Molecular epidemiology of multi-drug resistant *Mycobacterium tuberculosis* strains in Swaziland

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Authors declaration

This study represents the author's original work. It has not been submitted previously for any qualification to this or any other tertiary institution. Where use of work of others was made, such help has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Moneni Tuberculosis Referral hospital, Mbabane Government hospital, Nhlangano Health Centre, Swaziland National Tuberculosis Reference Laboratory, University of Pretoria Faculty of Health Sciences (Department of Microbiology), South Africa.

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Abstract

Background:

The tuberculosis (TB) epidemic remains a major global health problem, noting that the Swaziland government had declared the disease a national emergency. Hence the study was aimed to determine the prevalence of TB in Swaziland and further to ascertain whether the circulating susceptible *M. tuberculosis* strain later develops to MDR-TB on the same patient or whether patients acquire a completely new multi-drug resistant strain.

Methods: Participants were recruited from four TB testing facilities (n=560), which are regional TB referral facilities in Swaziland. Willing participants who had been selected using a systematic random sampling method, and who met the inclusion criteria, were included in this quantitative descriptive cohort study upon signing an informed consent form (n=103). Sputum samples collected from these participants (at baseline and at follow-up) were tested for the presence of *Mycobacterium tuberculosis* using the GeneXpert® MTB/RIF assay (Cephied, USA). When found to be positive, the samples were then cultured on the BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA), which was also utilised for drug sensitivity testing and thereafter DNA was extracted for spoligotyping, using a GenoLyse (Hain Life Science, Germany). The study results were analysed using the Statistical Package for the Social Sciences (SSPS) version 25, Epi Info (version 3.5.1, 2008) and STATA 13.0 software packages. Frequencies of multiple-drug resistant-TB (MDR-TB) amongst all the genotype families' categories based on spoligotyping were compared among the participants from the four regions using a Fisher Exact and Chi-square statistical methods, where associations with a p-value of < 0.05 were considered statistically significant. The spoligotyping findings were entered into an MS Excel spreadsheet as a binary code signifying either a positive or negative hybridization outcome. The spoligotyping results were then entered into SITVIT2 database (Pasteur Institute of Guadeloupe) to generate spoligotype families.

Results: The prevalence of TB infection in this study population was 26.41% (n=103/390) and MDR-TB prevalence was 33.01% (n=34/103). Notably, the TB infection was high among males (64%; n=103) and among the young adults of both genders (18-35 years, with a mean age of 34.4 years). Most of the strains at both baseline and follow-up (58.74%) were susceptible to all of these anti-TB drugs, followed by those strains classified as MDR-TB, with 28.64% and poly-resistant and RIF mono-resistant strains being 8.25% and 2.91%% respectively, although with no statistical significance (p=0.786). The Beijing sub-lineage (lineage 2) was the predominant

sub-lineage in 28.82% isolates (n=49/170), followed by lineage 4 (Euro American) the S and T1 sub-lineages (of lineage 4) in 20.0% and 11.76% isolates, respectively. The emerging U sub-lineage was isolated as well with n=1 (2.2%) p=0.001. We then compared sub-lineage changes of baseline and follow-up specimens, together with DST patterns. A total of 33 (53.23%) participants with a pattern of 'changed sub-lineage and the same DST pattern' followed by n=16 (25.81%) participants showing 'changed sub-lineage with changed DST pattern'. The least represented categories were of seven (11.29%) participants with 'same sub-lineage with the same DST pattern' category as well as the 'same sub-lineage with changed drug sensitivity pattern' category with n=6 (9.68%). The Beijing genotype was significantly detected in the group with the 'changed sub-lineage and the same DST pattern' ($p \leq 0.001$).

Conclusion: Since high prevalence of TB infection was observed in this current study, mainly among men and young adults, hence mass TB screening and testing campaigns in all health centres and workplace wellness centres are recommended, to reduce the high TB prevalence. A key finding of this study was n=24/103 (23.30%) participants who were susceptible to all first line anti-TB drugs at baseline developed MDR-TB at follow-up. A significant proportion of those developed MDR-TB were infected with the Beijing sub-lineage, which is linked with MDR-TB outbreaks in many regions. Notably, Swaziland has a high *M. tuberculosis* lineage diversity, with eighteen sub-lineages noted. A significant proportion of the TB infected participants had the Beijing sub-lineage, which is linked with MDR-TB outbreaks in many regions. In addition, the emerging U sub-lineage, also linked to MDR-TB, was noted. Since different strains are reported to uniquely respond to treatment, therefore, it is hence recommended that the various genotypes of *M. tuberculosis* strains circulating in Swaziland be investigated and monitored, so as to improve on the TB treatment outcomes, control and prevention programs and detect timeously the drug resistant TB strains in Swaziland.

Key Words: Tuberculosis, Multi-drug resistant tuberculosis, Drug sensitivity testing, Deoxy-ribonucleic acid, Spoligotyping, *M. tuberculosis* lineages, *M. tuberculosis* sub-lineages

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Operational and conceptual definitions

Term	Operational and conceptual definitions
Cured	Treatment completed as recommended by the national policy without evidence of failure AND three or more consecutive cultures taken at least 30 days apart are negative after the intensive phase
Treatment success	The sum of cured and treatment completed
Treatment completed	Treatment completed as recommended by the national policy without evidence of failure BUT no record that three or more consecutive cultures taken at least 30 days apart are negative after the intensive phase
Treatment failed	Treatment terminated or need for permanent regimen change of at least two anti-TB drugs.
Died	A patient who dies for any reason during the course of treatment
Lost to follow up	A patient whose treatment was interrupted for two consecutive months or more
Not evaluated	A patient for whom no treatment outcome is assigned (This includes cases "transferred out" to another treatment unit and whose treatment outcome is unknown)
Non-adherence	A patient is said to be non-adherent if they miss three or more doses of the prescribed anti-TB drugs
Adherence	A patient is said to be adherent if they do not miss the prescribed anti-TB drug doses.
MDR-TB	Are strains which are resistant to both RIF and INH
Mono-resistant	Are strains which are resistant to only one of the first-line anti-TB drugs.
Poly-resistant	Are strains which are resistant to more than one first line anti-TB drugs, other than INH and RIF.

Abbreviations/acronyms

DNA	-	Deoxy-ribonucleic acid
DS-TB	-	Drug susceptible tuberculosis
DST	-	Drug sensitivity testing
EMB	-	Ethambutol
INH	-	Isoniazid
MDR-TB	-	Multi-drug resistant tuberculosis
M. tuberculosis	-	*Mycobacterium tuberculosis*
NTCP	-	National TB Control Program
PCR	-	Polymerase chain reaction
RIF	-	Rifampicin
SDS	-	Sodium dodecyl sulphate
SHLS	-	Swaziland Health Laboratory Services
STR	-	Streptomycin
TB	-	Tuberculosis
TB-DR	-	Drug resistant tuberculosis
WHO	-	World Health Organisation
XDR-TB	-	Extensively drug-resistant tuberculosis

CHAPTER 1

Background of the study

1.1 Chapter Introduction

This chapter addresses the background of the subject at hand, which is to investigate whether the circulating susceptible *M. tuberculosis* strain in Swaziland later developed to MDR-TB on the same patient or whether patients acquired a completely new multi-drug resistant (MDR) strain utilizing the spoligotyping technique.

1.2 Background

The tuberculosis (TB) epidemic remains a major global health problem, responsible for ill health among millions of people each year (Sharma *et al.* 2013; Smith *et al.* 2013; World Health Organisation 2018). An estimate of 10.0 million of the global population developed TB in 2018, where 5.7 million were men, 3.2 million women and 1.1 million were children (World Health Organisation 2019). The sub-Saharan region is not spared from this epidemic (Stuckler *et al.* 2011). Swaziland, which has since been renamed to Eswatini is not excluded from the mostly affected countries. When the study was conceptualised and ethical clearance obtained thereafter, the country was still known as Swaziland, hence this dissertation used Swaziland as the name of the country. Swaziland has an estimated TB incidence of 565 per every 100,000 population of individuals affected with both drug susceptible TB (DS-TB) and drug resistant TB (DR-TB) (Swaziland National Tuberculosis Control Program 2017). Hence, in the year 2011, the Prime Minister of Swaziland declared the disease a national emergency (Swaziland 2011; World Health Organisation *et al.* 2011).

Compounding to the TB challenge in most developing countries, human immunodeficiency virus (HIV), another pandemic is co-existent (Padmapriyadarsini *et al.* 2011a). In 2017, 36.9 million individuals globally were living with HIV, whereby 19.6 million were in Eastern and Southern Africa region (UNAIDS 2018). Of those individuals infected with HIV, 1.2 million of them were co-infected with TB (UNAIDS 2018). Swaziland is one of the countries in this region

that has high prevalence of HIV, affecting 31% of individuals in the 18–49 years age group (Mchunu *et al.* 2016). In addition, about 69% of the HIV infected individuals in this country are co-infected with TB (Stop TB partnership 2017).

HIV infection is associated significantly with lower treatment success in TB patients, which may lead to drug resistant tuberculosis (DR-TB) (Karo *et al.* 2016). Drug resistance is the natural ability of a typical organism to remain unaffected by harmful causes in its environment (Davies *et al.* 2010). Furthermore, multidrug resistance or multiple drug resistance refers to a occurrence seen where cells have developed natural resistance to a single cytotoxic drug as well as to other structurally unrelated chemotherapy agents (Wu *et al.* 2008; Müller *et al.* 2013). In addition to the multidrug resistance definition, the World Health Organisation specifies the cytotoxic drugs to be rifampicin and isoniazid and should be resistant to both (World Health Organisation 2019).

The lower TB treatment success associated with HIV infection may be due to reasons such as anti-TB drug and antiretroviral (ARV) drug interactions and thus overlapping adverse-effect profiles and co-toxicities (Cohen *et al.* 2011a), as well as the occurrence of paradoxical reactions after the initiation of effective antiretroviral therapy (Onyebujoh *et al.* 2007). A drug interaction in the co-administration of TB and ARV drugs happens at the level of absorption and hepatic elimination (Gengiah *et al.* 2011). The ARV drugs alter the pH of the gastro-intestinal tract and thus affect the absorption of other drugs and that may have an unfavourable impact on TB treatment effects (Gengiah *et al.* 2011; Manosuthi *et al.* 2016).

Globally, more than 50% of individuals co-infected with HIV and TB are sputum smear negative (Chamie *et al.* 2010; Padmapriyadarsini *et al.* 2011b) due to low sputum bacillary loads that may occur in the co-infection (Zumla *et al.* 2000; Padmapriyadarsini *et al.* 2011b). HIV-TB co-infected individuals in most cases produce sputum samples containing less than 10^4 per microlitre (10^4/µL) of *Mycobacterium tuberculosis* (*M. tuberculosis)*, the causative organism of TB (Kehinde Aderemi *et al.* 2013). This low bacillary load may also be caused by TB being extra-pulmonary (Heunis *et al.* 2017), a phenomenon that has been associated with HIV infection (Shivakoti *et al.* 2017)

The investigation of the impact of HIV-TB coinfection was not part of the current study objectives however, the contribution of the HIV-TB coinfection to the TB treatment failure and the risk of developing DR-TB has been highlighted above. The effects of HIV-TB co-infection on a sputum smear may result in delayed or inaccurate diagnosis and treatment of TB infection due to unusual presentation and lower rates of sputum smear positivity (Sasindran *et al.* 2011a; Hoang *et al.* 2015). This therefore means that the *M. tuberculosis* acid fast bacilli may not be detected in these individuals on the sputum smear with conventional smear microscopy, stained with the Ziehl-Neelsen stain. This staining technique is a rapid low-cost method commonly used in countries with limited resources, and it requires an infection dose of more than 10^4/µL of *M. tuberculosis* in a sputum specimen for detection (Chen *et al.* 2012; Kehinde Aderemi *et al.* 2013). This may then lead to the development of DR-TB, since the patient may not be diagnosed and treated on time for TB, especially in instances where the smear microscopy is the only method of detection of TB that is used. Hence, accurate and timeous *M. tuberculosis* laboratory diagnosis is vital in the detection, effective management and treatment of TB infection to avoid mutations which can result to DR-TB (Hmama 2013). Timely diagnosis of TB infection and early start of chemotherapy is essential for a successful treatment outcome (Pinto *et al.* 2011; Chen *et al.* 2012; Jeon 2015).

The presence of *M. tuberculosis* is screened with various laboratory tests and techniques. These tests include sputum smear microscopy; tuberculosis lipoarabinomannan (TB LAM) assay; serologic tests such as TB interferon gamma release assay as well as using the molecular assay-based instruments, which include GeneXpert® MTB/RIF assay (Cephied, USA) and TrueNat. Furthermore, *M. tuberculosis* can be cultured and analyzed for drug sensitivity using the thin layer agar (TLA), Lowenstein Jensen media (LJ) and BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) for phenotypic culture and drug sensitivity methods (Brodie *et al.* 2005; World Health Organisation 2014; Dunna *et al.* 2016). TB culture methods have higher sensitivity than smear microscopy (Kehinde Aderemi *et al.* 2013). The mycobacteria produces carbon dioxide after utilising oxygen, thus allow radiometric sensors of the automated liquid culture systems to detect growth of mycobacteria within one to two weeks (Padmapriyadarsini *et al.* 2011b). Out of all these internationally recognised *M. tuberculosis* diagnostic methods, in Swaziland only the sputum smear screening method and GeneXpert® MTB/RIF assay (Cephied, USA) are used to diagnose the TB presumptive patients.

Furthermore, culture and drug sensitivity of the detected TB strain is determined using the TLA, LJ and BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) methods (Swaziland National Tuberculosis Control Program 2017). These techniques detect *M. tuberculosis* but not the family strain genotype, which is more informative. It is viewed as essential that the actual infecting *M. tuberculosis* strain be identified for effective TB control strategies, to avoid the drug susceptible TB evolving to drug resistant TB.

The GeneXpert® MTB/RIF assay (Cephied, USA) diagnoses only the rifampicin resistant TB cases. Other types of resistant TB strains such as those with isoniazid resistance (INH-mono-resistance) are not detected with the GeneXpert® MTB/RIF assay (Cephied, USA) (Hmama 2013; Rahman *et al.* 2016). Globally, the prevalence of INH- resistance is higher (13.3%) than rifampicin resistance (6.3%) (Jenkins *et al.* 2011). On an individual level, patients with INH-mono-resistant disease are at a theoretically greater risk of developing MDR-TB than those with drug-sensitive TB (Johnson 2009; Stagg *et al.* 2016). Thus, it is vital to detect all circulating TB strains, to aid in the reduction of TB mortality using effective diagnostic methods. Notably, *M. tuberculosis* has seven main phylogenetic lineages, which are Indo-Oceanic (lineage 1), Beijing (lineage 2), Central Asian Strain (lineage 3), Euro-American (lineage 4), M. africanum I (lineage 5), M. africanum II (lineage 6) and Aethiops vetus (lineage 7). For example, if the lineage is of the Beijing family strain, which is known to be associated with MDR-TB outbreaks (Kato-Maeda *et al.* 2011b; Hove *et al.* 2012; Sankar *et al.* 2013), then strategies to detect and control the strain may be easily developed when it is known. The inability of the diagnostic and culture methods in identifying the actual TB strain thus necessitates the exploration of techniques which may be used in the strain detection such as molecular epidemiological techniques.

Advancements in molecular epidemiological techniques which have been reported to expand the ability to investigate and understand the tuberculosis epidemic are documented (Martinson *et al.* 2011; Hove *et al.* 2012; Cudahy *et al.* 2016; Nurwidya *et al.* 2018). The commonly used techniques are:

- ✓ IS6110-based restriction fragment length polymorphism analysis,
- ✓ Mycobacterial interspersed repetitive unit-variable number tandem repeat analysis
- ✓ Spoligotyping technique

- ✓ Ligation-mediated polymerase chain reaction (LM-PCR)
- ✓ Fluorescent amplified-fragment length polymorphism (FAFLP)
- ✓ Whole genome sequencing and single nucleotide polymorphism (SNP).

These molecular epidemiological techniques can detect *M. tuberculosis* and assign TB isolates to the major phylogenetic lineages and sub-lineages, utilising the SITVIT2 fourth international spoligotyping database (SpolDB4) (Ouafae Lahlou *et al.* 2012). Phylogenetic lineage classifications are based on the presence or absence of regions of difference and smaller deletions in the *M. tuberculosis* genome (Nebenzahl-Guimaraes *et al.* 2016). Notably, these molecular techniques are not conventionally used in resource-limited settings such as in Swaziland (Lawn 2015). Hence, the current study aimed to investigate the circulating MDR-TB strains in Swaziland using the spoligotyping technique.

Hypothesis

The circulating susceptible *M. tuberculosis* strain in Swaziland later develops to MDR-TB on the same patient.

Aim

To determine whether the circulating susceptible *M. tuberculosis* strain in Swaziland later develops to MDR-TB on the same patient or whether patients acquire a completely new multi-drug resistant strain.

Objectives

1. To determine drug susceptibility of the *M. tuberculosis* using the BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) proportional method
2. To determine the *M. tuberculosis* strain that infected the patients through spoligotyping technique.
3. To determine whether susceptible *M. tuberculosis* strains develop into multi-drug resistant strains by comparing baseline spoligotyping results with the follow up spoligotyping findings at six months or the last positive sample from converted patients.

Potential study benefits

The current study was expected to generate data on the circulating strain, which may inform the Swaziland Ministry of Health policies or guidelines in an attempt to contribute to the reduction of the TB incidence, where treatment strategies may be aligned with the particular strain. Research evidence supports the use of specific treatment regimens for the different strains as opposed to administering general treatment to all the strains (Matteelli *et al.* 2014; Gröschel *et al.* 2018).

The TB incidence reduction is key in the campaign to attain the End TB Strategy target, which is to End TB by 2030. The specific targets for 2030 set inside the End TB Strategy are a ninety percent reduction within the absolute quantity of TB deaths and an 80% reduction in TB occurrence (new cases per 100 000 populace according per year) as compared to the ranges said in 2015 (World Health Organisation 2018).

When the circulating TB strain has been ascertained, and whether it mutates to DR-TB within the same infected individual, the Swaziland TB Control programme may then utilise that data to come up with more strategies to control its transmission and mutation.

1.3 Summary of chapters

Chapter 1:

This chapter discusses the background information of the study which is the overview of what the investigation is all about. The importance of the study was detailed and further explained how it would benefit the different stakeholders which include patients infected with *M. tuberculosis*. The goal of the take a look at, that is the spine of the research, was stated whereby the objectives were drawn.

Chapter 2:

This chapter reviews literature and will critically analyze as well as evaluate previous research available on the subject in relation to the current study. This is where current TB epidemiology statistics to note the extent of the disease burden would be dealt with in depth. With HIV being the main perpetrator of the high TB mortality rate (World Health Organisation 2018), studies on the effects of HIV on TB progression will be reviewed. The chapter will also discuss the various immune responses to *M. tuberculosis* and how the failure thereof may lead to the evolution to DR-TB. This chapter will also highlight the key role played by the laboratory in the accurately diagnosing TB cases. Hence, different TB diagnosing methods will be reviewed together with molecular epidemiological techniques, which yield the TB lineages.

Chapter 3:

This chapter discusses data collection, management and analysis strategies. It also addresses issues of validity, reliability and ethics considerations.

Chapter 4:

The chapter presents only the study findings. These are the findings which will indicate whether the circulating susceptible *M. tuberculosis* strain in Swaziland later developed to MDR-TB on the same patient or whether patients acquired a completely new multi-drug resistant strain.

Chapter 5:

This chapter discussed the study findings and also analysed the results in-depth to accept or reject the study hypothesis.

Chapter 6:

Study recommendations and conclusions were made in this chapter based on the study findings.

CHAPTER 2

Literature Review

2.1 Mycobacterium complex overview

The disease tuberculosis (TB) it an respiratory tract disease caused be *M. tuberculosis* which is part of mycobacterium complex, which is a highly clonal group pathogen of both humans and other animals (Knechel 2009). The mycobacterium complex includes the species *M. tuberculosis, M. africanum, M. bovis, M. canettii, M. caprae, M. microti, and M. pinnipedii* (Riojas et al. 2018*).* The *M. tuberculosis* which causes infection in humans is a rod shaped, non-spore-forming, aerobic acid-fast-bacillus *Mycobacterium tuberculosis*, measuring 0.5 μm by 3μm (Djelouadji *et al.* 2008; van Ingen *et al.* 2012; Landolt *et al.* 2019). Typical symptoms of TB include: a persistent cough that lasts more than three weeks and usually brings up phlegm, which may be bloody, weight loss, night sweats, high temperature, tiredness and fatigue, loss of appetite and swellings in the neck (Campbell *et al.* 2006; Keshtkar Jahromi *et al.* 2014; Cudahy *et al.* 2016).

2.2 Tuberculosis epidemiology

Due to the TB burden worldwide, the World Health Organization (WHO) declared the disease a global emergency in 1993, which is still presently considered as such (Dirlikov *et al.* 2015; Hassarangsee *et al.* 2015).

In 2018, the actual estimates of the global tuberculosis (TB) incidence were 132 per 100 000 population (World Health Organisation 2019). Meanwhile, the average change of decline in the global TB incidence rate remains low, at 1.6% per year between 2000 and 2018, and 2% between 2017 and 2018 (World Health Organisation 2019). This is an indicator for the need to accelerate efforts to decrease the TB incidence rate by 4–5% per year by 2020, as set by WHO in the End TB Strategy, to realize the milestones for drops in cases and deaths (World Health Organisation 2018).

An incidence of 301 per 100 000 population was estimated in South Africa in 2018, and that of Lesotho and Mozambique were 13 per 100 000 population and 162 per 100 000 population respectively (World Health Organisation 2019). Namibia has also shown to be among countries with the highest TB incidence rate with 524 per 100 000 population in 2018 (World Health Organisation 2019). Although high TB incidence rates have been reported in some regions, there are regions where declines in TB infection have been noted. The fastest declines have been observed in the WHO European Region at 5% per year, followed by WHO Africa region with 4% per year from 2013 - 2017 (World Health Organisation 2018). The Southern African countries (Swaziland, Lesotho, Zambia, Zimbabwe, South Africa) have a collective impressive reduction of 4 - 8% (World Health Organisation 2018). Although the declines in the region are reported, the rate of reduction in Swaziland as a country has not reached the target as set out by the End TB Strategy, which is a 4 – 5% decline per year by 2020 (World Health Organisation 2018). Notably, World Health Organisation (2019) estimated the incidence of TB infection in Swaziland to be 329 per 100,000 population in 2017. Mchunu *et al.* (2016) stated that the expansion of high-quality TB services and the timely case detection rate, which was not observed in Swaziland, has contributed to the declines in the neighboring countries. Swaziland is still experiencing some challenges with TB mortality rate of 14-16% (Mchunu *et al.* 2016; Swaziland National Tuberculosis Control Program 2017) Yet in 2018, the mortality rate was 18% in Swaziland (World Health Organisation 2019). Declines in TB incidence will help achieve the 2020 milestone which is 35% reduction in TB deaths from the death rate of 17% in 2015 to 10% (World Health Organisation 2018). This suggests that Swaziland, which is among countries with high TB incidence rates (Figure 1), may have to review their TB control strategies.

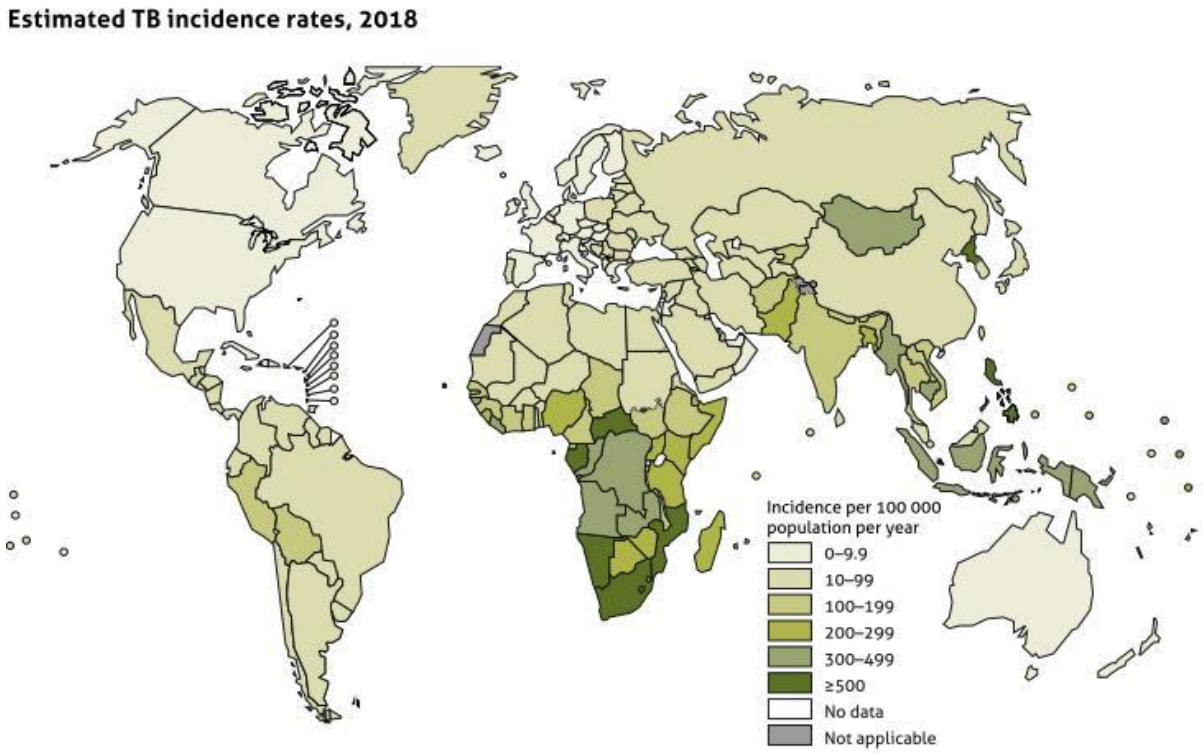

Figure 1:The global estimates of TB incidence rates in 2018 indicating Swaziland among countries with high TB infection rates, with TB incidence greater than or equals to 300 per annum (World Health Organisation 2018).

2.2 HIV and TB co-infection epidemiology

Human immunodeficiency virus (HIV) infection is another pandemic, which may co-exist with TB, due to the geographic overlap of these two infectious diseases (Barnabas *et al.* 2011; Padmapriyadarsini *et al.* 2011a). Globally in 2017, approximately 36.9 million individuals were living with HIV, where 1.2 million of them were co-infected with TB (UNAIDS 2018). Swaziland is one of the countries that has high prevalence of HIV, affecting 31% of individuals between the age of 18 – 49 years (Mchunu *et al.* 2016). Of these HIV infected individuals, 66% are co-infected with TB (World Health Organisation 2019) (Figure 2). According to Mchunu *et al.* (2016), despite a global antiretroviral (ART) uptake of as much as 88%, no reduction in mortality among notified TB cases happened in Swaziland between 2008 - 2012, but rather a trend towards a higher mortality. HIV infection compromises the immune system, and may therefore increase the probability of acquiring TB (Alland *et al.* 1994; Ngowi *et al.* 2008; Heunis *et al.* 2017). TB is the most common opportunistic infection (OI) amongst HIV-infected people,

causing exacerbation of viral load and reduced CD4 be count (Montales *et al.* 2015), which results in the worsening of the TB infection.

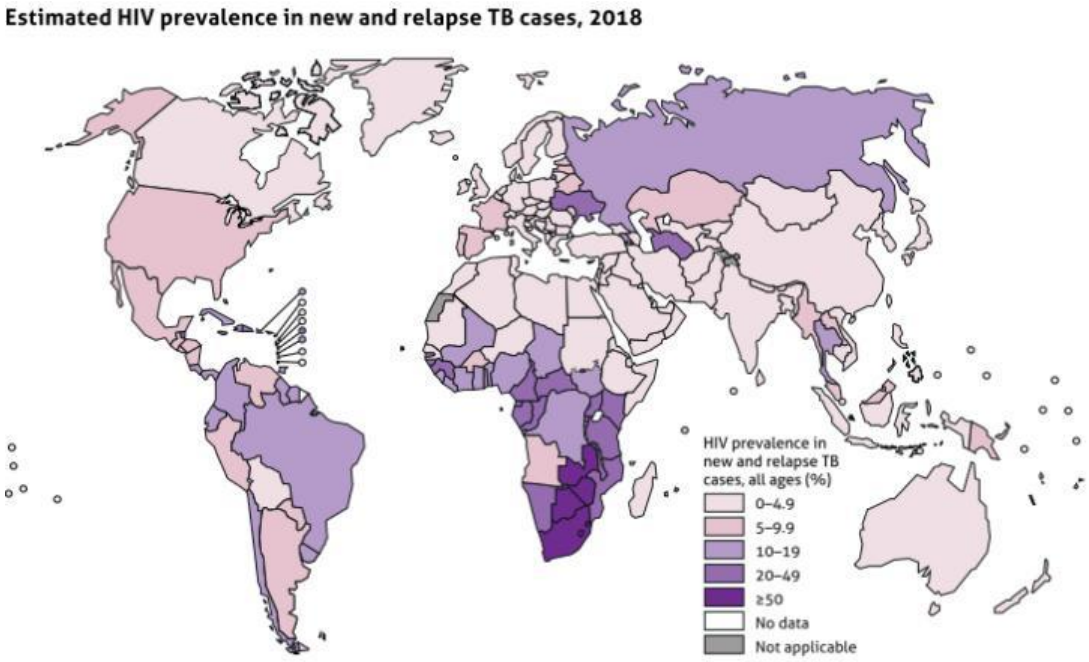

Figure 2: The global estimates of HIV prevalence in new and relapse TB instances in 2018 indicating Swaziland among countries with high HIV prevalence in new and relapse TB cases (greater than or equals to 50%).

HIV-TB co-infected individuals have high mortality rate due to the speedy progression of TB (Padmapriyadarsini *et al.* 2011a). This challenge of TB progression is compounded by the fact that patients may not present themselves to health care facilities timeously for early TB detection and treatment. Factors contributing to this include (i) the anticipated HIV-related stigma which may undermine optimal care seeking behaviour; (ii) the perception of TB as a disease of the poor and 'dirty'(Royce *et al.* 2017; Badu *et al.* 2018); (iii) the intricate link between HIV infection with drug abuse, previous imprisonment, being a sex worker, which acts as a major barrier to routine testing and the provision of optimal care (Trinh *et al.* 2015); (iv) low confidence in health care practitioners concerning the ensuring of patient confidentiality (Atif *et al.* 2016). These highlighted perceptions have the potential of influencing early detection and thus induce mutation of the *M. tuberculosis* in infected individuals by not presenting themselves at health care facilities and treating the infection early.

2.3 The role of immune responses to *Mycobacterium tuberculosis* infection

M. tuberculosis bacilli, either susceptible or drug resistant, always try to find a way to survive within the host (Forrellad *et al.* 2013). Although *M. tuberculosis* develops different systems and strategies to defeat the host's immune system, the host tirelessly fights to clear the bacilli. The human host elicits cell-mediated immunity to control *M. tuberculosis* infection, where the activation of both $CD4^+$ and $CD8^+$ T cells is observed in active TB (Woodworth *et al.* 2006; Lin *et al.* 2015). T cells recruited to the site of infection control the pathogen by means of generating interferon gamma (IFN-γ) in response to mycobacterial antigens provided by using macrophages (Yang *et al.* 2018). In turn, IFN-γ activates macrophages to kill the intracellular bacteria through reactive nitrogen and oxygen intermediates, and with the aid of inducing phagolysosome formation (Herbst *et al.* 2011) (Figure 3).

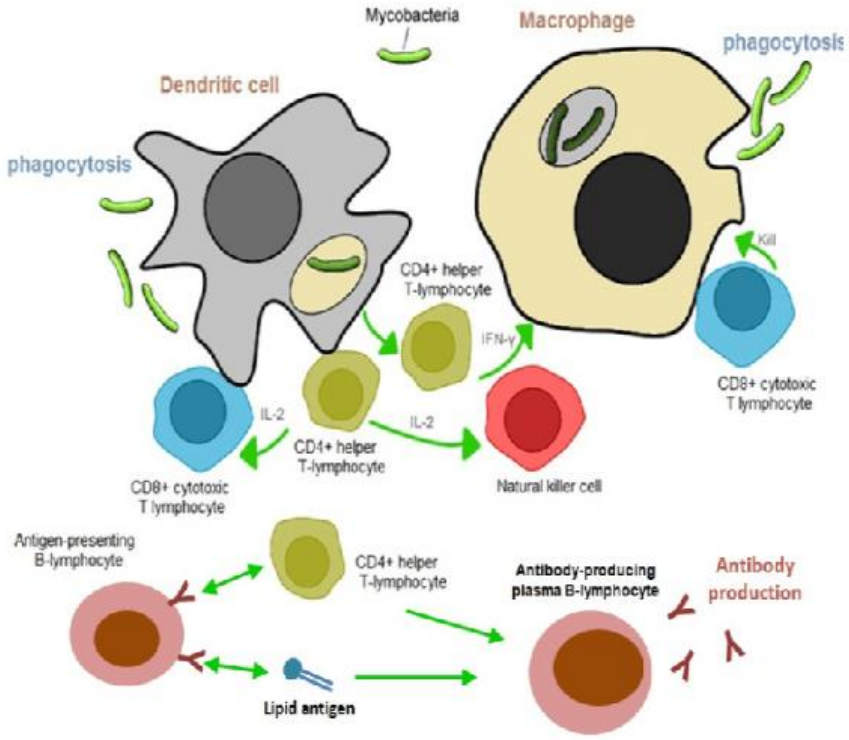

Figure 3: An overview of adaptive cell-mediated responses to *M. tuberculosis* infection indicating the internalization and processing of the mycobacteria and the activation of the $CD4^+$ and $CD8^+$ effector cells (Arroyo *et al.* 2012).

