Genetic characterization of drug resistant clinical isolates of *Mycobacterium tuberculosis* circulating within the Copperbelt province and Northern regions of Zambia.

By

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Declaration

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Abstract

The emergence and spread of drug resistant (DR) tuberculosis (TB) strains in the form of multidrug resistant (MDR)- and extensively drug resistant (XDR)-TB is a major threat to the global fight against TB. Treatment for these forms of TB is prolonged, up to 24 months, and involves the use of a combination of highly toxic, less potent anti TB drugs. In 2015 alone, the World Health Organisation (WHO) estimated 580,000 new MDR-TB cases across the world. Nine African countries are listed as high MDR-TB burden countries by the WHO.

A review of published research revealed that diverse genotypes are associated with DR TB in Africa, and demonstrated that DR TB strains are associated with community and nosocomial outbreaks. Furthermore, the role of migration in the transmission of DR TB strains has been demonstrated in certain parts of Africa. Of concern is the under-use of molecular epidemiological tools, resulting in gaps in knowledge of the transmission dynamics of DR TB on the continent. This study aims to address some of these gaps by describing the molecular epidemiology of DR TB in regions of the Copperbelt province and Northern regions of Zambia.

We used molecular strain typing tools of whole genome sequencing (WGS), Sanger (targeted gene) sequencing, insertion sequence *6110*-restriction fragment length polymorphism (IS*6110*-RFLP) and spoligotyping to describe the genotypes of DR *Mycobacterium tuberculosis* (*M.tb*) strains circulating within parts of Zambia. We demonstrated that a variety of genotypes are associated with DR TB in Zambia. The predominant genotype was lineage 4, with majority of strains belonging to Latin American Mediterranean (LAM). Other lineages belonged to 2 and 3. The genotyping analysis showed clustering of strains among patients being from different regions of the country thereby suggesting that DR TB is possibly widespread across the country. In addition, this analysis also identified household

transmission of MDR-TB between two household contacts, placing emphasis on the need for routine tracing of MDR-TB patient contacts in Zambia.

Further analysis of WGS and Sanger sequencing data identified 8 pre-XDR-TB cases. These belonged to lineage 4.6.1 (Uganda lineage), lineage 2.2 (Beijing genotype) and lineage 4.3 (LAM), giving a preliminary first insight into the genotypes associated with pre-XDR-TB in Zambia. Alarmingly, transmission of these pre-XDR-TB strains was demonstrated, with clustered strains sharing identical drug resistance-conferring mutations and low nucleotide variance differences. This finding emphasises the need for more comprehensive drug susceptibility testing, as failing to identify second line resistance may place the patient at risk of acquisition of additional resistance when treated with a standardised MDR-TB regimen.

Nosocomial transmission of DR TB has not been described in Zambia, despite the high risk of transmission in health care facilities. Assessment of the knowledge, attitudes and practices of health care workers at MDR-TB health care facilities in Ndola district revealed knowledge gaps and administrative deficiencies which could be placing these critical personnel at risk of acquiring DR TB at the work place. Findings highlighted continuous infection prevention and control trainings and provision of adequate personal protective equipment (PPE) as key areas of improvement.

The current study provides a first insight into the genetics of DR TB strains circulating in Zambia. These findings address knowledge gaps and contribute to our understanding of DR TB in Africa. To address the DR TB epidemic in Zambia, the TB control program need to expand the Xpert test-and-treat diagnostic strategy to all people entering healthcare facilities with symptoms of TB. More comprehensive drug susceptibility testing needs to be done to ensure patients are adequately treated. Following diagnosis of DR TB patients need to be counselled to initiate treatment and families and close contacts should be screened for TB.

Opsomming

Die opkoms en verspreiding van middelweerstandige (DR) tuberkulose (TB), spesifiek die multi weerstandige (MDR-TB) en uiters weerstandige (XDR-TB) vorme van TB is 'n groot bedreiging vir die globale stryd teen TB. Behandeling vir hierdie vorms van DR TB word verleng, tot 24 maande, en behels die gebruik van 'n kombinasie van hoogs toksiese en swakker anti-TB-middels. In 2015 het die Wêreldgesondheidsorganisasie (WGO) beraamd daar is 580,000 nuwe MDR / rifampisien weerstande (RR) -TB gevalle regoor die wêreld. Nege Afrika-lande word deur die WGO as hoë MDR-TB lande gelys.

'n Literatuuroorsig het aan die lig gebring dat diverse genotipes met DR TB op die vasteland geassosieer word, en getoon dat DR TB-stamme geassosieer word met gemeenskaps- en hospitaal uitbrake. Verder is die rol van migrasie in die oordrag van DR TB-stamme in spesifieke dele van Afrika gedemonstreer. Daar is kommerwekkend min molekulêre epidemiologiese studies, met ‘n gevolglike gebrek in kennis oor die transmissie dinamika van DR TB op die vasteland. Die doel van hierdie studie is om sommige van hierdie leemtes aan te spreek deur die transmissie dinamika van DR TB in dele van Zambië te beskryf.

Ons het genotipiese onderskeidings tegnieke, spoligotipering, IS*6110*-restriksie fragmentlengte polimorfisme (IS*6110*-RFLP), heel genoomvolgorde bepaling (WGS) en Sanger volgordebepaling gebruik om die genotipes van DR *Mycobacterium tuberculosis* te beskryf wat in dele van Zambië sirkuleer. Ons het gewys dat 'n wye verskeidenheid genotipes geassosieer word met DR TB in Zambië. Drie van die vernaamste stamme is gevind (linie 2, 3 en 4) met die oorheersende genotipes wat behoort in linie 4 (Latyns-Amerikaanse Mediterreens (LAM)). Groepering van stamme onder pasiënte uit verskillende streke van die land is getoon, wat daarop dui dat DR TB moontlik wydverspreid oor die land voorkom. Hierdie analise het ook huishoudelike oordrag van MDR-TB geïdentifiseer, wat klem lê op

die behoefte aan roetine opsporing van MDR-TB-pasiënt kontakte in Zambië.

Verdere analise van WGS en Sanger volgorde bepalingsdata het 8 pre-XDR-TB gevalle geïdentifiseer, wat aan linie 4.6.1 (T1 genotipe), linie 2.2 (Beijing genotipe) en linie 4.3 (LAM) behoort. Dit is die eerste beskrywing van genotipes wat verband hou met pre-XDR-TB in Zambië. Oordrag van hierdie stamme is gedemonstreer, deurdat groepe dieselfde weerstandsmutasies het, asook beperkte variasie in die heelgenoomdata toon. Hierdie kommerwekkende bevinding beklemtoon die behoefte aan meer omvattende middelweerstandigheidstoetse, aangesien versuim om tweede-linie weerstand te diagnoseer, die pasiënt se risiko verhoog om addisionele weerstand op te bou, indien ‘n gestandaardiseerde MDR-TB-regimen gebruik word.

Hospitaaloordrag van DR TB is nog nie voorheen in Zambië beskryf nie, ten spyte van die hoë risiko van oordrag in gesondheidsorgfasiliteite. Assessering van die kennis, houdings en praktyke van gesondheidswerkers by MDR-TB gesondheidsorgfasiliteite in Ndola-distrik, het gebrekkige kennis en administratiewe tekortkominge onthul, wat hierdie kritieke personeel in gevaar sou stel om DR TB in die werkplek op te doen. Ons bevindings beklemtoon die belang van deurlopende infeksie voorkomings- en beheerpraktyke en die voorsiening van voldoende persoonlike beskermingstoerusting (PPE) as sleutelareas van verbetering.

Hierdie studie is die eerste beskrywing van die genetika van DR TB-stamme in omloop in Zambië. Hierdie bevindings vul ons kennis aan en dra by tot ons begrip van DR TB in Afrika. Om die DR TB -epidemie in Zambië aan te spreek, moet die TB-beheerprogram die Xpert toets-en-behandel strategie uitbrei sodat alle mense met TB simptome bereik word. Meer omvattende middelweerstandigheidstoetsing moet gedoen word om te verseker dat pasiënte effektief behandel word. Na die diagnose van DR TB by pasiënte moet beraadslaag word om behandeling te begin en gesinne en naby kontakte moet vir TB gesif word.

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Above all, I thank God, for indeed I can do all things through Christ who strengthens me (Philippians 4:13).

List of symbols and abbreviations

ºC	degrees Celsius
Am	amikacin
Amx-Clv	amoxicillin-clavulanate
BCG	Bacillus Calmette–Guérin
Bdq	bedaquiline
BGI	Beijing Genomics Institute
BWA	Burrows-Wheeler Aligner
CAF	Central Analytical Facilities
CAM	Cameroon
CAR	Central African Republic
CAS	Central Asian
CDC	Centers for Disease Control and Prevention
Cfz	clofazimine
CHW	community health workers
Cm	capreomycin
Cs	cycloserine
Dlm	delamanid
DNA	deoxyribonucleic acid
DR	direct repeat
DST	drug susceptibility testing
E	ethambutol
EAI	East African Indian
EAI1_SOM	East African Indian_Somalia
EDTA	ethylenediaminetetraacetic acid
et al.	*et al*ii (and others)
ETH	Ethiopia
Eto	ethionamide
FQ	fluoroquinolone
GATK	Genome Analysis Tool Kit
Gfx	gatifloxacin
H or INH	isoniazid
HCWs	health care workers

HIV	human immunodeficiency virus
HREC	Health Research Ethics Committee
HRP	horseradish peroxidase
ID	identification
IMWM	internal molecular weight marker
Indels	insertions and deletions
IPC	infection prevention and control
Ipm	imipenem-cilastain
IPT	isoniazid preventative therapy
IS*6110*	insertion sequence *6110*
Km	kanamycin
KAPs	knowledge, attitudes and practices
KZN	KwaZulu-Natal
LAM	Latin American Mediterranean
LCC	low copy clade
MAF	*Mycobacterium africanum*
LAM	Latin American Mediterranean
LPA	line probe assay
Lfx	levofloxacin
LTBI	latent tuberculosis infection
MDR-TB	multidrug resistant TB
Mfx	moxifloxacin
MGIT	Mycobacteria growth indicator tube
MIC	minimum inhibitory concentration
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units – Variable Number of Tandem Repeats
MLVA	multiple loci VNTR analysis
mL	millilitre
MoH	Ministry of Health
Mpm	meropenem
M.tb	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
NALC	n-acetyl-L-cysteine
NaOH	sodium hydroxide

NATs	Nucleic acid tests
NGS	next generation sequencing
NTH	Ndola Teaching Hospital
NTLP	National TB and Leprosy control programme
PAS	para-aminosalicylic acid
PAS-Na	para-aminosalicylate sodium
PCR	polymerase chain reaction
PPE	personal protective equipment
PGG	principle genotypic groups
Pto	prothionamide
QRDR	quinolone-resistance-determining region
Ref	reference
R or RIF	rifampicin
RFLP	restriction fragment length polymorphism
RR	rifampicin resistant
RRDR	rifampicin resistance determining region
S	streptomycin
SAMTools	Sequence Alignment/Map tools
SDS	sodium dodecyl sulphate
SIRE	streptomycin – isoniazid – rifampicin – ethambutol
SIT	spoligo international type
Spoligotyping	spacer oligonucleotide typing
SNV	single nucleotide variants
TB	tuberculosis
TBE	trisaminomethane-borate-ethylenediaminetetraacetic acid
TDR	totally drug resistant
TDRC	Tropical Diseases Research Centre
TE	trisaminomethane-ethylenediaminetetraacetic acid
Trd	terizidone
TDRC	Tropical Diseases Research Centre
TGS	targeted gene sequencing
TO	transfer out
Tris-HCl	trisaminomethane- hydrochloride
Tx	treatment

USAP	Universal Sequence Analysis Pipeline
UTH	University Teaching Hospital
WGS	whole genome sequencing
WHO	World Health Organisation
WT	wild type
XDR	extensively drug resistant
Xpert MTB/RIF	Genexpert *Mycobacterium tuberculosis*/rifampicin
XXDR	extremely drug resistant
Z	pyrazinamide

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Chapter 1: General Introduction

1.1 Global burden of drug resistant tuberculosis

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (*M.tb*) remains a major cause of death worldwide and it is the number one cause of death amongst HIV infected individuals (1). The global TB epidemic is driven by factors such as development of drug resistance, the HIV epidemic, poverty and weak health care systems (1). In 2015 alone, the World Health Organisation (WHO) estimated that there were 10.4 million new TB cases of which the highest burden (60%) was seen in just six countries; India, Indonesia, China, Nigeria, Pakistan and South Africa (1). An estimated 2.7 million TB patients live in Africa where 16 of the 30 high TB burden countries are found (1). This region continues to experience high HIV/TB co-infection rates, with some regions in Southern Africa experiencing co-infection rates higher than 50% (1). Zambia has recently (2016) been included amongst the top 30 high TB burden countries, other countries recently included are Angola, Central African Republic, Congo, Democratic People's Republic (DPR) of Korea, Lesotho, Liberia, Namibia, Papua New Guinea and Sierra Leone (2). Of concern is that 9 out of 10 countries recently included amongst the high TB burden list are found within the WHO Africa region (2).

Exposure to *M.tb* has varying outcomes dependent on host genetic and immunological factors, the pathogen strain and environmental factors (1, 3). Exposure to *M.tb*, defined as contact with aerosolized bacilli, can result in either active infection, latency or no infection. Latent TB infection (LTBI) is described as the presence of *M. tuberculosis* in the body without clinical signs and symptoms, or radiographic or bacteriologic evidence of TB disease (4). Exposure to *M.tb* results in infection in approximately 20-50% of individuals. From these infected individuals, 2-10% will develop active disease while 90-98% remain latently infected with a lifetime reactivation risk of 5% (4, 5). Infection with *M.tb* can result in pulmonary and/or extra-pulmonary TB (1).

Treatment must be guided by the phenotypic drug susceptibility pattern of the infecting strain (Table 1.1), however globally most treatment is started without phenotypic drug susceptibility testing (DST) data. Treatment of drug susceptible TB relies upon a daily combination of anti-TB drugs over a period of 6 months, with a 2 month intensive phase followed by a 4 month continuation phase (6). Multidrug resistant (MDR)- and extensively drug resistant (XDR)-TB are treated over longer periods of time with a daily combination of anti-TB drugs which are less potent and more toxic than first line anti-TB drugs (Table 1.1) (7). Treatment adherence is a challenge due to the length of the treatment regimens, especially in the absence of a fully functioning TB control program. Strains resistant to all currently recommended anti-TB drugs, termed as "totally drug resistant" (TDR) TB and "extremely drug resistant" (XXDR) TB, have been described in various parts of the world, with initial cases identified in Italy and later described in Iran, India and South Africa (8-11). However, currently WHO does not recommend the use of the terms "TDR-TB" and "XXDR-TB" to describe strains showing *in vitro* resistance to all first and second line anti-TB drugs (12). This is due to the technical challenges of phenotypic DST for some second line anti-TB drugs and there is not enough data supporting a correlation between phenotypic DST results and the clinical response/outcome to treatment (12). Furthermore, the initial cellular and molecular classification of TDR-TB isolates, which was based on microscopic findings, is unclear and untestable (13).

Table 1.1: WHO recommendations for treatment of active drug sensitive and drug resistant TB, subject to phenotypic DST results (6, 7).

Regimen	Anti-TB drugs	Duration of treatment
Drug susceptible TB (first line treatment)	H, R, E, Z or S	**6 months** 2 months intensive phase (RHZE) followed by 4 months continuation phase (RH) **8 months** retreatment; 3 months intensive phase 2 months S(RHZE)/ 1 month (RHZE) followed by 5 months continuation phase (RHE)
Longer WHO approved MDR-TB regimen (second line treatment)	**Injectable drugs:** Km or Am or Cm **FQs:** Lfx or Mfx or Gfx **Other core agents:** Eto, Cs, Pto, Trd, PAS, PAS-Na, Cfz **Add on agents:** Z, E, high-dose H, Bdq, Dlm, Ipm, Mpm, Amx-Clv	**20 months** 8 months intensive phase e.g. (Km-Lfx-Eto-Cs-Z) and 12 months continuation phase e.g. (Lfx-Eto-Cs-Z)
Short WHO approved MDR-TB regimen	Gfx or Mfx, Km, Pto, Cfz, high-dose H, Z and E.	**9-12 months** 4/6 months intensive phase (Gfx or Mfx, Km, Pto, Cfz, high-dose H, Z, E) and 5 months continuation phase (Gfx or Mfx, Cfz, Z, E)
XDR-TB treatment	Km, or Am or Cm, Lfx or Mfx or Gfx. Eto, Cs, Pto, Trd, PAS, PAS-Na, Cfz. Z, E, high-dose H, Bdq, Dlm, Ipm, Mpm, Amx-Clv	**24 months** Drug combination is subject to DST results

Abbreviations: Am, amikacin; Amx-Clv, amoxicillin-clavulanate; Bdq, bedaquiline; Cm, capreomycin; Cfz, Clofazimine; Cs, cycloserine; Dlm, delamanid; E, Ethambutol; Eto, ethionamide; FQs, Fluoroquinolones**;** Gfx, gatifloxacin; H, Isoniazid; Ipm, imipenem-cilastain; Km, kanamycin; Lfx, levofloxacin; Mpm, meropenem; Mfx, moxifloxacin; PAS, para-aminosalicylic acid; PAS-Na, para-aminosalicylate sodium; Pto, prothionamide; Z, pyrazinamide; R, rifampicin; S, streptomycin; Trd, terizidone.

Drug resistant TB can either be as a result of infection with an already drug resistant strain, termed primary resistance, or can be acquired during the course of treatment, termed secondary resistance (1, 14). Drug resistant TB, in the forms of MDR/rifampicin resistant (RR)- and XDR-TB, continues to be a major public health concern globally (1). MDR-TB is defined as resistance to isoniazid and rifampicin, the most potent anti-TB drugs, while XDR-TB is defined as MDR-TB with added resistance to any of the second line injectable drugs (aminoglycosides) and any fluoroquinolone (FQ) (1, 14). Rifampicin resistance is defined as a proxy for MDR-TB and rapid detection of resistant strains is recommended (1). There were an estimated 480,000 new MDR-TB cases and 100,000 new RR-TB cases reported across the

world in 2015 with China, India and Russia accounting for 45% of cases (1). Nine additional countries, 7 of which are found within the WHO Africa region, were included in the high MDR-TB burden list namely; Angola, DPR Korea, Kenya, Mozambique, Papua New Guinea, Peru, Somalia, Thailand and Zimbabwe (14). A total of 117 countries have reported XDR-TB globally (1). It has been estimated that 9.5% of MDR-TB cases are XDR-TB, however the case detection rates for both MDR-TB and XDR-TB remain poor (1). Co-morbidity with HIV and other diseases such as diabetes mellitus worsens the progression of these diseases (1). However the molecular epidemiology of both TB and HIV are not well characterised in Zambia (1). The TB epidemic in Zambia is largely driven by the HIV epidemic, with a HIV/TB co-infection rate as high as 60% (1).

1.2 *Mycobacterium tuberculosis* genetics

The genus *M.tb* belongs to the *M.tb* complex (MTBC), a genospecies with a high level of homology. The other members of the complex are *Mycobacterium africanum*, *Mycobacterium bovis* (including the Bacillus Calmette–Guérin (BCG) strain), *Mycobacterium caprae*, *Mycobacterium microti*, *Mycobacterium mungi*, *Mycobacterium orygis*, *Mycobacterium pinnipedii*, *Mycobacterium suricattae* and the dassie bacilli (15, 16). A former member of the MTBC, *Mycobacterium canettii*, which is part of the 'smooth tubercle bacilli' has been described as sharing the most recent common ancestry with species of the MTBC (17). Species of the complex share 99.9% similarity at genome level (15, 18). However, there are differences in host range, pathogenicity and phenotypes (15). Exclusive human pathogens are *M.tb*, *M. africanum* and *M. canettii* (15, 19), it is however likely that other members of the complex are yet undiscovered.

Phylogenetic markers have been identified and whole genome sequencing (WGS) of *M.tb* has enabled better understanding of the organism (18). Knowledge of phylogenetic markers coupled with molecular typing tools is beneficial in investigating *M.tb* evolutionary and transmission events (18, 20, 21, 22). Molecular epidemiological studies of MDR- and XDR-TB reveal the major genotypes in circulation across the globe to be the Euro-American (Haarlem, LAM and T), East Asian (Beijing, Beijing-like) and East African-Indian (CAS) (Figure 1.1) (11, 19, 23, 24, 25). Genotypes such as the Beijing family are widespread across the world from its initial origin in Far-East Asia (26, 27, 28). Furthermore, the Beijing genotype family has been widely associated with MDR- and XDR-TB outbreaks across the globe (11, 26, 27, 29).

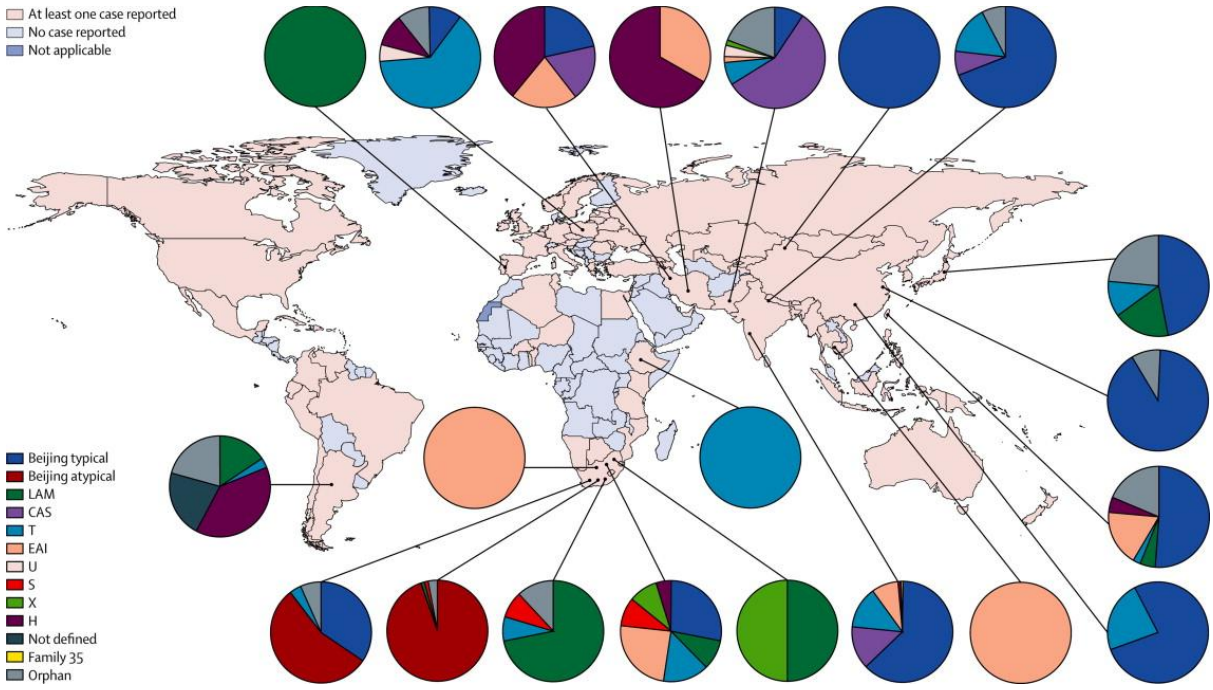

Figure 1.1: The global distribution of extensively drug resistant TB genotypes (24).

Since mid-1990s, several techniques have been validated for use in molecular epidemiological investigations of *M.tb* strain diversity and clustering including spacer oligonucleotide typing (spoligotyping), insertion sequence *6110*-based restriction fragment length polymorphism (IS*6110*-RFLP) and Mycobacterial Interspersed Repetitive Units – Variable Number Of Tandem Repeats (MIRU-VNTR) (20, 21, 22). Furthermore, next generation WGS of *M.tb* clinical isolates provides invaluable knowledge on genetic diversity and microevolution of the *M.tb* genomes in circulation (18). Whole genome sequencing is preferred to other typing techniques due to the robustness and high resolution offered by the technique (18). It however does not negate the usefulness of other typing tools due to limitations experienced in resource limited countries such as Zambia. These include the lack of expertise to set up libraries and to analyse sequencing data, the cost of equipment and the general running cost.

The burden of DR-TB and drug susceptible TB is highest in resource constrained communities across the world. It is in these regions that the molecular epidemiology as well as the transmission dynamics of *M.tb* is largely unknown. Genetic diversity has been demonstrated amongst isolates associated with DR-TB across the world with certain genotypes being more predominant in particular regions and population groups (19. 24*)*. Six major global lineages have been described, with varying distributions across the world (Table 1.2). Treatment success of DR-TB has also been associated with the infecting genotype of *M.tb*, with particular genotypes being strongly associated with high rates of resistance as well as development of MDR- and XDR-TB (30, 31).

Knowledge of DR-TB strains in circulation within a population group is particularly important for the national TB control program as it gives a better understanding of transmission dynamics, whether drug resistant TB is being acquired or transmitted, and

allows for better management of outbreaks in the population (11, 27, 29). The usefulness of a standard TB regimen can be guided through molecular investigations by identifying resistance-conferring mutations in key drugs. Furthermore, findings will guide diagnostic developers and drug/vaccine development efforts by defining strains present in the study population.

Table 1.2: the major global MTBC lineages and families (19).

Linage number	Lineage name	Family
1	Indo-Oceanic	EAI
2	East Asian	Beijing, none-Beijing
3	East-African-Indian	CAS, CAS1-Kili, CAS1-Delhi
4	Euro-American	LAM, Haarlem, S, Uganda, Cameroon, H37Rv-like, X
5	West-African 1	AFRI_2, AFRI_3
6	West-African 2	AFRI_1

Abbreviations: CAS, Central Asian; EAI, East African Indian; LAM, Latin American Mediterranean.

1.3 The global epidemiology of drug resistant TB

Case detection of DR TB remains low across the world. Of the 580,000 incident cases of MDR/RR-TB estimated in 2015, only half were started on treatment, falling short of the WHO target of 75% of MDR-TB cases being started on treatment (1). Trends in drug resistance are poorly characterised as there is very limited drug resistance surveillance in countries across the world. From the 30 high TB and high MDR-TB burden countries, only 50% had repeated a drug resistance survey to assess MDR-TB trends (1). Resistance trends for XDR-TB are even more poorly evaluated with only 6 out 30 (20%) high TB and MDR-TB burden countries establishing continuous national surveillance for XDR-TB (1). It has further been demonstrated that as high as 51% of MDR-TB cases are resistant to at least one fluoroquinolone or injectable agent or both (1), reinforcing the need to improve efforts in case detection and treatment of MDR/RR- and XDR-TB globally.

The global spread of MDR- and XDR-TB has been attributed to several factors including transmission within the community and inadequate infection control measures (1). Transmission of drug resistant TB has been described in vulnerable population groups including HIV positive individuals and hospital transmission has been described amongst patients as well as health care workers (HCWs) (31, 32). It is estimated that over 50% of new MDR-TB cases occur among individuals without prior TB infection and treatment (1). In some modelling studies estimates are as high as 95% (33, 34), implying that a large proportion of drug resistant TB is being transmitted.

According to WHO, the continents and countries with the highest burden of MDR- and XDR-TB burden are Africa (DR Congo, Ethiopia, South Africa, Nigeria), Asia/Eurasia (Armenia, Azerbaijan, Bangladesh, China, India, Indonesia, Kazakhstan, Kyrgyzstan, Myanmar, Pakistan, Philippines, Tajikistan, Uzbekistan, Viet Nam) and Europe (Belarus, Bulgaria,

Estonia, Georgia, Latvia, Lithuania, Moldova, Russia, Ukraine) (1). The global spread of specific *M.tb* genotypes is attributed largely to immigration and travel (19, 35). Drug resistant TB strains are mainly introduced to developed countries by economic migrants from developing nations (36, 37, 38). Molecular epidemiological investigations have revealed a strong association between *M.tb* genotype and geographical distribution/population groups (19, 30).

In parts of Africa, with an exception of South Africa were the population structure and transmission dynamics of drug resistant TB have been extensively described (11, 32, 37, 38), there is very limited knowledge on the molecular epidemiology of DR-TB. For instance, currently there is no published data on the genotypes associated with DR-TB in Zambia. This is concerning as it means that the transmission dynamics of drug resistant TB are largely unknown for Africa as a whole. This research aims to bridge some of the gaps in knowledge, with a specific focus on Zambia.

1.4 National TB and Leprosy Control Program (NTLP) Zambia

In the first national prevalence survey, conducted in 2013-2014, the prevalence of all forms of TB was estimated to be 455/100,000 population (39). In 2015, WHO estimated 1,500 MDR/RR-TB cases (1). From the estimated MDR/RR-TB cases, only 196 (13%) laboratory-confirmed cases were reported and only 50% of the laboratory confirmed cases were started on treatment (1). These statistics are similar to trends seen in previous years with only 13% of the estimated MDR-TB cases being notified in 2012 for Zambia (40). A review of national TB laboratory records over a period of 11 years showed that the incidence of MDR-TB is steadily rising with 18 cases notified in 2000 compared to 85 cases in 2011 (41). These statistics also highlight a poor case detection rate of MDR/RR-TB in Zambia with detected

cases falling short of WHO estimates (1, 40). This places emphasis on the likely presence of a large pool of undiagnosed and untreated MDR/RR-TB cases across the country and calls for improved case detection efforts.

The country experienced an increase in TB incidence in the 1990s to early 2000 due to the HIV epidemic (40). The National TB and Leprosy control programme (NTLP) and the Ministry of Health (MoH) recommend that patients with signs and symptoms of pulmonary TB provide sputum for smear microscopy for the diagnosis of TB (40). Zambia has over 360 diagnostic laboratories with the capacity to provide TB smear microscopy services for diagnosis and monitoring of treatment outcome, and 2000 treatment centres offering first line directly observed treatment short-course (DOTS) at no cost to the patient (40, 42). Zambia implemented the WHO recommended DOTS strategy in 2001 and has since attained a reported 100% coverage in all government-run health facilities (40). All new and retreatment TB patients diagnosed by smear microscopy, Xpert MTB/RIF, culture or chest x-ray are treated with the first line drugs rifampicin, isoniazid, ethambutol and pyrazinamide or streptomycin (Table 1.1), according to WHO recommendations (40).

There are 10 provinces in Zambia which are serviced by three TB reference laboratories providing culture and first line DST, namely Chest Diseases Laboratory (CDL; Lusaka district, Lusaka province), University Teaching Hospital (UTH; Lusaka district, Lusaka province) and Tropical Diseases Research Centre (TDRC; Ndola district, Copperbelt province), (Figure 1.2) (40, 42). The country is further serviced by two specialist MDR-TB wards at UTH and Ndola Teaching Hospital (NTH; Ndola district) where confirmed MDR/RR-TB patients are admitted for some part of second line treatment and treatment response is monitored (40). The standard first choice MDR/RR-TB regimen prescribed by the NTLP in Zambia is administered for a minimum of 20 months, 8-Km-Lfx-Eto-Cs-Z/12-Lfx-

Eto-Cs-Z (Table 1.1) (40). Currently, second line phenotypic DST is not routinely performed in Zambia, however there are two centres with the capacity of offering first and second line molecular line probe assay (LPA) and CDL TB reference laboratory has the capacity to perform DST on capreomycin, kanamycin and ofloxacin for MDR-TB patients (40).

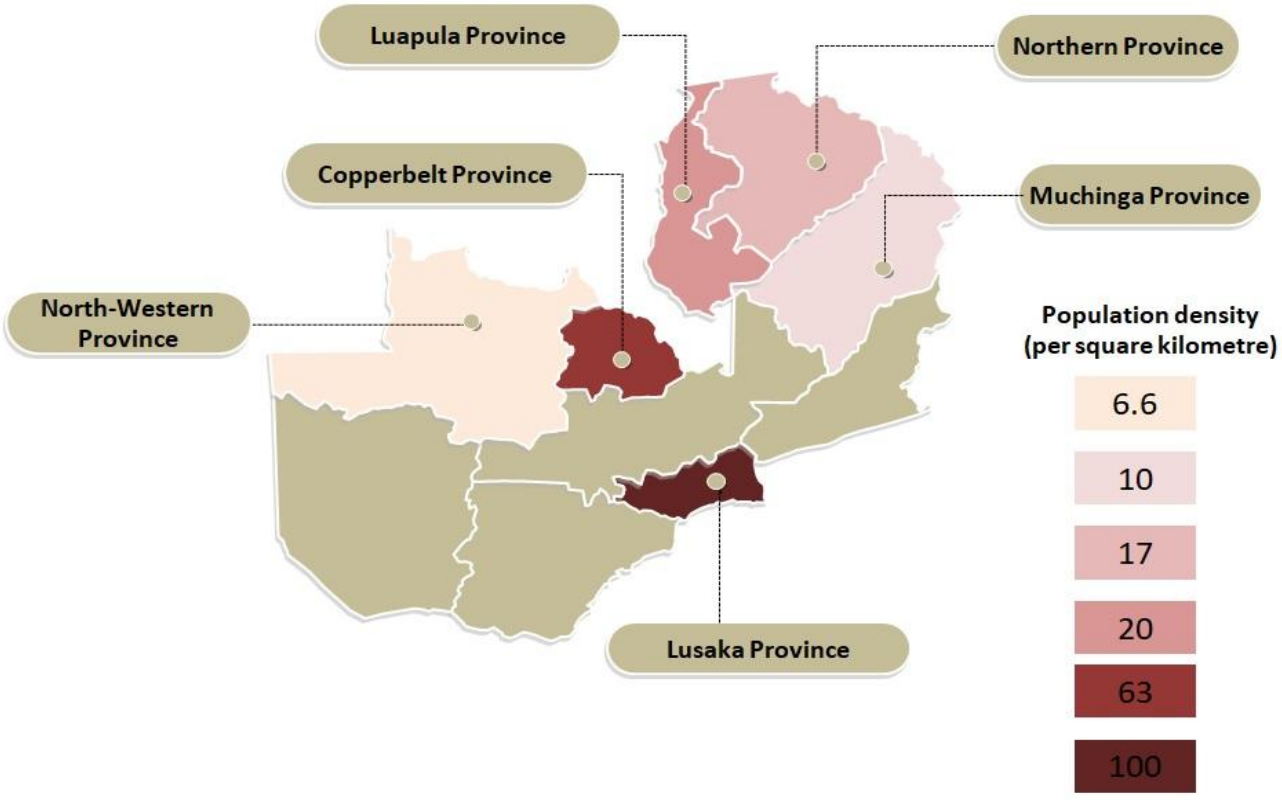

Figure 1.2: The map of Zambia highlighting patient residences by province (Copperbelt, Luapula, Muchinga, Northern and North-Western provinces) and the sites of the 3 national TB reference laboratories; Copperbelt (TDRC TB reference laboratory; Ndola district) and Lusaka province (CDL and UTH; Lusaka District).
Abbreviations: CDL, Chest Diseases Laboratory; TB, tuberculosis; TDRC, Tropical Diseases Research Laboratory; UTH, University Teaching Hospital.

The 10 provinces in Zambia are further subdivided into administrative districts. Diagnosis at district level is limited to smear microscopy with some designated TB diagnostic centres offering the Xpert MTB/RIF assay for simultaneous detection of *M.tb* and rifampicin

resistance (40). Due to the increased likelihood of HIV-positive patients having a smear negative TB result, the MoH in Zambia recommends the use of the Xpert MTB/RIF assay for high risk patients as in the case of HIV-positive individuals suspected of having TB, patients that have failed retreatment and for diagnosis of TB in children (40).

Culture based phenotypic DST is recommended for diagnosis of MDR-TB by the MoH and NTLP based on suspicion of MDR-TB (Figure 1.3) (40). According to the MoH and NTLP, TB patients who fail the WHO category II/retreatment regimen as well as TB patients from high MDR-TB burden facilities such as prisons have a high suspicion of MDR-TB while TB patients who remain smear positive at the end of treatment have a low suspicion of MDR-TB (40). However, the capacity to perform DST on all MDR-TB suspects is currently not available. For instance, TB in new cases is diagnosed by smear microscopy and it is only in the case that a patient remains smear positive after re/treatment that Xpert MTB/RIF assay and first line DST is performed.

