45, 46, 2). Factors that contribute to the development of drug resistance in MTB are man-made through erratic drug supply, patient non-compliance during disease treatment (possibly due to high tablet volume and/or lengthy treatment duration), improvement of patient's symptoms and socio-economic circumstances (45, 24). However, mathematical modelling and molecular epidemiology suggest that the majority of MDR-TB cases are due to ongoing transmission of multidrug-resistant strains than acquired resistance (94).

1.5.3 Resistance to Rifampicin

RIF is one of the principal first-line drugs used to treat MTB. It is a lipophilic rifamycin derivative and is the most important member of the rifamycin family currently in clinical use. RIF is active against both slowly metabolising (non-growing)

and actively growing bacilli (37). The drug binds to the β-subunit of the RNA polymerase (encoded by *rpo*B gene) and consequently interferes with transcription and inhibits the elongation of messenger RNA (mRNA). Resistance is provided by chromosomal mutations that alter the binding site, which then has lowered affinity for RIF, the organism escapes the inhibiting action and consequently the development of resistance (37). Selective toxicity is based on the far greater affinity for prokaryotic polymerases than for the equivalent human enzymes.

Mutations in a single gene seem responsible for almost every case of RIF-resistance. Nearly 90-95% of RIF-resistant strains carry either a small in-frame insertion/deletion or missense mutations within the 81-bp Rifampicin Resistance Determining Region (RRDR) of the *rpo*B gene mainly involving codons 531, 526 and 516 (47). RIF resistance may occur alone or in combination with isoniazid and other drugs. It has been reported in greater than 95% of MDR-TB strains (48) (49) such that the presence of resistance to RIF alone may serve as a proxy/surrogate marker for MDR-TB (50, (51, 52).

1.5.4 Multi-Drug Resistant TB

MDR-TB is defined as simultaneous resistance to RIF and INH with or without resistance to any other drug (3, 5). MDR-TB is classified as a global pandemic more deadly than AIDS, with a potential to destabilize society because of the large financial implications of the treatment and spread of MDR-TB strains (5, 53, 54, 55, 56).

Misuse of second-line drugs makes them ineffective against MDR-TB, thereby leading to the development of TB strains with amplified resistance called XDR-TB, which severely limits the chance of treatment success. With XDR-TB, the frequency of adverse events and death is high because treatment options are challenging and extremely limited (32). The WHO recommends that drug susceptibility testing (DST) for fluoroquinolones and second-line injectable agents be performed on all documented MDR-TB patients to determine the presence of XDR-TB (1).

Table 1: Molecular mechanisms of drug resistance in first-line treatment drugs for *Mycobacterium tuberculosis* (57, 37)

Antimicrobial	Mode of Action	Gene target	Role of gene product	Drug properties
Rifampicin	Inhibits RNA synthesis	*rpo*B	RNA polymerase β-subunit	Most effective sterilising agent. Bactericidal in less than 1hour. High potency.
Isoniazid	Inhibits mycolic acid biosynthesis & other multiple effects	*ahp*C *inh*A *kat*G *kas*A ndh	Alkyl hydroperoxide Enoyl ACP reductase Catalase-peroxidase β-ketoacyl-ACP synthase NADH dehydrogenase	Bactericidal after 24 hours. Kills >90% bacilli in first few days of treatment. High potency.
Pyrazinamide	Disrupts plasma membranes	*pnc*A or *rps*A	Pyrazinamidase / Nicotinamidase	Sterilising effect occurs within 2-3 months. Low potency Bactericidal
Ethambutol	Inhibits arabinogalactan	*emb*B	Arabinosyl transferase	Minimises the emergence of drug resistance Bacteriostatic. Low potency.
Streptomycin	Inhibits Protein synthesis	rrs *rps*L *gid*B	16S rRNA S12 ribosomal protein rRNA methyltransferase (G527 in 530 loop)	Bactericidal. Low potency.

Table 2: Molecular mechanisms of drug resistance in second-line treatment drugs for *Mycobacterium tuberculosis* (57, 37, (2)

Antimicrobial	**Mode of Action**	**Gene target**	**Encoded protein or RNA product**	**Role**
Ethionamide	Inhibits mycolic acid synthesis, an essential component of the bacterial cell wall Nicotinic acid derivative related to INH	*inh*A *eta*A/*et*A	Enoyl ACP reductase Flavin monooxygenase	Drug target
Amikacin/Kanamycin	Inhibits Protein synthesis	rrs	16S rRNA	Drug target
Capreomycin	Inhibits Protein synthesis	*tly*A	2'-O-methyltransferase	Drug target
Moxifloxacin	Inhibits DNA gyrase (topoisomerase II and IV)	*gyr*A *gyr*B	DNA gyrase subunit A DNA gyrase subunit B	Drug target Pro-drug conversion
Terizidone / Cycloserine	Competitively inhibits cell wall synthesis.	rrs	L-alanine racemase and D-alanine ligase	Drug target. Bacteriostatic
P-aminosalicylic acid [PAS]	Inhibits DNA precursor synthesis	*thy*A	Thymidylate synthase	Drug activation
Linezolid (member of the Oxazolidinones)	Inhibits protein synthesis Inhibits monoamine oxidase	*rplV* *rplD* *erm-37* *whiB7*	23S rRNA	Drug target Bacteriostatic

1.6 TB Diagnosis and Detection of Drug Resistance

One of the greatest obstacles to successful TB treatment and control is the ability to diagnose the disease and determine drug susceptibility (24). In low and middle-income countries, sputum smear microscopy remains the most common primary method used to diagnose active TB (24, 58). Some advantages of this method include its low cost, speed, simplicity and high specificity in high TB burden areas (59). However, this method has a modest sensitivity of less than 60% (59, 60, 61, 62) and the sensitivity decreases among HIV co-infected TB individuals to between 38% – 54% (63, 64). The number of bacilli secreted into sputum by HIV-positive patients with PTB is fewer than in HIV-negative patients which makes TB diagnosis difficult using conventional microscopy (65). In addition, smear microscopy has no ability to differentiate between drug-sensitive and drug-resistant MTB (66)

Culture for mycobacterium is still considered the gold standard for TB diagnosis and drug susceptibility evaluations (3). Culture detects as low as 10 – 100 viable bacteria/ml and is considered a more sensitive test in detecting MTB as compared to sputum smear microscopy which requires approximately 5,000 - 10,000 bacteria/ml of sputum for detection (24). With MTB culture, the efficacy of diagnosing treatment failure cases and detecting TB in HIV-infected individuals improves considerably

since culture techniques detect fewer bacilli. Furthermore, culture provides sufficient material for identification tests and DST (67). However, culture takes up to six weeks to obtain results, requires a high degree of technical expertise and specialised (containment) facilities such as Biosafety Level 3 laboratory (BSL3). Additionally, culture for mycobacterium is prone to contamination and is labour intensive (24).

1.7 Molecular Diagnostics and Drug Susceptibility Testing

The WHO has prioritized the development of new tools to fight and control the TB epidemic with bold aims of improving TB diagnosis, treatment, and vaccination in order to eliminate the disease by 2035 (5).

There are a number of commercial molecular diagnostic assays available for detection of the MTB complex although many countries still rely on older TB diagnostics. Other than the Xpert MTB/RIF, there is considerable interest in new technologies i.e. NAAT or molecular diagnostics which can rapidly detect MTB and determine drug resistance (Table 3) (68). Molecular diagnostics can be robust with fewer manipulations and may be adapted for high throughput. Furthermore, these diagnostics are fast, do not need viable bacilli, require simple-to-use equipment and less technical skill as compared to culture-based methods (24).

The clinical significance of molecular diagnostics in predicting drug resistance is challenging due to the limit of detection (LOD) when testing hetero-resistant populations considering that the predominant population is likely to produce a positive signal (24, 69).

Table 3: Tuberculosis Diagnostic Technologies and/or the detection of drug resistance (2, 52, 68, 70, 71, 72, 73)

Technology*	**Principle**	**Target region**	**Time**	**Intended use (Diagnosis and/or Drug resistance)**
Xpert MTB/RIF	-NAAT (qPCR) -Automated (extraction, amplification, detection) quantitative MTB/RIF test, based on a single-use disposable cartridge	*rpo*B	2 hrs	-Diagnose MTB and genotypic resistance to RIF -Increase in TB case notification and RIF-resistant TB case detection rates
GenoType MTBDR*plus* (First-line LPA)	NAAT (LPA) Transcription-mediated amplification	*rpo*B *kat*G *inh*A	1 – 2 days	-Diagnosis of active TB -Detection of genotype resistance to RIF and INH -Rapid and sensitive compared to smear microscopy and culture -Interrogates a large number of targets -Comparatively low cost as compared to culture-based DST
BD Probe Tec	Strand displacement amplification	*IS 6110*	4 hrs	-Direct NAA assay -uses processed sputum specimen
Amplified MTD	Transcription-mediated amplification	16S rRNA	3.5 hrs	-Direct NAA assay -uses processed sputum specimen

Inno-LiPA Rif TB test	-PCR -Reverse hybridisation	16S-23S DNA spacer region	1 – 2 days	-Detection of MTB and other potentially pathogenic mycobacteria -Detection of RIF resistance -Simultaneous detection of mixed and contaminated mycobacterial cultures
Amplicor PCR	PCR based	16S rRNA	6.5 hrs	-Direct NAA assay -uses processed sputum specimen
GenoType MTBDR*sl* (Second-line LPA)	NAAT (LPA)	*gyr*A *emb*B *rrs*	1 – 2 days	Detects drug resistance to fluoroquinolones, plus second-line drugs
Loopamp MTB Complex assay (LAMP)	NAAT Loop-mediated isothermal amplification	*gyr*B 16S rRNA	Same day	-Diagnosis of active TB -uses processed sputum specimen -Simple and rapid -High specificity -Minimal training requirement

*Not an exhaustive list; TB, tuberculosis; DST, drug susceptibility testing; qPCR: quantitative PCR; LPA, line probe assay; NAAT, nucleic acid amplification test; LAMP, loop-mediated isothermal amplification; MTB, Mycobacterium tuberculosis; RIF, Rifampicin; WHO, World Health Organisation; INH, Isoniazid;

Detection of drug resistance in MTB using Genotypic DST methods target well characterized resistance associated mutations (Table 3). Molecular detection of resistance to RIF is particularly suitable because 95% of mutations associated with RIF resistance are present in the RRDR (74). For other anti-TB drugs, molecular detection of resistance mutations is complex because it requires detection of resistance mutations in multiple genes for good concordance with phenotypic results e.g. INH and some second-line drugs (Table 1 and 2). Genotypic DST will be a more powerful technique in future with improvements in multi-analyte detection and multiplex-PCR technologies (75).

1.7.1 Xpert MTB/RIF

The WHO endorsed the Xpert® MTB/RIF assay in December 2010 as a novel, rapid, automated, cartridge-based NAAT that simultaneously detects TB and RIF resistance (50, 74). Xpert MTB/RIF is a hemi-nested real time PCR (rt-PCR) assay which utilises the molecular beacon technology which recognises and reports the presence or absence of the *rpo*B gene sequence of the wild-type TB (76, 77, 78). Xpert MTB/RIF amplifies the RRDR of the *rpo*B gene and the five overlapping probes hybridise to the amplified region (Figure 4).

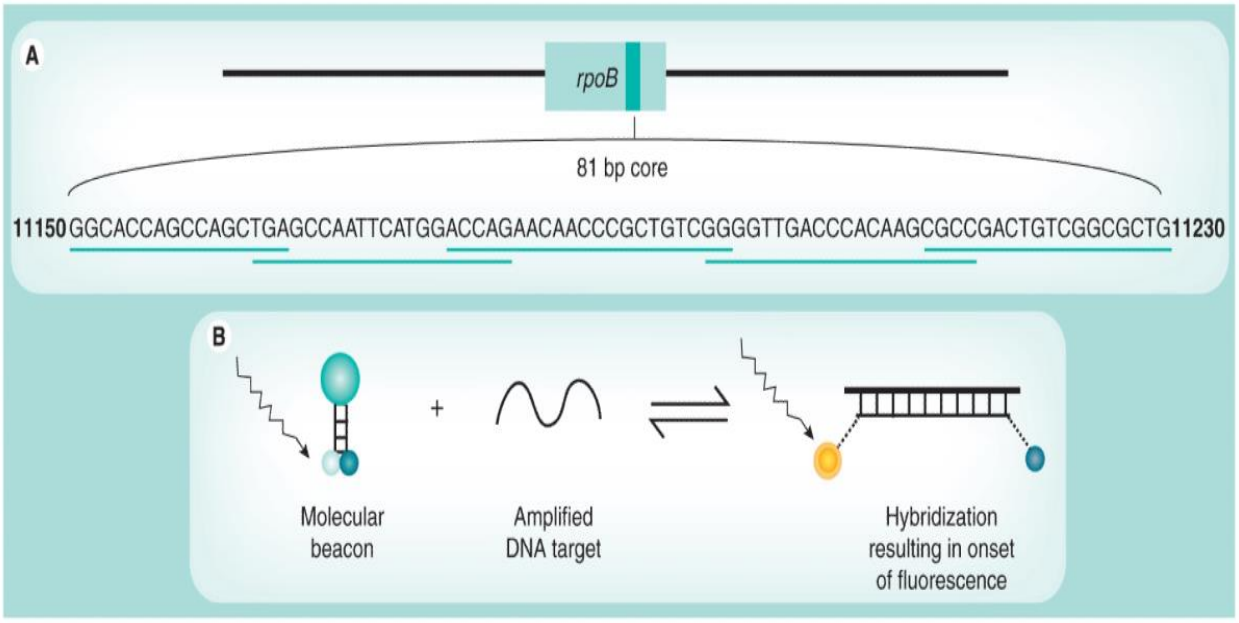

Figure 4: Target sequence of the *rpo*B gene core region and molecular beacon technology (A) shows the *rpo*B gene, nucleotide sequence of the 81bp core region of *rpo*B gene and the position of the complementary molecular beacon probes that span the entire RRDR (B) Structure of a molecular beacon and onset of fluorescence as per (79).

Identification of MTB is determined when at least two probes hybridise while RIF-resistance is detected when 1 or more probes do not hybridise (called a drop out) or when there is a delayed binding of the beacon to the matching sequence [called a delayed] (Figure 5) (74, 50).

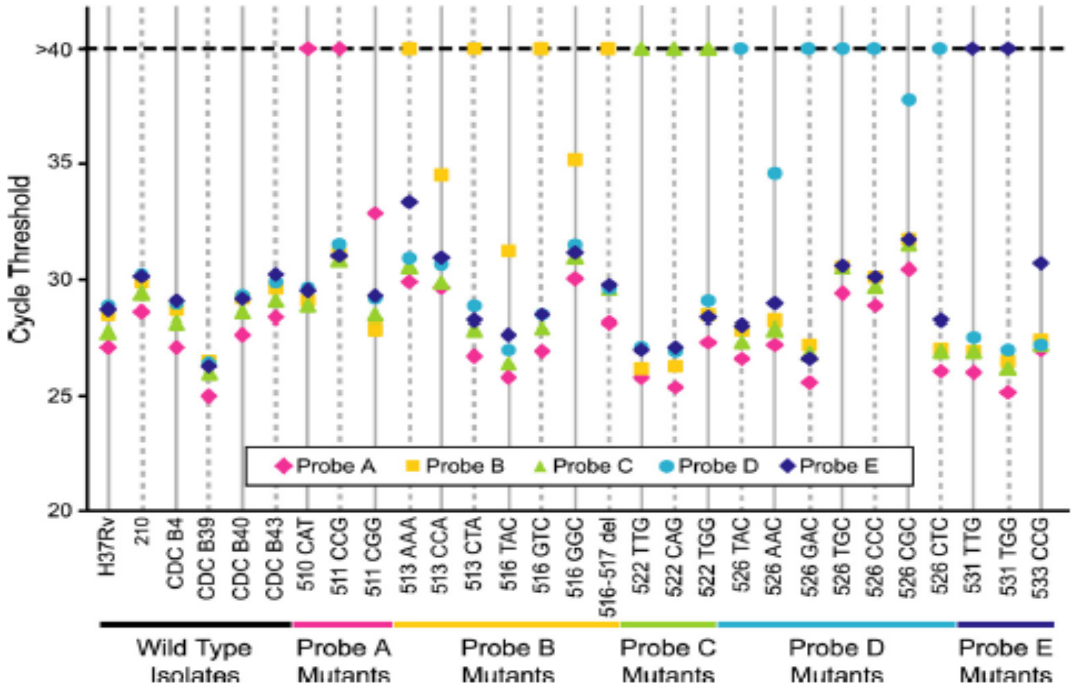

Fig 5: Showing mutations detected in the RRDR by probes A - E. Each sample result is indicated by a single vertical line showing all the five *rpo*B probes, with ΔC_T values ≥ 3.5 cycles indicating RIF Resistance (50).

This technology has emerged and remains one of the most promising tools for rapid TB diagnosis and screening for resistance to RIF (80, 81, 82, 83). It can potentially be performed at or near point-of-care (POC) (50) and the turnaround time for TB detection and RIF resistance can be shortened from a median of 7 or 21 days for MTBDR*plus* assay (version 2) and phenotypic DST respectively to around 2 hours for Xpert MTB/RIF after placing the cartridge in the instrument (Figure 6) (84, 85). The TB diagnostic landscape has made a significant shift from sputum smear microscopy to the Xpert MTB/RIF owing to the superior accuracy of the latter (25, 86, 87, 88, 89).

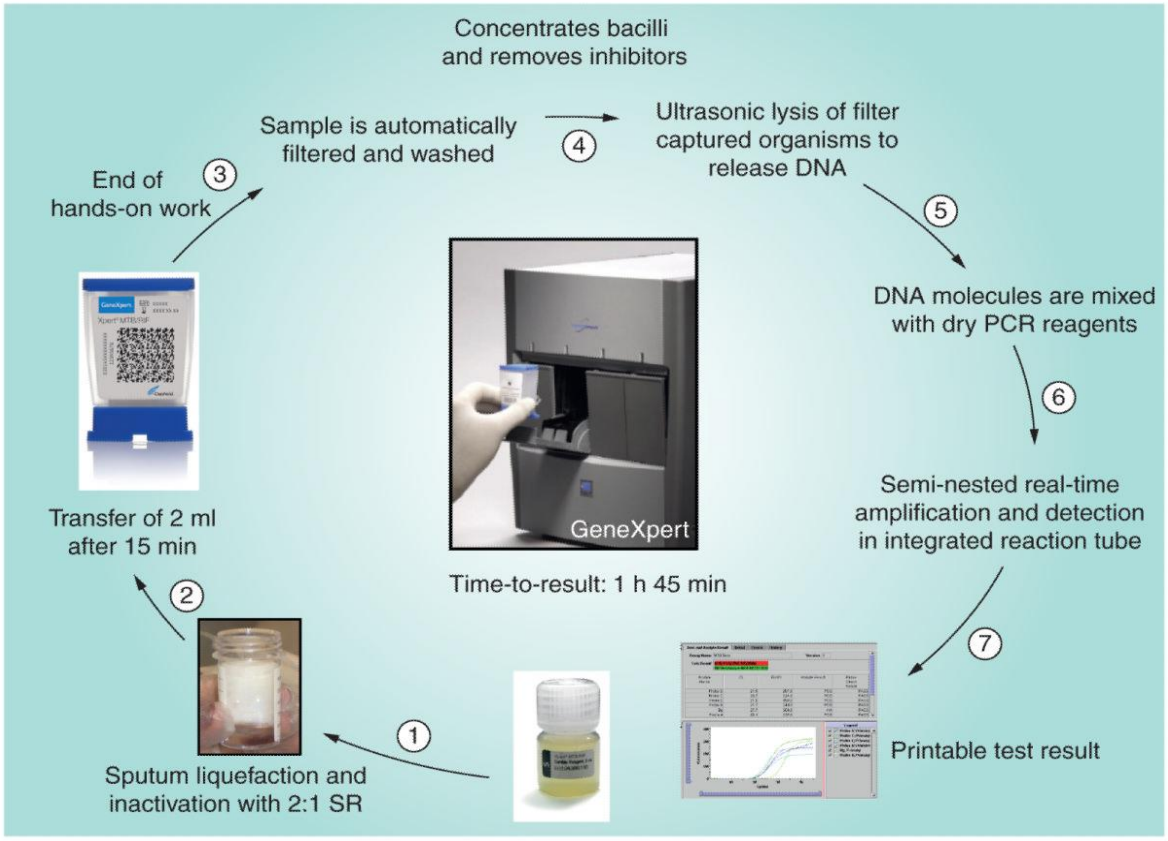

Figure 6: A graphical representation of the assay procedure for the Xpert MTB/RIF Test; showing both the manual and automated processes (79)

Xpert MTB/RIF is reported as having a pooled sensitivity of 94%, which is much higher than smear microscopy but is similar to that of solid culture in detecting TB (5, 90) and a specificity of 98% for the detection of RIF resistance (80). Among smear-positive PTB samples, Xpert MTB/RIF has a pooled sensitivity of 98.7% (95%CI 98.0% – 99.2%), but has a substantially lower sensitivity of 75% (95%CI 72.0% - 77.8%) among smear-negative PTB samples. The overall pooled sensitivity in diagnosing for

RIF-resistant PTB is 94.1% (95%CI 91.6% - 96.0%) and 97.0% (95%CI 96% - 97.7) pooled specificity (80).

