Genotypic and phenotypic diversity of *Mycobacterium tuberculosis* strains in patients with concomitant pulmonary and extra-pulmonary tuberculosis

Doctor Busizwe Sibandze

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by

Doctor Busizwe Sibandze

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DECLARATION

I, **Doctor Busizwe Sibandze**, hereby declare that the work on which this dissertation is based, is original and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree at this or any other university or tertiary education institution or examination body.

.....

Signature of candidate

.....06/06/2019.....

Date

“Exert your talents and distinguish yourself, do not think of retiring from the world until the world will be sorry that you retire” -**Samuel Johnson**

"...Our deepest fear is not that we are inadequate. Our deepest fear is that we are powerful beyond measure. It is our light, not our darkness that most frightens us. We ask ourselves, who am I to be brilliant, gorgeous, talented, fabulous? Actually, who are you not to be? You are a child of God. Your playing small does not serve the world. There is nothing enlightened about shrinking so that other people won't feel insecure around you. We are all meant to shine, as children do. We were born to make manifest the glory of God that is within us. It's not just in some of us; it's in everyone. And as we let our own light shine, we unconsciously give other people permission to do the same. As we are liberated from our own fear, our presence automatically liberates others." –**Marianne Williamson**

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ADA	Adenosine deaminase
BMA	Bone Marrow Aspirate
CTB	Centre for Tuberculosis
CDC	Centre for Disease Control and Prevention
C/M	Culture and Microscopy
CNS TB	Central Nervous System Tuberculosis
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic acid
DR	Direct Repeat
DST	Drug susceptibility testing
EAI1_SOM	Eastern African Indian 1 Somalis
EHLS	Eswatini Health Laboratory Services
EPTB	Extrapulmonary TB
FL-LPA	First Line Line Probe Assay
FNAC	Fine Needle Aspiration Cytology
HIV	Human immunodeficiency virus
INH	Isoniazid
IUATLD	International Union Against Tuberculosis and Lung Disease
IS*6110*-RFLP	IS*6110* Restriction Fragments Length Polymorphism
LAM	Latin-America-Mediterranean
LIS	Laboratory Information System
MDR-TB	Multi-drug resistant tuberculosis
MIRU-VNTR	*Mycobacterial* interspersed repetitive units of variable number tandem repeats
MOTT	Mycobacterium other than tuberculosis
MTBC	*Mycobacterium tuberculosis* complex
NHLS	National Health Laboratory Services
NICD	National Institute for Communicable Disease
nsSNP	nonsynonymous Single Nucleotide Polymorphisms
NTRL	National Tuberculosis Reference Laboratory
PCR	Polymerase Chain Reaction
PTB	Pulmonary tuberculosis
RNA	Ribonucleic acid
RIF	Rifampicin
RSA	Republic of South Africa

SRLN	Supranational Reference Laboratory
sSNP	synonymous Single Nucleotide Polymorphisms
PGRS	Polymorphic GC Rich Repetitive Sequence
TAD	Tshwane Academic Division
TBM	Tuberculosis Meningitis
TB	Tuberculosis
t-NGS	Targeted Next Generation Sequencing
UPGMA	Unweighted-Pair Group Method with Arithmetic
XDR-TB	Extensively drug resistant tuberculosis
WGS	Whole genome sequencing
WHO	World Health Organization

LIST OF ARTICLES AND CONFERENCE CONTRIBUTIONS

Publications 2015-2019

1. Lesibana Malinga, **B. Sibandze**, R. Tsireledzo, B. Magazi. **Performance evaluation of AnyplexII MTB/MDR/XDR for detection of first and second-line drug resistance in** *Mycobacterium tuberculosis.* Abstract: *International Journal of Infectious Apr 2016.*
2. **Doctor B. Sibandze**, Halima Said, Gugu Maphalala, Muyalo G. Dlamini, Barney Mitton, Sindi Dlamini, Shaheed V. Omar, Nontombi M Mbelle, Remco P.H. Peters. Genotypic and phenotypic diversity of *Mycobacterium tuberculosis* strains in patients with concomitant pulmonary and extra-pulmonary tuberculosis. Manuscript: *Journal of Infectious Diseases*.

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3. **Sibandze DB**, Mbelle M.N, Maningi NM, Malinga L, Magazi BT: Disseminated **bacilli Calmette-Guerin disease from the Northern Region of South Africa.** Poster Presentation, *African Society for Laboratory Medicine* conference, Cape Town, South Africa, 3-8 December 2016.

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7. Tfwala Z, Sikhondze W, Dlamini T, **Sibandze B,** Simelane M, Dlamini M **Factors associated with negative MTB culture results among gene Xpert positive MTB patients**, 49th Union World Conference, The Hague, The Netherlands, 24-27 October 2018.
8. **Doctor B. Sibandze**, Halima Said, Muyalo G. Dlamini, Barney Mitton, Sindi Dlamini, Shaheed V. Omar, Nontombi M Mbelle, Remco P.H. Peters **Heterogeneity of *Mycobacterium tuberculosis* infection in patients with concomitant pulmonary and extrapulmonary tuberculosis**. Poster Presentation, *European Society of Clinical Microbiology and Infectious Diseases* Conference, 13 – 16 April 2019, Amsterdam, Netherlands.

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By

Doctor Busizwe Sibandze

SUPERVISOR: Professor R.P.H Peters

CO-SUPERVISORS: Professor N.M. Mbelle & Ms. G. Maphalala

DEPARTMENTS: Medical Microbiology, Faculty of Health Sciences, University of Pretoria & National Tuberculosis Reference Laboratory, Eswatini Health Laboratory Services

DEGREE: Master of Science (Medical Microbiology)

SUMMARY

To date, the occurrence of extrapulmonary TB (EPTB) is considered to be caused by the same genotypic strain that infects the pulmonary site. Genetic heterogeneity of *M. tuberculosis* in patients with pulmonary tuberculosis (PTB) has been associated with adverse outcomes such as relapse and increased mortality due to presence of different strains with conflicting drug susceptibility testing (DST) patterns. It remains unknown whether heterogeneity of infection also occurs within a single host concomitant with extrapulmonary and pulmonary TB. In this study, we performed genotypic and phenotypic analysis of *M. tuberculosis* strains isolated from patients with both forms of TB to determine the rate of genotypic concordance and heterogeneity as well as the occurrence of phenotypic and genotypic hetero-resistance. Patients with microbiologically

confirmed extrapulmonary and pulmonary TB within a one-month period were identified in the laboratory information system; we included patients with *M. tuberculosis* isolated from blood or bone marrow aspirate, cerebrospinal fluid, lymph node and pleural fluid as EPTB specimens. Strains were retrieved retrospectively from the biobank at the National TB Reference Laboratory of the Health Laboratory Services in Eswatini (n = 17) and collected prospectively at the Tshwane Academic Division of the National Health Laboratory Services in South Africa (n = 15). Drug susceptibility for isoniazid and rifampicin was determined using the First Line Probe Assay (FL-LPA) and MGIT system. Genotyping of strains was done using spoligotyping and standard 24-loci mycobacterial interspersed repetitive units (MIRU) variable number of tandem repeats (VNTR) analysis. A dendrogram was generated to determine genetic relatedness. A total of thirty-two patients with concomitant extrapulmonary and pulmonary TB were included in this analysis. The most prevalent lineages were lineage four (Euro-American: n = 40, 63%) followed by Lineage two (East Asian: n = 20, 31%) and lineage three (East African Indian: n = 4, 6.3%). Genotypic analysis showed that 94% (30/32) had *M. tuberculosis* strains with the same spoligo type and MIRU VNTR pattern isolated from each body site. Heterogeneous infection was identified in 4 (12%) patients. Two (6%) patients showed presence of discordant strains: in both patients their extrapulmonary isolates showed presence of T1 strains and while unknown spoligo types were isolated from their pulmonary isolates; MIRU VNTR profiles were also discordant however, drug susceptibility patterns on FL-LPA and MGIT were the same. In another two patients (6%), their extrapulmonary and pulmonary isolates showed presence of the same genotype however, their drug susceptibility patterns showed presence of heteroresistance. This study confirms that heterogeneity infection occurs in patients with concomitant extrapulmonary and pulmonary TB in a setting of high incidence where patients may present with two overlapping infections at the same time. The rate at which heterogeneity occurs suggests that presence of different genotypes in different specimes may be clinically relevant and therefore, microbiological testing of specimens from both body sites in these patients is highly recommended. Our cohort study, moreover add to the scant literature which challenges the old dogmas related to TB immunity, pathogenesis and progression from latent to active disease. In

future, more robust or higher-resolution genomic typing tools, such as target Next Generation Sequencing (t-NGS) or metagenomic analysis are recommended to improve our understanding of the complexity of infections and for detection of both minority and major variants within extrapulmonary and pulmonary specimens for better estimation of the extent of *M. tuberculosis* genotypic and phenotypic heterogeneity.