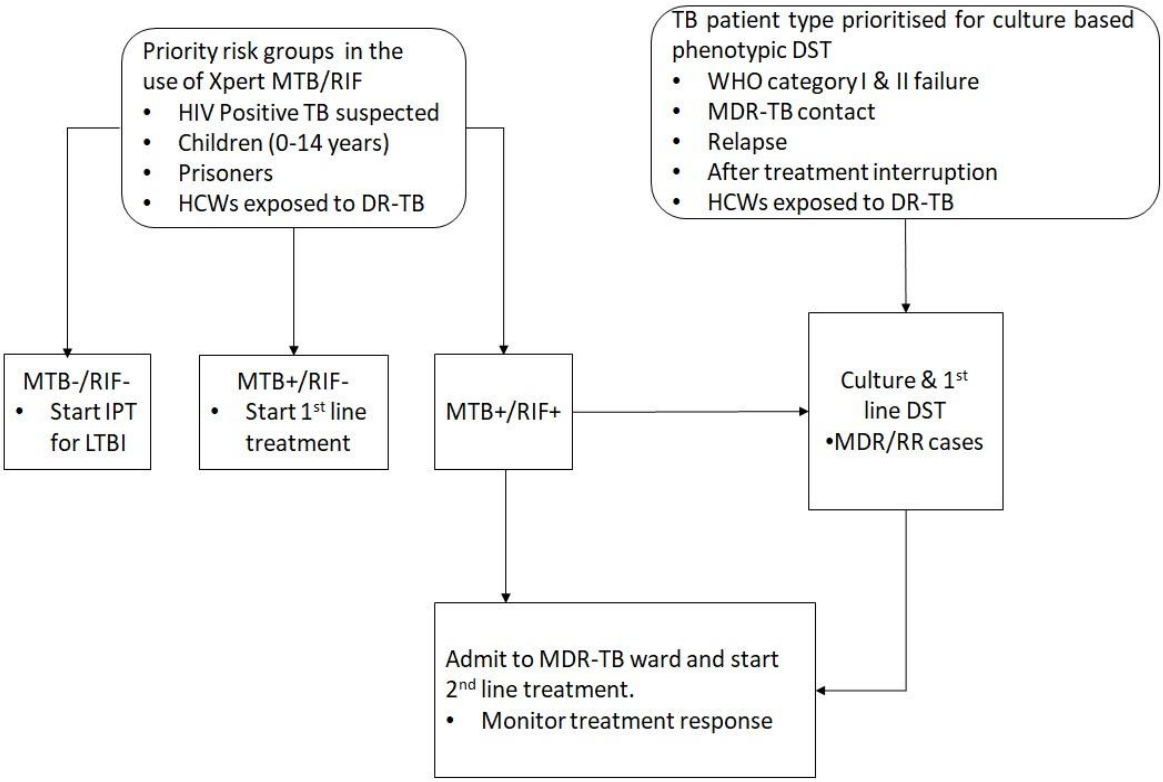

Figure 1.3: Recommended algorithm for the management of MDR/RR-TB patients in Zambia (40). Abbreviations: DR-TB, drug resistant tuberculosis; HCWs, health care workers; IPT, isoniazid preventative therapy; LTBI, latent TB infection; MTB, *M. tuberculosis*; RIF, rifampicin.

Ndola district is the provincial headquarters for the Copperbelt province and is the site for the TDRC TB reference laboratory and the NTH MDR-TB ward (40). The TDRC TB reference laboratory provides culture and first line DST to 3 provinces out of the 10 provinces, namely; Copperbelt, North-Western and Luapula provinces (Figure 1.2) (40). The three provinces have a combined population of 4.1 million out of the national population of 16 million (43).

The molecular epidemiology, that is the transmission and genotypes, of DR TB and drug susceptible TB in Zambia is poorly understood, with few reported studies. Only 3 molecular epidemiological studies have been published and these were largely focused on drug

susceptible TB in Ndola district, Namwala district (animal to human transmission was investigated) and one study collected samples from across Zambia (44, 45, 46). The main molecular typing tools used in these studies were a combination of spoligotyping, MIRU-VNTR, LPA and targeted gene sequencing (44, 45, 46, 47, 48). From these studies, the major genotypes were LAM (predominately LAM11_ZWE), T, CAS, *M. bovis* and X (44, 45, 46). Genotypic susceptibility testing to second line anti-TB drugs, with LPA, in one study in Zambia identified 1 XDR-TB and 1 pre-XDR-TB case out of 113 evaluated cases, however the associated genotypes were not described (47). In another study evaluating 16 samples from the capital city, Lusaka, one isolate was found to have variation at codon 73 in *gyrA* (which has not been associated with FQ drug resistance), however 13 out of 16 isolates had mutations in the "quinolone-resistance-determining region" (QRDR) of the *gyrA* gene (48). In these studies, the genotypes associated with drug resistance were not described and the studies are further disadvantaged by poor sampling coverage (44, 45, 46, 47, 48). The usefulness of WGS in understanding transmission dynamics has been demonstrated in Zambia (49), with one study using WGS data to differentiate relapses and re-infection through single nucleotide variant (SNV) analysis (49). The study found that 33 out of the 36 patients (92%) had TB due to relapse, that is recurrence of disease due to endogenous strains (49).

1.5 Rationale, aims and objectives of this study

1.5.1 Rationale

In Zambia, management and treatment of DR-TB remains in its infancy (1). By advancing knowledge of DR-TB epidemiology, it is anticipated that findings from this study will better inform the national TB control program on the *M.tb* genotypes that are circulating within the

Copperbelt province and Northern regions of Zambia. This study will provide a measure of the efficacy of the TB control program and guide intervention, to make better use of limited resources and will likely provide a better understanding of the transmission dynamics of drug resistant TB as well as the genetic mechanisms of resistance. Further, the findings will add to national, regional and global data on drug resistant TB genotypes that are in circulation.

1.5.2 Aim

This research aims to describe the molecular epidemiology of drug resistant *M.tb* clinical isolates circulating within the Copperbelt province and Northern regions of Zambia, diagnosed at the TDRC TB reference laboratory in Ndola district.

1.5.3 Objectives:

Objective 1 - To describe the genotypes and distribution of drug resistant *M.tb* clinical isolates circulating within the Copperbelt province and Northern regions of Zambia.

Zambia is amongst the top 30 high TB and high HIV burden countries in the world, however there is a lack of knowledge on the molecular epidemiology (transmission dynamics and genotypes) of TB with no data on the genotypes associated with DR TB in the country. The findings of this study will inform the national TB control program on the population structure and distribution of DR TB isolates from the Copperbelt province and northern regions of Zambia, molecular typing findings will be correlated to patient demographic data, including residential township. (Addressed in chapter 4.)

Objective 2 - To describe the transmission dynamics of drug resistant TB within the Copperbelt province and Northern regions of Zambia, in terms of acquisition vs. transmission.

Drug resistant TB can either be acquired during the course of treatment (secondary resistance) or it can be transmitted (primary resistance) (1). There is no data on the transmission dynamics of drug resistant TB for Zambia. Therefore molecular typing tools will be used to investigate clustering amongst clinical isolates. This will be analysed in conjunction with the clinical data. Clustering and primary drug resistance would be suggestive of recent transmission while unique patterns and secondary resistance would suggest acquired resistance. (Addressed in chapter 4 and 5.)

Objective 3 - To analyse the transmission of drug resistant *M.tb* using whole genome sequence analysis

Alongside using spoligotyping and IS*6110* DNA fingerprinting, next generation WGS data for 86 DR TB isolates will be used to investigate genetic diversity and transmission. A phylogenetic tree will be constructed using WGS data, this will be used to investigate strain relatedness and the evolution of drug resistant isolates from the Copperbelt province and northern regions of Zambia. (Addressed in chapter 5.)

Objective 4 - To compare genotypes of drug resistant *M.tb* from the Copperbelt province and northern regions of Zambia with isolates circulating within a high TB-incidence area of Cape Town, South Africa.

The molecular epidemiology of DR-TB in Cape Town has been systematically described and particular strains have been associated with MDR- and XDR-TB (11, 50). WGS data conveniently available at Stellenbosch University for strains that have previously been

described in Cape Town will be compared to WGS data for strains of the same lineage from this study. Migration has been documented within the region, mainly due to political instability, for instance cross border movement between South Africa, Zimbabwe and Zambia has been described (51). There however is very limited data on the role of migration on the transmission of TB within the region. In this study, we anticipate that strain relatedness would suggest that migration is playing a role in the transmission of drug resistant TB strains within the region and the continent (52). (Addressed in chapter 5.)

Objective 5 - To investigate the distribution and transmission of XDR-TB amongst *M. tuberculosis* isolates circulating within the Copperbelt province and northern regions of Zambia.

A case of XDR-TB and one case of pre-XDR-TB have previously been described in one study in Zambia (47). The lack of routine in-country second line DST could imply that a pool of XDR-TB cases remain undetected and is a potential source of future XDR-TB cases in Zambia and the surrounding region. In order to investigate the presence of XDR-TB in the Copperbelt province and northern regions of Zambia, WGS and targeted gene sequencing (TGS) will be used to determine the presence of mutations conferring resistance to second line anti TB drugs. This will give a first insight into the genotypes associated with XDR-TB in Zambia. (Addressed in chapter 6.)

Objective 6 - To describe the genetic mechanisms of resistance and relationship to phenotype.

Using TGS and next generation WGS, mutations conferring resistance will be investigated and findings will be compared to phenotypic DST results. (Addressed in chapter 6.)

Objective 7 - To determine the knowledge, attitudes and practices (KAPs) of health care workers, in MDR-TB diagnostic and treatment facilities, toward TB infection, prevention and control (IPC) practices.

Health care workers (HCWs) have an increased risk of acquiring TB due to exposure at the work place and in the community. HCWs play an important role in the transmission and the control of MDR-TB. During sample collection and initial processing, observations were made of HCWs not always adhering to TB IPC practices. The barriers in adhering to TB IPC practices in HCWs working at the Ndola Teaching Hospital (NTH) MDR-TB ward and the TDRC TB reference laboratory have not been evaluated. This research will provide an insight into the knowledge, attitudes and practices (KAPs) of this group of HCWs and the barriers to adhering to IPC practices and policies. The findings will identify key areas of training and safety and guide IPC practices in this at risk population group. (Addressed in chapter 7.)

1.5.4 Thesis structure

Chapter 1. Introduction

The general introduction chapter sets the tone for the thesis by introducing the background to the study. The chapter highlights the growing global concerns over the emergence and spread of drug resistant TB in the form of MDR- and XDR-TB. It further highlights the knowledge gaps on the genotypes that are associated with drug resistant TB in Zambia.

Chapter 2. Molecular epidemiology of drug resistant *Mycobacterium tuberculosis* in Africa

This chapter in the form of literature review summarises the molecular epidemiology of drug resistant TB across Africa. It further highlights the gaps in knowledge and the deficiencies in management of drug resistant TB across the continent.

Chapter 3. Materials and Methods

This chapter describes the study design, the materials and methods used to meet the objectives of this research. The study population group and ethical considerations are defined in this chapter.

Chapter 4. Drug resistant *Mycobacterium tuberculosis* clinical isolates collected from the TDRC TB reference laboratory in Ndola district: Genetic diversity, demographic and clinical characteristics

The genetic diversity observed in drug resistant clinical isolates of *M. tuberculosis* diagnosed at the TDRC TB reference laboratory is described in this chapter as part of the results. These

findings are significant as they give a first insight into the genotypes that are associated with drug resistant TB in parts of Zambia. This chapter addresses Objectives 1.

Chapter 5. Molecular analysis of transmission

This chapter describes the transmission of drug resistant clinical isolates of *M. tuberculosis* diagnosed at the TDRC TB reference laboratory using WGS analysis. Further, the chapter describes strain relatedness between strains of the same lineage from the study population and strains associated with drug resistance in Cape Town South Africa, whose WGS data is conveniently available at Stellenbosch University, to assess the impact of migration on transmission of drug resistant TB strains in the region. This chapter addresses Objectives 2, 3 and 4.

Chapter 6. Genetic mechanisms of drug resistance

In this chapter the genetic mechanisms of drug resistance in relation to the phenotype, are described in the study population. Further, the chapter gives a first insight into genotypes associated with pre-XDR and XDR-TB in Zambia. This chapter addresses Objectives 6.

Chapter 7. Occupational risk of transmission of drug resistant TB in healthcare workers: knowledge, attitudes and practices

This chapter describes the knowledge, attitude and practices of health care workers toward TB IPC practices. Health care workers are at an increased risk of acquiring drug resistant TB and TB in general due to increased exposure. The findings in this chapter were gathered through literature review and self-administered questionnaires to health care workers from MDR-TB health care facilities in Ndola district, Zambia. This chapter addresses Objective 7.

Chapter 8. General conclusion

The general conclusion brings the thesis together by describing the genotypes of drug resistant TB diagnosed at the TDRC TB reference laboratory. It further sums up factors that are driving drug resistant TB and the deficiencies in management of drug resistant TB in Zambia.

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Chapter 2: Molecular epidemiology of drug resistant *Mycobacterium tuberculosis* in Africa

2.1 Burden of drug resistant tuberculosis in Africa

The WHO reports that globally, of the 6.4 million global TB patients, 1.3 million (20%) live in Africa and 16 out of the 30 high TB burden countries are in this continent (1). In Africa, countries with the highest burden of MDR-TB are; Angola, Democratic Republic (DR) Congo, Ethiopia, Kenya, Nigeria, Mozambique, Somalia, South Africa and Zimbabwe (1). In 2015, the incidence of MDR/RR-TB in Africa was estimated to be 42,000 (1). Of the estimated cases only 26,929 (64%) MDR-TB and 1,100 XDR-TB cases had laboratory confirmation with a further 69% MDR-TB and 72% XDR-TB laboratory confirmed cases being started on second line treatment (1). The highest proportion of TB/HIV co-infection is also seen in this region (31% on average), with some regions having co-infection rates higher than 50% (1, 3). It is therefore important to identify TB/HIV co-morbidity in these high risk areas. A further factor driving the TB epidemic in Africa is non-communicable "lifestyle diseases", such as diabetes mellitus, which are on the rise within the continent and pose a potential source of at-risk individuals in high TB burden settings (4). It is in this light that recommendations have been made to screen for diabetes in TB patients in Africa (4).

The gold standard for the diagnosis of drug resistant TB is the use of culture-based phenotypic drug susceptibility testing (DST) which has been recommended by the WHO since 2007 (1, 5). Other WHO approved methods include nucleic acid tests (NATs) such as the Xpert MTB/RIF assay and the molecular line probe assay (LPA), which provide a more rapid diagnosis but are limited in the range of drug susceptibility that can be detected (1). The molecular LPA and Xpert MTB/RIF assay were recommended for rapid screening of patients at risk of drug resistant TB by the WHO in 2008 and 2010, respectively (1). More recently, in 2017, WHO endorsed the use of the next-generation Xpert MTB/RIF Ultra assay, which is said to have increased sensitivity in detecting MTB compared to the Xpert MTB/RIF assay

(6). The running costs associated with these techniques, the need for expertise and the lack of availability at point of care could however result in low uptake of these rapid diagnostic tools across Africa.

The recommended treatment regimen for drug susceptible TB, which includes streptomycin, isoniazid, rifampicin, ethambutol, or pyrazinamide, differs from the treatment regimen of MDR- and XDR-TB. The WHO recommends that MDR-TB be treated with a standard regimen of second line anti-TB drugs which includes a combination of an injectable drug, a fluoroquinolone, other core anti-TB agents as well as the first line anti-TB drugs pyrazinamide and ethambutol, subject to DST results (Table 1.1, page 4) (7). These drugs are however less potent, more toxic and require a prolonged treatment period of up to 24 months. More recently however, the WHO has endorsed a shorter 9-12 month regimen which has been demonstrated to be more effective in the treatment of MDR-TB and consists of a combination of anti-TB agents (Table 1.1, page 4) (8, 9). The initial phase of the newly endorsed regimen involves treatment with kanamycin, moxifloxacin, prothionamide, clofazimine, pyrazinamide and high dose isoniazid, for 4 to 6 months, followed by a 5 month continuation phase with moxifloxacin, clofazimine, pyrazinamide and ethambutol (9). The usefulness of including the first line drugs pyrazinamide and ethambutol in treatment of drug resistant TB is however challenged by the high association of some of these anti-TB drugs with MDR-TB (10, 11), placing emphasis on the need for routine DST prior to MDR-TB treatment.

Since 2014, at least 12 countries have introduced this short MDR-TB regimen in Africa (9). Inappropriate implementation of the shorter MDR-TB treatment regimen however poses a risk of acquiring additional resistance in affected patients, as currently observed for the longer MDR-TB treatment regimen (8, 9). It is in this light that the WHO recommends DST

before commencement of treatment and that the shorter regimen only be made available to patients that have not received prior MDR-TB treatment (9).

The molecular mechanisms of drug resistance as well as the evolution of drug resistant strains have been widely studied using a combination of genotyping tools (12, 13). This has given a better insight into the transmission dynamics of drug resistant TB. Most studies under review have used spacer oligonucleotide typing (spoligotyping) to describe the molecular epidemiology of drug resistant TB in Africa although there are a number of studies which have used highly discriminatory methods which include insertion sequence *6110*-restriction fragment length polymorphism (IS*6110*-RFLP), Mycobacterial interspersed repeat units-variable number of tandem repeats (MIRU-VNTR) and whole genome sequencing (WGS).

There is no existing review on the molecular epidemiology of drug resistant TB in Africa. This review aims to synthesise available knowledge of drug resistant TB in Africa, with a particular focus on molecular epidemiological studies.

2.2 Drug resistance tuberculosis surveillance

Routine and frequent epidemiological surveillance is critical for understanding the burden of drug resistant TB in a given region and for planning and policy development and implementation. The major drug resistance TB surveillance methods that have been used in Africa include case notifications combined with expert opinions, prevalence surveys, and capture-recapture to estimate incidence (1, 2). However, the most effective drug resistance monitoring tool has been demonstrated to be continuous surveillance of TB patients through DST and systemic analysis of routinely collected data (1). It is a concern that there is scanty data on the prevalence of drug resistant TB across Africa (Figure 2.1 and Table 2.1) (2).

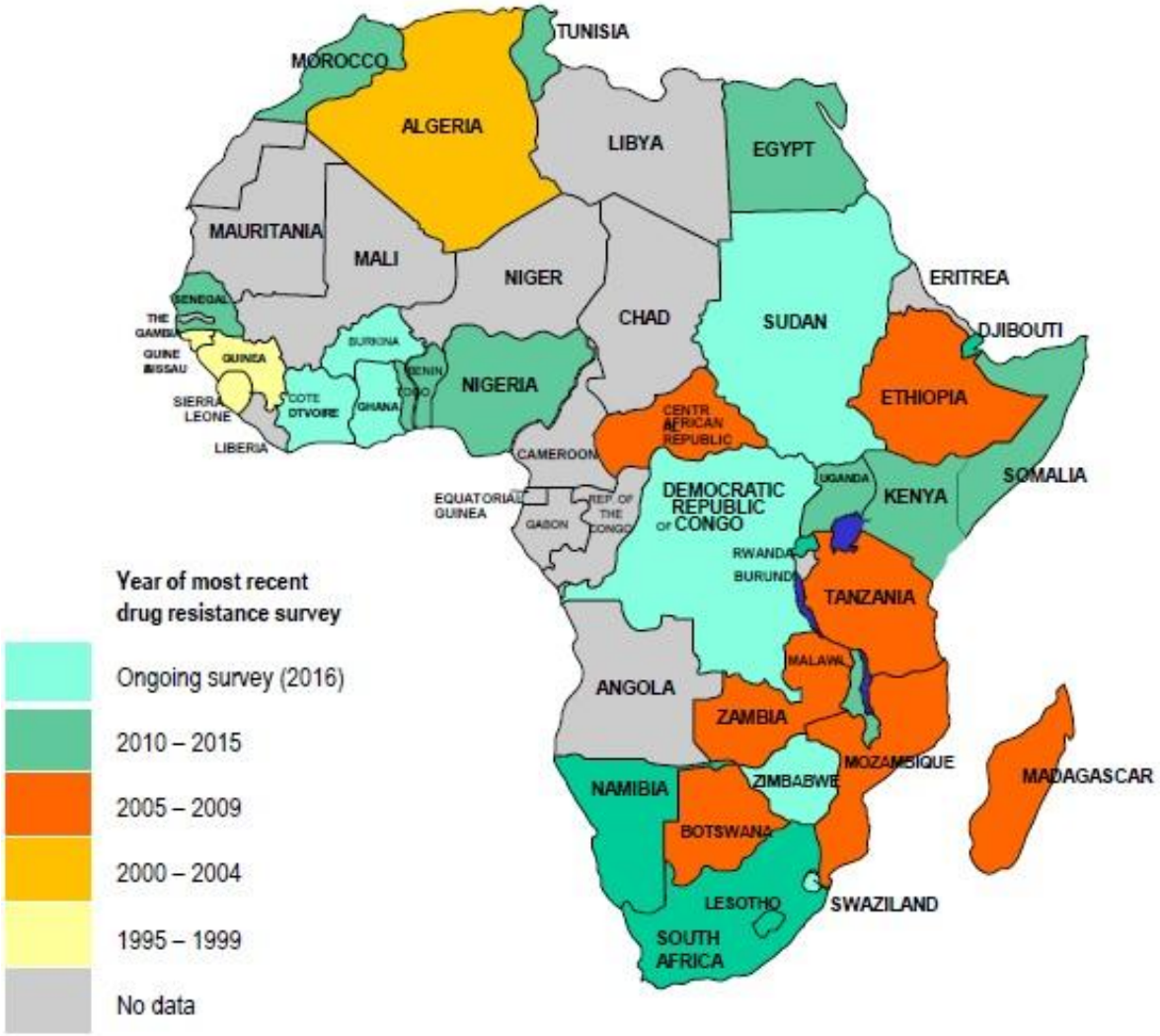

Figure 2.1: An illustration of the year of the most recent drug resistance survey for African countries. Adapted from WHO (1).

Between 2010 and 2015, only 16 of 54 African countries (30%) completed national drug resistance prevalence surveys namely; Benin, Djibouti, Egypt, Kenya, Lesotho, Malawi, Morocco, Namibia, Nigeria, Senegal, Somalia, South Africa, Rwanda, Togo, Tunisia, and Uganda (1, 2). Older drug resistance survey data is available from 8 countries for the period 2005 and 2009, namely Botswana, Central African Republic (CAR), Ethiopia, Madagascar, Mozambique, Tanzania, and Zambia (1). Since 2016, there were drug resistance TB surveys on-going in Burkina Faso, Cote D'Ivoire, DR Congo, Ghana, North-Sudan, Swaziland, and

Zimbabwe (1). Fourteen countries in Africa currently do not have any survey data (Figure 2.1). From the countries with repeat drug resistance survey data (Table 2.1), some countries have reported an increase in the prevalence of MDR-TB and drug resistant TB in general (14, 17). Other countries have demonstrated no significant changes in prevalence rates of drug resistant TB (15, 16, 18).

Table 2.1: Repeat drug resistance survey trends for African countries with published data.

Country	Survey periods	Trends	Ref
Botswana	1995-1996, 1999, 2002 and 2007-2008	• The proportion of new cases with MDR-TB tripled since the previous survey in 2002, from 0.8% to 2.5%. • There was a rise in R, H, E & S resistance in new cases in 2007 compared to 2002. • Prevalence of MDR-TB in retreatment cases was lower in 2007-2008 at 6.6% compared to 10.4% in 2002.	14
Mozambique	1998 and 2007-2008	• The prevalence of MDR-TB amongst new and previously treated cases was 3.5% and 11.2% during the 2007-2008 survey. • No significant differences in prevalence with the first survey.	15
South Africa	2001-2002 and 2012-2014	• National prevalence rate of MDR-TB was estimated to be 2.8% ○ 2.1% in new and 4.6% in retreatment cases. • MDR-TB prevalence has remained stable over a period of 10 years. • A doubling in R resistance from 1.8% in 2001-2002 to 3.4% in 2012-2014 survey. • Highest MDR-TB rate, 5.1%, seen in Mpumalanga province in both surveys	16
Swaziland	1995 and 2009-2010	• An 8.5- and 3.5-fold increment in previously treated cases and new MDR-TB cases, respectively, between the two surveys. • 0.9% in new cases and 9.1% in retreatment cases in 1995 compared to 15.3% and 33.8% in 2009-2010.	17
Zambia	2001 and 2008	• In 2001 the prevalence of MDR-TB in new cases and previously treated cases was 1.2% and 1.8%, respectively compared to 0 and 6.5% in 2008 • There was no significant increase in the prevalence of MDR-TB over a period of 8 years.	18

Abbreviations: MDR-TB, multidrug resistant tuberculosis; R, rifampicin; H, isoniazid; E, ethambutol; S, streptomycin.

High rates of resistance have been documented with some studies reporting over 50% of all cases as having some form of resistance with the majority of cases being MDR-TB (19, 20, 21, 22). This has been attributed to factors such as inadequate therapy due to lack of adherence to a combination therapy (20). The high rates of drug resistance observed pose a serious public health threat across the continent. There are however countries that have reported low levels of primary drug resistance, as low as 4.9% in Burkina Faso (3.3% isoniazid mono-resistant and 1.6% MDR-TB) (23), implying that the current treatment regimen is effective in managing drug resistant TB. Further, there is limited clustering of drug resistant isolates in parts of Africa, suggesting that drug resistance is largely acquired in some regions (24, 25). It is however worth noting that drug resistant isolates could possibly be under-estimated due to poor surveillance and low case detection rates seen across Africa.

African countries share similar challenges in the management of drug resistant TB mainly due to poorly funded TB programs and weak health care systems (1, 2). This is reflected by the poor surveillance and the poor treatment outcomes that are seen across the continent (1). The average MDR/RR-TB case detection rate for the 47 WHO African member states is 64% (1). From all laboratory confirmed MDR/RR-TB and XDR-TB cases 69% and 72% are started on treatment, respectively, across Africa. There is an average treatment success rate of 54% for MDR/RR-TB and 24% for XDR-TB (1), however there are variations from region to region (1). Across Africa, the MDR/RR-TB case detection rates range from 7% to 87% (1). This implies that drug resistant TB cases are perhaps underreported in the region.

2.3 Population structure of drug resistant TB genotypes

Sporadic molecular mycobacteriological studies have been conducted within Africa (Figure 2.2, Figure 2.3), with South Africa having the vast majority of data in the continent. Diverse genotypes have been associated with drug resistant TB (Figure 2.2, Table 2.2), with particular genotypes being more predominant (26, 27, 28, 29). For instance, the Beijing genotype is widespread across parts of Africa (30, 31, 32, 33, 34). The population structure of drug resistant TB is however not homogeneous, with certain strains being more predominant in specific population groups (35, 36, 37). For instance, the Haarlem and CAS genotypes are predominantly associated with drug resistance including MDR-TB in parts of North and East Africa while in Southern and West Africa the Beijing and LAM genotypes are highly associated with drug resistance (38, 39, 40, 41).

Associations between drug resistant TB strains and HIV have been noted, with high mortality rates being observed in these population groups (33, 42, 43, 44). Genotypes such as Beijing, Haarlem and LAM have been associated with high levels of drug resistance and high mortality rates in both HIV seropositive and seronegative individuals (38, 45, 46, 47). A clear distinction has been observed in the population structure of genotypes associated with mono-resistance, MDR- and XDR-TB (Table 2.2). In parts of South Africa the F15/LAM4/KZN and Beijing genotypes have been associated with XDR-TB while LAM11_ZWE is associated with MDR-TB in parts of Zimbabwe (41, 48, 49). The genetic diversity observed across the continent could be influenced by host genetics and migration, which is discussed further in section 3.4 (50).

High cluster rates of drug resistant TB isolates have been observed in parts of Africa (19, 51, 52), this is of great concern as it implies that there is recent and ongoing transmission of drug resistant TB strains within the region. Further, a correlation between the adult population and

children has been demonstrated (53), suggestive of adult to child transmission. There is however very limited molecular typing data on drug resistant TB amongst children and household contacts of drug resistant TB patients in Africa to confirm this.

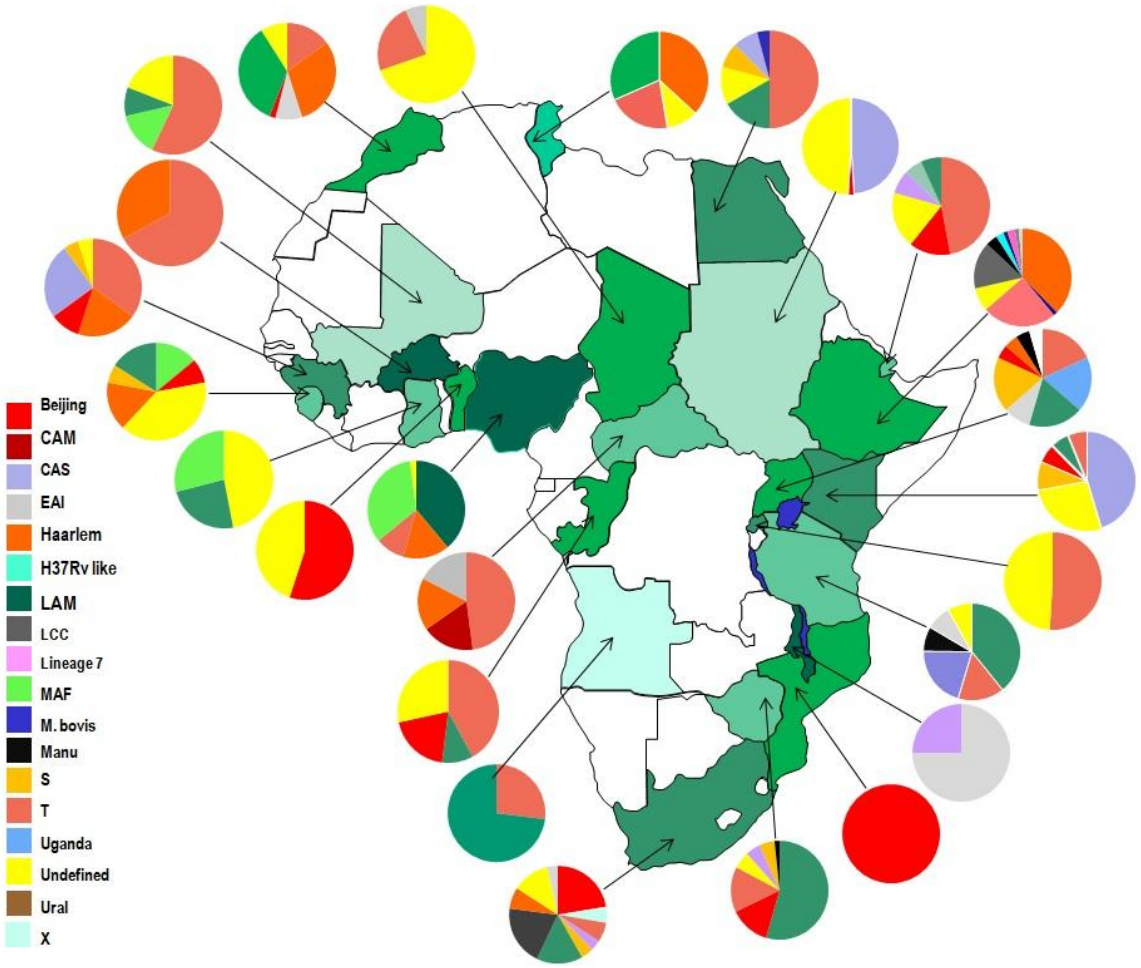

Figure 2.2: Genotypic distribution of drug resistant *Mycobacterium tuberculosis* isolates characterised across Africa. Varying genotyping tools were used to characterise isolates including spoligotyping, MIRU-VNTR, PCR typing, and WGS, further described in Table 2.3. Figure generated in Microsoft PowerPoint from references listed in Table 2.3.

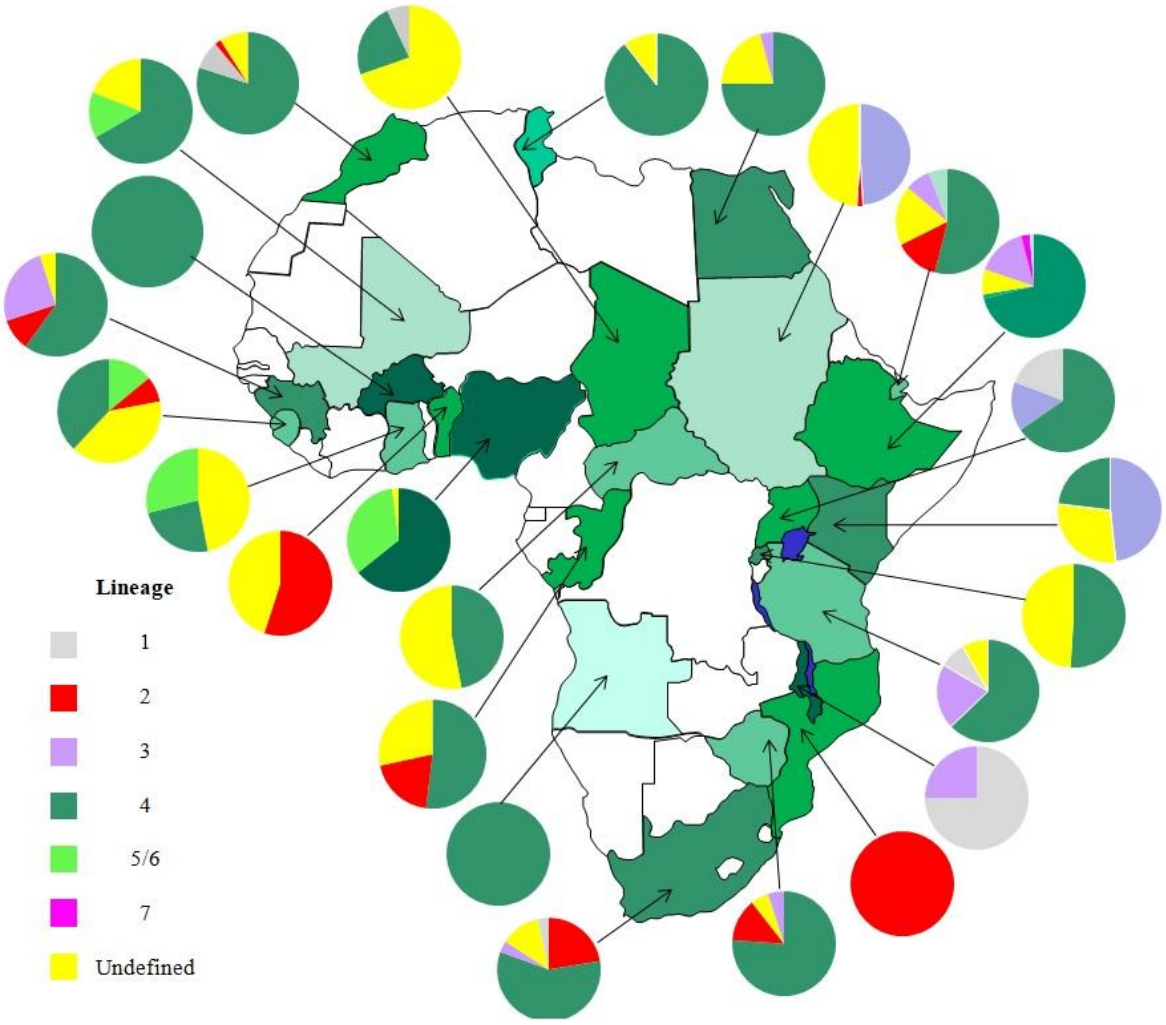

Figure 2.3: Distribution of *M. tuberculosis* strains according to the 7 major lineages. Figure generated from references listed in Table 2.3.

The appearance of modern lineages (East Asian, EAI and Euro American) has resulted in a shift in genotypes associated with resistance in Central and West Africa (22), regions predominantly associated with *Mycobacterium africanum* (MAF) (19, 54, 55). In North and East Africa, the most predominant genotypes associated with drug resistance are T, CAS and Haarlem (44, 56, 57, 58, 59) while in central and Southern Africa the lineages East Asian, Euro-American and East African Indian are more prevalent. Lineage 5 (West-Africa 1) and 6 (West-Africa 2) continue to predominate in West Africa and are not associated with drug resistance. However, modern strains are being observed in the region and are associated with drug resistance (60, 61, 62, 63). Overall, there is a high degree of diversity in the genotypes

associated with drug resistant TB across Africa with certain genotypes being more prevalent in specific regions (24, 56). Further, clustering of these strains has been observed, implying that there is ongoing transmission of multiple drug resistant TB strains within the continent. Additional studies are required to better understand the epidemiology and associated factors of drug resistant TB in Africa as a whole.

Table 2.2: Genotypes associated with drug resistant TB across Africa.