Xpert MTB/RIF is strongly recommended as an initial diagnostic test for use on pulmonary specimens from people suspected of having HIV-associated TB and those at risk of MDR-TB (5, 91, 92). Furthermore, the WHO recommends the use of Xpert MTB/RIF as an initial diagnostic test for Cerebral Spinal fluid (CSF) in patients suspected of TB meningitis and as a follow-on-test to microscopy in adult TB suspects not at risk of MDR-TB or HIV-associated TB where further testing of smear-negative specimens is necessary (76). Details of the performance characteristics of Xpert are detailed in Table 4.

Table 4: Performance characteristics of Xpert MTB/RIF assay (25, 92, 93)

Diagnostic	Performance characteristics	Advantages	Disadvantages
Xpert **MTB/RIF**	**<u>Pulmonary TB</u>** As initial diagnostic: Pooled sensitivity and specificity of 88% and 99% respectively when used in place of smear microscopy As an add-on test: pooled sensitivity and specificity of 68% and 99% respectively following smear-negative microscopy In PLHIV: pooled sensitivity of 79% Pooled sensitivity in diagnosing for RIF-resistant PTB is 94.1% and 97.0% pooled specificity **<u>Extra-pulmonary TB</u>** <u>(compared against culture)</u> In CSF: 79.5% pooled sensitivity and 98.6% pooled specificity Gastric lavage and aspirate: 83.8% pooled sensitivity; 98.1% pooled specificity Pleural fluid: 43.7% pooled sensitivity; 98.1% pooled specificity	Simultaneous detection of tuberculosis and RIF resistance Lower biosafety requirements Simpler contamination control Widespread application can increase the case detection rates Minimal hands-on technical time Integrated sample processes in a single, self-enclosed cartridge Uses both processed and unprocessed sputum specimens	Capital cost for GeneXpert instrument is significantly higher Requires uninterrupted and stable power supply Temperature control (maximum of 30^0C) Yearly GeneXpert instrument calibration Need for confirmation of RIF resistant result by culture and DST

The Xpert MTB/RIF has been rapidly adopted by many countries, including Malawi, as an initial diagnostic test (5) and by 2015, 69% of countries had recommended its use in individuals at risk of DR-TB while 60% recommended it for use in HIV-positive cases (5). As of December 2015, a cumulative total of 4,672 GeneXpert instruments had been procured in countries eligible for concessional pricing which comprised of 21,549 modules. Over 6.2 million Xpert MTB/RIF test cartridges were procured by the 122 countries in 2015 alone (1).

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CHAPTER 2

Overview of Tuberculosis in Malawi and Research Objectives

2.1 Introduction

Malawi is a landlocked country with an area of 118,484 km^2, of which 94,276 km^2 is land. The country is located south of the equator and shares boundaries with Tanzania in the north, Mozambique in the south, southwest and the east, and Zambia in the west. Malawi is divided into three administrative regions namely Northern, Central and Southern Regions and there are 28 administrative districts (Figure 1). Malawi's population was estimated at 16.5 million people in 2014, of which, 85% live in rural areas (1).

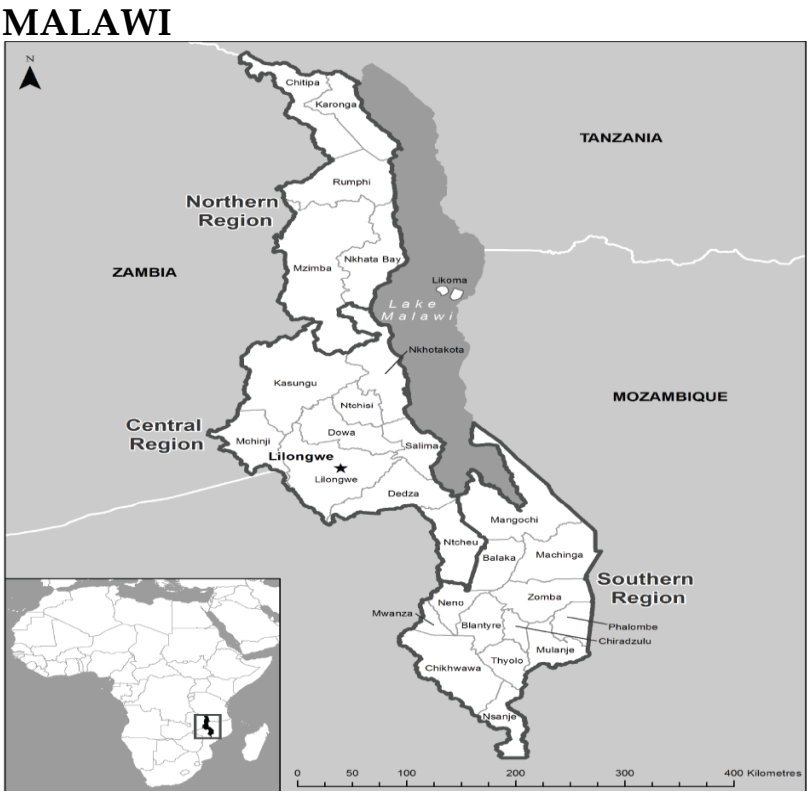

Figure 1: Map of Malawi illustrating its borders, Regional boundaries and the 28 administrative districts. Insert - Map of Africa showing Malawi's location on the continent (1)

2.2 Tuberculosis in Malawi

TB remains one of the main priority diseases of public health significance among the adult population in Malawi despite significant progress in the fight against the epidemic. The TB/HIV burden in Malawi is one of the highest in the sub region and drug-resistant TB strains have emerged over the years (2). Malawi's overall TB incidence rate is estimated at 193/100,000 with a mortality rate of 52/100,000 (HIV-negative and HIV-positive people) (3).

There has been a 2 - 5% annual decline of notified TB cases since 2005, reaching 42.9/100,000 for new smear-positive cases and 121/100,000 in 2013 for all forms of TB which is consistent with the WHO estimates that there is a downward trend for TB prevalence in Malawi since 2000 (4, 2). The number of new reported smear-negative cases has been surpassed by new smear-positive cases since 2011 (Figure 2) probably due to improved AFB smear microscopy with the introduction of LED fluorescence microscopes (5). Additionally, this could be due to a change in the case definition where one sputum smear-positive result is required to define a smear-positive case by either conventional or fluorescence microscopy (2).

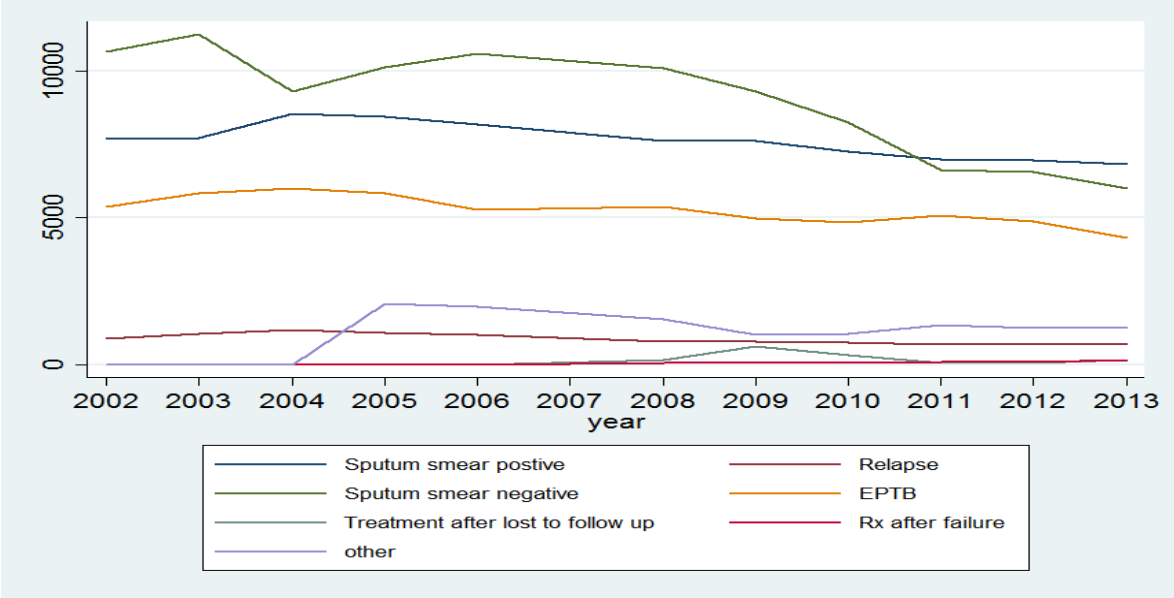

Figure 2: Trend of total notified TB cases by category of disease for the period 2002-2013 which shows that notification has consistently declined over the 8 year period in Malawi (2).

2.2.1 TB/HIV Co-infection in Malawi

Malawi is listed among the 30 high TB/HIV burden countries by the WHO and has consistently reported that ≥ 90% of TB patients know their HIV status. In Malawi, the rate of TB/HIV co-infection has declined from 69% in 2005 to 56% in 2013 which has been attributed to an increase in the proportion of HIV tested presumptive TB patients from 51% to 92% in the same period (2, 6). Additionally, the observed decline in TB case notification rate among HIV infected individuals is also attributed to an increase in ART uptake which increased from 54% in 2010 to 94% in 2015 (6).

Figure 3 shows trends in absolute numbers of notified TB patients in relation to HIV status and ART uptake.

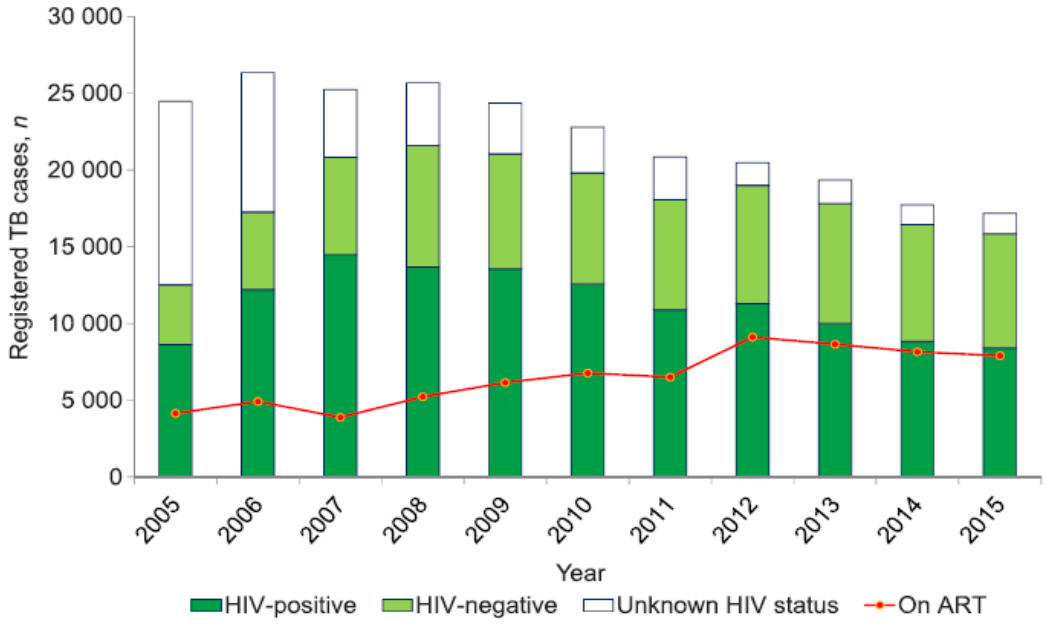

Figure 3: Trends of notified TB patients in relations to HIV testing, HIV status and ART uptake in Malawi between 2005 and 2015 (6)

Blantyre and Chiradzulu districts have higher rates of TB/HIV co-infection than all other districts in the country (2). The WHO estimates that the TB incidence rate among HIV-positive individuals in Malawi is at 104/100000 population and the total number of deaths among people infected with HIV is estimated at 7000 (4).

2.2.2 Tuberculosis Test Algorithm for Malawi

Sputum microscopy remains the mainstay for TB diagnosis in Malawi (Figure 4). Presumptive TB patients in Malawi submit "spot" and "morning" sputum specimens for testing. The spot specimen undergoes smear microscopy on the day it is collected while the second specimen undergoes microscopy on the day of receipt in the laboratory. If both specimens are negative for AFB, Xpert MTB/RIF is performed on the residual sputum from the second specimen. All MDR-TB suspects and treatment experienced cases submit sputum specimens for Xpert MTB/RIF and for culture and DST for first-line drugs (Figure 4) before starting TB treatment (5). Once RIF resistance is detected, second-line TB treatment is immediately initiated in order to prevent transmission of MDR-TB, avoid treatment delays in true positive cases and the benefits of preventing death, despite the risk of a false-positive result considering the low background prevalence of MDR-TB in Malawi (5).

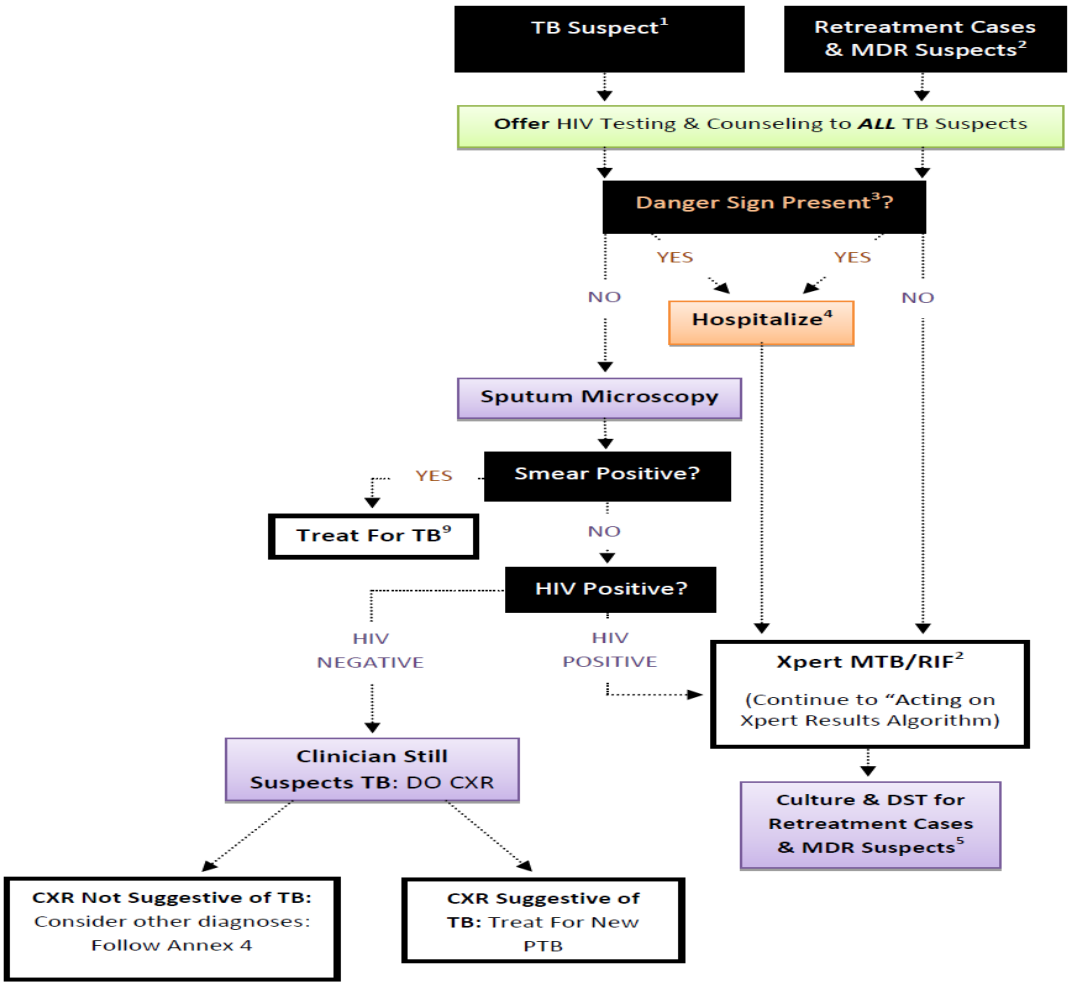

Figure 4: Algorithm for Malawi showing stages in management of presumptive and retreatment Tuberculosis patients which includes Xpert MTB/RIF testing (5).

2.2.3 Tuberculosis Treatment in Malawi

The Malawi National TB Programme (NTP) treatment guidelines for first-treatment patients include RIF, INH, EMB, PZA which come in a fixed dose combination tablet (FDC) and STR (injectable). For previously treated patients, the therapy includes STR,

INH, RIF, EMB and PZA. MDR-TB patients receive 6 months of treatment with Levofloxacin (LFX), CAP, Cycloserine (CS), Ethionamide (ETO) and PZA which is followed by a community based 18 months treatment with LFX, CS and ETO (5). Table 1 explains in details the treatment regimens used in Malawi for different forms of TB.

Table 1: Current guidelines for TB treatment in Malawi (5)

First line			
All new patients needing treatment except TB meningitis	Intensive Phase	Continuation phase	
	RIF, INH, EMB, PZA for 2 months	RIF, INH for 4 months	
TB meningitis	STR, RIF, INH, PZA, EMB for 2 months	RIF, INH for 7 months	Corticosteroids are given to reduce the risk of death
Previously treated for TB	STR, RIF, INH, PZA, EMB for 2 months, then RIF, INH, PZA, EMB for 1 month	RIF, INH, EMB for 5 months	Patient under DOT throughout the whole treatment course
Second line			
	Intensive Phase	Continuation phase	
Multi-Drug resistant TB	CAP, LFX, ETO, CS, PZA for 6 months	LFX, ETO, CS for 18 months	

RIF-Rifampicin; INH-Isoniazid; STR-Streptomycin; PZA-Pyrazinamide; EMB-Ethambutol; CAP- Capreomycin; CS-Cycloserine; ETO-Ethionamide; LFX-Levofloxacin; TB-Tuberculosis; DOT-Directly observed treatment

2.2.4 Drug Resistance in Malawi

2.2.4.1 Definition of an MDR-TB Patient

Presumptive MDR-TB patients in Malawi are defined among others as those with previous contact with a known MDR-TB patient, all patients who remain smear-positive after either 3, or 5 months of treatment (Table 2), patients previously successfully treated for TB and new patients who visited high MDR-TB prevalence areas like certain parts of South Africa, Lesotho etc (5).

2.2.4.2 Screening for Drug Resistance in Malawi

Xpert MTB/RIF is the initial screening test for DR-TB in Malawi since 2012. Sputum specimens from all smear-positive and smear-negative MDR-TB suspects are submitted for Xpert MTB/RIF testing and if RIF resistance is detected, the specimen is sent for culture and phenotypic DST at the National TB Reference Laboratory (NTRL) to confirm the resistance. RIF susceptible sputum specimens are also sent for culture and DST if RIF and other TB drug resistances are highly suspected clinically. Malawi NTP recommends that sputum specimens eligible for culture and DST should reach NRTL within 4 days of collection (5).

In 2011, a national wide TB drug resistance survey indicated that the prevalence of MDR-TB was 0.4% among new smear-positive TB cases with a prevalence of 0.3% for RIF mono resistance and 2.7% for INH mono resistance. MDR-TB prevalence was 4.8% among previously-treated TB cases (5).