Keywords: *Mycobacterium tuberculosis,* hetero-resistance, heterogeneity, extrapulmonary tuberculosis, pulmonary tuberculosis, concomitant infection, genotyping analysis, genotypes, *M. tuberculosis* lineages, drug susceptibility testing

CHAPTER ONE: INTRODUCTION

1.1 BACKGROUND

Tuberculosis (TB) manifests itself as either pulmonary tuberculosis (PTB) or extrapulmonary tuberculosis (EPTB). The later occurs as a result of haematogenous dissemination of *M. tuberculosis* from the lungs parenchyma to highl vascular areas such as lymph nodes, meninges, kidney, spine and growing ends of the bones (Norbis et al., 2014, World Health Organization, 2018).

In 2017, EPTB represented 14% of the 6.4 million incident notified cases, ranging from 8% in the WHO Western Pacific Region to 24% in the WHO Eastern Mediterranean Region (World Health Organization, 2018). In South Africa, EPTB is thought to occur at a rate of 15 - 20% of all TB cases in immune-competent patients and 20 - 70% of all TB in patients who are co-infected with HIV (Karstaedt, 2013, Hoogendoorn et al., 2017, Gounden et al., 2018). At least 10%-50% of EPTB patients have concomitant pulmonary involvement and it is highly recommended that all suspected cases of EPTB should be assessed for concomitant PTB (Lin et al., 2013, Nabueera-Burungi et al., 2014, Lee, 2015). In many cases, extrapulmonary and pulmonary lesions may be present in the same patient, which implies that this patient has concomitant EPTB and PTB. Nevertheless, such patients are classified as only PTB cases (World Health Organization, 2018). Probably, this is one among other reasons why EPTB data is lacking especially in developing countries including Eswatini.

Previously, TB was thought to be caused by reactivation of endogenous infection rather than by a new, exogenous infection. However, characterization of the genotype of *Mycobacterium tuberculosis* (*M. tuberculosis*) by deoxyribonucleic acid (DNA) fingerprinting studies have shown that some of these recurrences represent reinfection with a different strain from the first episode as opposed to treatment failure (van Rie et al., 1999, Lambert et al., 2003). In high TB burden settings with diverse *M. tuberculosis* genotypes a single host can be repeatedly infected with different *M. tuberculosis* genotypes (Cohen et al., 2012). To date, the occurrence of EPTB

is assumed to be caused by the same strain that infect and cause disease at the pulmonary site (Zumla et al., 2013).

Genetic heterogeneity of *M. tuberculosis* strains causing pulmonary disease in both HIV infected and HIV un-infected patients from both high and low TB incidence settings is well documented (Shamputa et al., 2004, Bernard et al., 2014, Ssengooba et al., 2015, Cohen et al., 2016). Genetic heterogeneity of *M. tuberculosis* in patients with PTB has been associated with adverse outcomes such as drug resistance, relapse and increased mortality rate (Cohen et al., 2011, Zetola et al., 2014). This holds true for diversity of *M. tuberculosis* genotypes as well as diversity in drug resistance profiles of bacilli occurring at the same time in a single host (Bernard et al., 2014, Zetola et al., 2014).

Mycobacterium tuberculosis genotypes have different virulence factors; hence the presence of multiple *M. tuberculosis* genotypes can have a negative impact outcome on clinical management of patients. Moreover, the proportions of patients with mixed-genotypes infections among HIV infected individuals have been estimated to be as high as 38% and 17% among those uninfected with HIV which could imply that impaired cell-mediated immunity may increase the probability of harbouring multiple infections (Cohen et al., 2012). The association of clinical outcome and prognosis in EPTB heterogeneity remains unknown.

To assess heterogeneity of *M. tuberculosis* infection, the IS*6110*-based restriction fragment length polymorphism (IS*6110* RFLP) analysis, spacer oligonucleotide genotyping (spoligotyping) and the mycobacterial interspersed repetitive unit variable number of tandem repeats (MIRU VNTR) typing can be used (Shamputa et al., 2004, Bernard et al., 2014, Ssengooba et al., 2015, Cohen et al., 2016). The IS*6110* RFLP analysis is the gold standard for genotypic characterization of *M. tuberculosis* strains, however available literature shows that this assay is cumbersome and has low discriminatory power among strains with less than six IS*6110* copies (van Embden et al., 1993). The combination of spoligotyping and MIRU VNTR has been

shown to be less cumbersome and has same discriminatory power as gold standard (Shamputa et al., 2004).

1.2 RATIONALE

Extrapulmonary TB is highly heterogeneous in nature with regards to its clinical presentations since it can potentially affect any organ in the body. The pathogenicity of EPTB is thought to seed from the haematogenous dissemination of *M. tuberculosis* strains from the lung parenchyma to other organs. Therefore, the assumption would be that the genotypic and phenotypic characteristics of the disseminated bacilli should be identical to that of the parent strain in the lung parenchyma. However, it remains unknown if this assumption stands as more molecular epidemiology studies are emerging show that a single host can be repeatedly infected with different *M. tuberculosis* strains (van Rie et al., 1999, Lambert et al., 2003). Furthermore, it remains unknown whether drug susceptible profiles of specimens collected from concomitant extrapulmonary and pulmonary TB are identical. Previously, diverse genotypic *M. tuberculosis* strains within a single host have been shown to result in conflicting phenotypic and molecular drug susceptibility testing (DST) results (Bernard et al., 2014). It would be beneficial to know if heterogeneity occurs in patients that have concomitant extrapulmonary and pulmonary TB disease to ensure patients received the correct treatment.

1.3 Aim of the Study

The aim of the study was to determine the rate of genotypic concordance and heterogeneity as well as the occurrence of phenotypic and genotypic hetero-resistance *M. tuberculosis* strains isolated in patients with extrapulmonary and pulmonary TB.

1.4 Objectives

To determine the proportion bacteriologically confirmed extrapulmonary isolates (Cerebrospinal fluid, pleural fluid, blood or bone marrow aspirate and lymph nodes) that have concomitant pulmonary tuberculosis disease

To determine the frequency of genotypic heterogeneity of *M. tuberculosis* isolates in patients with concomitant pulmonary and extrapulmonary infection by comparing specimen from the different anatomical sites by using spoligotyping assay, MIRU VNTR

To determine the frequency genotypic and phenotypic heteroresistance of *M. tuberculosis* isolates in patients with concomitant pulmonary tuberculosis and extrapulmonary tuberculosis by using phenotypic DST data collected from the Laboratory Information System

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CHAPTER TWO: LITERATURE REVIEW

2.1 HISTORY OF TUBERCULOSIS IN PERSPECTIVE

Despite being a curable disease, tuberculosis (TB) has been associated with high mortality and morbidity rates over the past centuries. *Mycobacterium tuberculosis* (*M. tuberculosis*) the causative agent for TB may have killed more persons to date than any other known microbial pathogen (Daniel, 2006, Sarmah et al., 2018). Available data estimate that the genus Mycobacterium originated more than 150 million years ago (Gutierrez et al., 2005, Daniel, 2006). Molecular genetics and molecular epidemiological studies through sequencing of the genome of several strains of *M. tuberculosis* and based on its mutation rate has made it possible to estimate the time of origin of mycobacteria. Literature on *M. tuberculosis* shows that much of the present diversity among these strains originated between 20,000–15,000 years ago (Hirsh et al., 2004, Gutierrez., 2005, Gagneux, 2012, Daniel, 2006).

Available literature depicts East Africa as the ancestral home of both tubercle bacilli and its human hosts. While, archaeological evidence for any disease is generally lacking in most African countries, Pott's deformities and skeletal abnormalities typical for TB in Egypt alone documents the disease to be more than 5000 years old (Gagneux, 2012, Daniel, 2006). The evidence of the existence of *M. tuberculosis* is further confirmed by deoxyribonucleic acid (DNA) amplification studies from tissues of Egyptian 5400 years old mummies associating the disease with early skeletal disease (Crubezy et al., 1998). To add, this theory is further supported by multi-locus sequence data analysis from 108 global *M. tuberculosis* strains that has confirmed that human *M. tuberculosis* originated in Africa (Hershberg et al., 2008).