Country	Province	DST phenotype	Genotype	Genotyping method	Ref
Angola	Luanda	MDR-TB, mono- & poly-resistant	T1, T2, LAM9, LAM1, LAM2, LAM6	Spoligotyping, MIRU-VNTR	64
Benin	Cotonou	S mono resistant	Beijing	MIRU-VNTR	65
Burkina Faso	Ouagadougou	MDR-TB, mono- & poly-resistant	T, Haarlem	Spoligotyping, MIRU-VNTR	23
CAR	Bangui	MDR-TB	T, Cameroon, H, EAI	Spoligotyping	54
Chad	N'Djame´na	I mono-, E mono-, P mono-, I & E poly-resistant	T, Cameroon, H37Rv, undefined	Spoligotyping	63
Congo Brazzaville	Brazzavile & Pointe Noire	MDR-TB, mono-resistant	T, Beijing, LAM	MIRU-VNTR, DNA sequencing	19
Djibouti	Countrywide	MDR-TB	T, Beijing	WGS, MLVA, Spoligotyping, MIRU-VNTR, IS6110-RFLP	22 66
	Djibouti city	XDR- Pre XDR-, MDR-TB, mono- & poly-resistant	T, Beijing, CASKILI-1, CAS-Delhi, LAM, EAI		
Egypt	Suez canal region	MDR-TB, I & S mono resistant	T, LAM, *M. bovis*, CAS, S, undefined	IS*6110*-RFLP, Spoligotyping, DNA sequencing	25 56
	Assiut				
Ethiopia	Jimma	I mono resistant	T3_ETH	Spoligotyping, DNA sequencing, MLPA assay, MIRU-VNTR	57 67 68
	Meskenena Mareko district	I mono resistant	Haarlem		
	Oromia, SNNRPS, Harari	MDR-TB, mono- & poly-resistant	Ethiopia_3 CAS1_Delhi, EA, ETH_H37Rv like, lineage 7, H, Ural, X, EAI		
Ghana	South-west Ghana	MDR-TB, mono- & poly-resistant	Cameroon, MAF, undefined	Spoligotyping, IS6110-RFLP, DNA sequencing	61
Guinea	Conakry	MDR-TB, mono- & poly-resistant	T, H, LAM, Beijing, EAI, S, Cameroon, MAF WA2,	Spoligotyping	55
Kenya	North west	MDR-TB	CAS1, Beijing	Spoligotyping	28 34
		Mono & poly resistant	CAS1, S, LAM, T2		

Country	Province	DST phenotype	Genotype	Genotyping method	Ref
Malawi	Karonga district	R & I mono resistant	EAI	WGS	43 51
Mali	Bamako	MDR-TB, mono- & poly-resistant	T, MAF2, LAM10	Spoligotyping	37
Morocco	Casablanca	MDR-TB, mono- & poly-resistant	H, LAM9, EAI, Beijing, T	Spoligotyping, MIRU-VNTR	69 70
	Countrywide				
Mozambique	Countrywide	MDR-TB	Beijing	Spoligotyping, MIRU-VNTR, IS*6110*-RFLP	33
Nigeria	Ibadan, Nnewi and Abuja	MDR-TB, mono- & poly-resistant	Cameroon, T, LAM, MAF	Spoligotyping, MIRU-VNTR	40 60
	Cross river state	MDR-TB & S mono-resistant	Cameroon		
Rwanda	Countrywide	MDR-TB, R mono-, I mono-resistant	T2, CAS1_Delhi	RD analysis, Spoligotyping	21
Sierra Leone	Western area & kenema district	MDR-TB, mono- & poly-resistant	MAF, LAM, H, Sierra Leone1/2, Beijing, S	Spoligotyping, IS*6110*-RFLP, MIRU-VNTR	62
South Africa	Eastern Cape	XDR-TB	Beijing, LAM, MANU, S	Spoligotyping, IS*6110*-RFLP, DNA sequencing	45 46
		Pre- XDR TB	Beijing, LAM4		
		MDR-TB	Beijing, LAM, H, MANU, S, T, U, X		
	Gauteng	XDR-TB	Beijing, LAM, T, H, EAI_SOM, X	Spoligotyping, MIRU-VNTR	27 35 48 71
		Pre-XDR-TB	Beijing, LAM, S, H, EAI_SOM		
		MDR-TB and mono-resistant	Beijing, LAM, T, H, S, EAI_SOM, X		
	KZN	XDR-TB & Pre-XDR-TB	LAM4 (F15/LAM/KZN), S, T	Spoligotyping, WGS, targeted gene sequencing, IS*6110*-RFLP	13 42 47 71
		MDR-TB	LAM4 (F15/LAM/KZN), Beijing, S, T		
		Mono- & poly-resistant	LAM4 (F15/LAM/KZN)		
	Limpopo	XDR-TB	LAM4, X1	Spoligotyping, MIRU-VNTR	27
		Pre-XDR	Undefined genotype		
		MDR-TB	Beijing, EAI1_SOM, T, S, LAM9, LAM10, X3, T2,		
	Mpumalanga	XDR-TB	None reported	Spoligotyping, MIRU-VNTR	27
		Pre-XDR	Beijing, EAI_SOM, T, S, LAM, X, H, T		
		MDR-TB	Beijing, EAI1_SOM, T, S, LAM, X, H, MANU		
	North-West	XDR-TB	None reported	Spoligotyping, MIRU-VNTR	27
		Pre-XDR	EAI1_SOM		
		MDR-TB	Beijing, EAI1_SOM, T, S, LAM		

	Western Cape	XDR-TB	Beijing, T	Spoligotyping, IS*6110*-RFLP, targeted gene sequencing	30 31 32 41 53
		Pre- XDR-TB	Beijing, LAM4, S, T, X		
		MDR-TB	Beijing, Haarlem, LAM, X.		
		mono- & poly-resistant	Beijing		
Sudan	Omdurman, Khartoum & Port Sudan	MDR-TB, mono resistant	CAS1_Delhi, T, Beijing, H, CAS, LAM7_TUR, U, T2, S, LAM9	Spoligotyping, MIRU-VNTR	72
Tanzania	Chagga and Masai tribes	MDR-TB, mono- & poly-resistant	CAS, LAM, T, EAI, MANU, Beijing	Spoligotyping	44
Tunisia	Bizerte	MDR-TB	Haarlem3, undefined	Spoligotyping, MIRU-VNTR, PCR typing	38 73
	Bizerte, Tunis, Zaghouan	MDR-TB, mono- & poly-resistant	H3, T1, H1, LAM9, U, unknown		
Uganda	Mubende district	R mono-, E mono-, I mono-resistant	T2, Uganda, LAM3 & S, CAS, CAS1_Delhi, LAM11_ZWE, undefined	Spoligotyping, MIRU-VNTR, RD analysis	29 58 59 74
	Mbabara district	MDR-TB, R mono-, I mono-resistant	T2, Uganda, CAS_Delhi		
	Kampala district	MDR-TB, R mono-, I mono-resistant	T2, Uganda, LAM9, Beijing		
Zimbabwe	Countrywide	Pre-XDR	Undefined	Spoligotyping	49
		MDR-TB	LAM11_ZWE, LAM other, Beijing, CAS1 KILI, CAS1 Delhi, T, S, MANU		

Abbreviations: XDR-TB, extensively drug resistant tuberculosis; MDR-TB, multidrug resistant tuberculosis; R, rifampicin; H, isoniazid; E, ethambutol; S, streptomycin; WGS, whole genome sequencing; MLVA, Multiple loci VNTR analysis; IS*6110*-RFLP, Insertion Sequence *6110*-Restriction Fragment Length Polymorphism; Spoligotyping, Spacer oligonucleotide typing; MIRU-VNTR, Mycobacterial interspaced repeat units-variable number of tandem repeats; CAS, Central Asian; EAI_SOM, East African Indian_Somalia; KZN, KwaZulu-Natal; LAM, Latin American Mediterranean; MAF, *Mycobacterium africanum*; H, Haarlem; ETH, Ethiopia; SNNRPS, Southern Nations, Nationalists and Peoples Regional State.

2.4 Application of molecular methods to describe transmission dynamics of drug resistant tuberculosis in Africa

2.4.1 Acquired MDR- and XDR-TB

Although there are few molecular epidemiological studies from Africa, evidence suggests that to an extent drug resistant strains endemic in parts of Africa are as a result of ongoing and recent transmission (75, 76). These methods have also been demonstrated however that acquisition of MDR-and XDR-TB also plays an important role in the burden of drug resistant TB in endemic regions of Africa (76, 77). Inadequate treatment has been shown to be a significant driving force in the development of drug resistant TB, driven by factors such as poor adherence to treatment, diagnosis delay and low quality anti-TB drugs (78, 79).

The WHO recommends the use of a standardized TB treatment regimen which has been adopted by most countries in the region (7). In the absence of laboratory monitoring and surveillance, mainly due to poor infrastructure and lack of resources, the risk of acquiring resistance is heightened in high TB burden settings (78, 80). Further, standardized TB treatment has been shown to be unsuccessful in preventing the spread of drug resistant TB (79, 81). Therefore, there is a need to implement routine DST and surveillance, supported by molecular epidemiology, in order to guide effective TB treatment in high risk population groups.

2.4.2 Outbreaks

Drug resistant strains of *M. tuberculosis* have been linked with outbreaks in parts of Africa (31, 42, 63, 65). Outbreaks are characterised by sporadic spread of a particular strain of drug resistant TB unlike ongoing transmission which is characterised by constant spread of strains

over a longer period of time. In Africa, there is very limited information on outbreaks of drug resistant TB; this is largely due to poor surveillance and monitoring systems.

A prominent outbreak in Tugela Ferry KZN (mostly amongst HIV positive individuals) involving the F15/LAM4/KZN lineage, brought global focus onto XDR-TB and revealed that XDR-TB strains are transmissible (42). The main factors associated with the outbreak were an inadequate TB control program coupled with a high HIV prevalence in the affected population (42). This stresses the need for improved TB infection prevention and control (IPC) measures, together with rapid diagnostics in the successful control of XDR-TB and TB in general. Outbreaks in vulnerable population groups of institutionalized and HIV positive individuals have been documented (13, 42) (and are further discussed in section 2.3.3). High clustering rates of drug resistant isolates was observed in a mining community which had a high rate of HIV sero-positive individuals (78). The outbreak was as a result of an inefficient TB control program and diagnosis delay with the biannual chest radiography screening only diagnosing 30% of TB cases in this group of miners (78). Recommendations have since been made to improve detection and to promote parallel treatment of TB and HIV in high risk groups (78).

Community outbreaks of MDR-TB in HIV sero-negative, non-institutionalized individuals have also been reported (31, 65). Molecular investigations have revealed diversity in genotypes associated with outbreaks of drug resistant TB. For instance the Beijing genotype was associated with an outbreak in parts of South Africa, while the Haarlem genotype was linked with outbreaks in Tunisia (31, 82). Genotypes initially identified to be responsible for drug resistant TB outbreaks have been demonstrated to re-emerge in communities as was the case in Tunisia (82). A subsequent MDR-TB Haarlem strain outbreak was reported amongst the post-outbreak patients' population group in which the same strain was identified as the

progenitor (82). Inefficient treatment and lack of effective control measures of outbreaks pose a major threat as outbreak strains are likely to become endemic in parts of Africa. The findings of these drug resistant TB outbreak studies emphasise that MDR-TB and indeed other drug resistant TB outbreaks are not limited to specific population groups such as the immunocompromised and the institutionalized (32, 82).

There is some evidence that particular bacterial genotypes are associated with outbreaks. The Beijing genotype for instance, which is endemic in parts of South Africa, was linked to an outbreak of MDR-TB at a school in the Western Cape Province (31). Molecular characterization confirmed that all isolates belonged to cluster R220 (31). The genotype was further associated with a streptomycin-resistance outbreak in Benin and an MDR-TB outbreak in Djibouti (65, 66). The occurrence of an outbreak caused by the Beijing genotype in East and West Africa further highlights the emergence of "modern" strains in this region which appear highly virulent and pose a potential threat to TB control efforts in the region. Further, lineage 4 has been demonstrated to be widespread across Africa. Genotypes from this lineage have been associated with drug resistant TB and have been implicated in ongoing outbreaks of MDR-TB in parts of East Africa (29).

While host and strain genetics may play a role in driving outbreaks, inappropriate treatment, non-compliance to treatment and delays in diagnosis are amongst risk factors that have been linked to outbreaks within the continent (28, 31, 32, 42, 65). This highlights the urgent need for development and implementation of TB IPC policies in high-risk population groups and also calls for strengthening of outbreak response measures.

2.4.3 Nosocomial transmission

Hospital-acquired drug resistant TB has been reported in Africa. An outbreak of the XDR-TB F15/LAM4/KZN strain was described in a district hospital in Tugela Ferry, KZN, South Africa (83). Epidemiological links for 82% of the patients were made and clustering was observed in 92% of strains (83). The major risk factors that have been associated with hospital-acquired drug resistant TB are lack of proper IPC measures such as overcrowded wards, poor ventilation and delayed diagnosis (83). This coupled with the high HIV prevalence experienced in most TB endemic regions makes nosocomial transmission a significant driving force in the transmission of drug resistant TB strains.

Rather than a single point-source outbreak, social network analysis has revealed that patients linked to nosocomial transmissions have a high degree of community interconnectedness (83, 84). This implies that transmission is occurring both in the community and in the health care facilities. Prolonged exposure to patients with drug resistant TB and frequent, concurrent hospital admissions were common in most XDR-TB patients providing strong evidence that nosocomial transmission had occurred (83, 84).

Transmission of TB and drug resistant TB in particular is not only limited to patients receiving care and treatment in health care facilities but has been described in healthcare workers (HCWs) (85). Healthcare workers are at an increased risk of acquiring drug resistant TB at the work place, especially in the absence of effective IPC measures (86). It has been demonstrated that diabetes mellitus and HIV infection are common co-morbidities in HCWs that were infected with MDR-TB in a teaching hospital in South Africa (85). Other factors that have been associated with occupational acquisition of drug resistant TB and TB in general include: increased contact with patients who typically present to the health care

facility when they are highly infectious, complacency and low awareness of self-risk typically seen in longer-serving HCWs (85, 86).

Recommendations made towards improved control measures are to prevent transmission through early diagnosis of resistant TB, minimize congregation areas in hospitals by redesigning wards and out-patient areas and use of personal protective equipment (84, 85, 86). The highly limited data on nosocomial transmission of drug resistant TB in Africa is alarming and places emphasis on the need for molecular epidemiological studies in these high risk settings.

2.4.4 Migration

Migration has been demonstrated to play a critical role in the spread of drug resistant TB strains globally, with the majority of cases being reported in high-income countries originating from economic migrants from high TB burden countries (87). There is abundant literature from high-income countries owing to excellent TB surveillance and monitoring (87). In Africa however, there is very limited information on the impact of migration on transmission of drug resistant TB; this is mainly due to poor surveillance and monitoring. Further, migrant populations have poor access to health care and social structures.

Lineages and strains that had previously not been described in particular population groups have been hypothesised to have been introduced to various regions by immigrants (50, 81, 87). However, the absence of baseline data makes it rather difficult to prove this hypothesis as there is very limited data on genotypes that are in circulation within Africa. On the other hand, migration is rife in Africa, mainly due to political instability, civil wars and poverty, and it poses a major concern in the fight against TB and drug resistant TB in particular (88).

Drug resistant strains with streptomycin resistance were detected in a refugee camp in Kenya (50). Upon comparison to strains in the general populace, the refugee strains were unique to the camp (50). The nomadic nature of refugees means that they are highly capable of spreading drug resistant strains. There is a higher possibility of refugees failing to complete treatment due to their drifting nature and instability. Further, there is a possibility that the transmission of drug resistant strains is facilitated by a poor TB control program in the country of origin and/or in the refugee camp (50, 88).

Migration is not only an important factor in transmission of drug resistant TB across country borders and across continents, it has also been demonstrated to be an important means of transmission within countries as a result of movement to new cities and provinces in search of better employment opportunities and better health care facilities (89). For instance, the F15/LAM4/KZN has been shown to be widespread both in districts of KZN and in surrounding areas (89). Further, transmission of drug resistant TB strains has been demonstrated between provinces and districts in South Africa (77, 90, 91). This stresses a need for rigorous screening of migrants coming from high TB endemic regions and also calls for development and implementation of TB IPC polices in congregate settings in high TB burden regions.

2.5 Summary and discussion

The emergence and spread of drug resistant TB strains in the form of MDR-and XDR-TB continue to hinder global efforts to curb the disease. The application of molecular epidemiological tools has enabled a better understanding of the global phylogeography of TB (12). In Africa however, there is very limited and sporadic data for the genotypes associated with drug resistant TB. Knowledge of the lineages that are driving drug resistant TB gives a

better understanding of whether drug resistant TB is being acquired or transmitted within specific populations. Further, routine surveillance better informs TB control programs on the incidence of drug resistant TB in a given population. It is important for African countries to implement rigorous drug resistant TB surveillance systems as it has been demonstrated that 16 out of the 30 global high TB and MDR-TB burden countries are found within the continent (1).

Genetic diversity of *M. tuberculosis* strains has been demonstrated across Africa implying that diverse genotypes are driving the epidemiology of drug resistant TB across the continent. The diverse genotypes seen in parts of Africa suggest that drug resistant strains are possibly being introduced to the region. There are variations from region to region and particular genotypes have been demonstrated to be more predominant in certain countries and regions. There is a high degree of genetic diversity in the predominant strains in West Africa with both ancient and modern strains being associated with drug resistant TB (37, 40, 55).

The Beijing and LAM genotypes are widespread across Africa demonstrating the ability of these "modern strains" to adapt and spread easily (28, 31, 49, 64). It is however worth noting that the strain relatedness or transmission dynamics of these genotypes are not fully understood due to the lack of highly discriminatory tools of WGS in the reviewed studies. In contrast, the "ancient strains" such as the Cameroon and MAF strains are largely restricted to West Africa where these strains are mostly associated with drug susceptible TB (37, 40, 60). A similar observation is made with the Haarlem genotype which is associated with drug resistant TB in North and East Africa (22, 38).

The drug resistant TB epidemic in Africa has been attributed to several drivers, including socio-economic factors (poverty, overcrowded living conditions) and inefficient TB IPC policies (inappropriate treatment, lack of surveillance, diagnostic and treatment delay). MDR-

TB case finding and treatment remain a challenge in Africa with high MDR-TB burden countries, such as Nigeria, falling short on treatment enrolment of new MDR-TB cases, mainly due to the lack of adequate DST (1).

There remains a large pool of MDR- and XDR-TB cases that are untreated and are a potential source of drug resistant TB in the various communities (1). There is a need for united efforts from the continent to improve case detection and treatment for prevention and control of drug resistant TB. Further, high mortality rates have been observed in MDR- and XDR-TB patients and this is worsened by co-infection with HIV (42). This places emphasis on the need to strengthen the integration of HIV/TB screening and treatment in Africa.

The main challenge for TB activities across the continent is the lack of adequate funding. The majority of countries receive limited funding toward the national TB program with almost a third of the budget being unfunded on average in Africa (1). Addressing this shortcoming will require collaborative efforts from global funders as well as domestic support from local government. Concerns regarding international funding have arisen following the proposed budget cuts after the election of Donald Trump as the president of the USA and after the ”Brexit” vote in the UK (92, 93). These changes from the major global TB funders could result in the disintegration of already weak TB control programs in developing countries across the world.

Political instability is a source for concern as it leads to failing of health care infrastructure which in turn results in poor surveillance and treatment efforts. This has been demonstrated in migrant population groups with high rates of untreated drug resistant TB being found in these groups (88). There is a need to develop and implement rigorous TB screening and treatment of migrants and TB suspects across Africa. This is however made difficult by the

poor laboratory infrastructure such as lack of rapid diagnostic techniques for these highly mobile population groups.

Through molecular epidemiology, it has been demonstrated that drug resistant TB which is endemic in parts of Africa is both acquired and transmitted. Acquired drug resistant TB is largely driven by inadequate treatment, as seen in the case of standardized treatment in the absence of DST results, and non-adherence to treatment. On the other hand, drug resistant TB has been demonstrated to be transmitted in communities and hospital outbreaks have been reported mainly due to poor IPC measures. On average, the treatment success rates for MDR- and XDR-TB are low for Africa, 54% and 28% respectively. There are lessons to be learnt from model countries on the continent that report higher than average treatment success rates, as high as 80%, in Tunisia (1).

The gap in knowledge on the transmission dynamics and molecular epidemiology of drug resistant TB across the continent is a hindrance in the management of drug resistant TB and calls for improved surveillance efforts. Molecular epidemiological studies play an important role in understanding the transmission dynamics of drug resistant TB across Africa, and will play a part in addressing this knowledge gap.

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Chapter 3: Materials and methods

3.1 Study setting

This study involved molecular characterisation of drug resistant clinical strains of *Mycobacterium tuberculosis* (*M.tb*) collected from the Tropical Diseases Research Centre (TDRC) tuberculosis (TB) reference laboratory in Ndola district on the Copperbelt province of Zambia (Figure 1.2, page 12). The TDRC TB reference laboratory has a catchment of 3 out of 10 provinces; Copperbelt, Luapula and North-Western provinces (Figure 1.2, page 12). The centre provides culture-based first line drug susceptibility testing (DST) for all presumptive multidrug resistant (MDR)-TB cases (approximately 50 cultures per month); these are confirmed TB cases suspected of having MDR-TB either due to treatment failure or contact with a confirmed MDR-TB patient (Figure 1.3, page 14). The centre offers diagnostic services to government and privately run clinics and hospitals that are within the catchment area at no cost to the patient (1).

Ndola district is also the site for one of the two national MDR-TB wards at Ndola Teaching Hospital (NTH) where laboratory confirmed MDR/rifampicin resistant (RR)-TB patients are quarantined for part of their second line treatment, until culture negative, and treatment response is monitored (1). Similar to the TDRC TB reference laboratory, the NTH MDR-TB ward has a catchment of 3 provinces namely; Copperbelt, Luapula and North-Western provinces (Figure 1.2, page 12) (1).

All drug resistant samples for patients diagnosed at the TDRC TB reference laboratory were included as part of the study. This included samples from the TDRC TB reference laboratory catchment area and samples from outside the catchment area; Muchinga and Northern provinces (Figure 1.2, page 12). Samples from children (15 years and below) were excluded.

Initial sample processing of culture and first line DST to streptomycin, isoniazid, rifampicin and ethambutol (SIRE) was conducted at the TDRC TB reference laboratory, a level 2

biosafety laboratory with level 3 practices, as per routine. In the recent past, TDRC TB reference laboratory has been involved in national surveillance of both drug susceptible and drug resistant TB. Molecular typing methods including whole genome sequencing (WGS) and Sanger targeted gene sequencing (TGS), insertion sequence *6110*-restriction fragment length polymorphism (IS*6110*-RFLP), spacer oligonucleotide typing (spoligotyping), described in sections 3.5 and 3.6, and all respective data analysis were performed at the Division of Molecular Biology and Human Genetics Stellenbosch University.

3.2 Ethical considerations

Permission to access clinical specimens and patient records was sought from the Ministry of Community Development, Mother and Child Health, and Ministry of Health in Zambia. Ethical approval was obtained from the TDRC ethics committee, ethics number: TDRC STC 2015/9, and the Stellenbosch University Health Research Ethics Committee (HREC), ethics number: S15/08/172. Informed consent was obtained from all study participants that were followed up for sputum collection by community health workers (CHW) and consent was obtained from health care workers (HCWs) that participated in the TB infection prevention and control (IPC) knowledge, attitude and practices questionnaire. A waiver of consent was obtained for the analysis of samples collected through programmatic diagnosis from the TDRC TB reference laboratory.

In order to uphold patient anonymity, patients were assigned a unique study identification (ID) number and were not identified by their name during data analysis and presentation of findings.

3.3 Organism selection and data collection

Based on convenience sampling a sample size of 170 out of 184 (92%) drug resistant clinical isolates were available following culture based phenotypic DST of 581 samples over a period of 15 months from January 2016 to April 2017, including samples stored at the TDRC TB reference laboratory. Samples found to be phenotypically drug susceptible were not processed further as part of this study. Fourteen samples either did not yield positive cultures during subsequent re-culturing at Stellenbosch University (6 samples) or were cultured at the University Teaching Hospital in Lusaka and isolates were not available for this study (8 samples). Primary diagnostic cultures for samples found to be resistant to any first line drugs (mono-resistant, poly-resistant and MDR-TB) through phenotypic DST at the TDRC TB reference laboratory were collected (Figure 3.1), and stored for subsequent processing. Twenty samples previously archived at the TDRC TB reference laboratory, from August 2014 to December 2015, which had corresponding clinical data were revived through culture. To increase the number of samples captured, smear positive sputum samples were collected from the busiest TB diagnostic centres in Ndola district namely; New Masala clinic and NTH. Community health workers were hired to follow up known MDR-TB patients that were lost to follow up by the national TB program and did not have a sample stored at the TDRC TB reference laboratory or whose archived sample had no growth after culture revival. Samples collected from consenting patients were cultured as described in section 3.4 as per routine at the TDRC TB reference laboratory.

Following approval of waiver of consent to access patient records, clinical and demographic data were collected from TB registers at the various TB diagnostic sites and in the case of patient follow up, through one-on-one interviews with patients with the aid of CHWs. The data collected included; patient name, TB number, age, gender, address, referring

clinic/hospital, TB history, Patient type (new, failure, treatment after interruption, relapse, re-treatment, and defaulter), treatment status and HIV status.

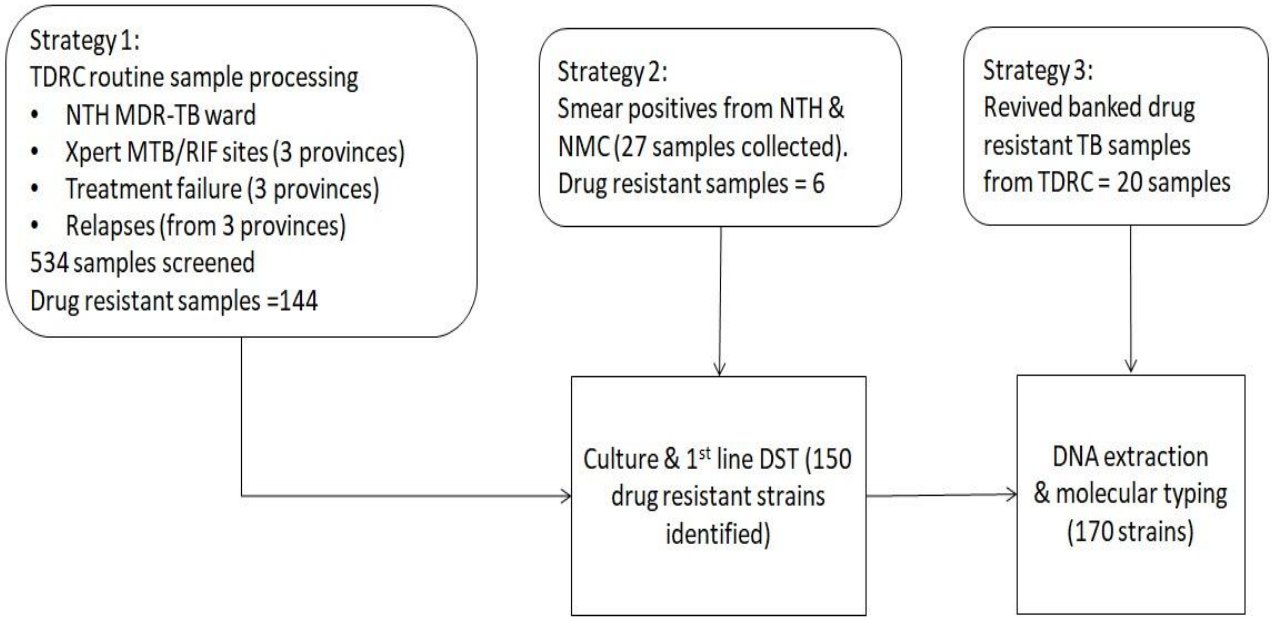

Figure 3.1: sample collection strategy used in the study. Abbreviations: NTH, Ndola Teaching Hospital; NMC, New Masala Clinic; TDRC, Tropical Diseases Research Centre.

3.4 *Mycobacterium tuberculosis* Culturing; phenotypic drug susceptibility testing and DNA extraction

A bacteriologic confirmation of *M. tuberculosis* was made at the various TB diagnostic centres through fluorescence smear microscopy using Auramine O staining and grading was done according to the National TB and Leprosy Control Program (NTLP) and World Health Organisation (WHO) recommendations (1). This was followed by digestion and decontamination of sputum using 1% N-acetyl-L-cysteine (NALC) sodium hydroxide (NaOH) reagent as per manufacturer's instructions (BD BBL MycoPrep kit). Decontaminated sputum samples were then inoculated into Mycobacteria Growth Indicator Tubes (MGIT) culture media and incubated in the BACTEC MGIT 960 culture system (Becton Dickinson Diagnostic Instrument Systems, Towson, Maryland, USA) until a culture flagged either positive or negative (no growth detected after 42 days of culture). Positive cultures were

confirmed to be *M. tuberculosis* complex using Capilia TB assay (TAUN, Numazu, Japan) and cultures found to be positive for *M. tuberculosis* were subjected to phenotypic DST.

Phenotypic drug susceptibility testing to the first line drugs streptomycin, isoniazid, rifampicin and ethambutol (SIRE) was performed using the BACTEC MGIT 960 SIRE kit, as per manufacturer's instructions, at the TDRC TB reference laboratory and for some isolates (70 isolates) at the Division of Molecular Biology and Human Genetics, Stellenbosch University, as per routine. A sample volume of 1 mL was stored at -80ºC for any sample that showed resistance to any of the first line drugs. DNA extraction and molecular typing was done at the Division of Molecular Biology and Human Genetics.

DNA extraction was performed as per routine and according to published methods (3). Briefly, MGIT cultures were grown from stock and positive cultures were re-grown in 7H9 Middlebrook broth (Becton, Dickinson and Company, Sparks, USA) supplemented with glycerol and oleic albumin dextrose catalase (OADC) for approximately 7 days or until opaque. One millilitre of the culture was transferred onto 7H11 Middlebrook (Becton, Dickinson and Company, Sparks, USA) agar plates for approximately 2-3 weeks or until a confluent lawn of *M. tuberculosis* culture was obtained. The *M. tuberculosis* cultures on 7H11 agar plates were heat killed at 80ºC for 2 hours. Bacilli were carefully scraped off the media into 6 mL of extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 5% mono-sodium glutamate, pH 7.4) in a 50 mL polypropylene tube containing approximately 20 glass beads (5 mm diameter) and vortexed for approximately 2 minutes or until homogenous. To degrade the cell wall, lysozyme (50 mg Roche, Germany) was added to each bacterial suspension and incubated at 37ºC for 2 hours after which 300 µl of a 10 mg/mL stock solution Proteinase K (Sigma-Aldrich, St. Louis, USA) and a 10X Proteinase K buffer (100 mM Tris-HCl, 50 mM EDTA, 5% SDS, pH 7.8) was added and incubated at 45ºC overnight (16 hours). In order to obtain pure DNA, the suspension was treated with 5 mL phenol/chloroform/isoamylalcohol

(ration 25:24:1) and incubated at room temperature for 2 hours with gentle mixing every 30 minutes. The suspension was centrifuged at 3000 x g for 20 minutes and the upper aqueous phase was aspirated into a new polypropylene tube containing 5 mL chloroform/isoamylalcohol (24:1). The suspension was gently mixed and centrifuged at 3000 x g for 20 minutes, after which the upper aqueous phase was aspirated into a new polypropylene tube containing 700 µl 3M sodium acetate pH 5.2. DNA was precipitated with 7 mL ice cold isopropanol, collected on a glass rod and washed in 70% ethanol for 10 minutes after which it was air dried overnight. The air-dried DNA was resuspended in 300 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and allowed to redissolve overnight at 4ºC after which it was stored at -20ºC until further use. The DNA concentration for individual samples was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

3.5 Spoligotype analysis

In order to determine the genotypes associated with drug resistant TB and whether isolates were clustered, spoligotyping and IS*6110*-RFLP were applied in parallel for all 170 drug resistant isolates, as per routine at the Division of Molecular Biology and Human Genetics, Stellenbosch University, as previously described (4, 5). Spoligotyping is a polymerase chain reaction (PCR) based technique that detects variations in the direct repeat (DR) region of *M. tuberculosis,* which consists of multiple 36 bp repetitive sequences interspersed by non-conserved sequences of about 35-41 bp in length (4). Primers used for spoligotyping were DRa (biotin-labelled) and DRb (Appendix 1) which amplify the DR region of the *M. tuberculosis* genomic DNA. The PCR reaction mix for individual samples consisted of 12.5 µl Kapa Taq PCR ReadyMix (Kapa Biosystem, Wilmington, Massachusetts, United States), 2 µl of each primer (DRa and DRb), 6.5 µl PCR grade water and 2 µl of template DNA (20

ng/µl) making a final volume of 25 µl. The PCR mix with genomic DNA was amplified in a thermal cycler with the PCR conditions listed in Table 3.2.

Table 3.1: Spoligotyping PCR reaction mix

Component	Volume
Kapa Taq ReadyMix	12.5 µl
DRa (biotin-labelled)	2 µl
DRb	2 µl
PCR grade water	6.5 µl
Template DNA (20 ng/µl)	2 µl
Final volume	25 µl

Table 3.2: Spoligotyping PCR conditions

Step	Temperature (ºC)	Time (minutes, unless stated)	Cycles
Initial denaturation	95	3	1
Denaturation	94	1	30
Annealing	55	1	30
Extension	72	30 seconds	30
Final extension	72	10	1
Hold	4	∞	1

M. tuberculosis H37Rv and *M. bovis* Bacillus Calmette–Guérin (BCG) were amplified as positive controls, and negative controls consisted of the PCR mix with PCR grade water. Following amplification of the *M. tuberculosis* DR region for individual samples, 150 µl 2xSSPE/0.1%SDS was added to 25 µl of the PCR product. This was followed by heat denaturation at 99ºC for 10 minutes and immediate cooling on ice. In parallel a nylon membrane consisting of 43 immobilized spacer oligonucleotide sequences (mapmygenome.in, Hyderabad, India) was washed in 2xSSPE/0.1%SDS at 60ºC for 5 minutes. The washed nylon membrane was placed on a support cushion in a mini-blotter with 40 sample slots. Residual fluid was aspirated out of the slots and slots were filled with the diluted denatured PCR product, with care to avoid bubbles. The genomic DNA was hybridized to the nylon membrane by incubating at 60ºC for 60 minutes. Following hybridization, samples were aspirated from the mini-blotter and washed twice in 2xSSPE/0.5%SDS at 60ºC for 10 minutes. Subsequently, the nylon membrane was incubated

in 40 mL of 2xSSPE/0.5%SDS containing 10 µl Strepavidin-peroxidase (Roche, Basel Switzerland) at 42ºC for 60 minutes. The nylon membrane was washed twice in 2xSSPE/0.5%SDS for 5 minutes at 42ºC, followed by a final 5 minute wash in 2xSSPE at room temperature.

To detect the presence of spacers, the nylon membrane was incubated with the ECL-detection system (Amersham GE Healthcare, Chicago USA) for 90 seconds. The nylon membrane was exposed to an x-ray film for 5 minutes and developed to identify the presence of spacers. Black squares on the x-ray film represent the presence of a spacer at that particular position while a clear area represents the absence of spacers in the particular position. Spoligotyping data was compared to the SITVIT database to determine the lineage and spoligo international type (SIT) based on existing patterns in the online SITVIT database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). Spoligotype patterns that matched with existing patterns on the online SITVIT database were assigned the corresponding SIT and clade while patterns that did not exist on the database were defined as orphans, not in SITVIT.

3.6 IS*6110*-RFLP analysis

Following DNA extraction and purification, genomic DNA for individual isolates (6 µg) was digested using the restriction enzyme PvuII (30 units). To test that the PvuII digestion was complete, gel electrophoresis was set up using 1% agarose gel (3g agarose in 300 mL 1x Tris-borate-EDTA (TBE), pH 8.3) and 8 µl of PvuII-digested genomic DNA was aliquoted into a 0.5 mL tube containing 4 µl 6x loading buffer. The PvuII-digested DNA was separated by electrophoresis at 120V for 4 hours after which it was stained by shaking for 30 minutes at room temperature in 500 mL 1xTBE containing 1 µg/ml ethidium bromide. The stained gel was visualized by trans-illumination at 245nm using a UV light box and the image was

captured by photography. Upon confirmation of complete digestion (DNA appeared as smears with evident banding patterns on the test gel), the intensity of the test gel was scored for each sample, from 12 (faintest bands) to 20 (brightest). This score was used to facilitate even loading on the final gel (see below).

For the final gel to be used in the Southern transfer, the remaining PvuII digested DNA was precipitated by adding 10 µl 3M sodium acetate pH5.2 and 300 µl ice cold 100% ethanol, mixed and incubated at -20ºC for 16 hours. The DNA was then pelleted by centrifugation at 14 000xg for 30 minutes at 4ºC. The supernatant was aspirated without disturbing the DNA pellet and 500 µl ice cold 70% ethanol was used to wash the DNA followed by a final centrifugation step at 14 000xg for 30 minutes at 4ºC. The supernatant was aspirated without disturbing the DNA pellet after which the DNA was allowed to dry for 16 hours (overnight). The DNA was resuspended with an internal molecular weight marker (IMWM) (volume dependent on the intensity of bands in the PvuII digestion test gel, to achieve even loading) and incubated at 65ºC for 4 hours with mixing every hour. A final gel (up to 20 samples per gel) was set up, the steps followed are as described for the test gel above with minor changes. Changes to the final gel included the use of 0.8% agarose gel (2.4g agarose in 300 mL 1x TBE, pH 8.3), the loading of 10µl of the PvuII-digested DNA for each sample with an external marker *M. tuberculosis* reference strain Mtb14323 (4), and separation by electrophoresis at 65V for 16 hours. The DNA was Southern transferred onto a nylon membrane (Hybond N+, GE Healthcare Amersham, Chicago USA). To fix the DNA to the nylon membrane, the membrane was baked at 80°C for 2 hours. To identify IS*6110* elements in the genomic DNA, the nylon membrane was hybridized with a horseradish peroxidase (HRP) nucleic acid labelling system (GE Healthcare Amersham, Chicago USA) labelled IS*6110* probe (3), to allow visualisation of the IS*6110* element following chemiluminescence. Briefly, to label the probe, 200 ng probe DNA was added to a 0.5 mL eppendorf tube and 15

µl nuclease free water was added. This was followed by a 5 minute incubation step at 100ºC to denature the probe DNA, and DNA was snap cooled on ice for 5 minutes. To label the probe, 15 µl of the HRP labelling mix was added to the probe DNA and mixed, after which 15 µl of gluteraldehyde solution was added and mixed, to aid in conjugation of HRP. This was followed by a 10 minute incubation step at 37ºC. To enhance visualisation the probe was used immediately. Hybridisation was done using the ECL Gold hybridization buffer according to manufactures instructions (GE Healthcare Amersham, Chicago USA).

To visualise the IS*6110* fingerprints, the probe hybridized DNA was detected using the ECL detection system (Amersham GE Healthcare, Chicago USA) and subsequently exposed to an x-ray film. The presence of bands in the developed films represents the presence of an IS*6110* element in the particular position while a clear region (lack of bands) represents the absence of an IS*6110* element in the specific position. Similar probe labelling, hybridization and visualisation steps were followed for the internal molecular weight marker, which is used to obtain accurate and standardized IS*6110* RFLP results during computer assisted analysis.