2.2.4.3 Management of Patients with Multi-Drug Resistant Tuberculosis

In Malawi, MDR-TB is managed under outpatient and community-based strategy where patients receive treatment in their homes through treatment supporters/health workers and undergo longitudinal clinical follow-up at zonal DR-TB referral clinics. Sputum specimens are collected monthly both during intensive and continuation MDR-TB treatment phases (Table 4) to monitor progress (5). The Malawi strategy for managing MDR-TB patients poses a threat of transmission of MDR-TB strains into the community.

2.2.5 Treatment outcomes

Malawi's treatment success rate (TSR) was estimated at 84.3% in 2013 for sputum smear-positive cases with drug-susceptible TB. The TSR was lower in smear-negative than in new smear-positive TB patients. In previously treated individuals with the exclusion of relapse cases, TSR was at 75%. For MDR/RR-TB cases, the TSR was estimated at 53% (2, 3).

Table 2: Actions on treatment and laboratory diagnosis for both smear-negative and smear-positive sputum at different time points (5)

Treatment period	Smear Result	Action
End of 2 months	Negative	Continuation treatment phase
	Positive	Continuation treatment phase, repeat smear at 3 months
End of 3 moths	Negative	Proceeding on continuation phase
	Positive	Perform Xpert MTB/RIF, Culture & DST. Empirical MDR-TB treatment is considered for seriously ill patients
End of 5 months	Negative	Proceeding on continuation phase
	Positive	Perform Xpert MTB/RIF, culture & DST, starts retreatment regimen as treatment failure. Empirical MDR-TB treatment is considered for seriously ill patients

Smear microscopy is performed on sputum samples from retreatment patients at 3, 5 and 7 months of treatment. Any previously-treated cases that remain smear-positive at 5 or 7 months of therapy are initiated on empiric MDR-TB treatment with second-line drugs (Table 1) in consultation with NTP while waiting for DST results.

2.3 Molecular Genotyping of Tuberculosis in Malawi

Spoligotyping and IS*6110*-RFLP have previously been used to determine lineages/families of TB strains circulating in Malawi (7). About 75% of TB strains belong to lineage 4, with most being of the LAM family, 46% of which belong to LAM11 despite the existence of all MTB lineages (8, 9, 10). Recent studies have shown an increase in the Beijing genotype (lineage 2) over time and suggest that this genotype has stabilized at around 4% of TB cases in the northern Malawi district of Karonga (8) (Figure 5). A variety of restriction fragment length polymorphism (RFLP) patterns have been identified from patients in the district, suggesting several different introductions of the Beijing genotype strains whose origin is not known but might have come from areas where this genotype is commonly found e.g. from Asia, through Chinese agricultural advisors who had previously worked in the district (10, 11).

Although an epidemiologic link was not established in most of the cases except for 2 in a study by Glynn *et al* (10), RFLP patterns suggested an outbreak of the Beijing genotype. Temporal analysis indicated that the Beijing genotype had increased significantly in comparison to other genotypes primarily due to the KPS332 strain (10). Findings on the Beijing genotype strains from studies conducted in Malawi correlate with other studies in South Africa which showed that the Beijing genotype was increasing significantly and that it was the most prevalent genotype family (12, 13, 14).

Karonga District where Beijing genotype TB strains were predominantly found due to KPS332 strain

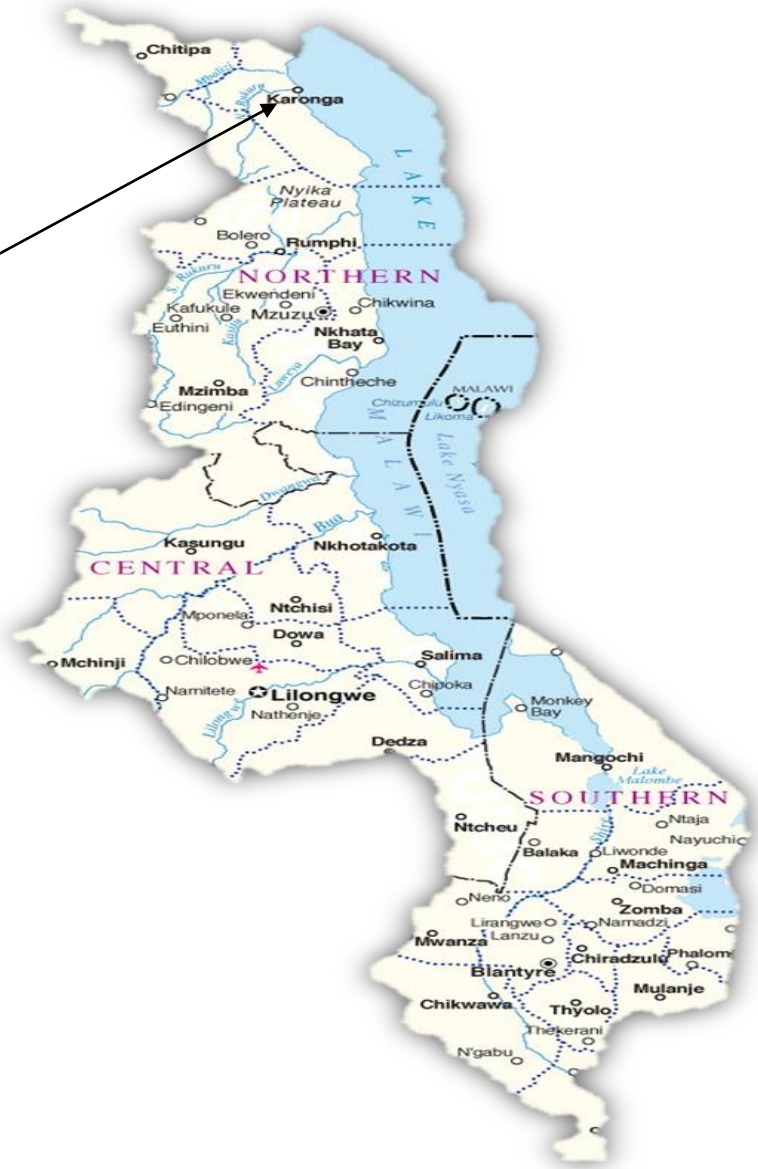

Figure 5: Map of Malawi showing Karonga District where the Beijing genenotype is suspected to have come from Chinese Agricultural Advisors (1)

2.4 Choice of Study area

2.4.1 Bwaila Hospital Specimen Description

Bwaila Hospital (Bwaila) is located in Lilongwe district in the central region of Malawi (Figure 6). Lilongwe district covers 6,159 km^2 and a total population of about 1,346,360. In 2008, a total of 428 patients were treated for TB at Bwaila of which 131 (30%) were first treatment patients while the remainder were previously treated (15). Presumptive TB patients attending service at Bwaila are either transferred from Kamuzu Central Hospital or from surrounding health facilities within the district. Between April 2011 and July 2012, our site (UNC Project, Lilongwe) conducted an observational study comparing the Mycobacterium Growth Indicator Tube (MGIT) and Hain GenoType MTBDR*plus* among outpatients initiating TB treatment at Bwaila (Chapter 3; published). From this study, sputum samples were frozen for further evaluations (Chapter 4).

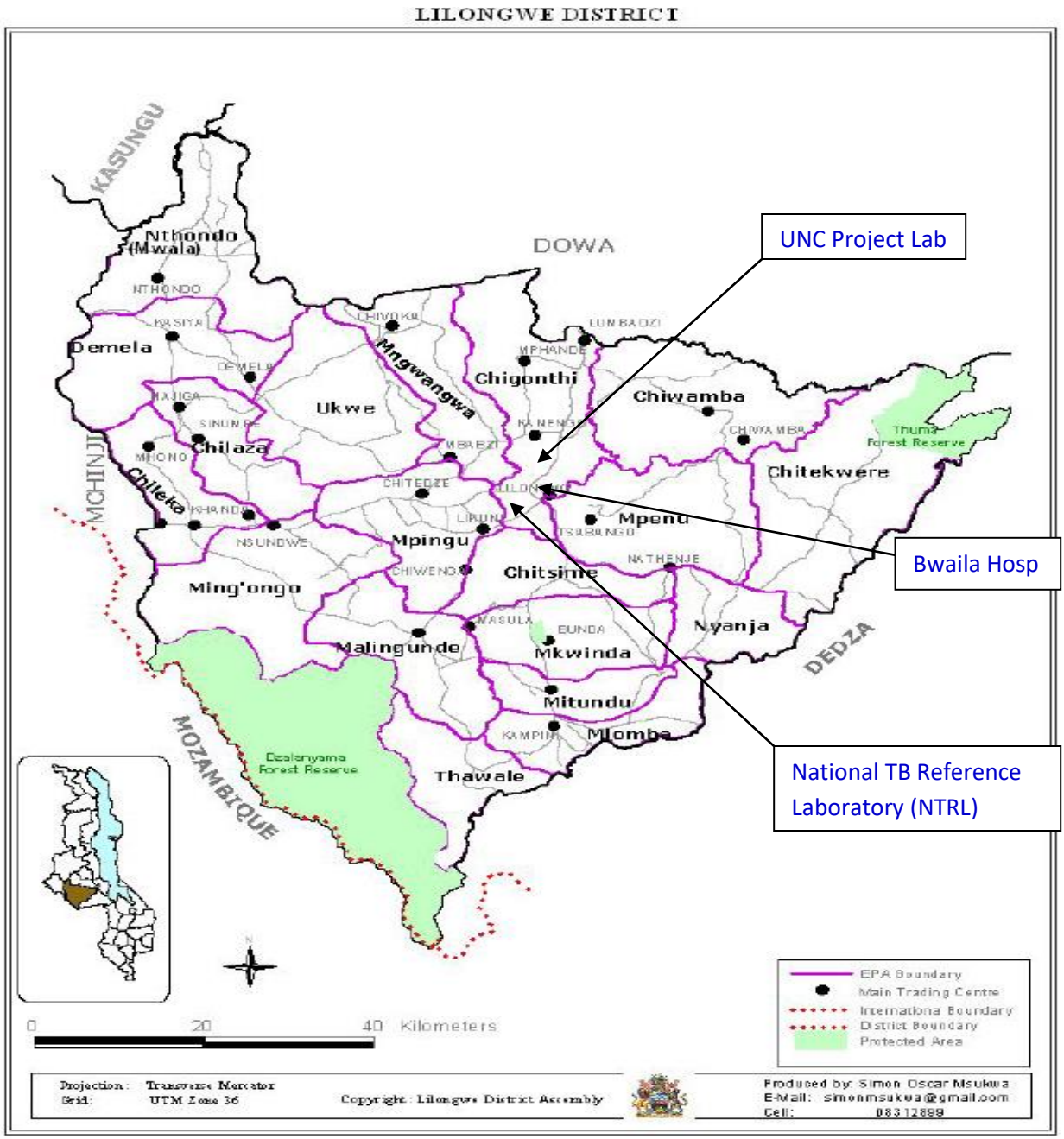

Figure 6: Map of Lilongwe District showing the location of Bwaila Hospital, UNC Project Laboratory and the National Tuberculosis Reference Laboratory.

2.4.2 National TB Reference Laboratory Specimen Description

Specimens in this category included pellets from TB patients' sputum which was referred to NTRL for Mycobacterial culture and DST. NTRL is located in Lilongwe (Figure 6) and receives sputum specimens from districts across Malawi. Work performed on these specimens is described in Chapter 5 which included DNA sequencing.

2.5 Research Objectives

The Malawi NTP recommends using the Xpert MTB/RIF assay as diagnostic tool for TB and detection of RIF resistance especially in sputum smear-negative and HIV infected individuals based on the previously described findings on sensitivity and specificity and that the median time to TB treatment is reduced from 56 days to just 5 days when using the Xpert® MTB/RIF assay for smear-negative TB (16). Since 2012 when the Malawi NTP recommended rolling out of the GeneXpert, there are currently over 50 GeneXpert instruments distributed across 40 of the 315 public and private diagnostic centres (laboratories) with Acid Fast Bacilli testing capacity in the country (2). However, to the best of our knowledge, no study has evaluated the performance of Xpert MTB/RIF in adult presumptive TB outpatients in Malawi. In

addition, the prevalence of *rpo*B gene mutations is not known in RIF-resistant MTB strains circulating in the country.

Thus, this study was aimed at determining the prevalence of drug-resistant TB in outpatients and evaluating the performance of Xpert MTB/RIF as a diagnostic tool for TB in a Malawian setting. Additionally, the study was aimed at assessing the RRDR of the *rpo*B gene for mutations responsible for RIF resistance in MTB strains and establishing the prevalence of such mutations in TB infected Malawians.

This was achieved by the following:

- Evaluating the sensitivity and specificity of Xpert MTB/RIF in comparison with fluorescence smear microscopy and culture (liquid and solid) for the diagnosis of MTB in presumptive TB outpatients at Bwaila Hospital in Lilongwe.
- Comparing the sensitivity and specificity of Xpert MTB/RIF with Hain MTBDR*plus* version 2 in detecting resistance to RIF.
- Comparing RIF-resistance, as defined by Xpert MTB/RIF, with nucleotide sequence data and determining the prevalence of *rpo*B gene mutations.

2.6 Ethics approvals

Ethical approvals for this study were granted by the following institutions;

a) University of the Witwatersrand Human Research Ethics Committee: # M120256 (Appendix 1)

b) National Health Sciences Research Committee (NHSRC) in Malawi: NHSRC # 999 (Appendix 2)

c) University of North Carolina (Chapel Hill) Institutional Review Board: # CID 1211 (Appendix 3)

d) National Health Sciences Research Committee (NHSRC) in Malawi (Change of title): NHSRC ≠ 999 (Appendix 4)

e) National Health Sciences Research Committee (NHSRC) in Malawi (Continuing renewals): NHSRC # 999 (Appendix 5)

f) UNC Project: Permission to conduct research at UNC Project Laboratories (Appendix 6)

g) Malawi National Tuberculosis Control Programme: Permission to access samples from NTRL (Appendix 7)

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CHAPTER 3

Prevalence of drug resistant tuberculosis among outpatients at an HIV/Tuberculosis clinic in Lilongwe, Malawi

Brian Barnett, Runa Gokhale, Robert Krysiak, Creto Kanyemba, **Tarsizio Chikaonda**, Maximina Kalikhoka, Mphatso Bokosi, Maggie Chirwa, Cornelius Mukuzunga, Friday Saidi, James Mpunga, Sam Phiri, Irving Hoffman and Mina C. Hosseinipour

Trans R Soc Trop Med Hyg 2015; 109: 763 – 768.

Summary

Recent estimates of MDR-TB in Malawi indicate that prevalence among patients with sputum smear-positive TB for both new and retreatment cases is low (0.4% in new cases and 4.8% retreatment cases). Additionally, RIF resistance is also low in Malawi. During the study period (2011), the WHO showed that in Malawi, the measured percentage of TB cases with MDR/RR-TB was 0.75% among new TB cases and 6.4% among previously treated TB cases.

In the present study, the main objective was to determine the prevalence of MDR-TB among presumptive TB outpatient presenting for treatment for the first time and also those previously treated at the Martin Preuss Centre at Bwaila in Lilongwe, Malawi. TB was culture confirmed in 375 of the patients enrolled in the study which comprised of both new (n=349) and retreatment (n=26) cases.

Our findings show predominance of INH mono-resistance (2.9%, 10/349), highlighting the need for rapid detection of INH mono-resistance to prevent development of additional RIF resistance and subsequent MDR-TB. Prevalence of MDR-TB in this study was 0.3% (1/349) among new cases and 3.8% (1/26) among retreatment cases. Findings from our study show a similar trend to that provided by the WHO during the study period.

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doi:10.1093/trstmh/trv092

Prevalence of drug resistant TB among outpatients at an HIV/TB clinic in Lilongwe, Malawi

Brian Barnett[a,b,*], Runa H. Gokhale[a], Robert Krysiak[a], Creto Kanyemba[a], Tarsizio Chikaonda[a], Mphatso Bokosi[c], Cornelius Mukuzunga[a], Friday Saidi[a], Sam Phiri[c], Irving F. Hoffman[d] and Mina C. Hosseinipour[a,d]

[a]*University of North Carolina Project, Lilongwe, Malawi;* [b]*Vanderbilt University School of Medicine, Nashville, Tennessee, United States;* [c]*Light House Clinic Trust, Lilongwe, Malawi;* [d]*Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States*

*Corresponding author: Present address: 115 Mill Street, Belmont, MA 02478, USA; Tel: +1 615 294 1279; Fax: +1 877 297 5901; E-mail: bbarnett@partners.org

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Background: We sought to determine the prevalence of drug resistant TB among outpatients initiating TB treatment in Lilongwe, Malawi.

Methods: This was a prospective cohort study of patients 18 years and older initiating TB treatment at Martin Preuss Centre, the primary integrated HIV/TB clinic in Lilongwe, Malawi, from April 2011 to July 2012. Procedures included questionnaires, physical exam, chest x-ray, full blood count and sputum collection. Sputum samples underwent acid-fast bacilli (AFB) smear testing and culture by Lowenstein-Jensen (LJ) and liquid Mycobacteria Growth Indicator Tube (MGIT) methods. Drug sensitivity was investigated using the Hain GenoType MTBDR*plus* line probe assay.

Results: Of the 702 patients, 219 (31.2%) were female and 653 (93.0%) were presenting for first-time TB treatment. HIV co-infection was present in 420 (59.8%) cases, with 137 (32.6%) of those patients receiving antiretroviral therapy at presentation. TB was culture-confirmed in 375 (53.4%) patients, 349 of which were first time treatment and 26 retreatment. Ten cases of isoniazid-resistant TB (2.9% of culture confirmed cases of newly treated TB), one of rifampin-resistant TB (0.3% culture confirmed cases of newly treated TB) and one of multi-drug resistant TB (MDR-TB) (3.8% of culture confirmed cases of retreatment TB) were detected.

Conclusions: MDR-TB prevalence is low among outpatients initiating TB treatment in Lilongwe.

Keywords: Africa South of the Sahara, Epidemiology, Isoniazid, Multi-drug resistant, Rifampin, Tuberculosis

Introduction

Despite efforts to control the spread of TB in Africa, there are 2.3 million new cases that lead to a quarter million deaths annually.[1] To further complicate this situation, Africa is currently experiencing a nearly 6% annual increase in cases of multi-drug resistant TB (MDR-TB), the fastest of any WHO region. Given that most countries in Sub-Saharan Africa have limited laboratory capacity and public health care infrastructure, resources which are already under significant strain from the longstanding HIV epidemic, the region has become a potentially fertile breeding ground for MDR-TB. Despite this concern, data on drug resistance in Sub-Saharan Africa is scarce outside of South Africa, which has a more advanced public health care system than its surrounding countries.

Malawi, a country of 14 million people, has been struck hard by both HIV and TB. Despite successful expansion of the antiretroviral therapy programme with marked reductions in HIV mortality and prevalence[2] HIV prevalence remains high at 10.6%, and 68% of TB cases are HIV co-infected.[3] Meanwhile, MDR-TB remains a concern for this vulnerable population. The WHO estimates that there were up to 990 cases of MDR-TB in Malawi in 2009, though only six of those cases were identified and enrolled in treatment.[4]

Previous studies of drug resistant TB in Malawi have focused on inpatients being treated for TB[5] or only patients undergoing treatment for recurrent smear positive pulmonary TB.[6] However, inpatients make up only a small portion of the total population of those with TB, and smear negative pulmonary TB is common among HIV co-infected patients.[7] Given the significant numbers of TB patients who are treated as outpatients and the high burden of HIV/TB co-infection, a broader investigation of drug resistance in Malawian patients living with TB is needed. In this study we evaluate the prevalence of drug resistant TB in Lilongwe, Malawi, among outpatients with new and retreatment TB, as well as smear positive and smear negative TB.

Methods

Study setting

In Malawi all patients diagnosed with TB are registered for treatment at TB clinics. Martin Preuss Centre, located on the campus of Bwaila Hospital, serves as Lilongwe's primary integrated HIV/TB treatment clinic and contains Malawi's largest TB registry. Patients arrive by two different routes, one being referral from surrounding facilities for commencement of treatment once they have been diagnosed with TB and the other being self-referral for sputum submission for those who have experienced chronic cough, defined as cough lasting longer than 3 weeks.