History shows that early people began to move out of Africa as early as 1.7 million years ago, considering the airborne infectious nature of TB, it is thought these "out of Africa" migrants took with them their diseases, including TB (Gagneux, 2012, Daniel, 2006). It is thought that while other *M. tuberculosis* lineages left Africa and spread into Euro-Asia where the three phylogenetically 'modern' lineages seeded Europe, India and China, respectively, two

phylogenetically 'ancient' *M. tuberculosis* lineages remained in Africa (Hershberg et al., 2008). Literature documents that the wake of the "out- of Africa" migration, Europe, India and China experienced strong human population growth and as a consequence, the *M. tuberculosis* populations in these areas were on the rise, and concomitantly spread globally through waves of human exploration, trade and conquest (Hirsh et al., 2004, Gagneux, 2012). An interesting aspect of TB of scrofula is documented somewhere in the middle age. It is said that affected individuals by the so called scrofula would be touched by European monarchs as a form of treatment (Daniel, 2006). History shows that this went on for at least hundreds years, and among those was the famous English scholar Samuel Johnson who is believed to have died of TB (Daniel, 2006). René Laennec a French physician is considered to be the first person to classify TB into either pulmonary or extra-pulmonary. Laennec mostly known for his invention of the stethoscope. Laennec is thought to have further explicitly defined the pathogenesis of TB, physical signs of pulmonary disease and introduced terms which are still in used to date (Laënnec, 1962, Daniel, 2006). However, it was on March 24, 1882 where the history of TB changed dramatically. On this date Hermann Heinrich Robert Koch made his famous presentation, Die Aetiologie der Tuberculose, to the Berlin Physiological Society (Daniel, 2006). Koch demonstrated the tubercle bacillus he had identified and postulated how infection occurs. This concept was termed the Koch-Henle postulates, which to date is used to set the standard for the demonstration of infectious aetiology (Daniel, 1997). Koch was bestowed with a Noble Prize in Medicine or Physiology later in 1905 for his significant contribution in the aetiology of TB. A century later in 1982, 24th March was officially announced World TB Day in a meeting organized by the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) and this day has been used to date to raise awareness and commemorate lives of victims of TB globally (Centres for Disease Control and Prevention, 2017).

The discovery of antibiotics such as streptomycin brought hope in the fight of eliminating TB in 1944 (Woodruff, 2014). However, several factors; such as the complicated standard treatment against TB, as it involves a six-month regimen with multiple antibiotics, patient non-adherence or drug shortages led to the development of drug resistance (Herzog, 1998). Anti-tuberculosis

drugs, such as para-aminosalicylic acid, streptomycin (SM), Isoniazid (INH), pyrazinamide (PZA), cycloserine and rifampicin were introduced as combination therapy in the 1950s (Herzog, 1998). According to Herzog (1998) by the 1980s there was 98% chance of cure, unfortunately, the very success of the drug treatment of TB has been the medium for the emergence of a new wave of drug resistance (Davies and Pai, 2008). Today reports on emergence of drug-resistant strains of *M. tuberculosis* strains are fast increasing (Schaaf et al., 2006, Cohen et al., 2011, Comas and Gagneux, 2011). Initially patients could take their medication home completely unsupervised, drugs were singularly administered, and patients did not use all the prescribed drugs, more robust interventions were warranted. In an attempt to halt and reverse the global spread of TB in 1993, WHO introduced numerous control programs such as the Stop TB Strategy and the Directly Observed Treatment Short Course (DOTS) and TB was declared a "global emergency", the first pathological disease to be given such recognition (World Health Organization, 2000).

However, the onset of the Human Immunodeficiency Virus (HIV) epidemic in the early 1980s drastically changed the history of TB (Daniel, 1997, Daniel, 2006, Gagneux, 2012). The emergence of HIV has fuelled the TB incidence, particularly in sub-Saharan Africa. The TB epidemic continues unabated hence at least ten million new TB cases and 2 million deaths are estimated to occur each year, more than any time in history (World Health Organization, 2018).

2.2 TAXANOMIC CLASSIFICATION OF MYCOBACTERIUM TUBERCULOSIS

The genera *Mycobacterium*, *Corynebacterium*, *Actinomyces* and *Nocardia* fall under same texa While the *Mycobacterium* is a genus under the *Actinobacteria* class, assigned as the *Mycobacteriaceae* family. The classification of *M. tuberculosis* is shown in Table 2.1.

Table 2.1: Taxonomic classification of *Mycobacterium tuberculosis* (Palomino et al., 2007).

Kingdom	**Bacteria**	
Phylum	Actinobacteria	
Class	Actinobacteria	
Subclass	Actinobacteridae	
Order	Actinomycetales	
Suborder	Corynebacterineae	
Family	Mycobacteriaceae	
Genus	*Mycobacterium*	
Species	*M. tuberculosis*	

The genus *Mycobacterium* comprises of 71 identified species which can be split on the basis of growth rate into two major groups: the fast growers and slow growers (Palomino et al., 2007, Mahon et al., 2015). The fast growers (e.g. *M. fortuitum* and *M. abscessus*) produce visible colonies within seven days under favourable conditions. While the slow growers (*M. tuberculosis*, *M. bovis*, *M. avium*, *M. leprae* and *M. kansasii*) take up to 8 weeks to grow and are more pathogenic in human and animals and causes chronic diseases (Thorel et al., 1990, Vishnevski and Steklova, 2008). In general, it is thought that a majority of these mycobacterial

species exist as saprophytes, with only a relatively a few of these thought to be being pathogenic (Thorel et al., 1990).

2.2.1 Aetiology of tuberculosis and the *Mycobacterium tuberculosis* Complex

Tuberculosis in humans is primarily caused by *M. tuberculosis* and *M. africanum*, the latter is a phylogenetic variants of *Mycobacterium tuberculosis* complex (MTBC) thought to be limited to West Africa. The *M. tuberculosis* and *M. africanum* members of the MTBC are responsible for a significant number of cases of human TB infections (Vishnevskii and Steklova, 2008, Reddington et al., 2011). The MTBC, in addition to the typical human pathogens *M. tuberculosis* and *M. africanum* comprises of other variants affecting various wild and domestic animal species. At present, MTBC the circulating strains are thought to fall into six major lineages, or clades, their global distribution varies (Figure 2.1) (Gagneux, 2012).

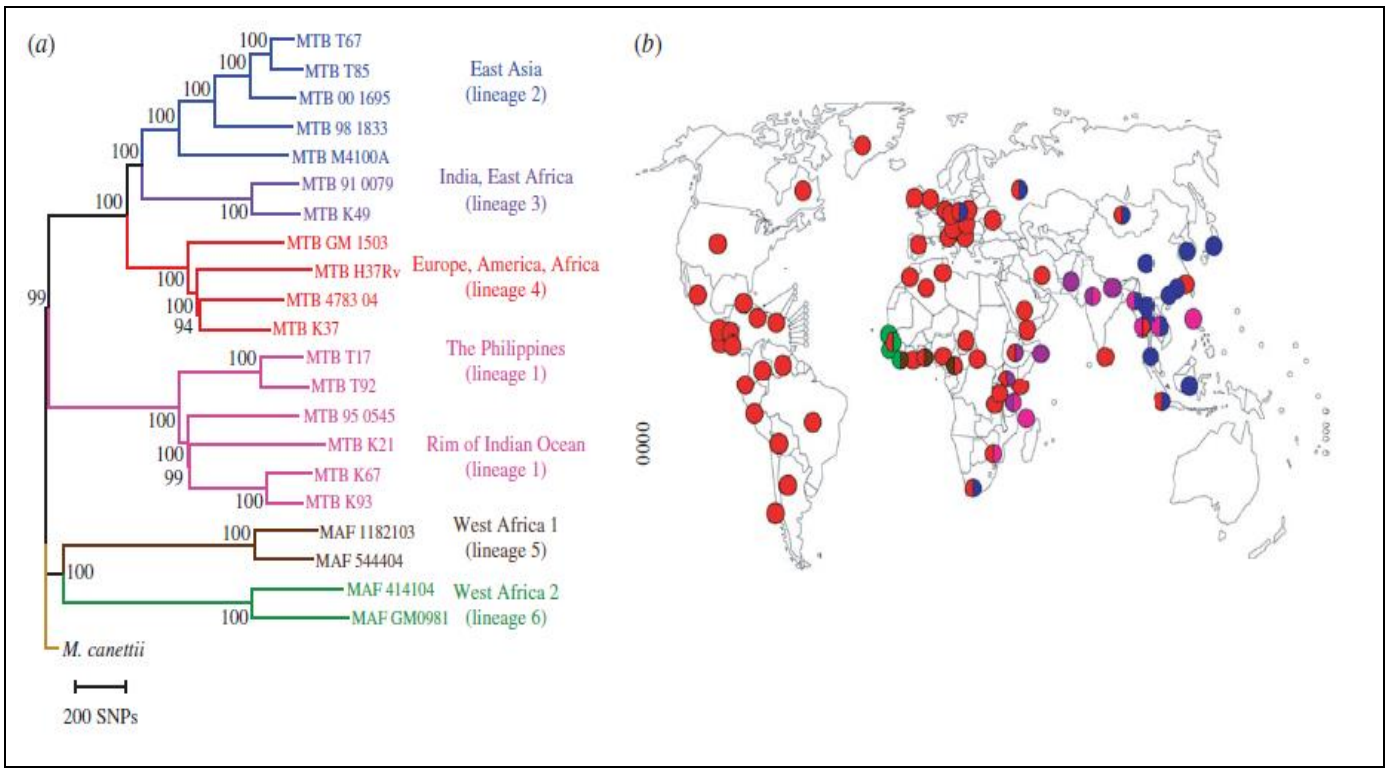

Figure 2.1: Global phylogeography distribution of MTBC. (a) Global phylogeny of human-adapted MTBC based on 22 whole-genome sequences (b) Global distribution of the six main human-adapted lineages of MTBC. The coloured dots represent the dominant MTBC lineages in each continent or country (Gagneux, 2012)**.**

These different circulating strains of MTBC are considered to be best adapted to their particular host species. The animal pathogens include *M. bovis* (a pathogen of cattle), *M. caprae* (goats and sheep), *M. microti* (voles), and *Mycobacterium pinnipedii* (seals and sea lions) (Hirsh et al., 2004a, Daniel, 2006, Gagneux, 2012). Recently a new member of the MTBC was discovered: *M. mungi*. *Mycobacterium mungi* is a novel species that is the causative agent of TB in banded mongooses which are found in close association with the human species in Botswana (Alexander et al., 2010).