The resulting autoradiographs were analysed using gel-analysis computer software, Gelcompar (Applied Maths, Kortrijk, Belgium). Fingerprints were assigned an IS*6110* cluster number, based on the positions and copy numbers of IS*6110* element, and strains were further assigned to an existing family that shared identical IS*6110* patterns. Dendograms were constructed based on similarity coefficients (6), that is according to the number and position of the IS*6110* elements identified. Strains sharing identical patterns were defined as clustered, suggestive of recent transmission.

3.7 Whole genome sequencing

To determine whether recent transmission had occurred, all 91 strains with purified genomic DNA at the window of opportunity for sequencing were sent for WGS. Due to financial constraints, not all strains could be analysed using WGS, the remainder of isolates without WGS data (84 isolates) were characterised using TGS, described in section 3.8. Whole genome sequencing, using the next generation sequencing (NGS) platform Illumina HiSeq 2000, was performed at the Centers for Disease Control and Prevention (CDC) Atlanta Georgia (90 strains) or Beijing Genomics Institute (BGI) Tech Solutions Hong Kong (12 strains) using the Illumina HiSeq 2500/4000 NGS platform as per routine. For validation and quality control (QC) purposes 11 strains were sequenced both at CDC and BGI.

3.7.1 Data analysis

WGS data were analysed using a data analysis pipeline developed in the Stellenbosch University Division of Molecular Biology and Human Genetics, Universal Sequence Analysis Pipeline (USAP) (Figure 3.2).

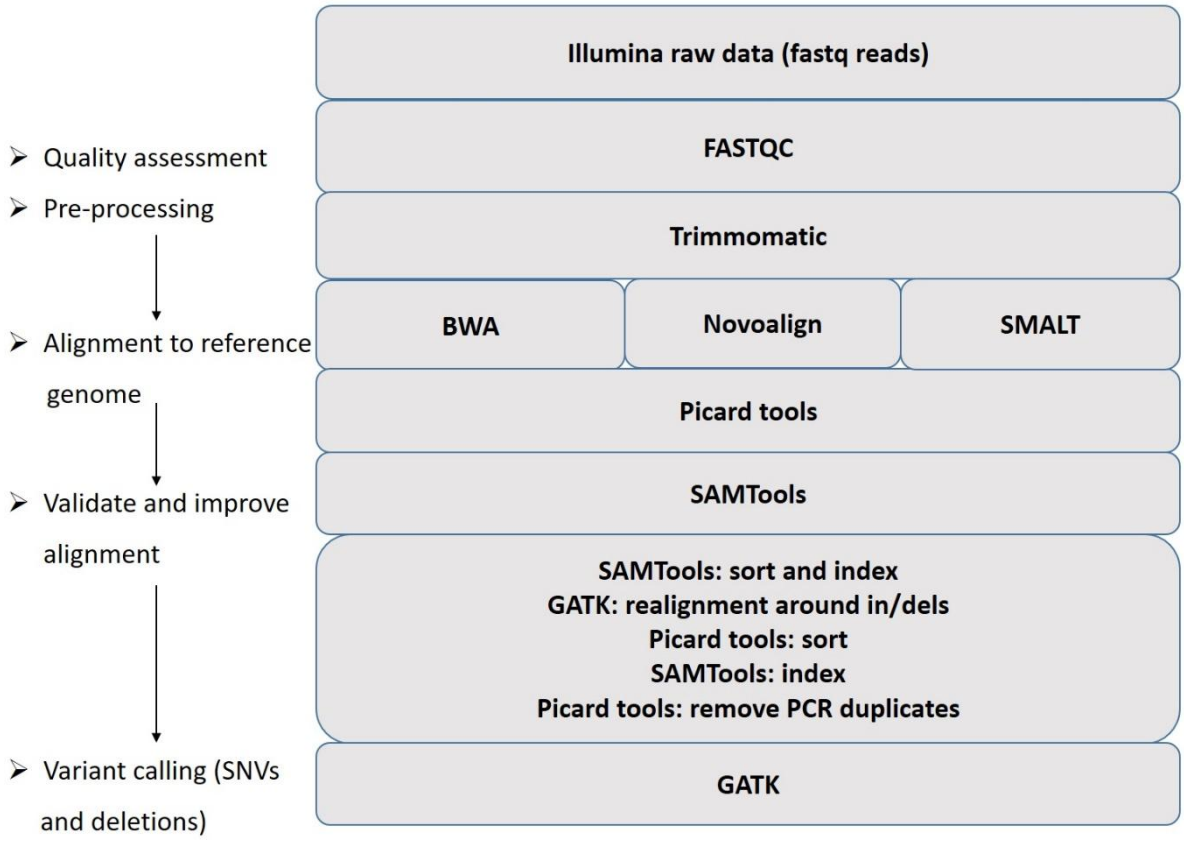

Figure 3.2: Universal Sequence Analysis Pipeline (USAP) genome sequence data analysis process (7). Abbreviations: BWA, Burrows-Wheeler Aligner; GATK, Genome Analysis Tool Kit; in/del, insertions/deletions; PCR, polymerase chain reaction; SAMTools, Sequence Alignment/Map tools; SMALT, Sequence Mapping and Alignment Tool.

3.7.2 Alignment and variant detection

The NGS data analysis pipeline is comprised of various open-source NGS analysis tools combined with in-house Python scripts (7). Briefly, as depicted in Figure 3.2, quality assessment was performed using FastQC after which reads were pre-processed by removing adapters and low quality (phred quality < 20, sliding window of 4 bases) bases using Trimmomatic (8). In order to identify genomic variants, high quality reads were aligned to the reference *M. tuberculosis* genome, H37Rv (GenBank NC000962.2), using a combination of 3 alignment tools; Burrows-Wheeler Aligner (BWA), Novoalign and Sequence Mapping

and Alignment Tool (SMALT) (9, 10). The alignments were validated with Picard tools and SAMtools was used to retrieve alignment statistics. The Genome Analysis Tool Kit (GATK) was used to detect single nucleotide variants (SNVs) and insertions and deletions (indels) according to the GATK Best-Practices and only variants identified in all 3 alignments were considered for downstream analyses (7, 11).

A high confidence variant file was generated for SNVs that were identified in all 3 alignment tools by GATK and the file was used for further analysis.

Using USAP, known mutations conferring resistance to any of the first and second line drugs were investigated. Isolates were classified according to lineage and in order to investigate strain relatedness (12), the number of SNVs between clustered isolates was determined using pairwise comparisons for isolates that were clustered following phylogenetic analysis (described further in 3.7.3). Upon pairwise comparison of strains sharing the same terminal branches, genomic clusters were defined as strains separated by 12 or fewer SNVs suggestive of recent transmission, as previously described (13, 14).

3.7.3 Phylogenetic analysis

A phylogenetic tree was generated to investigate the evolution and relatedness of 86 isolates that were sequenced, 5 failed sequencing and were not included in analysis. Previously described *M. tuberculosis* isolates, representative of the *M. tuberculosis* complex, were included in the phylogenetic tree for reference purposes (Appendix 2). Variants identified in repetitive regions, including the *pe/ppe* and *pe_pgrs* genes, were excluded for phylogenetic analysis. To determine the phylogenetic relationship between the sequenced isolates, high confidence variable coding and non-coding SNV sites were used with Randomized Axelerated Maximum Likelihood (RaxML), as previously described (7, 15). The general time

reversal model of nucleotide substitution (determined with ModelGenerator) was applied to construct a maximum likelihood phylogeny with RaxML with 1000 bootstrap pseudo-replicates.

3.8 Sanger (targeted gene) sequencing and analysis

Sanger sequencing was performed to identify resistance-conferring mutations to the first line drugs rifampicin (*rpoB* locus), isoniazid (*katG* and *inhA*; mutations in these genes results in high level and low level resistance, respectively), ethambutol (*embB*), streptomycin (*rrs*) and pyrazinamide (*pncA*). To investigate the presence of XDR-TB in the study population, Sanger sequencing was performed to identify mutations conferring resistance to fluoroquinolones (*gyrA*) and second line injectable drugs (*rrs* and *tlyA*). In total 8 loci associated with drug resistance in *M. tuberculosis* were sequenced and analysed. We performed targeted Sanger sequencing for 84 strains (and an additional 6 strains which had both WGS and Sanger sequencing performed, for validation purposes). Genomic DNA, either crude or purified DNA (25ng/µl), was used to amplify up to 8 genes associated with drug resistance using the CFX96 real-time PCR system (Bio-Rad, California, USA), as previously described (16, 17). The PCR reaction mix and conditions for individual genes are listed in appendix 1. Briefly, a 25 µl PCR mix was set up for each sample (90 samples by 8 loci). The reaction mix consisted of nuclease free water (making up 25 µl), 2.5 µl of 10x PCR buffer, 0.5 µl magnesium chloride ($MgCl_2$), dNTPs (2.5 mM each), forward and reverse primers specific for each loci (0.05 mM per primer), Taq polymerase (0.625 U; Qiagen, Germany), 1 µl Syto 9 green fluorescent nucleic acid stain (0.05 mM; Thermal Fisher Scientific, Waltham, Massachusetts, USA) and either 2.5 µl crude DNA or 1 µl purified DNA (25 ng/µl) was added. Q solution (Qiagen, Germany) was added to the PCR mix for amplification of *gyrA*, *rrs* and *tlyA*, to

facilitate amplification of G-C rich templates. The *M. tuberculosis* genome, H37Rv, was used as a positive control and nuclease free water was added to the negative control. The PCR products were sequenced at the Central Analytical Facilities (CAF), Stellenbosch University, as per routine, using the corresponding primers and amplification conditions listed in Appendix 1.

To identify single nucleotide variants (SNVs) and indels, ABI Sanger sequence data was aligned to the *M. tuberculosis* H37Rv (GenBank NC000962.2) reference genome using the sequence alignment editor BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) and chromatograms were viewed in Chromas (http://www.technelysium.com.au) in relation to the reference genome. To verify the findings, 6 strains were sequenced with both WGS and Sanger (targeted gene) sequencing. Mutations identified as resistance-conferring through sequencing were validated through comparisons with mutations listed on the TBdream database (https://tbdreamdb.ki.se/Info/Default.aspx).

3.9 Knowledge, attitudes and practices of health care workers on TB IPC measures

A cross sectional study was conducted to assess the knowledge, attitudes and practices (KAPs) of HCWs at the NTH MDR-TB ward and the TDRC-TB reference laboratory in Ndola district. A self-administered questionnaire (appendix 3) was developed, in consultation with an IPC expert, which captured demographic data and KAPs of the HCWs at the named facilities. The target population included clinicians, laboratory personnel (technologists and scientists), nurses, students, data entry clerks and cleaners who were directly involved in sputum collection and processing and/or had contact with MDR/RR-TB patients. Eleven out of 17 health care workers, 5 from the NTH MDR-TB ward and 6 from the TDRC TB reference laboratory, gave consent to participate in the survey in September 2017.

Participants were required to provide consent before filling out the questionnaire and questionnaires were anonymised to encourage participants to be forthcoming in their responses and for confidentiality. The survey was designed using previously described methods from published studies and based on guidelines from the WHO and the NTLP of Zambia (18, 19, 20, 21). The questionnaire included 34 questions based on demographics (age, gender, profession and highest education level), knowledge of TB transmission, attitude towards IPC policies and IPC practices. Ethical approval for this study was obtained from the TDRC ethics committee, ethics number: TDRC STC 2015/9.

3.10 References

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Chapter 4: Drug resistant tuberculosis cases from the Copperbelt province and Northern regions of Zambia: Genetic diversity, demographic and clinical characteristics.

4.1 Introduction

This chapter describes the genotypes associated with drug resistant TB within the Copperbelt province and Northern regions of Zambia diagnosed at the Tropical Diseases Research Centre (TDRC) TB reference laboratory in Ndola district as well as the demographic and clinical characteristics of patients. Currently, there is very limited knowledge on the genotypes and transmission dynamics of TB in Zambia as a whole with no published studies describing the genotypes associated with drug resistant TB in the country. Therefore this study aims to bridge the gaps in knowledge by describing the genetics and transmission dynamics of drug resistant TB for isolates collected from the TDRC TB reference laboratory in Ndola district, which has a catchment of 4.1 million (25% of the national population). Understanding the genotypes associated with drug resistant TB will be beneficial to the national TB control program as it provides a better insight into the population structure of drug resistant TB, transmission of these strains and associated risk factors.

Overall, 170 out of 184 (92%) phenotypically resistant isolates (any form of drug resistance) were available for genotyping using spacer oligonucleotide typing (spoligotyping) and insertion sequence *6110*-based restriction fragment length polymorphism (IS*6110*-RFLP) as described in sections 3.5 and 3.6.

4.2 Demographic and clinical characteristics of patients included in the study

Demographic and clinical data was directly captured into a study register and included age, gender, TB history, HIV status, DST results, residential address and treatment status, as described in section 3.3. As indicated in Table 4.1, over half of the cases were male (57%) and the age range was between 16-63 years old, with 50% of patients falling within the 30 to 49 years age group. The HIV-TB co-infection rate for patients with a known HIV status was 64% (79/123); comparable to the WHO estimate of 60% for Zambia, and 47 (28%) patients had an unknown HIV status. Close to half of the cases (49%) had failed prior first line treatment (either first or retreatment), and a large proportion were new cases (24%), that is patients without prior TB treatment or patients with prior TB treatment for less than 1 month. The majority of patients (78%) were residents of the Copperbelt province while the remainder were diagnosed and/or treated in Ndola, but resided outside the Copperbelt province, Ndola district specifically (Figure 4.1).

Less than half (35%) of the cases included in the current study were still receiving second line treatment at the time of sample analysis, while 23% of patients were not started on second line treatment, since these cases were not diagnosed with MDR/RR-TB prior to this study. A further 12% had completed treatment while close to 3% were lost to follow up and 9% had died during the course of treatment. The treatment status for 18% of patients was unknown, as data was not recorded in the registers.

Missing clinical and demographic data were observed for all the variables included in the current study. The largest gaps were seen in the HIV status (28% of patients had an unknown HIV status), TB history (15% of patients had an unknown TB history), and treatment status (18% of patients had an unknown treatment status). Gaps in the TB registers observed during data collection at the TDRC TB reference laboratory and the NTH MDR-TB ward are

concerning and point towards a poor health care system resulting from insufficient resources and staff to accurately record routine notification and follow up of MDR-TB cases.

The drug resistant profiles for isolates included in the study are illustrated in Figure 4.2. From these strains 93 were MDR-TB, 32 had added resistance to ethambutol and streptomycin, while 13 had added resistance to ethambutol only and 18 to streptomycin only (Figure 4.2). There were 12 rifampicin and 23 isoniazid mono resistant cases and 5 streptomycin and 3 ethambutol mono resistant TB cases.

Table 4.1: Basic demographic and clinical characteristics of patients with drug resistant TB diagnosed at the TDRC TB reference laboratory in Ndola district.

Variable		Number of patients (total=170)	%
Gender	Male	97	57
	Female	67	39
	Unknown*	6	4
Age	16-29	49	29
	30-49	85	50
	50-63	13	8
	Unknown*	22	13
HIV status	Positive	79	46
	Negative	44	26
	Unknown*	47	28
TB history	New	41	24
	Relapse	15	10
	After failure of 1st line treatment	45	26
	After failure of 1st line retreatment	43	25
	Unknown*	26	15
Treatment status	Treatment completed	21	12
	Treatment ongoing	60	35
	Lost to follow up (Defaulter)	5	3
	Died	15	9
	2nd line treatment not started	39	23
	Unknown*	30	18
Residence by Province	Copperbelt	133	78
	Luapula	16	9
	Northern	11	6
	North-Western	3	2
	Muchinga	1	1
	Unknown*	6	4

Abbreviations: TDRC, Tropical Diseases Research Centre; TB, tuberculosis. *not indicated in the TB register.

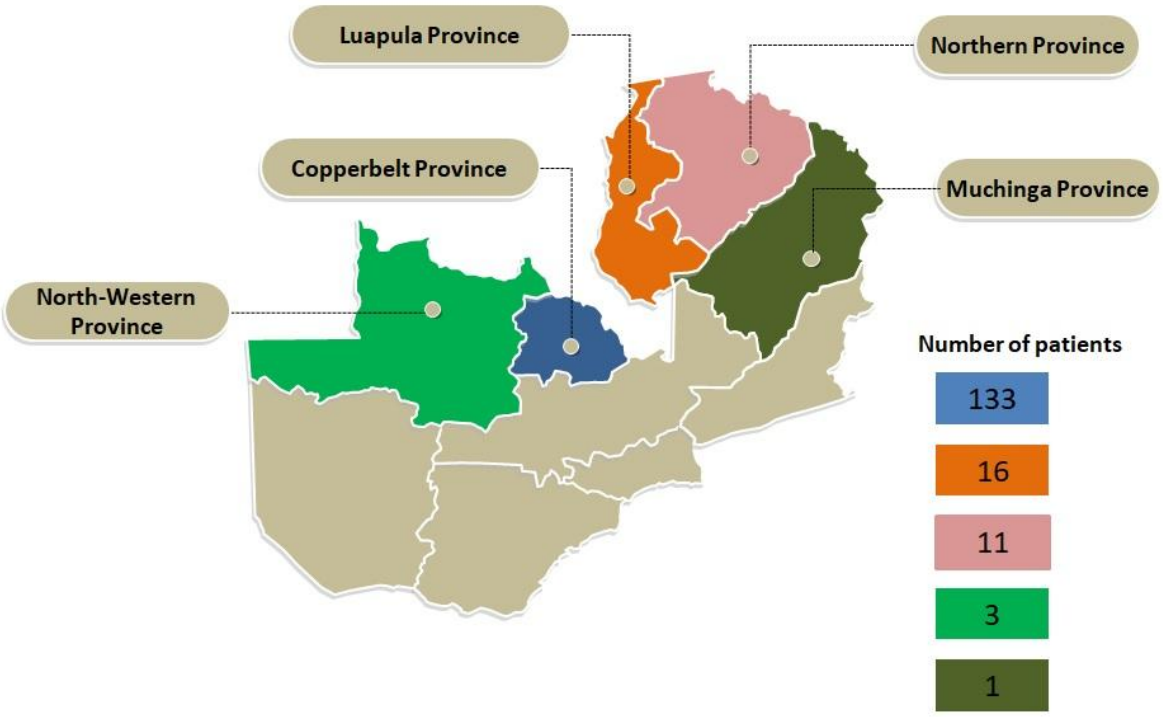


Figure 4.1: Distribution of drug resistant TB cases diagnosed at the TDRC TB reference laboratory in Ndola district Copperbelt province (highlighted in blue). The distribution of cases is according to Province of patient origin and does not include the 6 patients with an unknown residence.

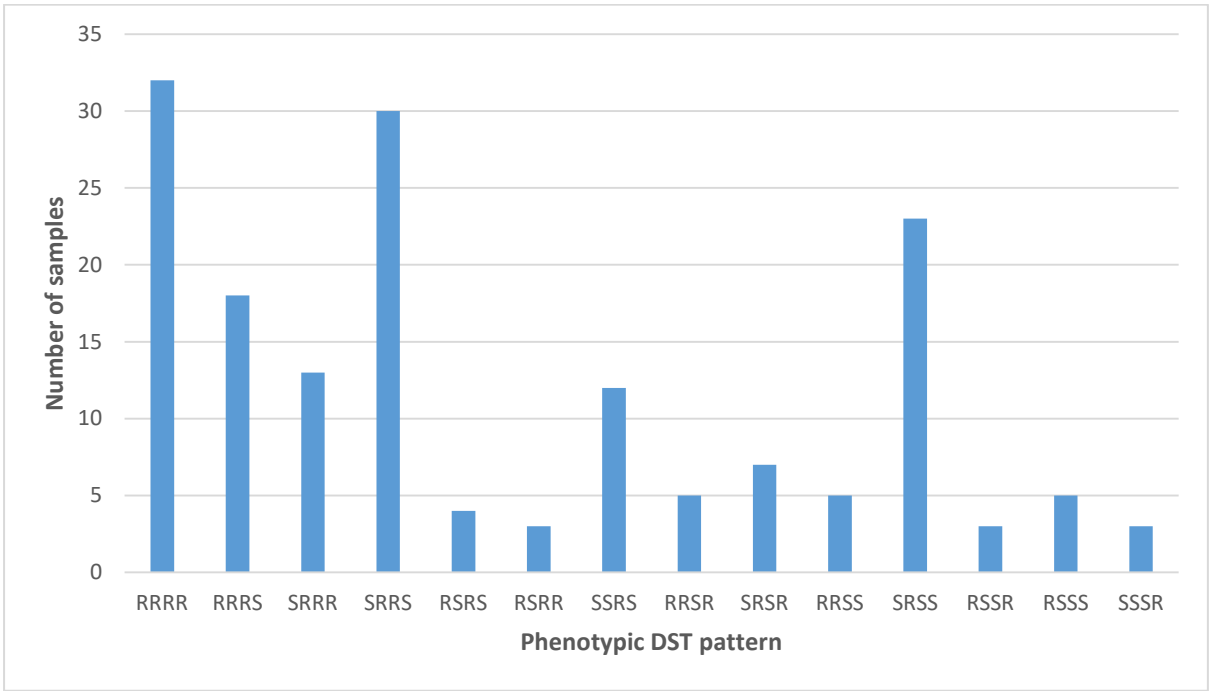


Figure 4.2: First line drug resistance patterns for individual isolates in the order SIRE (Streptomycin, S; Isoniazid, I; Rifampicin, R; Ethambutol, E). Abbreviations: R, resistant S, sensitive.

4.3 Analysis of spoligotype patterns

Spoligotype results were compared to existing patterns in the online SITVIT database (1) and isolates that shared identical patterns were assigned the existing spoligo international type (SIT) number while isolates that had a unique pattern not previously described in the SITVIT database were defined as orphans, not in SITVIT. Spoligotype analysis reveals that diverse genotypes are associated with drug resistant TB in the study population (Table 4.2 and Figure 4.2). Three major lineages (lineages 2, 3 and 4) were described with the major clades belonging to LAM, T and X (Figure 4.3). There were a total of 18 clusters, with 2 to 26 strains per cluster; the largest clusters belonged to LAM11_ZWE, SIT 815 (24 strains).

Previous molecular studies in this region demonstrated that the LAM11_ZWE, T, CAS and X were driving the epidemiology of drug susceptible TB in Ndola (2). The findings of the current study imply that genotypes associated with drug susceptible TB are evolving to become drug resistant in this population. In addition, genotypes not previously described in Zambia but other countries such as South Africa, have been identified amongst drug resistant *M.tb* clinical isolates in the study population (3). For instance, lineage 2 isolates belonging to the Beijing clade designated SIT 1 (4 strains) and an undefined non-Beijing lineage 2 with the SIT 955 (3 strains) were described in the current study. Nine strains (6%) did not exist on the online SITVIT database and were therefore defined as orphans.

Table 4.2: Summary of the *M. tuberculosis* spoligotype patterns for drug resistant TB cases diagnosed at the TDRC TB reference laboratory (total number of patients=170).

Clade	SIT	Spoligotype pattern (spacers 1-43)	n	*Strains from a previous study (2)
LAM11_ZWE	815	■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■□■■■	26	25
LAM11_ZWE	59	■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■■	24	61
LAM11_ZWE	2173	□■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■■	2	None
LAM11_ZWE	2490	■■■■■■■■□□□□□□□■■■■■□□□□■■□□□□■■□□□□■■■□■■■	1	None
LAM11_ZWE	2488	■■■■□□□□■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■■■■	1	None
LAM11_ZWE	2385	■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■□□■■■■	1	None
LAM9	42	■■■■■■■■■■■■■■■■■■■■■■■□□□□ ■■■■■■■■□□□□■■■■■■■	12	7
LAM1	20	■■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	6	4
LAM1	961	■□□□□□■■■■ ■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	4	None
LAM1	2522	■■□■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■□□□	1	None
LAM4	811	■■■■■■■■■■■■■■■■■■■■■■■□□□□■□□□□□■■□□□□■■■□■■■	3	2
LAM3	1685	■■■■■■■■□□□□■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	1	None
LAM3	719	■■■■■■■■□□□■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	1	None
T1	53	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	11	22
T1	244	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■	9	None
T1	52	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	6	1
T1	1926	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□□□■	4	None
T1	926	■■■■□■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	2	None
T1	291	■□□□□□■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■	1	None
T1	306	■■■■■■■■■■■■■■■■■■■■□□□□□□■■□■■■□□□□■■■■■■■	1	None
T3 T2	73	■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■□■■■	3	1
T1	Orphan	■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■□□□□■■■□□□■	1	None
T1	Orphan	■■■■□□□■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■□□□□	1	None
X1	1080	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■■□□□■	1	None
X2	137	■■■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■□□□□■	13	12
X2	476	■□■■■■■■■■■■■■■■■□■■■■■■■■■■■■■■■□□□□■■□□□□■	1	None
CAS1_KILI	21	■■■□□□□■■□■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■	7	1
Manu3	Orphan	■■■■■■■■■■■■□■□□■■■■■□□□■■■□■■■■■□□□□■■■■■■■	1	None
Beijing	1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■	5	None
Undefined	955	□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■■■■■■■■■	3	None
Not in SITVIT	Orphan	■□■□□□□□□□□□■□□■■□■□□□□□■□□□□□■□□□□■■□■■□■	2	None
Not in SITVIT	Orphan	■■■■■■■■■■■■■■□■■□■■□□□□■■□□□□■■□□□□■■■□■■■	1	None
Not in SITVIT	Orphan	■■■■■■■■■■■■■■■■■□□□□□□□□□□□□□□□□■■■■■■■■■	1	None
Not in SITVIT	Orphan	□■■■■■■■■■■■■■■■■■■■□□□□■■□□□□■■□□□□■■■■□■■	1	None
Not in SITVIT	Orphan	■■■□□□□■■■■■■■□■■■■■□□□□□□□□□□□□□□□□□■■■■■	1	None
Not in SITVIT	Orphan	■■■□□□□□□□□■■■■■□■■■■■■■■■■■■■□□□□□□■■■■■■	1	None
Not in SITVIT	Orphan	■□■■■■■■■■■■■□□■■■■■□□□□■■□□□□■■□□□□■■□■■□■	1	None
Not in SITVIT	Orphan	■■■■■■■■■■□■□■□□■■■■■□■■■■■■■■■□□□□■■□■■□■	1	None

Abbreviations: CAS, Central Asian; LAM, Latin American Mediterranean; SIT, spoligo international type. Orphans are defined as patterns that are not found in the SITVIT database. *The number of strains described for spoligotyping findings in a previous study are listed in comparison with findings from this study.

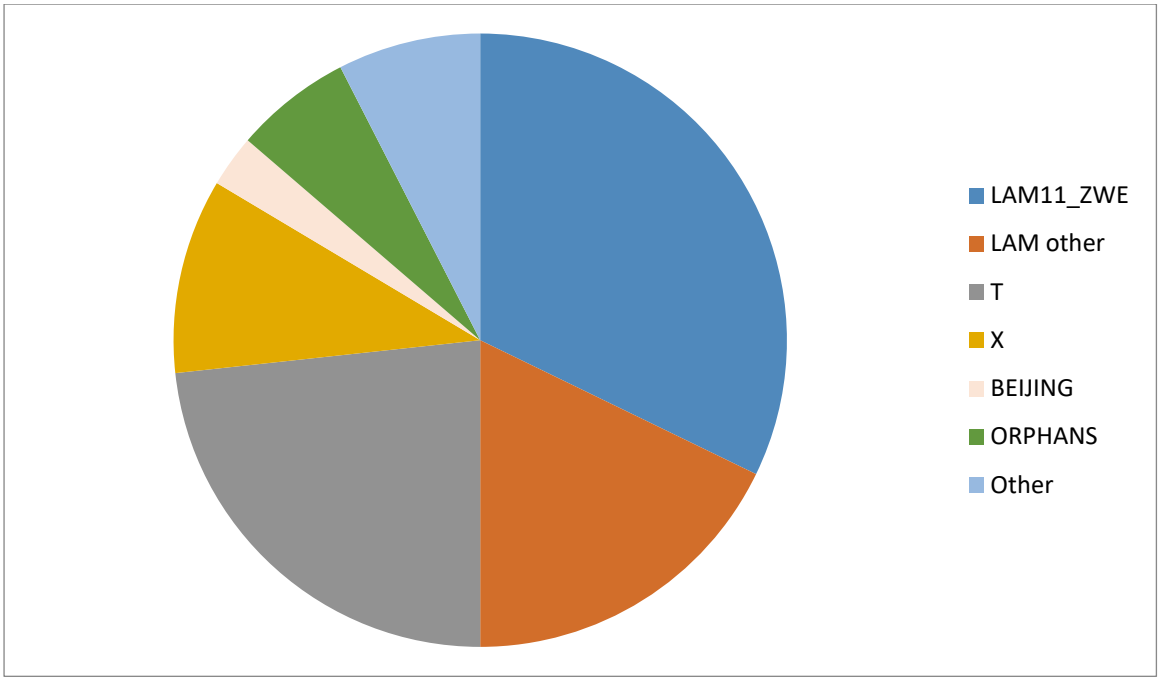


Figure 4.3: **Proportion of strains identified through spoligotype analysis.**

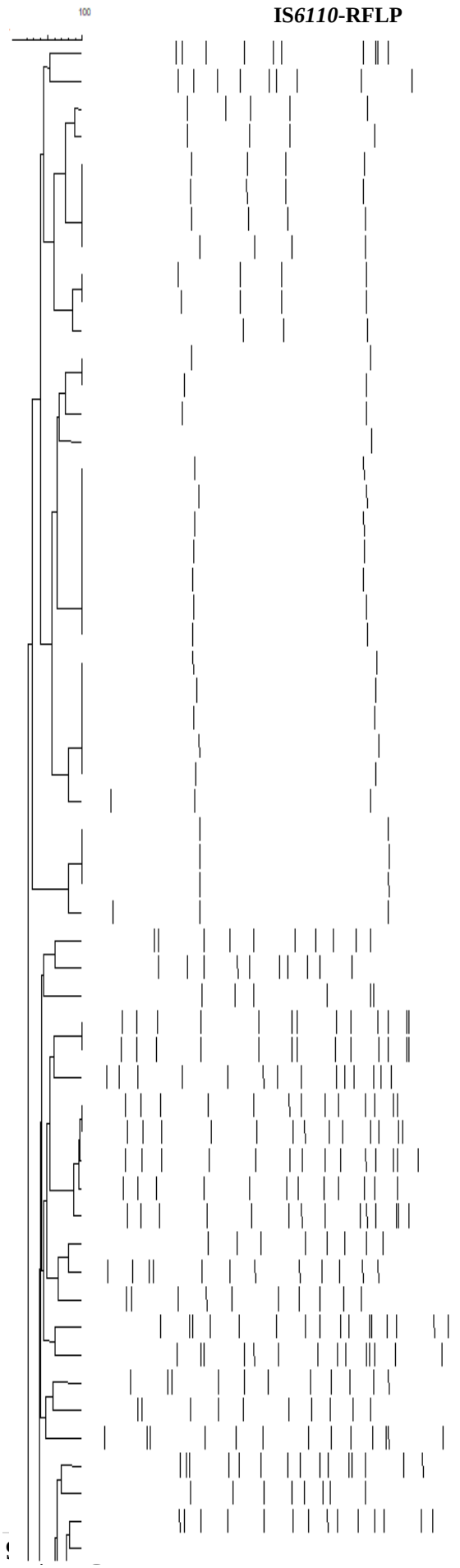
4.4 Analysis of IS*6110*-RFLP fingerprints

DNA fingerprinting using IS*6110*-RFLP has been demonstrated to have superior discriminatory power over spoligotyping (4, 5). We therefore applied IS*6110*-RFLP fingerprinting in parallel with spoligotyping in order to further differentiate strains. Fingerprints were assigned an IS*6110* cluster number, based on the positions and copy numbers of IS*6110* element, and strains were further assigned to an existing family that shared identical IS*6110* patterns (4), based on those existing on the Stellenbosch University Division of Molecular Biology Gelcompar database. Cluster numbers without an existing family on the Stellenbosch University Division of Molecular Biology Gelcompar database were left blank in the family assignment.

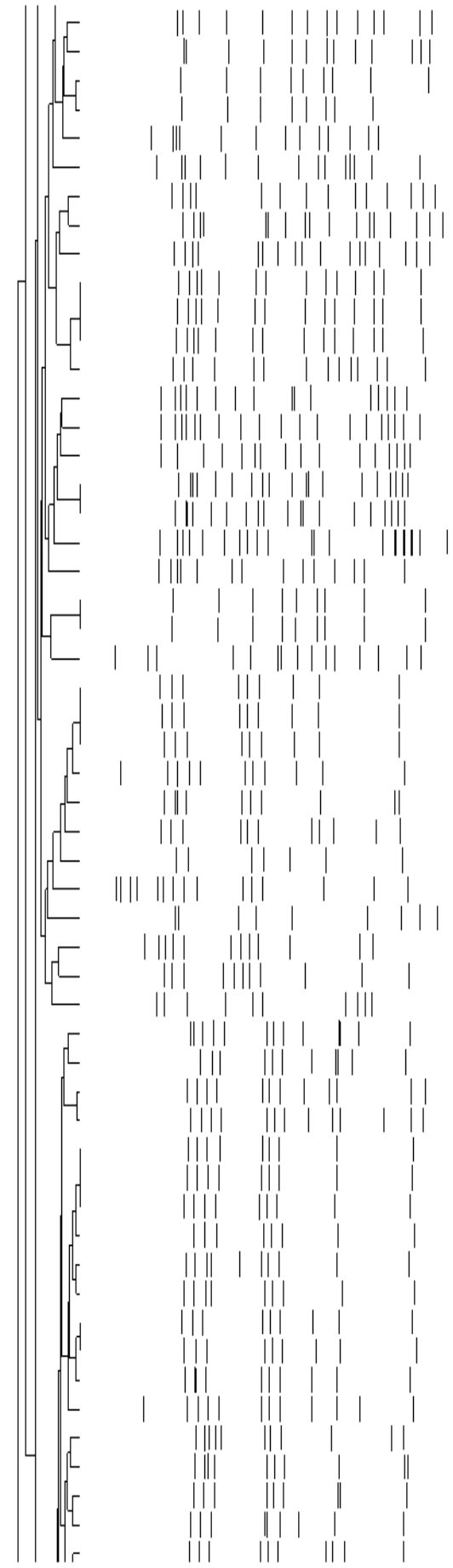
Similar to spoligotyping findings, IS*6110*-RFLP demonstrates that diverse genotypes are associated with drug resistant TB in the sampled regions of Zambia, implying that various strains are driving drug resistance in Zambia (Figure 4.4). According to IS*6110*-RFLP, 11

clusters with 2-5 strains per cluster were identified (Figure 4.4), where clusters were defined as strains sharing identical IS*6110*-RFLP patterns (in terms of band number and position).

The clustering of strains suggests that drug resistant TB is being transmitted in the study population; however there is a need for more detailed analysis and classical epidemiological links to support these findings. In contrast, 79 strains had unique patterns that were not clustered (Figure 4.4). This implies that drug resistant TB is both acquired and is being transmitted in the study population. The presence of unique patterns however could also be attributed to the poor case detection rate observed in Zambia, resulting in some cases being missed thereby impacting the estimate of transmission.