Study design and population

This was a prospective observational cohort study including all adult (age 18 years and older) patients registering for TB treatment at Martin Preuss Centre from April 2011 to July 2012. Originally, only patients presenting for TB treatment for the first time were enrolled. However, in November 2011 the study protocol was amended to allow for the additional enrollment of patients who had previously defaulted (been lost to follow-up), experienced treatment failure or whose TB had relapsed. Exclusion criteria included being less than 18 years of age, being a prisoner, or already being on TB treatment initiated at another facility. Once registered, patients were treated according to the Malawi National Tuberculosis Control Programme manual,[8] with first-treatment patients receiving isoniazid, rifampicin, pyrazinamide and ethambutol, and retreatment patients receiving streptomycin, isoniazid, rifampicin, pyrazinamide and ethambutol. Those with MDR-TB were admitted to the hospital and received capreomycin, levofloxacin, ethionamide, cycloserine and pyrazinamide for 6 months, followed by 18 months of levofloxacin, ethionamide, and cycloserine at home through a community based approach.

Enrollment procedures

Study personnel obtained written informed consent from each patient and administered a questionnaire that covered demographics, past medical history, symptoms, and diagnostic details (acid-fast bacilli [AFB] smear status and radiographic details). We collected blood for full blood count, sputum samples, and performed a chest x-ray on each patient. We offered rapid HIV testing and counseling to patients with an unknown HIV status at the time of enrollment. HIV infected individuals received a CD4 count and, if on antiretroviral therapy for greater than one year, a plasma HIV RNA test. Highly active antiretroviral therapy was initiated in those HIV infected individuals not yet on therapy, according to Malawi's national guidelines for the treatment of HIV.[9]

Laboratory procedures

All specimens were analyzed at the University of North Carolina Project laboratory, which has been given a four star ranking on the WHO Stepwise Laboratory Improvement Process Towards Accreditation (SLIPTA) Tier of Recognition by the African Society for Laboratory Medicine.

Sputum samples were visualized microscopically after auramine O staining and were considered to be smear positive if bacilli were observed. Additionally, they were cultured on Lowenstein-Jensen (LJ) and BACTEC™ Mycobacteria Growth Indicator Tube (MGIT; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For culture-positive samples, the presence of *Mycobacterium tuberculosis complex* (MTBC) was confirmed, and rifampicin and isoniazid resistance determined, using the GenoType MTBDR*plus* line probe assay (Hain Lifescience GmbH, Nehren, *Baden-Württemberg*, Germany). If the presence of MTBC was not confirmed using the GenoType MTBDR*plus*, samples were tested with the Genotype Mycobacterium CM (Hain Lifescience GmbH) to identify other strains of *Mycobacterium*. Drug susceptibility testing is not included as part of Malawi's standard of care.

Full blood count was performed using Beckman Coulter AcT 5diff Cap Pierce Hematology Analyzer, CD4 counts by BD FACSCount System, and HIVRNA levels by Roche AMPLICOR HIV-1 MONITOR Test version 1.5.

Statistical analysis

Data were double-entered into a Microsoft Access 2007 database (Microsoft Corp., Redmond, WA, USA) and statistically analyzed using Stata version 11.0 (Stata Corporation, College Station, TX, USA). Standard χ^2, Fisher's exact tests (two tailed) and t-tests (two tailed, unpaired) were used to determine associations between drug resistance and patient characteristics. A $p<0.05$ was considered statistically significant.

Ethical considerations

Study approval was obtained from the University of North Carolina at Chapel Hill institutional review board and the Malawi National Health Science Research Committee.

Results

Enrollment

Between April 2011 and July 2012, 2531 patients were registered for TB treatment at Martin Preuss Centre. Of these, 1640 patients were eligible to enroll in the study and 702 completed the enrollment process. The most common reasons for ineligibility were the patient not being physically present at registration (303), the patient being retreated for TB (184) during the initial portion of the study when retreatment patients were excluded, and the patient being less than 18 years of age (174). Patients that were ineligible for our study due to not being present at time of registration were admitted at local hospitals and were therefore not part of the outpatient population under investigation. Of eligible patients, the most common reasons for not joining were not being asked to join the study due to staffing constraints (402), being unable to produce a sputum sample (316) and reported lack of time to participate in the study (120) (Figure 1).

Patient characteristics

The median age of study patients was 35 years old (range 19–80), 219 (31.2%) were female, and 653 (93.0%) were presenting with TB for the first time (Table 1). Characteristics of both first time

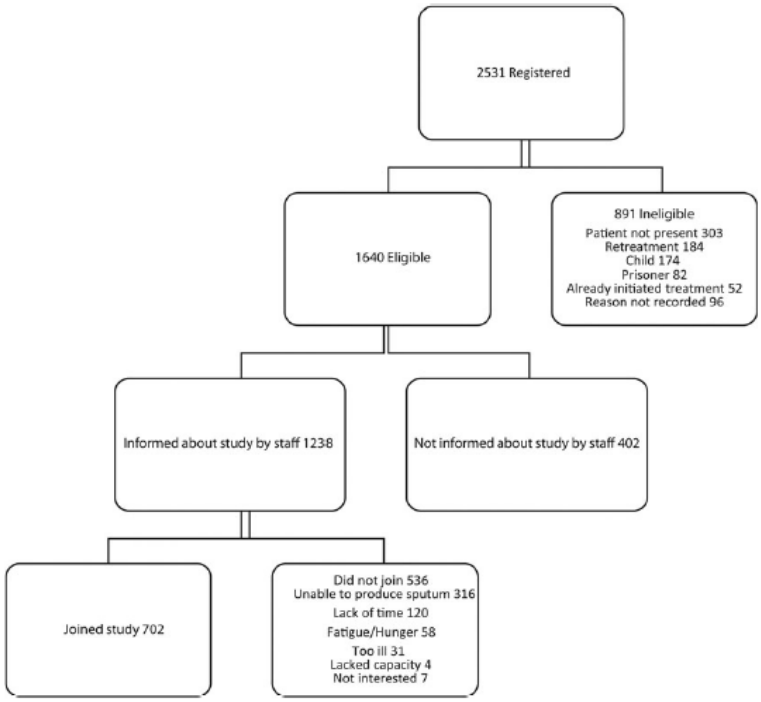

Figure 1. Study enrollment.

treatment patients and retreatment patients did not demonstrate statistically significant differences, except for smear positivity of sputum samples being more likely in retreatment patients (p=0.02). Pulmonary TB was the predominant form of TB, being diagnosed in 614 (87.5%) cases. Abnormal findings on chest x-ray were observed in 415 (59.1%) patients. HIV co-infection was present in 420 (59.8%) cases, but only 137 (32.6%) of those patients were receiving antiretroviral therapy at presentation, since most patients with HIV were diagnosed on the day of enrollment in the study. Among HIV infected patients, those in the retreatment group (p<0.001) and females (p=0.02) were more likely to be receiving antiretroviral therapy (Table 2). Of the 402 eligible patients that were not asked to join the study, 380 (94.5%) were receiving first time TB treatment, which was not statistically different from the percentage of enrolled patients receiving first time TB treatment (p=0.66).

Laboratory findings

Of the 702 sputum samples collected from patients for laboratory analysis, seven were inadequate in volume and one was lost, leaving a total of 694 samples that were cultured for mycobacteria. Of these 694 samples, AFB smear testing was positive in 244 (35.2%) patients. Three hundred and seventy nine samples (54.6%) were positive for mycobacteria by either LJ or MGIT culture. For MGIT culture, four samples were discarded due to contamination and three due to technical failure, leaving 687 MGIT cultured samples. Of these, 368 samples were culture positive (53.6%). For LJ culture, 20 samples were discarded due to contamination and 25 were unculturable due to LJ medium being out of stock, leaving 649 LJ cultured samples. Of these, 333 samples were culture positive (51.3%).

Of the 379 samples that were either LJ or MGIT positive for mycobacteria, testing by the Genotype Mycobacterium CM demonstrated that three samples were positive for *Mycobacterium intracellulare* and one for *Mycobacterium avium*. The remaining 375 culture positive samples were confirmed to be MTBC using the GenoType MTBDR*plus*.

Both AFB and culture positivity differed according to HIV status. AFB smear was positive in 116 of 420 (27.6%) HIV positive patients versus 128 of 282 (45.4%) for HIV negative patients (p<0.01). LJ or MGIT culture was positive for MBTC in 204 of 420 (48.6%) patients with HIV versus 171 of 282 (60.6%) of patients without HIV (p<0.01) (Table 3). Of the 204 HIV positive patients with culture confirmed mycobacteria, 89 (43.6%) had sputum samples that were AFB smear negative.

The 375 MTBC culture positive samples were assessed for drug resistance using the GenoType MTBDR*plus* line probe assay. Ten cases (2.6%) of isoniazid mono-resistant TB were detected, all from patients being treated for TB for the first time. All ten of these cases were also positive by LJ and MGIT culture; however, three (30%) were AFB smear negative. Three hundred and

Table 1. Demographic and clinical characteristics of patients

Characteristic	All patients (n=702)	First treatment (n=659)	Retreatment (n=43)	p-value
Median age with range (years)	35 (19–80)	34 (19–80)	37 (20–71)	NS
Female	219 (31.2%)	203 (30.8%)	16 (37%)	NS
Smear positive	244 (34.8%)	222 (33.7%)	22 (51%)	0.02
Pulmonary TB	614 (87.5%)	579 (87.9%)	35 (81%)	NS
Extrapulmonary TB	88 (12.5%)	80 (12.1%)	8 (19%)	NS
Meningitis (% EPTB)	1 (1%)	1 (1%)	0	NS
Lymphadenitis (% EPTB)	17 (19%)	16 (20%)	1 (13%)	NS
Pericarditis (% EPTB)	10 (11%)	8 (10%)	2 (25%)	NS
Pleuritis (% EPTB)	54 (61%)	49 (61%)	5 (63%)	NS
Arthritis (% EPTB)	1 (1%)	1 (1%)	0	NS
Spinal (% EPTB)	2 (2%)	2 (3%)	0	NS
Miliary[a] (% EPTB)	8 (9%)	8 (10%)	0	NS
Abnormal CXR[b]	415 (59.1%)	392 (59.5%)	23 (53%)	NS
Unilateral infiltrate (% Abnormal CXR)	156 (37.6%)	151 (38.5%)	5 (22%)	NS
Bilateral infiltrate (% Abnormal CXR)	201 (48.4%)	188 (48.0%)	13 (57%)	NS
Adenopathy (% Abnormal CXR)	69 (16.6%)	68 (17.3%)	1 (4%)	NS
Miliary (% Abnormal CXR)	31 (7.5%)	30 (7.7%)	1 (4%)	NS
Cavitary lesion (% Abnormal CXR)	96 (23.1%)	92 (23.5%)	4 (17%)	NS
Pleural effusion (% Abnormal CXR)	68 (16.4%)	61 (15.6%)	7 (30%)	NS
Pericardial effusion (% Abnormal CXR)	11 (2.7%)	10 (2.6%)	1 (4%)	NS
HIV positive	420 (59.8%)	393 (59.6%)	27 (63%)	NS
On ART (% of HIV+)	137 (32.6%)	121 (30.8%)	16 (59%)	<0.001
Median CD4 Count among positives (IQR)	167 (81–293)	160 (76–292)	185 (86–253)	NS
VL suppressed to <400 (% of HIV+on ART) (n=53 VLs)	38 (72%)	33 (75%)	5 (56%)	NS

ART: antiretroviral therapy; CXR: chest X-ray; EPTB: extrapulmonary TB; NS: not significant; VL: viral load.
[a] More than one Extrapulmonary category; [b] Total sums to >100% because patients often had more than one finding on CXR.

Table 2. Tests for association between patient characteristics in patients with HIV (n=420) and ART usage

	On ART (n=137)	No ART (n=283)	p-value
Mean age with range (years)	37 (19–71)	36 (19–76)	NS
Female	42 (30.7%)	30 (10.6%)	0.02
Smear positive	28 (20.4%)	27 (9.5%)	NS
Pulmonary TB	82 (59.9%)	86 (30.4%)	NS
Extrapulmonary TB	18 (13.1%)	14 (4.9%)	NS
Abnormal CXR	63 (46.0%)	63 (22.3%)	NS
Any resistance	3 (2.2%)	3 (1.1%)	NS

ART: antiretroviral therapy; CXR: chest x-ray; NS: not significant.

Table 3. Acid-fast bacilli smear and Lowenstein-Jensen culture positivity in HIV positive and negative patients

	HIV positive (n=420)	HIV negative (n=282)	p-value
AFB smear positive	116 (27.6%)	128 (45.4%)	<0.01
LJ or MGIT culture positive	201 (47.9%)	171 (60.6%)	<0.01

AFB: acid-fast bacilli; LJ: Lowenstein-Jensen; MGIT: Mycobacteria Growth Indicator Tube.

forty nine of MTBC culture positive patients were being treated for TB for the first time, while 26 were receiving re-treatment. This resulted in a calculated prevalence of 2.9% (10/349) for isoniazid mono-resistance in newly treated and culture confirmed cases of MTBC. One case of rifampicin mono-resistant TB was detected, from a patient being treated for TB for the first time. This case was AFB smear positive and also positive by LJ and MGIT cultures, resulting in a prevalence of 0.3% (1/349) in newly treated and culture confirmed cases of MTBC. One case of MDR-TB (resistant to both isoniazid and rifampicin) was detected, originating from a patient being retreated for TB. It was detected by LJ and MGIT cultures, but was AFB smear negative, resulting in a prevalence of 3.8% (1/26) in retreatment culture confirmed cases of MTBC.

Table 4. Tests for association between patient characteristics and any type of drug resistance

	Pansensitive (n=363)	Any drug resistance[a] (n=12)	p-value
Mean age (years)	34	36	NS
Female	106 (29.2%)	3 (25%)	NS
First treatment	335 (92.3%)	11 (92%)	NS
Retreatment	25 (6.9%)	1 (8%)	NS
Abnormal X-ray (n=155 and n=7 x-rays)	147 (94.8%)	7 (100%)	NS
Pulmonary TB	332 (91.5%)	11 (92%)	NS
Extrapulmonary TB	28 (7.7%)	1 (8%)	NS
Smear positive	234 (64.5%)	8 (67%)	NS
HIV positive	195 (53.7%)	6 (50%)	NS
Not on ART (% of HIV+)	130 (66.7%)	3 (50%)	NS
Mean CD4 count among HIV+	203	148	NS
Mean absolute neutrophil count among HIV+	3689	3117	NS

ART: antiretroviral therapy; NS: not significant.
[a] Includes 10 samples with isoniazid mono-resistance, one sample with rifampin mono-resistance and one sample with multi-drug resistance (both isoniazid and rifampin).

Drug resistance was not associated with age, gender, TB treatment status, TB site, AFB smear result, chest X-ray results or HIV status (Table 4).

Discussion

Though others have reported low rates of MDR-TB in Malawi,[5,6] our study is one of the first to demonstrate that the presence of MDR-TB in Malawi is very low among both first time and retreatment outpatient cases, as well as among those who are sputum positive or negative. This latter point is important as our study confirmed a high rate of smear negative, LJ culture confirmed TB (44%) among HIV co-infected patients, who represented 60% of our study population. Because TB is the leading cause of death among HIV-infected persons,[10] it is especially important to understand the prevalence of MDR-TB in this population. Our results are in line with a recently published nationally representative cross sectional survey assessing drug resistant TB in Malawi conducted from February 2010 to March 2011[11] that also demonstrated low prevalence of MDR-TB (0.4% in new cases and 4.8% retreatment cases), among patients with sputum smear positive TB.

Malawi currently appears to be faring better than some of its neighboring countries in the MDR-TB epidemic, such as Swaziland (prevalence of 0.9% of new cases and 9.1% of retreatment cases), South Africa (prevalence of 1.8% of new cases and 6.7% of retreatment cases) and Mozambique (prevalence of 3.5% of new cases and 11.2% of retreatment cases).[4] This can be partially attributed to the country's well-managed national TB control programme, which has used the directly observed treatment short-course (DOTS) strategy since 1964.[12] Treatment success rates have continually improved, rising from 65% in 1995 to 88% in 2010,[1] putting the country's performance above the WHO's target of 85%. Though the importance of future evaluation of prevalence in Malawi cannot be understated, our study demonstrates that prevention of MDR-TB is possible, even in a low resource country, through proper planning, implementation, regular monitoring and evaluation of treatment administration.

The predominance of isoniazid mono-resistance in our study is important due to the recent advent of the Xpert MTB/RIF assay, which allows for the detection of both MTB and rifampicin resistance simultaneously in less than two hours.[13,14] The assay was endorsed by the WHO in December 2010 and has since been specifically recommended as the initial diagnostic test in patients with suspected drug resistant TB or HIV associated TB. As a result, since June 2012, two-thirds of countries struggling with a high prevalence of TB have included the assay in their national TB control programmes. However, due to the predominance of isoniazid mono-resistance in our study results, the Xpert assay will underestimate overall TB drug resistance since it only detects rifampicin resistance. Given the low prevalence of rifampicin resistance in Malawi, the positive predictive value of the Xpert MTB/RIF assay is low. Therefore cases of rifampicin resistance detected by this assay should also be confirmed using drug susceptibility testing.[15] The 3% prevalence of isoniazid resistance in patients being newly treated for TB indicates a need for rapid detection of this mono-resistance so that treatment regimens can be modified quickly to prevent development of additional rifampicin resistance and subsequent MDR-TB.

Prior studies have found associations between previous treatment for TB, treatment not directly observed by a healthcare worker and exposure to a known case of MDR-TB and the development of MDR-TB.[16,17] Associations have also been observed between HIV co-infection and the development of MDR-TB; however, a recent meta-analysis of studies from Sub-Saharan Africa revealed no association.[16] We found no statistical associations between patient characteristics and TB drug resistance. However, our study was handicapped to show a statistical difference, given that there were so few drug resistant cases. Other limitations of this study include the initial exclusion of patients

seeking retreatment for TB, which likely contributed to underestimation of MDR-TB prevalence since these individuals have the highest risk for MDR-TB, and the sizable number of potential patients enrolling for TB treatment that were not approached by our study staff due to staffing shortages. Among those not approached, there was no difference in the proportion that were registering for first time TB treatment or retreatment, but we do not have access to other information such as age, gender and HIV status for further comparison. Regardless, we feel a systematic bias is unlikely. Prisoners were also excluded from our study due to concerns about their ability to give consent. Given that these individuals live in an environment known to have a higher prevalence of MDR-TB than the general population, it is possible that their exclusion contributed to underestimation of MDR-TB prevalence.

Conclusions

MDR-TB prevalence is low among outpatients seeking treatment in Lilongwe, emphasizing the effectiveness of Malawi's national TB control programme. However, future evaluation will be necessary to identify cases of isoniazid and rifampicin resistance in order to monitor TB control programme performance and prevent establishment of MDR-TB strains in the country.

Authors' contributions: BB was involved in study design, oversaw patient enrollment and study implementation, analyzed data and wrote the manuscript. RHG was involved in study design, gained institutional study approval and oversaw enrollment. CK, TC and RK carried out laboratory analyses of specimens and interpreted data. MB enrolled patients and collected/interpreted data. CM and FS interpreted laboratory/radiographic results. SP, IFH and MCH assisted with study design, study implementation, data interpretation and manuscript preparation. All authors read and approved the final paper. BB is the guarantor of the paper.

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Competing interests: None declared.

Ethical approval: Study approval was obtained from the University of North Carolina at Chapel Hill institutional review board and the Malawi National Health Science Research Committee.

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CHAPTER 4

Performance of Xpert MTB/RIF among presumptive TB outpatients in Lilongwe, Malawi

4.1 Summary

Diagnosis of TB requires diagnostics which have the potential to improve the turnaround time for both detection of tuberculosis and determination of drug susceptibility. Before implementing such technologies, there is a need to assess their performance in the environment in which they will be used. The following manuscript highlights the performance of the Xpert MTB/RIF in a Malawian setting using sputum pellets from presumptive TB patients at Bwaila hospital (an HIV/TB clinic). This manuscript has been published by the African Journal of Laboratory Medicine (AJLM) and a summary was presented as an oral abstract at the following conference:

- Tarsizio Chikaonda, 2013: Introduction and Evaluation of the Xpert® MTB/RIF assay in Lilongwe, Malawi. Health Research Capacity Strengthening Conference, Lilongwe, Malawi, 24th – 26th July

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Performance of Xpert® MTB/RIF among tuberculosis outpatients in Lilongwe, Malawi

Authors:
Tarsizio Chikaonda[1,2]
Nelson Nguluwe[2]
Brian Barnett[2]
Runa H. Gokhale[2]
Robert Krysiak[2]
Isaac Thengolose[2]
Nora E. Rosenberg[2]
Christopher Stanley[2]
James Mpunga[3]
Irving F. Hoffman[2,4]
Mina Hosseinipour[2,4]
Lesley Scott[1]
Wendy Stevens[1]

Affiliations:
[1]Department of Molecular Medicine and Haematology, Faculty of Health Sciences, School of Pathology, University of the Witwatersrand, Johannesburg, South Africa

[2]University of North Carolina Project, Lilongwe, Malawi

[3]Malawi National Tuberculosis Programme, Lilongwe, Malawi

[4]University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States

Corresponding author:
Tarsizio Chikaonda,
tarchikaonda@gmail.com

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Background: Xpert® MTB/RIF is a molecular test for the detection of *Mycobacterium tuberculosis* and rifampicin resistance. It is considered to be a great advance over smear microscopy and culture. However, there is very little information regarding the performance characteristics of Xpert MTB/RIF in Malawi.