Mycobacterium canettii (*M. canettii*) is one particular member of MTBC that deserves special mention. *Mycobacterium canettii*, previous described as 'smooth TB bacilli' (Gutierrez et al., 2005, Fabre et al., 2010, Gagneux, 2012). *Mycobacterium canetti* was first discovered in 1969,

and a full description followed after being isolated from a 2-year-old Somali patient suffering from lymphadenitis and so far, only about 60 isolates have been reported (van Soolingen et al., 1997b, Fabre et al., 2010). Interestingly, a large proportion of these isolates were recovered from TB patients in Djibouti or from individuals who spent some time at the Horn of Africa prior to developing TB. *Mycobacterium canettii* differs in many classical differences from members of MTBC. This pathogen is known to produce smooth and shiny colonies on solid growth media, which are distinct from the rough colony morphology characteristic of classical MTBC, hence its name, "smooth TB bacilli". In addition, *M. canettii* is thought to harbour more genetic diversity compared with classical MTBC and shows clear evidence of ongoing horizontal gene exchange, which does not occur in classical MTBC (figure 2.2) (Supply et al., 2003, Hirsh et al, 2004, Gagneux, 2012). According to Gagneux (2012) the above mentioned features in combination with the lack of evidence for human-to-human transmission and the fact that *M. cannettii* clinical isolates are rare, suggests that this organism is an opportunist. The environmental reservoir for *M. canettii* is surmised to exist exclusively in the Horn of Africa (van Soolingen et al., 1997a, Supply et al., 2003, Hirsh et al, 2004, Fabre et al., 2010, Gagneux, 2012).

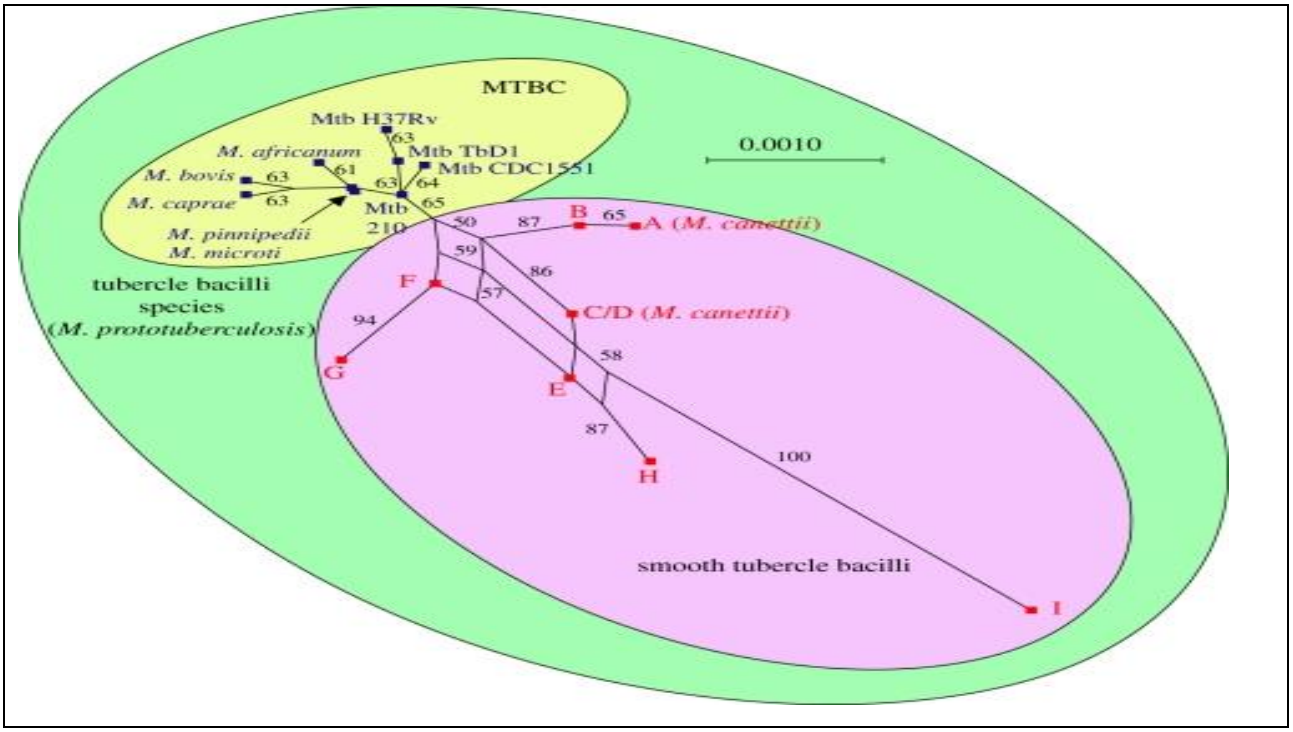

Figure 2.2: Phylogenetic relationship between 'smooth' tubercle bacilli and classical MTBC. Multi-locus sequence data of six housekeeping genes show that the smooth tubercle bacilli are more genetically diverse than MTBC (Gutierrez et al., 2005).

2.2.2 General Microbiological Characteristics of Mycobacterium Genera

The Mycobacterium species is characterized by slender, straight or slightly curved Gram positive bacilli about 0.2 x 10 µm that do not form spores and are non-motile non-spore forming bacilli actinomycetes occasionally forming branches (Rastogi et al., 2001, Cole, 2002). *Mycobacterium tuberculosis* complex are obligate pathogens growing most successfully at a temperature of 37^{o} C, in tissues having the highest partial pressure of oxygen, such as the lung apices, which explains why MTBC can remain dormant in the lungs of healthy individuals (Todar, 2005). In the environment, mycobacterium species are found in soil like most actinomycetes, and some of mycobacteria species are encapsulated like *M. tuberculosis* (Rastogi et al., 2001).

The cell-wall structure of *M. tuberculosis* is unique. More than 60% of the cell-wall contains lipid which consists of three major components: mycolic acids, cord factor and Wax-D (Todar. 2005). Mycolic acids are exclusively found in the cell-walls of the Mycobacterium and Corynebacterium species (Rastogi et al., 2001). These acid-fast Gram positive bacilli that contain peptidoglycan (murein) in their cell-wall and do not have the chemical characteristics of either Gram-positive or Gram-negative bacteria are characterized as acid fast bacilli (Hett and Rubin, 2008). The mycolic acids can be defined as *ß*-hydroxyl fatty acids with a long α-alkyl side chain and are thought to contribute to 50% of the dry weight of the cell envelope of Mycobacterium species (Huang et al., 2002, Takayama et al., 2005).

Mycolic acids are strong hydrophobic molecules that form a lipid shell around the bacteria and protects mycobacteria from agents such as cationic proteins, lysozyme and oxygen radicals in the phagocytic granule (Todar, 2005). The cell envelope of acid fast bacilli , such as mycobacteria contains an additional layer beyond the peptidoglycan that is exceptionally rich in unusual lipids, glycolipids and polysaccharides (Hett and Rubin, 2008). Cell-wall components, such as mycolic acids and arabinogalactan are thought to contribute to the mycobacterial longevity as these trigger inflammatory reactions in hosts (Hett and Rubin, 2008). The virulent strains of *M. tuberculosis* are known to produce a 'cord factor' causing the bacterium to grow in a rope-like fashion, which is called 'serpentine cords' (Huang et al., 2002, Hooja et al., 2011). These serpentine cords allow the bacterium lingers in the lungs for decades after infection waiting for a decrease in the host's defence mechanism (Noble, 2006, Julian et al., 2010).

The high lipid content in the cell wall renders mycobacteria resistant to staining by basic stains such as Gram staining that is commonly used in diagnostic laboratories to distinguish Gram positive organisms from Gram negative ones (Mahon et al., 2015). Staining is therefore achieved by an increase in staining time or application of heat. Mycobacteria are known to resist decolourization by acid-ethanol once they have absorbed the primary stain (Rastogi et al., 2001, Mahon et al., 2015). Such organisms are said to be acid fast bacilli (AFB). Stains such as Ziehl Nielsen's and auramine-O that are used to stain mycobacterium species and these are based on

the acid fastness aspect of the organism (Bishop and Neumann, 1970, Hooja et al., 2011). These microscopy techniques are used in diagnostic laboratories to differentiate AFB from non-acid fast organisms. Moreover, microscopy is the gold standard to quantifying the bacterial load in clinical specimens hence this technique is widely used in treatment monitoring (Lee, 2005).

