Molecular characterization of *Mycobacterium tuberculosis* isolates and their association to multidrug resistance in Lusaka, Zambia

（ザンビア共和国Lusaka市で分離された患者由来結核菌株の遺伝学的特徴と多剤耐性との関連に関する研究）

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ABBREVIATIONS

AIDS	Acquired Immuno deficiency virus
BCG	Bacille Calmette-Guerin
DOTS	Directly Observed Therapy Short Course
DR	Direct Repeat
HIV	Human immuno deficiency Virus
IS *6110*	Insertion Sequence 6110
LSP	Large Sequence Polymorphism
MDR	Multi Drug resistance
MDR-TB	Multidrug resistant tuberculosis
MIRU VNTR	Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeats
MoH	Ministry of Health
mRNA	Messenger Ribonucleic Acid
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length polymorphism
RR	Rifampicin resistant
RRDR	Rifampicin Resistance Detection Region
STH-PAS	Single-strand Tag Hybridization Chromatographic Printed Array Strip
TB	Tuberculosis
WHO	World Health organization

PREFACE

It has been over twenty years after declaring tuberculosis (TB) a disease of public health emergence by WHO in 1993, but TB has continued to inflict mankind. Globally, TB is one of the top 10 causes of death, and the world's top infectious disease killer (above HIV/AIDS) (1). Geographically, most people who developed TB in 2019 were in the WHO regions of South-East Asia (44%), Africa (25%) and the Western Pacific (18%) (Figure 1) (1).

TB claims more than a million lives each year and affects millions more, with enormous impact on families and communities. The disease typically affects the lungs (pulmonary TB) but can also affect other organs of the body (extra pulmonary TB). TB can affect anyone and anywhere, but most people who develop the disease (about 90%) are adults. There are more cases among men than women. Men aged 15 years and above accounted for 56% of the people who developed TB in 2019. Women accounted for 32% while children, aged less than 15 years accounted for 12% (1).

Worldwide TB incidence and deaths are falling. However, the pace is not fast enough to reach global TB targets set by WHO End TB strategy which include 90% and 95% reduction by 2035 in the TB incidence rate and annual TB deaths, respectively (Figure2).

The bacteria that cause TB belong to a group known as *Mycobacterium tuberculosis* (MTB) complex which comprises of closely related microorganisms. The members of MTBc are as follows; *Mycobacterium tuberculosis*, *Mycobacterium canetti*, *Mycobacterium africanum, Mycobacterium bovis*, *Mycobacterium caprea*, *Mycobacterium pinnipedii* and *Mycobacterium microti.* The natural host for *Mycobacterium tuberculosis* is mainly humans and this organism is responsible for the typical human TB disease in most part of the world (2).

TB is curable and preventable. Most people (about 85%) who develop TB disease can be successfully treated with a 6-month drug regimen (1). However, a number of factors have been elucidated as drivers of this disease. These risk factors include HIV infection, drug addition, diabetes, malnutrition, smoking and air pollution (3).

Furthermore, the emergency of drug resistant TB cases (organisms can grow in the presence of one or more anti-TB drugs) is threatening global efforts to control TB and threatens to reverse global progress made in TB control so far (4). While the incidence and mortality of drug susceptible TB is generally on the decline (Figure 2), the incidence of drug resistant TB has been described to be variable by country (1), with increasing incidences observed in some countries across the world (Figure3).

Worldwide in 2019, an estimated 465,000 people developed rifampicin-resistant TB (RR-TB), of which 78% had multidrug-resistant TB (MDR-TB) (defined as resistance to rifampicin and isoniazid). An estimated 182,000 deaths resulted from MDR/RR-TB. (10). In rates terms, an estimated 3.3% of new cases and 18% of previously treated cases had MDR/RR-TB globally in 2019 (1).

Factors associated with the emergence of drug resistance in new TB cases have been reported to include poor adherence to treatment, limited effective drugs and inadequate health care systems while primary drug resistance has been attributed to poor infection prevention and control measures leading to increased transmission (5).

In Zambia (a Sub Sahara African country) , despite the implementation of Directly Observed Therapy Short Course (DOTS) program and universal BCG vaccination, TB remains a disease of major public health concern and the country is enlisted among the 30 high TB burden countries by WHO due to its high TB incidence (333/100,000 population) (Figure 4).

Most recently (2021), WHO has reclassified Zambia as one of the high MDR/RR-TB burden countries (6). This is consistent with the in-country trend analyses which have demonstrated increasing threat of MDR-TB burden. For instance, the review of national data by Kapata et al (2013) highlighted four-folds increase in the number of MDR-TB patients notified by National TB control program (NTP) between 2000 and 2011 (7) (Figure 5). By 2019, the number of MDR/RR-TB notified by Zambia National TB control Program had increased to 500 cases per annum (8).

In the region, similar trends have been observed in the neighboring Botswana where the 4th national TB drug resistant survey reported a three-fold increase in MDR-TB rates (among new patients) relative to the preceding surveys (Figure 6) (9). By 2019, the proportion of MDR-TB in new cases in Botswana had risen to 3.6% (8).

Furthermore, high MDR-TB prevalence rates (7.7% in new cases and 33.8% in previously treated patients) have been reported from Swaziland by the national TB drug resistance survey [10]. While HIV/AIDS and social economic determinants have been linked to the high TB burden in Zambia and other countries in the region (11), factors driving the increase in MDR-TB burden are still unclear.

Although it is unquestionable that most factors responsible for TB pandemic are related to socioeconomic dynamics and insufficient health care systems among others, factors directly

related to the microorganism itself are also significant but they are less studied in the third world countries (12). Molecular understanding of a causative agent (*M. tuberculosis*) can provide an important study platform to investigate possible association of its strains with clinical and epidemiological characteristics.

Today, several techniques have been developed for molecular epidemiological investigations of *M. tuberculosis* strain diversity and these include 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) (13–15). Additionally, next generation whole genome sequencing (WGS) of *M. tuberculosis* clinical isolates provides invaluable knowledge on genetic diversity and evolution of drug resistance in the *M. tuberculosis* genomes in circulation (15). Whole genome sequencing is preferred to other typing techniques due to its robustness and high resolution, however, it does not negate the usefulness of other typing tools due to limitations experienced in resource limited countries.

Although spoligotyping is less discriminatory compared to IS*6110*-RFLP and MIRU-VNTR, this assay is rapid, inexpensive and robust therefore it is often used as a first-line genotyping method. It is the basis for the differentiation of major genotypes of *M. tuberculosis* such as Beijing, Euro-American sub-lineages, and Central Asian (CAS) families (13).

Some of the spoligotype families are distinctively distributed in specific geographical regions (17). This could signify that they are probably better adapted to certain human populations in those areas (18). For instance, Beijing spoligofamily is predominantly found in far-east Asia whereas Euro-American sub-lineages are predominant in Africa, Europe and the Americas (19).

The Latin-American Mediterranean (LAM) family has been described as the most prevalent *M. tuberculosis* lineage globally, accounting for approximately 15% of the global TB burden [19]. However, little is known about its epidemiology, biological behavior and disease patterns [12]. Different *M. tuberculosis* genotypes have been linked to cause drug resistant diseases and TB outbreaks in various regions (20).

There are strong indications that various lineages of *M. tuberculosis* have different biological characteristics which may influence the TB epidemiology (21). For instance, the Beijing genotype is suggested to be a possibly resistant clone against BCG vaccination, highly

pathogenic, transmissible and prone to becoming drug resistant (22). Despite this growing body of knowledge, most TB control strategies are generic with the supposition that all *M. tuberculosis* strains are equal in terms of transmission dynamics, virulence and drug resistance (22).

In various parts of Africa, diverse *M. tuberculosis* genotypes are driving the epidemiology of drug resistant TB and varied genotypes have been reported across the continent (Figure 7) (23).

M. tuberculosis is intrinsically resistant to many antibiotics, limiting the number of compounds available for treatment. This intrinsic resistance is due to a number of mechanisms including a thick, waxy, hydrophobic cell wall and the presence of drug degrading and modifying enzymes. However, by employing various modes of drug action, a number of drugs including rifampicin and isoniazid (two most powerful first line TB-drugs) have shown efficacious in the treatment of *M. tuberculosis* disease (Table 1). Eventually, resistance to those drugs (active against *M. tuberculosis*) has emerged and is conferred mainly by genetic polymorphism (24). For instance, resistance to rifampicin and isoniazid has been associated to mutations in *rpo*B and *kat*G genes of *M. tuberculosis,* respectively (25, 26). These chromosomal mutations may confer drug resistance via modification or over-expression of the drug target, as well as by prevention of prodrug activation (24) (Table1).

Furthermore, studies have reported that the rate of mutations causing drug resistance varies according to the lineage to which the strain belongs. For instance, the Beijing family has demonstrated increased mutation rates *in vitro* compared to the estimated probabilities for the acquisition of resistance by spontaneous mutation which is approximately 1 in 10^8 bacilli for rifampicin and 1 in 10^6 bacilli for isoniazid (27).

Broad understanding of those mutations encoding resistance in a specific geographic setting is valuable knowledge for the development and application of new vaccines, drugs and molecular diagnostic tools and understanding the epidemiology of drug resistant TB (28).

Although the overwhelming burden of TB is in developing countries, molecular characteristics of *M. tuberculosis* have been studied more in industrialized countries than in non-industrialized nations. For instance, in the United States of America nearly each newly identified culture-positive case of tuberculosis is genotyped whereas in the third world

countries, where the burden of TB and drug resistant TB is relatively high, genotyping of identified *M. tuberculosis* strain is not routinely done.

In Zambia, *M. tuberculosis* genotyping has locally been conducted by two studies, namely; Mulenga et al (2010) in Ndola district and Malama et al (2014) in Namwala district (29, 30). Both studies reported LAM family as the predominant *M. tuberculosis* genotype circulating in the studied districts. However, neither of those two studies analyzed detailed information relating to the Spoligotype International Types (SIT) of the identified *M. tuberculosis* families nor did they contextualize identified genotypes in relation to drug resistance. Furthermore, the *M. tuberculosis* genotypes reported by Mulenga et al and Malama et al were specific for the studied districts. To my knowledge, the *M. tuberculosis* genotypes isolated in Lusaka (Zambia's capital city) and their association to multi-drug resistance has not been analyzed. Besides, *M. tuberculosis* mutational patterns and frequencies encoding drug resistance to rifampicin and isoniazid were un-investigated in Zambia before my study.

For my PhD project, I have utilized spoligotyping to genotype *M. tuberculosis* cultures isolated from TB patients mainly residing in Lusaka city and stored at the University Teaching Hospital, TB laboratory in Lusaka. Furthermore, I sequenced *M. tuberculosis* genes associated with drug resistance encoding mutations to rifampicin and isoniazid among MDR-TB isolates from Lusaka and compared the identified mutational frequencies and patterns to those reported in the African region.

Lusaka has the population of 3.3 million people and is both a commercial and administrative town. In addition, it is a getaway connecting the country's four main highways to the neighboring countries on the north, south, east and western part of the continent and hosts the main international airport connecting the country to the globe (Figure 8).

During the 2013 –2014 national TB prevalence survey, Lusaka reported a high prevalence of bacteriologically confirmed TB of 932/100,000 population [31]. Furthermore, Lusaka recorded the highest proportion of MDR-TB patients in the country notified by the national TB program in 2019 (Ministry of Health, 2020).