ID	Family	IS*6110*	Spoligotype pattern	SIT
N 41		29706		53
N 246	16	29773		Not in SITVIT
N 446		29713		53
N 74	140	381		ORPHAN
N 341		29695		1926
N 359		29695		1926
N 422		29695		1926
N 456		29695		137
N 307	140	29709		Not in SITVIT
N 225	140	29709		1926
N 330	140	29708		Not in SITVIT
N 133		29696		137
N 241		29710		137
N 481		29711		137
N 67		325		244
N 338		29696		52
N 420		29696		244
N 283		29696		1301
N 352A		29696		244
N 352B		29696		244
N 271		29696		244
N 332		29696		244
N 464		29696		137
N 66		29696		137
N 407		29696		137
N 482		29696		137
N 89		29696		137
N 55		29712		476
N 67	120	191		244
N 89	120	191		137
N 113	120	191		137
N 55	120	191		476
N 577		14323		53
N 239		29714		53
N 37		29715		21
N ND		11527	.	
N ND		11527	.	
N 50		29675		21
N 424		29774		21
N 273		29775		21
N 247		29776		21
N 462		29777		21
N 50		29778		21
N 577		14323		
N 577		14323		
N 577		14323		
N 71	18	29623		53
N 105	19	29726		291
N 577		14323		
N 577		14323		
N 577		14323		
N 431		29679		Not in SITVIT
N 465		29721		42
N 110	18	29722		53



IS*6110* RFLP	ID	Family	IS*6110*	Spoligotype pattern	SIT
	N 110	18	29722	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	53
	N 474	18	29723	■■■■■■■■■□■■□□■■■■■■■■■■■■□□□□■■■■□■■■	Not in SITVIT
	N 60	18	29724	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	42
	N 249	18	29725	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	42
	N 476	18	29705	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	53
	N 423	18	29678	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	53
	N 53		29767	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	73
	N 308		29768	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	73
	N 365		29766	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	73
	N 176		29765	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	52
	N 339		29765	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	52
	N 63		29765	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	52
	N 62		29769	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	52
	N 38	29	29676	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	1
	N 59	29	29719	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	1
	N 43	29	29720	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	1
	N 25	29	29718	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	1
	N 26	29	29718	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	1
	N	29	208		
	N 176		29691	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	52
	N 181		29704	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	42
	N 279		29704	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■■	42
	N 478	34	29699	□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■■■■■■■■	955
	N 75		29685	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 119		29685	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 56		29685	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 199		29686	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 139		29687	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	811
	N 70		29688	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 192		29689	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 76		29690	■■■■■■■■■□■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	Not in SITVIT
	N 244	28	29716	■■■□□□■■□■■■■■■■■■□□□□□□□□□□□□□□□■■■■■■■■	21
	N 101		29692	■■□■■■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	20
	N 3		29693	■□□□□□□■■■■■■■■■■■■■■□□□□■■■■■■■■□□□□■■■■■■■■	961
	N 41		29694	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■	53
	N 258		29727	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 331		29728	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 396		29729	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 306		29730	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 240		29682	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 369		29682	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 370		29682	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 375		29731	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 262		29732	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 57		29733	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59
	N 427		29734	■■■■■■■■■□□□■■■■■■□□■■□□■■□□■■■□■■■	184
	N 64		29734	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 357		29735	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 346		29736	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	815
	N 282		29737	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■□■	2385
	N 434		29738	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	811
	N 312		29739	■■■□□□■■■■■■■■■■■■□□□□■■□□■■□□■■■■■■■■	2488
	N 322		29740	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□	244
	N 284		29741	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■□□■■■■■■	59

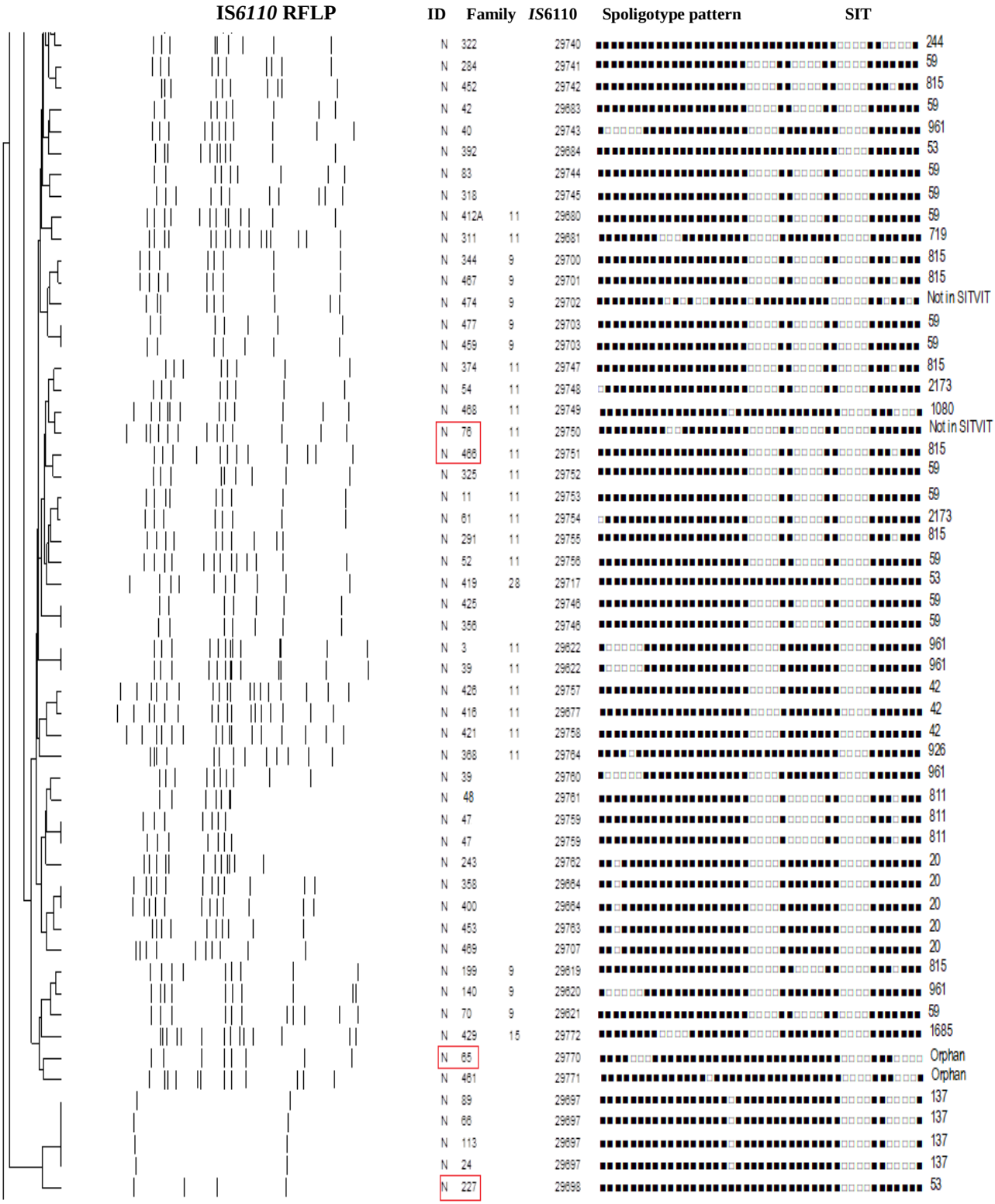


Figure 4.4: **IS*6110*-RFLP dendogram for *M. tuberculosis* strains showing strain relatedness.** Sample IDs begin with N (starting with N41). The external marker MTB14323 (N 577) was included as reference. Highlighted in red are strains with no resistance-conferring mutations identified (further discussed in section 6.5). Strains were assigned IS*6110* cluster numbers based on the positions and copy numbers of IS*6110* element, and strains were further assigned to an existing family that shared identical IS*6110* patterns. Cluster numbers without an existing family on Gelcompar were left blank in the family assignment.

4.5 Comparison of RFLP- IS*6110* clusters and spoligotyping clusters

Comparison of IS*6110* clusters revealed that 9 out of 11 clusters were in agreement with spoligotyping results (Table 4.3). Strains belonging to low copy clades (LCC) were poorly differentiated by IS*6110*-RFLP (Figure 4.4 and Table 4.3). For instance, strains belonging to family 140 (assigned according to an existing family on the Stellenbosch University Division of Molecular Biology Gelcompar database) and cluster number 29696 were clustered according to IS*6110*-RFLP, and were better differentiated by spoligotyping (Figure 4.4), confirming the low discriminatory power of IS*6110*-RFLP in differentiating LCCs and the superiority of spoligotyping in differentiating these clades (6). From the IS*6110*-RFLP clustered strains, 14 out of 30 (47%) of patients were residents of Ndola district, which would be suggestive of a higher transmission rate in this district, however the majority of strains in the study were from Ndola district. In total 57% of patients with clustered strains had failed treatment, either retreatment or first treatment, while 13% of cases were relapses (Table 4.3).

Compared to spoligotype clusters (18 clusters), IS*6110*-RFLP identified fewer clusters demonstrating the higher resolution of IS*6110*-RFLP. This illustrates the importance of combining spoligotyping with a higher discriminatory molecular technique for samples originating from this population.

Table 4.3: Comparison of RFLP-IS*6110* clusters with spoligotyping clades and characteristics of patients with clustered strains.

Sample ID	IS*6110*	SIT number	TB history	Year of diagnosis	Patient address (district/province)
N341	29695	1926	After failure of 1st treatment	2016	Ndola/CB
N359	29695	1926	After failure of re- treatment	2016	Samfya/LP
N422	29695	1926	After failure of re- treatment	2014	unknown/LP
N307	140	Not in SITVIT	After failure of 1st treatment	2016	Ndola/CB
N225	140	1926	Relapse	2016	Ndola/CB
N3A	29622	961	After failure of 1st treatment	2015	Kitwe/CB
N39	29622	961	Relapse	2015	Ndola/CB
N464	29696	137	New	2017	Kitwe/CB
N66	29696	137	Unknown	2016	Ndola/CB
N407	29696	137	New	2016	Ndola/CB
N482	29696	137	After failure of re- treatment	2016	Kitwe/CB
N89	29696	137	After failure of 1st treatment	2014	Ndola/CB
N338	29696	52	After failure of 1st treatment	2016	Ndola/CB
N430	29696	Not in SITVIT	After failure of re- treatment	2016	Chililabombwe/CB
N352	29696	244	New	2016	Ndola/CB
N332	29696	244	After failure of 1st treatment	2016	Mpongwe/CB
N176	29765	52	Relapse	2016	Ndola/CB
N339	29765	52	New	2016	Ndola/CB
N63	29765	52	Relapse	2016	Kitwe/CB
N75	29685	815	Relapse	2016	Kitwe/CB
N119	29685	815	Unknown	2014	Luanshya/CB
N56	29685	815	After failure of 1st treatment	2015	Masaiti/CB
N369	29682	815	After failure of 1st treatment	2015	Kitwe/CB
N370	29682	815	After failure of re- treatment	2016	Chililabombwe/CB
N477	29703	59	After failure of re- treatment	2017	Ndola/CB
N459	29703	59	After failure of re- treatment	2016	Mpika/MP
N425	29746	59	New	2016	Ndola/CB
N356	29746	59	After failure of re- treatment	2016	Samfya/LP
N358	29664	20	After failure of re- treatment	2016	Samfya/LP
N400	29664	20	After failure of 1st treatment	2016	Ndola/CB

Abbreviations: CB, Copperbelt province; ID, identification; LP, Luapula province; MP, Muchinga province, SIT, spoligo international type; TB, tuberculosis.

4.6 Conclusion

Over half of the cases included in the study were either retreatment failure cases (WHO category 2) (25%), or first line treatment failures (26%) (Table 4.1). These findings point to an inefficient TB control program. There is a need to implement routine phenotypic DST or nucleic acid tests such as Xpert assay or the line probe assay for all confirmed TB cases for early case detection of drug resistance and to enable appropriate treatment. These findings

further support WHO recommendations of discontinuing with the category 2 retreatment regimen in settings with the capacity to perform routine DST, it has been demonstrated that adding one drug to a failing regimen promotes selection of resistance (7). Twenty four percent of patients were categorised as “new”, that is patients that have not received prior TB treatment for longer than one month. Together with clustering of strains, this implies that drug resistant TB is being transmitted in the study population. The gaps in demographic and clinical data such as HIV status, and the inadequate specimen banking system at TB referral level is of great concern as analysis of these data and isolates could lead to misrepresentation of the transmission dynamics and evolutionary events of drug resistant TB in the study population and Zambia as a whole (8).

A high level of genotype diversity of drug resistant *M. tuberculosis* clinical strains has been observed within parts of Zambia. This suggests that multiple strains are responsible for drug resistant TB, and that acquisition is at least partially driving the burden of drug resistant TB in Zambia. It is however worth noting that drug resistant TB strains were possibly missed during sample collection due to the poor case detection rate reported for MDR/RR-TB in Zambia. Further, identical fingerprinting patterns have been observed amongst these isolates suggesting that there is a level of recent transmission of drug resistant TB, 84% and 28% of isolates share identical patterns according to spoligotyping and IS6110-RFLP, respectively.

A previous molecular study which focused largely on drug susceptible TB strains in Ndola district revealed that the predominant genotypes belonged to LAM, (specifically LAM11_ZWE), T1, X2, and CAS (2). The current study demonstrates that these genotypes are further driving drug resistant TB in Ndola district and the surrounding region. This implies that the current TB control program is not efficient in preventing the emergence and transmission of drug resistant TB in the study population.

There are however genotypes that were previously not described in this population group such as the lineage 2 Beijing genotype (which has been associated with MDR- and XDR-TB outbreaks across the world) and the non-Beijing SIT 955 (3, 9). The presence of Beijing genotypes in the study population suggests that migration from high Beijing prevalent areas, for example cross border movement between South Africa and Zimbabwe and Zambia, could possibly be playing a role in transmission of drug resistant TB strains. A further explanation for the identification of lineage 2 genotypes in the study population could be the high influx of Chinese workforce involved in road and other construction works in Zambia and the surrounding region, with an estimate of up to 18,000 Chinese in Zambia in 2015 (10). The LAM11_ZWE, T and X clades, on the other hand, have been demonstrated to be widespread in the sampled provinces, in the current study and previously (1, 11). These genotypes have further been described to be prevalent in neighbouring countries including Tanzania and Zimbabwe (11). There is however need to further evaluate the degree of strain relatedness amongst clustered strains which have been observed in the current study, and in so doing determine the extent of transmission in the study population and the surrounding region.

4.7 References

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Chapter 5: Molecular analysis of transmission of drug resistant tuberculosis from the Copperbelt province and Northern regions of Zambia

5.1 Introduction

In this chapter, whole genome sequence (WGS) analysis was applied to further investigate the degree of strain relatedness amongst clustered strains. Whole genome sequencing has been demonstrated to have a higher discriminatory power compared to traditional genotyping methods in molecular epidemiological studies of *Mycobacterium tuberculosis* (*M.tb*) (1). WGS has been used to classify outbreaks which conventional genotyping methods are unlikely to discriminate accurately as these techniques mostly probe less than 1% of the genome (1). It has further been demonstrated to be useful in understanding evolutionary events in *M.tb* to identify drug resistance through analysis of known drug resistance-conferring mutations (2, 3). The use of WGS in *M.tb* molecular investigations is rapidly becoming available across the world due to decreased cost, increased throughput and advancement of the technology. However, the lack of expertise and financial constraints mean that is has not been fully applied in low to middle income countries such as Zambia.

The usefulness of WGS for understanding transmission dynamics and as a rapid TB screening tool has been demonstrated in two studies in Zambia (4, 5). In one study, WGS data was used to differentiate relapses and re-infection through single nucleotide variant (SNV) analysis; this study identified low SNV differences in serial patient samples which was suggestive of relapse (4). In another study, WGS analysis was used to screen for *M.tb* in paediatric pneumonia patients from University Teaching Hospital (UTH) in Lusaka, highlighting the diagnostic value of WGS (5). To date, the current study provides the most extensive use of WGS to describe strain relatedness and drug resistance-conferring mutations in *M.tb* clinical isolates from Zambia.

5.2 Analysis of whole genome sequencing data

WGS was performed on 91 out of 170 clinical isolates (54%) with purified DNA (section 3.3). Genomic DNA was sequenced either at the Centers for Disease Control and Prevention (CDC) Atlanta Georgia (90 strains) or Beijing Genomics Institute (BGI) Tech Solutions Hong Kong (12 strains) using the Illumina HiSeq next generation sequencing (NGS) platform as per routine. For validation and quality control (QC) purposes 11 strains were sequenced both at CDC and BGI. Targeted Sanger sequencing was done on the remainder of strains to identify resistance-conferring mutations to first and second line drugs (described in chapter 6). Five samples failed initial QC due to low depth of coverage (less than 20x). Therefore WGS data for 86 (51%) strains were analysed and are described in this chapter.

The FASTQ data files generated from WGS were analysed using the customised in-house WGS analysis pipeline, Universal Sequence Analysis Pipeline (USAP), as described in section 3.7. The resulting high confidence variant files generated from USAP were used here to describe the transmission and evolutionary events in the genomes of drug resistant TB isolates diagnosed at the TDRC TB reference laboratory in Ndola district, Zambia. Thereby addressing Objectives 2, 3 and 4 (section 1.5.3).

5.3 Summary of whole genome sequencing data

Eighty-six clinical strains showed coverage of more than 20x therefore passing WGS QC (Table 5.1). The depth of coverage for the sequenced genomes ranged from 24x to 758x and for these isolates, over 96% of the sequencing reads aligned to the *M.tb* H37Rv (GenBank NC000962.2) reference genome.

The USAP pipeline includes a feature which uses lineage-defining SNVs to assign lineages based on WGS data (6). Similar to spoligotyping and RFLP-IS*6110* findings (supplementary

Table 1, https://tinyurl.com/Zambia-DRTB), the majority of strains belonged to lineage 4.3 (LAM; 42 strains). Other strains belonged to lineage 4.9 (H37Rv like; 18 strains), 4.1.1.3 (Haarlem; 6 strains), 4.6.1 (Uganda; 5 strains), lineage 2.2 (Beijing; 5 strains), lineage 3 (CAS; 2 strains), lineage 4.3.2 (LAM3; 2 strains), and an undefined sub-lineage 4 (6 strains) (Table 5.1).

A further feature of the USAP pipeline is an automated drug resistance classification, based on mutations known to confer drug resistance; application of this tool revealed that 16 of the sequenced strains were wild type (WT) in known drug resistance conferring genes. These were retained in the subsequent analyses for comparative purposes. A total of 58 MDR-TB strains were identified amongst the sequenced strains and a further 12 drug resistant strains (either mono- or poly-resistant) were identified.

Table 5.1: Summary of WGS data for 86 clinical strains sequenced at the CDC and BGI Tech solutions.

Sample ID	Date of Culture	Average Coverage	Mapped reads	Lineage	Family	DR classification	Spolpred octal code
N003	2015	122	99.04%	4.3	Euro-American (LAM)	MDR	407777607760771
N011	2015	60	98.68%	4.3	Euro-American (LAM)	DS	675556606060371
N024	2015	50	98.57%	4.9	Euro-American H37Rv like	MDR	337756677720601
N025	2016	55	98.15%	2.2	East-Asian (Beijing)	MDR	000000000003551
N026	2016	58	99.08%	2.2	East-Asian (Beijing)	MDR	000000000003751
N037	2015	67	97.97%	4.9	Euro-American H37Rv like	DR	777375777640771
N038	2015	70	98.25%	2.2	East-Asian (Beijing)	MDR	000000000003731
N039	2015	50	98.94%	4.3	Euro-American (LAM)	MDR	406777607660651
N040	2015	59	99.02%	4.3	Euro-American (LAM)	MDR	407737407620711
N041	2015	59	99.09%	4.9	H37Rv like	DS	737377137420671
N042	2015	50	98.94%	4.3	Euro-American (LAM)	MDR	633677406040771
N043	2015	62	98.61%	2.2	East-Asian (Beijing)	MDR	000000000003251
N050	2014	65	99.62%	3	East-African-Indian (CAS)	MDR	301335400001571
N052	2014	82	99.25%	4.3	Euro-American (LAM)	MDR	323756606060751
N053	2015	54	99.16%	4.6.1	Euro-American (Uganda)	MDR	452307637720531
N054	2014	86	99.06%	4.3	Euro-American (LAM)	MDR	377777406060771
N055	2014	67	99.02%	4.9	Euro-American H37Rv like	DR	477772777760601
N056	2015	60	99.29%	4.3	Euro-American (LAM)	MDR	777767606060131
N057	2015	79	99.04%	4.3	Euro-American (LAM)	DS	777337406060771
N059	2015	56	98.10%	2.2	East-Asian (Beijing)	DR	000000000003451
N060	2016	57	99.46%	4.9	Euro-American H37Rv like	MDR	673572203460751
N061	2016	62	98.72%	4.3	Euro-American (LAM)	MDR	377755606060570
N062	2014	61	99.10%	4.6.1	Euro-American (Uganda)	MDR	676561737640731
N064	2016	101	96.02%	4.3	Euro-American (LAM)	MDR	777777606060331
N065	2016	76	97.27%	4.9	Euro-American H37Rv like	DS	774000017640731
N066	2016	42	99.12%	4.9	Euro-American H37Rv like	MDR	725102416720201
N067	2014	61	98.90%	4.9	Euro-American H37Rv like	MDR	763766776760601
N070	2016	74	98.96%	4.3	Euro-American (LAM)	MDR	777773606060651
N071	2016	63	99.37%	4	Euro-American	MDR	777773677760770

				(UNSP)	H37Rv like		
N074	2016	89	98.54%	4.1.1.3	Euro-American Haarlem	DR	777776737740701
N075	2016	84	99.29%	4.3	Euro-American (LAM)	MDR	777777606060331
N076	2016	71	99.18%	4.3	Euro-American (LAM)	DS	733177606000771
N083	2016	24	97.48%	4.3	Euro-American (LAM)	MDR	401200204000000
N089	2014	103	98.52%	4.9	Euro-American H37Rv like	DR	777776777760401
N101	2016	39	98.77%	4.3	Euro-American (LAM)	MDR	674573406500671
N105	2016	46	99.18%	4 (UNSP)	Euro-American H37Rv like	MDR	527125214500150
N110	2016	66	98.23%	4 (UNSP)	Euro-American H37Rv like	MDR	761737737560571
N113	2016	87	98.85%	4.9	Euro-American H37Rv like	DR	577776577760601
N119	2014	110	98.68%	4.3	Euro-American (LAM)	MDR	777773606060731
N133	2016	60	98.53%	4.9	Euro-American H37Rv like	DR	767572657760401
N139	2016	82	99.21%	4.3	Euro-American (LAM)	MDR	777777204060531
N140	2016	47	99.18%	4.3	Euro-American (LAM)	MDR	633705004060701
N176	2016	63	99.16%	4.6.1	Euro-American (Uganda)	MDR	777635377620710
N192	2016	96	98.91%	4.3	Euro-American (LAM)	MDR	777777606060771
N199	2016	79	99.08%	4.3	Euro-American (LAM)	MDR	737777606060731
N227	2016	50	98.70%	4.1.1.3	Euro-American Haarlem	DS	737362616000001
N240	2016	58	99.12%	4.3	Euro-American (LAM)	DS	753573206040571
N262	2016	40	99.06%	4.3	Euro-American (LAM)	DS	377304406020530
N271	2016	44	98.98%	4.9	Euro-American H37Rv like	DS	573702615400600
N283	2016	45	99.00%	4.9	Euro-American H37Rv like	DS	573426637240000
N292	2016	38	79.82%	3	East-African-Indian (CAS)	MDR	702322400001001
N296	2016	36	99.26%	4.3	Euro-American (LAM)	DS	226171406060111
N311	2016	50	98.85%	4.3.2	Euro-American (LAM3)	DR	132005407500311
N332	2016	78	98.52%	4.9	Euro-American H37Rv like	MDR	777776737740601
N338	2016	67	98.37%	4.9	Euro-American H37Rv like	MDR	577676737500601
N339	2016	53	99.15%	4.6.1	Euro-American (Uganda)	MDR	657417677700321
N341	2016	53	98.31%	4.1.1.3	Euro-American Haarlem	MDR	736716375300401
N346	2016	56	99.76%	4.3	Euro-American (LAM)	DS	656217206060311
N352	2016	56	98.66%	4.9	Euro-American	MDR	077636757760601

					H37Rv like		
N354	2016	62	96.33%	4.9	Euro-American H37Rv like	MDR	467752777560600
N356	2016	59	99.19%	4.3	Euro-American (LAM)	MDR	573135606040551
N357	2016	90	98.88%	4.3	Euro-American (LAM)	MDR	777777606060731
N358	2016	48	98.61%	4.3	Euro-American (LAM)	MDR	061715407620550
N359	2016	48	98.13%	4.1.1.3	Euro-American Haarlem	MDR	071416036400001
N360	2016	47	99.55%	4 (UNSP)	Euro-American H37Rv like	MDR	633313637420770
N365	2016	51	88.56%	4.6.1	Euro-American (Uganda)	DR	723737456060711
N368	2016	62	98.66%	4.3	Euro-American (LAM)	DR	777753603540071
N369	2016	68	98.86%	4.3	Euro-American (LAM)	MDR	774637606060731
N370	2016	56	98.81%	4.3	Euro-American (LAM)	MDR	765373406060730
N373	2016	59	99.21%	4.3	Euro-American (LAM)	DR	776007606040610
N374	2016	39	99.26%	4.3	Euro-American (LAM)	MDR	775111006040431
N375	2016	50	99.37%	4.3	Euro-American (LAM)	MDR	761037406040531
N392	2016	51	99.28%	4.3	Euro-American (LAM)	MDR	675543206060551
N400	2016	45	99.17%	4.3	Euro-American (LAM)	MDR	657340607420540
N412A	2015	56	98.94%	4.3	Euro-American (LAM)	DS	236572406040741
N416	2016	66	98.75	4.3	Euro-American (LAM)	MDR	757747607160330
N420	2016	49	98.94%	4.9	Euro-American H37Rv like	MDR	175742117040001
N422	2016	52	98.78%	4.1.1.3	Euro-American Haarlem	MDR	473156517320101
N423	2016	56	98.99%	4 (UNSP)	Euro-American H37Rv like	MDR	575565716620111
N425	2016	63	90.90%	4.3	Euro-American (LAM)	MDR	737633606040740
N426	2016	67	98.60%	4.3	Euro-American (LAM)	MDR	677373407640671
N427	2016	758	97.93%	4.3	Euro-American (LAM)	DS	777417606060731
N429	2017	67	98.73%	4.3.2	Euro-American (LAM3)	DR	426137007740770
N430	2016	92	98.85%	4.3	Euro-American (LAM)	MDR	777777606060531
N431	2016	79	99.05%	4 (UNSP)	Euro-American H37Rv like	DS	777771777760761
N446	2014	88	98.63%	4.1.1.3	Euro-American Haarlem	DS	777576777720771

Abbreviations: DR, drug resistant; DS, drug susceptible; ID, identification; MDR, multidrug resistant; UNSP, unspecified.

5.4 Validation of whole genome sequencing data

Eleven isolates, from the same DNA stock, were independently resequenced for validation purposes at the CDC and BGI Tech solutions. All strains passed QC with an average depth of coverage of over 38x and more than 98% of the reads aligned to the *M.tb* H37Rv reference genome (Table 5.2). USAP analysis of the resulting independent sequences yielded corresponding SNV results. Crucially, there were no unique SNVs identified between duplicate samples sequenced at the CDC and BGI.

Table 5.2: Comparison of sequencing data for 11 strains resequenced at the CDC and BGI Tech solutions.

Patient ID	Average coverage		Mapped read (%)		Lineage	
	CDC	BGI	CDC	BGI	CDC	BGI
N038	70	89	98.57	89	2.2	2.2
N043	70	103	98.61	97.65	2.2	2.2
N059	56	104	98.80	95.20	2.2	2.2
N369	68	105	98.86	98.11	4.3	4.3
N370	56	116	98.81	99.06	4.3	4.3
N373	59	100	99.21	96.45	4.3	4.3
N374	39	102	99.26	95.03	4.3	4.3
N375	50	101	99.37	96.40	4.3	4.3
N392	51	101	99.28	96.54	4.3	4.3
N412A	56	115	98.94	98.93	4.3	4.3
N430	92	115	98.85	98.90	4.3	4.3

Abbreviations: BGI, Beijing Genomics Institute; CDC, Centers for Disease Control and Prevention; ID, identification.

5.5 Phylogenetic analysis of whole genomes

To determine the phylogenetic relationship between the 86 sequenced isolates, high confidence variable coding and non-coding SNV sites were used to construct a maximum likelihood phylogeny with Randomized Axelerated Maximum Likelihood (RaxML), and the resulting phylogeny was viewed and edited in FigTree (7). Previously described *M.tb* isolates, representative of lineages 2, 3 and 4 of the *M.tb* complex, were included in the phylogenetic tree for reference purposes (appendix 2). Strains without known resistance-conferring mutations (as identified by USAP) were included for comparative purposes. In agreement

with the lineage assignments described above, the resulting tree shows that 42 (92%) of the sequenced isolates belong to lineage 4, predominantly LAM, while other genotypes belong to lineage 2 and 3 (Figure 5.1). There were no isolates from lineages 1, 5, 6 and 7 identified in this study population.

Similar to spoligotyping and IS*6110*-RFLP findings, WGS suggested that there is a high degree of genetic diversity amongst drug resistant TB strains from this study population. Furthermore, initial phylogenetic analysis (Figure 5.1) demonstrates a level of clustering. To determine how closely related strains were, pairwise comparisons were made (described further in section 5.6).

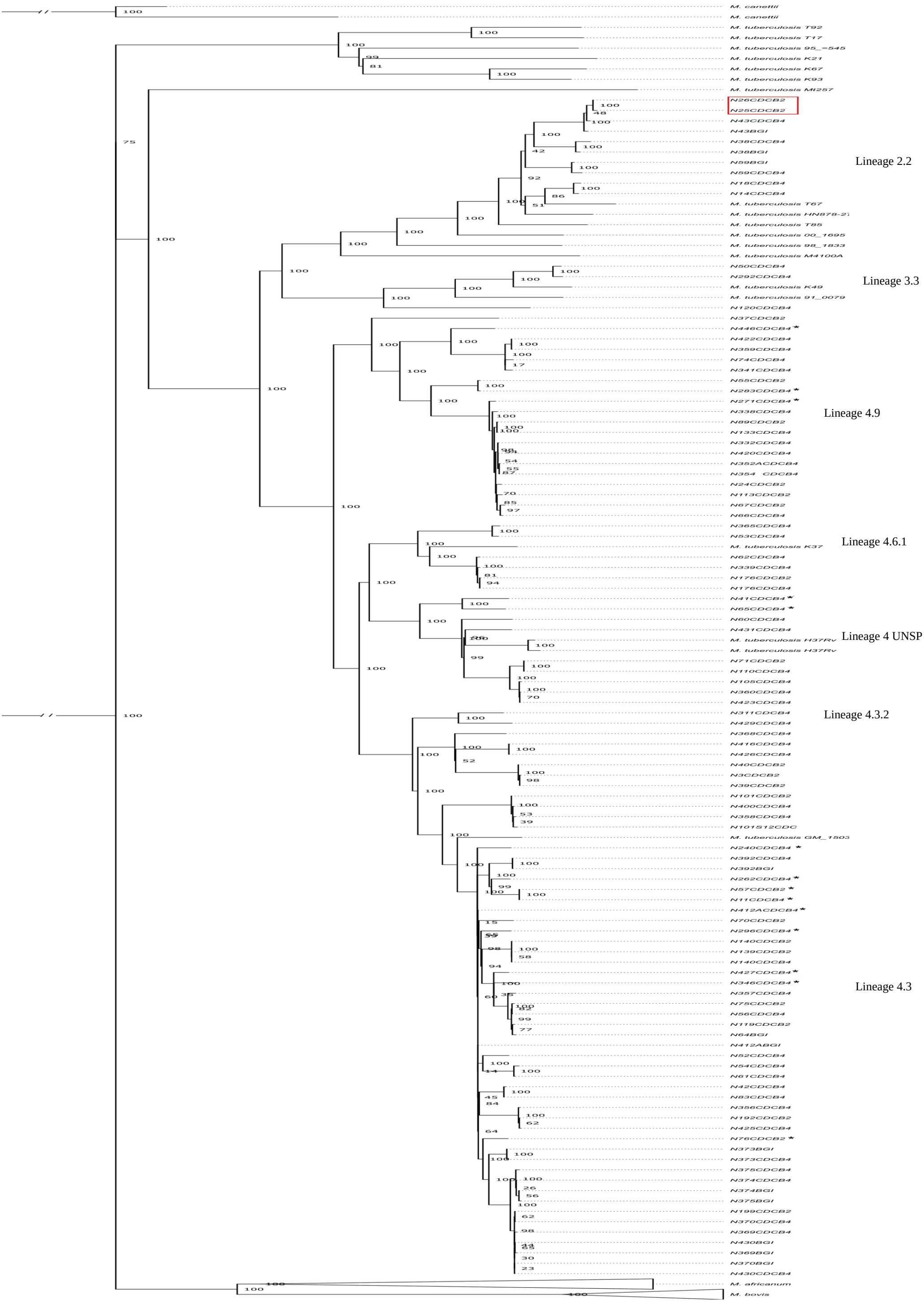

Figure 5.1: Genome-wide single nucleotide variant (SNV) based phylogenetic analysis of 86 *M.tb* clinical isolates collected from the TDRC TB reference laboratory. To determine the phylogenetic relationship between the sequenced isolates, high confidence variable coding and non-coding SNV sites were used with Randomized Axelerated Maximum Likelihood (RaxML) (7). The replicate tree percentage for clustered taxa in the bootstrap test (1000 replicates) is shown next to the nodes. Sample codes from this study begin with N followed by the isolate ID (starting with N26). Published *M.tb* genomes (Appendix 2; *M. canettii*, *M. africanum*, *M. bovis*, *M. tuberculosis* T92, T17, 95_=545, K21, K67, Mt257, T67, HN878-27, T85, 00-1695, 98_1833, M4100A, K49, 91_0079, K37, H37Rv, GM_1503) are included for reference purposes. The * after the sample ID represent strains genotypically identified as having no resistance conferring mutations. Serial isolates from the same patient (N025 and N026) are highlighted in red. CDC after the sample ID denotes samples sequenced at the CDC while BGI denotes samples sequenced at BGI. Abbreviations: BGI, Beijing Genomics Institute; CDC, Centers for Disease Control and Prevention; ID, identification; TB, tuberculosis; TDRC, Tropical Diseases Research Centre; UNSP, unspecified.

5.6 Single nucleotide variant (SNV) analysis of clustered strains

Following alignment to the *M.tb* H37Rv reference genome, an annotated variant file for the individual strains (86 strains) was generated for SNVs and used for pairwise comparison, to identify unique nucleotide variances between strains of the same lineage. Filters were applied to identity only SNVs identified in all 3 alignment tools by Genome Analysis Tool Kit (GATK). Low quality variants (depth of coverage less than 20x) and variants identified in repetitive regions, including the *pe/ppe* and *pe_pgrs* genes, were excluded.

To assess strain relatedness, phylogenetic tree-based clusters (hereafter referred to as “clusters”) were defined as strains within the same terminal branches (Figure 5.1). The number of unique SNVs between clustered strains was determined through pairwise analysis of clustered strains. Upon pairwise comparison of strains sharing the same terminal branches, genomic clusters were defined as strains separated by 12 or fewer SNVs which is suggestive of recent transmission, as previously described (8, 9).

5.6.1 Lineage 2.2 cluster analysis

In total there were 5 sequenced strains in lineage 2.2 (N025, N026, N038, N43and N059), Two strains however were serial isolates collected from the same patient (N025 and N026, at diagnosis and follow up, respectively) and as expected, the two isolates had low SNV differences between them (Table 5.3), one SNV in N025 (a deletion in Rv3479). Cluster analysis of the remaining lineage 2.2 strains identified a genomic cluster of two strains (N026 and N43). Analysis of the two clustered strains (N026 and N43) revealed low SNV differences (two unique non-synonymous SNVs) relative to *M.tb* H37Rv (Table 5.3). Both SNVs were found in N043, in Rv1747 (presumed to be involved in cell processes) and Rv2630 (a hypothetical protein of unknown function) (10). The two strains shared identical drug resistance-conferring mutations in *rpoB* (codon position 531, TCG-TTG), *katG* (codon position 315, AGC-ACC), *embB* (codon position 497, CAG-CGG; 1082, ACC-GCC) and *rpsL* (codon position 43, AAG-AGG), further validating how closely related the two strains were. The remaining two strains from lineage 2.2 (N038 and N059) were not clustered (Figure 5.1)

Patients N026 and N043 were diagnosed in 2016 and 2015, respectively, and both were receiving second line treatment at the time of data collection in 2016. Whereas spoligotyping data provided an indication of relatedness between these strains (both strains were assigned as belonging to the Beijing clade, SIT 1), IS*6110*-RFLP showed a level of differentiation; both strains were assigned to family 29, but strains had different IS*6110* cluster numbers (section 4.4). WGS provides a level of detail that supports very recent transmission between these patients. The low SNV differences identified in these two clustered strains and the similarities in drug resistance-conferring mutations is highly suggestive that lineage 2 strains are being transmitted in the study population. Both patients were residents of the Copperbelt

province, although in different districts, namely Mufulira district (N043) and Ndola district (N026). This further implies that drug resistant TB strains are possibly wide spread across parts of Zambia; alternatively nosocomial transmission is occurring during quarantine at the NTH MDR-TB ward. However, there is need for epidemiological data linking the patients to confidently draw any conclusions on transmission.

5.6.2 Lineage 3.3 cluster analysis

WGS data was obtained for two lineage 3.3 strains (N050 and N292; Figure 5.1); these strains had 30 SNVs differences between them (Table 5.3). Although they had the same resistance-conferring mutations in *katG* (codon position 315, AGC-ACC) (supplementary Table 1, https://tinyurl.com/Zambia-DRTB), the strains had additional, differing drug resistance-conferring mutations. For strain N050 these added mutations occurred in *rpoB* (codon position 516, GAC-GTC), *rpsL* (codon position 43, AAG-AGG) and *embB* (codon position 306, ATG-ATA). Strain N292 had added mutations in *rpoB* (codon position 526, CAC-GAC) and *embB* (codon position 306, ATG-TTG). The patients were admitted to the NTH MDR-TB ward in 2014 (N050) and in 2016 (N292). Treatment was completed in one (N050) while patient N292 died while on second line treatment. Patient N292 was a resident of Kitwe district on the Copperbelt province while patient N050 was a resident of Mwinilunga district in North-western province (Supplementary Table 1). Together, the available data suggests independent transmission events, with strains possibly evolving independently. However, more complete sampling would be required to accurately define the transmission and evolution of these strains.

5.6.3 Lineage 4 cluster analysis

There were two clusters of drug resistant strains belonging to lineage 4 (unspecified sub-lineage) which had two strains (N071 and N110) and three strains (N105, N423 and N360) per cluster (Figure 5.1). Two clustered strains (N041 and N065) were drug susceptible according to WGS and were therefore not included in the analysis. All clustered drug resistant strains in this unclassified sub-lineage shared the same mutations in *rpoB* (codon position 531, TCG-TTG), *katG* (codon position 315, AGC-ACC), *pncA* (codon position 35, CTG-CGG).