In general, the tubercle bacillus is a slow growing organism. The generation time of *M. tuberculosis* is typically 24 h when grown in a synthetic medium (Cook et al., 2009, Cole et al., 1998). Even with increased carbon dioxide (CO_2) concentration, mycobacteria still grow slower than most pathogenic organisms (Mahon et al., 2015). This is thought to be due to their hydrophobic cell surface; the organisms tend to clump and form cords which inhibit permeability of nutrients into the cell (Ryan et al., 2014). Cord formation is considered a virulent factor of *M. tuberculosis* and these cords can be easily seen in broth medium such as the Middlebrook 7H10 enriched medium (Julian et al., 2010).

Furthermore, the incubation time of this organism ranges between 2 to 6 weeks at optimum temperatures 35 to 37 °C for liquid medium and up to 8 weeks for solid medium (Mahon et al., 2015). Growth is stimulated by incubation in air with 5 to 10% added CO_2 and by inclusion of glycerol to 0.5% in the medium (Wayne et al., 1986). The long generation time of the bacillus contributes to the chronic nature of the disease. As a result, it imposes lengthy treatment regimens and ultimately, represents an obstacle to both public health policy makers and researchers (Cole et al., 1998). The phenotypic characteristics of *M. tuberculosis* described above are important for identification in the laboratory (Wayne et al., 1986, Lee, 2015).

2.3 GENOMICS OF *MYCOBACTERIUM TUBERCULOSIS*

Studying the genome of *M. tuberculosis* provides important insight into the biology of the species and the presence of sequence diversity. Sequential data provide a basis for understanding the pathogenesis, immune mechanisms and bacterial evolution of *M. tuberculosis* (Fleischmann

et al., 2002). The *M. tuberculosis* laboratory strain H37Rv was completely sequenced and genomic analysis depicts that it comprises of 4 411 532 base pairs (bp) and has a mean Guanine (G) + Cytosine (C) content of 65.6 mol % (Cole, 2002; Fleischmann *et al*., 2002). The genome is thought to contain roughly 4 000 genes and account for >91% of the potential coding capacity according to a study conducted by Cole (2002). At least 51% of the genes is thought to have arisen because of gene duplication or domain shuffling events while 3.4% of the genome is composed of insertion sequences (IS) and prophages (phiRv1 and phiRv2) (Cole, 2002). In total, Cole's study found that there about 56 copies of IS elements and these belonging to IS3, IS5, IS21, IS30, IS110, IS256 and IS*L*3 families (Cole, 2002).

Furthermore, genome sequences can be used for the development of diagnostic tools for the rapid and unambiguous identification of members of the MTBC. For example, comparative genomics of *Mycobacterium* spp aims to identify genes or loci that are different from virulent or attenuated strains (Cole, 2002). Data from such findings can be used to the characterise the strains and would define the molecular mechanisms of pathogenicity, as well as contribute to new information for vaccine development (Cole, 2002). Ravn and colleagues (2005) studied early secreted antigenic target 6 kDa protein and CFP-10 reported that these are contained within the RD1 (region of difference) of the mycobacterial genome. However, these proteins are absent in the TB vaccine strain *Mycobacterium bovis* bacillus Calmette-Guérin (*M. bovis* BCG) *M. avium* and most other nontuberculous mycobacteria making it easier to distinguishing between mycobacteria spp (Ravn et al., 2005).

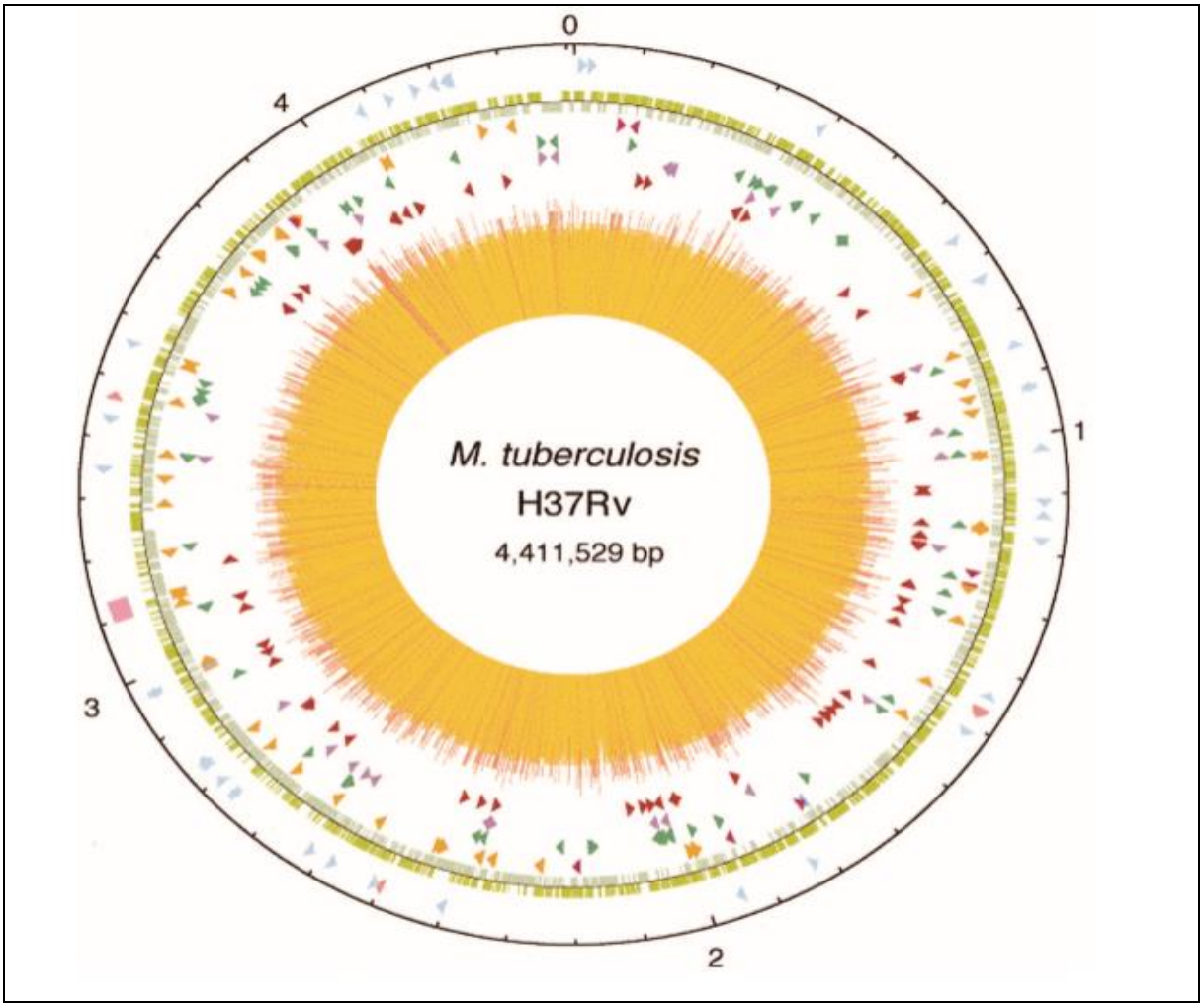

Figure 2.3: Map of the chromosome of *M. tuberculosis* H37Rv reference laboratory strain. The outer circle depicts the scale in mega bases, 0 is the origin of replication. The first ring from the exterior shows the positions of stable RNA genes (in blue are tRNAs, and in pink are others) and the direct-repeat region (pink cube) is the target for spoligotyping assay; the second ring depicts the coding sequence by strand (clockwise, dark green; anticlockwise, light green); the third ring shows repetitive DNA (insertion sequences [IS], orange; 13E12 REP family, dark pink; prophage, blue) these are targeted by the IS6110-based restriction fragment length polymorphism (IS6110 RFLF) assay; the fourth ring depicts the positions of the PPE family members (green); the fifth ring shows the positions of the PE family members (purple); and the sixth ring depicts the positions of the PGRS sequences (dark red) which are targeted by the Polymorphic GC rich repetitive sequence (PGRS) assay. The histogram represents the G+C content, with <65% G+C in yellow and >65%G+C in red (Cole et al., 1998).

2.4 TRANSMISSION OF TUBERCULOSIS

Globally, there is a large reservoir of latent TB cases. At least a third of the world is thought to have latent infected with *M. tuberculosis* (World Health Organization, 2018). Tuberculosis is transmitted through the production of aerosols from individuals with pulmonary or laryngeal disease through high-velocity exhalation actions such as coughing or sneezing. These aerosols produced are thought to rapidly get dehydrated and become droplet nuclei of about 1-5µm in diameter which contain *M. tuberculosis* bacilli that can remain suspended in the air (Wells, 1934, Riley et al., 1959, Frieden et al., 2003). Inhalation of these droplets results in colonization of the lungs, and in the lungs these droplets are subsequently taken up by resident dendritic cells and monocyte-derived alveolar macrophages (van Crevel et al., 2002). The bacilli replicate intracellularly and spread via the lymphatic system to the hilar lymph nodes (Frieden et al., 2003).