IFN-γ and tumour necrosis factor-α (TNFα), mainly formed by type-1 T-helper (Th1) cells, are main cytokines to fight off the *M. tuberculosis* infection. IFN-γ production is controlled by interleukin 12 (IL-12), which is secreted by stimulated macrophages (Trinchieri 2007; Xiaojing *et al.* 2015). Other important pro-inflammatory cytokines are IL-1β and IL-6, which may prime the Th17 differentiation, as a division of T cells vital for the formation of mature pulmonary granulomas (Timmermans *et al.* 2016; Tristão *et al.* 2017). The granuloma, a mass of granulation tissue, functions both as the niche in which the bacillus can grow or persist as well as the immunological environment in which host cells interact to control and prevent dissemination, resulting in latent TB (Gideon *et al.* 2011).

The host usualy strives for a equilibrium between immune activation and inflammation which influences the outcome of *M. tuberculosis* infection, which may either, remain in the latent stage or become an active infection (Sasindran *et al.* 2011b; O'Garra *et al.* 2013). The tireless immune activation may be self-defeating for the host, paving the way to active TB development and intensification (Alessandra *et al.* 2018). A significant production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and initiation of a functional autophagy progression is associated with a higher rate of replication in *M. tuberculosis* strains belonging to lineage 4 (Ferraz *et al.* 2006; Romagnoli *et al.* 2018). The autophagy process is the condition whereby the body consumes its own tissue as a metabolic process (Glick *et al.* 2010). Figure 4 depicts the *M. tuberculosis* infection cycle; which is how the bacilli defeats the immune system and cause an active infection as opposed to when the immune system manages to control the infection and result in a latent infection.

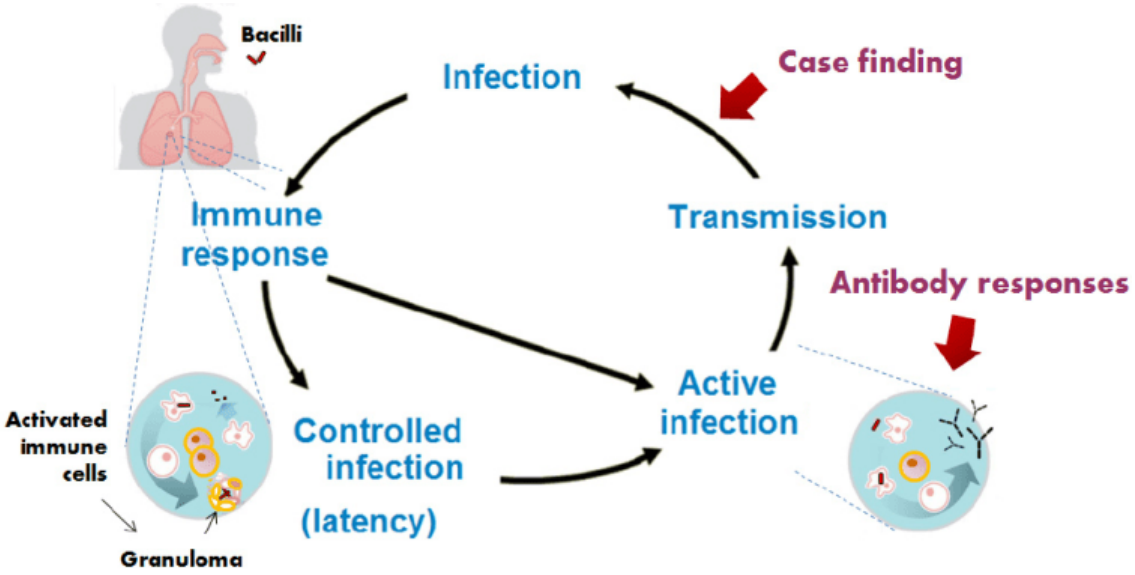

Figure 4**:** The pathogenesis of the *Mycobacterium tuberculosis* infection indicating the formation of latent and active stages of the infection (Arroyo *et al.* 2012).

HIV in a co-infection may cause the immune system to be unable to control the infection. To control *M. tuberculosis* infection, the human host activates the production of both $CD4^+$ and $CD8^+$ T cells (Woodworth *et al.* 2006; Lin *et al.* 2015). Yet the characteristic immunological effect of HIV is on CD4 cell count; which is decreased (Walker *et al.* 2013a). Thus, the immune system fails to control the infection. Reactivation of latent tuberculosis infection is also associated with reduction of $CD4^+$ T cells (Bucşan *et al.* 2019). The vital decline within the range of $CD4^+$ T cells is related to a lower in the number of reminiscence $CD4^+$ T cells ($CD27^+$ $CDRO45^+$) which can understand the *M. tuberculosis* antigens, a decrease in polyfunctional antigen-specific $CD4^+$ T cells and a relative increase in interferon gamma and CD 8^+ T cells, which may not be effective (Geldmacher *et al.* 2010; Sutherland *et al.* 2010). There is evidence from animals which support the importance of CD8 T cells in the immune response against *M. tuberculosis*, yet with humans it is much more difficult to determine the contribution of CD8 T cells to protection against tuberculosis infection (Lin *et al.* 2015).

In HIV-TB co-infection, TB may progress rapidly due to HIV-mediated impaired responses, which include: (i) the failure of the immune reactions to limit the growth of *M. tuberculosis* (Kwan *et al.* 2011; Sasindran *et al.* 2011a); (ii) the impaired responses that result in the

depletion of $CD4^+$ T cells, an significant contributor to the increased risk of rebirth of latent TB and susceptibility to new *M. tuberculosis* infection (Pawlowski *et al.* 2012; Rukumani *et al.* 2014); (iii) the up-regulation of *M. tuberculosis* access receptors on macrophages (Rosas-Taraco *et al.* 2006). *In vitro* research has validated that *M. tuberculosis* infection can up-regulate both HIV infection and duplication inside monocyte-derived macrophages (MDMs), increase the efficiency of virus transmission from infected MDMs to T cells, and favour replication of syncytium-inducing (SI) HIV (Pawlowski *et al.* 2012); (iv) furthermore, monocytes from HIV infected patients exhibition an impaired reaction to Toll-like receptors (TLR) ligands (Jiang *et al.* 2005); (v) the viral proteins can impede with both the maturation and function of MDM and dendritic cells (DC); (vi) suppressor of cytokine signalling 1 (SOCS1), which is inspired by infection with *M. tuberculosis* (Rosas-Taraco *et al.* 2006), has been shown to help the late duplication pathways of HIV infection and facilitate viral evasion of type I IFN anti-viral signalling, that allows the tuberculosis to be worse (Fenner *et al.* 2005); (vii) HIV-mediated impaired tumour necrosis factor (TNF)-mediated macrophage apoptotic reaction to *M. tuberculosis*, facilitating bacterial survival (Amaral *et al.* 2016). All these mentioned factors highlight the contribution of HIV to the exacerbated progression of TB.

2.3.1 The modulated immune responses to *M. tuberculosis* infection

Virulent *M. tuberculosis* species develop many strategies to evade the immune response one way or the other, in their favour for survival within the host (Forrellad *et al.* 2013). These virulence factors enable the *M. tuberculosis* to resist host immune system effects and, at the same time, chronically stimulate it (Smith 2003; Cambier *et al.* 2014). *M. tuberculosis* virulence determinants are divided into the following classes based on their purpose, molecular features or cellular localization: (i) the pathogen alters its lipid and fatty acid metabolism, including the catabolism of cholesterol (Ghazaei 2018). The lipid profile of the cell wall has been revealed to modulate the immune responses instigated by the host organism, especially in the suppression, or production of inflammatory factors, cytokines, and phagocytic cells, inclusive of dendritic cells and macrophages (Mukhopadhyay *et al.* 2012; Jia *et al.* 2017; Ghazaei 2018); (ii) the *M. tuberculosis* uses cell envelope proteins (cell wall proteins, lipoproteins and secretion systems) for persistence inside the hostile microenvironment of the host macrophages (Smith 2003; Maan *et al.* 2018); (iii) proteins protecting the pathogen against antimicrobial effectors of the

macrophage (Wilson *et al.* 2002), including those involved in responses to oxidative and nitrosative stresses, phagosome arresting and inhibition of apoptosis (Cumming *et al.* 2017); (iii) the pathogen also uses protein kinases to suppress phagosome-lysosome fusion (Zhai *et al.* 2019) (iv) proteases can also be utilised by the *M. tuberculosis* to defeat the host immune system. To illustrate this, it is reported that treatment with inhibitor bestatin strongly inhibited leucine aminopeptidase of *M. tuberculosis* activity, *M. tuberculosis* growth and macrophage infection (Smith 2003; Forrellad *et al.* 2013; Correa *et al.* 2017), and (v) other *M. tuberculosis* proteins of unknown function, including Pro-Glu (PE) and polymorphic CG-repetitive sequences (PE_PGRS) families inhibit treatment effect by neutralising the host immune response (Tian *et al.* 2010; Forrellad *et al.* 2013). The PGRS domain helps *M. tuberculosis* to modulate the release of TNF-α from macrophages, thus protecting itself from macrophages effects.

In addition to what the pathogen does to invade the host immune system, its cell wall anatomical structure has immunogenic lipoarabinomannan (LAM) which it uses to survive within the host. LAM is a major lipopolysaccharide component of the cell wall recovered in large quantities from *M. tuberculosis* facilitating survival within macrophages making TB treatment challenging (Joe *et al.* 2007). The LAM is a potent inhibitor of IFN-γ facilitated activation of murine macrophages. Studies of the ways by which this mycobacterial glycolipid reduces macrophage effector functions provide proof that LAM actions at several levels and that it can (i) search possibly cytotoxic oxygen free radicals (Roach *et al.* 1993), (ii) impede protein kinase C activity (Chan *et al.* 1991b), and (iii) block the transcriptional activation of gamma interferon-inducible genes in human macrophage-like cell lines (Diskin *et al.* 2018). The LAM can inhibit immune response by activation or triggering the cytocidal activity that may represent a chemically defined virulence factor contributing to the persistence of mycobacteria within the mononuclear phagocytes (Chan *et al.* 1991a; Attanasio *et al.* 2000; Chan *et al.* 2015).

In summary, the main immune evasion techniques adopted by *M. tuberculosis* are based on the inhibition of (a) phagolysosomes formation by the use of protein kinases and proteases; (b) antigen processing and presentation, to escape from T-cell surveillance by modulating the major histocompatibility complex (MHC) class I, class II and cluster of differentiation 1 (CD1) molecules (Baena *et al.* 2009); (c) IFN-γ-signalling pathway; and (d) autophagy which is a condition whereby the body consumes its own tissue as a metabolic process (Glick *et al.* 2010;

Alessandra *et al.* 2018). The survival and invading mechanisms developed by the *M. tuberculosis* to persist in the host cells can lead to multi-drug resistant TB or remain drug susceptible.

The drug susceptible TB implies that isolates are inhibited by the usually achievable concentrations of antimicrobial agents when the recommended dosage is used for the site of infection. In other words, the pathogen is capable of submitting to an action, process, or operation of the drug (World Health Organisation 2009). The purpose of antimicrobial susceptibility testing is to envisage the in vivo success or failure of antibiotic remedy. Tests are completed in vitro, to ration the increase response of an isolated organism to a specific drug or drugs. The investigations are accomplished beneath standardized laboratory settings in order that the outcomes are reproducible. The test effects ought to be used to manual antibiotic choice. The results of antimicrobial susceptibility examination have to be combined with clinical information and experience when choosing the maximum appropriate antibiotic for patients (World Health Organisation 2009).

Resistance is the natural capacity of a regular organism to stay unaffected by using noxious agents in its environment (Davies *et al.* 2010; Malinga *et al.* 2016a). Multidrug resistance or multiple drug resistance refers to a phenomenon realized in some malignant cell lines where cells have developed natural resistance to a single cytotoxic compound and are also unaffected by structurally distinct chemotherapy agents (Wu *et al.* 2008; Müller *et al.* 2013). Resistance can develop through spread (primary resistance) or under selective pressure of anti-tuberculous agents (acquired) (Tie *et al.* 1999). Primary resistance is defined as the presence of drug resistant TB in a patient with less than one month of treatment and acquired resistance is defined as resistance to anti-tuberculous drugs where a patient has received at least one month of anti-tuberculosis treatment (Lancelot *et al.* 2011).

There are two types of *M. tuberculosis* resistance, namely intrinsic and acquired. Intrinsic resistance of *M. tuberculosis* to most antibiotics, including macrolides which inhibits protein synthesis, is generally attributed to the low permeability of the mycobacterial cell wall (David-Bosne *et al.* 2000; Buria´nkova *et al.* 2004). This kind of resistance sometimes is referred to as

natural resistance. According to da Silva *et al.* (2011), drug efflux, which is the process of removing drugs before affecting the microorganism, has been described as an important mechanism for intrinsic and acquired drug resistance. Acquired drug resistance is generally mediated through horizontal transfer by mobile genetic elements, such as plasmids and transposons (Smith 2003). This is particularly resulting from spontaneous mutations in chromosomal genes, generating the selection of resistant strains at some stage in sub-most reliable drug therapy (Stephen 2002; Smith *et al.* 2013; Lee *et al.* 2015)

Drug susceptible TB may progress to becoming multi-drug resistant. There are many factors which may contribute towards the mutation of the drug susceptible TB to MDR-TB, such as the chemical modification of drugs by *M. tuberculosis* (Nguyen 2016). The bacteria shields itself against anti-tuberculous drugs through modification of their drug targets, thus reducing the antibiotic binding affinity (Smith *et al.* 2013). In this case, *M. tuberculosis* can inactivate antibiotics via direct chemical modifications (Nguyen 2016; Kerantzas *et al.* 2017; Zhang *et al.* 2018). There is also enzymatic degradation of drugs, molecular mimicry of drug targets and drug deportation by efflux pumps (Smith *et al.* 2013). Epigenetic drug tolerance mechanism has been observed in *M. tuberculosis* (Hameed *et al.* 2018; Sakatos *et al.* 2018). This involves the efflux as a mechanism for drug resistance, which means the *M. tuberculosis* pathogen recognize and pump toxic compounds from within the cell to the environment before they reach their targets (Venter *et al.* 2015). The drug efflux has been noted through studies as a serious threat mechanism for both natural and acquired resistance (Fange *et al.* 2009; Pagès *et al.* 2009; da Silva *et al.* 2011). *M. tuberculosis* evolution is inevitable to the development of drug resistant TB, which is very much harsh to the immune system.

When treatment is initiated, the *M. tuberculosis* is under pressure to produce changes in its survival pathways that functionally contribute to its resistance (Palomino *et al.* 2014; Fonseca *et al.* 2015). Many investigators pin down TB evolution to treatment non-compliance and prescription of incorrect anti-TB regimens as main contributors towards the *M. tuberculosis* evolution (Rabahi *et al.* 2002; Adane *et al.* 2013; Evans *et al.* 2015). Also, poor management of patients (Kaona *et al.* 2004), poor anti-TB drug quality, patient-dependent pharmacodynamics and pharmacokinetic properties can lead to TB strains with mutations conferring challenge to anti-tuberculous drugs (Kempker *et al.* 2015; Boru *et al.* 2016). Drug resistance in *M.*

tuberculosis was commonly believed to be caused by single-step mutations (Koch *et al.* 2014; Palomino *et al.* 2014; Dookie *et al.* 2018), however there is evidence to suggest that, at least for certain anti-tuberculous drugs, it is the result of a stepwise acquisition of mutations leading to a gradual decrease in susceptibility (Johnson *et al.* 2006). Initial steps in the cascade may involve a mutation that does not increase the minimum inhibition concentration of a drug above the breakpoint for clinical resistance, so such leads to drug resistance. (Reeves *et al.* 2013; Fonseca *et al.* 2015).

Furthermore, non-compliance to treatment contributes to worsening of the TB burden, not only by increasing incidence but also by initiating drug resistance (Salahuddin *et al.* 2013; Boru *et al.* 2016). Non-adherence (non-compliance) is a term used when a patient has missed three or more of the doses of prescribed anti-TB treatment drugs (Boru *et al.* 2016). Adherence is a dynamic issue and obstacles to observance are probable to change by individual perspective on treatment over time (Adane *et al.* 2013). Patients' compliance with medication is properly understood in the context of self-determination theory, since it considers personal and societal matrixes in explaining behavior (Stephen 2002). According to self-determination theory, society has determined effects on the inspiration and psychosomatic well-being of an individual (Stephen 2002). Patients' with chronic diseases including TB therapy defaulting habits is a worldwide problem of striking magnitude (Adane *et al.* 2013). In Brazil, a national study on tuberculosis drug compliance was conducted, and the rate of non-compliance was 12-16%. On the same study it was noted that TB patients in the major cities had a high non-compliance rate of 27-34% due to societal factors (Rabahi *et al.* 2002). There is quite a number of factors which contribute to non-compliance, which include forgetting to take the drugs timeously, the increased number of drugs to be taken in HIV co-infection and/or the experience of drug side-effects (Rabahi *et al.* 2002; Adane *et al.* 2013). Adherence to TB treatment is very important for one to consider to achieve cure, and decrease the risk of the strain acquiring resistance to that drug regimen (Adane *et al.* 2013). TB patients are said to be cured when they present with negative bacilloscopy in clinical respiratory samples and/ or show satisfactory clinical and X-ray improvements.

In addition, the TB drug prescription must be appropriate. It happens due to patient condition that treatment is initiated without drug sensitivity tests results, because such results takes long to be concluded (Chhabra *et al.* 2012; Prasad *et al.* 2018). In such cases, inappropriate prescription

might be issued. Correct TB chemotherapy is critical to avoid the development of intrinsic resistance, not ruling out the acquired resistance, as an appropriate anti-TB dosage is a factor that may contribute to drug resistance development (Fonseca *et al.* 2015). Each anti-TB treatment regimen should be regarded as the minimum effective therapy, based on laboratory results on drug minimum inhibition concentration (Tie *et al.* 1999; Fonseca *et al.* 2015). Compartmentalization of the treatment of the TB infection such as therapy decisions that are based on the detection of the bacilli, irrespective of the strain, makes it more likely that bacteria will be exposed to monotherapy, augmented by the patient receiving the inadequate therapy. This may arise due to an inadequate dosage because of insufficient prescription by the physician or non-adherence by the patient (Stephen 2002). Treatment of all forms of drug-resistant TB must be according to the DST results which address the specific form of resistance (Lancelot *et al.* 2011). Importantly, reduction of the TB burden depends in part on the accurate and rapid laboratory diagnosis of the active disease (Caulfield *et al.* 2016).

2.4 The diagnosis of *Mycobacterium tuberculosis*

The laboratory TB diagnostic or identification tests are important for screening TB presumptive patients and follow up the effectiveness of treatment against this microscopic organism. The TB diagnostic or identification tests used include: sputum smear microscopy, Chest X-ray, TB LAM, serologic tests (TB interferon gamma release assay, sero-diagnostic tests), molecular tests (LPA, Hain Life Science, GeneXpert MTB/RIF assay Cephied, TrueNat). *Mycobacterium tuberculosis* can be cultured and analyzed for drug sensitivity using the thin layer agar (TLA), Lowenstein Jensen media (LJ) and BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) phenotypic culture and drug sensitivity methods (Brodie *et al.* 2005; World Health Organisation 2014; Dunna *et al.* 2016). In Swaziland, the sputum smear, GeneXpert MTB/RIF assay Cephied and Line probe assay (Hain Life Science, Germany) methods are utilised to diagnose TB in presumptive patients. For culture and sensitivity, thin layer agar (TLA), Lowenstein Jensen media (LJ) and BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) methods are utilised and all yield reproducible results.

2.4.1 *Mycobacterium tuberculosis* laboratory diagnostic methods

2.4.1.1 Sputum smear microscopy

The sputum test is often considered as the initial TB laboratory diagnostic tool in countries with a high rate of TB infection (Tostmann *et al.* 2008). Sputum smear microscopy is economical, easy to perform, and personnel training can be done relatively quickly. In addition, the results are obtainable within an hour. It is based on the Ziehl-Neelsen (ZN) stain (Chen *et al.* 2012). *M. tuberculosis* is distinguished as a Gram negative, rod shaped bacilli under the microscope when analysing a ZN stained smear (Knechel 2009). The sensitivity of the sputum smear microscopy method is however reported to be only about 50-60% (Alfred *et al.* 2014). In countries with high prevalence of both pulmonary TB-HIV infection, the microscopic detection rate can be lower, since such individuals have a tendency to have a very low levels of TB bacteria in their sputum samples (Bruchfeld *et al.* 2015), and may therefore be recorded as sputum negative.

The use of fluorescent microscopy increases the microscopic detection rate (Desikan 2013). With a fluorescent microscope the smear is brightened with a quartz halogen or high pressure mercury vapour lamp, allowing a much greater area of the smear to be seen, resulting in more fast examination of the specimen (Nema 2012). Unfortunately, in resource limited settings, light microscopes are used instead of a fluorescent microscope, which may lower the rate of detection. Figure 5 shows how an *M. tuberculosis* bacillus appears on a sputum sample stained with the ZN stain under the light microscope. The size of the bacillus is approximately 0.5μm by 3μm under the light microscope using the 100X oil objective (Zhao *et al.* 2012). Its detection requires trained skilled personnel to process, stain and screen the sputum smears for accurate detection.

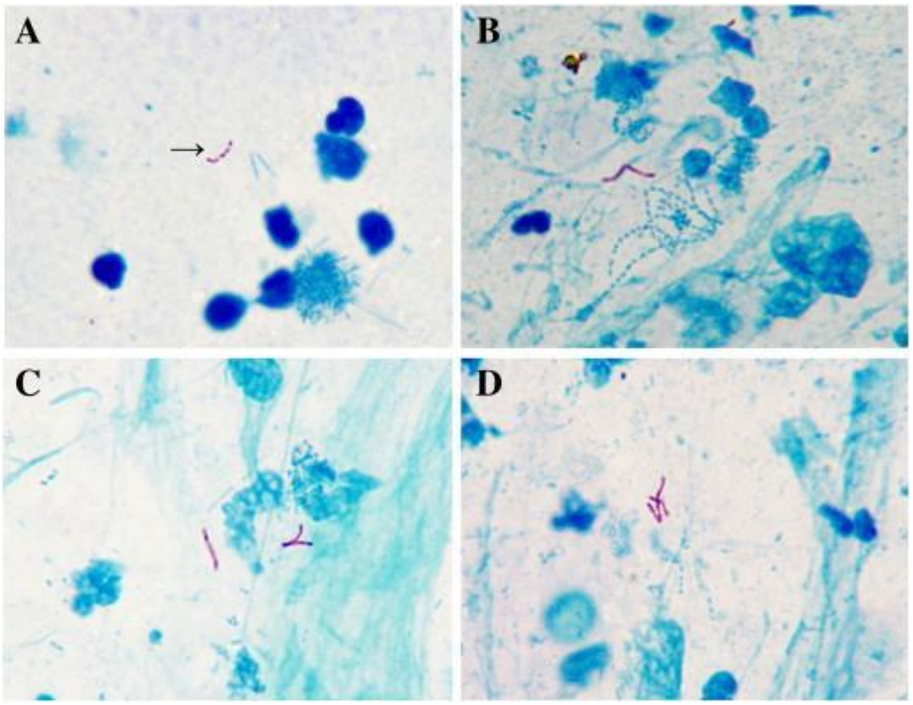

Figure 5: Sputum smears stained with Ziehl-Neelsen stain (ZN) depicting the morphology of the acid-fast bacillus *M. tuberculosis* in slides A-D (Zhao *et al.* 2012)

2.4.1.2 TB Interferon gamma release assays (IGRAs)

The Interferon Gamma Release Assays (IGRAs) are a new type of TB tests that are more accurate. IGRAs utilises blood as sample to measure a person's immune response to the bacteria that cause TB (Horvat 2015). The immune system produces the interferon gamma cytokine, and this TB tests work by detecting it (Yu *et al.* 2012; Wang *et al.* 2018). With an IGRA TB test requires patient to visit once to carry out the TB test and test findings are also obtainable within 24 hours like in sputum smear microscopy. Disadvantanges of IGRAs include the fact that (i) the blood sample must be processed within 8-16 hours, (ii) laboratory facilities are required, and (iii) the test is only for latent TB. It is also believed that the IGRAs are not accurate in HIV-TB infected people (Schluger *et al.* 2010; Padmapriyadarsini *et al.* 2011b).

Serological tests for TB detection, such as the IGRA, detect antibodies in the blood (Steingart *et al.* 2012; Maes 2017). However, detecting *M. tuberculosis* antibodies in the blood is very difficult due to low sensitivity and specificity (Singh *et al.* 2011; Nayak *et al.* 2012). That render them inaccurate and unreliable (Steingart *et al.* 2012). This is due to the instability of the test

kits in detection kits (Hwang *et al.* 2014). The kind of instability makes the kits not ideal in clinical application due to the fact that the quality of the antigen was not controllable. The kits would provide inconsistent and imprecise findings resulting in highly variable values for sensitivity and specificity (World Health Organisation 2011). Secondly, the method of antigen preparation, purity and the concentration affects the quality of the detection reagents (Bai *et al.* 2018). The World Health Organization (WHO) has warned against the use of these kits because of the aforementioned reasons (Bai *et al.* 2018).

2.4.2 Serological tests for TB (sero-diagnostic tests)

2.4.2.1 TB-LAM test

It is notably not easy to diagnose TB in people living with HIV, since some of them have extra-pulmonary TB or the individuals are too feeble to produce sputum samples (Chan *et al.* 1991a; Wood *et al.* 2012). A novel diagnostic with the potential to overcome these challenges is a urine-base rapid point of care lipoarabinomannan (TB-LAM) test (Dunna *et al.* 2016; García-Basteiroa *et al.* 2018). WHO recommends TB-LAM to diagnosis TB among HIV infected people with CD4 cell counts of ≤100 cells/μL and TB symptoms, or for any HIV-positive person who is very ill (World Health Organisation 2015; Lawn *et al.* 2016; Huerga *et al.* 2019). TB-LAM test has a potential to increase the number of people with confirmed TB by nearly 20% due to its sensitivity to diagnose the infection among the HIV-TB co-infected individuals (Hosek *et al.* 2006; Sun *et al.* 2013; Swaziland 2016a; Sahle *et al.* 2017). Coupling TB-LAM test results and clinical signs and symptoms has a diagnostic yield similar to an algorithm based on using clinical signs plus GeneXpert MTB/RIF assay (Cephied, USA) (76.9% versus 74.4%, respectively) (Sahle *et al.* 2017; Stop TB partnership 2017).

2.4.3 Molecular methods for TB identification

2.4.3.1 Line Probe Assay

Line probe assay uses polymerase chain reaction (PCR) and reverse hybridization methods for the rapid detection of mutations associated with drug resistance (Luetkemeyer *et al.* 2014; Soysal *et al.* 2017). Line probe assays (Hain Life Science) are designed to identify small amounts of *M. tuberculosis* complex and simultaneously stumble on mutations associated with drug resistance (Maningi *et al.* 2017; Soysal *et al.* 2017). Three steps are followed in this procedure: DNA extraction, amplification and hybridization. All three steps enhance binding of amplicons to specific oligonucleotide probes immobilized on a membrane strip. The membrane strip with bands is aligned in a worksheet. Bands are considered positive if they are approximately as strong as the amplification control (AC) except for the CC band (conjugate control) for *M. tuberculosis*. The locus control zones *rpoB, katG and inhA* must be present for results to be interpreted. Absence of signal with wild type probes may predict resistance, while positive hybridization signal with a mutation-specific capture probe (for common mutations only) may predict resistance. Presence of rare mutations may only be indicated by the lack of hybridization with one or more wild type probes. The main advantage for this technique, is that it can detect the presence *M. tuberculosis* and MDR-TB (resistance to both INH and RIF) at once (Madhuri *et al.* 2015; Desikan *et al.* 2017). One of the disadvantages with these assays is that it is not conducted in an enclosed instrument something which can lead to cross-contamination and an increased risk of false positive results (Ninan *et al.* 2016).

2.4.3.2 GeneXpert® MTB/RIF assay

The GeneXpert® MTB/RIF assay (Cephied, USA), is a molecular test for TB which diagnoses TB by detecting the presence of *M. tuberculosis* bacteria, as well as rifampicin (RIF) resistance by three automated steps; extraction, amplification and detection on a single system (Eddabra *et al.* 2018). It makes use of a sputum sample and it will deliver a result in less than an hour. The assay amplifies a portion of the "rifampicin resistance determining region" of the *rpoB* gene, the most common site for RIF mutations, in real-time, using two sets of primers. Fluorescent probes

are then used to differentiate between wild-type and mutant strains so that if one or more probes do not bind, this indicates the presence of a mutation and therefore RIF resistance.

2.4.3.3 TrueNat TB test

The TrueNat TB test is a molecular test that can diagnose TB and its RIF resistance in one hour (Nikam *et al.* 2013). It is conducted using a small handy instrument which is battery powered, which makes it useful in peripheral resource limited healthcare settings (Lee *et al.* 2019) The test is polymerase chain reaction (PCR) based, and it detects the DNA which defines *Mycobacterium tuberculosis* and targets the rifampicn gene to detect the bacterium resistance to drug rifampicin (Nikam *et al.* 2013; Jeyashree *et al.* 2020). TrueNat for TB diagnosis has advantages over smear microscopy or GeneXpert® MTB/RIF assay (Cephied, USA) as it improves linkage-to-care, increase life expectancy, and it is cost-effective (Nikam *et al.* 2014; Lee *et al.* 2019).

2.4.4 TB Culture

The TB culture is is considered the maximum correct test due to its excessive sensitivity and specificity, but the drawback is that it is labour intensive and slow to produce results. Clinical laboratories process cultures for six to eight weeks to achieve maximum sensitivity on solid media. To isolate *M. tuberculosis* on clinical samples, the liquid media (BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA)) is the most sensitive culture technique compared to solid culture (thin layer agar and Lowenstein Jensen media) (Tortoli *et al.* 1999). TB culture method is used conventionally in most settings (Lawn 2015; Dunn *et al.* 2016).

2.4.4.1 Thin layer agar (TLA)

Thin layer agar (TLA) uses a solid medium and is primarily based at the microscopic detection of early mycobacterial colonies (Boum *et al.* 2013; Asmar *et al.* 2015). This method is able to spot growth within nine – fourteen days and also allows the initial isolation of *M. tuberculosis* on the basis of its colony morphology. The sample is inoculated on a plate containing Middlebrook 7H11 and Middlebrook 7H11 enriched with PNB para-nitrobenzoic acid (PNB) (Somoskövi *et al.* 2000; Boum *et al.* 2013; Essawy *et al.* 2014). The two media help in the

identification and differentiation of *M. tuberculosis* complex since it is expected to grow on the media with Middlebrook 7H11 but not on Middlebrook 7H11 with PNB media, where there will be no growth (Naveen *et al.* 2012).

2.4.4.2 Lowenstein Jensen media

Lowenstein-Jensen (LJ) is the selective medium which is used to grow and isolation of *Mycobacterium* species. It was developed by Lowenstein who combined Congo red and Malachite green to inhibit unwanted bacteria (Naveen *et al.* 2012). The present formulation, a glycerated egg-based medium, is based upon Jensen's modification. Jensen's version excludes Congo red and uses a moderate concentration of Malachite green to prevent growth of the majority of contaminants enduring decontamination of the specimen (Kassaza *et al.* 2014). This formulation also boosts for the earliest possible growth of mycobacteria. It is used for the diagnosis of mycobacterial infections, testing antibiotic susceptibility of isolates, and is also used for distinguishing the species of mycobacterium (by colony morphology, growth rate, biochemical characteristics and microscopy).