The two strains from the same cluster (N71 and N110) had added mutations in *embB* (codon position 306, ATG-GTG; codon position 406, GGC-GCC) (Supplementary Table 1, https://tinyurl.com/Zambia-DRTB). These strains had 3 SNV differences between them, suggestive of very recent transmission events (Table 5.3). The nucleotide variant identified in N110 was in Rv1165 (probable GTP binding protein) and two SNVs were identified in N071 in Rv0358 and Rv0486 (10). Both patients were from the Copperbelt province, patient N071 from Ndola district and patient N110 from Mufulira district were diagnosed in 2016. One patient was admitted to the NTH MDR-TB ward while the other (N110) was not admitted at the time of data collection, raising concerns over MDR-TB patient enrolment to treatment. The low SNV difference identified between the two patient strains (3 SNVs) and the similarities in resistance conferring mutations is suggestive of recent transmission and implies that the sub-lineage is being transmitted in the study population.

The second cluster consisted of 3 strains N105, N423 and N360 with added mutations in *rpoB* (codon 633, CGC-TGC) and one strain (N360) had a mutation in *embB* (codon position 306, ATG-GTG). Nucleotide variance analysis revealed high SNV differences, compared to the genomic cluster described above, ranging from 22 to 43 SNV differences between strains

(Table 5.3). This data is suggestive of isolates evolving independently and is supported by spoligotyping and the IS*6110*-RFLP data which demonstrated that strains were not clustered (section 4.4). According to spoligotyping, all 5 sequenced strains from this lineage belonged to the T1 clade 3 strains belonged to the SIT 53 (N071, N110 and N423) while the other two strains belonged to SIT 926 (N360) and SIT 291 (N105) (section 4.4). IS*6110*-RFLP data was available for 4 strains out of the 5 with two families identified, family 18 (N071 and N423) and family 19 (N105 and N110), none of the strains were clustered according to IS*6110*-RFLP (section 4.4).

The data provided here demonstrates the presence of one genomic cluster in this lineage (N071 and N110), implying that drug resistant TB strains from this lineage are being transmitted in Zambia.

5.6.4 Lineage 4.3

In the current study, lineage 4.3 has been demonstrated to be the predominant genotype associated with drug resistant TB. From the sequenced strains, 42 (49%) belonged to lineage 4.3, and 11 clusters with 2 strains per cluster were identified and analysed further for strain relatedness (Figure 5.1).

Five genomic clusters were identified from the 11 clustered strains (Table 5.3), that is strains with 12 or fewer SNV differences between them (8, 9). The most closely related strains had 4 SNV differences between them N003 (3 SNVs) and N039 (1 SNV) (Table 5.3). Strains N003 and N039 shared similar resistance-conferring mutations in *rpoB* (codon position 526, CAC-AAC), *katG* (codon position 315, AGC-ACC) and *kasA* (codon position 269, GGT-AGT) (Supplementary Table 1, https://tinyurl.com/Zambia-DRTB). Both patients were diagnosed in 2015, one patient (N039) was admitted to the NTH MDR-TB ward while the second was not

admitted, however the treatment status for both patients is unknown. Both patients were from the Copperbelt province, Ndola district (N039) and Kitwe district (N003). Spoligotyping assigned both strains to LAM1, SIT 961, and IS*6110*-RFLP assigned both strains to family 14, indicating strain relatedness (section 4.4).

Strains N042 (2 SNVs) and N083 (3 SNVs) had low nucleotide variance differences between them and it was further demonstrated that strains shared similar mutations in *rpoB* (codon position 531, TCG-TTG) and *katG* (codon position 315, AGC-ACC). Both patients were admitted to the NTH MDR-TB ward in 2015 (N042) and 2016 (N083), treatment was ongoing for patient N083 at the time of data collection while patient N042 was lost to follow up. One patient was from Kitwe district on the Copperbelt province while the other was from Mpulungu district in Northern Province.

In the third genomic cluster strains had 7 SNV differences between them, N358 (4 SNVs) and N400 (3 SNVs), both patients had similar resistance-conferring mutations in *rpoB* (codon position 531, TCG-TTG) and *katG* (codon position 315, AGC-ACC). One patient (N358) was admitted to the NTH MDR-TB ward and was receiving second line treatment while the second patient (N400) was not admitted at the time of data collection. Patient N358 was from Samfya district in Luapula province while patient N400 was from Ndola district.

Strains N056 (2 SNVs) and N075 (7 SNVs) had 9 SNV differences between them and shared similar resistance-conferring mutations in *rpoB* (codon position 531, TCG-TTG), *katG* (codon position 315, AGC-ACC), *embB* (codon position 306, ATG-GTG) and in *pncA* (codon 58, TTC-TCC). One patient (N075) was defined as a relapse after completing second line treatment, while the second (N056) was not admitted to the NTH MDR-TB ward following diagnosis in 2015. Both patients were from the Copperbelt province, Kitwe district (N075) and Masaiti district (N056).

There were 12 SNV differences between strains N425 (4 SNVs) and N192 (8 SNVs), suggestive of recent transmission. These strains shared similar resistance-conferring mutations in *rpoB* (codon position 511, CTG-CGG; 516, GAC-TAC), *katG* (codon position 315, AGC-ACC), *embB* (codon position 306, ATG-ATA) and in *pncA* (codon 119, TGG-TGT). Both patients were admitted to the NTH MDR-TB ward in 2016, one patient (N192) was receiving treatment at the time of data collection while the treatment status for the second patient was unknown (not recorded in the register). Patient N192 was from Luapula province while patient N425 was from Ndola district on the Copperbelt province. Other genomic clusters identified in lineage 4.3 belonged to N374 (2 SNVs) and N375 (3 SNVs) (further discussed in section 5.7 below).

Genomic clusters with low SNV differences have been identified in lineage 4.3 using WGS analysis, these findings are complemented by spoligotyping and IS*6110*-RFLP data which suggests a level of strain relatedness amongst the clustered strains from lineage 4.3. The genomic clusters have been demonstrated to share similar resistance-conferring mutations. These findings are suggestive of recent transmission of these drug resistant strains in the study population. It is concerning that a proportion of MDR-TB patients were not admitted for second line treatment.

5.6.5 Lineage 4.6.1 cluster analysis

In total, 4 drug resistant strains were identified as belonging to sub-lineage 4.6.1 by WGS (Figure 5.1). Analysis of 3 clustered strains (N062, N176 and N339) from lineage 4.6.1 revealed that strains had low SNVs between them (Table 5.3). Comparison of N176 with N339 revealed that there were 12 SNV differences between them, 6 unique SNVs were identified in each strain. Comparisons between N062 and N176 revealed 13 SNV differences

between the strains, and comparisons between N062 and N339 identified 14 SNV differences, suggestive of some level of recent transmission of these strains (Table 5.3). Further analysis revealed that isolates from this cluster shared similar resistance-conferring mutations in *gyrA* (codon position 80, ACC-GCC), *katG* (codon position 315, AGC-ACC), *rpoB* (codon position 526, CAC-GAC) and one strain (N062) had a further insertion of G at position 450 in the *pncA* gene. Demographic data showed that all 3 patients from this cluster were from Ndola district and had previously been admitted to the NTH MDR-TB ward, in 2016 (N176 and N339) and 2014 (N062). One patient (N176) was receiving second line treatment during data collection, one had completed treatment (N062) and the other (N339) died while on treatment.

One strain (N053) was clustered with a strain (N365) identified as drug susceptible through WGS (Figure 5.1). The drug resistant strain (N053) had resistance-conferring mutations in *gyrA* (codon position 80, ACC-GCC), *katG* (codon position 337, TAC-TGC) and a double mutation in *rpoB* (codon position 516, GAC-GTC; 526, CAC-CAG). The patient (N053) was from Samfya district in Luapula province, and was diagnosed in 2015, however the patient died while receiving second line treatment.

The data presented here demonstrates that there is a level of recent transmission amongst strains from sub-lineage 4.6.1 in the study population with low SNV differences identified. The WGS findings are similar to IS*6110*-RFLP and spoligotyping findings which assigned the strains to respective clusters (section 4.4). Spoligotyping however assigned all 3 strains (N062, N176 and N339) as belonging to T2, SIT 52, N053 was assigned to T3T2, SIT 73 (Supplementary Table 1, https://tinyurl.com/Zambia-DRTB).

5.6.6 Lineage 4.9 cluster analysis

In lineage 4.9, two strains (N352 and N420) had low SNV differences (3 unique SNVs between the two strains), characteristic of a genomic cluster (8, 9). Both patients were diagnosed with MDR-TB in 2016 and were from the Copperbelt province, N352 was from Ndola district and N420 was from Mpongwe district. One patient (N352) died while receiving second line treatment while the second had an unknown treatment status. The findings of WGS analysis for these strains are similar to findings for IS*6110*-RFLP (low copy clade with 2 bands each) and spoligotyping (both strains were assigned to the clade T1, SIT 244), indicative of recent transmission of these strains in the study population.

Other clustered strains from this lineage had between 14 and 28 SNV differences between strains, possibly suggesting ongoing transmission of these drug resistant strains of TB (Table 5.3).

Table 5.3: Single nucleotide variant analysis and characteristics of patients with clustered strains identified through WGS analysis. This analysis determines the number of unique SNVs between each strain (column 2) and *M.tb* H37Rv (column 3), with the total number of SNV differences between strains shown in column 4 (bold indicates the number of SNVs in genomic clusters; that is strains separated by 12 or fewer SNV differences).

Cluster	Strain ID	Unique SNVs/strain in cluster	Total SNVs	Lineage	Treatment status	TB history
1	N025 N026	1 0	**1**	2.2	• 2nd line on going	• 1st line re-Tx failure
	N026 N043	0 2	**2**		• 2nd line on going • 2nd line on going	• 1st line re-Tx failure • 1st line Tx failure
2	N050 N292	14 16	30	3.3	• Completed • Died	• New • Relapse
3	N071 N110	2 1	**3**	4	• Completed • Unknown	• New • Unknown
4	N105 N423	10 12	22	4	• Unknown (TO) • 2nd line ongoing	• New • 1st line re-Tx failure
	N423 N360	16 11	27		• 2nd line ongoing • 2nd line ongoing	• 1st line re-Tx failure • 1st line re-Tx failure
5	N054 N061	11 3	14	4.3	• Unknown • Completed	• Unknown • 1st line re-Tx failure
6	N374 N375	2 3	**5**	4.3	• 2nd line ongoing • 2nd line ongoing	• 1st line re-Tx failure • 1st line re-Tx failure
7	N199 N370	8 12	20	4.3	• Completed • Died	• 1st line re-Tx failure • 1st line re-Tx failure
8	N056 N075	2 7	**9**	4.3	• Unknown • Completed	• 1st line Tx failure • Relapse
9	N358 N400	4 3	**7**	4.3	• 2nd line ongoing • Unknown	• 1st line re-Tx failure • 1st line Tx failure
10	N003 N039	3 1	**4**	4.3	• Completed • Unknown	• 1st line Tx failure • Relapse
11	N416 N426	7 11	18	4.3	• 2nd line ongoing • 2nd line ongoing	• 1st line Tx failure • 1st line re-Tx failure
12	N369 N430	8 10	18	4.3	• Died • Died	• 1st line re-Tx failure • 1st line Tx failure
13	N042 N083	2 3	**5**	4.3	• Defaulter • 2nd line ongoing	• Relapse • 1st line re-Tx failure
14	N139 N140	4 10	14	4.3	• 2nd line ongoing • 2nd line ongoing	• Relapse • 1st line re-Tx failure
15	N311 N429	105 112	117	4.3.2	• Unknown • 2nd line ongoing	• Unknown • New
16	N176 N339	6 6	**12**	4.6.1	• 2nd line ongoing • Died	• Relapse • New
	N062 N339	7 7	14		• Completed • Died	• New • New
17	N053 N365	20 15	35	4.6.1	• Died • Unknown	• New • Relapse
18	N066 N067	7 7	14	4.9	• Died • Completed	• Unknown • Relapse
19	N089 N133	11 7	18	4.9	• Unknown • 2nd line ongoing	• 1st line Tx failure • 1st line re-Tx failure
20	N352 N420	1 2	**3**	4.9	• Died • Unknown	• New • 1st line Tx failure
21	N359 N422	10 18	28	4.9	• 2nd line ongoing • 2nd line ongoing	• 1st line re-Tx failure • 1st line re-Tx failure

Abbreviation: ID, identification; SNV, single nucleotide variant; TO, transfer out; Tx, treatment.

5.6.7 Summary of cluster analysis

In total, 9 genomic clusters were identified, that is strains with 12 or fewer SNV differences between them (8, 9). Isolates from the 9 clusters accounted for 21% of the sequenced isolates (18/86). There were ten strains belonging to LAM and two of each strain belonging to Beijing, H37Rv-like strain, an unspecified sub-lineage and Uganda lineage (Table 5.3; Supplementary Table 1, https://tinyurl.com/Zambia-DRTB). The presence of clustered drug resistant TB strains with low SNV differences between them (as low as 2 SNVs) supports the hypothesis that drug resistant TB is being transmitted in Zambia. Of concern is that the majority of the genomic clusters are MDR-TB cases. Whole genome sequencing in combination with IS*6110*-RFLP and spoligotyping has demonstrated a high level of genetic diversity amongst drug resistant TB clinical strains diagnosed at the TDRC TB reference laboratory. This implies that diverse genotypes are driving drug resistant TB in parts of Zambia and that transmission of these strains is occurring.

Patients with clustered strains have been demonstrated to originate from different districts, implying that drug resistant strains are endemic in Zambia. Other possibilities that could explain clustered strains with low SNV differences are nosocomial transmission during quarantine at the NTH MDR-TB ward and social factors such as intercity travel for trading and social gatherings. To gain a better insight into the transmission of these drug resistant TB strains, detailed epidemiological investigations would be required.

5.7 Household transmission of MDR-TB

It has been demonstrated that TB is frequently transmitted outside the household with members of the same household being infected with different strains (11). In the current study, 2 strains (N374 and N375) were characterised from two members of the same household (father and daughter living in the same house) that were diagnosed with MDR-TB and admitted to the NTH MDR-TB ward for part of second line treatment in 2016. Initial typing revealed that both patient strains belonged to LAM11_ZWE (SIT 815) and strains shared identical IS*6110*-RFLP patterns (section 4.4). Whole genome sequence analysis was performed to investigate strain relatedness.

Whole genome sequence analysis revealed that the strains shared identical drug resistance-conferring mutations in *rpoB* (position 526, CAC-GAC), *katG* (codon position 315, AGC-ACC) and *embB* (codon position 1000, ATG-AGG) (Table 5.4). WGS data further revealed that the two strains were closely related with low SNV differences identified between them (5 unique nonsynonymous SNV). The 2 unique SNV identified in strain N374 were in genes Rv3198A (possible glutaredoxin protein with an unknown function) and Rv1699 (putative function in pyrimidine biosynthesis) (10). The unique SNVs identified in patient N375 were in genes Rv0001 (plays an important role in regulation of chromosomal replication), Rv2043c (whose product pyrazinamidase/nicotinamidase convert amides to corresponding acids) and Rv2173 (whose product is involved in lipid biosynthesis) (10). The presence of a SNV in Rv2043c confers resistance to pyrazinamide and therefore implies that this particular strain is developing resistance to pyrazinamide. Variation seen in the SNVs between the two strains could be suggestive of mutations that arose independently within the host.

Table 5.4: Genetic characteristics of strains from two household contacts.

Strain ID	Resistance-conferring mutations (WGS)	Number of SNVs	SNV position	Gene	Lineage
N374	*embB* (pos 1000: ATG-AGG); *katG* (pos 315: AGC-ACC); *rpoB* (pos 445: CAC-GAC).	2 (nonSyn)	Codon 112, Position 1, CAC-TAC	Rv3198A	4.3
			Codon 283, Position 1: AAT-CAT	Rv1699	
N375	*embB* (pos 1000: ATG-AGG); *katG* (pos 315: AGC-ACC); *rpoB* (pos 445: CAC-GAC).	3 (nonSyn)	Codon 1253, Position 2: GCT-GTT	Rv0001	4.3
			Codon 512, Position 2: GCG-GGG	Rv2043c	
			Codon 896, Position 2: CTG-CAG	Rv2173	

Abbreviations: ID, identification; SNV, single nucleotide variant; WGS, whole genome sequencing.

5.8 Molecular comparison of isolates from Cape Town and Ndola district

To determine how closely related isolates from Ndola district were to isolates from Cape Town, WGS data conveniently available at Stellenbosch University for strains that have previously been described in Cape Town was compared to WGS data for strains of the same lineage from the current study. The Beijing genotype which is prevalent in Cape Town and has been associated with MDR- and XDR-TB was compared to the Beijing strains from Ndola district. There was a high SNV difference between the Beijing strains with 210 SNVs between the most closely related strains. The findings suggest that the Beijing genotypes in circulation in the study population are genetically unrelated to genotypes in circulation in part of Cape Town South Africa.

It is however worth noting that the Beijing genotype makes up a small proportion of sequenced strains in the current study (5%), therefore extensive investigations would be required to identify further Beijing genotypes in the study population and in Zambia as a whole. It would be more significant to compare these genotypes to similar strains from closely neighbouring countries were high migration rates have been reported, such as Zimbabwe and Malawi.

5.9 Conclusion

There is very limited data on the transmission dynamics of TB in Zambia with no published data on drug resistant TB. Genotyping data available for Zambia from previous studies has been generated mostly from spoligotyping and MIRU-VNTR and based on these studies, clustering has been observed (14-17).

In this study, 86 out of 170 strains (51%) were analysed using whole genome sequencing. Even though not all strains were sequenced, the level of detail provided by WGS has demonstrated a likelihood of recent transmission amongst patients with drug resistant TB diagnosed at the TDRC TB reference laboratory. However to confidently conclude that transmission has occurred, there is a need for epidemiological data linking patients. Low SNV differences have been identified between strains from patients resident in different regions, implying that drug resistant TB is endemic in Zambia. Nosocomial transmission and intercity travel are possible reasons why clustered strains have been identified amongst patients from different regions. However there is need for more detailed epidemiological investigations. It has further been demonstrated that transmission of drug resistant TB is occurring amongst household contacts placing emphasis on the need for routine screening of MDR-TB contacts in Zambia, which is currently not implemented despite recommendations by the Ministry of Health (MoH) and the National TB and Leprosy Control Program (NTLP) in Zambia (12).

It is concerning that a significant proportion of patients with MDR-TB died while receiving second line anti TB treatment. These deaths raise concerns over treatment adherence, co-infection with HIV and co-morbidity with diabetes mellitus, which have been demonstrated to impact the progression of disease (18). It has been demonstrated that the majority of patients with clustered strains had failed first line treatment (first treatment or re-treatment),

this implies that the TB control program is not efficient in managing TB and as a result drug resistant TB is being acquired and transmitted in the study population.

Migration has been demonstrated to be a driver of the transmission of drug resistant TB and TB in general (13). In the current study, comparison of drug resistant strains belonging to lineage 2, which is prevalent in parts of South Africa and is further associated with XDR-TB (19), has revealed a high SNV difference (210 SNV differences between the most closely related strains). With the currently available data, we were not able to identify any suspected recent transmission events. However, we acknowledge the limitation of incomplete sampling. Extensive surveillance will be required to fully understand the role of migration in the transmission of drug resistant TB within the region.

The usefulness and application of genotyping tools in understanding *M.tb* transmission events has been demonstrated in the current study. Overall WGS provides a superior level of understanding strain relatedness and investigating resistance-conferring mutations in *M.tb* (discussed further in chapter 6), compared to IS*6110*-RFLP and spoligotyping. Spoligotyping has been demonstrated to be important in differentiating LCCs while IS*6110*-RFLP provides a good resolution of a particular subset of strains. In the current study, WGS has been demonstrated to provide the best resolution of *M.tb* strains, giving detailed insight into the level of nucleotide differences between strains. There is a need to implement the use of more rapid and accurate techniques alongside phenotypic culture based methods in order to fully understand the molecular epidemiology of drug resistant TB in Zambia and the surrounding region, for informed and targeted intervention measures. Data presented here represent a first step towards an improved understanding of the drug resistant TB endemic in Zambia.

5.10 References

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Chapter 6: Genetic mechanisms of drug resistance in *Mycobacterium tuberculosis* isolates from the Copperbelt province and Northern regions of Zambia

6.1 Introduction

In Zambia national guidelines recommend culture-based first and second line drug susceptibility testing (DST) for the diagnosis of drug resistant tuberculosis (TB) (1). However, in practice, first line DST is not readily available to all confirmed TB cases due to the lack of capacity, and therefore the Ministry of Health (MoH) and the National TB and Leprosy Control Program (NTLP) diagnostic algorithm prioritises high risk MDR-TB suspects for receiving first line DST (Figure 1.3) (1). The national TB reference laboratory at Chest Diseases Laboratory (CDL) has the capacity to perform DST on some second line drugs (kanamycin, capreomycin and ofloxacin) (1), however second line DST is not routinely performed for MDR-TB patients in Zambia. Overall, this has resulted in low multidrug resistant (MDR)-TB case detection rates and very limited knowledge on the efficacy of the current standardised MDR-TB treatment regimen used in Zambia. There is also very little to no information on the incidence of extensively drug resistant (XDR)-TB in the country with one study describing a case of pre-XDR-TB (*gyrA* mutation) and one case of XDR-TB (2). In addition, little is known regarding the transmission of these strains.

In this chapter, known mutations conferring drug resistance to any of the first and second line drugs are described for 170 clinical isolates collected over a period of 15 months from the Tropical Diseases Reference Centre (TDRC) TB reference laboratory in Ndola district. Further, a first insight is given into the genotypes associated with pre-XDR-TB in the study population.

6.2 Known resistance-conferring mutations in *Mycobacterium tuberculosis*

Several mutations in *Mycobacterium tuberculosis* (*M.tb*) have been demonstrated to be associated with drug resistance (Table 6.1) (3). For one of the core first line drugs, rifampicin (RIF), mutations in the *rpoB* rifampicin resistance determining region (RRDR), an 81bp region of the gene (spanning from codon 507 to 533), are most commonly associated with resistance to this drug (3). The rapid diagnostic tool GeneXpert MTB/RIF assay has exploited the RRDR by simultaneous identification of *M.tb* and mutations in this region which confer rifampicin resistance (4). While for isoniazid, the most common mutations associated with resistance are found in the *katG* gene and *inhA promoter*. For *katG*, mutations in codon 315 confer high level resistance, defined as a minimum inhibitory concentration (MIC) >1 μg/mL, while *inhA* promoter mutations (-15) confer low level resistance, defined as an MIC <1 μg/mL (3). Mutations commonly associated with resistance to the other first line drugs include those conferring resistance to streptomycin, (found in *rpsL*, codon 43 and *rrs*, codon 514), to ethambutol (*embB*, codon 306) and to pyrazinamide (*pncA;* mutations occur throughout the gene) (3).

In terms of second line drugs, specific resistance-conferring mutations to fluoroquinolones (FQs) have been described in *gyrA* and the most common mutations are seen in the quinolone-resistance-determining region (QRDR) in codon 90 and 94 (3). While the most common mutations associated with resistance to the second line injectable drugs kanamycin, capreomycin and amikacin are found in *rrs* (at position 1401, 1402 and 1484 in the gene), cross resistance is however not complete as mutations in *eis* gene and the *eis* promoter (position -10 and -35) have been demonstrated to confer resistance to kanamycin and not capreomycin or amikacin (3). There is full cross resistance in mutations associated with drug

resistance in capreomycin and viomycin, mutations throughout the open reading frame (ORF) are associated with drug resistance in *tlyA* (3, 4).

Conventional culture-based phenotypic DST is a laborious and time consuming approach to diagnosis of drug resistant TB (5, 6). Recommendations by the World Health Organisation (WHO) on the use of molecular line probe assays (LPA) and GeneXpert MTB/RIF assay have allowed rapid and simultaneous identification of *M.tb* and drug resistance in clinical samples (6). The use of DNA sequencing methods for known drug resistance-conferring mutations in *M.tb* , such as targeted gene sequencing (TGS) and whole genome sequencing (WGS) have been demonstrated to be superior in investigating genetic mechanisms of drug resistance (7, 8). The impediments to these DNA sequencing based techniques in low to middle income countries such as Zambia include the lack of expertise and the high costs associated with equipment purchase as well as running and maintenance costs. It is possibly due to these constraints that there are very limited molecular investigations occurring in Zambia and Africa as a whole.

Only a few studies have used molecular tools to elucidate the mechanisms of drug resistant TB in Zambia. One study used LPA to evaluate 113 MDR-TB strains (2). Another study used targeted gene sequencing to identify mutations conferring resistance to first and second line drugs, 13 out of 16 strains in the study had a Ser>Thr substitution at codon 88 (9). Two studies have demonstrated the usefulness of WGS as a rapid diagnostic tool and in understanding the transmission dynamics (differentiating relapse from reinfection) of TB in Zambia (10, 11). However one study had a small cohort size, 36 patients from 4 countries in Southern Africa including Zambia. Further, these studies were not centred on characterising genotypes of drug resistant TB. The current study has a particular focus on the genotypes of

drug resistant TB and included a larger cohort of patient isolates collected from routine diagnosis.

Table 6.1: The most frequently identified high confidence mutations conferring drug resistance in *M.tb* (3).

Drug	First/ Second line	Locus	Frequently mutated codon position
Streptomycin	First line	*rpsL*	43
		rrs (388-1084)	514
Isoniazid	First line	*katG*	315 (high level resistance)
		inhA promoter	-15 (low level resistance)
Rifampicin	First line	*rpoB*	531, 526, 516
Ethambutol	First line	*embB*	306
Pyrazinamide	First line	*pncA*	throughout gene
Fluoroquinolone	Second line	*gyrA*	94, 90
Kanamycin, Capreomycin, amikacin	Second line	*rrs* (1158-1674)	1401, 1402, 1484
Kanamycin	Second line	*eis*	-10, -35
Capreomycin, viomycin	Second line	*tlyA*	throughout ORF

Abbreviations: ORF, open reading frame.

6.3 Analysis of Sanger sequencing and whole genome sequencing data

To investigate the genetic mechanisms of drug resistance and the presence of XDR-TB in the study population, we performed targeted Sanger sequencing for 84 isolates (described in 3.8), extracted information from WGS data for 80 isolates, and applied both approaches to a further 6 strains (N024, N041, N064, N133, N139, N199). Similar mutations conferring resistance were identified in 5 of these strains by both WGS and TGS, there were no mutations identified in one strain (N041) (Supplementary Table 1, https://tinyurl.com/Zambia-DRTB).

Targeted gene sequencing for 8 genes (*rpoB*, *katG*, *inhA*, *embB*, *pncA*, *rrs*, *gyrA*, and *tlyA*) commonly associated with drug resistance in *M.tb* (Table 6.1) was performed at the Central Analytical Facilities (CAF), Stellenbosch University (as described in section 3.8). ABI Sanger sequence data was aligned to the *M.tb* H37Rv (GenBank NC000962.2) reference

genome in BioEdit and chromatograms were analysed for the presence of single nucleotide variants (SNVs) in relation to the reference genome.

The FASTQ data files generated from WGS were analysed using the customised in-house WGS analysis pipeline, Universal Sequence Analysis Pipeline (USAP), as described in section 3.7. The resulting WGS summary report, which included known mutations conferring drug resistance identified by USAP, was used to identify mutations conferring resistance to first and second line drugs. Mutations identified as resistance-conferring through sequencing were validated by comparing with mutations listed on the TBdream database (https://tbdreamdb.ki.se/Info/Default.aspx). The findings are used here to describe the genetic mechanisms of resistance and the relationship to phenotype as well as to describe the genotypes associated with XDR-TB in the study population, thereby addressing objectives 5 and 6 (section 1.5.3).

6.4 Overall profile of drug resistance-conferring mutations in study strains

In this chapter, TGS and WGS analysis was applied to profile drug resistance-conferring mutations in drug resistant *M.tb* isolates diagnosed at the TDRC TB reference laboratory during the study period. Among 170 isolates, nucleotide sequence analysis identified 23 (14%) strains which did not have any drug resistance-conferring mutations despite being classified as drug resistant through routine culture based phenotypic DST at the TDRC TB reference laboratory; this will be discussed further in section 6.5. One serial strain from the same patient (N025) was removed from analysis. In the remaining 146 strains, we did however identify high confidence mutations in genes associated with resistance to the first-line drugs rifampicin (*rpoB*), isoniazid (*katG, kasA*), ethambutol (*embB*), streptomycin (*rpsL, rrs*) and pyrazinamide (*pncA*), as well as to the second-line FQs (*gyrA*), kanamycin (*eis*), and

amikacin (*rrs*), there were no mutations identified in *tlyA* (Table 6.2, Figure 6.1, Supplementary Table 1, https://tinyurl.com/Zambia-DRTB). Further, the most common resistance-conferring high confidence mutations identified in this study are in keeping with previous studies (3, 5, 7).

Out of the 146 strains identified with drug resistance-conferring mutations 48 (33%) mono-resistant strains were identified. Of the 48 mono-resistant strains, 26 (54%) were resistant to rifampicin, 17 (35%) to isoniazid, 3 (6%) to streptomycin and one each (2%) to ethambutol and FQ (Supplementary Table 1). The high rate of mono-resistance is alarming and could point towards an inadequate TB control program and low treatment adherence (12). Genotyping identified 93 MDR-TB strains (64%), 53 of these had added resistance to one or more anti TB drugs (Supplementary Table 1). Eight (9%) pre-XDR TB cases were identified out of the 93 MDR-TB cases, comparable to WHO global estimate of 9.5% MDR-TB cases being XDR-TB (12). These are further discussed in section 6.6.

Of the 93 MDR-TB cases identified through genotyping, almost half of the patients were HIV positive (48%) while 18 (19%) had an unknown HIV status. Over half of the patients had failed first line treatment (20 (22%) failed first treatment while 27 (29%) failed retreatment), and 11 (12%) were defined as relapse cases, highlighting deficiencies in the TB control program. Of concern is that a further 27 (29%) were new TB patients, suggesting that MDR-TB is possibly being transmitted in the study population (Supplementary Table 1). With regards to the treatment status of MDR-TB patients identified in the current study, 39 (42%) were receiving second line treatment at the time of data collection, while 14 (15%) had completed treatment and 12 (13%) had died. Of concern is that a proportion of MDR-TB patients were lost to follow-up 3 (3%), had an unknown treatment status 19 (20%) and 7 (7%) were not enrolled on second line treatment at the time of data collection, similar to trends

observed in a previous study in Zambia (13). Therefore, there is a high risk that MDR-TB is being spread in the community by patients that are poorly managed by the TB control program.

One hundred and nineteen strains out of 170 (70%) had mutations identified in *rpoB* and in keeping with other studies (3, 5), the most commonly identified mutation in *rpoB* (63 strains) was in codon 531 (Table 6.2, Figure 6.1, Supplementary Table 1). Seven strains had double mutations in *rpoB*; codons 511 and 516 (3 strains), codons 516 and 526 (1 strain), codons 531 and 633 (3 strains) (Supplementary Table 1). Mutations conferring resistance to isoniazid were identified in 113 (66%) strains. Mutations in *katG* were the most common (103 strains), with *kasA* mutations only identified in 6 strains; 5 strains had multiple resistance-conferring mutations (*katG* and *kasA*), while 1 isoniazid mono-resistant strain had a mutation in *kasA* only which has been associated with drug resistance in previous studies (3). A deletion identified in codon 1737 of *katG* (N70) has previously not been identified as resistance-conferring (Table 6.2, Supplementary Table 1). There were no mutations identified in the *inhA* promoter region. In the other first line anti-TB drugs, mutations were identified in *embB* conferring resistance to ethambutol (55 strains; 4 strains had multiple resistance conferring mutations), while in *rrs* 15 strains were identified as having mutations, however only 5 of these mutations have previously been defined as conferring resistance to streptomycin, a further 4 strains had mutations in *rpsL* (Table 6.2). Targeted sequencing did not include the *rpsL* gene, data for this gene was only extracted from WGS data of 85 strains. Therefore, the incidence of mutations in *rpsL* is possibly underestimated. Seventeen strains had mutations in *pncA*, however only 10 mutations have previously been described as conferring resistance to pyrazinamide (Table 6.2). These findings are in keeping with other studies (3) describing the most commonly associated mutations conferring drug resistance to first line drugs to be found in *rpoB* (codons 531, 516 and 526), *katG* (315), *embB* (306), *rrs* (514), and *rpsL* (43).

Analysis of mutations associated with second line drug resistance identified 9 strains with mutations in *gyrA* conferring resistance to fluoroquinolones, 2 of these strains (N026 and N043) had multiple resistance-conferring mutations (Supplementary Table 1). One strain (N038) with a mutation in *rrs* (517, C-T) had a further mutation in the *eis* promoter region, which confers resistance to kanamycin, a core second line injectable drug which is part of the standard first choice MDR/RR-TB regimen recommended by the NTLP in Zambia (1). The most common mutation identified in 5 strains was in codon 80 (ACC-GCC) (Table 6.2, Figure 6.1). The characteristics of patients with resistance-conferring mutations to second line drugs are described in section 6.6. A mutation in *ethA* was identified at codon 1291, however resistance to ethionamide has not been confirmed.

The current findings demonstrate a high rate of rifampicin resistance amongst drug resistant TB strains (70%) highlighting the usefulness of the GeneXpert MTB/RIF assay in the rapid diagnosis of these drug resistant TB strains. Furthermore, all rifampicin resistant strains had mutations within the RRDR which the GeneXpert MTB/RIF assay detects, highlighting the usefulness of the technique in the study population. However a substantial number of strains that were not resistant to rifampicin were resistant to other drugs (27 strains), and could be missed by the GeneXpert MTB/RIF assay. The presence of pyrazinamide resistance amongst MDR-TB patients (approximately 11%; 10 out of 93 patients) is concerning as pyrazinamide is a key drug in treatment of MDR-TB in Zambia (1) and currently routine DST for this drug is not conducted in Zambia.

This data demonstrates the presence of mutations conferring drug resistance to core first and second line anti-TB drugs, placing emphasises on the need to implement routine first and second line DST for all TB patients in Zambia for early case detection.

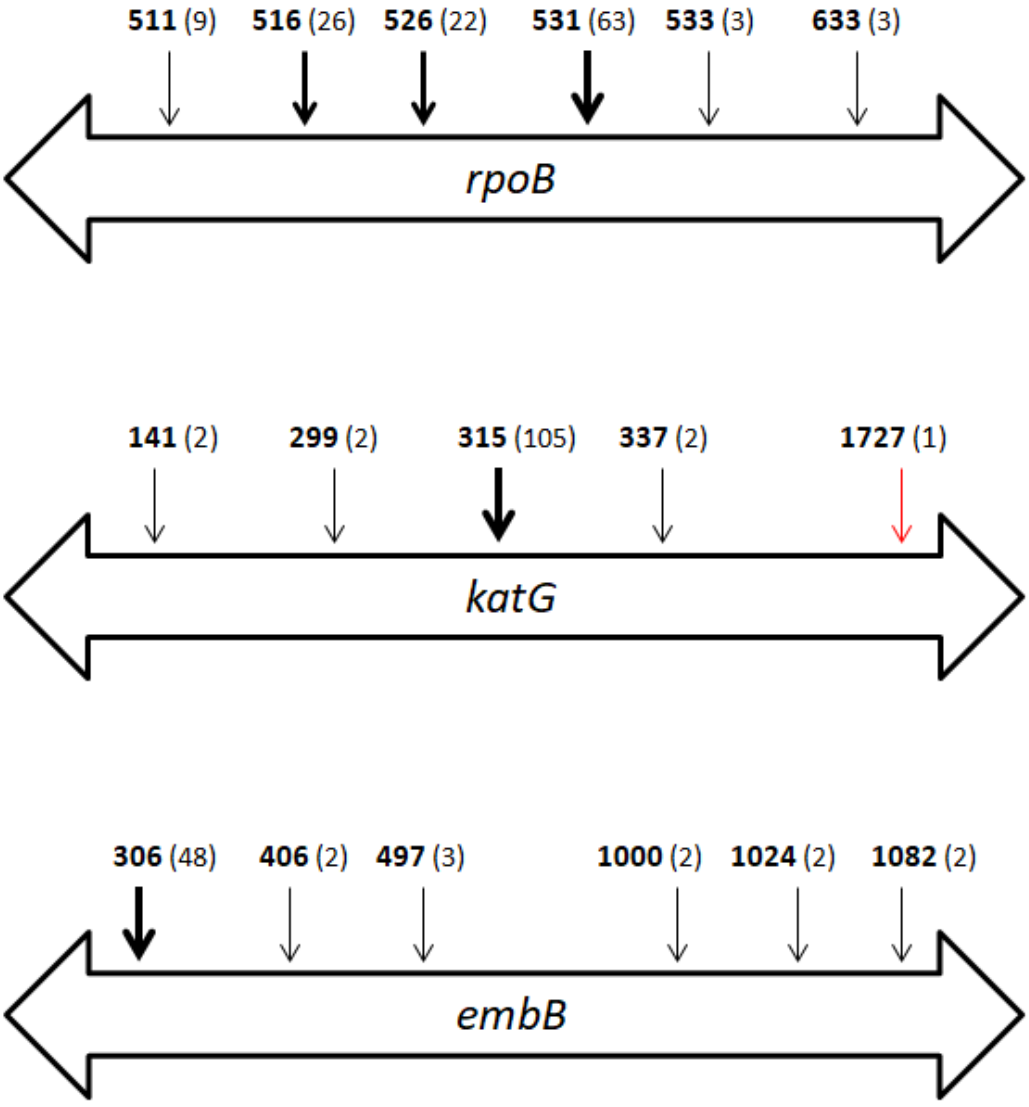
Table 6.2: Mutations identified in genes associated with drug resistance in clinical strains of *M.tb* diagnosed at the TDRC TB reference laboratory, using WGS and TGS data. Complete data set available at: https://tinyurl.com/Zambia-DRTB

Drug (number of strains)	Gene(s)	Codon position, nucleotide change	Number of strains	Confidence mutation confers resistance	Ref
Rifampicin (119 strains; 7 strains with multiple mutations)	*rpoB*	Codon 511, CTG-CCG	5	Yes	14
		Codon 511, CTG-CGG	4	Yes	14
		Codon 516, GAC-GTC	20	Yes	15
		Codon 516, GAC-TAC	6	Yes	15
		Codon 526, CAC-GAC	8	Yes	15
		Codon 526, CAC-AAC	5	Yes	15
		Codon 526, CAC-TAC	5	Yes	15
		Codon 526, CAC-CTC	3	Yes	15
		Codon 526, CAC-CAG	1	Yes	15
		Codon 531, TCG-TTG	63	Yes	15
		Codon 533, CTG-CCG	3	Yes	15
		Codon 633, CGC-TGC	3	Yes	16
Isoniazid (113 strains; 5 strains with multiple mutations)	*katG*	Codon 141, TTG-TTC	2	Yes	17
		Codon 299, GGC-AGC	2	Yes	17
		Codon 315, AGC-ACC	103	Yes	17
		Codon 315, AGC-AAC	1	Yes	17
		Codon 315, AGC-CGC	1	Yes	17
		Codon 337, TAC-TGC	2	Yes	17
		Deletion 1737, CGGTTT-C	1	ND	
	kasA	Codon 269, GGT-AGT	6	Yes	18
Ethambutol (55 strains; 4 strains with multiple mutations)	*embB*	Codon 306, ATG-GTG	28	Yes	17
		Codon 306, ATG-ATA	18	Yes	17
		Codon 306, ATG-ATT	1	Yes	17
		Codon 306, ATG-TTG	1	Yes	17
		Codon 406, GGC-GCC	2	Yes	19
		Codon 497, CAG-CGG	3	Yes	19
		Codon 1000, ATG-AGG	2	Yes	19
		Codon 1024, GAC-AAC	2	Yes	19
		Codon 1082, ACC-GCC	2	Yes	20
Streptomycin (19 strains)	*rpsL*	Codon 43, AAG-AGG	4	Yes	21
	rrs	Codon 431, CGG-CGT	1	ND	
		Codon 432, GGT-AGT	1	ND	
		Codon 438, CCC-TAC	1	ND	
		Codon 468, CGT-AGT	1	ND	
		Codon 469, CAT-CAC	2	ND	
		Codon 477, CCA-CCG	2	ND	
		Codon 482, TCG-TTG	2	ND	
		INS Codon 483, T	3	Yes	21
		Codon 486, TGT-CGT	2	ND	
		Codon 492, C-T	2	ND	
		Codon 514, A-C	1	Yes	3
		Codon 517 C-T	1	Yes	3
Pyrazinamide (17 strains)	*pncA*	Codon 35, CTG-CGG	6	No	22
		Codon 47, ACC-GCC	1	Yes	23
		Codon 58, TTC-TCC	3	Yes	17
		Codon 68, TGG-TGA	1	Yes	17
		Codon 102, GCG-GTG	1	Yes	23
		Codon 119, TGG-TGT	2	Yes	17
		Codon 120, CTG-CCG	2	Yes	23
		Insertion codon 450, G-GC	1	ND	

Table 6.2 continued.