2.4.1 Immune-mediated pathology response to Infection

Between 7 to 21 days after the initial infection, *M. tuberculosis* preferentially resides in the phagosome of macrophages (Todar, 2005a, Ahmad, 2011). In the phagosome, mycobacterial peptides produced bind directly to the Fc receptors of macrophages and interact with the major histocompatibility complex class II (MHC II) molecules that are shuttled to the cell surface (Ahmad, 2011). The tubercle bacillus can bind directly to the receptors on the macrophages or indirectly by means of complement receptors or Fc receptors (Todar, 2005a). Binding of tubercle bacillus to the MHC II molecules triggers the cluster of differentiation 4 (CD4) thymus cells (T-cells) (Kaufmann, 2001, Kaufmann and Parida, 2008). As a result, other macrophages begin to accumulate from peripheral blood, however these macrophages are inactivated and cannot destroy *M. tuberculosis* (Ryan et al., 2014, Andersen et al., 2000). Major histocompatibility complex class I (MHC I) molecules recognize *M. tuberculosis* and infiltrate the CD4+ T lymphocytes and Tcd-lymphocytes to produce cytokines, interferon gamma (IFN- γ), interleukin-2, tumour necrosis factor alpha (TNFα), and macrophage colony-stimulating factor (Todar, 2005b, Ahmad, 2011, Mahon et al., 2015). This is followed by the activation of macrophages to

inhibit *M. tuberculosis* intracellular growth. The T-cells and macrophages which are activated to then destroy the *M. tuberculosis* as shown in Figure 2.4 (Todar, 2005a). The IFN-γ plays an important role in this process. This is seen in individuals harbouring the genetic defects who lack of production of either IFN- γ or its cellular receptor. These individuals tend to develop severe and fatal TB disease (Kaufmann and Parida, 2008, Ahmad, 2011).

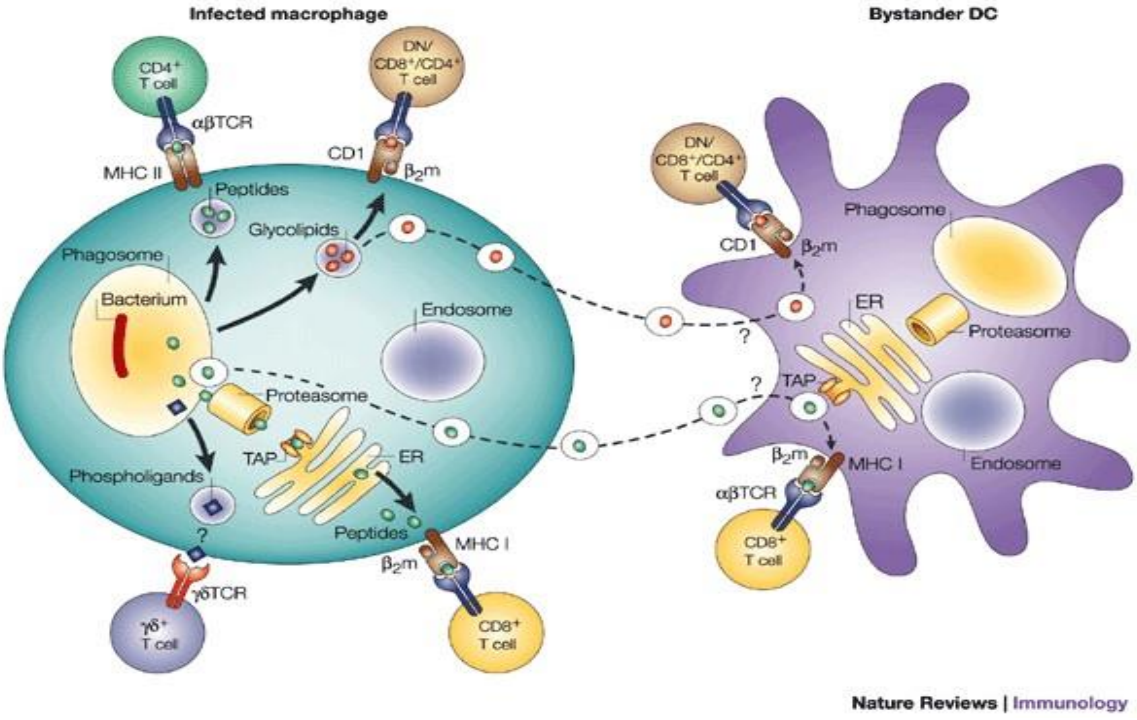

Figure 2.4. Different T-cell-processing pathways that triggers the activation of distinct T-cell populations in the immune response against *M. tuberculosis* (Kaufmann, 2001).

2.5 GLOBAL EPIDEMIOLOGY OF TUBERCULOSIS

Tuberculosis remains one of the main global public health problems, particularly in resource-limited settings. Tuberculosis is one of the top 10 causes of death, and the leading cause among single infectious agents. In 2017, WHO reported that at least 1.3 million deaths (range, 1.2–1.4 million) among HIV-negative people while among HIV infected adults an additional 300 000 deaths (range, 266 000–335 000) were estimated to have died from TB. New TB cases were approximately 10.0 million (range, 9.0–11.1 million) which are thought to translate to 133 cases (range, 120–148) per 100 000 population. Ninety percent of TB cases were adults (aged ≥15 years), 64% were male, and 9% cases were co-infected with HIV. In South Africa reported 567 TB cases per 100 000 population were reported while Eswatini reported 308 TB cases per 100 000 population (World Health Organization, 2018).

In the same year, EPTB represented 14% of the 6.4 million incident notified cases, ranging from 8% in the WHO Western Pacific Region to 24% in the WHO Eastern Mediterranean Region (WHO, 2018). However, literature shows that EPTB is underestimated and may vary among countries. A European survey of 2011 shows that at least 16,116 (22%) of 72,334 TB cases notified in the EU in were EPTB cases, with a wide variability range (4–48%) among the reporting countries (Solovic *et al*., 2013). At least 10%-50% of EPTB patients have concomitant pulmonary involvement and all suspected cases of EPTB should be assessed for concomitant PTB (Lee, 2015). In South Africa, EPTB is thought to occur at a rate of 15 - 20% of all TB in immune-competent and 20 - 70% of all TB in patients are co-infected with HIV (Karstaedt, 2013, Hoogendoorn et al., 2017, Gounden et al., 2018). There is no EPTB published data available in literature from Eswatini.

2.5.1 Human Immunodeficiency Virus and Tuberculosis Co-Infection

Africa is the epicentre for the world's AIDS pandemic. Among the 9% global HIV co-infected cases, 72% were Africa (World Health Organization, 2018). The increase in HIV infection rate has significantly fuelled the incidence of TB worldwide. Human immunodeficiency virus is the single most important risk factor for TB (Friedland, 2009). Co-infection with HIV has been associated with increasing the risk of developing active TB by over 100-fold, and such individual are more susceptible to developing resistant mycobacterial strains (Davies and Pai, 2008). Moreover, HIV-infected individuals are assumed to progresses faster from latent infection with *M. tuberculosis* to full-blown active TB disease (Valadas and Antunes, 2005). In Eswatini and South Africa, the co-infection rate is estimated to be 69% and 60% respectively (World Health Organization, 2018). However, one recent study conducted in a tertiary hospital in Durban reported a co-infection of 88.8% making co-infection the single most common risk factor for extrapulmonary TB (Gounden et al., 2018).

However, since the introduction of antiretroviral therapy (ART) for HIV infected individuals there has been significant decline in TB/HIV deaths (World Health Organization, 2018). Antiretroviral therapy is play a significant reduction the risk of progression from latent TB to active TB (Badri et al., 2002). One study conducted in four hospitals aimed to access the impart of ART scale up in South Africa showed a 13% decrease in overall number of TB cases between 2009 and 2013 (Hoogendoorn et al., 2017). Furthermore, the same study showed that the epidemiological profile of EPTB has changed; the proportion of miliary TB and disseminated TB decreased significantly ($P < 0{\cdot}01$), while TB meningitis and TB of bones showed a significantly increase ($P < 0{\cdot}01$ and $P = 0{\cdot}02$, respectively). However, TB pleural effusion and lymphadenopathy remained the same (Hoogendoorn et al., 2017).

2.6 CLINICAL MANIFESTATION OF TUBERCULOSIS

Tuberculosis manifests itself as either pulmonary tuberculosis (PTB) or extrapulmonary tuberculosis (EPTB). An immune compromised individual may show evidence of both PTB and EPTB, although in varying degrees. The WHO Global TB report of 2013 defines EPTB as a form of TB affecting any organ other than the lung parenchyma. Even though in many cases, pulmonary and extrapulmonary lesions are both present in the same patient which means these patients are concomitant with EPTB and PTB. These patients are nevertheless often misclassified as PTB (Norbis et al., 2014).