2.4.4.3 BACTEC™ MGIT™ 960 Mycobacteria Culture System phenotypic culture

The culture system considerably advances the isolation and cuts the time required to detect mycobacteria (Heifets *et al.* 2000). The automated BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) (MGIT) is an *in-vitro* diagnostic instrument designed and optimized for the rapid detection of mycobacteria from clinical samples (except blood) (Malinga *et al.* 2016b; Maningi *et al.* 2017). MGIT utilizes a modified 7H9 Middlebrook broth base with 0.25% glycerol (7ml) with an oxygen quenching fluorescent sensor embedded in silicon at the bottom to detect microbial growth directly from clinical samples. Species identification of all isolated mycobacteria can be done using a PNBA from MGIT positive vials (Somoskövi *et al.* 2000). Molecular epidemiological techniques produce evidence that strains of *M. tuberculosis* from different phylogenetic lineages may vary in virulence and pathogenesis.

2.4.5 Molecular epidemiologic approaches to detect *M. tuberculosis* strains

Molecular epidemiology refers to science that deals with the role of genetic and environmental risk factors (polymorphism) identified at a molecular and biochemical level, to the aetiology, distribution and control of disease in families and populations (Betsy *et al.* 2001). The molecular approaches have expanded the ability to investigate and understand the tuberculosis epidemic at strain level (Martinson *et al.* 2011; Hove *et al.* 2012). There are many genotyping techniques utilised in different settings (Viegas *et al.* 2010) including:

i) Spoligotyping technique
ii) Mycobacterial interspersed repetitive unit-variable number tandem repeat analysis
iii) IS6110-based restriction fragment length polymorphism analysis
iv) Ligation-mediated PCR (LM-PCR)
v) Fluorescent amplified-fragment length polymorphism (FAFLP)
vi) whole genome sequencing
vii) Single nucleotide polymorphism

2.4.5.1 Spoligotyping technique

Spoligotyping is referred to as phylogeographic classification (Sabban *et al.* 2002) and is based on deoxyribonucleic acid (DNA) polymorphism within the direct repeat (DR) locus of the *Mycobacterium tuberculosis* complex (Mehri *et al.* 2013). The DR locus of directly repeated sequences of 36 base pairs are interspaced with 35 – 41 base pairs of non-repetitive spacer sequences (Hove *et al.* 2012; Mehri *et al.* 2013). This method involves PCR amplification of the DR locus and hybridization to a membrane containing a series of DNA probes representing each of the unique spacer sequences in the DR locus (Kamerrbeek *et al.* 1997). This technique is fast, and is a highly reproducible method (Urvashi *et al.* 2004; Hove *et al.* 2012). Genotyping results from this technique has a simple binary format which permits the exchange of data and facilitates the construction of large collaborative and less DNA quantities are required (Filliol Ingrid *et al.* 2003; Abadia *et al.* 2009; Vitol *et al.* 2015). It has been demonstrated that

spoligotyping can aid in distinguishing whether a particular TB episode is due to relapse or re-infection (Warren *et al.* 2002).

SITVIT 2 and fourth international spoligotyping database (SpolDB4) tools are both utilised in a spoligotyping technique, to allocate isolates to major genetic lineages and sub-lineages (Ouafae Lahlou *et al.* 2012). This database defines 62 genetic lineages and sub-lineages which are often named after regions, countries, cities or places of high prevalence (Brudey *et al.* 2006b). These comprise definite signatures for the various *M. tuberculosis* complex members, as well as rules defining main lineages for *M .tuberculosis sensu stricto* (Viegas *et al.* 2010).

2.4.5.2 Mycobacterial interspaced repetitive units variable number of tandem repeats analysis

Mycobacterial interspaced repetitive units variable number of tandem repeats (MIRU-VNTR) analysis can be used as a tool to study the evolution of *Mycobacterium tuberculosis* genome (Supply *et al.* 1997). Mycobacterial interspaced repetitive units (MIRUs) are also a suitable marker for transmission studies in high incidence settings where clustering may be over represented. Polymorphism within certain locations enables the differentiation of *M. tuberculosis* strains since MIRU copy number differences are evident between non-related *M. tuberculosis* isolates, therefore this genotyping method is based on the variable number of tandem repeats (VNTRs) of MIRUs (Le Flèche *et al.* 2002). With this method, different sets of loci can be used for strain typing depending on the strain population and suitable set that exhibited better discriminatory power for genotyping isolates: 12 sets, 15 sets or 22 sets (Supply *et al.* 1997; Mazars *et al.* 2001; Pan *et al.* 2017).

2.4.5.3 IS6110-based restriction fragment length polymorphism analysis

The IS6110-based restriction fragment length polymorphism analysis (IS6110-RFLP) method is based on the detection of the IS6110 which is present in different copy numbers and integrated at different chromosomal sites in *M. tuberculosis* complex isolates (Doroudchi et al. 2000; Green et al. 2013). The fragments based on the IS6110 are highly polymorphic but stable

enough for epidemiological studies (Zheng et al. 2014; Mathuria et al. 2016). Strains with fewer copies of IS6110 are more homogenous and fingerprints are not as reliable concerning epidemiological links as of those containing multiple copies (van Soolingen et al. 1994).

2.4.5.4 Ligation-Mediated PCR (LM-PCR)

Ligation-mediated polymerase chain reaction (LM-PCR) is a genomic analysis technique for determination of (1) primary DNA nucleotide sequences, (2) cytosine methylation patterns, (3) DNA lesion formation and repair, and (4) *in vivo* protein–DNA footprints (Dai *et al.* 2000). LM-PCR is comparable to spoligotyping in terms both of feasibility with rapidly extracted DNA and of the generation of software-analysable images (Francesca Brunello *et al.* 2001). Moreover, LM-PCR group considerably fewer strains than spoligotyping (Bonora *et al.* 1999).

2.4.5.5 Fluorescent amplified-fragment length polymorphism (FAFLP)

Fluorescent amplified-fragment length polymorphism (FAFLP) typing identifies sequence differences across the genome. The genomic DNA is digested by two restriction enzymes and adaptors are ligated to the restriction fragments. PCR is performed with labelled primers directed to the adaptors, and the PCR products are separated on a denaturing polyacrylamide gel and detected on an automated sequencer (Kremer *et al.* 2005).

2.4.5.6 Whole genome sequencing

Whole genome sequencing generates complete information of a specific *M. tuberculosis* strain, including the evolutionary background, drug resistance mutations, virulence-associated polymorphism, and assessment of TB spread (Schürch *et al.* 2010; Cannas *et al.* 2016). The whole genome sequencing can identify genotypes predictive of drug-resistance phenotype within the entire region of microbial genome and has potential to determine genetic relatedness and identify transmission dynamics necessary in guiding clinical decisions (Katale *et al.* 2020). *M. tuberculosis* has a conserved genome with little genetic diversity between strains and no evidence of horizontal gene transfer, but more detailed analysis with whole genome sequencing has identified genetically separate bacterial subpopulations in sequential sputum samples

(Nimmo *et al.* 2019). *M. tuberculosis* whole genome sequencing is usually performed on fresh or stored frozen cultured isolates to obtain sufficient purified mycobacterial DNA (Brown *et al.* 2015). However, the culture process can change the population structure from that of the original sample due to genetic drift (random loss of lineages) and/or the selection of subpopulations more suited to growth in culture (Cabibbe *et al.* 2018; Meehan *et al.* 2019). However, the whole genome sequencing distinguishes between relapse and reinfection and describes disease outbreaks (Walker *et al.* 2013b).

2.4.5.7 Single nucleotide polymorphisms

Single nucleotide polymorphisms (SNPs) are a common form of genetic variation in the *M. tuberculosis* complex. Generally, SNPs represent single nucleotide differences between at least two DNA sequences. To generate data on SNP, it requires comparative sequencing of multiple genes or whole genome in two or more strains of interest. SNPs represent robust markers for inferring phylogenies and for strain grouping (Gagneux *et al.* 2007), due to the low frequency of SNPs and restricted ongoing horizontal gene transfer in the *M. tuberculosis* complex, resulting in low level of homoplasy (Comas *et al.* 2010).

Of all the above-mentioned molecular techniques, most of middle income countries including Brazil, Iran, Saudi Arabia, Tanzania, Uganda, Zimbabwe and South Africa utilise the spoligotyping technique to investigate the tuberculosis epidemic (Easterbrook *et al.* 2004; Eldholm *et al.* 2006; Sahal *et al.* 2007; Asiimwe *et al.* 2008; Mlambo 2011; Hove *et al.* 2012). Some investigators opt to rent both spoligotyping and IS6110 techniques in a bid to have added plenty-needed accuracy and precision in describing transmission dynamics, and to facilitate research of previously unresolved problems (Mathema *et al.* 2006; Viegas *et al.* 2010; Hove *et al.* 2012). A study conducted in South African gold mines, found a certain strain family which exhibited similar but non-identical IS6110 patterns, where they employed the IS6110-based restriction fragment length polymorphism analysis technique for genotyping (Mathema *et al.* 2015). In this study, spoligotyping was performed to further define each isolate and indeed the spoligotyping technique proved to be a sensitive genotyping technique. Spoligotyping is able to resolve the challenge of similar but non-identical IS6110 patterns encountered due to its high reproducibility (Eldholm *et al.* 2006). Therefore, due to the versatility of the spoligotyping

method, it has also allowed certain non-epidemiological questions to be answered, and it has shown to be a useful tool for rapid identification of laboratory error and contamination, even when used alone (Warren *et al.* 2002).

Notably, about a decade ago, the molecular markers available to look at the epidemiology of TB had been drug susceptibility profiles and phage types (Mathema *et al.* 2015). However, there have been some limitations to these methods and in current years, several genotyping methods had been evolved based totally on DNA polymorphisms inside the genome of the organism (Desikan *et al.* 2015) such as the spoligotyping method. These DNA polymorphisms are generally located in non-coding regions with unique frequencies validated between different strain lineages (Desikan *et al.* 2015). Thus, molecular epidemiology of *M. tuberculosis* utilises precise genetic markers within the *M. tuberculosis* genome to observe the distribution of strains as well as how the strains distribution modifies over a time frame (Chatterjee *et al.* 2010; Borgdorff *et al.* 2013). It represents an opportunity to apply new resolving power to broaden theories of sickness causation that are known to be complicated interactions within the health procedure (Betsy *et al.* 2001; Vineis *et al.* 2006). Polymorphism in mycobacterial repetitive elements has been drastically used in epidemiologic studies of tuberculosis (Kato-Maeda *et al.* 2011b; Ei *et al.* 2016). Molecular epidemiologic approaches have provided unique insights into the transmission dynamics of TB and have aided to refocus and refine control practices like the use of the N95 face mask, and not any other mask (Belay *et al.* 2013). Successful molecular epidemiology studies have the required accuracy and precision in clarifying transmission dynamics (Sankar *et al.* 2013). In addition, there may be evidence to signify that precise strains of *M. tuberculosis* belonging to distinct phylogenetic clusters or lineages may also range in virulence, pathogenesis, and epidemiologic characteristics, all of which may substantially affect TB management and vaccine improvement strategies (Comas *et al.* 2011; Sankar *et al.* 2013; Debebe *et al.* 2014).

2.5 *Mycobacterium tuberculosis* phylogenetic lineages

There is a growing interest in conducting phylogeographic studies on *M. tuberculosis* isolates (Nebenzahl-Guimaraes *et al.* 2016). The classification of phylogenetic lineages is based on the

existence or absenteeism of regions of difference and smaller deletions (Nebenzahl-Guimaraes *et al.* 2016). Studies show that there are different *M. tuberculosis* lineages according to region and support the notion that *M. tuberculosis* adapts to specific human populations (Ruth *et al.* 2008; Stefan *et al.* 2014). Quite a number of studies have shown that the human-adapted *M. tuberculosis* lineages show a strong phylogeographical population structure, with different lineages associated with distinct geographical regions (Sebastien 2012; Mireia Coscolla *et al.* 2014; Otchere *et al.* 2018a). *M. tuberculosis* has seven main lineages, which are: Indo-Oceanic (lineage 1), East Asian (lineage 2), Central Asian (lineage 3), Euro-American (lineage 4), West Africa 1 (lineage 5) and West Africa 2 (Lineage 6) and Aethiops vetus (lineage 7) (Comas *et al.* 2013). The SpolDB4, defines 1939 shared-types (STs) from 122 countries, which are tentatively classified into 62 lineages using a mixed expert-based and bioinformatical approach (Dou *et al.* 2008). The SpolDB4 update adds 26 new potentially phylogeographically-specific *M. tuberculosis* genotype families (Brudey *et al.* 2006a; Dou *et al.* 2008).

Figure 6 “diagrammatically shows the 62 lineages and sub lineages prototype patterns and statistical classification analysis of SpolDB4 (Brudey *et al.* 2006a). First column are Shared-type (ST) number: ST number of prototype pattern for the linage/sub lineage. Second column: lineage/sub lineage name. Third column: Binary spoligotype display with black-white squares for respectively hybridizing-non-hybridizing spacers. Fourth column: Octal code (in red: defining octal rule). Fifth column: total absolute number of isolates of the subclass when variant ST Spoligotypes are included (using SpolNet)” (Brudey *et al.* 2006a).

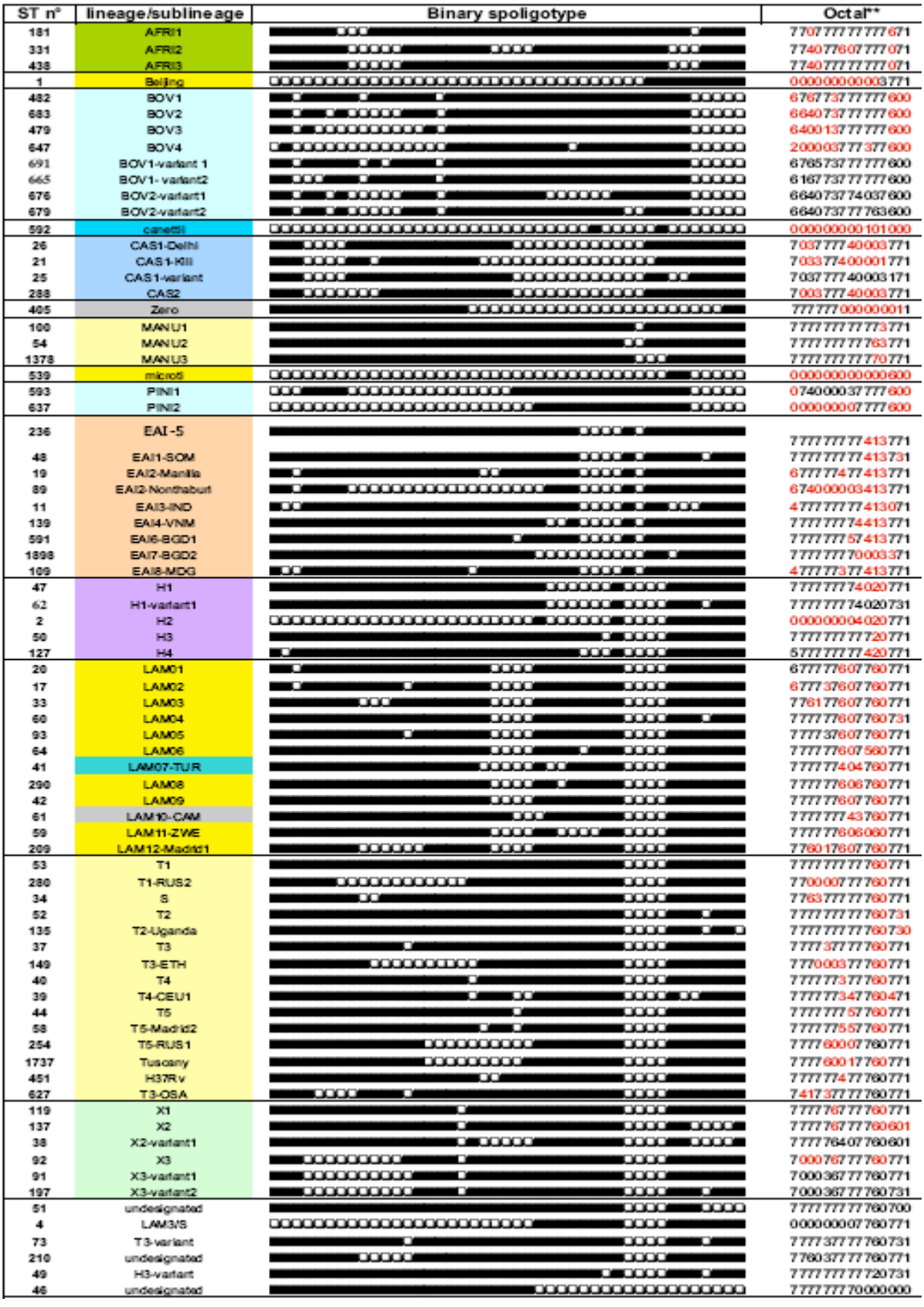

Figure 6: The bioinformatical and statistical classification analysis of SpolDB4, showing 62 sub-lineages prototype patterns (Brudey *et al.* 2006a)

2.5.1 Indo-Oceanic lineage (lineage 1)

The phylogenetic lineage 1 is prevalent in the East Africa, South Asia, and Southeast Asia (Minako Hijikata *et al.* 2017; Palittapongarnpim *et al.* 2018). This Indo-Oceanic lineage harbours the largest genetic diversity with an average of 930 single nucleotide polymorphisms (SNPs) between any two strains belonging to this lineage (Nebenzahl-Guimaraes *et al.* 2016). It is defined by the absence of spacers 29 to 32 and 34 and the presence of spacer 33 in the *M. tuberculosis* genome (Kato-Maeda *et al.* 2011a; Minako Hijikata *et al.* 2017). Spoligotype-defined EAI and MANU family strains belong to this lineage (Kato-Maeda *et al.* 2011a) (Figure 7).

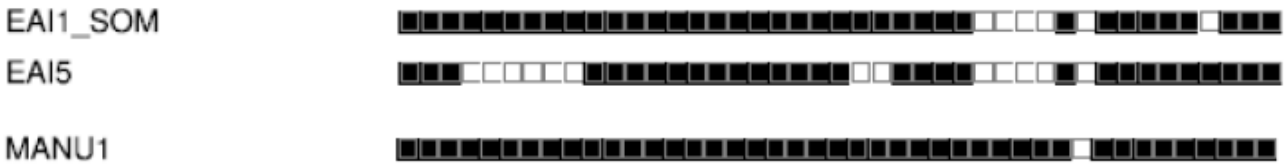

Figure 7: Spoligotype spacers which are characteristic of the lineage 1 (Ozcaglar *et al.* 2011; Thomas *et al.* 2011).

2.5.2 East Asian lineage (lineage 2)

M. tuberculosis strains from the Lineage 2, which are vastly predominant in East Asia and Russia, were found to elicit low-protective immune reaction in mice and are the most virulent (Tientcheu *et al.* 2017a). Lineage 2 is defined by deletion of spacers 1 to 34 in the direct repeat region (Kremer *et al.* 2005) (figure 8). The sub-lineage of this family is the Beijing strain. Several clinical trials have establish that Lineage 2 was linked with relapse, treatment failure, and fever during throughout early treatment (Burman *et al.* 2009; Haixia *et al.* 2017). Patients who are infected with the Lineage 2 strain are more likely to die of TB in comparison to patients infected with other lineages (Yimer *et al.* 2015; Haixia *et al.* 2017). It is therefore crucial to investigate the pathogenicity of the distinct lineage *M. tuberculosis*. This lineage consists of numerous families, however, the genotype of Beijing strain remains identical when using the spoligotyping method (Nikolayevskyy *et al.* 2006).

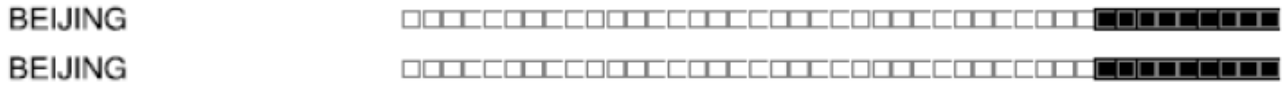

Figure 8: Spoligotype spacers which are characteristic of the lineage 2 (Ozcaglar *et al.* 2011; Thomas *et al.* 2011)

2.5.3 Central Asian Strain lineage (lineage 3)

Lineage 3 is generally disseminated in the Indian subcontinent and some countries in East Africa (Yimer *et al.* 2015). Lineage 3 is characterised by the absence of spacers 4-7 and 23-34 (Urvashi *et al.* 2004) (figure 9). *M. tuberculosis* lineage 3 is capable of evading the immune response, which contributes to the persistence and potential for outbreaks of this lineage among human populations (Chen *et al.* 2017). It has presented a relatively slow growth rate and a reduced ability to induce pro-inflammatory factors, permitting evasion of host immune responses (de Martino *et al.* 2019).

Figure 9**:** Spoligotype spacers which are characteristic of the lineage 3 (Thomas *et al.* 2011; Maharjan *et al.* 2018)

2.5.4 Euro-American lineage (lineage 4)

This lineage is commonly found in Europe, the Middle East, America, and some parts of Africa. (Kato-Maeda *et al.* 2011a; Yimer *et al.* 2015). The Latin American Mediterranean (LAM) strain is part of lineage 4 which is characterised by deletion of spacers 21-24 and 33-36, Haarlem (H1, H3); characterised by deletion of spacers 26-31, T- family (T1, T2, T3, T5), S-family; characterised by deletion of spacers 9-10 and 33-34, X and Ural (U) families. When compared to ancient lineage 1 and 5, *M. tuberculosis* strains belonging to modern lineage 4 show a higher rate of replication, associated to a significant production of pro-inflammatory cytokines (IL-1β,

IL-6, and TNF-α) and induction of a functional autophagy process (Alessandra *et al.* 2018). Haarlem family is characterised by the absence of spacers 29-31 and 33-36.

Figure 10: Spoligotype spacers which are characteristic of the lineage 4 (Ozcaglar *et al.* 2011; Maharjan *et al.* 2018).

2.5.5 West Africa I lineage (lineage 5)

Mycobacterium africanum causes a considerable percentage of TB in some countries of West Africa, but little is known on this pathogen (Yimer *et al.* 2015). Spoligotype patterns for this lineage tend to permit unambiguous classification, lacking spacers 8 through 12 and 37 through 39 (de Jong *et al.* 2010; Nuru *et al.* 2015). This lineage has been shown to elicit a robust early production of pro-inflammatory cytokines compared to the other lineages (Otchere *et al.* 2018b). The delayed pro-inflammatory immune response in the "modern" lineages might permit for more rapid disease advancement and transmission (Portevin *et al.* 2011).

Figure 11: The spoligotype spacers which are characterising for the West Africa I (lineage 5) (Ozcaglar *et al.* 2011)

2.5.6 West Africa II (lineage 6)

M. africanum West African II strain constitutes primeval lineage of the *M. tuberculosis* complex that generally causes human TB in West Africa and has an attenuated phenotype relative to *M. tuberculosis*. The spoligotype patterns for this lineage tend to permit unambiguous classification, lacking spacers 7 through 9 and 39 (de Jong *et al.* 2010; Nuru *et al.* 2015). This lineage is confined in West Africa (Yimer *et al.* 2015). *M. africanum* has lost genes, such as a recognized virulence gene and genes for vitamin synthesis, further to an intact reproduction of a gene which can affluent its susceptibility to antibiotics which can be insufficiently lively against *M. tuberculosis* (Bentley *et al.* 2012).

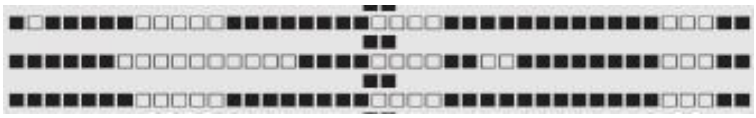

Figure 12: The spoligotype spacers which are characterising for the West Africa II (lineage 6) (Ozcaglar *et al.* 2011)

2.5.7 Aethiops vetus (lineage 7)

Lineage 7 was newly spotted in Ethiopia and among Ethiopian immigrants in Djibouti (Comas *et al.* 2015; Tulu *et al.* 2018). Whole genome sequencing (WGS) demonstrated that *M. tuberculosis* lineage 7 cells host a high number of mutations in genes involved in carbohydrate transport and metabolism, transcription, energy production and conversion, all of which contribute to the slow-growth phenotype (Tulu *et al.* 2018).

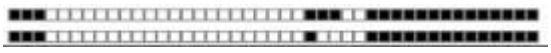

Figure 13: The spoligotype spacers which are characterising for the Aethiops vetus (lineage 7) (Tadesse *et al.* 2017)

2.6 Distribution of TB genotypes in African countries

The geospatial distribution of *M. tuberculosis* lineages in Africa has been documented (Groenheit *et al.* 2011; van Dijk *et al.* 2016). It is noted that all the lineages are isolated in the WHO Africa region with major substantial heterogeneity on the sub-lineages (Groenheit et al. 2011) (figure 14). Notably, Groenheit *et al.* (2011) did not specify sub-lineages which had prevalence of less than 3%; they were pooled together and shown as "other". Furthermore, all spoligotyping signatures that are not yet associated to a well-defined genotypic lineage in SITVIT2 were designated as "Unknown" in figure. Lineage distribution remains geographically restricted to specific regions/countries where they cause disease, that's what causes distribution variation (Tientcheu *et al.* 2017b).

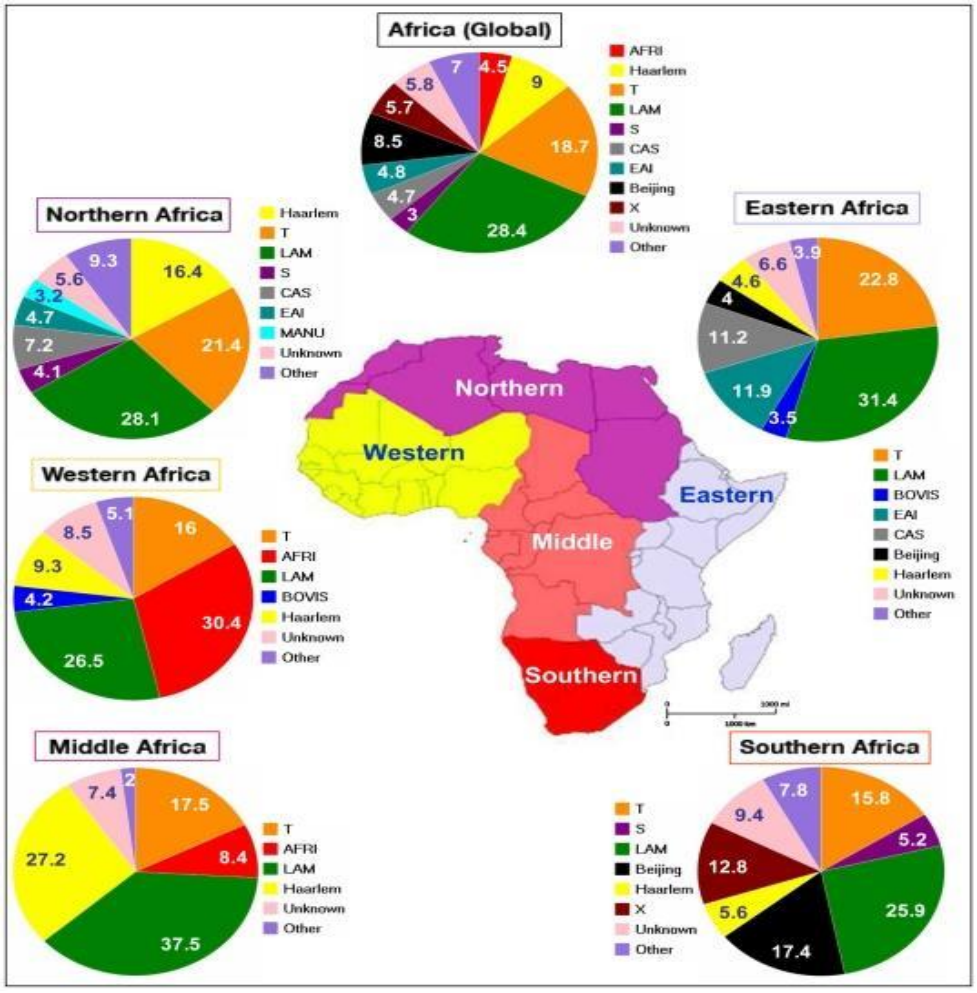

Figure 14: Geospatial distribution of *M. tuberculosis* lineages in Africa (Groenheit *et al.* 2011).

In a total of 125 isolates that were spoligotyped in South-West Uganda, the Uganda genotype (the lineage only found in Uganda) and CAS-Delhi (lineage 3) were found to be the most prevalent spoligotypes (Asiimwe *et al.* 2008). The prevalence of the Uganda genotype was 59.2% and that of the CAS1 genotype was 7.2%. Other genotypes were also found, although with low prevalence, which included the LAM (lineage 4) which was 6.2%, the EAI (lineage 1) which was 5.6%, the Cameroon family (lineage found only in Cameroon) which was 4%, and 2.4 % was the Ghana family (the lineage only found in Ghana) and 13% were not assigned to any family (referred to as orphans) (Asiimwe *et al.* 2008; Bazira *et al.* 2011). Patients infected with TB in Mozambique were found to be harbouring the spoligotypes that prevail in Eastern and Southern Africa; which was the LAM genotype (lineage 4) being 37% followed by EAI (lineage 1) with 29.7% and Beijing (Lineage 2) at 7% (Viegas *et al.* 2010).

In South Africa, the *M. tuberculosis* strains found in the provinces of Western Cape, KwaZulu-Natal, Gauteng, Mpumalanga, North-West, and Limpopo all indicated the Beijing genotype (lineage 2) to be most prevalent (Mlambo 2011; Hove *et al.* 2012; Anneke *et al.* 2016). Limpopo has a high diversity of strains, predominantly the non-Beijing lineages: lineage 4 sub-lineages LAM (LAM3, LAM9 and LAM11-ZWE), lineage 3 T-strains (T1, T2, T3 and T2/X1), Lineage 3 CAS sub-lineages (CAS1-Delhi and CAS1-KILI), MANU2 (lineage 1), U, X (X1, X2, and X3) (all in lineage 4), S including the Beijing (lineage 2) and Beijing-like families (Maguga-Phasha *et al.* 2017). The LAM genotype was found to be predominant in the Free State province (Anneke *et al.* 2016). After an extensive literature search, it is noted that there is no published data available for the types of strains in Swaziland.

2.7 Conclusion

Tuberculosis remains globally the deadliest communicable disease, fuelled by the HIV pandemic (Kwan *et al.* 2011). If the host immune system fails to control the infection progression, with other exacerbating factors contributing including the modulation of the immune responses to the *M. tuberculosis* infection by the pathogen, consequently the drug susceptible TB may develop to drug resistant TB. It is difficult to treat and control drug resistant TB (D'Ambrosio *et al.* 2015; Kurz *et al.* 2016). Hence, it is viewed to be essential to utilise the molecular techniques which diagnose *M. tuberculosis* accurately and further generate evidence

that different strains of *M. tuberculosis* from different phylogenetic lineages may differ in virulence and pathogenesis and possibly in their treatment. The next chapter will indicate the research study methods that were used to investigate whether the circulating susceptible *M. tuberculosis* strain in Swaziland later develops to MDR-TB on the same patient or whether the patient would acquire a completely new multi-drug resistant strain. It clearly captures the study design, sample size, data collection, data management and data analysis methods that were used in the current study.

CHAPTER 3

Research Methodology

3.1 Introduction

This chapter presents the research methodology that was used in conducting the present study. The study setting, the research design, research approach, the population, the sample and sampling techniques, data collection instruments, validity and reliability issues, ethical considerations as well as how the data were analysed are described.

3.2 Study setting and population

The study was conducted in Swaziland, which has four geographical regions named Hhohho, Manzini, Lubombo and Shiselweni regions. The country, which is located in Southern Africa, has a population of approximately 1.1 million (Swaziland Central Statistics Office 2007; World Population Prospects 2019). The Manzini region has the highest population, estimated at 355 945, followed by the Hhohho region with 320 651, then the Lubombo region with 212 531 and Shiselweni region with 204 111 individuals (Swaziland 2017). The population gender distribution is more or less balanced, with females being approximately 562 127 (52%) versus the male population of 531 111 (48%) (Swaziland 2017). Swaziland is a low to middle-income country with more than 60% of the population living below the poverty line, and 78.68% individuals living in the rural areas (Swaziland 2017; World Population Prospects 2019).

Currently there are 28 TB testing facilities in Swaziland, from which four were selected to be the study sites, based on the fact that they are regional TB referral facilities (Figure 15). These TB testing facilities were also selected after taking into consideration the quarterly TB statistics from the National TB Control programme, which indicated highest numbers of presumptive TB patients per region, therefore anticipating the finding of possibly varied circulating *M. tuberculosis* strains. The TB presumptive statistics for the last quarter of 2017, which was accessed from these TB facilities, showed that Good Shepherd Mission Hospital had 1009 patients, Moneni TB National Referral Hospital had 866, Mbabane Government Hospital had

1810 and Nhlangano Health Center had 544 patients. In an attempt to have the study findings to be representative of Swaziland, one TB testing facility from each region was included in the study: Moneni TB Referral Hospital (Manzini region) and Mbabane Government Hospital (Hhohho region), which are in an urban area as well as Good Shepherd Mission Hospital (Lubombo region) and Nhlangano Health Center (Shiselweni), which are in rural areas.