In chapter I of this thesis, I have described *M. tuberculosis* sub-lineages and documented their correlation with MDR-TB. Furthermore, I have illustrated gene mutations in *rpo*B and *kat*G genes and *inh*A operon conferring resistance to rifampicin and isoniazid, respectively, in chapter II. Additionally, I have shown comparisons in the frequencies of specific mutations identified among *M. tuberculosis* isolates from Lusaka with those reported by others in the region.

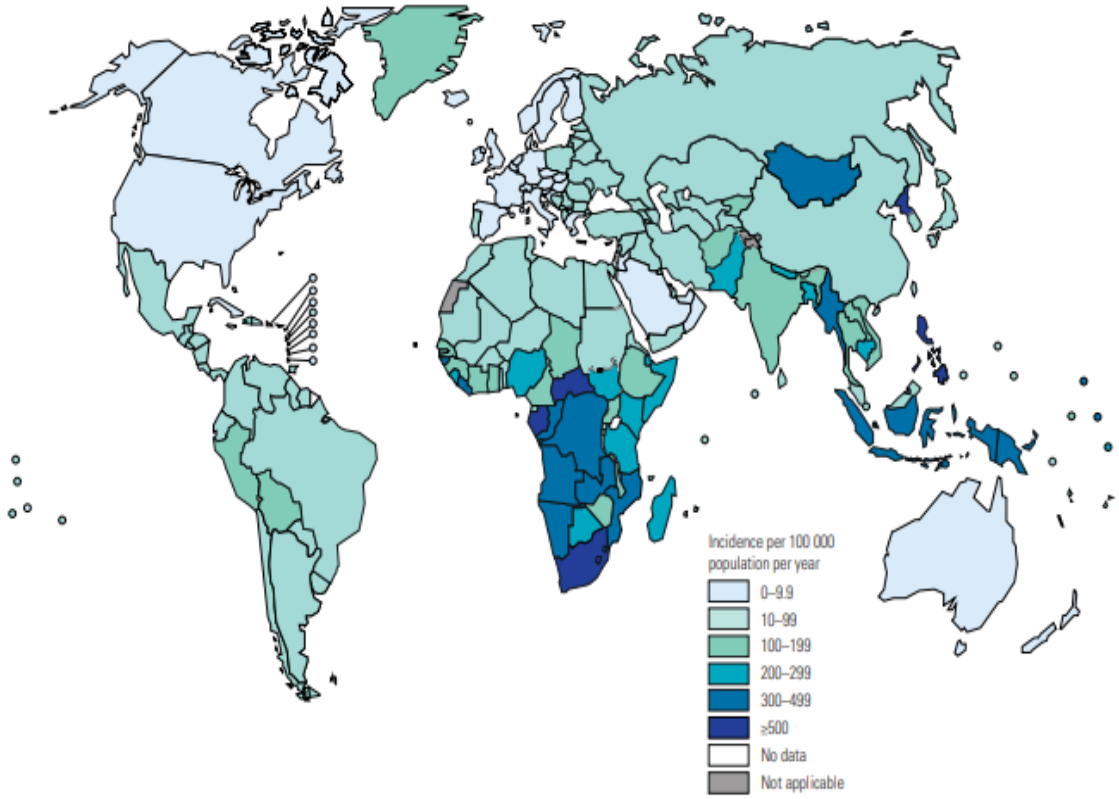


Figure 1. Estimated TB incidence rates in 2019 (WHO, 2020)

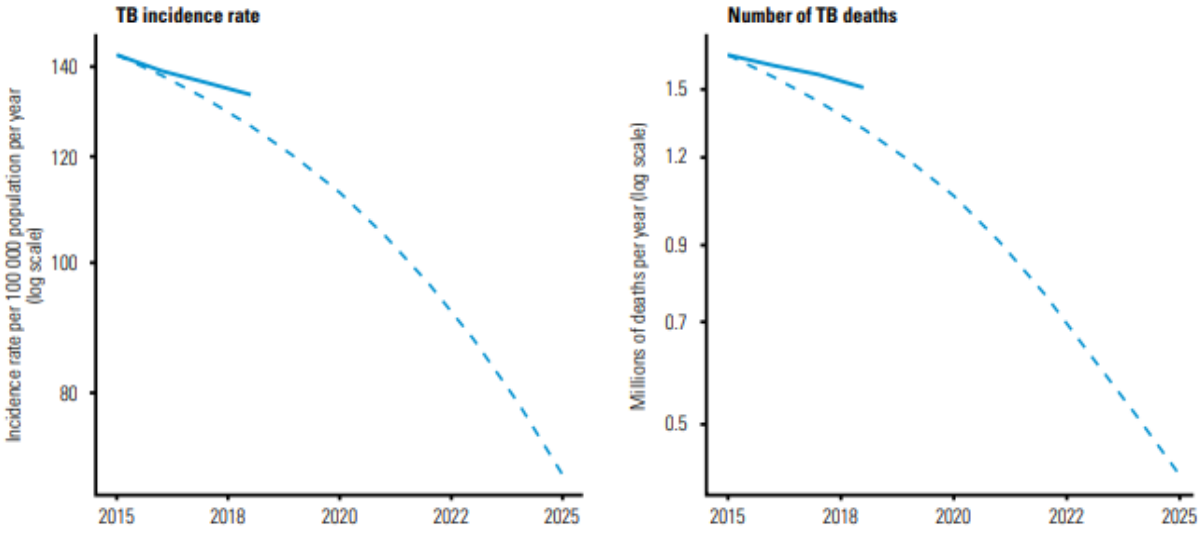
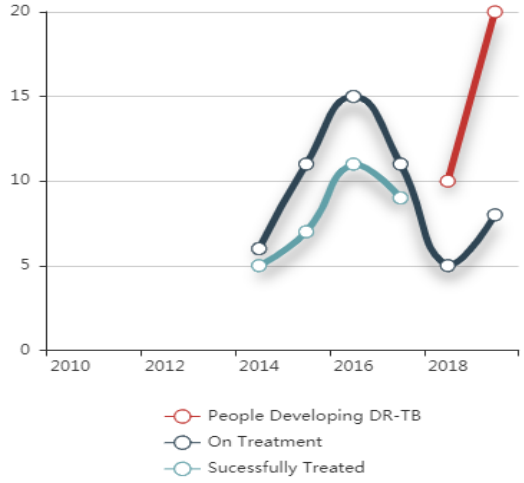
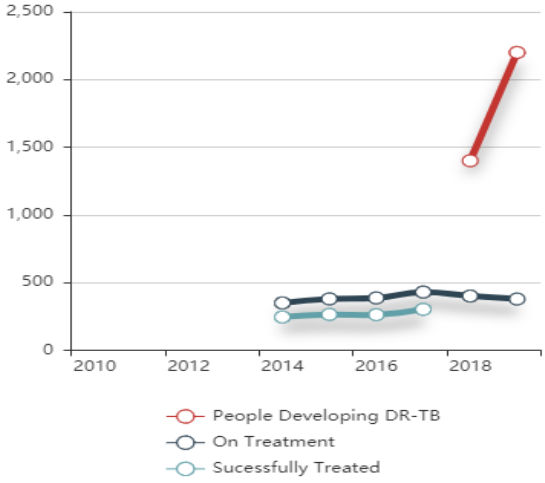


Figure 2. Global trends in the TB incidence rate and TB mortality (solid lines) compared with set milestones (at 2025) of the End TB strategy (dashed lines) (WHO 2020).

a) Netherlands



b) Nepal



C) Malawi

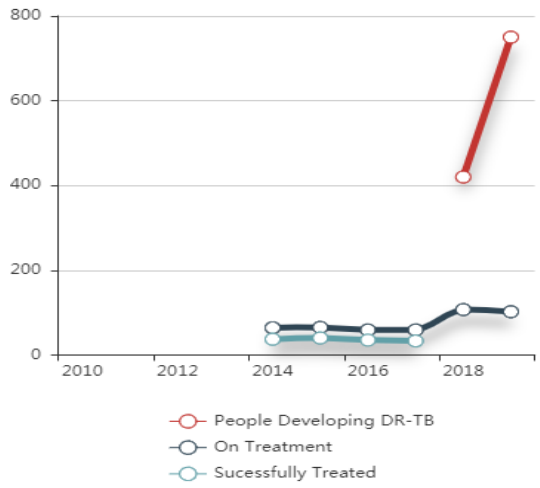


Figure 3. Countries with increasing incidence of DR-TB (STOP TB Partnership, 2020)

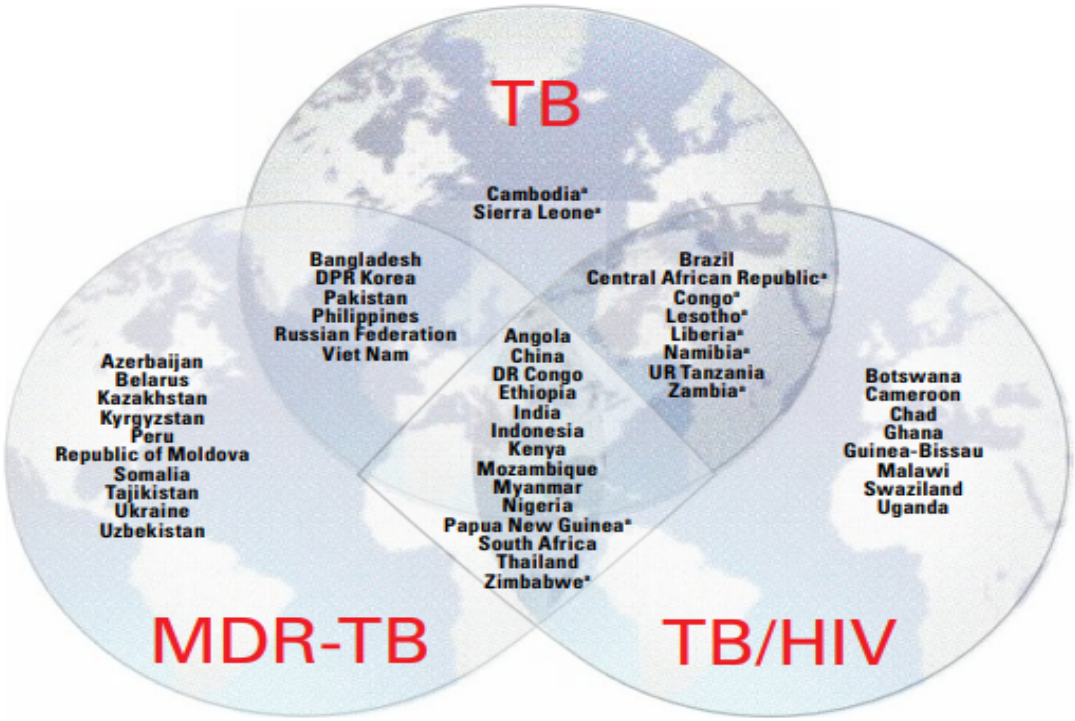


Figure 4. Countries in the three high-burden country lists for TB, TB/HIV and MDR-TB (WHO 2020)