Historically, EPTB is a neglected form of TB because of its limited epidemiological impact on the disease transmission with the notable exceptions of laryngeal TB and intrathoracic lymph node TB with bronchial fistulisation (Sevgi et al., 2013, Norbis et al., 2014). Therefore, EPTB does not constitute a public health threat according to experts. In addition to the fact that PTB is significant to public health, there is scant literature on EPTB as compared to PTB. However, since EPTB is significant risk factor among co-infected individuals the TB epidemiology has since changed. The sub-Saharan African region has the highest incident of HIV/AIDS; it is therefore crucial to know more about the epidemiology of EPTB for this region.

Several factors contribute to the poor prognosis of EPTB in resource limited countries. Firstly. Extrapulmonary TB is highly heterogeneous in the nature of clinical manifestations, it is generally associated with a challenging diagnosis, difficulty in obtaining clinical specimens and undiagnosed infection, and clinical expertise diagnosis (doctor) that may not be available at the primary healthcare or health centres (Norbis et al., 2014; Hoogendoorn et al., 2017; Gounden et al., 2018). Since EPTB is highly heterogeneous nature with regards to its clinical presentations, it can potentially affect any organ in the body. Although, there are known districts where it localizes more frequently that is: superficial and deep lymph nodes, pleura, bone and joints, central nervous system (CNS) and abdomen (Norbis et al., 2014). Most presentations of EPTB are observed in immunocompetent patients are located in the pleura, the lymphatic system, however the most affected site in HIV infected individuals is the lymphatic system (Norbis et al.,

2014). The fact that EPTB can potentially affect any organ in the body raises question on its nature if pathogenesis. In general, EPTB is known to occur as a result of haematogenous dissemination of *M. tuberculosis* from the lungs (caseous focus) to highly vascular areas such as lymph nodes, meninges, kidney, spine and growing ends of the bones (Sankar et al., 2013, Sevgi et al., 2013, Houston and Macallan, 2014, Norbis et al., 2014).

2.6.1 Lymphadenitis Tuberculosis

Lymphatic TB is thought to occur as a result of localization of TB to the lymphatic system and reflects a systemic involvement of TB infection. This is because *M. tuberculosis* bacilli is thought to follow the lymphatic drainage routes from a primary complex (most commonly located in the lung) to the systemic lymphatic circulation (Norbis et al., 2014).

Lymphadenitis is thought to be the most commonly occurring form and is estimated to constitute at least 40% of EPTB cases (Golden and Vikram, 2005, Sia and Wieland, 2011, Catano and Robledo, 2016). It was previously thought tuberculous lymphadenitis is a disease of childhood, until cases were documented across all ages. However, a greater proportion of lymph node TB is thought to be found in patients under 14 years. HIV infection is predisposing factor for Lymphadenitis and clinical presentation include cervical lymphadenitis historically known as scrofula (60–90% of lymph node TB cases) and other common sites are mediastinal, axillary, mesenteric, perihepatic and inguinal lymph nodes (Golden and Vikram, 2005; Sia and Wieland, 2011; Norbis et al., 2014).

2.6.2 Pleural Tuberculosis

Pleural TB or TB pleurisy is thought to result from either as a sequel of primary infection or as a manifestation of TB reactivation. This form of EPTB can occur even in the absence of patent PTB (Light, 2010). Pleural TB is thought to follow the rupture of a pulmonary caseous focus into the sub-pleural space. A delayed hypersensitivity reaction is initiated as result of the presence of mycobacterial antigens into the pleural space and this reaction triggers lymphocytic pleuritis and lymphocyte-rich exudative fluid production (Light, 2010, Norbis et al., 2014).

Pleural TB has been reported to account for roughly 4% to 5 % of all TB cases and is thought to be the second leading cause of EPTB (Golden and Vikram, 2005, Sia and Wieland, 2011). However, one study in Korea has reported pleural TB to be most common case followed by tuberculous lymphadenitis (Lee, 2015). Pleural TB may often present with an acute illness with cough, pleuritic chest pain, fever, or dyspnoea. Although rarely, TB pleurisy individuals can present as frank TB empyema, containing an abundance of mycobacteria (Houston and Macallan, 2014, Shaw et al., 2018).

2.6.3 Central Nervous System Tuberculosis

Tuberculosis of the central nervous system such as tuberculous meningitis (TBM) pathogenesis is caused by the inflammation of the meninges as consequence of *M. tuberculosis* presence into the subarachnoid space after the rupture of a sub-ependymal tubercle (Golden and Vikram, 2005, Norbis et al., 2014). Bone and joint TB occurrence is associated with via haematogenous dissemination of *M. tuberculosis* to the vertebral bodies as a result of bacilli. The infection further spreads to the adjacent discs and vertebral bodies under the longitudinal ligaments. Following that, the arachnoiditis encases both cranial nerves and penetrating vessels, leading to cranial nerve palsies and communicating hydrocephalus (Sia and Wieland, 2011, Houston and Macallan, 2014, Morteza et al., 2015).

The central nervous system TB occurs in approximately 1% of all TB cases and so far, the most fatal among EPTB cases. Central nervous system tuberculosis (CNS TB) includes tuberculous meningitis which is thought to be the most common presentation, intracranial tuberculomas, and spinal tuberculous arachnoiditis (Golden and Vikram, 2005; Sia and Wieland, 2011; Morteza et al., 2015). High levels of tuberculoproteins may cause meningismus. Cerebral edema has been associated with impairment of consciousness, seizures, and raised intracranial pressure, whereas tuberculomas can manifest as space-occupying lesions (Golden and Vikram, 2005; Sia and Wieland, 2011). A high index of suspicion is recommended for timely diagnosis and prompt initiation of therapy (Golden and Vikram, 2005; Sia and Wieland, 2011).

2.6.4 Miliary Tuberculosis

Miliary TB is the lymphatic and haematogenous spread of TB also referred to as any progressive, disseminated form of tuberculosis; the disease can occur during primary dissemination or after years of untreated tuberculosis (Golden and Vikram, 2005, Mert et al., 2017). Miliary TB is estimated to occur in 10% of individuals who have HIV infected and PTB, and in 38 % of those who have HIV infected and EPTB (Golden and Vikram, 2005). Individuals may present with fever, chills, night sweats, weight loss, and anorexia while the clinical manifestations depend on the organs involved. Cases such as fulminant disease including septic shock, acute respiratory distress syndrome, and multi organ failure have been previous described (Golden and Vikram, 2005; Sia and Wieland, 2011; Mert et al., 2017). Important laboratory indicators may include normochromic anaemia, leukopenia or leukocytosis, elevated sedimentation rate, and hyponatremia (Golden and Vikram, 2005). Typical specimen for diagnosis may include: sputum, bronchoalveolar lavage, gastric washings, CSF, blood culture, or biopsies of liver and bone marrow (Golden and Vikram, 2005; Sia and Wieland, 2011; Mert et al., 2017).

2.6.5 Other Clinical Manifestation of Extrapulmonary tuberculosis

The pathogenicity of Genitourinary (GU) TB appears to occur in a more stepwise manor. Genitourinary TB is thought to always occur secondary to the spread of TB localized elsewhere in the body. That is the *M. tuberculosis* bacilli spreads haematogenous to the kidney, the epididymis or the female genital organs and from then its spread towards the GU system through continuous structures (calyx, renal basin, ureter, bladder, urethra, reproductive organs, sometimes contralateral kidney) (Norbis et al., 2014). The pathogenesis of gastrointestinal (GI) and peritoneal TB is thought to be linked to at least 3 methods 1) by swallowing of infected sputum in PTB patients, (2) by ingestion of contaminated food this can include unpasteurized milk or dairy products), (3) by lymphatic or (4) haematogenous spread and, rarely, (5) by spread from adjacent organs such as the fallopian tubes (Houston and Macallan, 2014, Giouleme et al.,

2011). As a results, GI can affect any part of the digestive tract from the oesophagus to the rectum (Giouleme et al., 2011; Norbis et al., 2014).

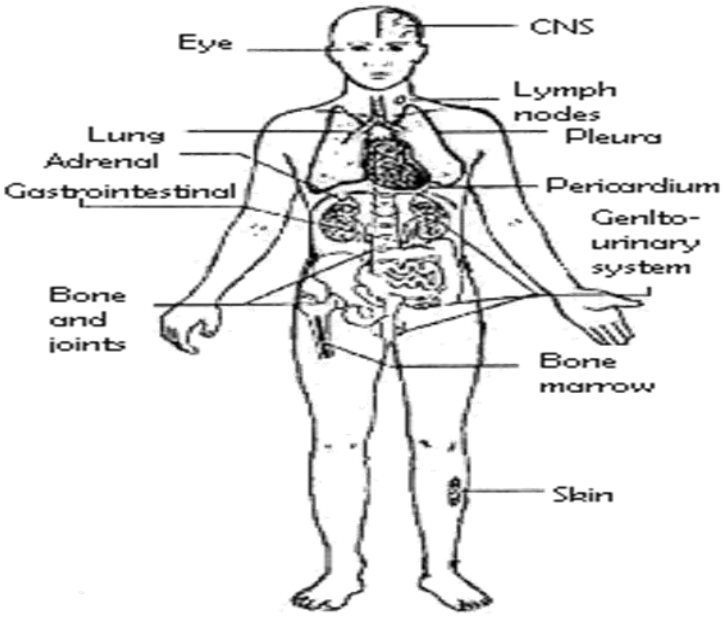

Figure 2.5: Sites most affected by extrapulmonary tuberculosis.