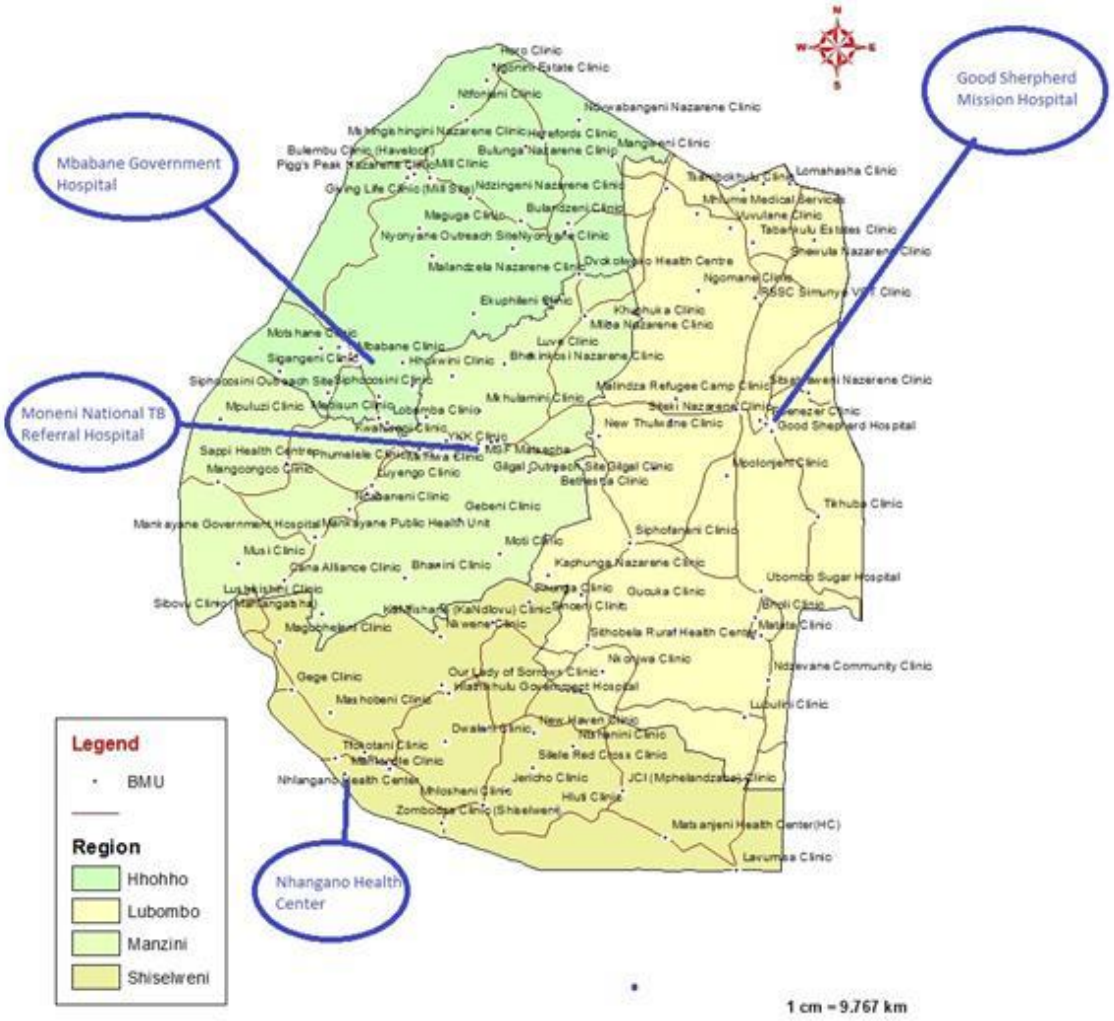

Figure 15**:** A map which shows the Swaziland geographical regions and their TB testing facilities, including the location of those which were selected as the study sites (Swaziland National Tuberculosis Control Program 2017).

3.3 Study design

This quantitative descriptive cohort study was conducted between November 2017 and January 2019. This study design was selected as it allows the researcher to follow participants over a

given period and use the data routinely collected. Follow up samples were collected six months after the baseline samples. The study investigated whether the circulating susceptible *M. tuberculosis* strain in Swaziland later developed to MDR-TB on the same patient or whether patients acquired a completely new multi-drug resistant strain.

3.4 Sample size calculation

A sample size of 103 participants was calculated with 95% power and statistical significance level of 1.96. The desired absolute precision (which is the size of difference in clinical importance) was expected be lower than 20% from the first proportion. The first proportion (0.5) was the expected proportion of MDR-TB from available data and the second proportion (0.34) was to account for expected losses (such as when the culture would not grow, when there would be contamination, or the drug sensitivity testing (DST) would be unreadable).

Following is the formula that was used to calculate the sample size (Centre for Clinical Research and Biostatistics 2019)

$$n = \frac{z^2_{\alpha/2} * [\frac{1 - P_1}{P_1} + \frac{1 - P_2}{P_2}]}{[\ln(1 - \varepsilon)]^2}$$

α The probability of type I error (significance level) is the probability of rejecting the true null hypothesis

P_1 The proportion of the exposed group (0.45)

P_2 The proportion of the non-exposed group (0.4)

ε The relative risk precision (0.273)

n Required sample size for one of the exposure group

n Total sample size needed for the cohort study

$n = (1.96)^2 \times [(0.55/0.45) + (0.6/0.4)] / [\ln (1-0.273)]^2 = 103$

n = 103

The sample size was thus determined to be 103 participants.

3.5 Recruitment and selection of the study participants

3.5.1 Ethical considerations and gatekeeper permissions

Ethical clearance to conduct the study was obtained from Durban University of Technology (IREC 111/17) (Appendix 1) and from Swaziland National Health Research Review Board (MH/599C/ IRB 0009688/NHRRB012/16) (Appendix 2). Support to conduct the study was sought and received from Swaziland TB Control program (Appendix 3) and from Swaziland Health Laboratory Services (Appendix 4) and Medical Research Council (Appendix 5), which means that data collection was carried out from the identified facilities with their full assistance and cooperation. Permission was also granted by the Mbabane Government Hospital (Appendix 6), National TB Referral Hospital (Appendix 7), Good Shepherd Mission Hospital (Appendix 8) and Nhlangano Health Centre (Appendix 9) to collect data from the study sites.

3.5.2 The recruitment strategy

The nurses and the matron and/ or the sister in-charge in each study site were briefed on the study protocol: on the aim of the study, objectives, possible benefits, possible participants' risks or discomforts and the participants' rights to withdraw from the study if they so wished. Thereafter, letters of information in English (Appendix 10) and in Siswati (Appendix 11) were distributed in all the study sites: National TB Referral Hospital, Siphofaneni Clinic/ Nhlangano Health Centre, Good Shepherd Mission Hospital and Mbabane Government Hospital in readiness for the participants' recruitment process.

On recruitment, the researcher, nurses and the sister in-charge presented the study information to the target population, who were presumptive TB patients. These were patients who presented with signs and symptoms suggestive of TB, who were previously referred to as TB suspects. These symptoms included coughing, sputum production, haemoptysis, breathlessness during coughing, weight loss, anorexia, fever, malaise, wasting, and terminal cachexia figure in various combinations (Campbell *et al.* 2006). These information sessions were held in the waiting area of the Outpatients Department. Further one-on-one individual information sessions were held

with those who were interested in participating in the study. After understanding the benefits and risks of the study, the potential participants then gave written informed consent for participation in the study, either in English (Appendix 12) or in Siswati (Appendix 13). Five hundred and sixty potential participants were purposively recruited from the study sites. Purposive homogeneous sampling method was a method of choice for this study, since it is effective when limited numbers of people can serve as primary data sources. The advantage of using this sampling method was that it made it possible to continue recruiting to replace those potential participants who were excluded for not meeting the inclusion criteria, until the sample size was obtained. Of the 560 potential participants recruited, 170 were from the Hhohho region, 182 from the Manzini region, 124 from the Lubombo region and 84 were from the Shiselweni region.

One hundred and three consenting adult participants, who were 18 years of age and above, as well being TB presumptive, regardless of gender were enrolled after the exclusion of those who were not infected with TB based on clinician's screening (n = 155) and the GeneXpert® MTB/RIF assay (Cephied, USA) results (n = 287). In addition, those who could not produce sputum samples on the day of recruitment even after the coughing officers' intervention, as per the national guidelines, were provided with universal containers to take away for the collection of the sputum sample in the morning. They had to bring this sample within three days after enrolment. Of those who did not produce a good quality sputum sample on the day of enrolment and still brought in a salivary sample were excluded (n = 15). Figure 16 depicts the recruitment and sampling strategy that was employed in the study, including the participants who were excluded from participating in the study. Although, participants who did not meet the inclusion criteria were excluded from participating in the study, they were attended to in the health care centers for the ailments they had presented in these facilities with.

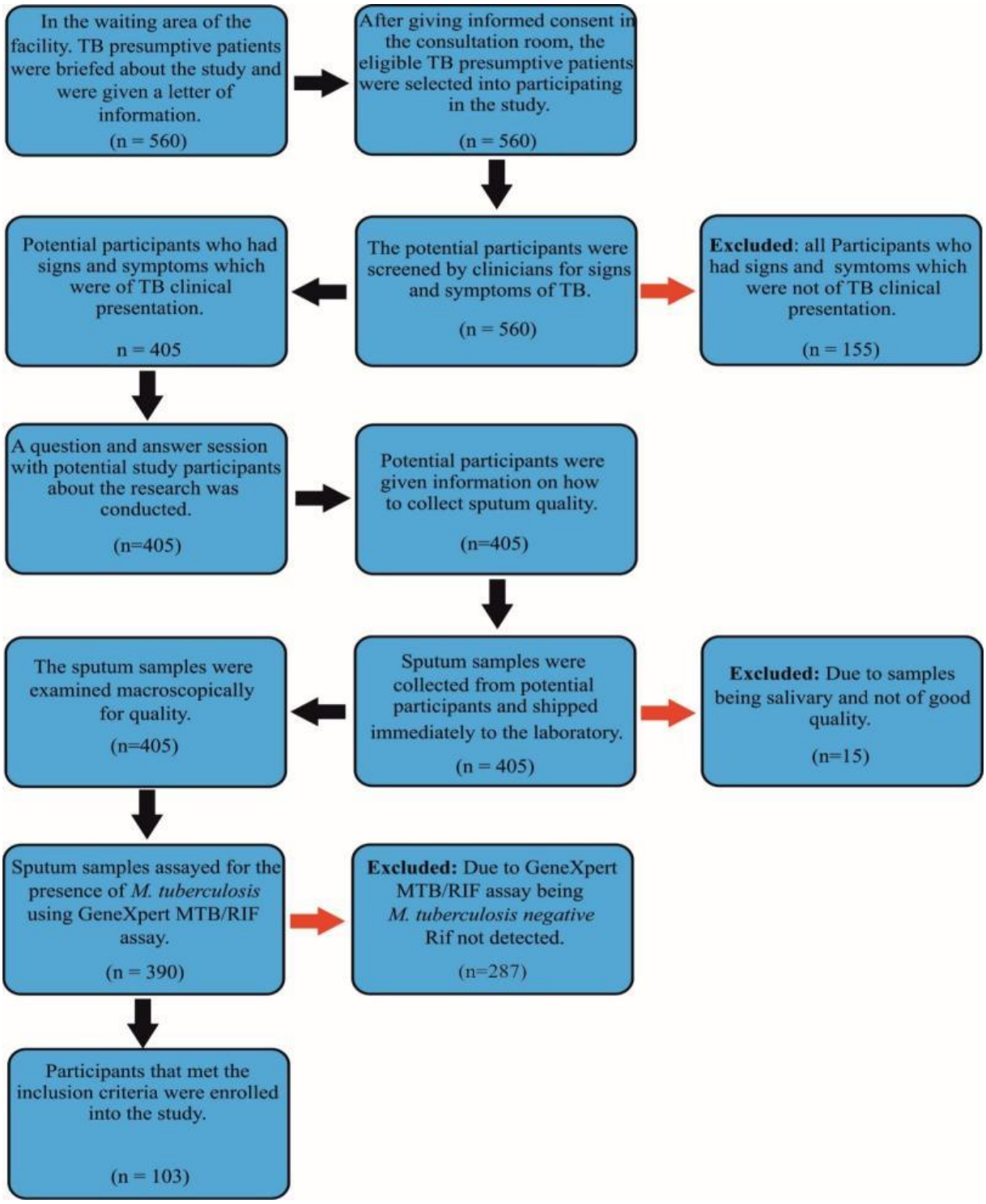

Figure 16**:** The recruitment and enrolment strategy process of the study participants which shows that 103 participants were enrolled into the study after the exclusion of potential participants who did not meet the inclusion criteria

3.6 Data collection

Sputum samples were collected by the researcher from each participant before enrolment in the study sites. These baseline samples were collected by trained coughing officers into universal containers. Those samples that were brought in by the participants, who had to collect the samples at the comfort of their homes, needed to be taken in the morning and be brought immediately after collection. Then the researcher shipped these samples in a package using the triple layer packaging method to the TB screening laboratory for immediate analyses.

3.6.1 Laboratory analyses for the presence of *M. tuberculosis*

Study methods and procedures used for the detection of the *M. tuberculosis* were aligned with the guidelines of the World Health Organization and Ministry of Health in Swaziland. In carrying out laboratory procedures for the collection of data, quality control was observed at all times. For every test method performed, a control test was performed together with the test so that the results could be comparable, valid and reliable. Furthermore, all reagents used in the study were checked for the expiry date and for their quality. Expired reagents were not used as they may affect the integrity of the results. In the performance of the tests, laboratory standard operating procedures (SOPs) and manufacturer's instructions were followed. TB cultures were processed in the BioSafety cabinet class II in the BioSafety level 3 laboratory at the accredited National TB culture referral laboratory in Mbabane (appendix 14). Analyses were conducted by the researcher using the GeneXpert® MTB/RIF assay (Cephied, USA), Ziehl-Neelsen method, BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) for TB culture and drug sensitivity testing (DST), Mycobacterium Protein 64 (MPT64) assay, GenoLyse® method, Hain Life Science for DNA extraction and a spoligotyping molecular technique.

3.6.1.1 The GeneXpert® MTB/RIF assay (Cephied, USA)

Sputum samples were processed for the detection of *M. tuberculosis* using the GeneXpert® MTB/RIF assay (Cephied, USA) (n = 405). The GeneXpert® (Cepheid), is a closed, self-

contained platform for the extraction, amplification and detection of *M. tuberculosis* complex from unprocessed samples. The method that was used was according to the manufacturers' instructions (Appendix 15). The GeneXpert® system is able to generate a result within two hours. The GeneXpert® MTB/RIF assay (Cephied, USA) allows for the rapid detection of *M. tuberculosis* and rifampicin (RIF) resistance by combining automated extraction, amplification and detection on a single system. The assay amplified a portion of the "rifampicin resistance determining region" of the rpoB gene, the most common site for RIF mutations, in real-time, using two sets of primers. Fluorescent probes were then used to differentiate between wild-type and mutant strains so that if one or more probes do not bind, this indicates the presence of a mutation and therefore RIF resistance. A sample processing control (SPC) consisting of spores from *Bacillus globigii*, was included in the assay as an internal control to ensure adequate processing of the sample as well as to monitor the presence of PCR inhibitors. A probe check control (PCC) verified reagent rehydration, PCR tube filling in the cartridge, probe integrity and dye stability. All samples that were positive with the GeneXpert® MTB/RIF assay (Cephied, USA) irrespective of RIF results were then cultured for the growth of TB.

3.6.1.2 TB Culture

All samples that had *M. tuberculosis* detected by the GeneXpert® MTB/RIF assay (Cephied, USA) (n=103) were cultured in an automated BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) liquid method. The method that was used was according to the manufacturers' instructions (Appendix 16). The *Myco*-Prep™ (made up of equal quantities of sodium hydroxide (NaOH) and N-acetyl-L-cysteine (NALC)-citrate) and sputum were mixed, to digest the sputum to liquid. NALC has a mucolytic effect and decontaminates the sample by killing other micro-organisms either than the mycobacteria, while NaOH only kills the other micro-organisms either than *M. tuberculosis*.

The decontaminated samples were then inoculated in the Middlebrook 7H9 liquid media tube. This tube has a fluorescent compound which is sensitive to the presence of oxygen dissolved in the broth, embedded in a silicone in the bottom. The tubes are automatically monitored hourly by the BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) for

increasing fluorescence to determine if the tube is instrument positive; which means that the test sample contains viable organisms. Fluorescence was recorded by the automated instrument

All BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) culture positive samples could be due to *M. tuberculosis* or non-Tuberculosis *Mycobacterium*. The Mycobacterium Protein 64 (MPT64) assay which detects the MPT64 antigen which is highly specific for *M. tuberculosis*, using a an immunochromatographic assay principle was used (Appendix 17). The sample flow laterally through the membrane, the antibody-colloidal gold conjugate binds the MPT 64 antigen in the sample. The complex then flows further and binds to the mouse monoclonal anti-MPT 64 on the solid phase in the test line, producing a red to purple color band. In the absence of MPT 64, there is no line in the test band region. The control area shows the efficiency of the gold binding therefore, valid results are always guaranteed.

3.6.1.3 Drug sensitivity testing

For all MPT64 positive samples, DST was carried out to note the drug sensitivity patterns of the isolated *M. tuberculosis*. Five BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) tubes were inoculated with the test culture for each of the samples (n=103). A known concentration of a test drug was then added into each of the Streptomycin, Isoniazid, Rifampin and Ethambutol, (SIRE) MGIT tubes, and growth was compared with the MGIT tube without the drug (which was used as a growth control). If the test drug were active against the isolated mycobacteria, it would inhibit the growth and thus suppression of fluorescence would occur, while the growth control would grow uninhibited and show increasing fluorescence. Growth was hourly monitored automatically by the BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) and results were interpreted as either susceptible or resistant to the drug. If the patient's isolate grew in the control but did not grow in the presence of the drug, it was considered susceptible. On the other hand, if the *M. tuberculosis* grew in both the tubes, then it was considered to be resistant to that drug. Appendix 18 details the drug and sensitivity testing procedure. Bacterial DNA was later extracted from all samples with positive DST results using the GenoLyse® Hain Life Science for the spoligotyping assay. GenoLyse allows extraction of highly purified genomic bacterial DNA in only three quick steps; pelleting of cells for removal of sample liquid, followed by lysis under alkaline conditions at elevated

temperatures and lastly neutralization. Appendix 19 describes the DNA extraction method. The extracted DNA was frozen at -20^{o}C while the shipping logistics were being arranged.

3.6.1.4 Spoligotyping

The extracted *M. tuberculosis* DNA was couriered frozen in a -20^{o}C container, to the Medical Research Council TB platform in Pretoria where spoligotyping was conducted by the researcher. This extracted DNA was used in the spoligotyping procedure following the manufacturers' instructions (Appendix 20).

The spoligotyping data was used to address the main objective of the study, which was to determine whether susceptible *M. tuberculosis* strains develop into multi-drug resistant strains by comparing baseline spoligotyping results with the follow up findings at six months.

The spoligotyping method was based on the detection of the polymorphism at one particular genomic region, the so-called direct repeat (DR) locus. The entire DR locus was amplified by PCR, using two inversely oriented primers complementary to the sequence of short DRs. The PCR products, of different sizes, were then hybridized to a membrane with 43 covalently bound synthetic oligonucleotides representing the polymorphic spacers identified in *M. tuberculosis* H_{37}Rv (spacers 1–19, 22–32, and 37–43) and *Mycobacterium bovis* BCG (spacers 20-21 and 33–36). The hybridization signals were detected by chemiluminescence through biotin labelling of the PCR products (one of the primers was biotinylated) and a streptavidin-peroxidase conjugate system and then visualized by autoradiography. The spoligotype patterns were converted into binary and octal formats and entered into the open source spoligotype database available at the website http://www.pasteur-guadeloupe.fr:8081/SITVIT ONLINE/tools.jsp. The shared international spoligotype (SIT) number and lineages/ sub-lineages were retrieved from the database. The results were compared with the existing designations in the SITVIT2 database of Institute Pasteur *de la* Guadeloupe. Individual strains were differentiated by the number of the spacers that were missing from the complete 43-spacer set.

In summary, the methods that were used to process and analyse baseline and follow up sputum samples that were collected from the patients are shown in Figure 17. The data collection

methods undertaken by the researcher included the processing of the sputum samples by their decontamination, followed by BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) phenotypic TB culture, and thereafter the detection of MPT64 antigen on TB culture positive samples. Drug sensitivity testing (DST) was then done on all the samples that were positive for the MPT64 antigen detection. Thereafter, spoligotyping was conducted on these samples. Follow up samples were collected six months after the baseline samples, which was six months after adherence to the anti-TB treatment.

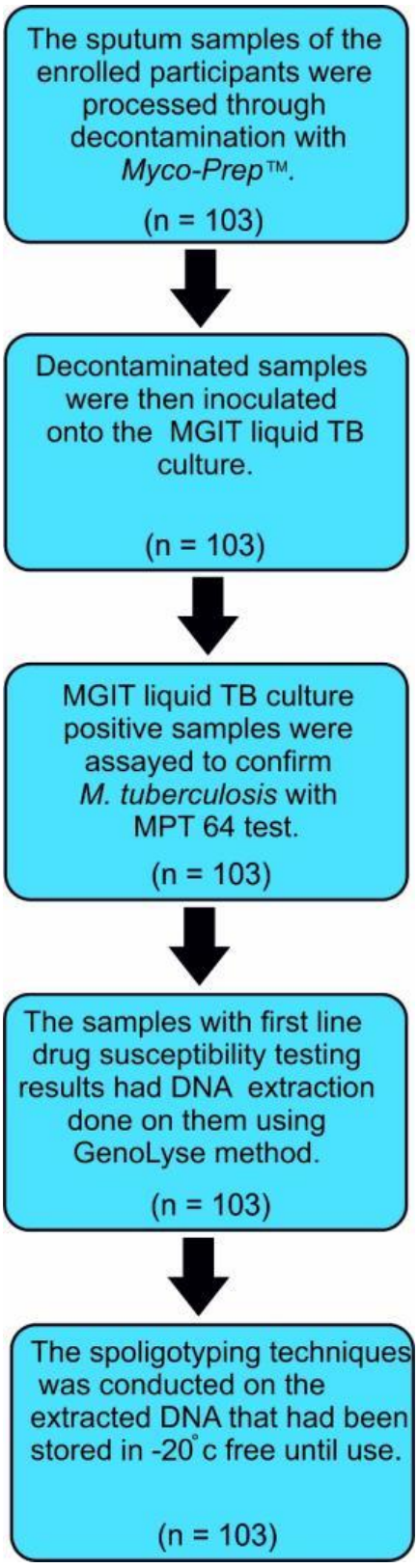

Figure 17: The data collection methods used in the study.

3.6.2 Review of participants' medical records

In addition, participants' medical records were reviewed by the researcher for the purpose of determining whether the patients were adhering to prescribed anti-TB treatment doses or not. Swaziland has embraced the delivery of directly observed therapy system (DOTS) as a programme to increase the adherence of patients to treatment. A pool of community treatment supporters are identified and given intensive core training on TB, DOTS, infection control and adverse effects management (Sanchez-Padilla *et al.* 2013; Swaziland National Tuberculosis Control Program 2017).

The participants were going to be stratified into two groups based on what would on medical records: those participants who did not miss any dose of the prescribed anti-TB drugs or missed one or two doses but not three were classified as compliant, and those who missed three or more doses of the prescribed anti-TB drugs were classified as non-compliant (Tang *et al.* 2015).

3.7 Data confidentiality

The data collected was encrypted and saved in a password protected computer and in compact discs (CDs) to ensure confidentiality. The CDs were locked in the researchers' office. Passwords were known only by the researcher and the research supervisors in order to ensure privacy of the data.

3.8 Data analysis

The genetic, genotype, and DST data sets were analysed on Statistical Package for the Social Sciences (SSPS) version 25. Epi Info (version 3.5.1, 2008) and STATA 13.0 software. Descriptive statistics were used to depict the demographic and clinical variables. Frequencies of multiple-drug resistance (MDR-TB) among different genotype families based on spoligotyping were compared with a chi-square test and Fishers exact to determine whether the circulating susceptible *M. tuberculosis* strain in Swaziland later developed to MDR-TB on the same patient or not. Associations with *p*-values of less than 0.05 were considered statistically significant. The

p-value and Fisher's exact were used to measure whether the research findings were meaningful or not. More specifically, whether the statistics closely matched the value one would expect to find in an entire population. If the Fisher's Exact Test p-value (Exact Sig. (2-sided) was < 0.05, then it implied that there was a significant relationship between the variables, and if it was > 0.05, there was no significant relationship. Spoligotyping results were entered into an MS Excel spreadsheet as a binary code representing either a positive or negative hybridization result. The spoligotyping results were then entered into SITVIT2 database (Pasteur Institute of Guadeloupe) in order to generate spoligotype families.

3.9 Summary

The chapter described the research study materials and methods. It clearly captured the study design, sample size, data collection, data management and data analysis. Lastly, ethical considerations that protected the researcher and the participants were indicated. Those who were excluded from participating in this study were not put in any jeopardy but were attended to in the health care facilities the same as any hospital or clinic attendee.

The next chapter (chapter 4) presents the findings that were generated in the current study. Chapter 5 discusses the study findings and highlighted whether susceptible *M. tuberculosis* strains develop into multi-drug resistant strains by comparing initial spoligotyping results with the follow up spoligotyping findings at six months or the last positive sample from converted patient as per the study objectives.

CHAPTER 4

Study findings

4.1 Introduction

This chapter presents the findings from the investigation that was conducted in the current study, based on the research methodology indicated in the previous chapter. In this chapter, the collected and analysed data is organised systematically and interpreted. The primary objective of the study was to use spoligotyping to determine whether the circulating susceptible *M. tuberculosis* strain in Swaziland later develops into drug-resistant tuberculosis (DR-TB) on the same patient or whether patients acquire a completely new DR-TB strain, and to assign all the strains in the study to the major clades in the SITVT2 database. Data was collected from patients who were diagnosed with TB infection on the first visit sputum sample, referred to as the baseline sample as well as six months after the initiation of anti-TB treatment. The hypothesis of the study was that the circulating susceptible *M. tuberculosis* strain in Swaziland later develops to MDR-TB on the same patient.

Pulmonary TB isolates that were collected from enrolled patients (n = 103) from the four regions of Swaziland were investigated for the type of strains of TB. The presence of *M. tuberculosis* was detected on the 103 samples using GeneXpert® MTB/RIF assay (Cephied, USA) at baseline. All the enrolled participants had their TB drugs adherence cards reviewed for compliance to the anti-TB drug doses at six months post-treatment. To achieve the aim of the study, all the sputum samples were cultured in a conventional BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA). Then all BACTEC™ MGIT™ 960 Mycobacteria Culture System (Becton Dickinson, USA) culture positive samples confirmed with MPT64 antigen test had DST done and later DNA extracted from all of the positive MPT64 antigen tests. The extracted DNA was used for genotyping.

4.2 Characteristics of the study participants

Of the 390 potential participants that were investigated for the presence of *Mycobacterium tuberculosis* in this current study, 26.41% (n=103) were detected as positive with TB infection. Of the 103 participants, the majority of them were males, with 64% (n=66) noted, compared to 36% (n=37) who were females (Figure 18).

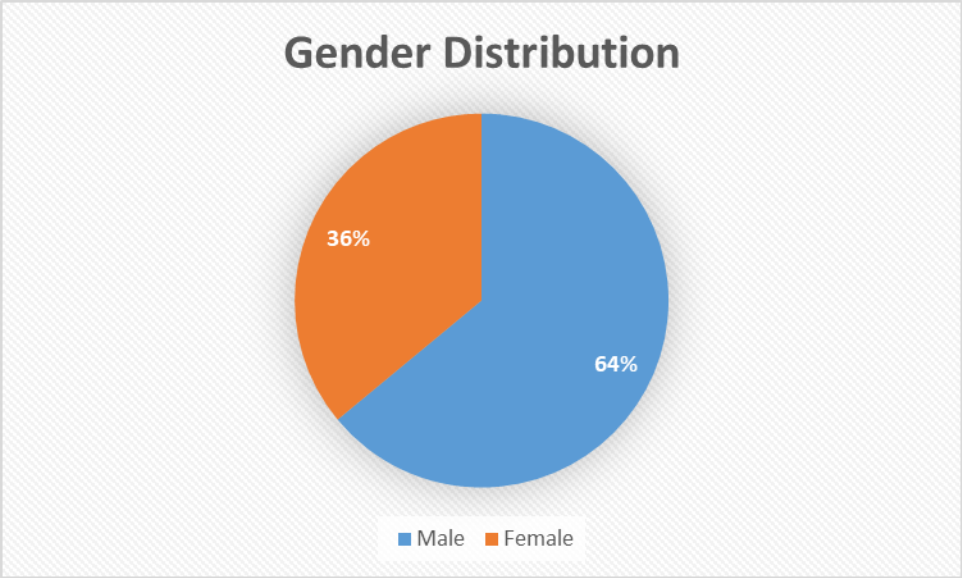

Figure 18**:** The gender distribution of the study participants, indicating that that were more male participants than females.

Furthermore, the mean age among the 103 participants was 34.4 years, with the mean age for males being 36.8 and females being 30.5 years of age (Figure 19). The aged ranged from 18 to 65 years of age.

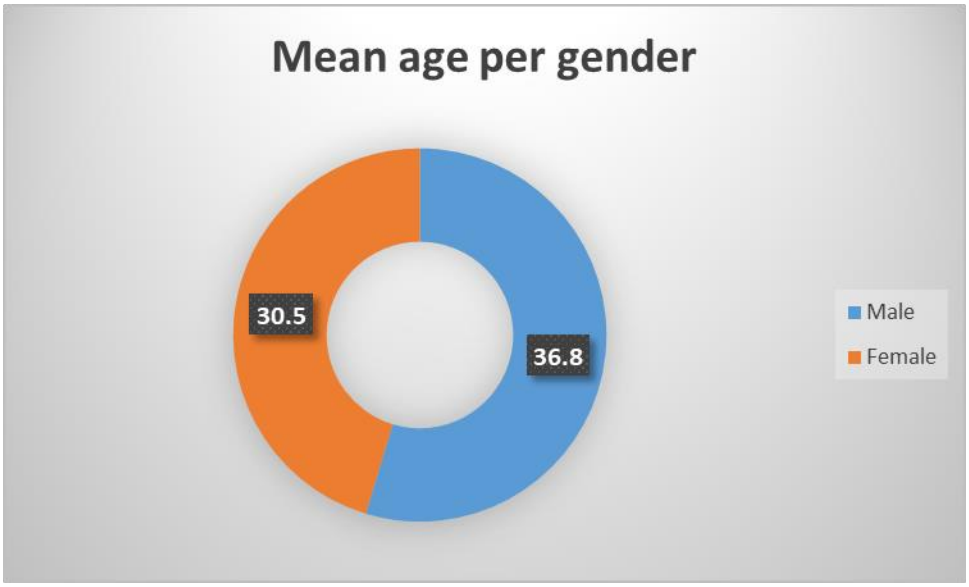

Figure 19: The age differences in years among the participants by gender, indicating that males were older than the female participants.

The geographical distribution of the study participants among the four regions of Swaziland (n=103) had 54.37% of them (n=56) from the Manzini region, 20.39% (n=21) from Hhohho region with Shiselweni region and Lubombo region sharing 25.24% equally (n=13) (Table 1).

Table 1: The geographical distribution of the study participants among the four regions in Swaziland (n=103), showing that most of them were from the Manzini region.

Variable		**Distribution n (%)**
Geographical regions	Manzini	56 (54.37)
	Hhohho	21 (20.39)
	Lubombo	13 (12.62)
	Shiselweni	13 (12.62)

Furthermore, the gender distribution among all the regions of Swaziland were noted to be mostly males, with 69% from in the Lubombo region, followed by the Manzini region (66%) with Hhohho (62%) and the least from the Shiselweni region with 54%. Notably, mostly females

were not from the Lubombo region, but from the Shiselweni region (46%), followed by Hhohho (38%), Manzini (34%) and Lubombo (31%) (Figure 20).