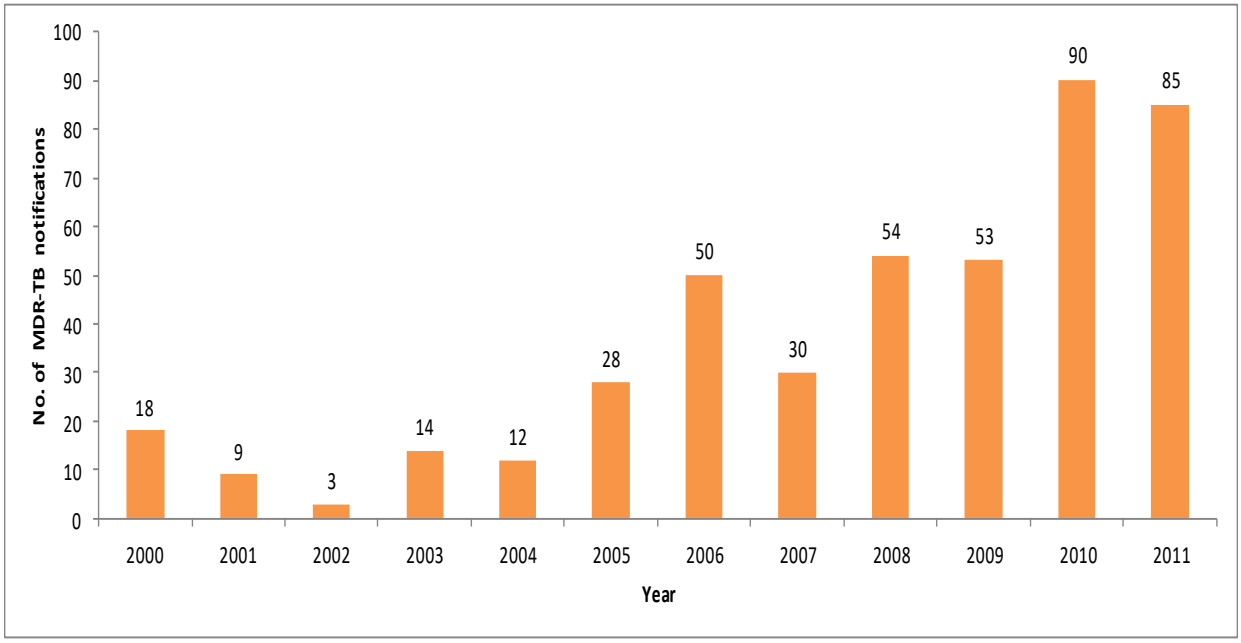


Figure 5. MDR-TB notifications in Zambia (Kapata et al, 2013)

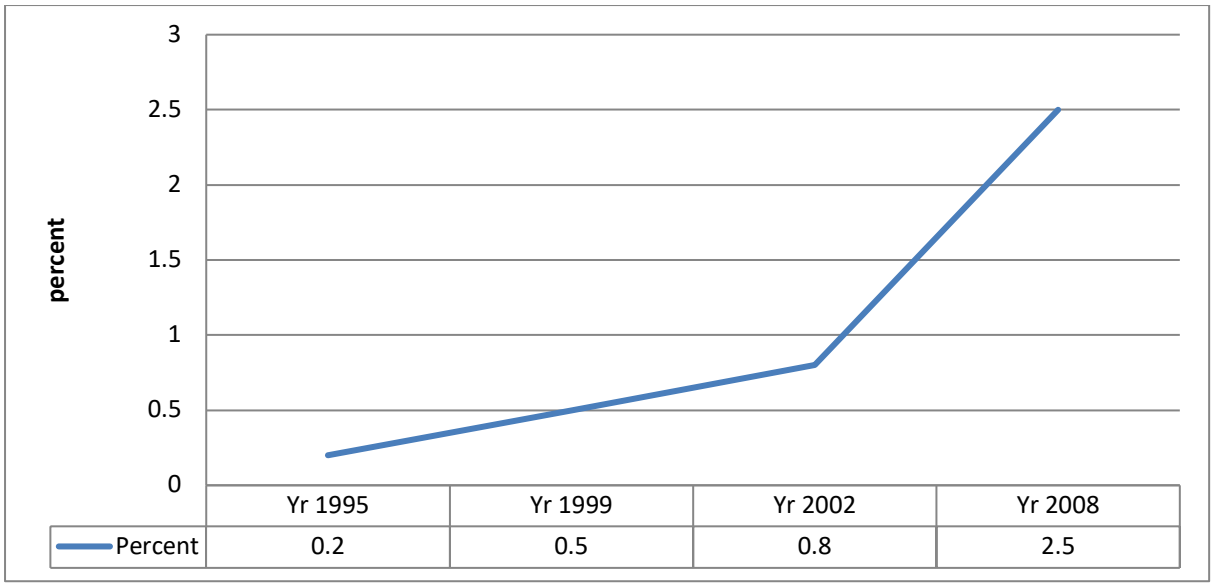


Figure 6. Trends in proportion of patients with MDR-TB in Botswana's four national TB drug resistance surveys, 1995–2008 (Menzies et al, 2014)

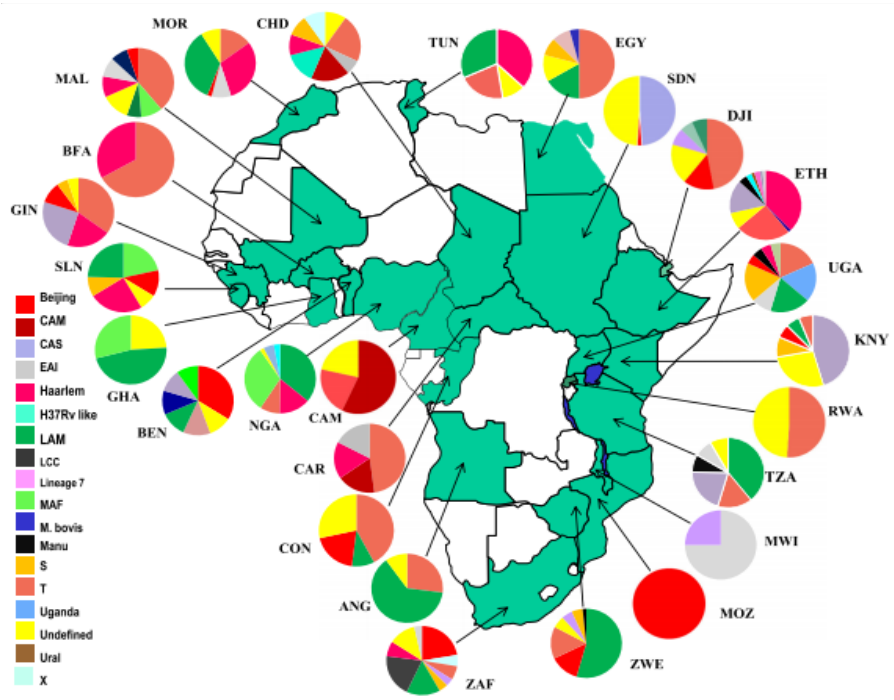


Figure 7. *M. tuberculosis* genotypes associated with drug resistant TB in Africa (Chisompola et al, 2020)

Table 1. Mode of action for rifampicin and isoniazid and the mechanisms of acquiring drug resistance by *M. tuberculosis*.

Drug	Mode of Action	Mechanisms of resistance
Rifampicin	Binds to β-subunit of RNA polymerase. Inhibition of elongation of mRNA.	Mutations in *rpo*B gene (codons 507-533).
Isoniazid	Pro-drug Activated by catalase peroxidase to reactive Isoniazid–NAD products.	Mutations in *kat*G gene (which encodes catalase peroxidase enzyme).
	Inhibits mycolic acid synthesis via disruption of the NAD-dependent enoy-acyl carrier protein reductase.	Mutations in *inh*A promoter region of *inh*A gene.

Zhang et al, 2009 (25)

Figure 8. Location of Lusaka city, Zambia

CHAPETER I

Characterization of *Mycobacterium tuberculosis* genotypes and their correlation to multidrug resistance in Lusaka, Zambia

Introduction

Worldwide, an estimated 10 million people fell ill with tuberculosis (TB) in 2019. Of this figure, most TB cases occurred in the WHO regions of South-East Asia (44%), Africa (25%), and the Western Pacific (18%) (1). An estimated 1.2 million deaths occurred due to TB among HIV negative people and 251,000 deaths among HIV-positive individuals (1). Globally, TB is one of the top ten causes of death and the leading cause from a single infectious agent above HIV/AIDS (1). With early case detection and adequate health care, TB is curable and preventable.

The emergence of multidrug-resistant (MDR) *M. tuberculosis* strains, simultaneously resistant to rifampicin and isoniazid, is among complex factors impeding the control of TB. Globally, 3.4% of new TB cases and 18% of previously treated cases had MDR-TB or rifampicin-resistant TB (MDR/RR-TB) in 2019 (1).

In Zambia, a high TB burden nation, the proportions of MDR-TB have risen from 0.3% among new cases and 8.1% among previously treated cases in 2014 to 2.8% in new cases and 18% in previously treated cases in 2019 (1). Local studies have also reported an emerging threat of MDR-TB in the country. For instance, a survey in Zambian prisons reported the MDR-TB rate of 9.5% (32). Another study that reviewed TB national data observed an increasing trend (four-fold between 2000 and 2011) of MDR-TB cases being detected by the national TB control program (7). Presently, the factors associated with this increase are not clear.

Although evidence on the association between MDR-TB and HIV infection is scanty, suggestions linking HIV infection with primary MDR-TB has been made (33). Researchers in sub-Sahara Africa have speculated that in populations where HIV infection is coupled with socioeconomic challenges, poor treatment adherence and lack of access to proper treatment may contribute to the development of acquired drug-resistant TB (10). Secondly, people living with HIV/ AIDS are likely to be exposed to MDR-TB patients due to increased hospitalizations in settings with inadequate infection control standards resulting in nosocomial MDR-TB infections (34). Furthermore, biological studies have demonstrated poor absorption of rifampicin and isoniazid in patients with HIV/AIDS (35), leading to drug resistance due to sub-

therapeutic drug concentrations. Zambia is one of the HIV-endemic countries, and that might have an association with the incremental increase of MDR-TB. However, to identify the associated factors of the increase of MDR-TB, we should first know the genotypes of the prevalent *M. tuberculosis* strains and their population structures in the area.

The establishment of molecular tools has improved the understanding of the circulating strains of *M. tuberculosis* in various geographical locations. Spoligotyping (spacer oligonucleotide typing) is a widely employed molecular method for studying *M. tuberculosis* genotypic structures. It is a PCR based method and employs a reverse hybridization rationale by determining the presence or absence of the 43 specific DNA spacer sequencers in the direct repeat (DR) region of *M. tuberculosis* (13). Spoligotype data can be encoded in a numerical format and interpreted using an international database, SITVITWEB (36).

Using spoligotyping, the global mapping of major genotype families of *M. tuberculosis*, such as Beijing, Haarlem, T, X, S, East African-Indian (EAI), Latin-American Mediterranean (LAM), and Central Asian (CAS) families has been achieved (37). Some of these genotypes have been linked to drug resistance, hypervirulence, and increased transmissibility (38). For instance, a subfamily of the LAM genotype (LAM4) was reported as the leading cause of extensively drug-resistant (XDR) TB in KwaZulu Natal, South Africa (39).

In line with the national TB guidelines, the mainstay for routine examination of presumptive TB patients in Zambia is Xpert MTB/ RIF and smear microscopy. TB culture and drug susceptibility testing (DST) is recommended for patients with poor treatment outcomes such as treatment failure and relapse cases (40). In Zambia, TB culture laboratories do not routinely genotype recovered *M. tuberculosis* isolates. This can probably be attributed to limited resources.

Therefore, genotypic profiles of *M. tuberculosis* strains circulating in most parts of the country are unknown as only few studies have been conducted (29, 30). Furthermore the possible correlation of those genotypes with drug resistance was un-investigated before my study. In this chapter, I have described the genotypic structure of *M. tuberculosis* strains in Lusaka, Zambia, and evaluated their association with anti-TB drugs. This knowledge is valuable to design effective MDR-TB control strategies and provides baseline information for surveillance of *M. tuberculosis* genotypes in the country.