Objective: We aimed to evaluate the performance of Xpert MTB/RIF in a Malawian setting.

Methods: Stored sputum pellets were processed on Xpert MTB/RIF between June 2012 and May 2014. Results were compared to mycobacteria growth indicator tube and Löwenstein-Jensen cultures, LED fluorescent microscopy and GenoType® MTBDR*plus* assay. Rifampicin resistance was confirmed by DNA sequencing.

Results: Of the 348 specimens with valid Xpert MTB/RIF results, 129/348 (37%) were smear-positive and 198/348 (57%) were culture-positive. Xpert MTB/RIF demonstrated a sensitivity of 93.8% (95% CI 89.4% – 96.8%) and specificity of 97.4% (95% CI 93.5% – 99.3%), with a positive predictive value of 97.8% (95% CI 94.6% – 99.4%) and a negative predictive value of 92.6% (95% CI 87.4% – 96.1%). Xpert MTB/RIF correctly identified 185/186 (99.5%) rifampicin-sensitive and 2/2 (100%) rifampicin-resistant *M. tuberculosis* strains. Mutations were not detected by sequencing in one isolate which was rifampicin resistant on Xpert MTB/RIF but sensitive on MTBDR*plus*. Four non-tuberculous mycobacteria grew from four smear-negative specimens, namely, *M. avium* (n = 1) and *M. intracellulare* (n = 3). No cross-reactivity was observed with any of the non-tuberculous mycobacteria when using Xpert MTB/RIF.

Conclusion: When fully implemented, Xpert MTB/RIF may have an impact on patient care in Malawi. The increased diagnostic yield of Xpert MTB/RIF over smear microscopy can increase laboratory-confirmed tuberculosis detection and ensure that treatment is given to appropriate individuals or groups.

Introduction

Tuberculosis remains a major health challenge that has worsened with the emergence of multi-drug resistant (MDR) tuberculosis strains that are resistant to rifampicin and isoniazid. Globally, the impact of tuberculosis is significant, with an annual estimate of 9.6 million tuberculosis cases and over 1.5 million deaths due to tuberculosis in 2014.[1] Preliminary data show that the prevalence of tuberculosis in Malawi is 286/100 000, which is higher than previous estimates by the World Health Organization.[2] Diagnosis of tuberculosis continues to be a major challenge in Malawi due to the widespread use of diagnostics with poor sensitivity, such as sputum smear microscopy. Although more sensitive than smear microscopy, tuberculosis culture, when available, can take days to weeks before a result is available. Due to limitations of both smear and culture, pulmonary tuberculosis is often diagnosed late or presumptively. Low utilisation of laboratory confirmation and widespread use of empirical treatment can either lead to true tuberculosis cases being missed or to the initiation of tuberculosis treatment in people without the disease.[1]

Malawi has a high HIV burden, with an estimated one million HIV-positive people. The major cause of morbidity and mortality of people living with HIV is tuberculosis. It is estimated that of all tuberculosis cases, 41% are smear-negative and 64% are HIV co-infected.[3] Diagnosis of tuberculosis may be delayed or missed in HIV-positive, smear-negative tuberculosis patients. As such, it is imperative to rapidly diagnose and treat tuberculosis cases in people living with HIV.[3,4] Introduction of simple and rapid diagnostic methods, such as the Xpert® MTB/RIF (Cepheid, Sunnyvale, California, United States), could benefit smear-negative patients in countries like Malawi, where culture is limited to the National Tuberculosis Reference Laboratory. Currently,

the Malawi National Tuberculosis Programme (NTP) is using Xpert MTB/RIF to increase tuberculosis case detection and to detect rifampicin resistance as a proxy for MDR tuberculosis followed by culture (liquid and solid) and drug-susceptibility testing (DST) as confirmatory tests.[2]

DST is not routinely performed in Malawi, with the exception of retreatment cases and individuals at risk for MDR tuberculosis.[3] This can lead to the development of under-reporting of drug resistance, unnecessary suffering, additional costs for patients, and treatment with suboptimal regimens.[5] Consequently, this might increase the spread of drug-resistant tuberculosis in the population.[1]

The Xpert MTB/RIF can both detect tuberculosis and identify rifampicin resistance in a single, rapid assay,[6,7] and is reported as having excellent sensitivity and specificity in detecting *Mycobacterium tuberculosis* and rifampicin resistance.[6,8] Some recent evaluations have demonstrated that Xpert MTB/RIF accurately detects 72.5% of smear-negative and 98.2% of smear-positive cases, and rifampicin resistance is detected with a specificity of 100% and sensitivity of 99.1%.[6,9] The test has an overall pooled sensitivity of 90.4% (95% CI 89.2% – 91.4%), which is much higher than smear microscopy but is similar to that of solid culture in detecting tuberculosis.[1,7] Among smear-positive tuberculosis samples, Xpert MTB/RIF has a pooled sensitivity of 98.7% (95% CI 98.0% – 99.2%), but has a substantially lower sensitivity of 75% (95% CI 72.0% – 77.8%) among smear-negative tuberculosis samples. The overall pooled sensitivity in diagnosing for rifampicin-resistant tuberculosis is 94.1% (95% CI 91.6% – 96.0%) and 97.0% (95% CI 96% – 97.7) pooled specificity.[7] Furthermore, use of Xpert MTB/RIF has been linked to improved diagnosis, resulting in early appropriate treatment.[10]

The Malawi NTP recommended rolling out Xpert MTB/RIF in 2012 to increase detection of tuberculosis, especially in sputum smear-negative and HIV-positive individuals. While Xpert MTB/RIF has been approved for clinical use, its optimal use in the clinical algorithm for Malawi has not been established. There are currently over 50 GeneXpert® instruments distributed across 40 of the 315 public and private diagnostic centres (laboratories) with acid-fast bacilli testing capacity in the country.[2] Xpert MTB/RIF's performance is not known in the adult outpatient population in Malawi.

In this study, we evaluated the performance of Xpert MTB/RIF in detecting *M. tuberculosis* complex and determining resistance to rifampicin among both HIV-positive and HIV-negative outpatients at Bwaila Hospital in Lilongwe, Malawi.

Methods

Ethical considerations

Approvals for this study were granted by the University of the Witwatersrand Human Research Ethics Committee (M120256), National Health Sciences Research Committee (NHSRC) in Malawi (NHSRC # 999) and the University of North Carolina (Chapel Hill) Institutional Review Board (CID 1211).

Laboratory methodologies

We used frozen stored sputum pellets (n = 351) obtained from an observational cohort study which collected 702 samples between April 2011 and July 2012 at Bwaila in Lilongwe, Malawi. The study was looking at the prevalence of drug-resistant tuberculosis among adult outpatients (≥ 18 years) with laboratory-confirmed or clinically-diagnosed tuberculosis registering for tuberculosis treatment at this HIV/tuberculosis clinic.[11] Pellets were selected at random for use in this study without any special criteria to eliminate bias. Testing of these pellets using Xpert MTB/RIF started in June 2012 at the University of North Carolina (UNC) Project laboratory, Lilongwe, Malawi. Pellets were re-suspended in 1.5 mL phosphate buffer and an aliquot of 0.5 mL was processed on the Xpert MTB/RIF assay. In the primary study, all sample processes were performed at the UNC Project laboratory. In brief, sputum smears were prepared and stained with auramine-O stain. Smear microscopy was performed using LED fluorescent microscopy. Mycobacterial cultures (reference standard) were performed using both BACTEC mycobacteria growth indicator tube media (liquid) and Löwenstein-Jensen slants (solid). The reference standard was considered positive if there was growth of *M. tuberculosis* on either of the media and negative if both media were negative. Drug susceptibility was investigated using the GenoType® MTBDR*plus* assay (version 2) (Hain Lifesciences GmbH, Nehren, Germany). Data collected from the above processes were included with data in this evaluation.

Xpert MTB/RIF

Samples for Xpert MTB/RIF testing were prepared as per manufacturer's instructions. Sample reagent was added to 500 µL of the re-suspended pellet in the ratio of 3:1 (sample reagent:specimen) as described previously.[12] Results were available within two hours of sample loading.

DNA extraction

Genomic bacterial DNA was extracted using a GenoLyse® kit (Hain Lifescience GmbH, Nehren, Germany) from mycobacteria growth indicator tube liquid media by transferring 1.0 mL to a Sarstedt micro-centrifuge tube. The tube was centrifuged for 15 minutes at 14 000 rpm. The supernatant was carefully removed and the pellet was re-suspended in 100 µL lysis buffer, vortexed thoroughly and incubated for five minutes at 95°C in a heating block. At the end of the five-minute incubation, tubes were briefly centrifuged to remove condensation. Neutralization buffer (100 µL) was added to each tube, vortexed for five seconds and then centrifuged for five minutes at 14 000 rpm. Supernatant was transferred to a new tube and the pellet discarded. Samples were stored at -80°C for use as DNA template.

Line probe assays

The GenoType® MTBDR*plus* (version 2) (Hain Lifesciences GmbH, Nehren, Germany) was performed on positive culture isolates according to manufacturer's instructions to identify *M. tuberculosis* complex and to determine drug susceptibility to rifampicin and isoniazid as described previously.[13] Genotype® CM (Hain Lifesciences GmbH, Nehren, Germany) was used to identify other common *Mycobacterium* species in samples which tested negative for *M. tuberculosis* complex on GenoType MTBDR*plus*.

PCR and DNA sequencing for the rifampicin resistance determining region

Determination of eligibility for DNA sequencing was based on detection of resistance to rifampicin by Xpert MTB/RIF. All eligible *M. tuberculosis* strains were shipped to the HIV Genotyping Laboratory (University of the Witwatersrand) and were processed as described below.

DNA amplification

Amplification of the 450 bp *rpo*B gene fragment which included the rifampicin resistance determining region was carried out by using forward primer *rpo*BF2 (5'-GAG GGT CAG ACC ACG ATG AC-3') and reverse primer *rpo*BR2 (5'-GAG CCG ATC AGA CCG ATG T-3') in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, California, United States). The total volume of the reaction mix was 50 µL, which contained 5 µL of 10X high fidelity buffer, 1 µL of 10 mM dNTP mix, 2 µL of 50 mM $MgSO_4$, 1 µL of each primer, 0.2 µL of Taq HiFi (Invitrogen, Carlsbad, California, United States), 36.8 µL of molecular grade water and 3 µL of genomic DNA. Amplification conditions were set as follows: 94°C for 2 minutes (initial denaturation); followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 40 seconds; followed by a 10-minute final elongation at 68°C. Amplified products were visualised on a 1% agarose gel after staining with ethidium bromide.

Sequencing

PCR products, after purification by GeneJet PCR purification kit (Thermo Scientific, Waltham, Massachusetts, United States), were subjected to DNA sequencing by automated DNA sequencer (ABI 3700, Applied Biosystems, Foster City, California, United States). PCR sequencing was carried out with a BigDye terminator V3.1 sequencing kit according to manufacturer's instructions, using forward primer *rpo*BS (5'-GCA GAC GTT GAT CAA CAT CC-3') and reverse primer *rpo*BR2 (5'-GAG CCG ATC AGA CCG ATG T-3'). The resulting sequences were analysed using Sequencher software V4.8 (Genecodes Corporation, Ann Arbor, Michigan, United States).

Statistical analysis

All data were double entered into a Microsoft Excel spreadsheet (Microsoft, Redmond, Washington, United States). Discrepancies were checked against source records for completeness and consistency. Data analyses were done in Stata Version 12 (StataCorp, College Station, Texas, United States). The following statistics were calculated: sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and 95% confidence intervals (CI). Mycobacterium culture was considered the reference standard.

Results

A total of 351 sputum pellets were processed both on culture and Xpert MTB/RIF. However, only 348 sputum pellets had valid Xpert MTB/RIF results. The remaining three samples were not included in the final analysis due to an error ($n = 1$) and an invalid result ($n = 2$) on Xpert MTB/RIF. Of the 348 sputum pellets, 200 (57%) were from HIV-positive individuals, and 148 (43%) were HIV-negative. Among all samples, 219/348 (63%) were smear-negative and 129/348 (37%) were smear-positive. As shown in Table 1, 70/219 (32%) of smear-negative and 128/129 (99.2%) of smear-positive samples were culture positive.

Xpert MTB/RIF detected *M. tuberculosis* in 58/219 (27%) of smear-negative and 127/129 (98.5%) smear-positive cases. Xpert MTB/RIF correctly detected *M. tuberculosis* in 181/198 (91.4%) of the culture-positive samples. Of the remaining 17 samples, 1/198 (0.5%) was positive while 16/198 (8.1%) were negative for acid-fast bacilli on smear microscopy. A total of 150 samples were culture-negative regardless of their smear status. Among the 150 culture-negative samples, *M. tuberculosis* was detected by Xpert MTB/RIF in four (2.7%) samples which were smear-negative on microscopy. Xpert MTB/RIF did not detect *M. tuberculosis* in 12/70 (17%) of smear-negative, culture-positive samples. We further observed that 145/219 smear-negatives were culture negative and Xpert MTB/RIF negative. Out of these, 93/145 (64%) were from HIV-positive individuals. Four non-tuberculous mycobacteria grew from a total of four smear-negative specimens: *M. avium* ($n = 1$) and *M. intracellulare* ($n = 3$). No cross reactivity was observed with any of the non-tuberculous mycobacteria when using Xpert MTB/RIF (Table 1).

TABLE 1: Comparison of Xpert MTB/RIF and culture diagnostic test results in relation to smear microscopy for outpatients at a single hospital in Lilongwe, Malawi (2011–2012).

Smear (n = 348)	Tuberculosis culture positive			Tuberculosis culture negative		Total
	Xpert MTB/RIF positive	Xpert MTB/RIF negative (NTM)	Xpert MTB/RIF negative	Xpert MTB/RIF positive	Xpert MTB/RIF negative	
Smear positive	127 (36.5%)	0 (0%)	1 (0.25%)	0 (0%)	1 (0.25%)	129 (37%)
Smear negative	54 (15.5%)	4 (1.15%)	12 (3.5%)	4 (1.15%)	145 (41.7)	219 (63%)
Total	**181 (52%)**	**4 (1.15%)**	**13 (3.7%)**	**4 (1.15%)**	**146 (42%)**	**348 (100%)**

NTM: Non-tuberculous mycobacteria; *n*: number.

When compared to culture, the overall sensitivity for Xpert MTB/RIF was 93.8% (95% CI 89.4% – 96.8%) and specificity was 97.4% (95% CI 93.5% – 99.3%), with PPV of 97.8% (95% CI 94.6% – 99.4%) and NPV of 92.6% (95% CI 87.4% – 96.1%). Among smear-negative individuals, the overall sensitivity of Xpert MTB/RIF was 81.8% (95% CI 70.4% – 90.2%) and specificity was 97.4% (95% CI 93.4% – 99.3%), with PPV of 93.1% (95% CI 83.3% – 98.1%) and NPV of 92.5% (95% CI 87.3% – 96.1%) (Table 2).

Stratified by HIV status, sensitivity for smear microscopy was 78.9% (95% CI 69.4% – 86.6%) among HIV-negative individuals, with a specificity of 100% (95% CI 93.3% – 100%). Among HIV-positive individuals, sensitivity was 51.5% (95% CI 41.4% – 61.4%) and specificity was 99.0% (95% CI 94.4% – 100%). The sensitivity (90%; 95% CI 82.4% – 95.1%) and specificity (96%; 95% CI 90.1% – 98.9%) of Xpert MTB/RIF were lower in HIV-positive individuals, with PPV of 95.7% (95% CI 89.5% – 98.8%) and NPV of 90.6% (95% CI 83.3% – 95.4%). This was compared to HIV-negative participants, which showed a sensitivity of 97.9% (95% CI 92.5% – 99.7%) and specificity of 100% (95% CI 93.4% – 100%), with PPV of 100% (95% CI 96.1% – 100%) and NPV of 96.4% (95% CI 87.7% – 99.6%). Among smear-negative, HIV-positive individuals, the sensitivity of Xpert MTB/RIF was 78.7% (95% CI 64.3% – 89.3%) with a specificity of 96% (95% CI 90% – 98.9%) (Table 2).

DST using GenoType MTBDR*plus* was performed on 188/348 tuberculosis strains. When compared to Genotype MTBDR*plus*, Xpert MTB/RIF correctly identified 185 of 186 (99.5%) rifampicin-sensitive *M. tuberculosis* and 2/2 (100%) rifampicin-resistant *M. tuberculosis*. A single point mutation was detected in each of the two rifampicin-resistant strains (S531L and D516V). It was observed that for both strains, the corresponding Xpert MTB/RIF probes gave cycle threshold (Ct) value = 0 and were also detected as resistant to rifampicin by GenoType MTBDR*plus* line probe assay. One (0.5%) smear-negative, culture-positive tuberculosis strain was resistant on Xpert MTB/RIF but sensitive on GenoType MTBDR*plus* (Table 3). A delayed amplification on probe B was observed in this strain, with a Ct value of 24.4. Further testing on the isolate using DNA sequencing revealed no *rpo*B gene mutation.

Discussion

In the present study, the Xpert MTB/RIF assay detected 58 more tuberculosis cases than smear microscopy. The paucibacillary nature of samples cultured from tuberculosis/HIV co-infected individuals revealed a more serious inability to detect tuberculosis using smear microscopy than was the case for Xpert MTB/RIF. Among HIV-positive individuals registering for tuberculosis treatment, a relatively larger percentage of those who were smear-negative, were also Xpert MTB/RIF and culture negative, and thus had no laboratory confirmation of disease. Overall analysis revealed expected results using the Xpert MTB/RIF assay, proving more sensitive and specific than fluorescent microscopy in detecting *M. tuberculosis* when using culture as the reference standard and there was no overlap of the confidence intervals. The sensitivity of Xpert MTB/RIF was lower in HIV-positive than in HIV-negative individuals. The Xpert MTB/RIF correctly distinguished *M. tuberculosis* from non-tuberculous mycobacteria. Among the non-tuberculous mycobacteria, three were isolated from HIV-positive individuals, and one was from an HIV-negative individual.

Findings from our study are in agreement with studies by Rahman et al.[14] and Barnard et al.[15] who demonstrated an overall agreement of 92.4% and 100%, respectively, between Xpert MTB/RIF and MTBDR*plus* for the detection of rifampicin susceptibility. Other studies have shown results in which rifampicin resistance was detected with a specificity of 100% and sensitivity of 99.1%.[16,17] Steingart et al. demonstrated a pooled sensitivity of 94% and a specificity of 98% for the detection of rifampicin resistance.[18]

The sensitivity of Xpert MTB/RIF (93.8%) observed in this study is in contrast to Dorman et al. and Geleta et al., who reported 62.6 % in South Africa and 65.5% in Ethiopia when compared with culture.[19,20] The possible explanation for this discrepancy could be differences in study design, considering that the current study used processed pellets while the other studies performed their evaluations using unprocessed sputum.

TABLE 2: Sensitivity, specificity, positive predictive value and negative predictive value of smear microscopy and Xpert MTB/RIF at 95% CI compared with sputum culture for outpatients at Bwaila Hospital, Lilongwe, Malawi (2011–2012).

Variables	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Sensitivity for SPTB	Sensitivity for SNTB
Smear microscopy						
Overall	64.6 (57.6–71.3)	99.3 (96.3–100)	99.2	68.0	-	-
HIV-positive (n = 200)	51.5 (41.4–61.4)	99.0 (94.4–100)	98.1	65.8	-	-
HIV-negative (n = 148)	78.9 (69.4–86.6)	100 (93.3–100)	100	72.6	-	-
Xpert MTB/RIF						
Overall	93.8 (89.4–96.8)	97.4 (93.5–99.3)	97.8	92.6	100 (97.2–100)	81.8 (70.4–90.2)
HIV-positive (n = 200)	90.0 (82.4–95.1)	96.0 (90.1–98.9)	95.7	90.6	100 (93.3–100)	78.7 (64.3–89.3)
HIV-negative (n = 148)	97.9 (92.5–99.7)	100 (93.4–100)	100	96.4	NA	89.5 (66.9–98.7)

NA, Not applicable NPV, Negative predictive value; PPV, Positive predictive value; SNTB, Smear-negative tuberculosis; SPTB, Smear-positive tuberculosis.