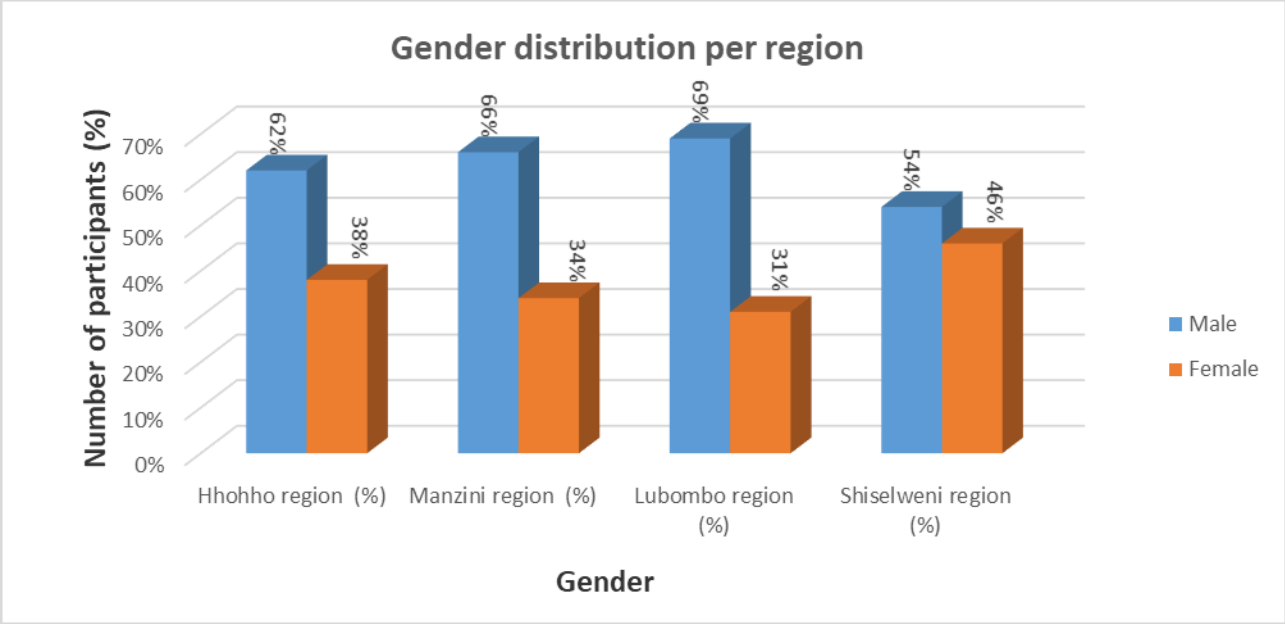

Figure 20**:** Gender distribution of the participants among the Swaziland geographical regions, showing that majority of males were from the Lubombo and most females from Shiselweni regions.

Participants per geographical region were of different ages per gender according to the different age categories: 18 – 35, 36 – 45 and above 46 ages. The 18 – 35 years age bracket was in the majority in all the regions, with 61% in the Hhohho region, 57% in the Manzini region, 46% and 54% in the Lubombo and Shiselweni regions, respectively. The least represented was the 46 and above age category, whereby few participants in that bracket were recorded from the Hhohho region (9.5%), the rest of the regions were around 15%: Manzini (16.07%), Lubombo (15.39%) and Shiselweni (15.39%) (Figure 21).

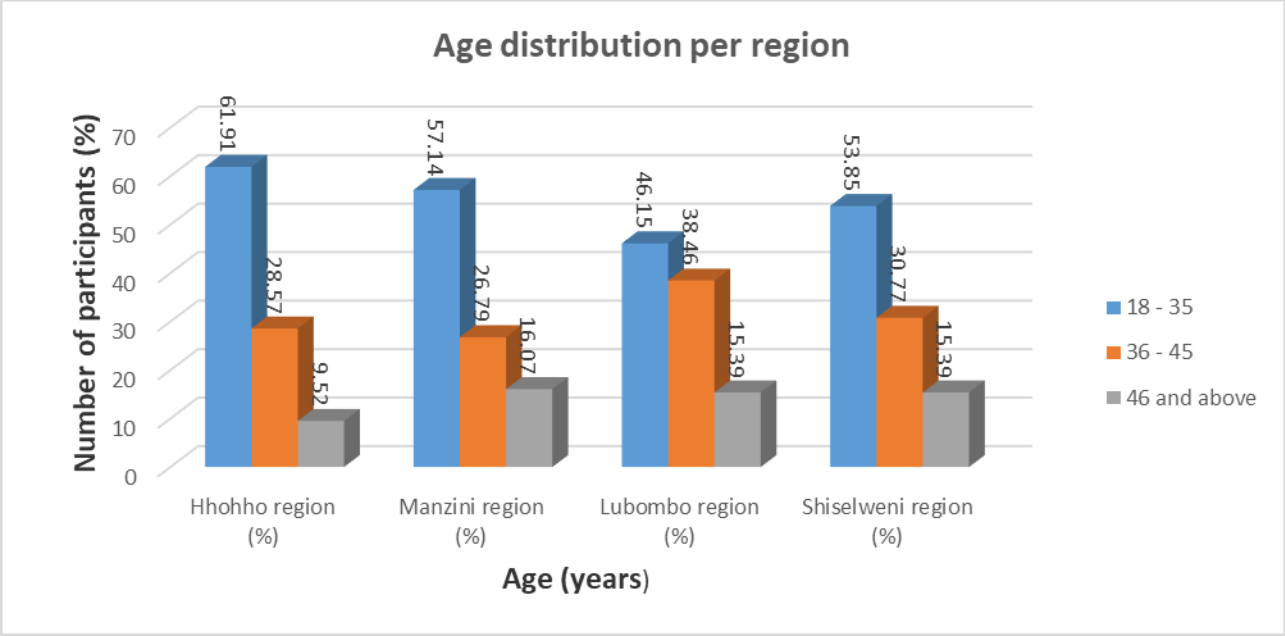

Figure 21: The age distribution of the participants in percentage per region among the various age categories in the four regions in Swaziland (n=103), indicating that the proportion of participants in the 18- 35 years age group were in the majority in all the regions

Furthermore, the age distribution of the participants among the four regions indicates that the male participants were older (Figure 21). The males from the Hhohho region had a mean age of 35.2 years, and females had a mean age of 26.9 years. The mean age for males from the Lubombo region was 38.3 years and that of females was 29.5 years. The Manzini and Shiselweni regions had the mean age for males being 36.8 and 37 years of age respectively, and females mean age had been observed to be 31.3 years in the Manzini region and 34.3 years in the Shiselweni region. Regionally, the Shiselweni region had the highest mean age, with 35.8 years compared to the other regions. The Lubombo regiona mean age was 35.6 years, followed by the Manzini region mean age was 34.9 years and Hhohho region with 32 years mean age (Figure 22).

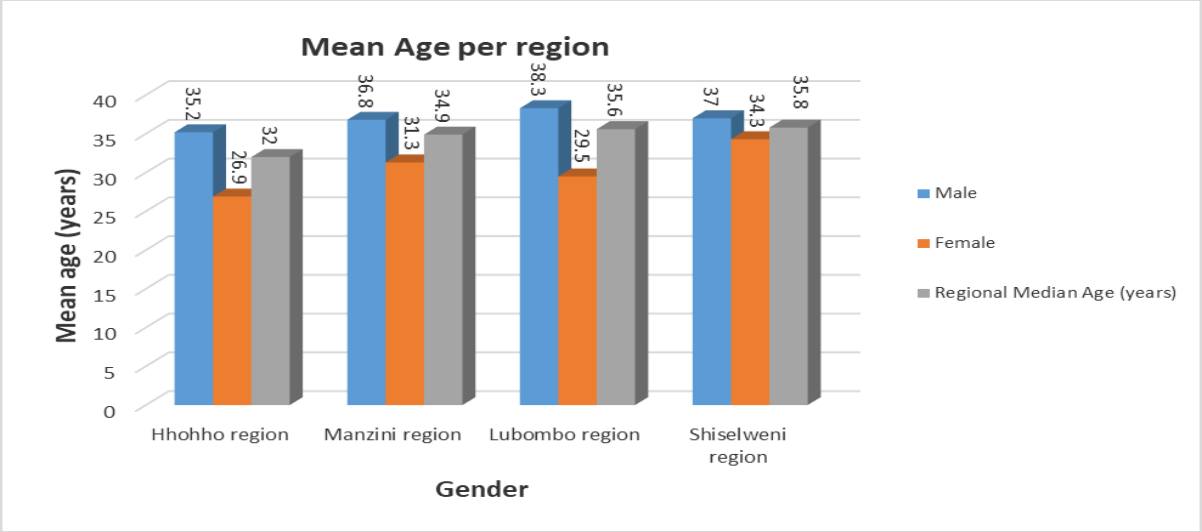

Figure 22: The mean age distribution per gender across the four regions of Swaziland (n=103), with most male participants being older compared to the median age of the participants in each region.

4.2.1 Drug susceptibility profile of the *M. tuberculosis* strains

The drug susceptibility test (DST) results that were generated at baseline (n=103) and at follow up, which was six months later (n=103) were classified into four possible drug susceptibility categories. The four categories were (i) susceptible, (ii) multi-drug resistant (MDR-TB), (iii) poly-resistant and (iv) mono-resistant *Mycobacterium tuberculosis* strains according to the drug susceptibility patterns of the 206 isolates (which include both baseline and follow up samples). The anti-TB drugs that were used to determine the drug susceptibility profile of the study strains were rifampicin (RIF), isoniazid (INH), Streptomycin (STR) and ethambutol (EMB). The strains that were classified as multi-drug resistant were those strains which were resistant to at both RIF and INH; those classified as poly-resistant were those which were resistant to more than one first line anti-TB drugs, other than INH and RIF and lastly, those classified as mono-resistant were resistant to only one of the first-line anti-TB drugs (Palmero *et al.* 2013; World Health Organisation 2016).

Although, most of the strains at both baseline and follow up (58.74%) were susceptible to all of these anti-TB drugs, there was a significant proportion of strains that were classified as RIF mono-resistant which were 28.16%. Furthermore, the poly-resistant strains were 8.25% and

those classified as INH mono-resistant were 4.85% (Table 2). Notably, the region with a high proportion of MDR-TB strains compared to the rest of the regions, although with no statistical significance (p=0.786) was Lubombo, with 38.46%.

Table 2: The DST classification of both the baseline (n=103) and follow up (n=103) *Mycobacterium tuberculosis* strains.

DST Classification	**Region**				**Total n (%)**	
	Hhohho n (%)	**Manzini n (%)**	**Lubombo n (%)**	**Shiselweni n (%)**		
Susceptible	25 (59.52)	67 (59.82)	11 (42.31)	18 (69.23)	121 (58.74)	
RIF- mono-resistant	0 (0.00)	3 (2.69)	1 (3.85)	1 (3.85)	6 (2.91)	**p-value =0.786**
Poly-resistant	2 (4.76)	12 (10.71)	2 (7.69)	2 (7.69)	17 (8.25)	
INH mono-resistant	1 (2.38)	0 (0.00)	2 (7.69)	0 (0.00)	3 (1.46)	
MDR	14 (33.33)	30 (26.79)	10 (38.46)	5 (19.23)	59 (28.64)	
Total	42 (100)	112 (100)	26 (100)	26 (100)	206 (100)	

Upon the analysis of the susceptibility patterns of the anti-TB drugs in all the strains between baseline and follow up isolates (n=206), it was revealed that the strains had increased resistance to all the drugs at follow up compared to baseline (Table 3). Resistant strains to RIF had significantly increased from 30.1% to 48.5% at follow up (p=0.005), with resistance to STR significantly increased from 27.2% to 39.8% (p = 0.038) and to EMB significantly increased from 21.4% to 36.9% (p = 0.011). Resistance to INH increased from 22.3% to 32%, however with no statistical significance (p = 0.079).

Table 3: The drug susceptibility patterns of the study strains at baseline (n=103) and follow up (n=103), showing increases in drug resistance at follow up.

Anti - TB drugs	DST classification	Baseline n (%)	Follow up n (%)	Total n (%)	p-value
Rifampicin	Susceptible	72 (69.9)	53 (51.5)	125 (60.7)	0.005*
	Resistant	31 (30.1)	50 (48.5)	81 (39.3)	
	Total	103 (100)	103 (100)	206 (100)	
Isoniazid	Susceptible	80 (77.7)	70 (68.0)	150 (72.8)	0.079
	Resistant	23 (22.3)	33 (32.0)	56 (27.2)	
	Total	103 (100)	103 (100)	206 (100)	
Streptomycin	Susceptible	75 (72.8)	62 (60.2)	137 (66.5)	0.038*
	Resistant	28 (27.2)	41 (39.8)	69 (33.5)	
	Total	103 (100)	103 (100)	206 (100)	
Ethambutol	Susceptible	81 (78.6)	65 (63.1)	146 (70.9)	0.011*
	Resistant	22 (21.4)	38 (36.9)	60 (29.1)	
	Total	103 (100)	103 (100)	206 (100)	

*: p-value significant at $p < 0.05$

The drug susceptibility findings were further analysed per region to determine the participant profiles among the different regions. Although most of the strains in all the regions were susceptible to the four anti-TB drugs (Table 4), the Lubombo region had the highest drug resistance rates with all the anti-TB drugs, although with no statistical significance. The resistance to RIF was 50% (p=0.557), to INH was 42.3% (p=0.197), to STR was 38.5% (p= 0.853) and to EMB was 34.6% (p=0.639) (Table 4).

Table 4: The drug susceptibility profiles according to the four regions of Swaziland, illustrating that the Lubombo region had the highest resistance in all the four anti-TB drugs.

		Region					p-value
Anti - TB drugs	**DST classification**	**Hhohho n (%)**	**Manzini n (%)**	**Lubombo n (%)**	**Shiselweni n (%)**	Total n (%)	
Rifampicin	Susceptible	25 (59.5)	69 (61.6)	13 (50)	18 (69.2)	125 (60.7)	0.557
	Resistant	17 (40.5)	43 (38.4)	13 (50)	8 (30.8)	81 (39.3)	
	Total	42 (100)	112 (100)	26 (100)	26 (100)	206 (100)	
Isoniazid	Susceptible	31 (73.8)	82 (73.2)	15 (57.7)	22 (84.6)	150 (72.8)	0.197
	Resistant	11 (26.2)	30 (26.8)	11 (42.3)	4 (15.4)	56 (27.2)	
	Total	42 (100)	112 (100)	26 (100)	26 (100)	206 (100)	
Streptomycin	Susceptible	28 (66.7)	74 (66.1)	16 (61.5)	19 (73.1)	137 (66.5)	0.853
	Resistant	14 (33.3)	38 (33.9)	10 (38.5)	7 (26.9)	69 (33.5)	
	Total	42 (100)	112 (100)	26 (100)	26 (100)	206 (100)	
Ethambutol	Susceptible	29 (69)	79 (70.5)	17 (65.4)	21 (80.8)	146 (70.9)	0.639
	Resistant	13 (31)	33 (29.5)	9 (34.6)	5 (19.2)	60 (29.1)	
	Total	42 (100)	112 (100)	26 (100)	26 (100)	206 (100)	

Notably, the analysed data suggests that a high proportion of participants infected with MDR-TB strains were from the Lubombo region. In addition, majority of these strains in this region were resistant to all the anti-TB drugs. This was not surprising, since this region together with Shiselweni region, had the smallest pool of isolates, thus influencing the percentages.

4.2.2 The distribution of the TB phylogenic lineages among the participants

Of the 206 *M. tuberculosis* DNA samples that were extracted from the TB culture positive samples for spoligotyping, all the 103 samples at baseline and 103 at follow up were deemed suitable for spoligotyping based on TB culture and drug susceptibility results. Upon analysis of the genotype data, it was noted that 170 isolates had distinct spoligotyping patterns: 35 isolates were from the Hhohho region, 92 from the Manzini region, the Lubombo and Shiselweni regions with 22 and 21 isolates, respectively. The remainder of the study isolates (n=36) had

spoligotyping findings missing due to DNA inadequacy (less than 10ng) as well as the spoligotype patterns which were not found in the SpolDB4 database, either at baseline or at follow up, hence they were excluded from the analysis.

The analysed spoligotyping patterns (n=170) revealed a total of four lineages: Indo-Oceanic (lineage 1), East Asian (lineage 2), Central Asian (lineage 3), and Euro-American (lineage 4) and eighteen sub-lineages (EAI1_SOM, EAI5, MANU1, BEIJING, CAS_DELHI, CAS_KILI, T1, T5_RUS1, T1_T3, S, X2, X3, U, LAM1, LAM3, LAM4, LAM9 and H37Rv) among the study participants. The S sub-lineage was noted to be predominant at baseline and the Beijing sub-lineage being the most prevalent at follow up (Figure 23). The Manzini and Hhohho regions had most of the sub-lineages (11 of 18 each), followed by the Lubombo region with *n*=10 and Shiselweni region with *n*=7 of 18, respectively.

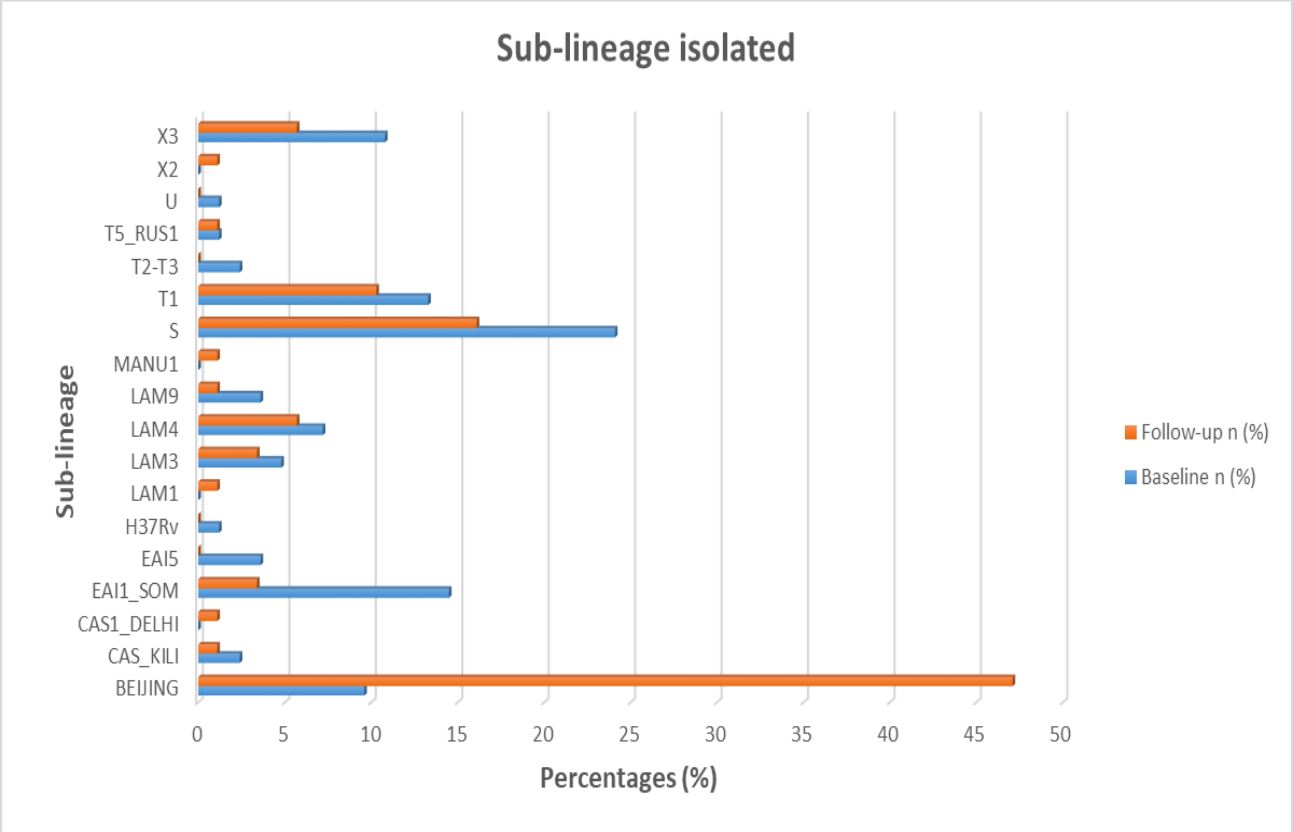

Figure 23: The sub-lineages that were detected at baseline and at follow up among the study participants, indicating the S sub-lineage to be predominant at baseline and the Beijing sub-lineage being the most prevalent at follow up.

The distribution of the lineages was varied among the different regions (Table 5). The Euro-American (Lineage 4) was the most prevalent lineage with 57.6% (n=98), followed by the East Asian (Lineage 2) with 28.8% (n=49) and 11.2% (n=19) for Indo-Oceanic (Lineage 1) and 2.4% (n=4) for the Central Asian (Lineage 3) (Table 5).

Furthermore, a high proportion of the S sub-lineage (20%; n=34) and Beijing sub-lineage (28.8%; n=49) strains were noted (Table 5). The S sub-lineage was predominant in the Shiselweni region with 30% at baseline and the Lubombo region with 27.3% at follow up. Furthermore, the Beijing sub-lineage strain was predominant at baseline in the Hhohho region with 23.5% and Shiselweni had more of the Beijing strain at follow up with 54.5%.

Notably, in most regions a change in the frequency of strains between baseline and follow up isolates was observed, although in some instances the differences between baseline and follow up strains were not statistically significant (Table 5). Some of sub-lineages isolated at baseline were not isolated at follow up. In the Hhohho region all lineage 1 sub-lineages (EAI1_SOM and EAI5) and X3 of lineage 4 were isolated at baseline and were not isolated at all at follow up, although there was no statistical significance (p=0.51). Also, the Manzini region had the EAI5, T1_T3 and X3 sub-lineages isolated only at baseline (p=0.001). Likewise, in the Shiselweni region the CAS_KILI and T1 were only isolated at baseline, although with no statistical significance (p=0.157). On the other hand, in other regions some sub-lineages emerged at follow up. This was noted in the Manzini region with the T5_RUS1 sub-lineage (p=0.001) as well as in the Hhohho region with the CAS_DELHI and LAM 1 sub-lineages, although with no statistical significance (p=0.51). Likewise, the X3 sub-lineage in the Shiselweni region was increased at follow up (p=0.157). Interestingly, the frequency of the Beijing sub-lineage, was observed to be more at follow up across all the regions.

Some lineages were decreased in frequency at follow up compared to baseline. These were LAM 4 sub-lineage in the Hhohho region, the S sub-lineage in the Shiselweni region and EAI1_SOM, S, X3 and LAM3 sub-lineages in the Manzini region at follow up.

Moreover, some sub-lineages were the same frequencies between baseline and follow up. Notably, the Shiselweni region had no sub-lineages which had unchanged frequencies at both baseline and follow up. The T1 sub-lineage remained constant in Hhohho, Manzini and Lubombo regions. The LAM 4 sub-lineage had unchanged numbers at follow up in the Manzini and Lubombo regions, likewise the S sub-lineage in the Hhohho and Lubombo regions remained unchanged.

Table 5: The change in the frequency of *M. tuberculosis* lineages and sub-lineages among the participants in the different regions, indicating that some participants were successfully treated, and others may have acquired new strains at follow up.

Lineage	**Lineage distribution n (%)**	**Sub-lineage**	**REGIONS**							
			Hhohho *n* (%)		**Manzini *n* (%)**		**Lubombo *n* (%)**		**Shiselweni *n* (%)**	
			Baseline	**Follow up**	**Baseline**	**Follow up**	**Baseline**	**Follow up**	**Baseline**	**Follow up**
Indo-Oceanic (Lineage 1)	19 (11.2)	EAI1_SOM	2 (11.8)	0 (0.00)	9 (20)	3 (6.4)	1 (9.1)	0 (0.00)	0 (0.00)	0 (0.00)
		EAI5	1 (5.9)	0 (0.00)	1 (2.2)	0 (0.00)	1 (9.1)	0 (0.00)	0 (0.00)	0 (0.00)
		MANU1	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (9.1)	0 (0.00)	0 (0.00)
East Asian (Lineage 2)	49 (28.8)	BEIJING	4 (23.5)	9 (50.0)	1 (2.2)	23 (48.9)	1 (9.1)	3 (27.3)	2 (20.0)	6 (54.5)
Central Asian (Lineage 3)	4 (2.4)	CAS1_DELHI	0 (0.00)	1 (5.6)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		CAS_KILI	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (9.1)	1 (9.1)	1 (10.0)	0 (0.00)
Euro-American (Lineage 4)	98 (57.6)	T1	1 (5.9)	1 (5.6)	6 (13.3)	7 (14.9)	1 (9.1)	1 (9.1)	3 (30.0)	0 (0.00)
		T5_RUS1	0 (0.00)	0 (0.00)	0 (0.00)	1 (2.1)	1 (9.1)	0 (0.00)	0 (0.00)	0 (0.00)
		T1_T3	0 (0.00)	0 (0.00)	2 (4.4)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		S	3 (17.6)	3 (16.7)	11(24.4)	7 (14.9)	3 (27.3)	3 (27.3)	3 (30.0)	1 (9.1)
		X2	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (9.1)
		X3	2 (11.8)	0 (0.00)	5 (11.1)	2 (4.3)	1 (9.1)	1 (9.1)	1 (10.0)	2 (18.2)
		U	0 (0.00)	0 (0.00)	1 (2.2)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		LAM1	0 (0.00)	1 (5.6)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		LAM3	1 (5.9)	1 (5.6)	3 (6.7)	1 (2.1)	0 (0.00)	0 (0.00)	0 (0.00)	1 (9.1)
		LAM4	2 (11.8)	1 (5.6)	3 (6.7)	3 (6.4)	1 (9.1)	1 (9.1)	0 (0.00)	0 (0.00)
		LAM9	1 (5.9)	1 (5.6)	2 (4.4)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		H37Rv	0 (0.00)	0 (0.00)	1 (2.2)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
		TOTAL	17 (100)	18 (100)	45 (100)	47 (100)	11 (100)	11 (100)	10 (100)	11 (100)
p-Values			0.51		0.001*		0.834		0.157	

*: p-value significant at $p < 0.05$

To address the main aim of the study, which was to ascertain whether the circulating susceptible *M. tuberculosis* strain later developed to MDR-TB on the same patient or whether patients acquired a completely new strain at follow up, further analysis of the spoligotyping patterns was done. Although there was no statistical significance, it was noted that some of the lineages had no variation on the sub-lineage or DST pattern between baseline and follow up findings and others had variation on the sub-lineage or DST pattern between baseline and follow up findings (p=0.851) (Table 6).

Where there was no variation on the sub-lineage or DST pattern between baseline and follow up findings it was classified as "same sub-lineage with same drug susceptibility pattern". However, when there was a variation on the sub-lineage or DST pattern between baseline and follow up findings it was classified as "changed sub-lineage with changed drug susceptibility patterns". In situations where the sub-lineage had not changed yet the drug susceptibility pattern had variations, it was categorised as "same sub-lineage with changed drug susceptibility pattern". The last classifications were the "changed sub-lineage with same drug susceptibility pattern" whereby there was no variation on the DST yet the sub-lineage had changed to a different one. 'Changed sub-lineage' or 'changed DST' indicated a pattern of a sub-lineage or DST that was not the same between baseline and follow up for a participant; it compared between baseline and follow up findings.

The forty one of the study isolates which had no spoligotyping findings due to DNA inadequacy (less than 10ng) and or spoligotype patterns which were not found in the SpolDB4 database, either at baseline or at follow up, whom were excluded from analysis of data, reduced the number of participants for this part of analysis from 103 to 62.

Although with no statistical significance (p=0.851), the analysis revealed that the majority of the total isolates (n=62), had the 'changed sub-lineage with the same DST pattern' category with 53.23% (n=33), followed by the 'changed sub-lineage with changed DST pattern' category with 25.81% (n=16). The least represented categories with 9.68% (n=6) were the 'same sub-lineage with changed drug sensitivity pattern' category as well as the 'same sub-lineage with the same DST pattern" category with 11.29% (n=7).

Table 6: The association between sub-lineage and DST patterns at baseline and follow up per region.

Variables	Hhohho region n (%)	Manzini region n (%)	Lubombo region n (%)	Shiselweni region n (%)	Total n (%)	p-value
Same sub-lineage with same drug sensitivity pattern	1 (7.14)	3 (9.38)	2 (20.00)	1 (16.67)	7 (11.29)	0.851
Same sub-lineage with changed drug sensitivity pattern	2 (14.29)	4 (12.50)	0 (0)	0 (0.00)	6 (9.68)	
Changed sub-lineage with same drug sensitivity pattern	6 (42.86)	18 (56.25)	5 (50.00)	4 (66.67)	33 (53.23)	
Changed sub-lineage with changed drug sensitivity pattern	5 (35.71)	7 (21.88)	3 (30.00)	1 (16.67)	16 (25.81)	
TOTAL	14 (100)	32 (100)	10 (100)	6 (100)	62 (100)	

4.2.3 Treatment compliance

The study hypothesised that the circulating susceptible *M. tuberculosis* strain in Swaziland later develops to MDR-TB on the same patient due to non-compliance to treatment. Hence, the medical records were reviewed (n=103) to ascertain the level of compliance of the participants to the anti-TB treatment regimen, which composed of rifampicin, isoniazid, streptomycin and ethambutol.

Although there was no statistical significance, the pattern was that of all participants in the poly-resistant classification (which were those participants with isolates resistant to more than one first line anti-TB drugs, other than isoniazid and rifampicin) noted to be compliant to the

treatment. Furthermore, those participants in the mono-resistant category (which included those with isolates that were only resistant to the isoniazid drug), had 50% of them noted to be compliant (p=0.493) (Figure 24).

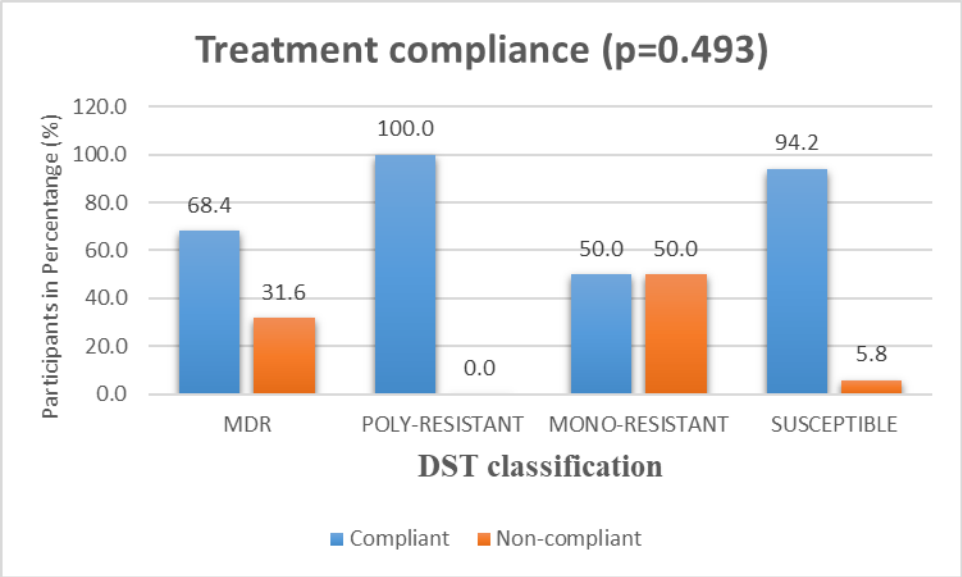

Figure 24: The level of compliance to the anti-TB treatment, indicating that the majority of the study participants were taking their anti-TB treatment accordingly compared to those who defaulted on their treatment.

Among the different regions, although with no statistical significance (p=0.493), 83.50% of the participants were noted to be compliant to the treatment. Furthermore, a high proportion of those who were non-compliant (30.77%) were noted to be from the Lubombo region (Table 7).

Table 7: The level of treatment compliance among the participants among the regions

Treatment Compliance	**Hhohho n (%)**	**Manzini n (%)**	**Lubombo n (%)**	**Shiselweni n (%)**	**Total n (%)**	**- p-value**
Compliant	18 (85.71)	47 (83.93)	9 (69.23)	12 (92.31)	86 (83.50)	0.493
Non-compliant	3 (14.29)	9 (16.07)	4 (30.77)	1 (7.69)	17 (16.50)	
TOTAL	21 (100)	56 (100)	13 (100)	13 (100)	103 (100)	

4.2.4 TB mixed infection

The aim of the study was to determine whether the circulating susceptible *M. tuberculosis* strain in a single patient in the cohort investigated would later develop to an MDR-TB strain or whether the patient would acquire a completely new strain at follow up. This was on the hypothesis that the investigated patient would have a single *M. tuberculosis* strain. This was based on the fact that TB is usually caused by a single *M. tuberculosis* strain (Gan *et al.* 2016). However, on the genotype data analysis it was found that some participants had TB mixed infections. TB mixed infection refers to an infection caused by more than one clonally distinct *M. tuberculosis* strain, either through a single transmission event involving more than one distinct strain or through multiple transmission events (super-infection) during a single-disease episode (Shamputa *et al.* 2004; McIvor *et al.* 2017). The mixed infections are noted by the overlapping spoligotype patterns upon analysis (Kargarpour Kamakoli *et al.* 2018). In this phenomenon, the sample would have additional spacers on the spoligotype pattern defining a certain sub-lineage (Guernier-Cambert *et al.* 2019).