Materials and methods

Study settings and design

This was a collaborative study between the University Teaching Hospital (UTH) in Lusaka, Zambia, and Hokkaido University in Japan. The study utilized *M. tuberculosis* isolates routinely collected over a period of five years (2013–2017) at the UTH in Lusaka. The isolates were previously recovered from different pulmonary TB patients, mainly living in Lusaka city, followed by phenotypic DST at the TB culture laboratory in the UTH. The TB culture laboratory participates in a TB DST proficiency testing scheme offered by the Uganda Supranational TB reference laboratory. All the available clinical *M. tuberculosis* isolates with determined DST profiles in the laboratory information system were revived from -80 ^{0}C storage freezers at the UTH in Lusaka, followed by DNA extraction. Extracted DNA samples were then transported to Hokkaido University in Japan for molecular examination. TB genotypes were established using spoligotyping and large sequence polymorphism (LSP), and sequencing of drug resistant associated genes was conducted to validate phenotypic resistance to rifampicin and isoniazid and determine associated mutations.

Drug susceptibility testing

Phenotypic DST to the first-line anti-TB drugs was previously performed using BACTEC^{TM} 960 MGIT^{TM} (Mycobacteria Growth Indicator Tube) system (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) in a Biosafety Level 3 (BSL3) laboratory. The critical drug concentrations of 1.0, 0.1, 1.0, and 5.0 µg/mL were employed for RIF, INH, streptomycin (STR), and ethambutol (EMB), respectively, as recommended by the kit manufacturer (Becton, Dickinson, and Company). *M. tuberculosis* H37Rv strain was routinely utilized as a control strain.

DNA extraction

DNA was prepared for PCR by transferring 1 mL culture broth from Mycobacteria Growth Indicator tubes into cryovials, followed by heating the aliquots at 90 ^{0}C for ten minutes in a dry heating block.

Spoligotyping

Spoligotyping was performed on all isolates, as described by Kamerbeek et al (1997). Briefly, a PCR-based reverse hybridization method was used in which the direct repeat (DR)

region of *M. tuberculosis* was amplified using a pair of primers. The PCR products were hybridized to a set of 43 oligonucleotide probes corresponding to each spacer on the DR region and covalently bound to the membrane. Obtained spoligo-patterns were then compared to the SITVIT-Web database (http://www.pasteurguadeloupe.fr:8081/SITVIT_ONLINE, http://www.pasteurguadeloupe.fr:8081/SITVIT2/files/SITVIT-KBBN_report_310313.xls) for identification of spoligo families and Spoligo International Types (SIT) (36).

Large Sequence Polymorphism (LSP)

Isolates that could not be classified by spoligotyping were further analyzed with a PCR-based method using specific primers for the expected region of difference (RD) for each lineage, as reported by Gagneux et al (18).

Sequencing of *rpo*B, *kat*G genes, and *inh*A regulatory region

PCR was performed with 20 µL of a mixture comprising 25 mM deoxy-ribonucleotide triphosphate (dNTP), 5 M betaine, 10 mM of each primer described by Poudel et al (41), 1 U of GoTaq DNA polymerase (Promega Corp, Madison, WI, USA), GoTaq buffer (Promega Corp) and 1 µL of DNA template. The reaction was carried out in a thermal cycler (Bio Rad Laboratories, Hercules, CA, USA) under the following conditions; pre-heating at 96 ^{0}C for one minute, 35 cycles for denaturation at 96 ^{0}C for ten seconds, attachment at 55 ^{0}C for ten seconds, and elongation at 72 ^{0}C for 30 s and a final extension at 72 ^{0}C for five minutes. Electrophoresis was then conducted using 2% agarose gel to separate the PCR products. DNA fragments were recovered from the gel and applied for sequencing according to the manufacturer's instructions with the BigDye Terminator v3.1 cycle sequencing kit (Life Technologies Corp, Carlsbad, CA, USA) on an ABI 3500 Genetic Analyzer (Life Technologies Corp). The obtained sequences were compared to wild type sequences of *M. tuberculosis* H37RV using BioEdit software version 7.0.9 (42).

Ethical clearance

The ethical approval to conduct this study was granted by The University of Zambia Biomedical Research Ethics Committee (No. FWA00000338 IRB00001131 of IORG0000774). The Zambia National Health Research Ethics Committee authorized the transfer of mycobacterial DNA to Hokkaido University (Japan) for molecular analysis.

Data analysis

Using VassarStats (http://vassarstats.net/) (43), an online tool, Fisher's exact test and a chi-square test were computed to determine significance for observed differences. A p -value $\leq$ 0.05 was considered significant.

Results

Phenotypic drug susceptibility profile

This study analyzed a total of 274 clinical *M. tuberculosis* isolates, of which 134 were MDR while 6 and 3 were isoniazid and rifampicin mono-resistant isolates, respectively (Table 2). Among the MDR-TB isolates, 31 were resistant to INH and RIF only, while 37 were resistant to all the four first-line drugs. One hundred and twenty-six isolates were pan susceptible (Table 2).

Genetic diversity of *M. tuberculosis* isolates

From the 274 isolates, 64 spoligotype patterns were observed and classified into major *M. tuberculosis* lineages; lineages 1, 2, 3, and 4 (18), as shown in Table 3. Adding to that, three *M. bovis* (1.1%, 3/274) isolates were also identified among the clinical samples. Unclassified spoligotype patterns by the SITVIT WEB database were identified by the recognition rule proposed by Filliol et al. (2002) or by LSP analysis. The observed frequencies of major spoligofamilies were the LAM family 54.4% (149/274), the CAS family 16.1% (44/274), and the T family 14.2% (39/274). In minor proportions, X, EAI, Harleem, S, and Beijing spoligofamilies were identified. Among the LAM family, 24 out of 29 spoligotypes, including orphans, showed a specific pattern of LAM11-ZWE (spacer deletion: 21–24, 27–30, and 33–36) and made up a majority (77.2%, 115/149) of the LAM family isolates. Likewise, CAS1-kili (SIT21) dominated among the CAS family (15%, 40/44) (Table 3).

Distribution of *M. tuberculosis* SITs among MDR-TB and non-MDR isolates

The majority of CAS1-Kili (SIT 21) isolates (33/40, 82.5%) were MDR and more predominant among total MDR-TB isolates (33/134, 24.6%) than non-MDR isolates (7/140, 5.0%) ($p = 0.0001$). Similarly, most of LAM1 (SIT 20) were MDR (16/18, 88.9%) and was more predominant among MDR-TB isolates compared to non-MDR isolates (11.9% vs. 1.4%, $p = 0.001$). Other families, including LAM11- ZWE (SIT 815), LAM11-ZWE (SIT 59), and T1 (SIT 53), did not show statistically significant differences in distribution between MDR-TB and non-MDR isolates (Table 4).

Frequencies of drug resistance-conferring mutations among *M. tuberculosis* isolates

Of the 134 MDR-TB isolates and three mono rifampicin resistant isolates, Ser531Leu was the most frequent substitution across all the SITs. CAS1-Kili (SIT 21) exhibited a high diversity of mutations, especially at codon His526. Four isolates from LAM11-ZWE (SIT 59) carried

double mutations of Asp516Tyr and Leu511Arg (Table 5). SITs with clustering (≥ 4 isolates) mutations showed frequencies higher than the global levels (Table 5) (44). Regarding isoniazid resistance-conferring mutations, all isolates except one carried a mutation at codon Ser315 in *kat*G, and the majority (136/139) was Ser315Thr. Two isolates had a mutation in the *inh*A promoter region (C-15 T) (45).

Discussion

Diversity of *M. tuberculosis* genotypes

In line with what has been previously reported in Zambia, this study identified the LAM family (54.4%) as the most prevalent spoligofamily among the examined isolates from Lusaka (29, 30). The CAS family was second at 16.1%, and T was third at 14.2% (Table 3). Ten isolates could not be identified by the SITVIT WEB database; however, upon the application of LSP and classification by the spoligotype patterns, they were grouped into lineages 4 and 1, as shown in Table 3. Both spoligotyping and LSP analysis confirmed the predominance of the lineage 4 strain.

The LAM genotype is dominant among countries in the Southern region of Africa, including Zimbabwe reported at 47.2% (46), Mozambique at 37% (47), Angola at 64.8% (48), and Malawi at 44.0% (49). The SITs identified at high prevalence were LAM11-ZWE (SIT 59), LAM11-ZWE (SIT 815), and LAM1 (SIT 20) (Table 3). Apart from Zambia, LAM11-ZWE has been reported to be a dominant subfamily in Zimbabwe (46). The origin of this subfamily (LAM11-ZWE) has been speculated to be Portugal (50). This genotype may have entered Southern Africa either through Angola or Mozambique, as both countries were colonized by Portugal. Zambia shares a common border with both Angola and Mozambique, and there were long standing civil wars in these two neighboring countries, which forced many immigrants to seek refuge in Zambia. Countries in this region are also linked by shared ethnicity and trade.

Another genotype identified at a relatively high proportion in the current study is the CAS family (16.1%). This rate is similar to what was reported in the Namwala District of Zambia (15%) by Malama et al (30). Among the CAS family, CAS1-Kili (SIT 21) was the main subfamily observed in the current study. CAS1-Kili (SIT 21) has been reported as the predominant subfamily in Tanzania (25.9%) and reported to have adapted to the local population in that country (51). The population of this spoligofamily has been reported to be expanding in neighboring Malawi, increasing by 12.2% between 2006 and 2008 (52). On the other hand, Mulenga et al (2010) observed a very low prevalence of the CAS family (0.7%) in Zambia's Ndola district. While the distribution of *M. tuberculosis* genotypes described in Ndola by Mulenga et al (2010) and in Namwala by Malama et al (2014) may be restricted to those locations, the observations in this study might have a national representation because the general population travels from different parts of the country to Lusaka for trade, administrative purposes and social visits.

The cosmopolitan nature of Lusaka city may also explain the observation of a Beijing isolate in the current study (Table 3). To my knowledge, the current study is the first to report the Beijing genotype in Zambia. Although the patient was a Zambian, the strain had likely been introduced by Asians, given the recent rapid increase in immigration from East Asia, including China. The appearance of the Beijing genotype among the studied population is worrisome as this strain has been associated with hypervirulence, drug resistance, and evasion of the Bacillus Calmette - Guerin (BCG) vaccine in some settings, including South Africa (53). The Beijing isolate identified in the current study exhibited a mono-resistance to streptomycin.

Another spoligofamily identified at a relatively high proportion in the current study was the T family (14.2%). This spoligofamily has been observed to be the second most prevalent family in Zambia and Zimbabwe (50). Specifically, T1 (SIT 53) was the most prevalent subfamily (Table 3), and among the neighboring countries, this SIT has been reported at a relatively high frequency in Angola (13.6%) (48).

Furthermore, the present study, as well as data reported by Malama et al (2014) identified *M. bovis* among isolates from human populations. Again, the heterogenic nature of Lusaka's population may account for the three *M. bovis* isolates observed in the current study as people living in rural parts of the country frequently visit the capital city for various activities. Although the frequency of cases observed in my study is low (1.1%), Malama et al (2014) identified two *M. bovis* isolates from 33 samples indicating a 6% prevalence of *M. bovis* among TB patients in the Namwala district. This figure is close to the 9% prevalence of *M. bovis* reported in the city of Amsterdam (the Netherlands) before the era of pasteurization of milk products (54). Within Zambia, Pandey et al. (2013) reported an 18.7% prevalence of *M. bovis* in cow milk sampled from tuberculin positive cattle (55). As *M. bovis* is intrinsically resistant against one of the first-line anti-TB drugs, pyrazinamide, identifying this specie is important to treat patients properly (56). Further studies involving genotyping of *M. tuberculosis* strains are required in other parts of the country to determine the burden of bovine TB and understand its transmission dynamics.