TABLE 3: Rifampicin susceptibility results (n=188) from both GenoType MTBDR*plus* and Xpert MTB/RIF for outpatients at Bwaila Hospital, Lilongwe, Malawi (2011–2012).

Total tested	Drug	Resistant		Susceptible	
188	Rifampicin	Xpert MTB/RIF	GenoType MTBDR*plus*	Xpert MTB/RIF	GenoType MTBDR*plus*
		3	2	185	186

Prior studies have shown greater accuracy of Xpert MTB/RIF as compared to smear microscopy[6] and sensitivities of Xpert MTB/RIF ranging from 78% to 100% for smear-positive/culture-positive samples.[21,22] Since Xpert MTB/RIF identified 58/219 (26.5%) of smear-negative samples, it can therefore assist in rapid detection of tuberculosis cases among smear-negatives and has the potential to impact patient care, although a small percentage (17%, 12/70) of the smear-negative/culture-positive cases was missed by Xpert MTB/RIF. In Malawi, smear-negative adult tuberculosis patients have poor treatment outcomes with high death rates, largely due to concurrent HIV infection.[23] It is of particular interest to note that 64% of smear-negative samples which did not grow on culture and where *M. tuberculosis* was not detected by Xpert MTB/RIF, were from HIV-positive individuals. This may be interpreted as significant incorrect diagnosis of tuberculosis with a likelihood of treating people for tuberculosis who do not have the disease. It is therefore of paramount importance that steps should be taken to improve tuberculosis diagnosis to avoid exposing non-tuberculosis patients to the adverse effects of the tuberculosis medication. Misdiagnosis of tuberculosis can be a contributing factor to the high death rate observed in HIV-positive individuals globally.[1] Due to the increased diagnostic yield and short turn-around time of Xpert MTB/RIF as compared to culture, Xpert MTB/RIF offers an opportunity to improve the diagnosis of tuberculosis, both in HIV-positive and HIV-negative individuals, if used as a first diagnostic for all presumptive tuberculosis cases in Malawi, considering the high proportion of misdiagnoses observed in this study.

Our results are comparable to other studies which have shown that Xpert MTB/RIF increased tuberculosis case detection by almost 31%, despite its low sensitivity with smear-negative samples.[20] Another study demonstrated that 89% of rifampicin-resistant tuberculosis patients started second-line treatment when Xpert MTB/RIF was introduced and that the average time to start second-line treatment was reduced from 1 – 1.5 months by culture to one week with Xpert MTB/RIF.[23] The current study does not highlight this for Malawi since Xpert MTB/RIF testing was performed retrospectively and the initial study did not have a long-term follow-up on patients, as it was only concentrated on diagnostics.

Results in this study show that Xpert MTB/RIF detected primary rifampicin resistance in 3/188 (1.6%) specimens. DNA sequencing of the rifampicin resistance determining region of the *rpo*B gene confirmed two mutations (S531L and D516V) and further helped to resolve the discordant result, since no mutation was detected. Mixed infection with multiple *M. tuberculosis* strains was excluded in this strain, given the wild-type *rpo*B gene sequence and no observed underlying peaks on the DNA sequence chromatogram. Prior studies demonstrated that DNA sequencing resolves discrepancies in favour of Xpert MTB/RIF,[6] but this was not the case in our study; a false rifampicin resistance was most likely. In Malawi, the Xpert MTB/RIF is widely used to identify MDR tuberculosis patients and to initiate MDR tuberculosis treatment until culture and DST results are obtained. Malawi has a low MDR tuberculosis prevalence; as such the expected low PPV of Xpert MTB/RIF could contribute to false-positive results and would require a second Xpert MTB/RIF test as per current international recommendations.[2]

False resistance to rifampicin on Xpert MTB/RIF has been reported previously, although in some of the studies hetero-resistance could not be excluded, because neither cartridge amplicons nor unprocessed sputum were sequenced,[17,25] which was also the case in our study. We performed DNA sequencing on isolates from culture, whereas Xpert MTB/RIF was performed on sputum pellets. Theron et al.[22] detected mutations in 5/6 cases which were rifampicin resistant on Xpert MTB/RIF but were susceptible on phenotypic DST. Marlowe et al.[26] further investigated a sample which was susceptible on phenotypic DST and no mutation was detected in DNA sequencing but was repeatedly resistant to rifampicin on Xpert MTB/RIF. Investigations carried out in these studies show how difficult it is to distinguish true-positive rifampicin resistance from false positives in clinical practice. Also, the samples run on Xpert MTB/RIF might have included both dead and live bacteria since the specimens were taken directly from pellets, whereas for DNA sequencing, DNA extraction was performed after observed growth on culture (live bacteria). It was demonstrated previously that Xpert MTB/RIF has a pooled sensitivity of 94.1% (95% CI 91.6% – 96.0%) and pooled specificity of 97.0% (95% CI 96.0% – 97.7%) in diagnosing rifampicin resistance.[7] In the present study, the sensitivity, specificity, PPV and NPV were not calculated due to the small number of rifampicin-resistant isolates identified.

The low rifampicin resistance detected in this study is similar to results obtained by Glynn et al., which identified 3/373 samples as resistant to rifampicin in Karonga, in the northern region of Malawi.[27] Low rifampicin resistance detection among incident cases observed in this study reflects well on the prevalence of rifampicin-resistant tuberculosis strains in communities surrounding Bwaila. However, it is debatable whether Xpert MTB/RIF results alone can be used for making a decision to start on second-line treatment in outpatients detected with resistance to rifampicin in Malawi, considering the number of isoniazid mono-resistances detected in previous studies.[11] It is therefore crucial to test tuberculosis strains for resistance to isoniazid as well, and to confirm rifampicin resistance detected by Xpert MTB/RIF before switching to second-line treatment. The success of the tuberculosis-control programme could be measured by the level of anti-tuberculosis drug resistance in a community. Consequently, the level of drug resistance gives future indications of suitable drug regimens.[27,28]

The Malawi NTP has phased in plans within the context of national strategic plan for tuberculosis to expand and maintain Xpert MTB/RIF as a primary test for active case finding at high-volume antiretroviral therapy services and among high risk and vulnerable populations. This will consequently lead to improved tuberculosis diagnosis and

initiation of appropriate treatment to patients, thereby reducing the spread of tuberculosis and/or transmission of resistant strains in the country. Additionally, Xpert MTB/RIF offers to help in reducing the time lost in the diagnostic pathway and potential loss of patients at each step of the pathway.[2] As part of the scale-up plan for the GeneXpert technology, the Malawi NTP plans to assess the concordance between phenotypic and genotypic technologies in order to inform interpretation of single rifampicin-resistance results. Systematic confirmation of MDR tuberculosis status of rifampicin-resistant patients diagnosed with Xpert MTB/RIF is lacking in the country.[2] Findings from the current study form a bank of data which can be beneficial to the NTP in its assessment of the Xpert MTB/RIF assay.

Limitations

With our limited sample size from a single outpatient location, the observed results may not apply across the country due to differences in the prevalence of HIV in rural districts. The relatively small number of patients with drug-resistant tuberculosis limits the ability to evaluate comparative performance of Xpert MTB/RIF. We recommend that future studies extend to other sites with an overall sample size and/or focus on evaluating Xpert MTB/RIF on raw sputum, since is it known that testing frozen stored specimens may influence results.[29]

In the current algorithm for Malawi, a residual specimen from the second sample of presumptive tuberculosis patients is tested on Xpert MTB/RIF if both samples are smear-negative. Xpert MTB/RIF is also performed on samples from all patients suspected of having MDR tuberculosis and retreatment cases, but not on new smear-positive cases.[3]

Conclusion

Our results demonstrate that Xpert MTB/RIF has the potential to increase the number of patients initiated early on appropriate treatment, given that additional tuberculosis cases were identified among smear-negative/culture-confirmed cases, one of which was resistant to rifampicin. It is expected that the use of Xpert MTB/RIF will offer a greater opportunity in increasing tuberculosis case detection among high-risk and vulnerable groups in Malawi where sputum smear microscopy is still widely used. We believe that results obtained by Xpert MTB/RIF may facilitate a proactive approach to tuberculosis by enhancing decisions towards treatment and ensuring that the tuberculosis programme is not treating people who do not have tuberculosis but are obviously sick. When this is achieved, it is anticipated that there will be a reduction in the burden of tuberculosis among people living with HIV.

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Competing interests

The authors declare that they have no financial or personal relationship(s) that may have inappropriately influenced them in writing this article.

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Authors' contributions

T.C. conceptualised the study. B.B. and R.H.G. oversaw patient enrolment in the primary study. T.C., N.N. and I.T. performed the laboratory experiments. M.H., L.S., W.S., R.K., J.M. and I.F.H. oversaw the operational aspects of the study. T.C., N.E.R. and C.S. conducted the data analysis, and T.C. drafted the manuscript. All authors revised the manuscript and approved the final draft.

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CHAPTER 5

Molecular Characterisation of Rifampicin Resistant *Mycobacterium tuberculosis* Strains from Malawi

5.1 Summary

Confirmation of mutations responsible for rifampicin resistance in *Mycobacterium tuberculosis* strains in Malawi was done by DNA sequencing of the Rifampicin Resistance Determining Region (RRDR) of the *rpo*B gene, with particular focus on strains which were define as resistant by the Xpert MTB/RIF. This work is presented in the following manuscript, which has been published by the African Journal of Laboratory Medicine (AJLM) and was also presented at the following conferences.

a) Tarsizio Chikaonda, E-poster presentation 2015: Sequencing-based detection of *rpo*B gene mutations in *Mycobacterium tuberculosis* strains from Malawi. 46th Union World Conference on Lung Health, Cape Town, 2nd to 6th December. E-Poster presentation.

b) Tarsizio Chikaonda, Oral presentation, 2016: *rpo*B gene mutations in rifampicin resistant *Mycobacterium tuberculosis* strains from Malawi. National TB Conference, Lilongwe, Malawi, 11th to 12th February. Oral presentation

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Molecular characterisation of rifampicin-resistant *Mycobacterium tuberculosis* strains from Malawi

Authors:
Tarsizio Chikaonda[1,2]
Irene Ketseoglou[1]
Nelson Nguluwe[2]
Robert Krysiak[2]
Isaac Thengolose[2]
Felix Nyakwawa[3]
Nora E. Rosenberg[2]
Christopher Stanley[2]
James Mpunga[3]
Irving F. Hoffman[2,4]
Maria A. Papathanasopoulos[1]
Mina Hosseinipour[2,4]
Lesley Scott[1]
Wendy Stevens[1]

Affiliations:
[1]Department of Molecular Medicine and Haematology, Faculty of Health Sciences, School of Pathology, University of the Witwatersrand, Johannesburg, South Africa

[2]UNC Project, Lilongwe, Malawi

[3]Malawi National Tuberculosis Programme, Lilongwe, Malawi

[4]University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, United States

Corresponding author:
Tarsizio Chikaonda,
tarchikaonda@gmail.com

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Background: Availability and access to the detection of resistance to anti-tuberculosis drugs remains a significant challenge in Malawi due to limited diagnostic services. The Xpert® MTB/RIF can detect *Mycobacterium tuberculosis* and resistance to rifampicin in a single, rapid assay. Rifampicin-resistant *M. tuberculosis* has not been well studied in Malawi.

Objectives: We aimed to determine mutations in the rifampicin resistance determining region (RRDR) of the *rpo*B gene of *M. tuberculosis* strains which were defined as resistant to rifampicin by the Xpert MTB/RIF assay.

Methods: Rifampicin-resistant isolates from 43 adult patients (≥ 18 years) from various districts of Malawi were characterised for mutations in the RRDR (codons 507–533) of the *rpo*B gene by DNA sequencing.

Results: Mutations were found in 37/43 (86%) of the resistant isolates in codons 511, 512, 513, 516, 522, 526 and 531. The most common mutations were in codons 526 (38%), 531 (29.7%) and 516 (16.2%). Mutations were not found in 6/43 (14%) of the resistant isolates. No novel *rpo*B mutations other than those previously described were found among the rifampicin-resistant *M. tuberculosis* complex strains.

Conclusion: This study is the first to characterise rifampicin resistance in Malawi. The chain-termination DNA sequencing employed in this study is a standard method for the determination of nucleotide sequences and can be used to confirm rifampicin resistance obtained using other assays, including the Xpert MTB/RIF. Further molecular cluster analysis, such as spoligotyping and DNA finger printing, is still required to determine transmission dynamics and the epidemiological link of the mutated strains.

Introduction

Tuberculosis remains an important public health problem especially in the developing world. The global impact of tuberculosis is significant, with an annual estimate of 9.6 million tuberculosis cases and over 1.5 million deaths due to tuberculosis in 2014.[1] The tuberculosis burden is worsened by the emergence and spread of multi-drug resistant (MDR) tuberculosis cases, defined as simultaneous resistance to at least rifampicin and isoniazid, with or without resistance to any other drug.[1]

In Malawi, there were 5564 new smear-positive cases of tuberculosis registered in 2014.[1] Patients diagnosed with tuberculosis are treated with the standard quadruple antibiotic therapy recommended for drug-susceptible tuberculosis (rifampicin, isoniazid, ethambutol and pyrazinamide). Rapid detection of drug resistance is crucial in choosing the most effective treatment to avert morbidity and mortality of infected individuals and reduce the risk of MDR tuberculosis transmission.[1]

Rifampicin, if the isolate is susceptible, is a very important component of the current tuberculosis treatment regimen and has proved to be effective to both susceptible strains and strains resistant to streptomycin and isoniazid.[1] However, there is growing resistance to rifampicin, largely due to particular genomic mutations in the *rpo*B gene of *Mycobacterium tuberculosis*.[2] The *rpo*B gene encodes the β subunit of RNA polymerase, which is involved in chain initiation and elongation. A signature sequence for *M. tuberculosis* identification is contained in this region.[3,4] Mutations in the rifampicin resistance determining region (RRDR) of this gene (codons 507–533) are associated with rifampicin resistance.[5] Detection of such mutations indicates rifampicin-resistant tuberculosis strains and can be used as a predictor for MDR tuberculosis, although not a complete surrogate marker.[6]

Globally, it is estimated that 3.3% of new cases and 20% of previously-treated cases have MDR tuberculosis and that 9.7% of these cases have pre-extensively drug resistant tuberculosis.[1] A substantial percentage (37%) of new tuberculosis cases and a staggering percentage (74%) of the global estimate of MDR tuberculosis incident cases were not reported or remained undiagnosed in 2014. Determination of the pattern of drug resistance is performed in less than 3% of people diagnosed with tuberculosis worldwide.[1,7,8]

Phenotypic drug susceptibility testing (DST) relies on detection of growth and is performed using an antibiotic susceptibility testing set consisting of a growth control and one tube for each anti-tuberculosis test drug, with known concentration.[9] Phenotypic DST is widely used in limited-resource settings. This method is inexpensive and accurate, though time consuming due to its reliance on the growth of *M. tuberculosis*, which takes a long time to obtain results.[10] Mutation(s) in the genes relevant to responses to each drug are associated with resistance to tuberculosis drugs.[10] Genotypic DST methods target well-characterised resistance-associated mutations. Genotypic DST determines such mutations in the tested gene region only and as such, unknown or less frequent mutations might be missed.[11,12] Molecular tools for rapid DST are developed upon a better understanding of mutations responsible for drug resistance in the bacterial genome.[13,14] Several molecular methods, including the Xpert® MTB/RIF (Cepheid, Sunnyvale, California, United States), have been developed for the detection of *M. tuberculosis* complex DNA and RRDR mutations associated with rifampicin resistance.[15,16,17] The World Health Organization strongly recommends the use of Xpert MTB/RIF as the initial diagnostic test for use on pulmonary specimens from adults and children suspected of having HIV-associated tuberculosis or MDR tuberculosis.[1]

The use of DNA sequencing complements the above assay in detecting new mutations, as well as confirming the presence of the most frequent mutations that could be associated with drug resistance, and has conferred excellent benefits to patient care due to the larger DNA fragment that is sequenced.[18,19] DNA sequencing determines mutations by comparing the differences between the gold standard (H37Rv reference strain) and the test nucleotide sequence.[20,21]

We aimed to assess the RRDR of the *rpo*B gene for mutations in *M. tuberculosis* strains that were defined as rifampicin resistant by the Xpert MTB/RIF assay and to establish the prevalence of such mutations in tuberculosis-infected Malawians.

Methods

Ethical considerations

Approvals for this study were granted by University of the Witwatersrand Human Research Ethics Committee (M120256), National Health Sciences Research Committee (NHSRC) in Malawi (NHSRC # 999) and the University of North Carolina (UNC; Chapel Hill) Institutional Review Board (CID 1211). Obtaining consent was waived because the study used previously-stored and residual sputum pellets, and consent from patients was given during recruitment and/or this was part of routine testing for tuberculosis drug resistance.

Study population

We conducted this study using processed sputum sediments (pellets) from new and previously-treated patients, ≥ 18 years of age. Demographic and clinical information was collected from the respective laboratory tuberculosis registers and entered in Excel (Microsoft, Inc., Redmond, Washington, United States) spreadsheets. Both retrospective and prospective pellets ($N = 995$) were used in the current study.

Retrospective pellets

Retrospective sputum pellets ($n = 351$) were collected between April 2011 and July 2012 from a study conducted in outpatients initiating tuberculosis treatment at Martin Preuss Centre at Bwaila Hospital (Bwaila) in Lilongwe, which was looking at the prevalence of drug-resistant tuberculosis at this HIV/tuberculosis clinic.[22] Sputum samples were processed for routine culture at the UNC Project laboratory following the N-acetyl-L-cysteine and sodium hydroxide (NALC-NaOH) method. Pellets were inoculated on both mycobacterium growth indicator tubes (MGIT) and Löwenstein Jensen media. DST was performed using the Hain MTBDR*plus* assay (Hain Lifescience GmbH, Nehren, Germany). Residual pellets were stored at -80°C and selected at random for use in this study without any special criteria to eliminate bias.

Prospective pellets

Prospective sputum pellets came from patients suspected of drug-resistant tuberculosis ($n = 644$) who presented consecutively to district hospitals in Malawi, and sputum samples were sent to the National Tuberculosis Reference Laboratory (NTRL) in Lilongwe for conventional DST between June 2012 and May 2014. Sputum was processed using the NALC-NaOH method and the pellet was split in two. The first sample was used for routine culture and conventional DST at the NTRL, while the other was stored for up to two weeks at 2°C – 8°C for Xpert MTB/RIF testing and MGIT culture at the UNC laboratory.

Mycobacterium cultures at the UNC laboratory

Pellets were re-suspended in 1.5 mL of phosphate buffer and split into different volumes. One part was processed for routine culture using MGIT and the other was processed on Xpert MTB/RIF (0.5 mL). MGIT tubes were inoculated with 500 µL of the re-suspended sample as previously described[23] and were monitored daily for growth using a hand-held BACTEC MicroMGIT Reader for up to 42 days. Smears were prepared from positive cultures, stained using Ziehl-Neelsen stain (Becton, Dickinson & Company, Sparks, Maryland, United States) and examined for

acid-fast bacilli. Determination of eligibility for DNA sequencing was dependent on results from the Xpert MTB/RIF assay (software version G4). DNA was extracted from corresponding MGIT cultures if rifampicin resistance was detected by Xpert MTB/RIF.

Xpert MTB/RIF assay

Samples (n = 995) were processed as per the manufacturer's instructions. A sample reagent buffer was added to 500 µL of the re-suspended pellet in the ratio of 3:1 (sample reagent buffer:specimen) as described previously.[24] The container was closed tightly and then vigorously shaken for 15 seconds. The mixed specimen was left to stand for 10 minutes followed by another vigorous shaking for 15 seconds and left to stand for a further five minutes. Thereafter, 2 mL of the mixed specimen was loaded into a single-use Xpert MTB/RIF cartridge. The closed cartridge was loaded into the GeneXpert® instrument, where extraction, amplification and detection of *M. tuberculosis* and screening for resistance to rifampicin were performed automatically and simultaneously. Results were available within two hours. The Xpert MTB/RIF probe and cycle threshold (Ct) value was documented for each tuberculosis strain that was detected as resistant to rifampicin.