Drug (number of strains)	Gene(s)	Codon position, nucleotide change	Number of strains	Confidence mutation confers resistance	Ref
Fluoroquinolone (10 strains)	*gyrA*	Codon 79, AGA-AGG	1	ND	
		Codon 80, ACC-GCC	5	Yes	24
		Codon 88, GGC-TGC	1	Yes	17
		Codon 90, GCG-GTG	2	Yes	17
		Codon 91, TCG-CCG	2	Yes	17
Kanamycin (1 strain)	*eis* promoter	Codon -10, C-T	1	Yes	3
Ethionamide (1 strain)	*ethA*	Codon 1291, AG-A	1	ND	

Abbreviations: ND, not defined; ref, reference; TB, tuberculosis; TDRC, Tropical Diseases Research Centre.



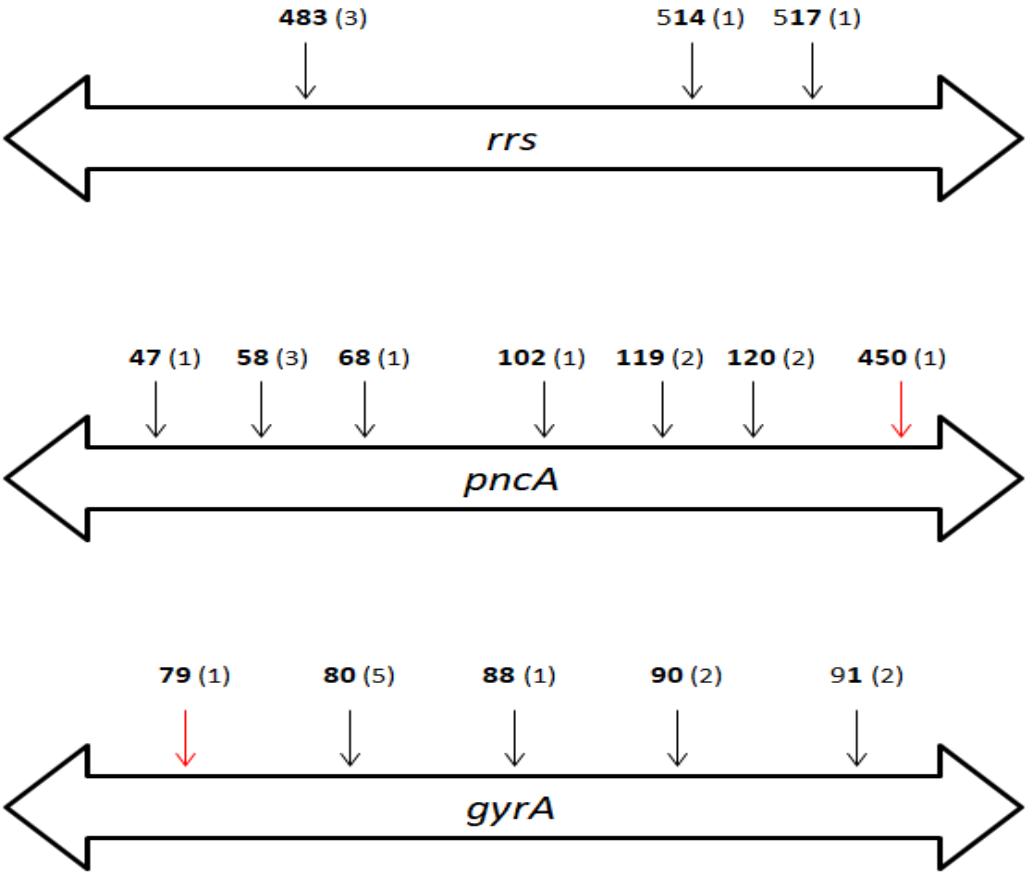


Figure 6.1: The most common mutations identified in genes associated with drug resistance in clinical strains of *M.tb* diagnosed at the TDRC TB reference laboratory. The arrows represent the codon positions (in bold) while the number of strains with a specific mutation are represented in brackets. Black arrows have previously been associated with resistance while red arrows have not been defined as resistance-conferring mutations.

6.5 Comparison of genotype and phenotype data

To assess the level of agreement between genotype and phenotype data, comparisons of phenotypic DST findings and genotype data were made. Phenotypic data was only available for the first line drugs, streptomycin, isoniazid, rifampicin and ethambutol and therefore comparisons could only be made for these data. In total, phenotypic data for 120/170 (71%) samples were in agreement with sequence data (Supplementary Table 1, https://tinyurl.com/Zambia-DRTB). Of the 50 (29%) samples that were not in agreement, 23 (46%) were scored as phenotypically resistant at the TDRC TB reference laboratory, however

no drug resistance-conferring mutations were identified according to sequence data. Repeat phenotypic DST was performed for 5 isolates out of the 23 at the Division of Molecular Biology and Human Genetics, Stellenbosch University and they were all scored as drug susceptible (Table 5.3, Supplementary Table 1).

Of the 23 isolates with no known drug resistance-conferring mutations identified the majority (96%) belonged to lineage 4, one strain belonged to lineage 3 (Table 6.3). Most patients in this group had failed first line treatment (13 out of 23) and a proportion (6 out of 23) had received second line treatment, either completed or treatment was ongoing during the study period (Table 6.3).

In the remaining 146 isolates, additional phenotypic resistance was identified in 15 strains (10%) compared to genotyping results. Further, 12 strains (8%) were over-scored by genotyping compared to phenotypic DST findings. Analysis revealed that WGS under-scored 8 phenotypically resistant strains while TGS under-scored 7 strains (Supplementary Table 1). Furthermore, WGS over-scored 7 strains while TGS over-scored 5 phenotypically resistant strains. Comparison of phenotype and genotype data for individual drugs revealed that phenotypic DST identified 5 fewer rifampicin resistant strains and over-scored 1 compared with genotyping. Phenotyping under-scored 1 and over-scored 2 isoniazid resistant strains than genotyping (Supplementary Table 1). For ethambutol and streptomycin genotyping under-scored 6 and 12 strains with resistant phenotypes, respectively (Supplementary Table 1). Furthermore, genotyping over-scored 3 streptomycin resistant strains and 7 ethambutol resistant strains compared with phenotypic DST findings (Supplementary Table 1)

The discrepancies identified could be attributed to a number of factors. For example, operational errors during sample processing (due to inadequate laboratory staff training and/or insufficient QC) could result in errors in data capture and sample mix ups. Biological

factors could include mixed infections or genetic heterogeneity (25); indeed analysis of TGS data identified 3 strains (N068, N094 and N452) with heterogeneous variants in *rpoB* and one (N094) had added variants in *katG* and *embB* (Supplementary Table 1). Other biological possibilities include unidentified gene targets which could be involved in the phenotypic resistance profiles identified. Furthermore, not all genetic resistance markers are included in the USAP and therefore resistance-conferring mutations could have been missed during analysis of sequence data.

Phenotypic DST for certain anti TB drugs has been demonstrated to be highly varied and challenging and discordant results have been demonstrated between phenotype and genotype (26, 27). For instance, phenotypic DST for ethambutol has been shown to be discordant with genotypic testing, with as high as 91% of ethambutol resistant strains being phenotypically undetected in one study (26), highlighting the need for more sensitive and accurate diagnostic tools for this drug. However, other studies have described ethambutol susceptible strains that harbour mutations in *embB*306 (28, 29), similar to findings in the current study for 9 isolates (N050, N068, N292, N332, N354, N392, N420, N459, N477). The *embB*306 mutation has further been associated with strains harbouring a wide range of drug resistance patterns and is associated with an increased ability of transmission of these strains (28, 29). These findings demonstrate the importance of complementing phenotypic DST with genotyping methods (25, 27, 30).

Table 6.3: Phenotypic characteristics of strains identified as having no known resistance-conferring mutations by WGS and TGS analysis. Complete data set available at: https://tinyurl.com/Zambia-DRTB

Isolate ID	Phenotypic DST SIRE (TDRC)	Phenotypic DST SIRE (SU)	Lineage	Treatment received
N011	SSRS	Not done	4.3	First line treatment completed
N041	SRSS	Not done	4.9	After failure of 1st line retreatment
N057	RSRR	Not done	4.3	After failure of 1st line treatment
N065	SRRR	Not done	4.9	2nd line treatment completed
N076	RSSS	Not done	4.3	After failure of 1st line treatment
N181	SSRS	**SSSS**	4	Not enrolled on second line treatment
N227	SRSS	Not done	4.1.1.3	After failure of 1st line retreatment
N240	SSRS	Not done	4.3	After failure of 1st line treatment
N246	SRSS	**SSSS**	4.9	After failure of 1st line treatment
N262	RSRS	Not done	4.3	After failure of 1st line treatment
N271	SRRS	Not done	4.9	2nd line treatment on going
N283	SSRS	Not done	4.9	After failure of 1st line treatment
N296	RSRS	Not done	4.3	After failure of 1st line treatment
N318	SRRS	**SSSS**	4	Not enrolled on second line treatment
N325	SSRS	Not done	4.3	2nd line treatment defaulter
N331	SSRS	**SSSS**	4.3	After failure of 1st line retreatment
N346	SSRS	Not done	4.3	2nd line treatment on going
N412A	SSRS	Not done	4.3	2nd line treatment completed
N424	SSRS	**SSSS**	3	After failure of treatment
N427	RSSR	Not done	4.3	After failure of 1st line treatment
N431	SRRS	Not done	4.3	2nd line treatment on going
N446	RSSR	Not done	4.1.1.3	After failure of 1st line treatment
N465	SSRS	**SSSS**	4.3	After failure of retreatment

Abbreviations: DST, drug susceptibility testing, TGS, targeted gene sequencing; WGS, whole genome sequencing. Drug resistance (SIRE) order; S, streptomycin; I, isoniazid; R, rifampicin; E, ethambutol.

6.6 A first insight into the genetics of pre-XDR in Zambia

Here we use sequencing data captured from the more sensitive techniques of targeted gene sequencing and WGS to describe the molecular characteristics of pre-XDR-TB identified from isolates collected from the TDRC TB reference laboratory in Ndola district, giving a first insight into the genotypes associated with XDR-TB in Zambia.

6.6.1 Pre-XDR-TB genotypes identified from drug resistant isolates diagnosed at the TDRC TB reference laboratory

From the sequenced strains, 8 were identified as genotypically pre-XDR-TB cases (MDR-TB with added genotypic resistance to a second line injectable drug or a FQ) with mutations either in *gyrA* (7 strains) or in *rrs* and the *eis* promoter (1 strain) and not both (Table 6.4). In total 4 out of the 8 strains (50%) belonged to lineage 4.6.1, there were 3 strains belonging to lineage 2.2 (37.5%), and 1 strain belonged to lineage 4.3 (N054). One strain (N063) had a mutation in codon 79 (AGA-AGG) of *gyrA*, which has not been described as conferring resistance to FQ. Four pre-XDR-TB strains from lineage 4.6.1 (N053, N062, N176 and N339) shared a similar mutation in codon 80 (ACC-GCC) of the *gyrA* locus. Furthermore, strains from this lineage shared similar mutations in *rpoB (*526, CAC-GAC) and *katG* (315, AGC-ACC), one strain (N062) had an added mutation in *pncA* (insertion codon 450, G-GC) which has not been associated with pyrazinamide resistance. One strain (N053) had mutations in *rpoB* (526, CAC-CAG; 516, GAC-GTC) and in *katG* (337, TAC-TGC).

The other predominant pre-XDR-TB strains belonging to lineage 2.2 (N026, N038 and N043) have previously not been described in Zambia. Two strains (N026 and N043) from this lineage shared similar mutations in *gyrA* (90 GCG-GTG; 91, TCG-CCG), *rpoB* (531, TCG-TTG) *katG* (315, AGC-ACC), *embB* (497, CAG-CGG), *embB* (1082, ACC-GCC) and *rpsL*

(43, AAG-AGG), Table 6.4. Overall, there was concordance between phenotype and genotype data for the pre-XDR-TB strains identified in the study (Supplementary Table 1).

Only one pre-XDR TB strain (N054) belonging to lineage 4.3 was identified. This lineage is the predominant genotype associated with drug resistance in the current study and has been associated with drug susceptible TB in previous studies in Zambia and the surrounding region (31, 32). Of interest is that the lineage represents a small proportion of pre-XDR TB cases identified in the study (11%), this could however be due to the low case detection reported for Zambia or additional unknown resistance-conferring gene targets.

From the eight pre-XDR-TB cases identified in the current study, 3 (37.5%) were receiving second line treatment at the time of data collection, 2 (25%) had completed treatment and a further 2 (25%) had died while on treatment. Of concern is that the treatment status for one patient was unknown, lost to follow up (Table 6.4). The pre-XDR TB genotypes have been detected in 4 districts and 2 provinces implying that these strains could be widespread across the country.

Table 6.4: Characteristics of pre-XDR-TB cases and strains with *gyrA* mutations identified amongst drug resistant TB patients diagnosed at the TDRC TB reference laboratory

Patient ID	Lineage	Locus	Mutations conferring resistance to 2nd line anti-TB drugs	Other SNVs	Treatment status	Residential address
N026	2.2	*gyrA*	Codon 90, GCG-GTG; codon 91, TCG-CCG	***rpoB*** 531, TCG-TTG; ***katG*** 315, AGC-ACC; ***embB*** 497, CAG-CGG; ***embB*** 1082, ACC-GCC; ***rpsL*** 43, AAG-AGG	Treatment ongoing	Ndola, Copperbelt
N038	2.2	*rrs*	Codon 517, C-T	***rpoB*** 531, TCG-TTG; ***katG*** 315, AGC-ACC; ***ethA*** 1291, AG-A: ***pncA*** 120, CTG-CCG;	Completed	Mansa, Luapula
		*eis*_promoter	-10, C-T			
N043	2.2	*gyrA*	Codon 90, GCG-GTG; 91, TCG-CCG	***rpoB*** 531, TCG-TTG; ***katG*** 315, AGC-ACC; ***embB*** 497, CAG-CGG; ***embB*** 1082, ACC-GCC; ***rpsL*** 43, AAG-AGG	Treatment ongoing	Mufulira, Copperbelt
N053	4.6.1	*gyrA*	Codon 80, ACC-GCC	***rpoB*** 516, GAC-GTC; ***rpoB*** 526, CAC-CAG; ***katG*** 337, TAC-TGC;	Died	Samfya, Luapula
N054	4.3	*gyrA*	Codon 88, GGC-TGC	***rpoB*** 526, CAC-CTC; ***katG*** 141, TTG-TTC; ***embB*** 1024, GAC-AAC; ***pncA*** 68, TGG-TGA	Unknown	Ndola, Copperbelt
N062	4.6.1	*gyrA*	Codon 80, ACC-GCC	***rpoB*** 526, CAC-GAC; ***katG*** 315, AGC-ACC; ***pncA*** Indel codon 450, G-GC	Completed	Ndola, Copperbelt
N063	4.6.1	*gyrA*	Codon 79, AGA-AGG	***rpoB*** 526, CAC-GAC; ***katG*** 315, AGC-ACC	Completed	Kitwe, Copperbelt
N176	4.6.1	*gyrA*	Codon 80, ACC-GCC	***rpoB*** 526, CAC-GAC; ***katG*** 315, AGC-ACC	Treatment ongoing	Ndola, Copperbelt
N339	4.6.1	*gyrA*	Codon 80, ACC-GCC	***rpoB*** 526, CAC-GAC; ***katG*** 315, AGC-ACC	Died	Ndola, Copperbelt
N365	4.6.1	*gyrA*	Codon 80, ACC-GCC	None	Unknown	Ndola, Copperbelt

Abbreviations: ID, identification; SNV, single nucleotide variant; TB, tuberculosis.

6.6.2 Transmission of pre-XDR TB in the study population

In the preceding chapter (section 4.4) molecular typing using spoligotyping and IS*6110*-RFLP demonstrated clustering amongst pre-XDR TB strains, which could be suggestive of recent transmission. However, the low discriminatory power of these tools limits the ability to conclusively determine whether transmission has taken place. To address this, we

examined WGS data. This revealed low nucleotide variant differences amongst these clustered strains (section 5.6). For instance, cluster analysis of strains belonging to lineage 4.6.1 showed that strains N62, N176 and N339 were genomic clusters with 12 SNVs between them (discussed in the previous chapter), indicative of recent transmission (33, 34). Furthermore, these strains share similar drug resistance-conferring mutations, discussed above. Two pre-XDR-TB strains (N026 and N043) from lineage 2.2 were clustered with a low SNV difference (2 unique non-synonymous SNVs). This is suggestive of recent transmission of these strains in the study population and calls for urgent action towards active case detection and treatment. Since sample collection was not comprehensive, there could be underestimation of transmission of XDR TB in the study population.

All 8 pre-XDR-TB patients and one MDR-TB patient (N063) with a *gyrA* mutation that has not been confirmed as resistance conferring had previously been admitted to the Ndola Teaching Hospital (NTH) MDR-TB ward for part of second line treatment; however patients had varying residential addresses (Figure 6.2). One patient that was not admitted to the NTH MDR-TB ward had a streptomycin resistant strain according to phenotypic DST, genotyping however only identified a mutation in *gyrA*. A possible explanation for the presence of a fluoroquinolone mono resistant strain could be the use of fluoroquinolones, which is rampant in Zambia, in the treatment of other bacterial infections (35).

Five out of the eight patients were residents of Ndola district on the Copperbelt province; there was a single patient each from Mansa district (Luapula province), Samfya district (Luapula province) and Mufulira district (Copperbelt province) (Figure 6.2). Two lineage 2.2 strains (0N26 and N043) shared very low SNVs (2 SNVs), even though patients originated from different districts. This could be explained by the high rate of intercity travel that is seen on the Copperbelt province and Zambia as a whole.

The incidence of pre-XDR-TB in the sampled patients (9%; 8 out 93 MDR-TB cases) is comparable with WHO global estimate of 9.5% MDR-TB cases being XDR-TB (12). The incidence of XDR- and pre-XDR-TB in the study population could be higher than identified in the current study, since *gyrA* is not the only gene target that confers resistance to FQs (3) and the USAP does not identify all resistance markers. Routine phenotypic DST would be essential to confirm the incidence of XDR-TB in Zambia. The presence of pre-XDR-TB in the study population is concerning as it implies that strains are developing added resistance to the current MDR-TB treatment regimen.

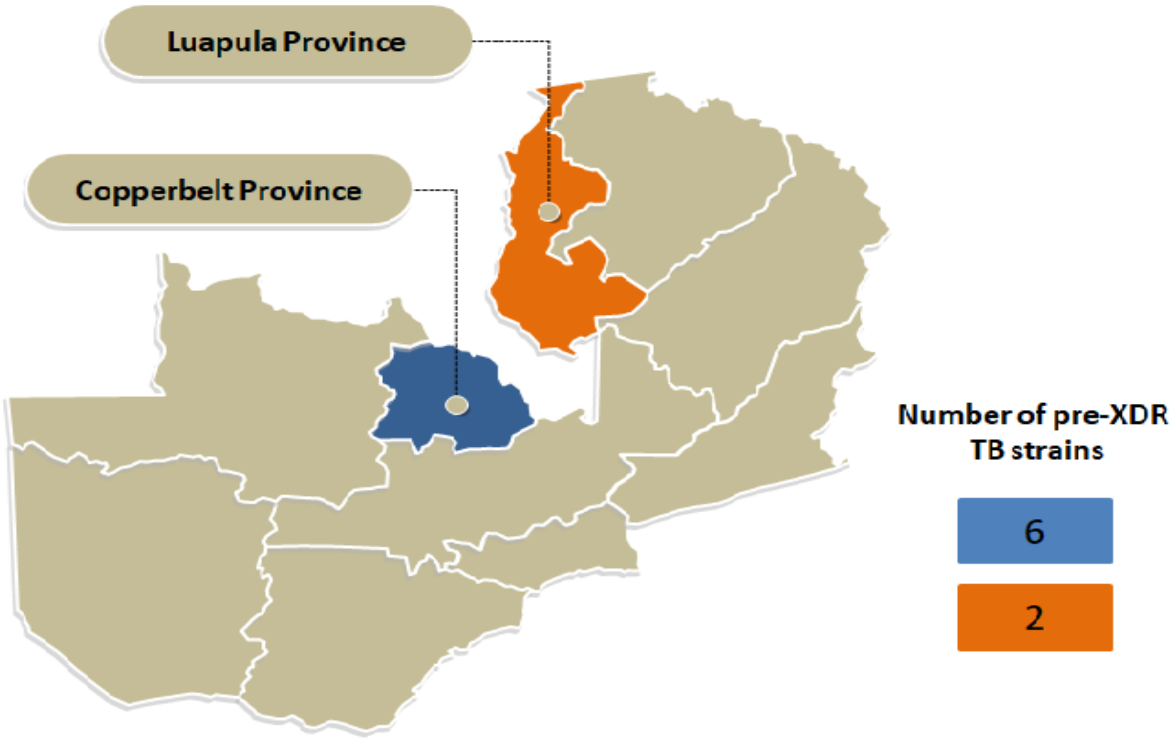


Figure 6.2: Geographical distribution of pre-XDR-TB cases for strains collected from the TDRC TB reference laboratory. 6 cases were identified on the Copperbelt province (5 in Ndola district and 1 in Mufulira district) and 2 cases were identified in Luapula province (1 in Mansa district and 1 in Samfya district).

6.7 Conclusion

In the current study, the prevalence of pre-XDR TB amongst drug resistant isolates was 5.5% (8 out 146 genotypically drug resistant TB strains). The incidence of pre-XDR-TB in MDR-TB patients (9%; 8 out of 93) is comparable to WHO estimates for the incidence of XDR-TB amongst MDR-TB cases (9.5%) (12). Of interest, the predominant genotype in the current study, LAM (lineage 4), is only accounting for 12.5% of pre-XDR, with the majority of pre-XDR isolates belonging to lineage 4.6.1 (Uganda) (62.5%) and lineage 2.2 (Beijing) (25%). This could be due to strains from these lineages (4.6.1 and 2.2) having a higher ability to develop resistance compared to strains from lineage 4.3. Other factors that could be attributed to the low rate of pre-XDR strains belonging to sub-lineage LAM11_ZWE are incomplete sampling and the current MDR-TB regimen is possibly successful in preventing development of added resistance in this sub-lineage.

Further, isolates belonging to lineage 4.6.1 and lineage 2.2 have been demonstrated to be in genomic clusters with low SNV differences (section 5.4), implying that these genotypes are being transmitted within the study population. Transmission of these lineages is not only limited to one district but appears to occur across multiple districts and provinces (Figure 6.1; Figure 6.2A and B), implying that drug resistant TB strains are endemic across the country. However, there is need for further evaluation to investigate the extent of transmission of these strains in Zambia. The Beijing genotype (lineage 2.2) has been associated with diverse drug resistance profiles including XDR-TB and what is termed as “Totally Drug Resistant” (TDR)-TB in parts of the world including South Africa (36-39). This genotype has previously not been described in Zambia. In the current study, we have demonstrated that the Beijing genotype is associated with drug resistant TB in Zambia and of great concern is that the

lineage is associated with pre-XDR-TB and is further being transmitted in the study population.

The lack of strains with mutations in the *tlyA* locus and the low level of *rrs* mutations in loci associated with second line resistance is encouraging as it demonstrates the usefulness of these second line drugs in the management of MDR-TB in the sampled patients. However, more extensive investigations are required to determine the extent of resistance to these critical second line anti TB drugs. Resistance to core anti TB drugs including rifampicin has been demonstrated to be high, raising concerns over the efficacy of the TB control program in managing both drug resistant and drug susceptible TB in Zambia. Mono-resistance to fluoroquinolones and streptomycin has been identified suggesting that the use of these key anti TB drugs in the treatment of other bacterial infections could possibly play a role in development of drug resistance in *M.tb* (40).

Discordant phenotype and genotype findings are concerning as this would lead to inappropriate treatment regimens. Although biological factors may play a role in the discordant samples (e.g. underlying genetic heterogeneity), operational factors should be strongly considered. There is an urgent need to implement quality assurance measures such as the use of genotyping techniques alongside phenotypic DST and inter-laboratory quality assurance checks. For instance, 5 out of the 23 isolates scored as drug resistant at the TDRC TB reference laboratory were scored as susceptible at the Division of Molecular Biology and Human Genetics, Stellenbosch University, demonstrating a level of phenotypic over-scoring at the TDRC TB reference laboratory. However, heterogeneous variants have been identified through targeted gene sequencing in 3 strains (N068, N094 and N452) in the current study, providing a possible explanation for some of the discordant strains (25). A further

consideration is not all drug resistance markers are identified by USAP, therefore some resistance-conferring mutations could have been missed.

The high rate of rifampicin resistant cases observed in this study (82%; 119 strains with confirmed SNV out of 146 drug resistant strains) highlights the need for early case detection through the use of rapid diagnostic tools such as the GeneXpert MTB/RIF assay, whose usefulness in diagnosis of rifampicin resistance in a high HIV prevalence setting has been demonstrated (40). However, the observation of drug resistant strains with no phenotypic or genotypic rifampicin resistance suggests the need for some caution moving forward. Further, there is a need for specialised training and QC in the various centres offering the service in order to minimise on false positive and negative results. The high rate of mono-resistant strains (33%) is alarming and is possibly due to deficiencies in the TB control program; such as inadequate chemotherapy, poor adherence to treatment (12). The resistance-conferring mutations identified in the current study are in keeping with previous studies (3, 4, 7). There is a need to conduct extensive molecular typing studies to acquire further knowledge of how widespread drug resistant strains identified in the current study are across Zambia. This would be made feasible in cooperation with national drug resistance surveillance.

6.8 References

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Chapter 7: Occupational risk of transmission of drug resistant TB in healthcare workers: knowledge, attitudes and practices.

7.1 Introduction

Healthcare workers (HCWs) play a critical role in the management and control of nosocomial transmission of tuberculosis (TB) (1, 2). At the same time, working in TB health care facilities such as hospital wards, diagnostic and treatment facilities increases the risk of acquiring TB due to occupational exposure in HWCs (3, 4, 5). The risk is further heightened in high TB prevalence populations as HCWs are exposed both occupationally and in the community (6, 7). The prevalence of TB in HCWs has been demonstrated to be higher than that of the general population (3, 8). Globally, it is estimated that HCWs are three times more likely to acquire TB, while in high TB burden countries such as South Africa, estimates are as high as six times more than the general population (3, 8). There is however very scarce data on the occupational transmission of multidrug resistant (MDR)-TB and associated risk factors in health care facilities in Africa as a whole, mainly due to poor surveillance and reporting (1). Furthermore occupational exposure to MDR-TB is likely to be minimal in African countries that have not been treating MDR-TB or have recently begun treating MDR-TB (9), resulting in fewer cases of MDR-TB in HCWs. In Zambia for instance, there are no reliable estimates of the incidence of MDR-TB in HCWs. However in one study aiming to assess the feasibility of TB screening in HCWs at TB diagnostic and treatment facilities in Ndola district, the incidence of TB was estimated to be 1% in HCWs (10), lower than the estimated incidence of 6.5% in the general population for Ndola (personal communication with the Ndola district TB focal person). Furthermore, the study recommended establishing screening algorithms for HCWs in Zambia (10).

The highest burden of MDR- and extensively drug resistant (XDR)-TB has been noted in resource-constrained countries, regions also experiencing a TB/HIV co-epidemic (11, 12). Infection with HIV has been associated with an increased risk of nosocomial transmission in

both HCWs and patients (3, 5, 13, 14). The increase in lifestyle diseases in Africa, such as diabetes mellitus, has also been demonstrated to be a further driver in progression of drug resistant TB in the general population and in HCWs (4, 14, 15).

Critical knowledge gaps on infection control measures have been demonstrated in HCWs (14, 15, 16). Infection control practices have been described to be influenced by staffing levels and patient load in TB health care facilities, with HCWs in high burden facilities being less likely to adhere to infection control practices (17, 18).

The usefulness of molecular epidemiological tools in investigating nosocomial transmission of TB in HCWs and patients has been described (6, 7). For instance, in South Africa an endemic XDR-TB strain (designated F15/LAM4/KZN) has been described in patients and HCWs, placing emphasis on the transmission of these highly resistant strains in health care facilities (19, 20). The genotype was further associated with high mortality rates amongst HIV infected patients and HCWs (20). These investigations have demonstrated that both patients and HCWs are at an increased risk of acquiring TB in the health care facility more especially in the absence of infection control and prevention (IPC) measures (6, 7). While no such examples were identified in the current study, the lack of routine screening and lack of data on nosocomial transmission of drug resistant TB in health care workers warrants further studies.

This chapter describes the knowledge, attitudes and practices (KAPs) of health care workers, towards IPC measures, at the Ndola Teaching Hospital (NTH) MDR-TB ward and the Tropical Diseases Research Centre (TDRC) TB reference laboratory in Ndola district. A self-administered KAPs questionnaire which included 34 questions based on demographics (age, gender, profession and highest education level), knowledge of TB transmission, attitude towards IPC policies and IPC practices was developed in consultations with an IPC specialist.

7.2 National TB and Leprosy Control Program (NTLP) Zambia: TB infection prevention and control (IPC) guidelines

The National TB and Leprosy Control Program (NTLP) in Zambia has prescribed guidelines to prevent the transmission of TB in the general population and in health care facilities (21). The guidelines define three core TB IPC measures centred on administration, environmental controls and the use of personal protective equipment (PPE) for all HCWs working in TB health care facilities (21). Administrative controls include the provision of up to date IPC policies, continuous staff training, patient education and community awareness as well as integration of HIV/TB care programs (21). Environmental control measures include safe sputum collection practices such as establishing designated sputum collection points preferably outdoors, increasing natural ventilation in congregate areas and the provision of high efficiency particulate air (HEPA) filters as well as ultraviolet germicidal irradiation (21). (21). The PPE recommended to be important in minimising exposure to aerosols containing bacilli consists of N95 masks/respirators, water proof laboratory gowns, disposable overshoes and gloves (21).

Despite these clear guidelines, personal observations during sample collection and initial processing for this study however suggested deficiencies in IPC practices at the TDRC TB reference laboratory and the NTH MDR-TB ward. Improper IPC practices observed included lack of full PPE during sample processing and patient consultation. In some cases, HCWs were observed using surgical masks instead of N95 masks. It is in this light that the study was developed with the aim of providing some preliminary knowledge on barriers to adhering to TB IPC recommendations. The information gathered in this study will guide larger future studies.

7.3 Data capture and analysis

The study assessed the knowledge, attitudes and practices of HCWs through a self-administered questionnaire which was designed in consultation with an IPC specialist, Dr Dramowski**,** Department of Paediatrics and Child Health Stellenbosch University. The survey questions were developed using previously described methods from published studies and based on guidelines from the World Health Organisation (WHO) and the NTLP guidelines (1, 17, 18, 21). The questionnaire included 32 multiple choice questions and 2 open ended questions based on demographics (age, gender, profession, service length in current employment and highest education level), knowledge of TB transmission, attitude towards IPC policies and IPC practices (appendix 3).

Six out of nine HCWs at the TDRC TB reference laboratory were available to participate in the survey, one was on study leave and two were out of station at the time of data collection. Five out of eight HCWs participated in the survey at the NTH MDR-TB ward, two HCWs were not willing to participate and one was unavailable at the time of data collection. Eight HCWs preferred to answer the questionnaire privately and gave a date for collection of answered questionnaires, while three participants required assistance with entry and descriptions. The captured data was entered and analysed in Microsoft excel.

7.4 Characteristics of health care workers that participated in the survey

Eleven out of 17 health care workers, 5 from the NTH MDR-TB ward and 6 from the TDRC TB reference laboratory, gave consent to participate in the survey in September 2017. The participants included 3 scientists, 3 nurses, 1 laboratory assistant, 1 data entry clerk and 3 cleaners (Table 7.1). The highest levels of education were 1 postgraduate degree (scientist) and 2 undergraduate degrees (scientists) and 4 diploma holders (3 nurses and 1 data entry

clerk), while the lowest level of education was grade 12 secondary school education (1 laboratory assistant and 3 cleaners). All participants at the NTH MDR-TB were female while there were 67% males and 33% females at the TDRC TB reference laboratory. The age range for participants was between 25 and 63 years and the longest serving HCW had served for 26 years at the TDRC TB reference laboratory. At the time of the study, there were no students on attachment at the two institutions. The laboratory manager of the TDRC TB reference laboratory and the clinician in charge of the NTH MDR-TB ward were unavailable to participate in the survey.

Table 7.1: Characteristics of health care workers that participated in the IPC knowledge, attitudes and practices survey.

	TDRC TB reference laboratory (%)	NTH MDR-TB ward (%)
Gender:		
Male	4 (67%)	-
female	2 (33%)	5 (100%)
Age:		
25-35	2 (33%)	-
36-45	3 (50%)	1 (20%)
46-55	1 (17%)	2 (40%)
56-65	-	2 (40%)
Highest level of education:		
Postgraduate degree	1 (17%)	-
Undergraduate degree	2 (33%)	-
Diploma	1 (17%)	3 (60%)
Secondary education (G12)	2 (33%)	2 (40%)
Profession:		
Scientist	3 (50%)	-
Nurse	-	3 (60%)
Laboratory assistant	1 (17%)	-
Data clerk	1 (17%)	-
Cleaner	1 (17%)	2 (40%)
Number of years in service:		
>5	-	1(20%)
6 – 10 years	4 (66.6%)	2 (40%)
11 – 20 years	1 (16.7%)	2 (40%)
21 – 30 years	1 (16.7%)	-

7.5 Knowledge, attitudes and practices of healthcare workers towards IPC practices

7.5.1 Knowledge of TB and symptoms

Assessment of TB knowledge indicates that all participants identified chronic cough and night sweats as symptoms suggestive of TB (Table 7.2). Eighty-two percent associated weight loss as a symptom suggestive of TB while 73% identified coughing up blood and fever as symptoms suggestive of TB. A further 64% identified weakness and chest pains as symptoms suggestive of TB. HIV was identified as a predisposing factor for TB by all participants while only 18% (MDR-TB ward nurses) identified both HIV and diabetes as predisposing factors. At birth *M. bovis* Bacillus Calmette–Guérin (BCG) vaccination was reported as not protective in adult TB by 73% of participants, 27% indicated that at birth BCG vaccination was protective against adult TB.