Correlation of SITs with MDR-TB

Besides the identification of *M. tuberculosis* genotypes, this study investigated an association between spoligotypes and MDR-TB. Interestingly, a positive correlation was observed between MDR-TB and two genotypes, namely CAS1-Kili (SIT 21) and LAM1 (SIT 20) (Table 4).

CAS1-Kili (SIT 21) was more prevalent among MDR-TB isolates than susceptible isolates (24.6% versus 5.0%, p = 0.0001). In fact, 33 of the 40 isolates of this subfamily identified in this study were MDR. Elsewhere, CAS1-Kili (SIT 21) was found to be associated with MDR-TB in Ethiopia (57). In India, all the five MDR-TB identified in the state of Madhya Pradesh exclusively belonged to the CAS family, and investigators attributed the burden of MDR-TB in that state to the CAS genotype (58). Similarly, I attribute the increasing burden of MDR-TB in Zambia to the growing population of *M. tuberculosis* families with the propensity to developing MDR. On the contrary, CAS1-Kili (SIT 21) did not demonstrate an association with drug resistance in Tanzania (59).

LAM1 (SIT 20) was another subfamily that showed association with MDR-TB (p = 0.001) in the current study (Table 4). Among neighboring countries, this SIT is prevalent in Namibia (78.5%), and Angola (18.2%) (48), but drug susceptibility data for this SIT was scarce in these two neighboring countries. Elsewhere, the LAM1 (SIT 20) genotype has been associated with MDR-TB in Portugal (60).

Apart from the two SITs discussed above, other genotypes identified in this study, including the predominant strains LAM11- ZWE (SIT 59) and (SIT 815), did not demonstrate a correlation with MDR-TB (Table 4). These results suggest that a lack of predilection for acquiring MDR by the predominant strains might explain the current low MDR-TB rates in Zambia. Similar assertions were presented before in Uganda when the predominant strain in that country (T2 SIT135) showed a negative correlation with anti-TB drug resistance (61). On the other hand, an earlier study conducted in Zambia, which only utilized phenotypic data, attributed the low MDR-TB burden in the country to a successful DOTS (Directly Observed Therapy Short Course) program (62). There is a need for NTP in Zambia to combine conventional TB epidemiological monitoring with molecular analysis to fully understand MDR-TB dynamics in the country.

Frequencies of *rpo*B drug resistance-conferring mutations among MDR-TB isolates

Ser531Leu was the most prevalent mutation found in *rpo*B among the MDR-TB isolates in this study (Table 5). These results are consistent with what has been observed by similar studies (63 - 65). On the contrary, Lipin et al (2007) reported unusual findings of the predominance of the Asp516Val mutation (75%) among the LAM family in Russia (66). Notably, in the current study, four isolates belonging to the LAM11-ZWE (SIT 59) subfamily exhibited double mutations of Asp516Tyr and Leu511Arg in the *rpo*B gene. Compensatory mutations have been explained to alleviate bacteria's fitness cost (67) and increase their transmissibility (68).

Therefore, this finding might suggest a clonal expansion of a LAM11-ZWE (SIT 59) MDR clone in Lusaka. In the LAM1 (SIT 20) subfamily, which showed a significant association with MDR-TB, a high ratio of Ser531Leu, 15 out of 16 isolates (93.8%), was observed. This higher ratio than expected might suggest a clonal expansion of a specific MDR strain, possibly possessing some compensatory mechanisms (44, 67, 68). Other clusters sharing the same rare mutations, five isolates with Ser531Trp in LAM11-ZWE (SIT 59) and four isolates with Asp516 deletion in CAS1_Kili (SIT 21) might also be suggesting primary MDR-TB cases (Table 5). An in-depth analysis of those strains seems to be needed to elucidate transmission dynamics.

Apart from the Ser531Leu mutation, His526Tyr was prevalent among CAS1-Kili (SIT 21) isolates. The diversity of mutations among this SIT was relatively high compared to other SITs, especially at the position His526. Two of them were found accompanying secondary mutations within RRDR (Table 5). This specific lineage might tend to have point mutations and thus acquire drug resistance like the Beijing lineage (39, 65). Further analysis employing whole genome sequencing will help to characterize this lineage.

This study has provided baseline information on the composition of different *M. tuberculosis* genotypes and their association with MDR-TB in Lusaka. Certain strains tend to acquire drug resistance, and some of those were shown to have already spread and caused MDR-TB, primarily in the studied area. To control such dangerous strains, an effective monitoring system using a genotyping method is required. My study's data has highlighted the specific need for policy formulation towards the attainment of universal TB drug susceptibility testing by the national TB control program and surveillance of TB genotypes circulating in the country.

Study limitations and future directions

Sampling of *M. tuberculosis* clinical isolates from a TB culture laboratory might have an inclination towards a particular category of patients. The current study only investigated clinical isolates whose availability was influenced by the national TB testing policy algorithm (MoH, 2017). Therefore, population-based studies are needed in Zambia to validate my findings, and multicenter studies are also needed to examine transmission links of MDR-TB in the country and the region at large. In the next step, my study collaborators plan to utilize more specific epidemiological methods such as Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) and whole-genome sequencing to

explore epidemiologic transmission dynamics of specific genotypes which have shown an association with MDR-TB in the current study.

Summary

Zambia is a high TB burden country (638/100,000) and worse more the burden of MDR-TB has shown an increasing trend. For instance TB information published by WHO has established that the proportions of MDR-TB had risen from 0.3% among new cases and 8.1% in previously treated cases in 2014 to 2.8% in new cases and 18% in previously treated cases in 2019. Currently, factors associated with this increase are not clear. Other than social economic dynamics, molecular studies have shown that specific *M. tuberculosis* genotypes can be linked to drug resistance. For example Beijing and LAM4 genotypes have previously been linked to causatives of MDR-TB and XDR-TB in South East Asia and South Africa, respectively.

In Zambia, previous studies genotyped *M. tuberculosis* isolated in Ndola town by Mulenga et al (2010) and in Namwala district by Malama et al (2014) but those studies did not attempt to associate identified genotypes to drug resistance. This study is the first to simultaneously characterize *M. tuberculosis* genotypes and investigate their association with MDR-TB.

Using spoligotyping, LSP and sequencing, I studied 274 *M. tuberculosis* isolates stored from 2013 to 2017 at the University Teaching Hospital in Lusaka. Of these, 134 were MDR-TB. Results showed the dominance of LAM family (54.4%) followed by CAS (16.1%) and T (14.2%). Other subfamilies observed in small proportions were X, S, Harleem and Beijing. Three isolates were identified as *M. bovis* (1.1%). Among the LAM genotypes, SIT 59 LAM11-ZWE (17.5%) and SIT 815 LAM11-ZWE (10.5%) were the most prevalent. SIT 21 CAS1-KILI (15.0%) dominated among the CAS family.

Upon further evaluation of these identified SITs for association to MDR-TB, CAS1-kili (SIT 21) and LAM1 (SIT 20) showed correlation to MDR-TB, P=0.0001 and 0.001, respectively. Other SITs did not show association with MDR-TB.

Study results have demonstrated that CAS1-kili (SIT 21) and LAM1 (SIT 20) have strong propensity for becoming MDR-TB suggesting that future increase in the population of these genotypes may be responsible for the proportional increase in the burden of MDR-TB in Zambia. Therefore NTP in Zambia need to monitor the phylogenetic population of *M. tuberculosis* genotypes, particularly those with predilection for MDR-TB and to formulate policies that encourage universal DST to allow for continuous surveillance of MDR-TB.

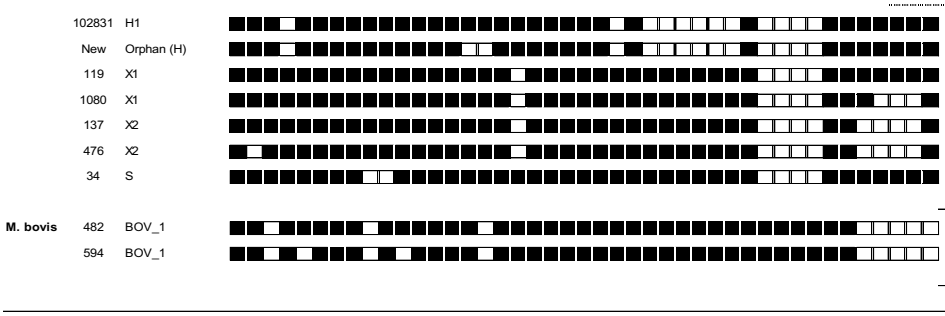
Table 2. Phenotypic drug susceptibility profile for *M. tuberculosis* isolates

Characteristics	Resistance Patterns	No. (%)
MDR	INH + RIF	31 (11.3)
	INH + RIF + EMB	24 (8.7)
	INH + RIF + STR	42 (15.3)
	INH + RIF + EMB + STR	37 (13.5)
non-MDR	INH	4 (1.5)
	INH + STR	2 (0.7)
	RIF	1 (0.4)
	RIF + STR	2 (0.7)
	STR	4 (1.5)
	EMB	1 (0.4)
Pan-susceptible[a]	None	126 (46.0)
Total		274 (100)

[a] susceptible to the four 1st-line anti-TB drugs; INH, RIF, EMB and STR
INH, isoniazid; RIF, rifampicin; EMB, ethambutol; STR, streptomycin

Table 3. Diversity of *M. tuberculosis* subfamilies in current study

Lineage	SIT	Subfamily	Spoligotype pattern (spacer 1 - 43)	Octal Number	No. of isolates	% *	method **
1	702	EAI6_BGD1		700775747413771	3	1.1	
	129	EAI6-BGD1		700777747413771	2		
	10	EAI8_MDG		477777277413771	1		
	New	Orphan		577777277413771	2		LSP
	New	Orphan		477777776010771	1		
	New	Orphan		743777777003731	1		LSP
				Lineage 1 sub total	**10**	**3.6**	
2	1	Beijing		3771	1		
				Lineage 2 sub total	**1**	**0.36**	
3	25	CAS1-Delh		703777740003171	1		
	2277	CAS		703377600001771	1		
	21	CAS1_KILI		703377400001771	40	15.0	
	3284	CAS1-Kili		703357400001771	1		
	102405	CAS1-Kili		703377400001731	1		LSP
				Lineage 3 sub total	**44**	**16.1**	
4	53	T1		777777777760771	17	6.2	
	373	T1		777777767760771	3	1.1	
	51	T1		777777777760700	2		
	102	T		777703777760771	2		
	154	T1		757777777760771	1		
	3291	T1		743777777760700	1		
	2067	T1		777771777760771	1		
	245	T1		777777777760671	1		
	105172	T		777777357760771	1		
	52	T2		777777777760731	4	1.5	
	317	T2		777777777360731	3	1.1	
	73	T		777737777760731	1		
	117	T2		777767777760731	1		
	New	Orphan		777777667760731	1		
				subtype T (lineage 4) sub total	(39)	(14.2)	
	20	LAM1		677777607760771	18	6.6	
	3457	LAM1		675777607760771	1		LSP
	33	LAM3		776177607761771	2		
	719	LAM3		776177407760771	1		
	42	LAM9		777777607760771	12	4.4	
	59	LAM11_ZWE		777777606060771	48	17.5	
	815	LAM11_ZWE		777777606060731	29	10.6	
	2173	LAM11_ZWE		377777606060771	6	2.2	
	184	LAM11_ZWE		777417606060731	4	1.5	
	811	LAM11_ZWE		777777604060731	3	1.1	
	814	LAM11_ZWE		77777606060671	2		
	1468	LAM11_ZWE		77777606060671	2		
	2488	LAM11_ZWE		741777606060771	2		
	103978	LAM11_ZWE		777177606060771	2		LSP
	105148	LAM11_ZWE		777777206060731	2		
	812	LAM11_ZWE		777777606060571	1		
	1607	LAM11_ZWE		601777606060771	1		
	1471	LAM11-ZWE		777737606060771	1		
	2017	LAM11-ZWE		677777606060771	1		
	2265	LAM11_ZWE		577777606060771	1		
	3292	LAM11_ZWE		777377606060771	1		
	100148	LAM11_ZWE		1777606060771	1		
	101412	LAM11_ZWE		577777606060731	1		
	105304	LAM11_ZWE		7777777606060730	1		
	New	Orphan		760000606060771	2		
	New	Orphan		177777606060771	1		
	New	Orphan		703770002060771	1		
	New	Orphan		777770606060731	1		
	New	Orphan		777777606060711	1		
				subtype LAM (lineage 4) sub total	(149)	(54.4)	