Although detecting the presence of mixed infection in the study participants was not part of the study objectives and hence not possible to determine the mixed strains, however a few isolates (6.48%; n=11) were noted to have mixed infections (Table 8).

Table 8: The prevalence of TB mixed strains among the study isolates (n=170)

Sub-lineage	Single Strain *n* (%)	Mixed Strain *n* (%)	Total *n* (%)
BEIJING	46 (27.06)	3 (1.76)	49 (28.82)
CAS_KILI	1 (0.59)	2 (1.18)	3 (1.77)
CAS1_DELHI	1 (0.59)	0 (0.00)	1 (0.59)
EAI1_SOM	13 (7.64)	2 (1.18)	15 (8.82)
EAI5	3 (1.76)	0 (0.00)	3 (1.76)
H37Rv	1 (0.59)	0 (0.00)	1 (0.59)
LAM1	1 (0.59)	0 (0.00)	1 (0.59)
LAM3	6 (3.53)	1 (0.59)	7 (4.12)
LAM4	11 (6.47)	0 (0.00)	11 (6.47)
LAM9	4 (2.35)	0 (0.00)	4 (2.35)
MANU1	1 (0.59)	0 (0.00)	1 (0.59)
S	32 (18.82)	2 (1.18)	34 (20.00)
T1	19 (11.17)	1 (0.59)	20 (11.76)
T2-T3	2 (1.18)	0 (0.00)	2 (1.18)
T5_RUS1	2 (1.18)	0 (0.00)	2 (1.18)
U	1 (0.59)	0 (0.00)	1 (0.59)
X2	1 (0.59)	0 (0.00)	1 (0.59)
X3	14 (8.23)	0 (0.00)	14 (8.23)
TOTAL	159 (93.52)	11 (6.48)	170 (100)

4.3 Conclusion

This chapter was able to illustrate in the main the type of strains and their DST patterns in the investigated cohort. Furthermore, chapter 5 interpreted chapter 4 findings and discusses their possible implications to public health in Swaziland. In addition, chapter 6 draws study recommendations and conclusions.

Chapter 5

Discussion

5.1 Introduction

The tuberculosis (TB) epidemic remains one of the main global communicable diseases responsible for ill health among millions of people each year (Sharma *et al.* 2013; Smith *et al.* 2013; World Health Organisation 2018). The common rate of decrease within the international TB incidence charge remained low, at 1.6% per annum between 2000 and 2018, and was 2% between 2017 and 2018 (World Health Organisation 2019). Notably, the rate of decline in TB incidence in Swaziland is still low, and has been documented to be 565 per every 100,000 population, which is 0.6% (Swaziland National Tuberculosis Control Program 2017). This is however high compared to that of neighbouring countries where in 2017 South Africa had incidence of 301 per 100 000 population, Lesotho and Mozambique were 13 per 100 000 population and 162 per 100 000 population respectively (World Health Organisation 2019). It is noted that the World Health Organisation (2018) had indicated that a decrease in the TB incidence rate between 4–5% per annum by year 2020 would be essential to achieve the End TB Strategy. Thus, any efforts to realise the decline targeted by the World Health Organisation must be explored. Hence, the study aimed to highlight if the circulating susceptible *M. tuberculosis* strain in Swaziland later develops to MDR-TB on the same patient or whether patients acquire a completely new multi-drug resistant strain, to contribute to the efforts of the government of Swaziland in realising the targeted rate of decline.

This burden of TB infection is unfortunately in the backdrop of the co-existent human immunodeficiency virus (HIV) infection in Swaziland, with about 69% of the HIV-TB co-infections (Stop TB partnership 2017). Lower treatment success among HIV-TB co-infected is one of the major contributing factors to the development of drug resistant tuberculosis (DR-TB) (Karo *et al.* 2016). It is also documented that HIV infection may render false negative sputum smear results during the investigation of the presence of TB, because of an unusual presentation and lower rates of sputum smear positivity associated with HIV, which may result in delay in the treatment of the TB infection (Sasindran *et al.* 2011a; Hoang *et al.* 2015). Hence, this current study intended to detect reliably the type of *M. tuberculosis* sub-lineages in the study participants who were diagnosed with TB infection by the utilisation of spoligotyping. We

employed spoligotyping, which is an epidemiological technique that investigated whether the TB infected patients in Swaziland (either with strains susceptible or resistant to anti-TB treatment) would acquire a different *M. tuberculosis* strain during the course of treatment.

The evolution of *M. tuberculosis* occurs from time to time (Brites *et al.* 2015) therefore presenting with emerging strains of unique resistance, which are classified as multi-drug resistant or extremely drug resistant, and are very difficult to treat (Chang *et al.* 2013; Pontali *et al.* 2019). The change of strain infecting an individual has the potential to impair responsiveness to anti-TB treatment. Hence, the current study findings highlighted the TB epidemiology and the development of MDR-TB in the Swaziland population at the time when the study was conducted.

5.2 Prevalence of TB infection in the study population

The prevalence of TB infection in this study population was 26.41% (n=103/390), which was diagnosed using the GeneXpert MTB/RIF assay (Cephied, USA). This prevalence was lower compared to the most recent published TB prevalence data in Swaziland, which was 40% (Mandalakas *et al.* 2017). Furthermore, the prevalence of multi-drug resistant-TB (MDR-TB) among those who were diagnosed with TB infection was 28.64% (n=29/103). The recent anti-TB drug resistance survey which was conducted in Swaziland in 2012 had reported an MDR-TB prevalence of 33.8% (Sanchez-Padilla *et al.* 2012). Although these findings may suggest that there was no significant change in the prevalence of MDR-TB infection in Swaziland when the study was conducted, however the slight reduction was noted, although it had not reached the desired output of containing the epidemic that has put enormous pressure on the health delivery system in Swaziland (University Research Co. LLC 2016). The desired outcome is to eliminate TB by 2050 by reducing the annual incidence of new cases to less than 1 per million population (World Health Organisation 2009). The epidemic impact of the disease has instituted the integration of TB and HIV services in health facilities of Swaziland from the year 2015 (University Research Co. LLC 2016). This integration has slightly improved access to diagnosis and treatment of TB when compared to the last TB prevalence survey in 2009 (Swaziland National Tuberculosis Control Program 2017), which may have attributed to the slight decline noted in this current study.

Furthermore, the results revealed that majority of the TB infected participants were male. This was not surprising, as the global TB disease burden is reported to be higher among adult males, with a 57% prevalence rate when compared to 32% of adult females (World Health Organisation 2019). This suggests that males could be the main transmitters of TB and could be considered to be a TB high-risk group (Horton *et al.* 2016). Of concern is the fact that males tour greater often; have greater social contacts; spend extra time in settings that may be conducive to transmission, consisting of bars; and have interaction in professions associated with a better danger for TB, such as mining (Boum *et al.* 2014; Nhamoyebonde *et al.* 2014).

In addition, the study found that the TB infection was more prevalent in the age category of between 18-35 years with a median age of 34.4 years, and a higher proportion of these participants being male. Several studies including those in the WHO Africa region concur with the current study findings, showing high TB incidence among adolescents and young adults (Wood *et al.* 2010; Snow *et al.* 2018; World Health Organisation 2019). Also, a study done in South Africa, which is a neighbouring country to Swaziland, showed the highest burden of TB within the 20 – 39 old year old category (Blaser *et al.* 2016). In Swaziland, this age bracket contributes to approximately 27% of the total population (Index Mundi 2018). Moreover, Swaziland is a traditional country, with socio-cultural norms on gender roles associated with men's predominant role in the household economy and limited employment for women (Russell 1986; International Labour Office 2016; Swaziland 2016b). When males are infected with TB, there may be loss of income in their households due to frequent travel to health centres for treatment, increased need for nutrition, hospitalisation and worse if there is a death (Onifade *et al.* 2010). Therefore, it is viewed important that TB is detected and treated timeously among the men of Swaziland in particular.

The study further observed that the majority of the participants were from the Manzini region followed by the Hhohho, Lubombo and Shiselweni regions. This geographic distribution aligned to the 2017 Swaziland Population census whereby Manzini was the most populous region with n=355,945 (32.6%) inhabitants, followed by Hhohho with a population of n=320,651 (29.3%). Lubombo was next with a population of n=212,531 (19.4%) and the least populated region is Shiselweni with n=204,111 (18.7%) inhabitants (The United Nations Population Fund 2017). The population densities influence the government of Swaziland to focus on major service

delivery decisions where the population density is high (Linard *et al.* 2012). The number of health facilities per region are therefore a mirror reflection of the population densities. The service deliveries which include TB testing sites are located more in the Manzini region as a result; where Manzini region has 130 health facilities while the Hhohho, Lubombo and Shiselweni regions has 100, 52 and 45 health facilities respectively (Swaziland National tuberculosis control programme 2019). Interestingly, the results exposed that most of the participants infected with MDR-TB were from the Lubombo region, even though this finding had no statistical significance. Moreover, the Lubombo region had participants with the highest drug resistance to all four widely used anti-TB drugs at follow up, which were rifampicin (RIF), isoniazid (INH), Streptomycin (STR) and ethambutol (EMB). Notably, the INH mono-resistance at 7.69% in the Lubombo region. It is well documented that IHN mono-resistance to these two drugs is used as markers for MDR-TB (Kurz *et al.* 2016; Mesfin *et al.* 2018; Swaziland National tuberculosis control programme 2019). These findings of significant proportions of MDR-TB strains in this region may probably be due to poor TB control issues (Nachega *et al.* 2003). In addition, the Lubombo region is a rural region (Swaziland Central Statistics Office 2007) and an association between high MDR-TB strains and a rural setting has been made. Mvelase *et al.* (2019) found the highest rate of MDR-TB cases among the South African northern districts of Umkhanyakude and Zululand, which are also rural, similar to the Lubombo region.

5.3 The development of drug resistant strains in the study participants

A significant proportion of the study population, which was 28.64% (n=59/206), was noted to be infected with MDR-TB strains at baseline and/or at follow-up. Further analysis revealed that the proportion of participants who had been detected with resistant strains had significantly increased at follow up in comparison to baseline. This could be due to the emergence of strains at follow-up which were not isolated at baseline. This was evident in the Manzini and Shiselweni regions, where at follow-up there was a statistically significant increase in participants infected with the Beijing sub-lineage of lineage 2, from eight to forty-one participants. The Beijing sub-lineage is a strain which is associated with high prevalence of MDR-TB (Glynn *et al.* 2006; Stavrum *et al.* 2009; Ribeiro *et al.* 2014). The Beijing sub-lineage was the second most prevalent sub-lineage after the S sub-lineage in the Lubombo region, and this region had the highest rate of MDR-TB at 38.46%. The second region with high MDR-TB

was the Hhohho with 33.33% and that region also had high prevalence of the Beijing sub-lineage. These findings support the suggestion that the Beijing sub-lineage is related to excessive occurrence of drug resistant TB (Ribeiro *et al.* 2014). In addition to the Beijing sub-lineage, there was also the Ural (U) sub-lineage of lineage 4, which was observed in the Manzini region. Although, this strain was noted in only 0.59% of participants, however the control this sub-lineage is viewed essential, as it may significantly contribute to the MDR-TB development rate in Swaziland. The U sub-lineage is also associated with the development of MDR-TB and XDR-TB (Mokrousov et al. 2015; Sinkov et al. 2018; Feyisa et al. 2019).

Further analysis revealed that some participants who had drug resistant strains at baseline or at follow-up, had shown sub-lineage and/or DST profile change between baseline and follow-up samples. A total of 53.23% participants (n=33/62) had shown changed sub-lineage with the same drug sensitivity pattern between baseline and follow-up, followed by 25.81% participants (n=16/62) with baseline and follow-up changes in both sub-lineage and drug sensitivity patterns. This was concerning as these findings were observed at six months after the administration of the conventional first line drug regimen, which was expected to subside the infection rate. Such findings are postulated to may have arisen due to the acquisition of mutations by the baseline *M. tuberculosis* strains, which may have occurred outside the common direct repeat (DR) region of the *M. tuberculosis* genome during the course of treatment (Kurz *et al.* 2016; Dookie *et al.* 2018; Mabhula *et al.* 2019). The possible reasons for these mutations cannot be explained, however it may not be the non-compliance to treatment. It is noted that 83.5% (n=85) of the study population were compliant to treatment. This was an interesting finding. This may therefore refute the association of MDR with non-compliance in this study cohort. The MDR may most likely be due to treatment failure, which may have been caused by newly acquired mutations due to the administration of lower drug dose and rapid evolution of *M. tuberculosis* that confer development of the anti-TB drug resistance (Takarinda *et al.* 2013; Tang *et al.* 2015; Nguyen 2016; Swaziland National Tuberculosis Control Program 2017; World Health Organisation 2018). The current study did not assay to check for mutations to note whether there was lateral genetic exchange between lineages or not. It is reported that genetic exchange is favourable for the attainment of autonomous pathogenic traits within TB lineages, and thus confers resistance to some anti-TB drugs (Gagneux *et al.* 2007; Nicol *et al.* 2008).

Further analysis of the findings on the resistance of the strains to the TB drugs highlighted that a substantial proportion of the participants were observed with different TB mono-drug resistance, where 2.91% (6/206) of those participants showed RIF mono-resistance and 1.46% (3/206) were INH mono-resistant. The participants who had poly-resistance (which were unaffected to more than one first line anti-TB cupsules, other than INH and RIF) were 8.25%. This may probably mean that there is a significant proportion of TB infected patients in Swaziland that may be resistant to at least one of the first line anti-TB drugs. In agreement with this view, it was interesting to note that a study that had been conducted in Swaziland among previously treated patients (n=281) by Sanchez-Padilla *et al.* (2012) had also found almost similar findings, where 2.5% of the participants were resistant to RIF and 4.3% were resistant to INH. Generally, the patients observed with resistance may have been infected with a strain that is resistant to the drug before they started treatment Currently, the standardised first line drug regimen which Swaziland uses, adopted from WHO, consists of two phases, an intensive phase (two months with isoniazid (INH), rifampicin (RIF), pyazinamide (PZA) or streptomycin (STR) and ethambutol) and followed by a continuation phase (four months by a combination of isoniazid and rifampicin) (World Health Organization 2014; Swaziland National tuberculosis control programme 2019). or it could be that they were administered with a lower dose of the drug or the patient may have been non-compliant to the drug (referred to as a defaulter). The finding of mono-resistance to each of the anti-TB drugs in the current study indicate that more may still need to be done to effectively combat the TB epidemic in Swaziland. Many cases of mono-resistant TB in the end make a contribution to the amplification of resistance and ultimately result in MDR (Liu *et al.* 2020). Although treatment may be administered to compliant patients however, it does not take into account the strain DST patterns, then that may lead to the development of MDR-TB (Mase *et al.* 2019). Correct remedy of mono-resistant TB can prevent genetic mutations that may cause the development of MDR-TB in Swaziland. Although treatment may be administered to compliant patients, it does not take into account the strain DST patterns, which may lead to the development of MDR-TB.

Furthermore, some sub-lineages (noted to be of lineage 1 and lineage 4) which were detected at baseline, were not detected at follow up specimens, such as EAI1_SOM to X3 in the Hhohho region and EAI5 in the Manzini and Lubombo regions. Moreover, some of the sub-lineages (also of lineage 1 and lineage 4) had shown trends of reduction at follow-up such as the

EAI1_SOM, X3, LAM3 and S sub-lineages in the Manzini region, LAM 4 and S sub-lineages in the Hhohho and Shiselweni regions respectively. The decrease in the number of participants infected by these sub-lineages may be suggestive of successful treatment (van Rie *et al.* 2005; McIvor *et al.* 2017). Lineage 1 strains (including EAI1_SOM and EAI5) cause less severe TB infection, and their transmission consistently indicate that there are less virulent than lineage 2 and lineage 4 strains, hence more treatable than the other sub-lineages (Coscolla *et al.* 2010). The X3, LAM3 and S sub-lineages are of lineage 4 which is associated with severe lung consolidation; however, these strains are less virulent as opposed to other sub-lineages, hence they respond well to treatment (Coscolla *et al.* 2010; Wampande *et al.* 2019).

5.4 The phylogenetic diversity of *M. tuberculosis* strains in the study population

The current study provided the most recent insights into the genetic assortment of *M. tuberculosis* strains that might be circulating in Swaziland. Of the seven known *M. tuberculosis* lineages, which are: Indo-Oceanic (lineage 1), East Asian (lineage 2), Central Asian (lineage 3), Euro-American (lineage 4), West Africa 1 (lineage 5) and West Africa 2 (Lineage 6) and Aethiops vetus (lineage 7) (Comas *et al.* 2013), the observed *M. tuberculosis* strains in the current study belonged to lineages 1 to 4. The Euro-American lineage (lineage 4) was the most common lineage, with 57.6% isolates noted with this strain, followed by the East Asian lineage (lineage 2) in 28.8% isolates, Indo-Oceanic lineage (lineage 1) in 11.2% and Central Asian lineage (lineage 3) in 2.4% isolates. The Beijing sub-lineage (lineage 2) was the predominant sub-lineage in 28.82% isolates, followed by the S and T1 sub-lineages (lineage 4) in 20.0% and 11.76% of isolates, respectively. This lineage heterogeneity in Swaziland may potentially render a challenge in putting in place an effective TB control programme, which would control the spread of the TB infection (Di Pietrantonio *et al.* 2011). All TB lineages have different virulence factors which may have effects on the anti-TB treatment outcomes (Coscolla *et al.* 2014; Zhan *et al.* 2020). It is generally accepted that the overall strain success to survive treatment effects relies upon the combination of strain virulence traits and host genetic elements (Di Pietrantonio *et al.* 2011; Pareek *et al.* 2013; Asante-Poku *et al.* 2015). Hence it is believed that this lineage diversity might be a contributing factor towards the development of MDR-TB and treatment failure (de Steenwinkel *et al.* 2012). For instance, the prevalent Beijing sub-lineage which is widely known to be frequently isolated where there is an MDR-TB outbreak (Ribeiro *et al.*

2014) may be associated with the high MDR-TB rate in Swaziland. Others have attempted to tie epidemiological data to mechanistic explanations. For example, the *M. tuberculosis* strains that have highly biological active lipid called polyketide synthase-derived phenolic glycolipid (PGL) which is which are believed to contribute to the *M. tuberculosis* phenotype (Krishnan *et al.* 2011), such as the East Asian widely known as Beijing, Indo-Oceanic and Euro-American strains which are believed to be more virulent or associated with increased progression to disease (de Jong *et al.* 2008; Thwaites *et al.* 2008). Overproduction of PGL by these strains inhibits the release of tumour-necrosis factor-alpha and interleukins 6 and 12 pro-inflammatory mediators (Reed *et al.* 2004; Krishnan *et al.* 2011).

The X2 sub-lineage was only isolated in the Shiselweni region of Swaziland (n= 1). The X2 sub-lineage is commonly isolated in the Americas population and lower proportions in few countries of southern Africa, Asia, and Europe (Stucki *et al.* 2016). Probably, the X2 sub-lineage might have been acquired in Swaziland through migration of people from the countries where it is dominant (Stucki *et al.* 2016). Furthermore, the CAS-Delhi, LAM1, LAM3, LAM9, U and T1_T3 sub-lineages of lineage 4 were found in few participants from the Hhohho and Manzini regions that are considered urban regions. It will be interesting to determine why these sub-lineages would infect preferentially a specific urbanized population, this needs further research. The Manzini and Hhohho regions are directly comparable to the Capricorn district of Limpopo in South Africa, which is an urbanized area with lots of industries. The Capricorn district also recorded the diverse lineages and sub-lineages T1 (7.0%), CAS-Delhi (1.0%), CAS-KILI (1.0%), LAM3 (7.4%), LAM4 (1%), LAM9 (1.9%), T3 (0.5%), X3 (1.0%) (Maguga-Phasha *et al.* 2017). This could suggest that these strains are associated with urban areas in the southern Africa region as seen in South Africa. Human population density and growth are significant predictors of historical emerging infectious disease events like *M. tuberculosis*, and thus urbanization is likely to have a profound effect on public health as rural pathogens adapt to urban conditions, and other pathogens emerge (or re-emerge) in urban areas (Hassell *et al.* 2017). For cities in developing countries like Swaziland, the epidemiological effects which promote transmission of TB infections are often concentrated in informal settlements, where population growth and density is highest (Neiderud 2015).

Furthermore, CAS-Delhi and CAS-KILI of lineage 3 strains (East African Indian) are associated with increased relapse and reinfection rates among the TB infected patients (Guerra-Assunção *et al.* 2014, 2015). According to Swaziland National Tuberculosis Control Program (2017), the Lubombo region had 5% TB infection relapse rate while the Hhohho region had a 2%, Manzini 2% and Shiselweni had 1% relapse rates, however the exact strains causing the relapse were not mentioned. Therefore, based on Guerra-Assunção *et al.* (2015) findings, the current study may point to the lineage 3 strains as a possible cause of relapse cases in the Hhohho, Shiselweni and the Lubombo regions in Swaziland.

In conclusion, the findings of the current study revealed that Swaziland has four of the seven lineages circulating, with lineage 4 being dominant. Moreover, the lineage 2 sub-lineage, the Beijing strain was also found to be predominant. The MDR-TB rate in Swaziland can be associated with this Beijing sub-lineage, which is commonly associated with outbreaks of MDR-TB. The Beijing sub-lineage was isolated along with seventeen more sub-lineages; EAI1_SOM, EAI5, MANU1, CAS_DELHI, CAS_KILI, T1, T5_RUS1, T1_T3, S, X2, X3, U, LAM1, LAM3, LAM4, LAM9 and H37Rv. The study findings hence highlighted the importance of detecting and epidemiologically analysing the strains that are circulating in Swaziland. The analysis revealed that some participants developed or acquired an MDR strain at follow up while others had MDR strains at both baseline and at follow-up. Interestingly, the MDR-TB infections significantly increased at follow-up. The increase may be alluded to the Beijing strain, which is known to be dominant where there is an MDR-TB outbreak, which also showed an increase at follow-up. On the other hand, there were strain that were only observed at baseline and were not noted at follow-up, thus were interpreted as they were successfully treated.

5.5 Mixed strain infection

In this present study, the prevalence of mixed infection was 6.48%. This identification of mixed (or polyclonal) infection, which is an infection with several different genotypes of *M. tuberculosis* in a single patient was an interesting finding. Mixed strain (mixed infection) is a TB disease caused by multiple strains of *M. tuberculosis* which is found to have infected an individual host at a single point in time (Cohen *et al.* 2011b; McIvor *et al.* 2017; Ndhlovu *et al.* 2019).

The mixed infection rate in the current study was not unique as the frequency of mixed infections in countries like Georgia, Taiwan, China, Central Asia, Uganda, Malawi and South Africa has been reported within a range between 2.8-19% (Hanekom *et al.* 2013). Swaziland is an African country in Southern Saharan region similar to Uganda, Malawi and South Africa. This could have influenced the mixed infection rate to be 6.48%, which is within the range observed in the aforementioned countries. In this study, the Beijing sub-lineage had a substantial rate of mixed strain with 1.8% (n=3), followed by the S sub-lineage EIA_SOM and CAS_KILI with 1.2% (n=2) each. It is suggested that in the African continent, the Beijing and S sub-lineages are most likely to be associated with mixed infections (Hanekom *et al.* 2013; McIvor *et al.* 2017). The challenges with a mixed infection is that it can lead to changing drug-susceptibility patterns during therapy (Zaoxian Mei *et al.* 2015). It is suggested that a mixed infection with both drug sensitive and drug resistant strains may lead to discordant drug-susceptibility testing profiles, which could complicate the treatment regimen (Gan *et al.* 2016) and lead to poor treatment outcomes (van Rie *et al.* 2005; Gan *et al.* 2016). However, the detection of the mixed infection in the study participants was not part of the study objectives, thus further investigation was not possible.

5.6 Study limitations

5.6.1 Study design

The nature of this cohort study design, which was cross-sectional and not longitudinal, was a limitation. The cross-sectional design of the study could not allow the generalisation of the findings to the general population of Swaziland. However, the findings were able to highlight the TB strain lineages circulating in Swaziland and their DST patterns.

In addition, the study utilised the purposive homogenous sampling method, a method which is too selective in the sampling process compared to that of the randomised methods. However, the purposive sampling was utilised to enable the recruitment and selection of participants that would best enable the answering of the research questions, that is, the patients infected with TB.

Moreover, the exclusion of potential participants (n=15) who were exhibiting signs and symptoms of TB due to their salivary samples, which were categorised as samples of poor sputum quality even on their second attempt of collection, was a limitation. Their inclusion in the study may have contributed to a more informative TB prevalence data based on the GeneXpert® MTB/RIF assay (Cephied, USA). However, a third or further attempt at good quality specimen collection was not possible due to study time constrains.

It is therefore recommended that future studies should be longitudinal in design and community based, with randomised sampling and large sample sizes. Such studies may be able to produce more realistic MDR-TB prevalence data and deduce re-infections in instances where some participants had shown different lineages/sub-lineages at baseline and at follow-up.

5.6.2 Sample size

Although the sample size of 103 participants was calculated appropriately for power prior to the commencement of the study, in consultation with a statistician, it was viewed as small for generalizability. The ability to determine the level of participants' homogeneity and degree of variability was affected by the small sample size. More participants could not be recruited due to the genotyping test kits and method consumables being costly and not affordable within the budgetary constraints. It is recommended that future studies of this nature should include more participants so that the characteristics of the strains circulating in Swaziland may be more generalizable.

5.6.3 Data collection methods

The fact that the study could not analyse the whole *M. tuberculosis* genome was a limitation. However, the spoligotyping which targets repeat regions that are prone to convergent evolution, did classify the *M. tuberculosis* strains into lineages and sub-lineages.

Similarly, the SITVIT database that we used to assign spoligotypes to phylogenetic lineages had a limited representative collection compared to the circulating strains. The fact that some of the isolates (n=36) were left unclassified was a limitation, since it reduced the findings that could be further compared at baseline and at follow-up.

Another limitation was the collection of data on the participants' compliance to treatment from medical records, and this collection method not triangulated with an interview or a questionnaire. Due to the exhaustion from waiting in long queues in the TB clinic, the consented participants were not willing to stay long inside the screening room for the interviews. However, since the direct observed treatment system is utilised in the health facilities to monitor treatment compliance, enhance the information that was extracted from the patients' files was trusted.

The fact that the study isolated mixed infections, although few, it was a limitation that could not be investigated further due to budgetary constraints. Moreover, this was not part of the study objectives.

Despite these above-mentioned limitations, the interpretation of the findings in the current study was cognisant of these limitations. The findings provided a "snapshot" of the circulating *M. tuberculosis* strains in the Swaziland cohort that was investigated, which require to be further investigated in a community based study, longitudinal in design with randomised sampling and large sample size.

Chapter 6

Study Conclusion and Recommendations

6.1 Study Conclusion

To the best of our knowledge, this was the first of such a study to be conducted in Swaziland, where the type of *M. tuberculosis* lineages and sub-lineages were investigated in TB presumptive patients. The lineages and sub-lineages were investigated with their first line anti-TB drugs patterns at baseline and at follow-up, after the six months of treatment.

The findings revealed that the prevalence of TB infection was high. The findings further indicated that majority of these TB infected population that was investigated of young adult age (mean age of 36.8 years for males and 30.5 years for females), noting that the majority of them were male. Therefore, it is suggested that Swaziland Ministry of Health and the National TB Control programmes have to consider including in the health screening programmes in the health care facilities and places of employment all men and young adult females, symptomatic or asymptomatic, the testing for TB for early detection and treatment initiation, which may help to reduce the TB prevalence. Also, advocacy campaigns to amplify the TB screening and testing in all health centres and workplaces for all men and young adults' females should be heightened. These campaigns may aid in the reduction of the prevalence of both TB and MDR-TB in Swaziland.

Furthermore, the fact that most participants infected with MDR-TB were from the rural Lubombo region, points to the need to increase the number of health care facilities in that region. This may aid in the accessibility of more of the TB screening facilities to a wider population which may contribute towards the reduction in the rates of MDR-TB.

The study also revealed that Swaziland has high *M. tuberculosis* lineage diversity, with eighteen sub-lineages noted. This was a key finding. This lineage heterogeneity in Swaziland may potentially render a challenge in putting in place an effective TB control programme, which would control the spread of the TB infection (Di Pietrantonio *et al.* 2011). The different strains uniquely respond to treatment, hence the use of standard treatment may induce mutations which may lead to the development of MDR-TB (Coscolla *et al.* 2014). It is therefore suggested that

the various genotypes of *M. tuberculosis* strains circulating in Swaziland be investigated and monitored, so as to understand the transmission dynamics of these clones in the geographic regions of Swaziland, to help improve the TB control and prevention programs (McIvor *et al.* 2017). Strain genotypes may play a role in the disease outcome, variation in vaccine efficacy and the emergence of drug resistance.

Another key finding was the observance of strains that are capable of the development of MDR outbreaks, which were the Beijing strain and the U strain. The fact that the Beijing sub-lineage was observed as the most common strain was concerning. In addition, the detection of the U strain, although it was only in a single participant, it was also concerning as, it was felt that this U strain could potentially result in MDR outbreaks, if not controlled. Hence, it is recommended that Swaziland implements measures to control these strains that are associated with MDR-TB. This may include the routine screening of all TB infected patients for the presence of these MDR strains, utilising the molecular epidemiological technique, the spoligotyping, to limit the spread.

Some of the participants had shown a change of sub-lineage and DST profile patterns during the course of TB treatment, which could lead to poor treatment outcome and/ or potentially develop to drug resistant TB strains in Swaziland. Therefore, to improve TB treatment outcomes in Swaziland, it is recommended that the Ministry of Health reviews their TB drug susceptibility testing guidelines, to allow for testing of the type of strains in the patients diagnosed with TB at least twice before the completion of treatment. The continual DST testing may be beneficial in detecting any change in the DST patterns, which would inform a change in the combinations of the anti-TB drugs prescribed to the patients. In turn, the revised DST monitoring system may reduce the TB prevalence in Swaziland.

6.2 Recommendations

In this study, the high prevalence of TB infection, in particular the MDR-TB infection as well as the DST patterns that changed between baseline and follow-up, warrants for the advocating of treatment regimens that would improve the treatment outcomes by administering anti-TB drugs that are aligned to the DST patterns. It is thus recommended that the Ministry of Health reviews

the TB drug susceptibility testing guidelines, to allow for testing at least twice before the completion of treatment.

It is further recommended that a future study utilising the Geographic Information System (GIS) mapping be done in all the regions of Swaziland. The GIS mapping will assist in contact tracing, to note the cluster position of the TB strains, and the epidemiological linking, to obtain a clear molecular epidemiological overview of the strains circulating in Swaziland. It is recommended that these studies which would utilise GIS should be longitudinal in design and community based, with randomised sampling and large sample sizes. It is further recommended that the Swaziland Ministry of Health should consider including the molecular epidemiological techniques in their TB infection diagnostic modalities since these techniques provide much expedient information which may be utilized to combat the burden of the TB infection.

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Appendices

Appendix 1: Durban University of Technology IREC Ethics Clearance

DUT
DURBAN UNIVERSITY OF TECHNOLOGY

Institutional Research Ethics Committee
Research and Postgraduate Support Directorate
2nd Floor, Berwyn Court
Gate 1, Steve Biko Campus
Durban University of Technology

P O Box 1334, Durban, South Africa, 4001

Tel: 031 373 2375
Email: lavishad@dut.ac.za
http://www.dut.ac.za/research/institutional_research_ethics

www.dut.ac.za

21 November 2017

IREC Reference Number: **REC 79/17**

Mr T C Dlamini
P O Box C609
Hub Manzini
Swaziland

Dear Mr Dlamini

Molecular epidemiology of multi-drug resistant *Mycobacterium tuberculosis* strains in Swaziland

I am pleased to inform you that Full Approval has been granted to your proposal REC 79/17.

The Proposal has been allocated the following Ethical Clearance number **IREC 111/17**. Please use this number in all communication with this office.

Approval has been granted for a period of two years, before the expiry of which you are required to apply for safety monitoring and annual recertification. Please use the Safety Monitoring and Annual Recertification Report form which can be found in the Standard Operating Procedures [SOP's] of the IREC. This form must be submitted to the IREC at least 3 months before the ethics approval for the study expires.

Any adverse events [serious or minor] which occur in connection with this study and/or which may alter its ethical consideration must be reported to the IREC according to the IREC SOP's.

Please note that any deviations from the approved proposal require the approval of the IREC as outlined in the IREC SOP's.