DNA extraction and amplification

Genomic bacterial DNA was extracted from cultures at the UNC laboratory using the HAIN GenoLyse kit (Hain Lifescience GmbH, Nehren, Germany). From MGIT liquid media, 1.0 mL was transferred to a Sarstedt micro-centrifuge tube. The tube was centrifuged for 15 minutes at 14 000 rpm. The supernatant was carefully discarded and the pellet was re-suspended in 100 µL Lysis Buffer from the kit, vortexed thoroughly and incubated at 95°C in a heating block for five minutes. Tubes were removed and briefly centrifuged to remove condensation. To each tube, 100 µL of Neutralization Buffer was added, vortexed for five seconds and then centrifuged for five minutes at 14 000 rpm. Supernatant was transferred to a new tube and the pellet was discarded. The extracted DNA was stored at -80°C for use as DNA template.

MTBDR*plus* line probe assay

The GenoType®MTBDR*plus* line probe assay (Version 2) was performed according to the manufacturer's instructions. In brief, an appropriate number of PCR tubes, one for each sample and one each for a PCR positive (*M. tuberculosis* DNA) and negative (dH_2O) control were labelled. A master mix was prepared for one reaction and the final volume adjusted according to the total number of samples, with some excess to allow for pipetting errors. The solution was mixed by inversion and an aliquot of 45 µL of the prepared master mix was transferred to each PCR tube. 5 µL of each sample or control DNA was added to the appropriate PCR tube. PCR was performed in a 9700 DNA thermocycler (Applied Biosystems, Foster City, California, United States), using the cycling protocol as described by the manufacturer.

DNA PCR

PCR amplification of the *rpo*B gene, which included the RRDR, was carried out using forward primer *rpo*BF2 (5'-GAG GGT CAG ACC ACG ATG AC-3'; nucleotide positions 1030 to 1049 according to H37Rv numbering, GenBank accession number CAB09390.1) and reverse primer *rpo*BR (5'-GAG CCG ATC AGA CCG ATG T-3'; nucleotide positions 1460 to 1478 according to H37Rv numbering) in a GeneAmp PCR system 9700 thermocycler (Applied Biosystems, Foster City, California, United States). The total volume of the PCR reaction used was 50 µL, containing: 5 µL of 10X high fidelity buffer, 1 µL of 10 mM dNTP mix, 2 µL of 50 mM $MgSO_4$, 1 µL of each primer, 0.2 µL of Taq HiFi (Invitrogen, Carlsbad, California, United States), 36.8 µL of molecular grade water and 3 µL of extracted genomic DNA. Amplification conditions were set at: 94°C for two minutes; followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 68°C for 40 seconds; followed by a 10-minute final elongation at 68°C. PCR products were purified using the GeneJet PCR purification kit (Thermo Scientific, Waltham, Massachusetts, United States) following the manufacturer's instructions. 5 µL of the amplified product was visualised on a 1% agarose gel.

DNA sequencing

DNA sequencing was performed in an automated DNA sequencer ABI 3700, (Applied Biosystems, Foster City, California, United States) using the BigDye terminator V3.1 sequencing kit with forward primer *rpo*BS (5'-GCA GAC GTT GAT CAA CAT CC-3') and reverse primer *rpo*BR (5'-GAG CCG ATC AGA CCG ATG T-3'). The total volume of the sequencing reaction was 20 µL, containing: 4 µL of BigDye terminator, 2 µL of 10X sequencing buffer, 2 µL of water, 1 µL of primer and 11 µL of sample. Amplification conditions were set for: one minute at 96°C; followed by 25 cycles of 96°C for 10 seconds, 50°C for five seconds, 60°C for four minutes; followed by a 10-minute final extension at 72°C. The completed sequencing reaction mixture was purified using 75% isopropanol as per the manufacturer's instructions.

The sample plate was loaded onto the ABI 3700 automated DNA sequencer and the resulting sequences were analysed and compared to the wild-type sequence of the well-characterised *M. tuberculosis* H37Rv reference strain using Sequencher software V4.8 (Genecodes Corporation, Ann Arbor, Michigan, United States). The well-defined RRDR region (codons 507–533) corresponded to codons 426–452 (nucleotides 1276–1356) in the downloaded H37Rv reference sequence.

Statistical analysis

All study data were entered into an Excel spreadsheet and then exported and analysed using Stata Version 12 (StataCorp, College Station, Texas, United States). We calculated proportions and percentages based on district, patient category, Xpert MTB/RIF probes and codons in the RRDR of the *rpo*B gene to determine resistance mutations and their prevalence.

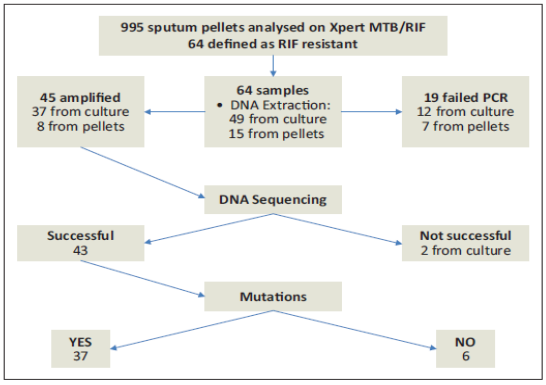
Results

The 644 specimens from NTRL were categorised clinically as previously treated cases (574/644; 89.1%), new (27/644; 4.2%), or not known (43/644; 6.7%). From Bwaila, 24/351 (6.8%) suspected tuberculosis cases were categorised as retreatment, and 327/351 (93.2%) were from patients presenting with tuberculosis for the first time.

The Xpert MTB/RIF assay detected rifampicin resistance in 64/995 (6.4%) specimens, which were selected for further DNA sequencing of the larger region of the *rpo*B gene. Three specimens (3/64) were from Bwaila, while the rest (61/64) were collected from NTRL. Most of the rifampicin-resistant cases detected by Xpert MTB/RIF were associated with probe B (23/64) and probe E (23/64). Fifteen (15/64) samples were detected by probe D, 2/64 by probe A, while probe C detected 1/64 samples (Table 1). Of these, 43/64 were successfully sequenced. The remainder (n= 21; 32.8%), all from NTRL, were not sequenced, either due to failure of PCR amplification (n = 19) or they failed sequencing (n = 2) (Figure 1). The PCR amplification failure could not be explained, although in part this could be due to insufficient template DNA, a an attempt was made to extract DNA from pellets (n = 7) which had not grown on culture.

TABLE 1: *rpo*B gene mutations associated with Xpert MTB/RIF probes used.

DNA sequencing mutations	Xpert MTB/RIF probes					Total
	A	B	C	D	E	
D516V	1	4	0	0	1	6
Failed sequencing	0	2	0	0	0	2
H526D	0	0	0	1	0	1
H526R	0	0	0	2	0	2
H526Y	0	1	0	10	0	11
L511P	1	0	0	0	0	1
Wild-type	0	4	0	0	2	6
Failed PCR amplification	0	10	0	2	7	19
Q513E	0	1	0	0	1	2
S512T	0	0	0	0	1	1
S513L	0	0	0	0	1	1
S522L	0	0	1	0	0	1
S531L	0	1	0	0	9	10
S531Y	0	0	0	0	1	1
Total	2	23	1	15	23	64



Abbreviations: RIF, rifampicin.

FIGURE 1: Workflow and isolate selection for DNA sequencing from tuberculosis cultures and processed sediments.

Seven different mutations were detected in 37/43 (86%) specimens in codons 511, 512, 513, 516, 522, 526 and 531. Mutations were common in codons 526 (38%), 531 (29.7%) and 516 (16.2%) (Table 2). No insertion, deletion or double point mutations were observed. However, mutations were not detected in 6/43 (14%) of the strains by DNA sequencing, despite being detected by Xpert MTB/RIF probe B (4/6) and by probe E (2/6) as rifampicin resistant. Repeat Xpert MTB/RIF testing was not done for these strains due to insufficient left-over pellets. Delayed Ct values between 17.0 and 32.7 were observed in 4/6 strains which had no mutations. The observed Ct values were markedly higher than the ΔCt max cutoff of > 4 for the automated detection of rifampicin resistance by the Xpert MTB/RIF assay.[25] The remainder (n = 2) gave an undetectable result on probe B (n = 1) and probe E (n = 1).

When stratified by district, the prevalence of mutations was high in tuberculosis patients from Ntchisi 2/5 (40%), Nkhotakota 4/13 (30.8%), Nsanje 5/20 (25%) and Balaka 2/12 (16.7%). Lilongwe, the capital city of Malawi, had the lowest prevalence of mutations 9/466 (1.9%), followed by Mulanje 2/57 (3.5%).

As expected, the number of mutations was highest among patients registered as retreatment 29/598 (4.8%), compared with new cases 8/354 (2.3%). There were no real trends of mutations across classified retreatment cases (recurrent, default, etc.) in relation to new tuberculosis cases. Mutations in codon 531 were found in almost all patient groups, except for those classified as recurrent; codon 526 in all groups, except in the 'new patient' category in which mutations at codon 516 were predominant. A single strain from a patient with no history of prior tuberculosis treatment was detected with a mutation at codon 522.

All 43 specimens that were successfully sequenced were also tested on GenoType MTBDR*plus* and two strains gave

TABLE 2: Distribution of mutations in codons 491–574 of the *rpo*B gene and amino acid changes in the rifampicin resistance determining region, Malawi, 2011 and 2014†.

Mutated codon	Amino acid change		Nucleotide change		Total isolates (n = 37)	Percentage
	From	To	From	To		
511	Leu	Pro	CTG	CCG	1	2.7%
512	Ser	Thr	AGC	ACC	1	2.7%
513	Gln	Leu	CAA	CTA	1	8.0%
		Glu	CAA	GAG	2	
516	Asp	Val	GAC	GTC	6	16.2%
522	Ser	Leu	TCG	TTG	1	2.7 %
526	His	Arg	CAC	CGC	2	38%
		Tyr		TAC	11	
		Asp		GAC	1	
531	Ser	Leu	TCG	TTG	10	29.7%
		Tyr		TAG	1	

†DNA sequencing results using sputum pellets and culture specimens of 37 tuberculosis-infected patients.

discordant results. Both strains showed susceptibility to rifampicin on GenoType MTBDR*plus*, but resistance to rifampicin on Xpert MTB/RIF. Mutations were not detected in these strains by DNA sequencing.

Discussion

This study is the first to characterise rifampicin resistance in Malawi. The majority of *rpo*B gene mutations in this study analysed by direct sequencing correlated well with rifampicin resistance observed on Xpert MTB/RIF. Our findings reveal that 86% of all rifampicin-resistant isolates harboured mutations in the RRDR of the *rpo*B gene with codon 526 (CAC → TAC) as the most frequent, followed by codon 531 (TCG → TTG/TAG). By contrast, mutations were not detected in 6/43 (14%) of the strains following nucleotide sequencing. False-positive rifampicin resistance was most likely considering the observed wild-type sequences in these strains. Data on drug-resistance mutations involving the RRDR of the *rpo*B gene, which relates highly to rifampicin resistance, is new for Malawi as no previous studies have analysed the *rpo*B gene among tuberculosis strains circulating in the country.

Previous reports demonstrated that strains requiring high rifampicin concentrations in phenotypic DST have been associated with mutations at codons 526, 516, and 531,[26,27] corroborating that these are the most prevalent *rpo*B mutations worldwide.[28] Several studies of rifampicin-resistant tuberculosis have reported the presence of novel and common *rpo*B gene mutations, especially in the RRDR.[29,30,31] In addition, other studies have documented the presence of common and novel *rpo*B mutations outside the RRDR.[29,32] Based on our amplicon size, we only evaluated the most frequently-mutated codons between positions 491–574, which include the RRDR. The contribution of additional mutations to resistance at positions outside the region that was sequenced, for example codon 176, cannot be ruled out. Future work should focus on sequencing the entire *rpo*B gene.

It is of particular interest that the Xpert MTB/RIF assay detected rifampicin resistance in six isolates, but partial sequencing of the *rpo*B gene did not detect any changes in the nucleotide sequences, such that the mechanisms/pathways of resistance of these strains remain unknown. Since unprocessed sputum was not sequenced, it was difficult to confidently exclude hetero-resistance in these strains. In low rifampicin-resistance prevalence areas, the Xpert MTB/RIF assay would be expected to falsely diagnose some cases,[8] which could be the case with these strains. Sequencing the entire *rpo*B gene might help to understand the mechanism of resistance of the strains. In contrast, results obtained in Swaziland show that the Xpert MTB/RIF assay did not detect the *rpo*B I491F mutation (outbreak strain) in 38/125 (30%) of the isolates tested as compared to DNA sequencing, raising fears about the assay's unreliability due to under-diagnosis and potential treatment inadequacy, since it does not detect mutations outside the RRDR.[33] Furthermore, a study by Theron et al. observed that five strains were rifampicin resistant on Xpert MTB/RIF, *rpo*B sequencing and/or GenoType MTBDR*plus*, but susceptible on phenotypic DST.[34] It should be noted, however, that PCR amplicons were different in the Xpert MTB/RIF assay versus what was used for the Sanger-based sequencing. The probes may have bound to minority variants in the Xpert MTB/RIF amplicons, resulting in a positive signal, whereas a combination of PCR bias and population-based Sanger sequencing (where the limit of detection is approximately 20% of the quasispecies and only the predominant population is reported) might have resulted in only the wild-type sequence being detected.[35] Moreover, different fitness of drug-resistant tuberculosis under culture conditions can allow for the outgrowth of wild-type tuberculosis,[36] accounting for the discordant results between the molecular and phenotypic assays.

Drug resistance was detected in some new tuberculosis cases and these results were confirmed by detecting mutations in the RRDR of the *rpo*B gene. Detecting resistance to rifampicin in new tuberculosis cases highlights the need for testing resistance to drugs in this category especially if the patient was in contact with a known MDR tuberculosis patient. Our results show that the prevalence of mutations associated with rifampicin resistance among new tuberculosis cases was 2.3%. In Malawi, the prevalence of MDR tuberculosis is low (0.4%) among new smear-positive cases and is 4.8% among retreatment cases. Testing for tuberculosis drug resistance in Malawi is not routinely done except for those highly suspected of drug resistance.[37]

Results obtained by the Xpert MTB/RIF assay were in agreement with GenoType MTBDR*plus* results on rifampicin resistance, with an exception of 2/43 (4.7%), which were sensitive on the GenoType MTBDR*plus* but rifampicin resistant on Xpert MTB/RIF. Ct values of 18.5 and 24.4 on Xpert MTB/RIF, both on probe B, were interpreted as rifampicin resistant. Mixed infection with multiple tuberculosis strains was excluded in these strains given the wild-type *rpo*B gene sequences and no observed underlying peaks on the DNA sequence chromatograms. Phenotypic DST is difficult to standardise and expensive and time-consuming to maintain routinely. In the absence of molecular techniques, several critical questions about tuberculosis remain unanswered, including recognition of acquisition of drug resistance resulting from gene mutations versus transmission of drug resistant strains.[38]

Limitations

The relatively small number of rifampicin-resistant samples observed in the current study limited our ability to establish the overall prevalence of *rpoB* gene mutations in tuberculosis strains circulating in Malawi. Moreover, we sequenced only an approximately 450 bp amplicon; thus, the contribution of mutations in regions outside of the amplicon sequenced to overall resistance could not be determined. With the available data, an assessment of the epidemiological link between the

rifampicin-resistant strains was not possible, as such further molecular cluster analysis is still required to determine transmission dynamics of the mutated strains. Future studies should consider performing phenotypic DST to detect mutations outside the RRDR (full length *rpo*B gene) and compare to DNA sequencing, considering that Sanger sequencing has a limit of detection where the minority population may not be detected.

Conclusion

The chain termination DNA sequencing employed in this study is a standard method for the determination of nucleotide sequences. It is conclusive and can be used to confirm rifampicin resistance obtained by other assays, including the Xpert MTB/RIF assay, despite having a limit of detection with minority resistant populations. Although phenotypic DST is recommended to verify rifampicin resistance when using Xpert MTB/RIF, DNA sequencing can be used to detect the frequency and exact site of mutations in the RRDR. All tuberculosis strains with mutations in the current study had one of the previously-described *rpo*B gene mutations containing nucleotide changes. No new mutations were identified. Findings from this study emphasise the need for a national representation of sputum specimens from tuberculosis-infected patients to assess the magnitude of tuberculosis *rpo*B gene mutations that are responsible for rifampicin resistance in Malawi.

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Competing interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

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Authors' contributions

T.C. conceptualised the study. T.C., I.K., N.N. and I.T. performed the experiments. M.H., L.S., W.S., F.N., R.K., M.A.P., J.M. and I.F.H. oversaw the operational aspects of the study. T.C., N.E.R. and C.S. conducted the data analysis and T.C. drafted the manuscript. All authors revised the manuscript and approved the final draft.

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CHAPTER 6

Conclusions and Future Perspectives

Conclusions and Future Work

6.1 Conclusions and Recommendations

TB and MDR-TB epidemics are unlikely to be controlled without better diagnostic tools especially in countries with high HIV prevalence. Malawi is listed among the 41 high TB burden countries worldwide and yet there is limited data about the transmission patterns and population structure of TB drug-resistant strains. Additionally, no information is available on the composition of DR-TB genotypes in most of the country's districts other than Karonga (1, 2). The work from this PhD study is the first to characterize the *rpo*B gene of RIF-resistant MTB strains from various districts of Malawi.

Resource limited countries like Malawi should aim at utilizing rapid, reliable and accurate methods for TB diagnosis and detection of drug resistance. In the current study, Xpert MTB/RIF proved to be a suitable tool in identifying MTB strains and detecting resistance to RIF. Our findings support the Malawi NTP decision to adopt the Xpert MTB/RIF to diagnose TB and DR-TB in spite of the DR prevalence being low (0.3% among new TB cases and 3.8% among retreatment cases) and there being a

2.9% prevalence of INH mono-resistance. Staff performing the Xpert MTB/RIF need to understand test performance characteristics and utilize confirmatory facilities for RIF resistance detected by Xpert MTB/RIF (3) considering the occurrence of silent mutations in the RRDR of the *rpo*B gene and the complexity of molecular mechanisms of RIF resistance in MTB (4).

The Malawi NTP recommends a repeat Xpert MTB/RIF and confirmatory DST on specimens with RIF resistance (3) which has implications on patient management. Between initial diagnosis and availability of DST results, the median delay is about 40 days using MTBDR*plus* to longer delays using phenotypic DST. With such delays, there is usually a clinical dilemma of which regimen to start (5). Delays in starting of treatment increase the risk of death and poses infection control issues. Starting on first-line drugs while awaiting confirmatory DST poses the risk of limiting future treatment option due to a possibility of amplification of resistance to pyrazinamide or ethambutol (6).

The use of DNA sequencing to confirm drug resistance proved to be challenging, mostly because of the lack of sufficient DNA quantities for amplification. Inadequate

DNA was extracted either from culture or directly from pellets resulting in amplification failure of 19 isolates. In resource limited areas, such technical difficulties can prove to be time consuming and also expensive.

The overall objectives of this study were met. In line with rolling out of Xpert MTB/RIF testing in Malawi, the study aimed at evaluating this technology as a diagnostic tool for TB and screening for drug resistance in the adult population by comparing its sensitivity and specificity to fluorescent smear microscopy, MTBDR*plus* V2 and mycobacterial culture. Furthermore, the study aimed at determining the prevalence of mutations responsible for RIF resistance in the RRDR of the *rpo*B gene among TB strains in the adult population in Malawi.

Chapter 3 addressed levels of drug resistance in presumptive TB outpatients starting on treatment at Bwaila in Lilongwe, emphasizing the effectiveness of the Malawi NTP. Differences were observed in AFB and culture positivity according to HIV status but drug resistance was not associated with AFB smear result, age, gender, TB treatment status or TB site. Chapter 4 used 351 stored sputum pellets selected from this cohort to evaluate the performance of Xpert MTB/RIF in a Malawian setting.

Introduction of the Genotype® Mycobacterium CM identified 4 isolates as NTM, 3 *Mycobacterium intracellulare* and one *Mycobacterium avium.* NTM can act as pathogens or colonisers as such they may have contributed to the patient's respiratory symptoms (7). NTM are not routinely identified in Malawi, suggesting more efficient technologies should be adopted such as the Genotype® Mycobacterium CM. However, cost of reagents and equipment might be some of the limiting factors to implementation of such new technologies.