	102831	H1	737777764020771	4	1.5	LSP
	New	Orphan (H)	737763764020771	1		LSP
	119	X1	777776777760771	2		
	1080	X1	777776777760701	1		
	137	X2	777776777760601	14	5.1	
	476	X2	577776777760601	2		
	34	S	776377777760771	4	1.5	
			Lineage 4 sub total	**216**	**78.8**	
M. bovis	482	BOV_1	676773777777600	1		
	594	BOV_1	656573777777600	2		
			M. bovis sub total	**3**	**1.1**	
			Total	**274**	**100**	

SIT: Spoligo International Type by SITVIT WEB and/or SITVIT-KBBN_report_310313, LSP: large sequence polymorphism

* Percentages of each spoligotype were shown when it is larger than 1.0 % (number of isolates > 2). ** Used genotyping method other than spoligotyping

Table 4. Correlation between observed *M. tuberculosis* SITs and MDR-TB

Spoligotype		MDR-TB isolates N=134	Non-MDR isolates N=140	Significance	
SIT	CLADE	Frequency No. (%)	Frequency No. (%)	Odds ratio (95% CI)	*p*-value
59	LAM11-ZWE	29 (21.6)	19 (13.6)	1.8 (0.4- 3.3)	0.1
815	LAM11-ZWE	14(10.4)	15 (10.7)	0.9 (0.4-2.1)	0.9
20	LAM1	16 (11.9)	2 (1.4)	9.4 (2.1- 41.5)	0.001[a]
42	LAM9	4 (3.0)	8 (5.7)	0.5 (0.1-1.7)	0.4
21	CAS1-Kili	33 (24.6)	7 (5.0)	6.2 (2.6-14.6)	0.0001[a]
3284	CAS1-Kili	1 (0.8)	0	-	-
2277	CAS	1 (0.8)	0	-	-
25	CAS1-Delh	0	1 (0.7)	-	-
137	X2	7 (5.2)	7 (5.0)	-	-
476	X2	0	2 (1.4)	-	-
119	X1	0	2 (1.4)	-	-
1080	X1	0	1 (0.7)	-	-
53	T1	8 (6.0)	9 (6.4)	-	-
52	T2	4 (3.0)	0	-	-
51	T1	0	2 (1.4)	-	-
117	T2	0	1 (0.7)	-	-
154	T1	0	1 (0.7)	-	-
373	T1	0	3 (2.1)	-	-
102	T1	0	2 (1.4)	-	-
245	T1	0	1 (0.7)	-	-
317	T2	2 (1.5)	1 (0.7)	-	-
2067	T1	0	1 (0.7)	-	-
73	T	1 (0.8)	0	-	-
34	S	1 (0.8)	3 (2.1)	-	-
2173	LAM11-ZWE	0	6 (4.3)	-	-
812	LAM11-ZWE	0	1 (0.7))	-	-
811	LAM11-ZWE	1 (0.8)	2 (1.4)	-	-
814	LAM11-ZWE	0	2 (1.4)	-	-
184	LAM11-ZWE	0	4 (2.9)	-	-
2488	LAM11-ZWE	0	2 (1.4)	-	-
1607	LAM11-ZWE	0	1 (0.7)	-	-
33	LAM3	0	2 (1.4)	-	-
1468	LAM11-ZWE	0	2 (1.4)	-	-
1471	LAM11-ZWE	0	1 (0.7)	-	-
2017	LAM11-ZWE	0	1 (0.7)	-	-
2265	LAM11-ZWE	0	1 (0.7)	-	-
719	LAM3	0	1 (0.7)	-	-
10	EAI8-MDG	1 (0.8)	0	-	-
702	EAI6-BGD1	0	3(2.1)	-	-
129	EAI6-BGD1	0	2 (1.4)	-	-
1	Beijing	0	1 (0.7)	-	-
482	BOV_1	0	1 (0.7)	-	-
594	BOV_1	0	2 (1.4)	-	-
-	Orphans[b]	11 (8.2)	17 (12.1)	-	-

-,not applicable; [a]positive correlation with MDR-TB; [b]Orphans by SITVIT WEB search

Table 5. Frequency of observed rifampicin-resistance associated mutations in *rpo*B gene among the four main SITs

Amino acid change	LAM11-ZWE SIT 59	LAM11-ZWE SIT 815	LAM1 SIT 20	CAS1-Kili SIT 21	Global frequency among MDR, Pre-XDR and XDR
	N=29(%)	N=14 (%)	N= 16 (%)	N =33 (%)	N =7347 (%)
Ser531Leu	15 (51.7)	12 (85.7)	15 (93.8)	10 (30.3)	4798 (65.3)
Ser531Phe	-	1 (7.1)	-	-	N/A
Ser531Trp	5 (17.2)	-	-	-	113 (1.5)
His526Tyr	-	1 (7.1)	-	8 (24.2)	326 (4.4)
His526Asp	-	-	-	7 (21.2)	266 (3.6)
His526Leu	-	-	-	1 (3.0)	N/A
His526Gln and Leu533Pro	-	-	-	1 (3.0)	N/A
His526Arg and Ser509Ile	-	-	-	1 (3.0)	N/A
Ser522Val	-	-	-	1 (3.0)	N/A
Asp516Val	2 (6.9)	-	-	-	N/A
Asp516Phe	1 (3.5)	-	-	-	N/A
Asp516Tyr	-	-	1 (6.2)	-	N/A
Asp516Tyr and Leu511Arg	4 (13.8)	-	-	-	17 (0.23)
Asp516 deletion	-	-	-	4 (12.1)	1 (0.1)
Leu511Arg	1 (3.5)	-	-	-	N/A
Ser531Trp and Glu504Ala	1 (3.5)	-	-	-	N/A

-;not observed

N/A; not computed

CHAPTER II

Mutations in *rpo*B and *kat*G genes and the *inh*A operon in multidrug-resistant *Mycobacterium tuberculosis* isolates from Zambia

Introduction

TB continues to threaten human health despite the availability of effective anti-TB drugs. The emergence and spread of drug-resistant strains of *M. tuberculosis* is among factors compounding the control of this disease (69). World Health Organization estimated that 558,000 new TB cases in 2017 were rifampicin-resistant TB (RR-TB), of which 82% were MDR-TB (70).

In Zambia, a sub-Saharan African country, the burden of MDR-TB is an emerging threat unless investment is made to improve the ability of the TB programme to detect and manage MDR-TB (7).

Early detection of patients with MDR-TB is an effective strategy to halt the rise in the MDR-TB burden and to avoid development of further resistance (71). Nowadays, rapid molecular tools have been developed and those offer a solution for early detection of MDR-TB cases (72). However, for optimal application of these tools and further development of new ones, knowledge regarding genetic determinants conferring resistance to anti-TB drugs in specific geographical regions is essential (73). In addition, this knowledge could help TB programmes to design effective control strategies.

Generally, studies have shown that resistance to rifampicin in >90% of cases is attributable to point mutations occurring in a specific region of the RNA polymerase β-subunit gene (*rpo*B), called the RIF resistance-determining region (RRDR) (25). For isoniazid, 50–95% of resistant organisms carry mutations in the *kat*G gene, encoding a catalase-peroxidase enzyme that transforms the prodrug isoniazid into reactive molecules including superoxide, nitric oxide and isonicotinic acyl radical (25, 74). In addition, mutations in the regulatory region of the *inh*A operon, encoding a putative enzyme (enoy-acyl carrier protein reductase) involved in mycolic acid biosynthesis, cause over expression of the InhA protein leading to isoniazid resistance through a titration mechanism (26). Other genes that have been linked with isoniazid resistance include *ahp*C locus, encoding alkyl hydroperoxide reductase enzyme which is involved in the

cellular response to oxidative stress, *kas*A gene (encoding β-ketoacyl carrier protein synthase) also involved in mycolic acid biosynthesis, *ndh* gene (coding for NADH dehydrogenase), *nat* gene (coding for N-acetyl transferase) and *msh*A (encoding glycosyl transferase) involved in the synthesis of mycothiol (75).

Although specific genes and mechanisms involved in *M. tuberculosis* resistance to rifampicin and isoniazid have been well established, the pattern and frequency of these mutations vary by geographic region (76 – 78). Therefore, in the current study I analysed the patterns and frequencies of resistance-associated mutations in the *rpo*B and *kat*G genes and the *inh*A regulatory region among MDR-TB strains isolated in Lusaka. Furthermore, I compared the frequencies of mutations identified in this study with those reported by others in the African countries.

Materials and methods

Mycobacterium tuberculosis isolates

A total of 99 MDR-TB and 49 pan-susceptible clinical isolates were purposefully selected from an archiving bank at a University Teaching Hospital TB laboratory in Lusaka, Zambia. Isolates were collected over a 4-year period (2013–2016). Phenotypic drug susceptibility testing to the first-line anti-TB drugs was previously performed using BACTECTM Mycobacteria Growth Indicator Tube 960 (MGITTM 960) System (Becton Dickson & Co., Franklin Lakes, NJ, USA) using *M. tuberculosis* H37Rv as a susceptible control strain in a biosafety level 3 (BSL3) laboratory. Critical drug concentrations of 1.0, 0.1, 1.0 and 5.0 µg/mL were employed for rifampicin, isoniazid, streptomycin and ethambutol, respectively, as recommended by the kit manufacturer.

DNA extraction

DNA was prepared for PCR by aliquoting 1 mL of culture broth from the MGIT tube into cryovials, followed by heating the aliquots at 90^{o}C for 10 minutes in a dry heating block.

Sequencing of *rpo*B and *kat*G genes and the *inh*A regulatory region

PCR was conducted with 20 µL of a mixture consisting of 25 mM deoxyribonucleotide triphosphate (dNTP), 5 M betaine, 10 mM of each primer as described by Poudel et al (41), 1 µL of GoTaq® DNA Polymerase (Promega Corp., Madison, WI, USA), GoTaq® buffer (Promega Corp.) and 1 µL of DNA template. The reaction was carried out in a thermal cycler

(Bio-Rad Laboratories, Hercules, CA, USA) under the following conditions: pre-heating at 96 ^{0}C for 1 minute; 35 cycles of denaturation at 96 ^{0}C for 10 s, annealing at 55 ^{0}C for 10 s and extension at 72 ^{0}C for 30 s; and a final extension at 72 ^{0}C for 5 minutes. PCR products were then separated by 2% agarose gel electrophoresis. DNA fragments were recovered from the gel and were applied for sequencing according to the manufacturer's instructions using primers TB *rpo*B Fw, TB *kat*G Fw and TB *inh*A Fw, respectively, and a BigDye™ Terminator v.3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, CA, USA) on an ABI 3500 Genetic Analyzer (Life Technologies). The obtained sequences were compared with wild-type sequences of *M. tuberculosis* H37Rv using BioEdit v.7.0.9 (42).