The knowledge of treatment length for drug susceptible and MDR/RR-TB was generally high amongst HCWs, 82% and 73% respectively, and 91% for knowledge of whether both forms of TB have similar routes of transmission. Two HCWs (cleaners) had not heard of XDR-TB and one indicated that MDR-TB was not as infectious as drug susceptible TB. Most gaps in knowledge were seen in cleaners, and this could be due to the lack of formal training in health sciences and TB IPC practices. This highlights the need to provide TB IPC training for all HCWs, including cleaners, that are in direct contact with patients or working in laboratory facilities.

7.5.2 Infection prevention and control training

All participants reported to have received TB IPC training with the majority (73%) stating that TB IPC training was conducted during induction at the start of the current employment (Table 7.2). Only three participants, scientists from the TDRC TB reference laboratory,

reported to have received TB IPC training after induction, two participants on the 5th of May 2017 and one in 2015. It is concerning that HCWs from the NTH MDR-TB ward and non-scientists at the TDRC TB reference laboratory did not participate in TB IPC training subsequent to that provided at induction.

7.5.3 TB screening and use of personal protective equipment (PPE)

All participants from the TDRC TB reference laboratory were screened for TB at the start of employment while three participants (one nurse and two cleaners) from the NTH MDR-TB ward were not screened at the start of the current employment (Table 7.2). All participants reported that they had not received annual TB screening due to the service not being provided for by their institution, although all were willing to receive annual TB screening.

Participants were further assessed on the use of PPE, 82% of participants reported that their institution provided N95 masks while 18% were unsure (Table 7.2). The majority of participants (64%) reported that they always used N95 masks during sample processing and patient consultation, while 36%, from the NTH MDR-TB ward, reported periodic use of N95 masks. The lack of ongoing TB IPC training at the NTH MDR-TB ward could explain the inconsistencies observed in the use of PPE by the HCWs at the MDR-TB ward and calls for frequent IPC training for HCWs. All participants reported not having received any fit testing for N95 masks. Assessment of HCWs attitudes towards TB IPC measures revealed that all participants were willing to receive TB and HIV screening if they had symptoms suggestive of TB. All participants indicated that they would be willing to disclose to their family and work colleagues if they were diagnosed with TB.

All HCWs that participated in the study indicated that indoor sputum collection areas were high risk areas for TB transmission and outdoor sputum collection areas were low risk transmission areas, this implies that the assessed HCWs have adequate knowledge on the

need for good ventilation as a TB IPC measure. A high proportion of HCWs (82%) showed knowledge that wards and consultation rooms were high risk areas for transmission of TB, this is possibly due to the ongoing campaign using “Stop TB keep doors open” stickers which encourages keeping doors and windows open at all times to allow for adequate air circulation. There are lessons to be learned from the efficiency of this campaign which has translated noticeable stickers in both English and vernacular languages at the various TB diagnostic and treatment facilities in Zambia.

Close to half of the participants indicated that the specimen processing laboratory was not a high risk area for transmission of TB (Table 7.2). An explanation for this could be complacency on the part of HCWs with the lack of direct contact with patients being deemed safer.

In conclusion, 82% of HCWs indicated that TB IPC practices were well implemented in their workplace, despite observed practices. Response bias is a possible limitation to the study. When asked about factors which make their work place unsafe 67% of laboratory personnel indicated that manipulation of live cultures made their work place unsafe and 40% of personnel at the MDR-TB ward (nurses) indicated that lack of staff rotation and low staffing levels made their work place unsafe as other nursing staff were unwilling to work at the MDR-TB ward in preference for lower risk hospital departments and wards.

Table 7.2: Knowledge, attitude and practices of HCWs at the NTH MDR-TB ward and the TDRC TB reference laboratory.

	Yes Number of participants (%)	No Number of participants (%)	Not sure Number of participants (%)
Prior TB diagnosis:	-	11 (100%)	-
Most recent TB IPC training in current job:	-	-	-
Less than 6 months ago	2 (18%)	-	-
2 years ago	1 (9%)	-	-
At induction	8 (73%)	-	-
None received	-	-	-
Knowledge of symptoms suggestive of TB:			
Chronic cough	11 (100%)	-	-
Fever	8 (73%)	-	-
Weight loss	9 (82%)	-	-
Chest pains	7 (64%)	-	-
Night sweats	11 (100%)	-	-
Weakness	7 (64%)	-	-
Coughing up blood	8 (73%)	-	-
Predisposing factors for TB:			
HIV	11 (100%)	-	-
Diabetes	2 (18%)	-	-
Not sure	-	-	-
Does birth BCG vaccine prevent TB in adults?	3 (27%)	8 (73%)	-
High risk areas for TB transmission:			
Wards	9 (82%)	2 (18%)	-
Sputum collection rooms	11 (100%)	-	-
Specimen processing laboratory	5 (45%)	5 (45%)	1 (9%)
Consultation office	9 (82%)	2 (18%)	-
Outdoor sputum collection area	-	11 (100%)	-
Are N95 masks provided for by institution?	9 (82%)	-	2 (18%)
Use of N95 masks when in contact with patients or during sample processing:			
Always	7 (64%)	-	-
Sometimes	4 (36%)	-	-
Has fit testing been done for current N95 mask?	-	11 (100%)	-
TB Screening:			
At the start of employment	8 (73%)	3 (27%)	-
Annual screening	-	11 (100%)	-
Annual screening not done due to service not being offered by employer	11 (100%)	-	-
Elective screening if TB suspected			
Elective HIV testing if TB suspected	11 (100%)	-	-
Willing to inform family, colleagues and manager if diagnosed with TB	11 (100%) 11 (100%)	- -	- -

Table 7.2 (Continued)

	Yes Number of participants (%)	No Number of participants (%)	Not sure Number of participants (%)
Knowledge of treatment length for drug sensitive TB	9 (82%)	-	2 (18%)
Knowledge of treatment length for MDR/RR-TB	8 (73%)	1 (9%)	2 (18%)
Is MDR-TB as infectious as drug susceptible TB?	10 (91%)	1 (9%)	-
Does MDR-TB spread the same way as drug susceptible TB?	11 (100%)	-	-
Are MDR-TB symptoms similar with drug susceptible TB symptoms?	10 (91%)	1 (9%)	-
Knowledge of XDR-TB	9 (82%)	2 (18%)	-

Abbreviations: BCG, *M. bovis* Bacillus Calmette–Guérin; IPC, Infection Prevention Control; MDR, multidrug resistant; NTH, Ndola Teaching Hospital; TB, tuberculosis; TDRC, Tropical Diseases Research Centre; XDR, extensively drug resistant.

7.6 Conclusion

Transmission of drug resistant TB strains in healthcare facilities is driven by several factors centred on lack of adherence to TB IPC practices, and in some cases the lack of existing IPC policies (1, 11). This is reflective of a poor TB national control program (1, 11). Effective administrative IPC measures require the development of policies and administrative support to ensure adherence to the established policies (1, 2). These administrative support measures include staff training and retraining, patient and community education, provision of mandatory PPE to staff and affected patients, and annual TB screening of HCWs (1, 2, 10). These measures together with environmental measures have been described as being effective in the management and control of TB (1).

In the current study, all HCWs indicated that they received TB IPC training with most (73%) reporting that training was received only during induction at the beginning of employment. This is concerning since most HCWs that participated in this study have served in their current employment for more than 5 years. Further, HCWs that reported training within the past 6 months were all scientists implying that HCWs with lower qualifications do not

receive ongoing IPC training. It is therefore recommended that HCWs at all levels be included in annual TB IPC trainings including cleaners (22), who are less knowledgeable but play a critical role in management of drug resistant TB.

There is strong evidence that additional administrative support is required to implement TB IPC policies in the management of drug resistant TB (1, 2, 23, 24). For instance, all HCWs had not received prior fit testing for N95 masks that were in use. This means that there is an increased risk of acquiring TB in the work place as HCWs are not adequately protected due to poorly fitting masks (25, 26). Variation in knowledge has been demonstrated which can be attributed to the different levels of education. There was a low level of knowledge of diabetes being a predisposing factor for TB, with only nurses indicating this point. This could be suggestive of the differences in training curriculum and patient exposure. This highlights the need to incorporate these gaps in curriculum into TB IPC training modules (27).

The absence of routine monitoring of these critical personnel has serious implications for transmission of drug resistant TB. There is a need to implement annual screening and routine TB IPC training. In the current study, it has been demonstrated that barriers for adhering to TB IPC practices in this group of HCWs are mainly due to the lack of administrative support. The HCWs that participated in this study have demonstrated willingness to implement TB IPC practices with the necessary administrative support in place. Overall HCWs had a good attitude towards participating in the survey and knowledge of TB symptoms was generally high. Key areas of improvement are the lack of administrative support mainly centred on provision of fit testing for N95 masks and annual TB screening for all HCWs. Transmission of MDR-TB and pre-XDR-TB has been identified in the study population (discussed in section 6.4.2) and these patients have received care and continue to receive care from HCWs at the NTH MDR-TB ward and the TDRC TB reference laboratory. The risk of transmission

of these drug resistant strains to HCWs is heightened in the absence of adequate IPC measures.

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Chapter 8: General conclusion

8.1 Summary of findings

The current study used molecular strain typing tools, including spoligotyping, IS*6110*-RFLP, whole genome sequencing (WGS) and Sanger (targeted gene) sequencing to describe the genetic characteristics of drug resistant *Mycobacterium tuberculosis* (*M.tb*) isolates diagnosed at the Tropical Diseases Research Centre (TDRC) TB reference laboratory in Ndola district, Zambia. The centre provides culture based phenotypic drug susceptibility testing (DST) to the first line drugs streptomycin, isoniazid, rifampicin and ethambutol (SIRE) to patients from the catchment area; Copperbelt, Luapula and North-Western provinces. Furthermore, an assessment of the knowledge, attitudes and practices (KAPs) of health care workers (HCWs) at the TDRC TB reference laboratory and the Ndola Teaching Hospital (NTH) multidrug resistant (MDR)-TB ward was conducted with the aim of identifying barriers in adhering to TB infection prevention and control (IPC) measures.

The current study identified a high level of genetic diversity amongst drug resistant TB strains diagnosed at the TDRC TB reference laboratory. The majority of strains belong to lineage 4 (LAM11_ZWE, T and X), discussed in section 4.3, which have previously been associated with drug susceptible TB in Ndola district and the surrounding region (1, 2). This implies that drug susceptible strains are probably evolving to become resistant in the study population. Other lineages associated with drug resistant TB were lineage 2 (Beijing and non-Beijing SIT 955) and lineage 3 (CAS1-Kili and Manu3), section 4.3. The lineage 2 genotype, which has previously not been described in Zambia, has been associated with MDR- and extensively drug resistant (XDR)-TB across the world and transmission of these strains has been documented in parts of Africa (3, 4, 5). There is a need for further investigations to determine the extent of transmission of these lineage 2 strains in Zambia and the surrounding region.

The current study demonstrated that genotypes that are driving drug resistant TB are widespread across the sampled districts in Zambia (section 4.2). A high degree of clustering amongst drug resistant TB strains was demonstrated using spoligotyping and IS*6110*-RFLP suggestive of recent transmission (section 4.4). Whole genome sequence analysis further demonstrates the level of strain relatedness with some clustered strains sharing low single nucleotide variant (SNV) differences, as low two SNVs in some genomic clusters (section 5.6). Patients with clustered strains have been demonstrated to originate from the same districts and in some cases from different districts, highlighting how widespread these drug resistant strains and their transmission could be in Zambia.

Alarmingly, transmission of pre-XDR-TB was identified in the current study, which provides a first preliminary insight into the genotypes associated with pre-XDR-TB in Zambia (section 6.6). The predominant genotypes associated with pre-XDR-TB in the study population are lineage 4.6.1 (Uganda), lineage 2.2 (Beijing clade) and lineage 4.3 (LAM). Household transmission was also demonstrated, placing emphasis on the need for routine contact tracing and screening of MDR-TB/rifampicin resistant TB contacts (section 5.7). Contact tracing would improve MDR/RR-TB case detection and allow for early quarantine; this should be de-centralised to district or provincial level allowing patients easier access to health care. The current MDR/RR-TB quarantine does not protect the community well enough as MDR/RR-TB patients are usually expected to travel long distances (over 900 km in some cases) for admission and review at the NTH MDR-TB ward and in most instances patients rely on public transport, placing unknowing passengers at risk of TB infection.

Quarantine of MDR/RR-TB patients in the absence of IPC measures poses a serious risk of nosocomial transmission to patients and HCWs (6). Previous studies have demonstrated that lack of adherence to TB IPC practices is a major driver of nosocomial transmission of drug

resistant TB and TB in general (6, 7). Low adherence to TB IPC practices, such as improper use of personal protective equipment, low knowledge of TB IPC measures and lack of continuous IPC training has been observed amongst health care workers at the MDR-TB facilities in Ndola district (section 7.5). Knowledge gaps have been observed to be higher in HCWs with informal training such as cleaners. And it has further been demonstrated that IPC trainings are not inclusive to all HCWs with laboratory scientists having more opportunities to attend trainings than nurses and other groups of health care workers. This calls for changes to the current TB IPC policy in Zambia to enable frequent IPC trainings that are inclusive of all HCWs. Improved administrative support, such as provision of annual TB screening and fit testing for N95 masks, is required to prevent transmission of drug resistant TB strains in HCWs at the MDR-TB facilities.

Weaknesses have been identified in the TB control program which could be impacting on TB IPC and transmission in Zambia. For instance, a large proportion of patients in the current study had failed treatment (47%), 22% of these patients failed retreatment raising concerns that patients are being provided with an inappropriate treatment regimen or patients are not adhering to treatment, resulting in resistance (section 4.2, Supplementary Table 1). Patients should therefore be subjected to routine DST before commencement of treatment.

In the current study, the incidence of pre-XDR-TB amongst MDR-TB patients (9%) is comparable with WHO estimates (9.5%) suggesting that the current MDR-TB regimen is not entirely efficient in treating MDR-TB in Zambia, section 6.6.2, (8). This places further emphasis on the need for routine second line drug susceptibility testing to monitor treatment response and trends in drug resistance. From the eight pre-XDR-TB cases identified, 37.5% were receiving second line treatment at the time of data collection, 25% had completed treatment, and a further 25% had died while on treatment. Of concern is that 12.5% of these

cases had an unknown treatment status and were lost to follow up by the NTLP (section 6.6.1).

High rates of resistance to other first line drugs pyrazinamide and ethambutol have been demonstrated in the current study. Of great concern is the drug resistance rate (11%) to pyrazinamide which plays an important role in management of MDR-TB in Zambia (9). Currently there is no routine DST for pyrazinamide in Zambia; therefore patients are likely to be placed on an inappropriate treatment regimen (10, 11).

Concerns have arisen over the management of MDR-TB patients with a proportion of patients being lost to follow up (3%) and not being enrolled on second line treatment (7%), similar to trends observed in a previous study in Zambia (12). The current patient clinical records archiving system, which is paper based, poses a high risk of loss of data and does not allow adequate transfer of records between health care facilities. Furthermore, there are gaps in knowledge with registers not being completely filled. In the current study, there were gaps in all variables that were assessed, for instance the HIV status for 26% of patients was not recorded (section 4.2). There is a pressing need to implement an isolate and record banking system for all drug resistant TB strains at referral level. This would allow for future molecular studies to be conducted and provide a better insight into the transmission dynamics of drug resistant TB in Zambia. The current study has demonstrated that drug resistant strains of TB are being transmitted in Zambia. This places emphasis on the need for routine surveillance to monitor drug resistance trends which will inform treatment and management of drug resistant TB in Zambia.

The usefulness of genotyping tools in understanding *M.tb* transmission events has been demonstrated in the current study. Overall WGS provides a superior level of understanding strain relatedness and investigating resistance-conferring mutations in *M.tb,* compared to

IS*6110*-RFLP and spoligotyping. Spoligotyping has been demonstrated to be important in differentiating LCCs while RFLP provides a good resolution of strains, other than LCCs. These techniques are therefore recommended for future studies in Zambia. There is an urgent need to build in-country capacity to enable molecular investigations to be conducted locally. This would require laboratory capacity and training of laboratory and research personnel and would be achieved through local and international funding.

8.2 Limitations

The case detection rate for MDR-TB is low in Zambia, 13% according to WHO (8). This means that there is a high proportion of undetected MDR-TB in the country and a possible underestimation of transmission of drug resistant TB in the current study. In order to improve case detection, there is need for the National TB and Leprosy Control Program (NTLP) in Zambia to initiate active case finding through universal DST for all TB patients, and an integration of HIV and TB care services which would encourage early case detection. Universal DST would however require increasing diagnostic capacity at the three TB reference laboratories that are currently offering first line DST.

Several operational errors were observed during sample processing, data capture and analysis which could impact the findings in the current study. For instance, discordant phenotypes and genotypes could lead to some drug resistant genotypes being missed since the sampling process relied up phenotypic DST findings to select only samples that were phenotypically resistant. Discordant phenotype and genotype findings (described in section 6.5) are concerning and could imply that patients are being over treated. However, the Universal Sequence Analysis Pipeline (USAP) used for WGS analysis in this study does not identify all

resistance-conferring mutations, therefore it is highly likely that some resistance-conferring mutations were missed.

Gaps in data were observed in the TB registers at the TDRC TB reference laboratory and the NTH MDR-TB ward, a challenge which stems from clinic level with clinicians and nurses not always completely filling in patient request forms. This calls for an electronic medical records system accessible by all TB diagnostic and treatment centres, similar to the "SmartCard care" system currently available for HIV management in Zambia (13). There is also a need to collect epidemiological data in order to strengthen molecular findings.

A poor specimen banking system at the TDRC TB reference laboratory meant that only a few strains with corresponding clinical records were revived for genotyping. This means that a wealth of genotype information has been lost for strains that have been associated with drug resistant TB in the past for the study population resulting in underestimation of transmission and genetic variability. There is a need to implement a reliable specimen backing system for drug resistant strains diagnosed at the TDRC TB reference laboratory which will allow future molecular studies to provide a better understanding of the transmission dynamics of drug resistant TB and TB in general in Zambia.

8.3 Future research

To address the operational errors observed in the current study, we aim to compare culture-based phenotypic findings with genotype over a prolonged period of time at the TDRC TB reference laboratory and inter-laboratory comparisons will be made with standard operating procedures. Further, recommendations will be made for retraining and quality control measures such as equipment calibration and assessment of contamination will be emphasised.

In order to get a better understanding of the transmission dynamics of drug resistant TB in Zambia as a whole, countrywide molecular typing of drug resistant *M. tuberculosis* strains will need to be conducted. However to provide a better representation of the transmission dynamics of drug resistant TB in Zambia, there is a need for active case finding. A pilot study is therefore proposed to screen contacts of MDR-TB patients in Zambia and from there determine the incidence of MDR-TB amongst contacts, in relation to strain relatedness.

An expansion on the findings reported in the current study (chapter 7) aiming to investigate the knowledge, attitudes and practices of health care workers towards TB infection prevention and control measures is recommended. It is envisioned that such a study will sample a larger cohort of health care workers and will be inclusive of diagnostic and treatment centres offering services to patients with drug susceptible TB. Furthermore, nosocomial transmission of TB should be investigated with a particular focus on health care workers. The study will also aim to estimate the incidence of latent TB infection amongst health care workers.

Currently, there is little to no information on the role of migration on transmission of drug resistant TB strains in Zambia. The current study was unable to identify strain relatedness between drug resistant strains belonging to lineage 2.2 (Beijing genotype) identified in the study with strains of the same lineage from Cape Town, South Africa (section 5.8). Therefore we aim to assess the role of migration in transmission of drug resistant *M. tuberculosis* strains, through collaborative studies with neighbouring countries in the region. Molecular typing methods will be used to assess strain relatedness amongst drug resistant *M. tuberculosis* strains identified in neighbouring countries and Zambia.

8.4 Conclusion

In the current study we have demonstrated that diverse genotypes are associated with drug resistant TB in parts of Zambia. Genotypes previously associated with drug susceptible TB have been identified to be driving drug resistant TB implying that these genotypes are evolving to become resistant. This is reflective of a weak national TB control program. Pre-XDR-TB has been identified in the current study with an incidence of 9% amongst MDR-TB patients, comparable to WHO estimates of 9.5%. The presence of pre-XDR TB in the study population is concerning as it suggests that the current MDR-TB regimen and management is not efficient in the treatment of MDR-TB.

Alarmingly, these forms of drug resistant TB are being transmitted in the study population with molecular typing techniques demonstrating clustering of these strains. Furthermore, WGS analysis has revealed low single nucleotide variant differences, as low as 2 in some clustered strains. Sanger (targeted gene) sequencing of genes associated with drug resistance has further revealed that some clustered strains have similar drug resistance conferring mutations, implying transmission of these strains. The current study has provided a first insight into the genetics of drug resistant TB strains in circulation in Zambia. These findings add to the gaps in knowledge for Zambia and Africa as a whole.

8.5 Summary of contributions

My contributions to this study included:

1. Protocol development

2. Obtaining necessary ethical approval from the TDRC ethics committee, the Health Research Ethics Committee Stellenbosch University and the National Health Research Authority at the Ministry of Health Zambia;
3. Obtaining approval to collect samples and access clinical data from the Ministry of Community Development Mother and Child Health, the Ndola District Medical Office and the Ndola Teaching Hospital management;
4. Study design and proposal development;
5. Development of a TB infection, prevention and control (IPC) questionnaire;
6. Sample and clinical data collection;
7. Experimental work: *M. tuberculosis* culturing, DNA isolation, spoligotyping, IS*6110* typing and targeted gene sequencing;
8. Data analysis and interpretation;
9. Thesis write up and editing

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Appendices

Appendix 1: PCR primers and melting temperatures for the amplification of genetic elements

Primer name	Primer sequence	Tm (ºC)	Target	Product size (bp)
DRa (reverse)	GGTTTTGGGTCTGACGAC	55	Direct repeat (DR) sequence	Varied 35 to 41
DRb (Forward)	CCGAGAGGGGACGGAAAC			
RTB 59	TGGCCGCGGCGGTCGACATT	78	*rpoB*	437
RTB 38	GGTCAGTGGCCAGCATCGTC	76		
inhA P5	CGCAGCCAGGGCCTCGCTG	55	*inhA* promoter	246
inhA P3	CTCCGGTAACCAGGACTGA			
emb 151	TCCACAGACTGGCGTCGCTG	64	*embB*	260
emb 131	TCCACAGACTGGCGTCGCTG			
rrs290F	TGCTACAATGGCCGGTACAA	62	*rrs*	290
rrs290R	CTTCCGGTACGGCTACCTTG			
pncAF	GGCGTCATGGACCCTATA	60	*pncA*	700
pncAR	GTGAACAACCCGACCCAG			
gyrA For	TGACATCGAGCAGGAGATGC	62	*gyrA*	344
gyrA REV	GGGCTTCGGTGTACCTCATC			
rrs290 F	TGCTACAATGGCCGGTACAA	62	*rrs*	290
rrs290 R	CTTCCGGTACGGCTACCTTG			
tlyA F	CTGGAGTCGGCGGAGAAG	62	*tlyA*	871
tlyA R	GGACGACCAGCAGAACACTG			

Abbreviations: DR, direct repeat; Tm, melting temperature.

Appendix 2: *M. tuberculosis* genomes included in phylogenetic analysis

Isolate name	Accession number	Original study
T92	SRX003589	1
T17	SRX005394	1
95_0545	SRX007721	1
K21	SRX002001	1
K67	SRX002004	1
K93	SRX002005	1
T67	SRX007715	1
T85	SRX003590	1
00_1695	SRX007716	1
98_1833	SRX007718	1
M4100A	SRX007719	1
91_0079	SRX007720	1
K49	SRX002002	1
GM_1503	SRX012272	1
4783_04	SRX007723	1
K37	SRX002003	1
M. canetti	SRX002429	1
HN878_27	PRJNA242362	2
M.bovis (ravenel)	SRR022532	SRA EBI Animal
Mt257	ERR181435	3
H37Rv	ERS153830	4

Appendix 3: Knowledge, attitudes and practices questionnaire: occupational transmission of MDR-TB in health care workers.

The following questionnaire aims to determine the knowledge, attitudes and practices of health care workers at the Ndola Teaching Hospital (NTH) MDR-TB ward and the Tropical Diseases Research Centre (TDRC) TB reference laboratory towards drug resistant TB infection prevention and control (IPC) measures. The study has been cleared by the TDRC ethics committee (ethics number: TDRC STC 2015/9) as part of a larger study which aims to characterise the genetics of drug resistant tuberculosis in Ndola. If you have any further queries concerning ethical approval of this study please contact the ethics secretary at TDRC on the 6th floor of the NTH.

Attached is a self-administered questionnaire which will take approximately 15 minutes to complete. Should you require assistance with filling in this questionnaire, please feel free to ask the researcher/assistant. You are required to answer the questions fully and with sincerity. There will be no personal identifying information used. By filling in this questionnaire you have demonstrated your willingness to take part in this study. Findings from this study will be disseminated to IPC policy makers and recommendations will be made for key areas of training and safety measures.

Questionnaire

Gender: Male ☐ Female ☐ **Age**: ☐

Profession: Doctor ☐ Scientist ☐ Laboratory technologist ☐ Nurse ☐

Data entry clerk ☐ Cleaner ☐ Other (specify) ☐

Highest level of education:

Secondary education (up to G9) ☐ Secondary education (G12) ☐ Diploma ☐

Undergraduate degree ☐ Postgraduate degree ☐

Number of years in service: ☐

Have you been diagnosed with active TB disease in the past? Yes ☐ No ☐

Have you received training on TB infection prevention and control in your current job?

Yes ☐ No ☐

If yes, how long ago was the training? ☐

What type of training was received? In-service ☐ Lecture ☐ Course ☐

Induction at start of employment ☐ Other (specify) ☐

Which of the following would you consider to be symptoms suggestive of active TB?

Chronic cough ☐ Fever ☐ Weight loss ☐ Chest pains ☐

Night sweats ☐ Weakness ☐ Coughing up blood ☐ Not sure ☐

Which of the following would you consider to be predisposing factors for TB disease?

HIV ☐ Diabetes ☐ Not sure ☐ Other (specify) ☐

Does birth vaccination with BCG prevent TB in adults?

Yes ☐ No ☐ Not sure ☐

Which of the following do you consider to be high risk areas for transmission of TB:

Wards ☐ Sputum collection rooms ☐ Specimen processing laboratory ☐

Consultation office/station ☐ Outdoor sputum collection area ☐

Other (specify) ☐

Are N95 respirators (masks) provided by your institution? (Refer to attachment for pictures of a surgical mask & N95 mask**)** Yes ☐ No ☐ Sometimes ☐

Do you use N95 respirators (masks) when in contact with TB patients and/or when processing specimens?

Always ☐ Sometimes ☐ No ☐ I use surgical masks ☐

If no, what are your reasons? ☐

If yes, have you received fit testing for the mask type you are currently using?

Yes ☐ No ☐

If yes, how long ago? Less than 2 years ☐ 2-5 years ☐ 5 years + ☐

Were you screened (x-ray, smear microscopy, culture, tuberculin skin test) for TB at the start of your current job? Yes ☐ No ☐

Do you receive annual TB screening? Yes ☐ No ☐ sometimes ☐

If no, why not? I do not want to be screened ☐ service is not offered ☐

Other (specify) ☐

Would you receive screening if you had symptoms suggestive of TB?

Yes ☐ No ☐ Not sure ☐

If diagnosed with TB, would you be willing to test for HIV?

Yes ☐ No ☐ Not sure ☐

Would you tell your family if you were diagnosed with TB?

Yes ☐ No ☐ Not sure ☐

Would you tell your manager/supervisor if you were diagnosed with TB?

Yes ☐ No ☐ Not sure ☐

Would you tell your work colleagues if you were diagnosed with TB?

Yes ☐ No ☐ Not sure ☐

If no, why not? ☐

What is the recommended treatment length for drug sensitive TB in Zambia?

1 month ☐ 3 months ☐ 6 months ☐ until a patient is culture or/and smear negative ☐ Until a patient feels better ☐ Not sure ☐

Which of the following is the recommended treatment length for MDR-TB in Zambia?

6 months ☐ 12 months ☐ 20 months + ☐ until a patient is culture or/and smear negative ☐ Until a patient feels better ☐ Not sure ☐

Do you think MDR-TB is as infectious as drug sensitive TB?

Yes ☐ No ☐ Not sure ☐

Dose MDR-TB spread the same way as drug sensitive TB?

Yes ☐ No ☐ Not sure ☐

Are MDR-TB symptoms similar to drug sensitive TB symptoms?

Yes ☐ No ☐ Not sure ☐

Have you heard of extensively drug resistant (XDR)-TB?

Yes ☐ No ☐

How well do you feel TB-IPC is implemented at your workplace? ☐

What are the factors that make your workplace an unsafe working environment for TB transmission? ☐

Appendix 4: Ethics clearance and research approval letters

TROPICAL DISEASES
Tel/Fax +260212 615444
P O Box 71769
tdrc-ethics@tdrc.org.zm
NDOLA, ZAMBIA

RESEARCH CENTRE

TDRC ETHICS REVIEW COMMITTEE
IRB REGISTRATION NUMBER : 00002911
FWA NUMBER : 00003729

TRC/C4/09/2015

25th September 2015
Namaunga Kasumu Chisompola
C/O Copperbelt University
School of Medicine, Ndola Central Hospital
P.O Box 71191, Ndola.

Dear Namaunga Kasumu Chisompola

RE: ETHICAL APPROVAL OF STUDY PROTOCOL – TDRC STC/2015/9

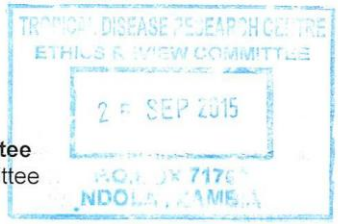
Reference is made to the above mentioned concerning your protocol entitled **"Genetic Characterization of Drug Resistant Clinical Isolates of Mycobacterium Tuberculosis Circulating within Ndola District: TDRC Registration number STC/2015/11."**

On behalf of the Chairman of the TDRC Ethics Review Committee, I am pleased to inform you that your protocol was reviewed and granted ethical approval.

You are required to submit at least two (2) progress reports annually. A final report to the Ethics Review Committee should also be submitted at the end of the study. The Committee shall not provide ethical renewal of on-going project in absence of progress reports. This approval is valid for the period 25th September 2015 to 25th September 2016. The Committee wishes you and your team success in the execution of the study.

Yours faithfully
TROPICAL DISEASES RESEARCH CENTRE

Shepherd Khondowe
SECRETARY – TDRC Ethics Review Committee
cc: Chairman – TDRC Ethics Review Committee



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Approved with Stipulations

New Application

11-Sep-2015
Chisompola, Namaunga N

Ethics Reference #: S15/08/172

Title: **Genetic characterization of drug resistant clinical isolates of Mycobacterium tuberculosis circulating within a high HIV prevalence district of Zambia.**

Dear Ms Namaunga Chisompola,

The **New Application** received on **12-Aug-2015**, was reviewed by Health Research Ethics Committee 1 via Committee Review procedures on **02-Sep-2015**.

Please note the following information about your approved research protocol:

Protocol Approval Period: **02-Sep-2015 -01-Sep-2016**

Present Committee Members:
Weber, Franklin CFS
Unger, Marianne M
Els, Petrus PJJS
Dosi, Alswald A
Lachman, Anusha A
Barsdorf, Nicola N
Botha, Paul JP
Rohland, Elvira EL
Hoek, Kim KGP
Glashoff, Richard RH
Philander, Cynthia C
Werely, Cedric CJ
Hendricks, Melany ML
Ferris, William WF
Welzel, Tyson T
Potgieter, Sunita S

The Stipulations of your ethics approval are as follows:
Kindly submit signed investigator declarations from Dr E Streicher, Prof R Warren and Prof S Sampson.

Please remember to use your **protocol number** (**S15/08/172**) on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review:
Please note a template of the progress report is obtainable on www.sun.ac.za/rds and should be submitted to the Committee before the year has expired. The Committee will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.
Translation of the consent document to the language applicable to the study participants should be submitted.

Federal Wide Assurance Number: 00001372
Institutional Review Board (IRB) Number: IRB0005239

The Health Research Ethics Committee complies with the SA National Health Act No.61 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 Part 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Contact persons are Ms Claudette Abrahams at Western Cape Department of Health (healthres@pgwc.gov.za Tel: +27 21 483 9907) and Dr Helene Visser at City Health (Helene.Visser@capetown.gov.za Tel: +27 21 400 3981). Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.
For standard HREC forms and documents please visit: www.sun.ac.za/rds

If you have any questions or need further assistance, please contact the HREC office at 0219399657.

Included Documents:
CV RM Warren
Application form_signature page
Declaration N Chisompola
Protocol
Checklist
Evaluation committee reponse letter - S Sampson
Declaration S Sampson
Participant information leaflet & consent form
Declaration M Tembo
Data capture sheet
Checklist
Declaration K Webster
MTA draft
Protocol Synopsis
CV N Chisompola
CV E Streicher
Application form
CV K Webster
Payment instruction form
Response to ERES Converge IRB from student
CV M Tembo
Declaration RM Warren
KNO Approval email
KNO feedback to candidate_supervisor
Declaration E Streicher
CV S Sampson
Cover letter
ERES Converge IRB feedback

Sincerely,

Franklin Weber
HREC Coordinator
Health Research Ethics Committee 1

THE NATIONAL HEALTH RESEARCH AUTHORITY
C/O Ministry of Health
Ndeke House
P.O. Box 30205
LUSAKA

MH/101/23/10/1
In reply please quote:

No.....

29 October, 2015

Mrs. Namunga Kasumu Chisompola
Copperbelt University
School of Medicine
P. O. Box 21692
Ndola

Dear Mrs. Chisompola,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Genetic characterization of drug resistant clinical isolates of *Mycobacterium* tuberculosis circulating within a high HIV prevalence district of Zambia."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been approved to carry out the above mentioned exercise on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Dr. I. Muteba
For/Director
National Health Research Authority

Republic of Zambia
MINISTRY of COMMUNITY DEVELOPMENT, MOTHER AND CHILD HEALTH
NDOLA DISTRICT COMMUNITY HEALTH OFFICE
P.O.BOX 70672, NDOLA, 1307 NAIDU CLOSE, KANINI, NDOLA
Tel: (260) 2 612819 Fax: (260) 2 612819

11th November, 2015

Mrs. Namaunga Kasumu Chisompola
Ndola Central Hospital
P. O. Box 21692
NDOLA

Dear Madam,

REF: **PERMISSION TO ACCESS DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS ISOLATES FOR THE PURPOSE OF PHD TRAINING**

My office has no objection to your request to access drug resistant mycobacterium tuberculosis clinic isolates and accompanying clinical data for Ndola. Kindly ensure you adhere to the approved protocol and keep the district as well as the Ministry informed about your research whenever necessary

Yours Faithfully
NDOLA DISTRICT MEDICAL OFFICE

Dr. Kakungu M. Simpungwe
DISTRICT MEDICAL OFFICER

REPUBLIC OF ZAMBIA

All correspondence should be addressed to the
Senior Medical Superintendent
Ndola Central Hospital
Postal Agency
NDOLA

Telephone: 611585-9
Fax: 612204
E-mail: *nch @ zammet.zm*

MINISTRY OF HEALTH
NDOLA CENTRAL HOSPITAL

20th October 2015

Mrs. Namaunga k. Chisompola
Copperbelt University
NDOLA

Dear Sir

RE: ACCESS TO DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATES.

Reference is made to your letter dated 13th October 2015 regarding the above mentioned subject matter.

I am pleased to inform you that Management has no objection for you to come and carry out your research at this institution.

While at this institution, you will not be entitled to any salary or accommodation. You will also be expected to abide by Ndola Central Hospital Disciplinary Code and Regulations.

Please report to the Human Resources Department as soon as you arrive at the hospital.

Yours faithfully
NDOLA CENTRAL HOSPITAL

CHIPO C.S.SIKE
SENIOR HUMAN RESOURCES MANAGEMENT OFFICER
For/ SENIOR MEDICAL SUPERINTENDENT

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH
SNR. H. R. MANAGEMENT OFFICER
NDOLA CENTRAL HOSPITAL

CC: Senior Medical Superintendent
HOD – Laboratory
HOD – Chest Clinic
Human Resources – Training

References

1. Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, *et al*. Human T cell epitopes of *Mycobacterium tuberculosis* are evolutionarily hyperconserved. Nat Genet 2010; 42: 498–503.
2. Domenech P, Rog A, Moolji JU, Radomski N, Fallow A, Leon-Solis L, et al. Origins of a 350-Kilobase Genomic Duplication in Mycobacterium tuberculosis and Its Impact on Virulence. Infect Immun. 2014; 82(7): 2902-2912.
3. Blouin Y, Hauck Y, Soler C, Fabre M, Vong R, Dehan C, *et al*. Significance of the identification in the Horn of Africa of an exceptionally deep branching *Mycobacterium tuberculosis* clade. PloS One 2012;7:e52841.
4. Coscolla M, Lewin A, Metzger S, Maetz-Rennsing K, Calvignac-Spencer S, Nitsche A, *et al*. Novel *Mycobacterium tuberculosis* complex isolate from a wild chimpanzee. Emerg Infect Dis 2013; 19: 969–976.