Yours Sincerely

Professor J K Adam
Chairperson: IREC

DUT DURBAN UNIVERSITY OF TECHNOLOGY
2017 -11- 21
INSITUTUTIONAL RESEARCH ETHICS COMMITTEE
P O BOX 1334 DURBAN 4000 SOUTH AFRICA

Appendix 2: Swaziland National Health Research Review Board Clearance

Research Protocol clearance certificate

Type of review	Expedited	X		Full Board			
Name of Organization	**TALENT DLAMINI**						
Title of study	**MOLECULAR EPIDEMIOLOGY OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS STRAINS IN SWAZILAND**						
Protocol version	**1.1**						
Nature of protocol	New			Amendment	X		
List of study sites	**MBABANE TB CLINIC, TB CENTER MANZINI, SIPHOFANENI HEALTH CENTER, AND GOOD SHEPHERD HEALTH CENTER AND AT NATIONAL TB REFERRAL HOSPITAL.**						
Name of Principal Investigator	**MR TALENT DLAMINI**						
Names of Co- Investigators	**N/A**						
Names of steering committee members in the case of clinical trials	**N/A**						
Names of Data and Safety Committee members in the case of clinical trials	**N/A**						
Level of risk (Tick appropriate box)	Minimal			High			
	X						
Clearance status (Tick appropriate box)	Approved	X		Disapproved			
Clearance validity period	Start date	**28/08/2017**		End date	**28/08/2018**		
Signature of Chairperson							
Date of signing	**29/08/2017**						
Secretariat Contact Details	Name of contact officers	**Ms Simangele Masilela**					
	Email address	kaluamasi@gmail.com					
	Telephone no.	**(00268) 24040865/24044905**					

MINISTRY OF HEALTH
30 AUG 2017
HEALTH RESEARCH REVIEW BOARD
SWAZILAND

Telegrams:
Telex:
Telephone: (+268 404 2431)
Fax: (+268 404 2092

MINISTRY OF HEALTH
P.O. BOX 5
MBABANE
SWAZILAND

THE KINGDOM OF SWAZILAND

28th July, 2016

Talent Dlamini
Principal Investigator
MBABANE

REF: MH/599C/ IRB 000 9688/NHRRB 012/16

Dear Mr. Dlamini,

RE: MOLECULAR EPIDEMIOLOGY OF MYCOBACTERIUM TUBERCULOSIS STRAINS: CASE STUDY IN 5 DIFFERENT SCREENING FACILITIES.

The committee thanks you for your submission to the Swaziland Scientific and Ethics Committee, an expedited review was conducted.

In view of the importance of the study and the fact that the study is in accordance with ethical and scientific standards, the committee grants you authority to conduct the study. You are requested to adhere to the specific topic and inform the committee through the chairperson of any changes that might occur in the duration of the study which are not in this present arrangement.

The committee requests that you ensure that you submit the findings of this study (**Electronic and hard copy) and the data set** to the Secretariat of the SEC committee.

The committee further requests that you add the SEC secretariat as a point of contact if there are any questions about the study on 24040865/24044905.

Yours Sincerely,

MINISTRY OF HEALTH Swaziland Scientific and Ethics Committee (SEC) 28 JUL 2016 P.O. Box 5 Mbabane H100 SWAZILAND

THE CHAIRMAN, SEC
cc: SEC members

Appendix 3: Letter of Support from Swaziland National TB Control Programme

Tel: (+268 404 2431)
Fax: (+268 404 2092

MINISTRY OF HEALTH
P.O. BOX 5
MBABANE
SWAZILAND

THE KINGDOM OF SWAZILAND

03 JUNE 2016

Dr Rudolph Maziya
The Chairman
Scientific and Ethics Committee
Mbabane, Swaziland

Dear Sir

Letter of support: Molecular epidemiology of Mycobacterium Tuberculosis strains: case study in 5 different TB screening facilities.

This letter serves to confirm that the National TB Control Program (NTCP) is in support of the above study conducted in collaboration with Mr Talent Dlamini in fulfillment of the requirements of the degree of Masters' of Biomedical and Clinical Technology: Health Sciences at the Durban University of Technology.

The NTCP's support of the proposed research is aligned to the Swaziland National Health Research Agenda as it falls within the following priority: Burden and determinants of TB disease in key populations, i.e. DR-TB. As per the Ministry of Health Research Unit requirements for conducting research in Swaziland, the study team is reminded of the following conditions:

1. The NTCP must be provided with the final approved protocol and SEC approval letter and should be always informed of any protocol amendments
2. The Ministry of Health remain the custodians of the data and upon study completion, datasets and reports must be furnished to the Research Unit

We trust this application will receive the Ethics Committee kind consideration.

Yours sincerely,

Dr Welile Sikhondze

NTCP Technical Adviser and Research Coordinator

Appendix 4: Letter of support from Swaziland Health Laboratory Services

Telegrams:

Telex:
Telephone: (+268 404 2190)
Fax: (+268 404 8157)

Swaziland Health
Laboratory Service
P.O. Box 6840
Mbabane
Swaziland

THE KINGDOM OF SWAZILAND

1st June, 2016

Dr.Rudolph Maziya
The Chairperson
Scientific and Ethics Committee
Ministry Of Health
Mbabane, Swaziland

Dear Sir

Letter of support: Molecular epidemiology of Mycobacterium Tuberculosis strains: case study in 5 different TB screening facilities.

This letter serves to confirm that the Swaziland Health Laboratory Services (SHLS) is in support of the above study conducted by a Durban University of Technology Masters' student. The SHLS support of the proposed research is aligned to the Swaziland National Health research Agenda as it falls with the following priorities: Burden of TB disease and drug-resistance strains.

The study will adhere to the Ministry Of Health Research Unit requirements for conducting research in Swaziland by ensuring: SHLS must be provided with the final approved protocol and SEC approval letter and should be always informed of any protocol amendments. The Ministry of Health remains the custodians of the data and upon study completion, datasets and reports will be furnished to the Health Research unit

We are in full support of the study as it has the potential to contribute significantly towards the global body of evidence for improving TB diagnosis and treatment. We trust this application will receive the ethics Committee kind consideration.

Sincerely

Ms Sindisiwe Dlamini
Chief Medical Laboratory Technologist
(Swaziland Health Laboratory Service)

SWAZILAND HEALTH LABORATORY SERVICE
P.O. BOX 6840
MBABANE H100
01 JUN 2016
CHIEF MEDICAL LABORATORY TECHNOLOGIST
MOH

Appendix 6: Letter of support from Mbabane Government Hospital

Telegrams:
Telex:
Telephone: (+268 2411 8000)
Fax: (+268 2404 6471)

Mbabane Government Hospital
P.O. BOX 8
MBABANE
SWAZILAND

THE KINGDOM OF SWAZILAND

31 October 2017

Mr Talent C. Dlamini

Principal Investigator

STUDENT NUMBER: 21649493

Mbabane

Dear Talent,

RE: MOLECULAR EPIDERMIOLOGY OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS STRAINS IN SWAZILAND

This letter serves to inform you that management has granted you permission to undertake the above mentioned research study in the facility. You are expected to adhere to all the ethical considerations in the study.

We would very much appreciate if the findings and recommendations of the study can be communicated back to the facility (electronic and hard copy).

Wishing you all the best in your study.

Yours sincerely

MBABANE GOVERNMENT HOSPITAL
P.O. BOX 8
MBABANE H100
2017 -11- 02
MATRON 1
SWAZILAND

MATRON T. MKHONTA (Senior Matron)

FOR: HOSPITAL MANAGEMENT

Appendix 5: Letter of support from Medical Research Council Tuberculosis Platform

TUBERCULOSIS PLATFORM:
PRETORIA
Private Bag x385
Pretoria
0001

September 19, 2016

To whom it may concern

Dear Sir/Madam,

Re: Visit of Mr Talent C Dlamini from the Southern Africa Nazarene University (SANU), Swaziland.

Mr Talent C Dlamini is well known to me and will be visiting South African for the following reason:

1. We are currently collaborating with Mr Talent C Dlamini of Department of Medical Laboratory Sciences SANU where he is employed as a junior lecturer, studying the genetic profiles of *Mycobacterium tuberculosis* for his MSc study. She will be working closely with me on projects of key interest to both of our labs.

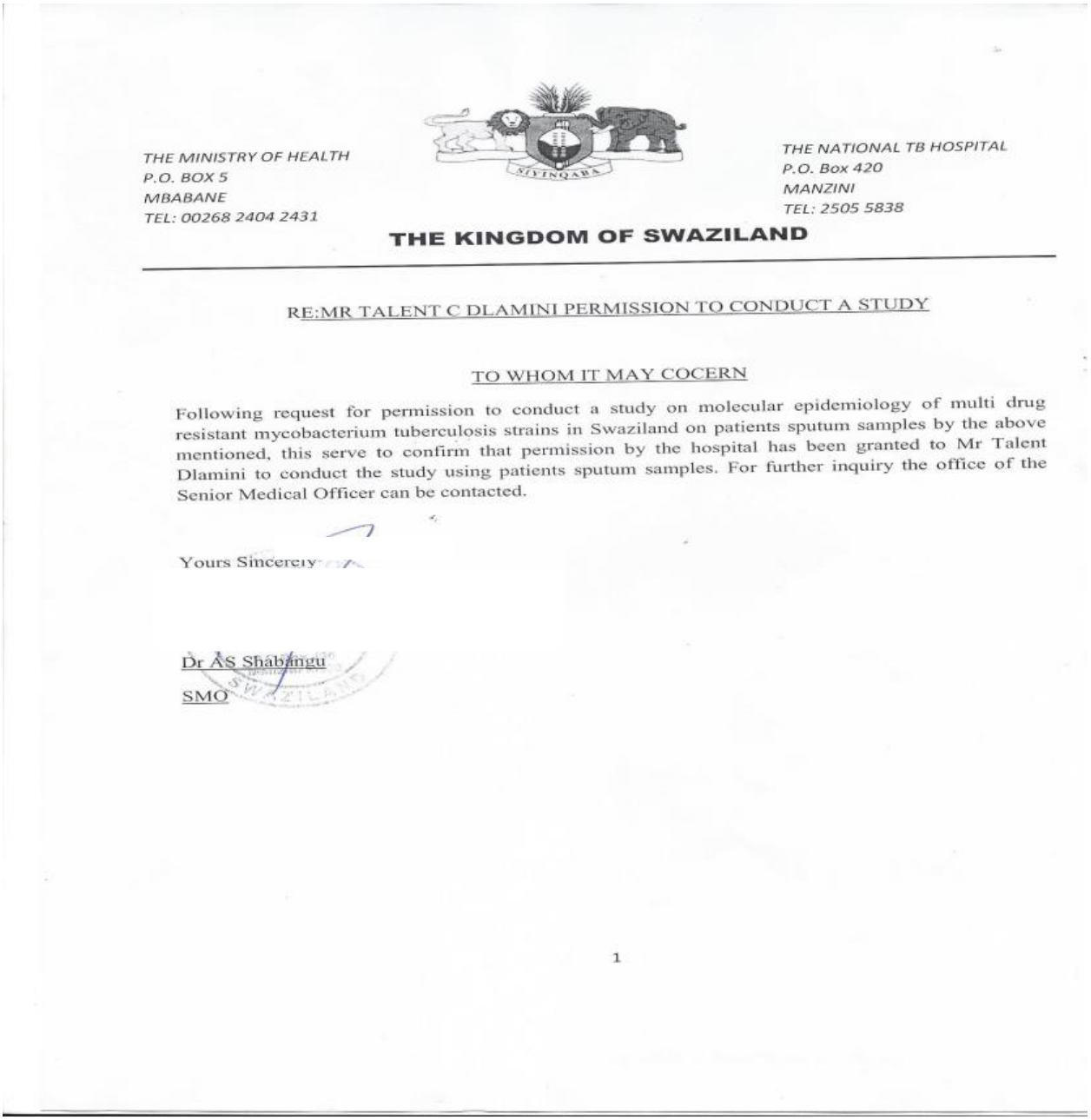
I am writing to grant Mr Dlamini permission for the purpose of conducting research experiments, discussions and collaborations at the South African Medical Research Council, TB Platform, Pretoria, South Africa.

I would be happy to deal with any queries should this be required.

Best wishes,

Lesibana Malinga, MSc.
Senior Scientist,
TB Platform, South African Medical Research Council
South Africa

Appendix 7: Letter of support from Moneni National TB Referral Hospital



THE MINISTRY OF HEALTH
P.O. BOX 5
MBABANE
TEL: 00268 2404 2431

THE NATIONAL TB HOSPITAL
P.O. Box 420
MANZINI
TEL: 2505 5838

THE KINGDOM OF SWAZILAND

RE:MR TALENT C DLAMINI PERMISSION TO CONDUCT A STUDY

TO WHOM IT MAY COCERN

Following request for permission to conduct a study on molecular epidemiology of multi drug resistant mycobacterium tuberculosis strains in Swaziland on patients sputum samples by the above mentioned, this serve to confirm that permission by the hospital has been granted to Mr Talent Dlamini to conduct the study using patients sputum samples. For further inquiry the office of the Senior Medical Officer can be contacted.

Yours Sincerely

Dr AS Shabangu
SMO

1

Appendix 8: Letter of support from Good Shepherd Mission Hospital

GOOD SHEPHERD
MISSION HOSPITAL

P.O. Box 2, Siteki
Swaziland, Southern Africa
Tel: 343 4133 / 4, 343 4467
Fax: 343 4003. 343 4064

"THE LORD IS MY SHEPHERD"

08 November, 2017

To.
Talent Dlamini
Junior Lecturer
SANU

Dear Dlamini

RE: MOLECULAR EPIDEMIOLOGY OF MULTI-DRUG RESISTANT MYCOBACTERIUM TUBERCULOSIS STRAINS IN SWAZILAND

I am writing this letter to inform you that after discussing with the relevant stake holders within the institution, you are permitted to conduct the study at our hospital.

In view of this important subject you chose as the case study, along with the approval from Swaziland Scientific and Ethics Committee, you can start your study at our hospital as early as possible.

I wish you all the best.

Yours Sincerely

GOOD SHEPHERD HOSPITAL
Dr. Kiron Koshy
Senior Medical Officer
P.O. Box 2. Siteki
Tel: 2343 6672/3

DR. K. KOSHY
SENIOR MEDICAL OFIICER

E-mail: admingsh@realnet.co.sz

Appendix 9: Letter of support from Siphofaneni / Nhlangano Health Centre

MEMORANDUM

To: Who it may concern

From: Siphofaneni Zonal Clinic

Date: 13/11/2017

RE: APPROVAL FOR MOLECULAR EPIDEMIOLOGY OF MULTI-DRUG RESTISTANT MYCOBACTERIUM TUBERCULOSIS STRAINS IN SWAZILAND

This serves to confirm that the Siphofaneni Clinic and Laboratory are in support of the above conducted by Durban University of Technology Master's student.

This study will adhere to the Ministry of Health Research Unit requirements for conducting research in Swaziland by ensuring: Swaziland Health Laboratory Services must be provided with the final approved protocol and SEC approval letter and should always be informed of any protocol and amendments, The MOH remains custodians of the data and upon study completion, dataset and report will be furnished to the Health Research unit.

We are in full support of the study as it has potential to contribute significantly towards the global body of evidence for improving TB diagnosis and treatment. We trust this application will receive the ethics Committee's kind consideration.

Sincerely

MINISTRY OF HEALTH
SIPHOFANENI CLINIC
13 NOV 2017
P.O. BOX 85, SITEKI

Mrs Dumsile L Gwebu
Clinic Supervisor
(Sister in Charge)

Appendix 10: Letter of Information

LETTER OF INFORMATION

Dear Participant

Title of the study: Molecular epidemiology of multi-drug resistant *Mycobacterium tuberculosis* strains in Swaziland.

Mr. Talent Colani Dlamini – Principal investigator B - Tech Biomedical Technology: (+268) 7615 4174
Dr. Brenda Mkhize – Supervisor, PhD: Medical Microbiology: (+27) 082 879 4923
Mr. Clive Sydney – Co-Supervisor, MMed Science: (+27) 083 783 865
Dr. Lesibana Malinga – Co-Supervisor PhD in Stem Cell Biology (+27) 081 252 0861

I Mr. Talent Colani Dlamini, a Masters in Health Sciences: Medical Laboratory Science student at Durban University of Technology am doing a study on Molecular epidemiology of *Mycobacterium tuberculosis* strains in Swaziland. There is little data on *Mycobacterium tuberculosis* strain types that are available in Swaziland. The aim of this study then is to investigate whether the Swaziland circulating susceptible *M. tuberculosis* strain will later develop to MDR-TB on the same patient or whether the patients acquire a completely new *M. tuberculosis* strain which is multi-drug resistant. Spoligotyping genotyping technique shall be used to define isolates (strains). The minimum number of 103 presumptive TB patients is expected to be enrolled on this study.

You will be requested to produce two sputum samples. In one sample, microscopy and/ or GeneXpert shall be done, your results then be released back to you by the clinic according to its protocol. If your results are positive for *Mycobacterium tuberculosis*, then the other sample shall be cultured for *Mycobacterium tuberculosis*. If culture positive, first line TB drug sensitivity will be performed, then results shall be released back to you once again according to facility protocol. After then DNA will be extracted from the sample and thereafter will be spoligotyped.

There will be no risks or discomforts associated with participating in this study.

You will not incur any cost, nor will you receive any money for participating in this study.

Knowledge and an understanding of the type of *Mycobacterium tuberculosis* strain(s) circulating in the country will be of great value in the improvement of the strategies to prevent and control transmission of TB epidemic, more especially the DR-TB.

Your name will not be attached to the information to be generated from the study, only unique identifiers shall be used to ensure confidentiality. Data collected will be kept in a secured, password encrypted computers and only the researchers will have access to them.

Participation in this study is voluntary. You will not be forced to partake in the study and you can withdraw from this study if you wish without any consequences or threats. Withdrawal will not jeopardize your access to the health care facility.

For any question or information regarding the study, you can contact the researchers (contacts above), the Institutional Research Ethics Administration at +27 31 373 2375 and or the Director: Research and Postgraduate Support, Prof S Moyo at +27 31 373 2577 or at moyos@dut.ac.za

Appendix 11: Consent form

CONSENT FORM

Statement of Agreement to participant in the research study:

- I hereby confirm that I have been informed by the researcher Mr. Talent Colani Dlamini, about the nature, conduct, benefits and risks of this study- Research Ethics Clearance Number: MH/599C/ IRB 0009688/NHRRB012/16 (Swaziland) and IREC 111/17 (DUT)**.**
- I have also received, read and understood the above written information (Participants letter of information) regarding the study**.**
- I am aware that the results of the study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a study report**.**
- In view of the requirements of research, I agree that the data collected during this study can be processed in a computerized system by the researcher**.**
- I may, at any stage, without prejudice, withdraw my consent and participation in the study.
- I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the study.
- I understand that that significant new findings developed during the course of this research which may relate to my participation will be made available to me.

…… ……. ……. ……

Full Name of Participant Date Time Signature/Right thumbprint

I, **Mr. Talent Colani Dlamini** herewith confirm that the above participate has been fully informed about the nature, conduct and risks of the above study.

Mr. Talent Colani Dlamini ---------- ----------

Full Name of the Researcher Date Signature

Appendix 12: INCWADZI YELWATI NGALELICWANGO

INCWADZI YELWATI NGALELICWANGO

Sikuvusele wena loyincenye yelucwaningo

Sihloko selucwaningo: Kwehlukanisa nekuhlatiya tibilini teligciwane lelibanga sifosesifubasengati Swaziland.

Babe Talent Colani Dlamini – Uncwaningi lomkhulu, kutemfundvo uphetse B - Tech Biomedical Technology: (+266) 7615 4174

Dr. Brenda Mkhize – Lobuke lolucwaningo, kutemfundvo uphetse PhD: Medical Microbiology : (+27) 082 879 4923

Mr. Clive Sydney – Lositana nalolalobuke lolucwaningo, kutemfundvo uphetse MMed Science: (+27) 083 783 865

Dr. Lesibana Malinga – Lositana nalolalobuke lolucwaningo, kutemfundvo uphetse PhD in Stem Cell Biology (+27) 081 252 0861

Libito lami ngu Talent Colani Dlamini, umfundzi eDurban University of Technology ephikweni lasegumbini lekucwaninga, ngenta i Masters. Lolucwaningo lubuke kwehlukanisa nekuhlatiya tibilini teligciwane lelibanga sifosesifubasengati Swaziland. Lwati ngaloluhlobo lwelucwaningo luncane Swaziland. Lotobuka kabanti kutsi labo labanalesifo, leligciwane legucugucuka kanjani. Loku kutobukwa ngebucwephesha be Spoligotyping. Lolucwaningo lubhekeke kutsi lungenelwe batfu labakhomba timphawu talesifo labangu 103.

Utocelwa kutsi ukhokhe tikhwehlela letimbili bonesi. Sinye sitopopolwa ngemshini we GeneXpert noma nge Microscope. Imiphumela yalololupopolo itobuyiselwa kuwe ngedlela sibhedlela lesiyibuyisela ngayo imiphumela. Nangabelibatjiwe lesigciwane lelibanga sifo sengati, lesilesinge sesibili sikhwehlela, titomikiswa eMbabane lapho siyopotjolwa ngalokujulile phindze kubukwenekutsi nguliphi liphilisi noma umjovo longelapha leligciwane. Phindze futsi ibuyiswelwe emuva imiphumela yakho, ifike kuwe ngendlela lesibhedlela ibuyisela ngayo imiphumela kubantfu lebuya eMbabane. Emvakwaloko, leligciwane litovulwa kukhokwe tibilini talo ticwaningwe kabanti ngalobucwephesha be Spoligotyping.

Kute kutsibameteka nama kungoti lobuhambelana nekubayincenye yalolucwaningo.

Angeke ubhadale noma ubhadalwe ngekubayincenye yalolucwaningo

Lwati ngeluhlobo leligciwane lelibanga sifo sesifuba sengati lutosita wena nelive kutsi litihlele kahle ngemacinga ekulwa nekuvikeka, nekwelapha sifo sengati kancono.

Libito lakho ngangeke livele kumiphumela yelucwaningo. Esikhundleni salo kutovela inombolo leyokwatiwa bacwaningi kuphela kutsi yakho. Imiphumela yalomsebenti itobekwa kunqondvo mshina lohlala ukhiyiwe, phindze uvulwe nge sikhiya setinombolo. Kanjalo kulamafayela lapho itobe ibutsiswe khona lemiphumela kutonesikhiya. Letikhiya tokhe titokwatiwa bagcwaningi kuphela.

Ungenela lolucwaningo ngekutsandza nangekukhetsa kwakho, awufaphocelelwa. Ungaphuma futsi nobe kunini. Nawuphuma, budlelwane bakho nesibhedlela angeke benteleke phansi noma bonakale.

Nawunemibuto mayelana nanobe yini ngalolucwaningo, tsintsa bagcwaningi kuletinombo ngetulu. Nobe utsintse lababukele kuvikeleka kwebantfu nemvelo enyuvesi enombolweni letsi +27 31 373 2375. Kantsi futsi na Prof S Moyo somaqhuzu kutelucwaningo neskusita labaquba tifundvo tabo ungamtsints ku +27 31 373 2577 noma ku; moyos@dut.ac.za

Appendix 13: IMVUMO YELUCWANINGO

IMVUMO YELUCWANINGO

Lokum'cokwa ngalolucwaningo ngisavuma:

- Ngiyacinisekisa kutsi ngatisiwe ngalolucwaningo lolwentiwa ngu babe Talent Colani Dlamini**.** Ngicaciselekile kutsi lutohasiphi, yini imbuyiselo yami kulo, nalokwekutsi lolucwaningo lubukiwe futsi lwabajabulisa labakuke kuphepha nekuvikeleka kwelutfu. Tinombolo talolucwaningo letikhomba kutsi lungaqhubeka lwentiwe: MH/599C/ IRB 0009688/NHRRB012/16 (from Swaziland Scientific and Ethics Committee) and IREC 111/17 (from Durban University of Technology ethic committee)**.**
- Ngiyifundzile, ngachazeleka futsi nagyicodza imininigwane yalocucwaningo
- Ngatisiwe kutsi inininigwane yami letosebentiswa kulolucwaningo itobayifihlo, angeke yatiwe noma inikekwe nobengubani.
- Ngekubuka nekucondza sidzingo salolucwaningo, ngiyavuma kutsi imiphumela nalenye imininigwane letobutfwa kulolucwaningo igcinwe kubongcondvo mshina yalabacwaningi.
- Ngaphandle kwekusatjiswa, kubandlululwa nobe kwecwaywa, ngingaphuma nobe ngiyekele kubayincenye yalolucwaningo
- Litfuba lekubuta ngilitfolile, netimphendvulo tangijabulisa. Nitikhandza ngisesigabeni sekutsingingaba yincenye yalolucwaningo.
- Ngiyacondza kutsi imiphumela lebalulekile lengase itfolakale kulolucwaningo ngitoyitfola.

…… ……. ……. ……

Libito lwaloyicenye yelucwaningo Lusuku sikhatsi Sitfupha

Mine **Babe Talent Colani Dlamini** ngiyacinisekisa kutsi ngim'chazele lona loyicenye yalolucwaningo ngalolucwanigo, nekutsi lutohamba njani nebungoti lobuyamelane nelucwaningo.

Appendix 14: Swaziland TB Referral Laboratory Accreditation certificate

SADCAS

SOUTHERN AFRICAN DEVELOPMENT COMMUNITY ACCREDITATION SERVICE

CERTIFICATE OF ACCREDITATION

NATIONAL TUBERCULOSIS REFERENCE LABORATORY

Company Registration No. 5/1969

Facility Accreditation Number: MED 036

is a SADCAS accredited Medical Laboratory
provided that all SADCAS conditions are complied with

This certificate is valid as per the scope stated in the accompanying schedule of accreditation, Annexure "A", bearing the above accreditation number for

MEDICAL TESTING LABORATORY
MYCOBACTERIOLOGY

The facility is accredited in accordance with the recognized International Standard

ISO 15189:2012

The accreditation demonstrates technical competency for a defined scope and the operation of a laboratory quality management system

DCAS is a subsidiarity organization of SADC. A memorandum of understanding between SADC and SADCAS serves as the basis for the recognition of SADCAS by SADC Member States as a multi-economy accreditation body

[redacted]

Mrs Maureen P Mutasa
SADCAS Chief Executive Officer

Effective Date (Issue No: 1): 20 August 2019
Certificate Expires: 19 August 2024

Appendix 15: GeneXpert MTB/RIF assay

Materials:

1. Surfanios disinfectant/3.5% or 5% Sodium hypochlorite for disinfection
2. Tap Water
3. 70% Ethanol
4. GeneXpert Kit including: Xpert cartridges, sterile disposable transfer pipettes, Sample Reagent (SR) buffer (Pro-Gen Diagnostics)
5. Sharps container
6. Biohazard medical waste bin
7. Screw-capped sputum containers
8. The GeneXpert Dx System includes GeneXpert instrument, computer and barcode scanner, UPS, printer (Pro-Gen Diagnostics
9. Stop watch/timer

Procedure:

Collection, Labeling and Registration specimen

1. Properly identify the patient with at least the Patient's name, Patient number, date of birth, and Doctor's name.
2. Wash hands with soap and water.
3. Explain the specimen collection procedure to the patient
4. Provide patient with the screw-capped container
5. Instruct patient to cough deeply and spit up sputum (phlegm) into the screw-capped sputum container.
6. Instruct patient to seal the specimen container tightly.
7. Ensure the sputum in the container is sent to the laboratory.
8. Label each specimen immediately with identifying information – patient identification number, date and time of collection.
9. Fill out lab request/report form for each specimen with date, time and initials of collector.
10. Check that lab serial number on the specimen container matches with that on the lab request form.

NOTE: This labeling and matching is the primary responsibility of the collector and must be done carefully and precisely.

11. Dispose of gloves and wash hands after each specimen collection/handling.
12. Log the sputum specimen in your laboratory log book/computer system

Sample preparation

NOTE: If there is a backlog of specimens, the unprocessed sputum specimen can be stored at 4°C for 4-10days

1. Check the sample integrity.
    a. If food particles are present – Proceed but be careful not to add food particles to the Xpert cartridge!
2. Visually inspect and estimate the volume of sputum in the collection container.
    a. Specimen needs to be more than 0.5ml
    b. If less than 0.5ml, request a second specimen
    c. If more than 4mls, split the specimen
3. Open the lid of the collection container and pour Sample Reagent (SR) buffer (supplied in kit) to the sputum specimen in a 2:1 (SR buffer: specimen) volume ratio.
4. Close the lid of the container tightly and swirl vigorously for 15 seconds.
5. Allow the mixed specimen to stand for 15 minutes.
6. Shake the container once during the 15 minute incubation i.e after 10 minutes.

NOTE: This mixture can be kept for up to 8 hours at 2-8ºC, in case repeat testing is required.

7. If any specimen is spilt at any stage, see appropriate SOP

REMEMBER!

Integrity of specimens – storage	
Unprocessed sputum specimen	@ 4°C for 10 days
	@ room temperature for ≤3 days
After addition of SR buffer to specimen	@ 2-8°C for up to 8 hours

Loading the specimen into the Xpert cartridge

1. After 15 minutes, remove an MTB/RIF® Xpert cartridge from its wrapper, **taking care NOT TO TOUCH the back of the cartridge**.

NOTE: If the sample is still viscous, shake the sample again and leave it for another 5 min until it is properly liquefied. Once the foil wrapper is removed, the specimen must be added to the cartridge within 30 minutes.

2. Label the side of the Xpert cartridge with the sample ID. Do not write or place sticker over cartridge barcode.
3. Open the lid of the cartridge.
4. Open the lid of the collection container containing the mixed sputum specimen.
5. Open a sealed sterile pipette (supplied in kit) without touching the tip. Use the pipette to aspirate >2ml of the specimen (just above 2ml mark on pipette) and slowly dispense it into the open port of the Xpert MTB/RIF cartridge.
    a. **Do not add less than 2ml** of mixed specimen to the cartridge.

NOTE: the test must be started within 30minutes of adding the specimen to the cartridge.

6. Close the cartridge lid firmly.
7. Dispose of the specimen collection container, pipette and leftover SR buffer into a suitable medical waste bin.
8. Take the Xpert cartridge to the bench with the GeneXpert instrument.

Cartridge Integrity	
After opening the foil of the cartridge	*You must add the specimen to the cartridge within 30 minutes*
After addition of the specimen to the cartridge	*You must load the cartridge into the Xpert instrument within 30 minutes*

Running the assay on the GeneXpert instrument

1. Switch on the GeneXpert at the back of the instrument.
2. Switch on the computer.
3. When prompted, log onto the computer with the ‘cphd’ password.
4. Double click ‘GeneXpert’ icon on desktop.
5. Log onto the GeneXpert Dx software using your username and password.
6. When asked whether or not you would like to ‘Perform Database Management Tasks’, such as backing up the database, checking the database integrity etc, choose ‘Yes’ or

'No'. If you are archiving weekly, database management tasks do not need to be performed.

6a) If you selected 'Yes', see SECTION G under MAINTENANCE – Database Maintenance

6b) If you selected 'No' (recommended), you will be prompted again when you close the GeneXpert Software

7. Click on 'Create test' on the GeneXpert system toolbar.
8. Enter the 'Sample ID Barcode' dialog box appears. Type in the sample ID barcode. Once the barcode is entered, select 'OK'
9. The 'Scan barcode' dialog box appears. Scan in the barcode of the MTB/RIF cartridge by placing the X of the barcode scanner in line with the barcode on the cartridge and hold until it beeps. Otherwise select 'Manual Entry' and type in the Cartridge barcode. Once it has been correctly entered, select 'OK'.
10. The software automatically fills in the Reagent lot ID, Cartridge SN, and expiration date, as well as Select Assay, Assay version number, Test Type and Sample type.
11. Should you wish change the sample type, fill in the desired information in the 'Other sample type' box. This is not a compulsory field.
12. Should you wish to add any additional notes about the test or specimen enter these in the Notes box. This is not a compulsory field.
13. Click on 'Start Test'
14. In the dialog box that appears type in your password again and press 'ENTER'.
15. A green light will start flashing above the empty module. Open the instrument module door with the **blinking light** and load the cartridge.
16. Firmly close the module door till you hear a click.
17. After a few seconds, the green light will stop blinking indicating that the test has started.
18. After completion of the run, the green light will switch off and the module lid will open automatically ejecting the cartridge.
19. Remove the cartridge and dispose of it a suitable biohazard medical waste bin.

Interpretation of results

1. After completion of the run, click on the 'View Results' icon on the system toolbar.
2. Click on 'View Test' at bottom of the result screen toolbar.