Results

Drug susceptibility profile

A total of 148 clinical isolates were analysed, of which 99 were MDR-TB and 49 were pan-susceptible isolates. Among the MDR-TB isolates, 21 were resistant to INH and RIF only, whilst 30 were resistant to all four first-line drugs (Table 6).

Mutations in the *rpo*B gene

Of the 99 MDR-TB isolates included in this study, 98.0% (97/99) showed point mutations in the *rpo*B gene, with 2 isolates having an Asp deletion at codon 516 and 1 isolate having an Asn deletion at codon 518. One isolate had a mutation outside the 81-bp RRDR at codon 505. The most altered codon in the *rpo*B gene was codon 531 (55.6%; 55/99), of which 52.5% (52/99) were a Ser531Leu amino acid substitution. The next most altered codons were 526 and 516, both at 18.2% (Table 7). None of the 49 pan-susceptible isolates had a mutation in the *rpo*B gene. The specificity for the molecular technique was therefore 100% (49/49)

Mutations in the *kat*G gene and the *inh*A regulatory region.

Among the MDR-TB isolates investigated, 96.0% (95/99) had point mutations, all of them involving the *kat*G gene. In addition to the *kat*G gene mutations, two isolates had simultaneous mutations in the *inh*A regulatory region. The most frequent point mutation among the INH-resistant isolates was *kat*G Ser315Thr, which occurred in 90.9% (90/99) of the mutants. The frequency of mutations in the *inh*A regulatory region was 2.0% (2/99) (Table 8).

Comparison of the frequencies of drug resistance-associated mutations among selected studies in Africa

To compare the frequencies of resistance-associated mutations with other African countries, published articles from six countries were reviewed. Among the African studies reviewed, all countries except Kenya reported codon 531 of the *rpo*B gene to be the most mutated among RIF-resistant *M. tuberculosis* isolates (Table 9). The highest frequency of mutations in this codon was reported in a study from Tunisia (100%), while the lowest was reported from Kenya at 40% (Table 9).

For INH-resistant isolates (Table 10), the highest frequency of mutations in the current study was observed in codon 315 of *kat*G (94.9%). A point mutation at position 15 in the *inh*A regulatory region was reported as the highest in a study from Cameroon (29.5%), whereas in the current study it was only observed in 2.0% of the isolates (Table 10).

Discussion

Molecular studies have established that *M. tuberculosis* resistance to rifampicin and isoniazid is due to spontaneous chromosomal mutations in specific genomic regions of the bacteria (26). Whilst the patterns and frequencies of genetic mutations within *rpo*B, *kat*G and *inh*A genes encoding drug resistance to rifampicin and isoniazid have been clearly determined in most countries, this knowledge was lacking in Zambia before this study. A total of 99 MDR-TB and 49 pan-susceptible *M. tuberculosis* isolates from Zambia were investigated and mutations were observed in 98.0% and 96.0% among rifampicin and isoniazid-resistant isolates, respectively. No mutations were identified in the pan-susceptible isolates.

The high frequency of rifampicin-resistant isolates (98%) with mutations in the RRDR of the *rpo*B gene observed in this study has been reported previously from other regions. For instance, phenotypically rifampicin-resistant isolates carrying mutations in the RRDR of the *rpo*B gene were reported at frequencies of 93.7% and 92% from Kazakhstan (81) and Russia (82), respectively. Generally, it has been observed that between 78–100% of rifampicin-resistant *M. tuberculosis* strains carry mutations in *rpo*B (26). The high level of correlation (98%) between phenotypic drug susceptibility testing and genotypic prediction of rifampicin resistance in this study suggests that molecular tools can be applied in Zambia for the detection of MDR/RR-TB in clinical specimens with acceptable sensitivity. One isolate had a mutation outside of the RRDR. This mutation was outside the target region of the widely available rapid molecular tool (GeneXpert®) in Zambia. Such a phenomenon has been reported previously in Swaziland where mutation (I572F) outside of the RRDR was reported in 30% of rifampicin-resistant isolates (83). However, in my study, mutation outside of the RRDR occurred at a low frequency. Furthermore, lack of mutations among the pan-susceptible isolates implies that molecular tools can exclude non-MDR/RR-TB cases among clinical specimens in Zambia with high specificity. These parameters endorse the applicability of molecular-based technologies for the identification of MDR/RR-TB in Zambia

Further analysis of rifampicin-associated mutations revealed that codon 531 of the *rpo*B gene (55.6%) was the most altered among the rifampicin-resistant isolates. This figure is consistent with data reported by others, including in Uganda (50%) (84), South Africa (55%) (85) and Zimbabwe (69.7%) (86). These findings suggest that molecular tools designed with a probe at codon 531 could detect approximately one-half of the rifampicin-resistant *M. tuberculosis* strains in Zambia and other sub-Saharan African countries.

The second most frequently mutated codon in the RRDR has widely been reported at codon 526 of the *rpo*B gene (84, 85, 87), but the current study found that codons 526 and 516 were altered at equal frequencies (Table 8). Other studies that could not determine the second most commonly altered codons in *rpo*B gene include studies from France (76) and Cameroon (88).

Other polymorphism observed in r*po*B gene of rifampicin-resistant *M. tuberculosis* isolates from Lusaka included those in codon 511, four isolates. Significance of mutations in that codon were previously disputed in literature as causing low level resistance to rifampicin. However World Health Organization has recently recommended that "borderline resistance" *rpo*B mutations, including L511P and I572F, need to be treated with an MDR-TB regimen (89). Further molecular studies are required in Zambia and indeed globally to clarify uncertain areas, to validate previous data and to enable surveillance of drug-resistant TB.

Although mutations in several genes of *M. tuberculosis* such as *kat*G, *inh*A, *ahp*C, *ndh*, *msh*A and *kas*A have been associated with isoniazid resistance, that resulting in the amino acid substitution S315T in the *kat*G gene is postulated to be favoured by the bacteria as it reduces activation of isoniazid while retaining 30–40% of catalase-peroxidase activity required for its virulence (90). In the current study, 96.0% of the isoniazid-resistant isolates had an amino acid change in the *kat*G gene, with 90.9% being the S315T amino acid substitution. Similar high proportions of *kat*G S315T substitution among isoniazid-resistant *M. tuberculosis* isolates have been reported from northwestern Russia (93.6%) (91), Lithuania (95%) (92) and Kazakhstan (98.4%) (81). Overall, a high prevalence of *kat*G S315T substitution has been observed to occur in high TB prevalence regions (93) and undeniably the TB prevalence in Zambia is quite high (638/100 000 population) as reported in the 2013–2014 national TB prevalence survey (94). Furthermore, mutations in codon 315 of *kat*G have been shown to confer a range of resistance levels to isoniazid (95). This fact is worrisome to countries in sub-Saharan Africa, as a high prevalence of this mutation is observed from studies reviewed in this region, including Kenya, (82.8%), Uganda (81.9%), Zimbabwe (73.0%) and South Africa (67.8%) (80, 84, 86, 96).

On the other hand, pronounced features such as the observed high *kat*G315 mutational frequencies (94.9%) among *M. tuberculosis* strains observed in this study can be exploited to design a low-cost molecular tool to complement techniques that only detect rifampicin resistance. Restrictively, such a tool may not be generically applicable in certain settings with relatively low mutational frequencies involving *kat*G315. For instance, a study in Tunisia reported a uniquely low mutation frequency (37.2%) in the *kat*G315 codon among isoniazid-resistant isolates (97).

Besides the *kat*G gene, polymorphisms in other genes have been elucidated to contribute to isoniazid resistance in *M. tuberculosis*, such genomic regions include *inh*A, *kas*A, *ahp*C and *oxy*R (76). Specifically, mutations in the *inh*A regulatory region have been described to occur in 10–35% of isoniazid resistant strains (98-100). Higher proportions of *inh*A mutations among MDR-TB isolates were observed in two locations of South Africa; Western Cape (48.4%) and Eastern Cape (62.4%) (101). Conversely, a relatively low frequency (2.0%) of mutations in the *inh*A regulatory region was observed in the current study. Low rates of mutations in the *inh*A regulatory region among isoniazid-resistant *M. tuberculosis* strains have also been reported from Ethiopia (0.8%) (102). Mutations in the *inh*A region have been described to confer low-level resistance in *M. tuberculosis* (88), but when they occur in combination with *kat*G mutations an increase in resistance levels to isoniazid has been observed (26). In the current study, two isolates with simultaneous mutations involving the *inh*A region and *kat*G gene were observed (Table 8). Besides causing isoniazid resistance, *inh*A mutations also confer cross-resistance to ethionamide, a second-line anti-TB drug (26). Therefore, ethionamide needs to be excluded in the clinical management of MDR-TB cases exhibiting *inh*A mutations.

This study could not detect any mutation in 4.0% of the phenotypically isoniazid-resistant isolates in the *inh*A and *kat*G genes. Two of these isolates had a minimum inhibitory concentration (MIC) to isoniazid of >0.40 mg/mL. Mutations in the *oxy*R–*ahp*C intergenic region have been hypothesised to be compensatory to *kat*G mutations, whilst the contribution of *kas*A mutations to isoniazid resistance requires further exploration because some studies have reported a low association with isoniazid resistance (103, 104).

Although *M. tuberculosis* strains in African nations have similar mutations types conferring drug resistance to rifampicin and isoniazid, variations in frequencies exist within the region. A predominance of *kat*G315 mutations (94.9%) associated with isoniazid resistance among MDR-TB isolates was observed in the current study. Relative to frequencies reported by others in the region, this figure is distinctively high. On the other hand, the involvement of the *inh*A regulatory region in conferring resistance to isoniazid was lower (2.0%) among MDR-TB isolates from Zambia than that reported elsewhere in the region (Table 10).

Based on these distinctive mutational frequencies observed among Zambian *M. tuberculosis* isolates, I speculate that cross border transmission of resistant strains might not be a significant factor contributing to the rising MDR-TB burden in Zambia. Local conditions such as poor treatment regimens and non-adherence have been established to facilitate the emergence of drug resistance in *M. tuberculosis* (26, 105).

However, further molecular analyses employing genotyping tools such as multiple-loci variable-number tandem repeat (MLVA), restriction fragment length polymorphism (RFLP) and whole-genome sequencing are required to compare other genetic markers for the MDR-TB strains circulating in Zambia and other countries in the region. The types of mutations conferring resistance to rifampicin and in *rpo*B, *kat*G and *inh*A genomic regions among MDR-TB isolates in Zambia are similar to those described globally.

Therefore, the TB programme in Zambia can customise diagnostic molecular tools designed to target these genetic markers for early detection of MDR/RR-TB. Examples of such tools include GeneXpert® MTB/RIF (Cepheid), GenoType MTBDRplus (Hain LifeScience) and MeltPro® TB/INH (Zeesan Biotech), among others. Furthermore, the relatively high prevalence of *kat*G315 gene mutations observed among isoniazid-resistant isolates from Zambia has recently been utilized by my research collaborators to innovate a rapid and simple molecular tool to detect isoniazid resistance and the technology has since been evaluated on *M. tuberculosis* strains from Lusaka (106).