Finally the study achieved its objective of determining the prevalence of DR-TB among presumptive TB outpatients at Bwaila.

Chapter 4 evaluated the performance of Xpert MTB/RIF in outpatients at Bwaila by determining its sensitivity, specificity, PPV and NPV which were 93.8%, 97.4%, 97.8% and 92.6% respectively compared to culture. Since culture was the reference standard, it was established that 4 additional TB cases were identified by Xpert MTB/RIF as compared to smear microscopy. Our findings suggest that in 70 individuals that were classified as smear-negative by microscopy, the sputum contained the bacilli that cause TB suggesting that this category of patients could be a

substantial source of TB transmission (8). Prior studies suggest that up 17% of new TB cases are acquired from smear-negative cases as such it is critical to break the TB transmission cycle both in health facilities and the community by early identification and treatment of smear-negative cases (3).

We believe that results obtained by Xpert MTB/RIF may facilitate a proactive approach to tuberculosis by enhancing decisions towards treatment and ensuring that the TB programme is not treating people who do not have tuberculosis but are obviously sick. When this is achieved, it is anticipated that there will be a reduction in the burden of tuberculosis among people living with HIV.

Chapter 5 established *rpo*B gene mutations in the 81-bp core region of RIF-resistant MTB strains circulating in Malawi. This included evaluating Xpert MTB/RIF in the detection of RIF resistance in comparison to DNA sequencing. Thus, the presence of mutations in 37/43 RIF-resistant strains was determined by amplifying the RRDR and performing partial DNA sequencing of the *rpo*B gene. Despite its advantages over phenotypic DST, DNA sequencing is expensive and requires skilled personnel. The

PhD candidate achieved such skills of performing DNA sequencing and analysing sequences as a result of this work.

One discrepant RIF-resistant strain (described in chapter 4) showed DNA sequencing resolved the discrepancy between MTBDR*plus* V2 and Xpert MTB/RIF in favour of MTBDR*plus* V2. The overall conclusion reached was Xpert MTB/RIF should be confirmed by other assays including but not limited to DNA sequencing. This could have cost implications but beneficial.

A single *M.tb* strain was associated with a Probe E failure on the Xpert MTB/RIF assay while the amino acid change derived from Sanger sequencing was D516V. Our finding was very unusual considering that mutation D516V is associated with failure of Probe B, which covers the positions 513 to 517 of the RRDR of the *rpo*B gene. Different probes are associated with specific codons within the RRDR of the *rpo*B gene as such a correlation would be expected between the probe that failed and the amino acid change (9). Repeat testing to verify the obtained results could not be performed due to insufficient funds.

Despite the limited sample size (n=43) analysed through DNA sequencing, this study has shed light on *rpo*B gene mutations among MTB isolates circulating in the country. The Malawi NTP should consider conducting a similar study at national level to detect the prevalence of mutations conferring resistance not only to RIF but all available drugs in use to treat TB. Despite efforts to have good sample selection criteria and a well-represented study specimen number, there were still some limitations in that equal number of isolates from each district could not be selected. This was probably due to the differences in the number of people failing on treatment in relation to the population size of each district.

There is a need to reduce the mean time of sample handling at NTRL in order to improve patient care and TB control. Specimens referred for culture and DST from district facilities are transported to NTRL by courier services. This increases cost to specimen transportation and storage in addition to the complexity of MDR-TB testing because of the requirement for significant logistical support and cold chain management. If Xpert MTB/RIF can be introduced in all facilities that handle specimens for suspected MDR-TB, only eligible specimens for DST (according to the algorithm) can be collected and sent to NTRL. Thus, placement of Xpert MTB/RIF in such facilities can help reduce transporting huge number of specimens thereby

reducing the workload at NTRL and significantly lowering cold chain and transportation costs.

6.2 Future Perspectives

Outcomes in this thesis form a basis for future research on TB in Malawi. The identified *rpo*B gene mutations in RIF-resistant TB strains allows for ongoing research to establish the genetic relatedness of these strains as well as with other TB isolates across the country and from neighbouring countries. Further analysis should be done using DNA fingerprinting and spoligotyping to identify TB strain families circulating in the country and establish if there is transmission of drug-resistant TB strains among individuals. Such information would help in monitoring dynamics of TB transmission within and around Malawi. Molecular testing such as DNA sequencing and spoligotyping should be established at NTRL (central level) in order to monitor disease transmission trends with a possibility of conducting more studies in future.

The ease of travel between countries experienced in recent times in central and Southern Africa poses a real and serious risk of MDR-TB transmission and outbreak into Malawi. Mutational analysis has been used in several studies across the globe to gain insight in MDR-TB strains of similar and/or different strain families (10). MTB

strains included in this study provide a bank of data that can be compared to those from neighbouring countries in monitoring the transmission of DR-TB strains.

Overall, technological advancements in molecular techniques such as Xpert Ultra (next-generation cartridge) and the GeneXpert Omni diagnostic platform will greatly improve TB case detection and determination of drug resistance. This, coupled with a concerted effort from infrastructural advancement programmes, funding agencies and politicians, molecular techniques will be made more accessible to patients requiring TB investigations in Malawi.

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CHAPTER 7

Appendices

Appendix 1

Ethical approval from University of the Witwatersrand (M120256)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Tarsizio Chikaonda

CLEARANCE CERTIFICATE	M120256
PROJECT	Exploring the Role and Accuracy of the Gene Xpert in TuberculosisDiagnosis and Drug Resistance Screening in Malawi
INVESTIGATORS	Mr Tarsizio Chikaonda.
DEPARTMENT	Internal Med/Molecular Med & Haematology
DATE CONSIDERED	24/02/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 24/02/2012 CHAIRPERSON .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Prof Wendy Stevens

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and ONE COPY returned to the Secretary at Room 10034, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the proposal to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix 2

Ethical Approval from the National Health Science Research Committee (NHSRC # 999)

Telephone: + 265 789 400
Facsimile: + 265 789 431
e-mail doccentre@malawi.net
All Communications should be addressed to:
The Secretary for Health and Population

In reply please quote No. MED/4/36c

MIN STRY OF HEALTH
P.O. BOX 30377
LILONGWE 3
MALAWI

25th May 2012

Tarsizio Chikaonda
UNC Project

Dear Sir/Madam,

RE: **Protocol # 999: Exploring the role and accuracy of the genexpert in Tuberculosis diagnosis and drug resistance screening in Malawi**

Thank you for the above titled proposal that you submitted to the National Health Sciences Research Committee (NHSRC) for review. Please be advised that the NHSRC has reviewed and **approved** your application to conduct the above titled study.

- **APPROVAL NUMBER** : **NHSRC # 999**
  The above details should be used on all correspondence, consent forms and documents as appropriate.
- **APPROVAL DATE** : **25/5/2012**
- **EXPIRATION DATE** : This approval expires on **25/05/2013**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the NHSRC secretariat should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING** :All serious problems having to do with subject safety must be reported to the National Health Sciences Research Committee within 10 working days using standard forms obtainable from the NHSRC Secretariat.
- **MODIFICATIONS:** Prior NHSRC approval using standard forms obtainable from the NHSRC Secretariat is required before implementing any changes in the Protocol (including changes in the consent documents). You may not use any other consent documents besides those approved by the NHSRC.
- **TERMINATION OF STUDY:** On termination of a study, a report has to be submitted to the NHSRC using standard forms obtainable from the NHSRC Secretariat.
- **QUESTIONS:** Please contact the NHSRC on Telephone No. (01) 789314, 08588957 or by e-mail on doccentre@malawi.net
- **Other:**
  Please be reminded to send in copies of your final research results for our records as well as for the Health Research Database.

Kind regards from the NHSRC Secretariat.

.....
FOR CHAIRMAN, NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE

Telephone: + 265 726 422
Facsimile: + 265 726 418
E-mail: mohdoccentre@gmail.com

All Communications should be addressed to:
The Chairperson

In reply please quote No. MED/4/36c
MINISTRY OF HEALTH
P.O. BOX 30377
LILONGWE 3
MALAWI

NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE

MATERIAL TRANSFER AGREEMENT (MTA) FORM

Protocol Number: 999

Title of protocol:

EXPLORING THE ROLE AND ACCURACY OF THE GENEXPERT IN TUBERCULOSIS DIAGNOSIS AND DRUG RESISTANCE SCREENING IN MALAWI

Intention and Justification of transfer:

DNA extracted from all rifampicin resistant will be shipped to the department of Molecular Medicine and Haematology at University of the Witwatersrand for the determination of the rpoB gene mutations by sequencing using an ABI prism 3100 capillary DNA sequencer which is currently not available at UNC Project. It is the University's recommendation that the student performs sequencing and sequence analysis by himself for this project.

Duration of storage:

DNA lysate will be stored for up to a maximum of six months depending on the time of detection of resistance to the required student period of stay at University of the Witwatersrand

Responsible Party:

Tarsizio Chikaonda
UNC Project
Tidziwe laboratory
P/Bag A-104
Lilongwe
Malawi

Location of stored samples:
UNC Project Tidziwe laboratory in -80^{0}C freezer P/Bag A-104 Lilongwe Malawi
Transportation of samples:
Samples will be transported through courier as per the country's regulations
Ownership of samples:
Ministry of Health- Malawi
After all laboratory testing has been completed: Describe what will happen to the samples
The samples will be discarded upon completion since my proposed study and the results to be obtained from it will be strictly academic as such only approved analyses may be done on the samples.
Appropriate informed consent authorising the exportation and importation of samples
This study will use previously stored sputum pellets and residual samples from "The In-patient Tuberculosis Ward at Bwaila Hospital, Lilongwe, Malawi: A Feasibility Study to Improve Laboratory Capacity and characterize the Population; Outpatient Extension" study as such there won't be a need to get consent from patients as it is assumed that consent was given during recruitment. A consent form used in the adult TB study (Protocol # 672) is attached to this form.
To whom will the samples be accessible
Other than the student, the samples will be accessible by supervisors where verification and/or retesting will be required
Who will be the controlling officers of the samples
Tarsizio Chikaonda

Signed by

Name of the PI:

Tarsizio Chikaonda

Name of Institution:

UNC Project

Signature:

Date Signed:

23/05/12

Name of Co – PI

Mina Hosseinipour

Name of Institution:

UNC Project

Signature:

Date Signed:

23-5-12

NHSRC APPROVAL

Name of the Chairperson:

DR D. KATHYOLA

Signature:

Date Signed: 25/05/12

Name of Secretary

R. MAJAMANDA

Signature:

Date Signed: 25/5/12

SECRETARY FOR HEALTH
2012 -05- 25
P.O. BOX 30377, CAPITAL CITY, LILONGWE 3

NHSRC STAMP OF APPROVAL:

Appendix 3

Ethical Approval from the University of North Carolina at Chapel Hill (CID 1211)

THE UNIVERSITY of NORTH CAROLINA at CHAPEL HILL

OFFICE OF HUMAN RESEARCH ETHICS
Medical School Building 52
Mason Farm Road
CB #7097
Chapel Hill, NC 27599-7097
(919) 966-3113
Web site: ohre.unc.edu
Federalwide Assurance (FWA) #4801

To: Irving Hoffman
Medicine

From: Biomedical IRB

Approval Date: 11/07/2012
Expiration Date of Approval: 11/06/2013

RE: Notice of IRB Approval by Expedited Review (under 45 CFR 46.110)
Submission Type: Initial
Expedited Category: 5.Existing or non-research data
Study #: 12-2077
Study Title: CID 1211 - Exploring the role and accuracy of the GeneXpert® in tuberculosis diagnosis and drug resistance screening in Malawi
Sponsors: National Commission for Science and Technology (Malawi)

This submission has been approved by the IRB for the period indicated. It has been determined that the risk involved in this research is no more than minimal.

Study Description:
Purpose: The aim of this study is to explore the Xpert® MTB/RIF assay as a diagnostic tool for tuberculosis and screening for drug resistance in a Malawian setting, and determine the prevalence of rpoB gene mutations among rifampicin resistant strains defined by the Xpert®.

Participants: Retrospective: Approximately 600 sputum pellets stored for future diagnostic testing including the Xpert® assay from a prospective study comparing MGIT and HAIN GenoType MTBDRplus among TB retreatment inpatients and newly diagnosed out patients in Malawi. Prospective: Residual samples from the "Bwaila/Kamuzu Central Hospital In-patient Tuberculosis Ward: A feasibility study to introduce additional diagnostic tests and characterize the re-treatment patient population? study will be used for this evaluation. The above study will collect data and analyse sputum from up to 400 TB suspects.

Procedures (methods): Pellets from both arms (retrospective and prospective) will be re-suspended, processed, and drug susceptibility testing will be performed using the BACTEC MGIT SIRE method and also GenoType® MTBDRplus line probe assay . All rifampicin resistant isolates will be stored and later shipped to the department of Molecular medicine and Haematology at University of the Witwatersrand for sequencing.

Regulatory and other findings:
The IRB has determined that the study-specific rationale provided by the investigator is sufficient to justify the waiver of informed consent according to 45 CFR 46.116(d) as consent was already given in the original study for the use of these stored samples for future use.

Investigator's Responsibilities:

Federal regulations require that all research be reviewed at least annually. It is the Principal Investigator's responsibility to submit for renewal and obtain approval before the expiration date. You may not continue any research activity beyond the expiration date without IRB approval. Failure to receive approval for continuation before the expiration date will result in automatic termination of the approval for this study on the expiration date.

Your approved consent forms and other documents are available online at http://apps.research.unc.edu/irb/irb_event.cfm?actn=info&irbid=12-2077.

You are required to obtain IRB approval for any changes to any aspect of this study before they can be implemented. Any unanticipated problem involving risks to subjects or others (including adverse events reportable under UNC-Chapel Hill policy) should be reported to the IRB using the web portal at http://irbis.unc.edu.

Researchers are reminded that additional approvals may be needed from relevant "gatekeepers" to access subjects (e.g., principals, facility directors, healthcare system).

This study was reviewed in accordance with federal regulations governing human subjects research, including those found at 45 CFR 46 (Common Rule), 45 CFR 164 (HIPAA), 21 CFR 50 & 56 (FDA), and 40 CFR 26 (EPA), where applicable.

CC:
Nazneen Howerton, Medicine
Tania Caravella, Center for Infectious Diseases

Appendix 4

Change of Tittle from the National Health Science Research Committee (NHSRC ≠ 999)

Telephone: +265 789 400
Facsimile: +265 789 431

All Communications should be addressed to:
The Secretary for Health and Population

In reply please quote No. MED/4/36c
MINISTRY OF HEALTH AND POPULATION
P.O. BOX 30377
LILONGWE 3
MALAWI

28th March 2013

Tarsizio Chikaonda
UNC Project

Dear Sir/Madam,

RE: Protocol # 999: Evaluation of the Xpert MTB/RIF assay and its impact on tuberculosis diagnosis and rifampicin resistance screening: Efforts to determine the prevalence of drug resistant TB and rpoB gene mutations in the Malawian adult population

Thank you for the above titled proposal that you submitted to the National Health Sciences Research Committee (NHSRC) for review.

The committee reviewed and **approved** the proposed changes made to the study.

Kind regards from the Secretariat.

.....

FOR: CHAIRMAN, NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE

Appendix 5

Annual Renewal from National Health Sciences Research Committee (NHSRC # 999)

Telephone: + 265 789 400
Facsimile: + 265 789 431
e-mail doccentre@malawi.net
All Communications should be addressed to:
The Secretary for Health and Population

In reply please quote No. MED/4/36c

MINISTRY OF HEALTH
P.O. BOX 30377
LILONGWE 3
MALAWI

28th March 2013

Tarsizio Chikaonda
UNC Project

Dear Sir/Madam,

RE: Protocol # 999: Evaluation of the Xpert MTB/RIF assay and its impact on tuberculosis diagnosis and rifampicin resistance screening: Efforts to determine the prevalence of drug resistant TB and rpoB gene mutations in the Malawian adult population

Thank you for the above titled proposal that you submitted to the National Health Sciences Research committee (NHSRC) for review. Please be advised that the NHSRC has **reviewed** and **approved** your application for **continuation** to conduct the above titled study along with the following documents;

APPROVAL NUMBER : **NHSRC # 999**
The above details should be used on all correspondence, consent forms and documents as appropriate.

- **APPROVAL DATE** : **28/03/2013**
- **EXPIRATION DATE** :This approval expires **on 28/03/2014**
  After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the NHSRC secretariat should be submitted one month before the expiration date for continuing review.
- **SERIOUS ADVERSE EVENT REPORTING** :All serious problems having to do with subject safety must be reported to the National Health Sciences Research Committee within 10 working days using standard forms obtainable from the NHSRC Secretariat.
- **MODIFICATIONS**: Prior NHSRC approval using standard forms obtainable from the NHSRC Secretariat is required before implementing any changes in the Protocol (including changes in the consent documents). You may not use any other consent documents besides those approved by the NHSRC.
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the NHSRC using standard forms obtainable from the NHSRC Secretariat.
- **QUESTIONS**: Please contact the NHSRC on Telephone No. (01) 789314, 0888344443 or by e-mail on doccentre@gmail.com
- **Other:**
  Please be reminded to send in copies of your final research results for our records as well as for the Health Research Database.

Kind regards from the NHSRC Secretariat.

.....
FOR CHAIRMAN, NATIONAL HEALTH SCIENCES RESEARCH COMMITTEE

PROMOTING THE ETHICAL CONDUCT OF RESEARCH
Executive Committee: *Dr.C.Mwansambo (Cheirman). Prof. E. Molyneux (Vice Chairperson)*
Registered with the USA Office for Human Research Protections (OHRP) as an International IRB
(IRB Number IRB00003905 FWA00005976)

Appendix 6

Permission from UNC Project to Conduct Research

UNC PROJECT
(University of North Carolina Project)
Lilongwe, Malawi

UNC PROJECT
Lilongwe, Malawi

Malawi Office
UNC Project
Private Bag A-104
Lilongwe, Malawi
Phone: 01 755056
Fax: 01 755954
Email: fmartinson@unclilongwe.org.mw

USA Office
Division Of Infectious Diseases
Bioinformatics Bldg., CB# 7030
Univ. of North Carolina at Chapel Hill
Chapel Hill, NC 27599-7030
Phone: (919) 966-6324
Fax (919) 966 8536
Email: heffron@med.unc.edu

January 19, 2012

To whom it may concern

RE: PERMISSION TO CONDUCT RESEARCH AT UNC PROJECT LABORATORIES

Permission has been granted to the bearer of this letter **Mr. Tarsizio Chikaonda** to conduct research at UNC Project laboratories as part of his PhD study at University of the Wilwatersrand.

The proposed research is **"Exploring the role and accuracy of the GeneXpert in tuberculosis diagnosis and drug resistance screening in Malawi"** which is expected to be completed in 3 years. The study will be conducted in microbiology and PCR laboratories.

Any assistance rendered to him will be greatly appreciated.

Sincerely,

Prof. Francis Martinson

Country Director.

Appendix 7

Permission from the National TB Programme to Use Samples from NTRL

Telephone: + 265 789 400
Facsimile: + 265 789 431

All Communications should be addressed to:
The Secretary for Health and Population

In reply please quote No.
MED/1/24.....

Ministry of Health,
P.O. Box 30377,
LILONGWE 3,
Malawi.

TO WHOM IT MAY CONCERN

RE: PERMISSION FOR USE OF TB SAMPLES/ISOLATES FOR RESEARCH TOWARDS PhD STUDY

Permission has been granted to the bearer of this letter Mr. Tarsizio Chikaonda to collect sputum pellets/isolates from the National TB Reference Laboratory at CHSU. The samples are from suspected drug resistant patients and will be included in his research at UNC Project laboratories in Lilongwe as part of his PhD study at University of the Witwatersrand as per his submitted research protocol.

The proposed research is "Exploring the role and accuracy of the GeneXpert in tuberculosis diagnosis and drug resistance screening in Malawi" which is expected to take a total of 3 years. The researcher will not have access to patient identifiers which can be linked to the specimens. The proposed study and results obtained from it will be strictly academic and will not be compared to any Xpert MTB/RIF assay results generated from the TB Reference laboratory.

Any assistance rendered to him will be greatly appreciated.

Yours faithfully,

Dr. James Mpunga
TB Control Programme Manager
For: SECRETARY FOR HEALTH