3. Select the patient test by clicking on the patient ID field. This will highlight the test.
4. Click ‘OK’ and the result screen will be displayed as one of the following:
    - ✓ MTB DETECTED – positive (with/out RIF resistance)
    - ✓ MTB NOT DETECTED - negative
    - ✓ INVALID – repeat test
    - ✓ ERROR – repeat test
    - ✓ NO RESULT – repeat test

Appendix 16: BACTEC MGIT ™ 960 culture

Reagents/Materials Required:

1. BSC, class II
2. Refrigerated centrifuge with safety shield, a minimum RCF of 3000*g*
3. Centrifuge tubes with screw-caps, resistant to RCF of >3000*g* RCF
4. Rack for tubes
5. Pasteur pipettes for 1.0 ml (with graduations)
6. Pipetting aids
7. Separate waste containers, autoclavable, for pipettes and disposals
8. Vortex mixer
9. Timer
10. Incubator
11. Mycoprep solution kit
12. Phosphate buffer, 0.067 mol/ litre, pH 6.8

Procedure:

Specimen processing

1. Clean the biological safety cabinet with disinfectant
2. Take out specimens from the refrigerator to reach room temperature
3. Set up all materials to be used in the biological safety cabinet
4. Break mycoprep glass ampule and mix contents gently
5. Add an equal volume of the NALC–NaOH solution to the quantity of the specimen and tighten the screw-cap
6. Vortex lightly for about 20 seconds. Invert the tube so the whole tube is exposed to the NaOH-NALC solution
7. Wait 20 minutes after adding the NaOH-NALC solution. Tubes can be put on a shaker to shake lightly during the whole time
8. Make sure the specimen is completely liquefied. If still mucoid, add 0.3g of NALC directly to the specimen tube and mix well

9. Add phosphate buffer (Ph 6.8) up to the 50ml mark
10. Invert several times to mix
11. Centrifuge the tubes at a speed of 3000g for 20 minutes at 4°C
12. After centrifugation carefully decant the supernatant into a suitable waste bucket inside the biosafety cabinet
13. Add 3ml of sterile phosphate buffer (ph 6.8) and resuspend the sediment with help of a vortex mixer
14. Leave tubes undisturbed for at least 15 minutes

Reconstituting MGIT PANTA

1. Bring the reagents to room temperature.
2. Reconstitute MGIT PANTA with 15ml MGIT growth supplement
3. Mix well until completely dissolved
4. Leave to stand for 5 minutes

Inoculation of MGIT medium

1. Label MGIT tubes with unique laboratory number and avoid covering the MGIT barcode
2. Unscrew the cap and aseptically add 800ul of MGIT growth supplement/PANTA to each MGIT tube
3. Using a separate sterile pipette tip, add 500ul of concentrated specimen to the appropriately labeled MGIT tube. Use separate pipette tip for each specimen
4. Immediately recap the tube tightly and mix by inverting the tube 2-3 times
5. Wipe the tubes and caps with damp tide wipe containing 5% sodium hypochlorite

Loading new tubes into the BACTEC™ MGIT™ 960 MYCOBACTERIA CULTURE SYSTEM (BECTON DICKINSON, USA) instrument

1. Tubes are loaded into the instrument for up to 42 days
2. Refer to the BACTEC BACTEC™ MGIT™ 960 MYCOBACTERIA CULTURE SYSTEM (BECTON DICKINSON, USA) System User's Manual Section 4.5 for detailed procedure

Appendix 17: MPT64 assay

Materials

1. Sterile filtered tips
2. A suitable pipette
3. MPT64 kit

Test Procedure:

1. Remove the test device from foil pouch and place on a flat and dry surface
2. Add 100µl of vortexed liquid culture to the sample well
3. Interpret the test after 15 minutes.

Results Interpretation:

- A color band will appear at left section of the results window to show that the test is working properly. This band is the control band.
- The right section of the results window indicates the test results. If another color band appears at the right section of the window, this band is the test band.
- **Negative Result**: the presence of only control band within the result window. Negative result is indicative of non-tuberculosis mycobacteria (NTM).
- **Positive Result**: the presence of two color bands within the result window. A positive test is suggestive for *M. tuberculosis* complex bacteria.
- **Invalid result**: No band is visible, or test band is visible in the absence of control band.

Appendix 18: BACTEC MGIT ™ 960 for Drug Susceptibility Testing

Reagents/Materials Required:

1. BACTEC BACTEC™ MGIT™ 960 MYCOBACTERIA CULTURE SYSTEM (BECTON DICKINSON, USA) Kit
2. Sterile distilled water
3. Sterile saline
4. Tube racks
5. Pipettes
6. Pipette tips
7. 15ml falcon tubes, sterile

Procedure:

Reconstitution of lyophilized drugs

1. Reconstitute each critical concentration drug vial with 4 ml of sterile distilled/deionized water.
2 Mix thoroughly and make sure the drug is completely dissolved

Inoculum from the MGIT tube:

Create a worksheet for DST

It is important that the growth is within the following recommended timeframe.

1. The day a MGIT tube is positive by the instrument is considered **Day 0.**
2. The tube should be kept incubated for at least one more day (**Day 1**) before being used for the susceptibility testing (may be incubated in a separate incubator at 37ºC + 1ºC).
3. A positive tube may be used for drug susceptibility testing up to and including the fifth day (**Day 5**) after it becomes instrument positive. A tube that has been positive for more than 5 days should be subcultured in a fresh MGIT tube supplemented with BACTEC™ MGIT™ 960 MYCOBACTERIA CULTURE SYSTEM (BECTON DICKINSON, USA) Growth Supplement and should be tested in a BACTEC™ MGIT™ 960 MYCOBACTERIA CULTURE SYSTEM (BECTON DICKINSON, USA) instrument

until it is positive. Use this tube from one to five days of instrument positivity as described above.

4. If growth in a tube is on **Day 1** or **Day 2**, mix well (vortex) to break up clumps. Leave the tube undisturbed for about 5-10 minutes to let big clumps settle on the bottom. Use the supernatant undiluted for inoculation of the drug set.
5. If growth is on **Day 3, 4, or 5**, mix well to break up the clumps. Let the large clumps settle for 5-10 minutes and then dilute 1.0 ml of the positive broth with 4.0 ml of sterile saline. This will be a 1:5 dilution. Use this well mixed diluted culture for inoculation.

Inoculation and incubation

1. Label 5 MGIT tubes for each test culture. Label one for GC (growth control, without drug), one for STR, one for INH, one for RIF, and one for EMB.
2. Aseptically add 0.8 ml of BACTEC 960 SIRE Supplement to each of the MGIT tubes. Use only MGIT SIRE Supplement and not MGIT Growth Supplement.
3. Aseptically add 0.1 ml (100 ul) or properly reconstituted STR drug in the STR labeled tube. Similarly, add other drugs in the other labeled tubes. It is important to add the correct amount of drug to each tube. If possible, use a well calibrated micropipette for each addition. Use a separate pipette or micropipette tip for each drug. Do not add any drug to the GC tube.
4. Aseptically add 0.5 ml of the well-mixed culture suspension (inoculum) into each of the drug containing tubes using a pipette. Do not add to the control.
5. For the control, first dilute the test culture suspension 1:100 by adding 0.1 ml of the test culture suspension to 10.0 ml of sterile saline. Mix well by inverting the tube 5-6 times. Use this diluted suspension to add 0.5 ml into the growth control tube.
6. Tighten the caps and mix the inoculated broth well by gently inverting the tube several times.
7. Place labeled tubes in the correct sequence in the set carrier (GC, STR, INH, RIF, EMB).
8. Enter the susceptibility set carrier into the BACTEC BACTEC™ MGIT™ 960 MYCOBACTERIA CULTURE SYSTEM (BECTON DICKINSON, USA) instrument using the susceptibility test set entry feature. (Refer to the BACTEC BACTEC™

MGIT™ 960 MYCOBACTERIA CULTURE SYSTEM (BECTON DICKINSON, USA) User's Manual, AST Instructions.) Ensure that the order of the tubes in the AST Set Carrier conforms to Set Carrier definitions. For example, GC, STR, INH, RIF, EMB for the SIRE standard testing.

Results Interpretation:

The instrument monitors the entered susceptibility test set. Once the test is complete (within 4 to 14 days), the instrument will indicate that the results are ready.

1. Scan the susceptibility Set Carrier and print the report.
2. The instrument printout indicates susceptibility results for each drug. Results are qualitative: Susceptible (**S**), Resistant (**R**) or indeterminate (**X**).

The instrument interprets results at the time when the growth unit (GU) in growth control reaches 400 (within 4-13 days). At this point, the GU values of the drug vial are evaluated.

- **S** = Susceptible – the GU of the drug tube is less than 100.
- **R** = Resistant – the GU of the drug tube is 100 or more.

X = Error – indeterminate results when certain conditions occur which may affect the test, such as GU of the control reaches >400 in less than 4 days. In such situations, the test should be repeated with pure, actively growing culture that is confirmed to be *M. tuberculosis* complex. Certain drug resistant strains grow very slowly in the medium and the results may not be achieved within 13 days with the standard inoculum. In such a case, the inoculum should be increased by decreasing the dilution of the culture suspension in order to get reportable results.

Appendix 19: DNA extraction

Materials

1. 70% alcohol disinfectant
2. 1% Sodium Hypochlorite disinfectant
3. Centrifuge
4. Conical 1.5ml PCR tubes
5. Ultrasonic bath

Test Procedure

1. Thoroughly clean all surfaces of hood as well as external surfaces of all equipment in hood with 1% Sodium Hypochlorite followed by 70% alcohol.
2. Spray gloves with 70% alcohol before work.
3. Pipette 1 ml of liquid culture directly to conical vial.
4. Centrifuge 15 min at 10000 x g.
5. Discard supernatant and suspend pellet in 100ul H_2O.
6. Incubate 20 min at 95°C in the hot plate.
7. Incubate 15 min in ultrasonic bath.
8. Centrifuge 5 min at maximum speed.
9. Take 80µl DNA supernatant to new 1.5 ml conical tube.
10. Add 5µl of DNA supernatant to corresponding PCR tubes.

Appendix 20: Spoligotyping

The whole DR region will be amplified with two primers, each of which is 18 nucleotides long, which annealed to the DR. Table 1 shows sequences of the primers used used in PCR. The DRa will be biotinylated at the 5' end.

Preparing Buffers

1. **2X SSPE**

   $C_1V_1 = C_2V_2$

   $20xV_1 = 2x\ 1000$

   V1 = 2000/20

   20x SSPE = 100ml

   De-mineralized water = 900 ml

2. **2X SSPE/0.1% SDS**

   $C_1V_1 = C_2V_2$

   20x SSPE = 100ml

   10% SDS = 10ml

   De-mineralized water = 890 ml

3. **2X SSPE/0.5% SDS**

   $C_1V_1 = C_2V_2$

   20x SSPE = 100ml

   10% SDS = 50ml

   De-mineralized water = 850 ml

4. **STRtavidin-peroxidase**

   2Xsspe/0.5%SDS = 10ML

   Streptavidin-peroxidase = 7.5µl

5. **ECL detection liquid**

   Solution 1 = 10ml

Solution 2 = 10ml

Table 9: Primers and their sequences to be used for PCR

Primers	Sequence	Size	T_A °C	Reference
DRa (biotinylated)	'5-CCAAGAGGGGACGGAAAC-3'	36bp	55	Spoligotyping User's Manual
DRb	'5-GGTTTTG GGTCTGACGAC-3'	36bp	55	Spoligotyping User's Manual

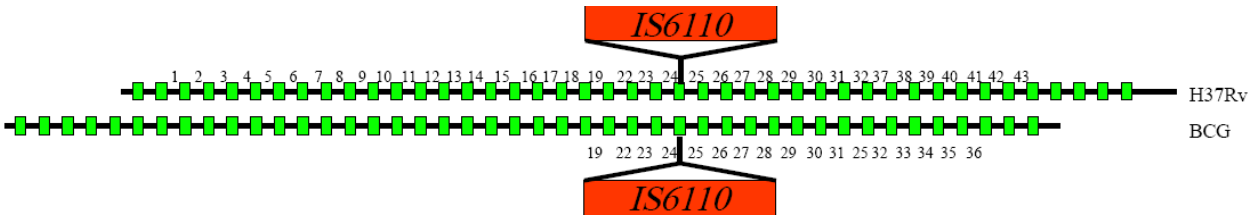


Figure 25: Structure of the DR locus in the genome of M. tuberculosis H37Rv and M. bovis BCG P3. The green rectangles depict the 36 bp Direct Repeat (DR) (Kremer *et al.* 2012).

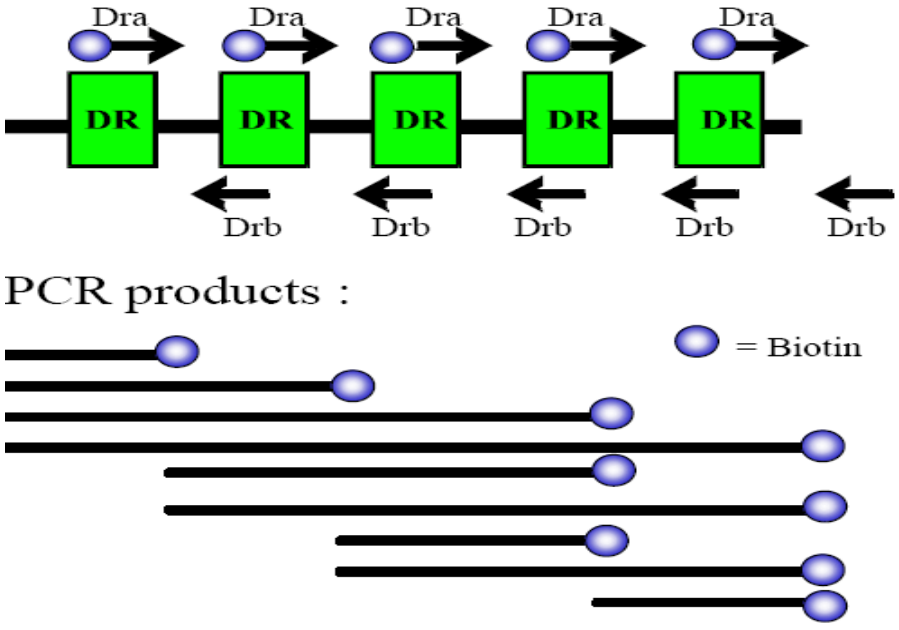


Figure 26: Principle of the in vitro amplification of DNA within the DR region of M. tuberculosis complex bacteria (Kremer *et al.* 2012).

The reaction mixture was prepared as shown in table 2 and the thermo-cycler was programmed as shown in table 3.

Table 10: Reaction mixture for PCR

Reagent	**Volume (µl) 1x Reaction**	**Volume (µl) 46x Reaction**
Primer Dra	4 µL	184
Primer DRb	4 µL	184
Taq PCR MasterMix	25 µL Taq polymerase	1150
dNTP-mixture	4µL	
MQ water	13 x µL	598
Positive control (cDNA of *M. tuberculosis* strain H37Rv and		
Positive control: *M. bovis* (BCG P3)		
Negative Control		
Template (DNA)	4 µL	
Total	50µL	

Table 11: Thermo-cycle program for PCR

	Temperature °C	**Time**	**Cycles**
Initial Denaturation	94	3 min	1
Denaturation	94	1 min	30
Primer annealing	55	1 min	30
Primer extension	72	30s	30
Final extension	72	7 min	1
Hold	4	Over night	

Hybridization with PCR Product and Detection

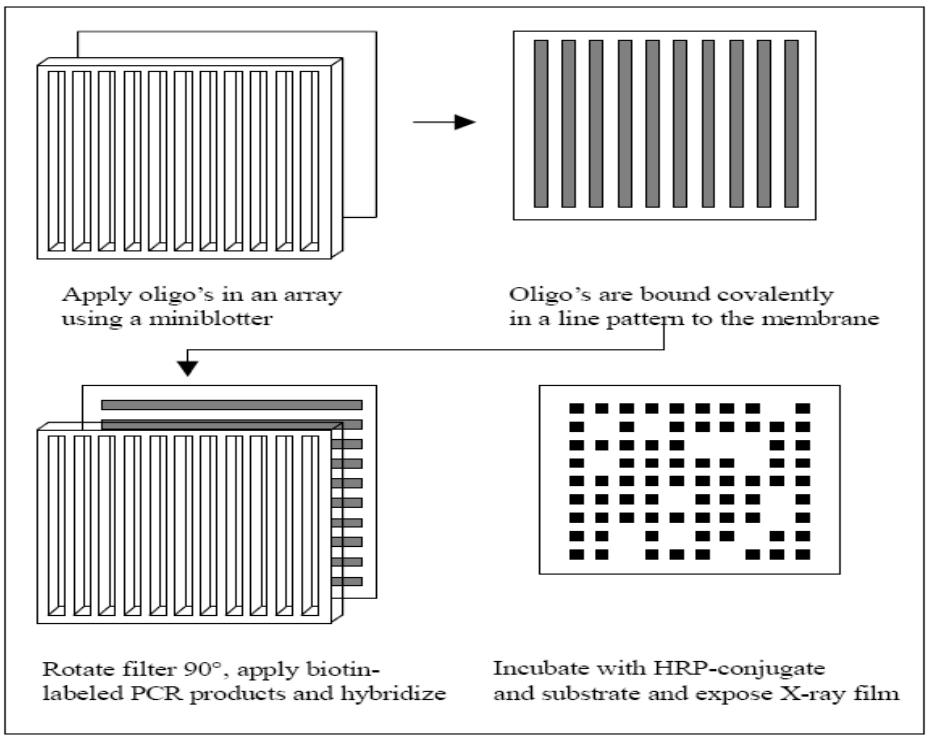


Figure 27: Overview of the spoligotyping method (Kremer *et al.* 2012)

Procedure:

1) All buffers should be pre-warmed before use. Prepare the following buffers from concentrated stocks, using de-mineralized water for dilution (quantities for one membrane):

- 250 mL 2xSSPE/0.1% SDS, 60ºC
- 250 mL 2xSSPE/0.5% SDS, 60ºC x 2
- 250 mL 2xSSPE/0.5% SDS, 42ºC x 2
- 250 mL 2xSSPE, room temperature
- EDTA Buffer

2) Add 20 µL of the PCR products to 150 µL2xSSPE/0.1% SDS.

3) Heat-denature the diluted PCR product for 10 min at 99°C and cool on ice immediately.

4) Wash the membrane for 5 min at 60°C in 250 mL2xSSPE/0.1% SDS.

5) Place the membrane and a support cushion into the miniblotter in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides. a)

6) Remove residual fluid from the slots of the miniblotter by aspiration.

7) Fill the slots with the diluted PCR product (avoid air bubbles!) and hybridize for 60 min at 60ºC on a horizontal surface (no shaking!). Avoid contamination of neighboring slots. b)

8) Remove the samples from the miniblotter by aspiration and take the membrane from the miniblotter using forceps.

9) Wash the membrane twice in 250 mL2xSSPE/0.5% SDS for 10 min at 60°C

10) Place the membrane in a rolling bottle and allow it to cool down to prevent inactivation of the peroxidase in the next step.

11) Add 2.5 µL STRtavidin-peroxidase conjugate (500U/ml) to 10 mL of 2xSSPE/0.5% SDS, and incubate the membrane in this solution for 45 to 60 min at 42°C in the rolling bottle.

12) Wash the membrane twice in 250 mL of 2xSSPE/0.5% SDS for 10 min at 42°C.

13) Rinse the membrane twice with 250 mL of 2xSSPE for 5 min at room temperature.

14) For chemiluminiscent detection of hybridizing DNA, incubate the membrane for 1 min in 20 mL ECL detection liquid. c)

15) Cover the membrane with a transparent plastic sheet or Saran-wrap and expose a light sensitive film to the membrane for 20 min. d)

16) If the signal is too weak or too strong, the membrane can be used again directly to expose another film for a shorter or longer period.

17) Put the hyperfilm in the developer reagent for 15-20 seconds

18) Rinse the hyperfilm in water

19) Put the hyperfilm in a fixer reagent for 30 seconds

Remarks

a) Do not reuse the support cushions. You can order more support cushions from us.

b) If less than 45 samples are applied to the miniblotter, fill one neighboring slot with 2X SSPE/0.1%SDS to prevent cross-flow.

c) Use a dedicated plastic container. Do not use this container for other purposes, since some reagents decrease the intensity of the Spoligo patterns.

d) If the result is unsatisfactory, you can try to improve this.

Black spots (background) possibly occur due to contamination during filter handling (e. g. touched with fingers). Start again from step 8.

Blank areas in the spoligo patterns possibly indicate that the membrane was not completely soaked with ECL detection liquid. Start again from step 13.

Regeneration of the Membrane

Purpose The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 5 times.

Procedure

1) Wash the membrane twice by incubation in 1% SDS at 80°C for 30 min.

2) Wash the membrane in 20 mM EDTA pH 8, for 15 min at room temperature.

3) Store the membrane at 4°C until use (sealed in plastic or wrapped in Saran-wrap, to avoid dehydration of the membrane).

Appendix 21: Permit to import *M. tuberculosis* DNA samples

health
Department:
Health
REPUBLIC OF SOUTH AFRICA

2030 NDP

Private Bag X828, PRETORIA, 0001 Civitas Building, c/o Struben and Thabo Sehume Streets
Tel (012) 395 8000, Fax (012) 395 8918
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E-Mail:importexportpermit@health.gov.za J1/2/4/14 No 01/18

IMPORT PERMIT

In terms of Section 68 of the National Health Act 2003 (Act No. 61 of 2003) –

Jeannatte Brand
Chief Research Technologist
Medical Research Council
No 1 Soutpansberg Rd
Arcadia
PRETORIA
0001
Tel: +27 12 339 8565 Email: Jeannette.brand@mrc.ac.za

is hereby authorised to import into the Republic of South Africa –

2 ml x 160 vials — DNA samples derived from TB cultures (Non-Infectious)

from –

Mr Dlamini Talent C.
Junior Lecturer
Southern Africa Nazarene University (SANU)
Faculty of Health Science
Medical Laboratory Science Department
PO Box 6800
Manzini
SWAZILAND
M200
Tel: (+268)76154174/ (+268)25055749 Email: talent@sanu.ac.sz

for – Research

This import permit is subject to the following conditions:

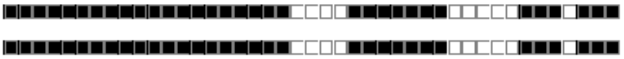
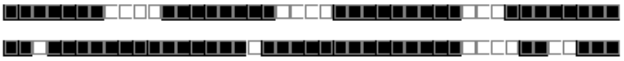
1. The material shall be exported from the country specified above, within the legal requirements of that country.
2. The material shall be imported into South Africa and handled in accordance with the provisions of the National Health Act 2003 and regulation made in terms of the Act.
3. The import permit shall not be used for any trade or advertising purposes.
4. **This import permit shall expire on 28 February 2019.**

DIRECTOR-GENERAL: HEALTH
Date: 22/02/2019

Ms P Netshidzivhani

Appendix 22: Phylogenetic Tree

102	tuberculosis	?-?	73	T2-T3	
27	tuberculosis	?-?			
3.1	tuberculosis	?-?	53	T1	
20.1	tuberculosis	?-?	53	T1	
29	tuberculosis	?-?	53	T1	
31	tuberculosis	?-?	53	T1	
33.1	tuberculosis	?-?	53	T1	
38	tuberculosis	?-?	53	T1	
40.1	tuberculosis	?-?	53	T1	
41.1	tuberculosis	?-?	53	T1	
43.1	tuberculosis	?-?	53	T1	
46.1	tuberculosis	?-?	53	T1	
87	tuberculosis	?-?	53	T1	
87.1	tuberculosis	?-?	53	T1	
58.1	tuberculosis	?-?	53	T1	
93.1	tuberculosis	?-?	53	T1	
54.1	tuberculosis	?-?	926	T1	
58	tuberculosis	?-?	926	T1	
22.1	tuberculosis	?-?	451	H37Rv	
11	tuberculosis	?-?	71	S	
12	tuberculosis	?-?	71	S	
12.1	tuberculosis	?-?	71	S	
17	tuberculosis	?-?	71	S	
18.1	tuberculosis	?-?	71	S	
21	tuberculosis	?-?	71	S	
4.1	tuberculosis	?-?	34	S	
18	tuberculosis	?-?	34	S	
19.1	tuberculosis	?-?	34	S	
24	tuberculosis	?-?	34	S	
29.1	tuberculosis	?-?	34	S	
32.1	tuberculosis	?-?	34	S	
33	tuberculosis	?-?	34	S	
39.1	tuberculosis	?-?	34	S	
40	tuberculosis	?-?	34	S	
42.1	tuberculosis	?-?	34	S	
43	tuberculosis	?-?	34	S	
48.1	tuberculosis	?-?	34	S	
49.1	tuberculosis	?-?	34	S	
51	tuberculosis	?-?	34	S	
55	tuberculosis	?-?	34	S	
55.1	tuberculosis	?-?	34	S	
60.1	tuberculosis	?-?	34	S	

61.1	tuberculosis	?-?	34	S	
62.1	tuberculosis	?-?	34	S	
66	tuberculosis	?-?	34	S	
70	tuberculosis	?-?	34	S	
76.1	tuberculosis	?-?	34	S	
84	tuberculosis	?-?	34	S	
89	tuberculosis	?-?	34	S	
90.1	tuberculosis	?-?	34	S	
96.1	tuberculosis	?-?	34	S	
98	tuberculosis	?-?	34	S	
100	tuberculosis	?-?	34	S	
23.1	tuberculosis	?-?			
17.1	tuberculosis	?-?	100	MANU1	
23	tuberculosis	?-?			
86.1	tuberculosis	?-?			
37	tuberculosis	?-?	60	LAM4	
47.1	tuberculosis	?-?	60	LAM4	
48	tuberculosis	?-?	60	LAM4	
57	tuberculosis	?-?	60	LAM4	
59.1	tuberculosis	?-?	60	LAM4	
63.1	tuberculosis	?-?	60	LAM4	
86	tuberculosis	?-?	60	LAM4	
99	tuberculosis	?-?	60	LAM4	
99.1	tuberculosis	?-?	60	LAM4	
100.1	tuberculosis	?-?	60	LAM4	
8	tuberculosis	?-?	42	LAM9	
25.1	tuberculosis	?-?	42	LAM9	
97	tuberculosis	?-?	42	LAM9	
70.1	tuberculosis	?-?			
91.1	tuberculosis	?-?			
88.5	tuberculosis	?-?	20	LAM1	
20	tuberculosis	?-?	33	LAM3	
25	tuberculosis	?-?	33	LAM3	
74	tuberculosis	?-?	33	LAM3	
92	tuberculosis	?-?	33	LAM3	
94	tuberculosis	?-?	33	LAM3	
101.1	tuberculosis	?-?	33	LAM3	
56	tuberculosis	?-?	719	T1	
81.1	tuberculosis	?-?			
85.1	tuberculosis	?-?			
14.1	tuberculosis	?-?			
30.1	tuberculosis	?-?			
4	tuberculosis	?-?	137	X2	
78.1	tuberculosis	?-?			
81	tuberculosis	?-?			

71	tuberculosis	?-?			
77.1	tuberculosis	?-?			
75.1	tuberculosis	?-?			
47	tuberculosis	?-?			
63	tuberculosis	?-?			
7	tuberculosis	?-?	92	X3	
28.1	tuberculosis	?-?	92	X3	
30	tuberculosis	?-?	92	X3	
34	tuberculosis	?-?	92	X3	
36.1	tuberculosis	?-?	92	X3	
37.1	tuberculosis	?-?	92	X3	
38.1	tuberculosis	?-?	92	X3	
44	tuberculosis	?-?	92	X3	
44.1	tuberculosis	?-?	92	X3	
46	tuberculosis	?-?	92	X3	
57.1	tuberculosis	?-?	92	X3	
59	tuberculosis	?-?	92	X3	
95.1	tuberculosis	?-?	549	X3	
102.1	tuberculosis	?-?	549	X3	
72.1	tuberculosis	?-?			
73	tuberculosis	?-?			
101	tuberculosis	?-?			
16	tuberculosis	?-?	354	U	
27.1	tuberculosis	?-?	48	EAI1_SOM	
39	tuberculosis	?-?	48	EAI1_SOM	
42	tuberculosis	?-?	48	EAI1_SOM	
3	tuberculosis	?-?	48	EAI1_SOM	
6	tuberculosis	?-?	48	EAI1_SOM	
9	tuberculosis	?-?	48	EAI1_SOM	
13	tuberculosis	?-?	48	EAI1_SOM	
15	tuberculosis	?-?	48	EAI1_SOM	
15.1	tuberculosis	?-?	48	EAI1_SOM	
69	tuberculosis	?-?	48	EAI1_SOM	
83.1	tuberculosis	?-?	48	EAI1_SOM	
88	tuberculosis	?-?	48	EAI1_SOM	
94.1	tuberculosis	?-?	48	EAI1_SOM	
96	tuberculosis	?-?	48	EAI1_SOM	
103.1	tuberculosis	?-?	48	EAI1_SOM	
60	tuberculosis	?-?	129	EAI5	
93	tuberculosis	?-?	702	EAI5	
26.1	tuberculosis	?-?			
6.1	tuberculosis	?-?	254	T5_RUS1	
10	tuberculosis	?-?	254	T5_RUS1	
5.1	tuberculosis	?-?			
5	tuberculosis	?-?	136	T1	
10.1	tuberculosis	?-?	136	T1	
19	tuberculosis	?-?	811	LAM4	

90	tuberculosis	?-?			
76	tuberculosis	?-?			
67	tuberculosis	?-?			
67.1	tuberculosis	?-?			
64.1	tuberculosis	?-?			
84.1	tuberculosis	?-?			
34.1	tuberculosis	?-?	26	CAS1_DELHI	
61	tuberculosis	?-?	21	CAS1_KILI	
66.1	tuberculosis	?-?	21	CAS1_KILI	
35	tuberculosis	?-?	1675	CAS1_KILI	
89.1	tuberculosis	?-?			
98.1	tuberculosis	?-?			
21.1	tuberculosis	?-?	4	LAM3 and S /convergent	
2	tuberculosis	?-?	1	BEIJING	
2.1	tuberculosis	?-?	1	BEIJING	
7.1	tuberculosis	?-?	1	BEIJING	
8.1	tuberculosis	?-?	1	BEIJING	
9.1	tuberculosis	?-?	1	BEIJING	
11.1	tuberculosis	?-?	1	BEIJING	
13.1	tuberculosis	?-?	1	BEIJING	
14	tuberculosis	?-?	1	BEIJING	
16.1	tuberculosis	?-?	1	BEIJING	
22	tuberculosis	?-?	1	BEIJING	
24.1	tuberculosis	?-?	1	BEIJING	
26	tuberculosis	?-?	1	BEIJING	
28	tuberculosis	?-?	1	BEIJING	
31.1	tuberculosis	?-?	1	BEIJING	
32	tuberculosis	?-?	1	BEIJING	
35.1	tuberculosis	?-?	1	BEIJING	
41	tuberculosis	?-?	1	BEIJING	
45	tuberculosis	?-?	1	BEIJING	
49	tuberculosis	?-?	1	BEIJING	
50	tuberculosis	?-?	1	BEIJING	
50.1	tuberculosis	?-?	1	BEIJING	
51.1	tuberculosis	?-?	1	BEIJING	
52	tuberculosis	?-?	1	BEIJING	
53	tuberculosis	?-?	1	BEIJING	
52.1	tuberculosis	?-?	1	BEIJING	
53.1	tuberculosis	?-?	1	BEIJING	
54	tuberculosis	?-?	1	BEIJING	
56.1	tuberculosis	?-?	1	BEIJING	
62	tuberculosis	?-?	1	BEIJING	
64	tuberculosis	?-?	1	BEIJING	
65	tuberculosis	?-?	1	BEIJING	
68.1	tuberculosis	?-?	1	BEIJING	
69.1	tuberculosis	?-?	1	BEIJING	
74.1	tuberculosis	?-?	1	BEIJING	

75	tuberculosis	?-?	1	BEIJING	
77	tuberculosis	?-?	1	BEIJING	
80	tuberculosis	?-?	1	BEIJING	
36	tuberculosis	?-?	1	BEIJING	
80.1	tuberculosis	?-?	1	BEIJING	
78	tuberculosis	?-?	1	BEIJING	
82	tuberculosis	?-?	1	BEIJING	
82.1	tuberculosis	?-?	1	BEIJING	
83	tuberculosis	?-?	1	BEIJING	
85	tuberculosis	?-?	1	BEIJING	
91	tuberculosis	?-?	1	BEIJING	
92.1	tuberculosis	?-?	1	BEIJING	
95	tuberculosis	?-?	1	BEIJING	
97.1	tuberculosis	?-?	1	BEIJING	
103	tuberculosis	?-?	1	BEIJING	
45.1	tuberculosis	?-?			
1	tuberculosis	?-?			
68	tuberculosis	?-?			
71.1	tuberculosis	?-?			
72	tuberculosis	?-?			
73.1	tuberculosis	?-?			
79	tuberculosis	?-?			
79.1	tuberculosis	?-?			
65.1	tuberculosis	?-?			
1.1	tuberculosis	?-?