Summary

TB continues to threaten human health despite the availability of effective anti-TB drugs. The emergency of drug resistant strains of MTB is among other factors compounding the control of this disease. WHO estimated that 558,000 new TB cases in 2017 were RR-TB, of which 82% were MDR-TB. In Zambia, MDR-TB burden has been described to be an emerging threat unless investment is made to improve the ability of the NTP to detect and manage MDR-TB.

Today, rapid molecular tools have been innovated and offer a solution for early detection of MDR cases. However, for optimal application of these tools and further development of new ones, knowledge about genetic determinants conferring resistance to TB drugs in specific regions is essential. The knowledge about mutations conferring drug resistance to rifampicin and isoniazid was lacking in Zambia before this study. I sequenced *rpoB*, *kat*G and *inh*A for 99 MDR-TB and 49 susceptible isolates stored at a University Teaching Hospital in Lusaka, Zambia.

Among rifampicin resistant isolates, 98% (97/99) showed point mutations in the RRDR of the *rpoB* gene. The most altered codon was 531 at 55.5 % (55/99) and out of this rate, 52.5% was Ser to Leu amino acid substitution. None of the 49 pan-susceptible isolates had a mutation. The specificity for the molecular technique was therefore 100% (49/49).

For isoniazid resistant isolates, 96% (95/99) had point mutations and all of them involved *katG* gene. The most frequent point mutation among the INH resistant isolates was *katG* S315T which occurred in 90.9% of the mutants.

Mutations observed in *rpo*B gene are similar to what has been described globally. This study therefore has validated the use of molecular TB diagnostics in Zambia as sensitivity and specificity for the genotypic analysis were 98% and 100%, respectively.

Isoniazid resistance isolates showed distinctively high mutational frequency of *kat*G gene (96%). Based on these findings, a simple and rapid molecular tool (STH PAS), fit for Lusaka population, has recently been developed by my research collaborators to detect isoniazid resistance by targeting k*at*G gene. This tool has the potential to be applied to confirm MDR-TB by complementing Xpert MTB/RIF which currently only detects rifampicin resistance.

Table 6. Clinical *M. tuberculosis* isolates included in the study

Characteristics	Resistance Patterns	No. (%)
MDR	INH + RIF	21 (14.2)
	INH + RIF + EMB	22 (14.9)
	INH + RIF + STR	26 (17.6)
	INH + RIF + EMB + STR	30 (20.3)
Pan-susceptible	None	49 (33.1)
Total		148 (100)

MDR; multidrug resistant

RIF; rifampicin

INH; isoniazid

EMB; ethambutol

STR; streptomycin

Table 7. Distribution of mutations in the RRDR of the *rpo*B gene

Mutated codon(s)	Amino acid change(s)	Nucleotide change(s)	No. of isolates RIF^r (N=99)	No. of isolates RIF^s (N=49)
505[a]	Phe to Leu	TTC to CTC	1	0
513	Gln to Glu	CAA to GAA	4	0
516	Asp to Val	GAC to GTC	10	0
	Asp to Tyr	GAC to TAC	1	0
	Asp deleted	GAC deleted	2	0
	Asp to Phe	GAC to TTC[b]	1	0
526	His to Tyr	CAC to TAC	9	0
	His to Asp	CAC to GAC	4	0
	His to Leu	CAC to CTC	2	0
	Hist to Cys	CAC to TGC[b]	1	0
522	Ser to Val	TCG to GTG[b]	1	0
531	Ser to Leu	TCG to TTG	52	0
	Ser to Trp	TCG to TGG	2	0
	Ser to Phe	TCG to TTC[b]	1	0
518	Asn deletion	AAC deleted	1	0
511 and 516	Leu to Arg and Asp to Tyr	CTG to CGG and GAC to TAC[c]	4	0
526 and 533	His to Gln and Leu to Pro	CAC to CAG and CTG to CCG[c]	1	0
509 and 526	Ser to Ile and His to Arg	AGC to ATC and CAC to CGC[c]	1	0
Wild type	None	None	1	49

[a] Outside the 81 bp RRDR
[b]Double mutations in one codon
[c]Double mutations in two different codons
RIF^r; phenotypically rifampicin resistant
RIF^s; phenotypically rifampicin susceptible

Table 8. Distribution of mutations in *kat*G gene and *inh*A regulatory region

Loc. Mutated Codon(s)	Amino acid change(s)	Nucleotide change(s)	No. of isolates INH^r (N=99)	No. of isolates INH^s (N=49)
315 *katG*	Ser to Thr	AGC to ACC	89	0
315 *katG*	Ser to Asp	AGC to AAC	4	0
315 *katG* and -15*inhA*	Ser to Thr	AGC to ACC and C to T	1	0
329 *katG* and -15*inh*A	Asp to Glu	GAC to GAA and C to T	1	0
W None	None	None	4	49

INH^r: phenotypically isoniazid resistant

INH^s: phenotypically isoniazid susceptible

Table 9.
Diversity of mutations in RRDR of the *rpo*B gene in phenotypically rifampicin resistant isolates reported by seven groups from north, west, east and southern Africa

Codon position	(%) mutations						
	(79) Tunisia (N=9)	(88) Cameroon (N=7)	(84) Uganda (N=90)	(96) Kenya Nairobi (N= 15)	(85) South Africa (N=87)	(86) Zimbabwe Harare (N=69)	current study (N=99)
504	-	-	-	-	1.0	-	-
505	-	-	-	-	-	-	1.0
513	-	-	-	-	3.4	-	4.0
516	-	-	12.5	-		-	14.1
518	-	-	-	-	-	-	1.0
522	-	-	-	-	-	-	1.0
526	-	14.3	28.1	60.0	17.2	6.1	16.2
531	70.0	71.4	50.0	40.0	49.4	69.7	5.6
509 and 526	-	-	-	-	-	-	1.0
511 and 516	-	-	-	-	-	-	4.0
516 and 572	-	-	3.1	-	-	-	-
526 and 531	30.0	-	-	-	-	-	-
526 and 533	-	-	-	-	-	-	1.0
None	0	0	11.4	0	11.5	11.6	1.0

N, number of isolates analysed.
[a] – indicates not reported

Table 10. Diversity of mutations in *kat*G gene and *inh*A regulatory region in phenotypically isoniazid resistant isolates reported by seven groups from north, west, east and southern Africa.

	(%) mutations						
Codon position	(97) Tunisia (N=43)	(88) Cameroon (N=44)	(84) Uganda (N=90)	(96) Kenya Nairobi (N= 29)	(80) South Africa (N=87)	(86) Zimbabwe Harare (N=69)	current study (N=99)
315 *kat*G	37.2	45.5	81.9	82.8	67.8	73.0	94.9
326 *kat*G	4.6	-	-	-	-	-	-
328 *kat*G	-	-	-	-	2.3	-	-
329 *kat*G	-	-	-	-	-	-	1.0
-15 *inh*A	27.9	29.5	1.6	-	-	16.2	2.0
*fab*G1.C-8T	-	-	1.6	-	-	-	-
None	27.9	27.0	44.4	17.2	29.9	-	4.0

N, number of isolates analyzed.
- indicates not reported

CONCLUSION

TB continues to threaten human health globally. In Zambia TB is a disease of public health magnitude even with the implementation of DOTs control program. Worse more the emergence of MDR strains are compounding control strategies aimed at controlling this disease. In Zambia, both WHO estimates and in-country reports have shown that the burden of MDR-TB is on the increase. So far suggested reasons are mostly related to social-economic factors but possible genetic determinants relating to the causative organism (*M. tuberculosis*) were uninvestigated before this study.

Today, rapid molecular tools have been innovated and offer a platform for studying the molecular characteristics of circulating strains and early detection of MDR-TB cases.
Understanding the *M. tuberculosis* genotypes circulating in a specific location is vital for designing effective control strategies as *M. tuberculosis* genotypes do not have same characteristics regarding transmission dynamics, virulence and acquisition of drug resistance. Furthermore, for optimal utilization of molecular tools and further development of new ones, knowledge about mutations conferring resistance to TB drugs in specific regions is essential.

Therefore to understand genetic characteristics of MDR-TB situation in Lusaka, this study conducted molecular analysis on 274 *M. tuberculosis* isolates stored (2013 – 2017) at the University Teaching Hospital in Lusaka.

In Chapter 1, I have differentiated and discussed *M. tuberculosis* genotypes among the Lusaka isolates and elucidated their association with MDR-TB, respectively. Both Spoligotyping and LSP reviewed that lineage 4 of *M. tuberculosis* was the most predominant lineage circulating in Lusaka. As reported by others, LAM family at 54.4% was the most dominant genotype followed by CAS (16.1%) and T (14.2%). Other genotypes I observed in small proportions were Harleem , X ,S, EAI and Beijing. Three (3) isolates were identified as *M. bovis* indicating human infection with this zoonotic specie of mycobacteria.

From SITVIT online web analysis, LAM11-ZWE SIT 59 was the most dominant SIT (17.5%) followed by CAS1-kili SIT 21 (15.0%) and third was LAM11-ZWE SIT 815 (10.6%). Others were LAM1 SIT 20 (6.6%) and T1 SIT 53 (6.0%). Further analysis of these SITs for correlation to MDR-TB revealed that CAS1-kili (SIT 21) and LAM1 (SIT 20) had significant correlation to MDR-TB. Other SITs including the dominant subfamily (LAM11-ZWE SIT 59) did not correlate to MDR-TB.

Therefore, future increase of MDR-TB burden in Zambia would be as a result of proportional growth in the phylogenetic profile of genotypes that have demonstrated propensity to becoming MDR-TB in the current study. There is need for continuous surveillance and monitoring of evolutionary expansion (by NTP) of those genotypes in Zambia. I recommend that population based studies (utilizing MIRU-VNTR and/or whole genome sequencing) are conducted in Lusaka to further understand transmission dynamics of MDR-TB in Zambia.

In Chapter 2, I have discussed the sequence analysis of *M. tuberculosis* genes known to confer resistance to first line anti-TB drugs (rifampicin and isonaizid). Sequence data for r*po*B gene on rifampicin-resistant isolates showed that 98% (97/99) had point mutations in the RRDR. The most altered codon was 531 at 55.6 % (55/99) of which 52.5% were Ser to Leu amino acid substitution.

For isoniazid-resistant isolates, 96% (95/99) had point mutations and all of them involved *katG* gene. The most frequent point mutation among the isoniazid resistant isolates was *katG* 315 Ser to Thr which occurred in 90.9% (90/99) of the mutants. I identified 2 isolates which had double mutations in *kat*G and *inh*A -15CT. None of the 49 pan-susceptible isolates had a mutation neither for rifampicin nor for isoniazid resistance.

Therefore sequencing of *rpo*B and *kat*G genes and *inh*A promoter region for resistance to rifampicin and isoniazid, has proved that genotypic testing methods can achieve 98% and 96% sensitivities for predicting drug resistance to rifampicin and isoniazid, respectively. The specificity for the molecular analysis was 100% compared to phenotypic gold standard drug susceptibility testing. My results have therefore validated the adoption and customization of rapid molecular diagnostics (targeting RRDR codons) by NTP in Zambia for efficient detection and management of MDR-TB in the country. The uniquely high *kat*G mutations (96%) conferring resistance to isoniazid observed in this study has been utilized by my research collaborators to develop a rapid and simple molecular tool (STH-PAS) for detection of isoniazid resistance. STH-PAS has been evaluated using *M.tuberculosis* isolates from Lusaka and has demonstrated potential for simple detection of isoniazid resistance. Such a tool can complement Xpert/MTB RIF for confirmation of MDR-TB in Lusaka.

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