2.7 DIAGNOSTIC CHALLENGE, TREATMENT AND MANAGEMENT OF EXTRAPULMONARY TUBERCULOSIS

2.7.1 Challenges in Extrapulmonary Diagnosis

The laboratory is the corner stone for the diagnosis and management of TB patients. The overall goal of TB laboratory is diagnosing active TB and to provide clinicians with guidance on which antibiotics they should or should not prescribe for their patients (Lee and Behr, 2016). Diagnosis of EPTB is often delayed or even missed due to insidious clinical manifestations, its pauci-bacillary nature, and the sites of infection may not be easily accessible for the collection of specimens suitable or adequate for laboratory diagnosis (Norbis et al., 2014, Lee, 2015b, Baqui et al., 2018). Diagnostic methods commonly used for diagnosis of EPTB are fine needle

aspiration cytology (FNAC), culture, histopathology, immunohistochemistry, polymerase chain reaction (PCR) and Xpert MTB/RIF assay.

While culture is the classical gold standard for TB diagnosis, with regards to EPTB its efficiently is questionable due to increased technical and logistical constraints in EPTB cases (Norbis et al., 2014). Culture sensitivity and specificity has been report to be as low as 47 and 75% respectively in EPTB. In EPTB culture, mycobacteria are thought to grow in only about 39–80% of cases (Prakash and Reiman, 1985, Baqui et al., 2018). This is because culture requires at least 101–102 bacilli/ml of sample to yield positive result which is often difficult to get as some specimens are needs to be collected in deep seated organs (Chakravorty et al., 2005, Sevgi et al., 2013).

Histopathology and immunochemistry are one of the vital techniques for diagnosis of EPTB. These techniques involve histomorphological analysis of EPTB sample and chemistry analysis of fluid from EPTB specimens (Norbis et al., 2014). Histologic findings such as typical caseous granuloma may be highly suggestive of tuberculosis, however histopathology and immunochemistry tests cannot provide bacterial confirmation therefore, requires to be used along other diagnostic tests or with a strong clinical suspicion (Chakravorty et al., 2005, Baqui et al., 2018). The PCR is probably the most modernized method for diagnosis of EPTB. The Xpert MTB/RIF assay test simultaneously identify *M. tuberculosis* complex and also can identify resistance to RIF in less than 2 hours. So far, the Xpert MTB/RIF has reported higher specificity such as 99.8% and higher sensitivity of 81.3%.

Most recently a developed next-generation Xpert MTB/RIF Ultra (GX-Ultra; Cepheid) assay aimed to overcome the limitations of Xpert MTB/RIF assay by increasing the sensitivity for detection of *M. tuberculosis* DNA when few bacilli are present in a clinical specimen (Perez-Risco et al., 2018). The GX-Ulta is a new and fully automated nested real-time PCR assay differs from Xpert MTB/RIF Assay in several ways: the larger PCR chamber with a total capacity of 50 µl, in contrast to 25 µl in the previous cartridge, the incorporation of two different multicopy targets that is IS*1081* and IS*6110* insertion sequences, and the optimization of PCR and thermal-

cycling parameters (Perez-Risco et al., 2018). The results are provided automatically in 77 min if the genetic material is amplified or in 66 min if it is not. The system classifies *M. tuberculosis* detection in the following semi quantitative results: high, medium, low, very low, and a new category named trace, and it classifies RIF resistance as detected, not detected, or indeterminate. Table 2.2 shows sensitivities of GX-Ultra for different specimens' groups were as follows: 94.1% in lymph nodes, 93.7% in nonsterile fluids, 86.6% in tissue specimens, 80% in stool material, 64.7% in abscess aspirates, and 60.5% in sterile fluids. Ten of 21 (47.6%) pleural fluid samples were GX-Ultra positive for the detection of *M. tuberculosis* DNA. GX-Ultra presented a specificity of 100% for the 40 clinical specimens with a negative *M. tuberculosis* culture and the 20 that were Non tuberculous Mycobacteria culture-positive.

Nevertheless, the sensitivity and specificity of these diagnostics methods differs from one EPTB specimen to the next and for that the search for "Gold standard method for EPTB" is still on-going (Norbis et al., 2014; Baqui et al., 2018). To date the diagnosis of EPTB still lies in integration of clinical medicine, microbiology and other diagnostic modalities, and a team of experts (Norbis et al., 2014; Lee, 2015; Baqui et al., 2018). Table 2.3 shows the most common EPTB clinical presentations, associated specimens and methods used for their diagnosis.

Table 2.2: Results obtained by the Xpert MTB/RIF Ultra assay according to the source and *M. tuberculosis* culture of the specimens (Perez-Risco et al., 2018).

Clinical sample	Total no. of samples	Samples MTUBC culture positive		Sensitivity (%)	Samples MTUBC culture negative		Specificity (%)
		GXU+[a]	GXU−[b]		GXU+	GXU−	
Sterile fluids	44			60.5			100
Pleural fluid	24	10	11	47.6	0	3	
Cerebrospinal fluid	4	3	0	100	0	1	
Joint fluid	9	7	1	87.5	0	1	
Ascitic fluid	3	1	2	33.3	0	0	
Pericardial fluid	4	2	1	66.6	0	1	
Nonsterile fluids	29			93.7			100
Gastric aspirate	5	3	1	75	0	1	
Urine	24	12	0	100	0	12	
Lymph nodes	25	16	1	94.1	0	8	100
Abscess aspirates	20			64.7			100
Cervical abscess	6	4	1	80	0	1	
Skin abscess	6	2	2	50	0	2	
Paravertebral abscess	3	2	1	66.6	0	0	
Osteitis pus	5	3	2	60	0	0	
Tissues	24			86.6			100
Skin biopsy	8	2	0	100	0	6	
Intervertebral disc biopsy	2	2	0	100	0	0	
Bone biopsy	4	2	0	100	0	2	
Pleural biopsy	2	2	0	100	0	0	
Rectal biopsy	1	0	1	0	0	0	
Costal cartilage biopsy	1	1	0	100	0	0	
Liver biopsy	1	1	0	100	0	0	
Cervical tissue	1	1	0	100	0	0	
Mediastinal tissue	1	0	1	0	0	0	
Synovial tissue	3	2	0	100	0	1	
Joint biopsy	18	0	0		0	18	
Stool	8	4	1	80	0	3	100
Total	168	82	26	75.9	0	60	100

[a]GXU+, positive results from GeneXpert MTB/RIF Ultra.
[b]GXU−, negative results from GeneXpert MTB/RIF Ultra.

Table 2.3: Most common EPTB forms, associated specimens and methods used for their diagnosis (Norbis et al., 2014).

Forms of EPTB	Laboratory specimens	Laboratory tests
Lymphatic TB	Material include draining sinus of FNA specimen or biopsy	C/M: Culture, microscopy on FNA and biopsy specimens, Histology on granulomatous inflammation Molecular (Xpert MTB/RIF) tests on FNA and biopsy specimens (PCR),
Pleural TB	Pleural fluid aspirates	C/M: Microscopy and culture perform poorly unless tuberculous emphysema or pleural biopsy specimens are used, molecular tests (PCR): Xpert MTB/RIF Histology: exudative (proteins >5 g/dl, high LDH levels, lymphocyte predominance, > 50% scarce mesothelial cells, ADA IN Pleural Fluid: if > 40 U/l in lymphocytic pleural effusion a suggestive of TB pleurisy, if < 40U/l a not likely TB
CNS TB (TBM)	CSF	C/M: Microscopy and culture: Microscopy on concentrated CSF useful for rapid diagnosis Cytology/histology: leucocytosis (>lymphocytes), proteins, CSF/plasma glucose ratio < 50% ADA on CSF: not recommended for routine diagnosis, IGRA: does not distinguish TB infection from TB disease

Bone and Joint TB	Lesions biopsies, FNA	C/M: Microscopy and culture on FNA specimens Histology: Biopsies on necrotizing epithelioid cells

2.7.2 Extrapulmonary TB Treatment Guidelines

Early treatment and appropriate diagnosis are keys to treatment and management of EPTB. However, WHO guidelines recommends that pulmonary and extrapulmonary disease should be treated with the same regimens (World Health Organization, 2010). Literature shows that the treatment of EPTB differs enormously in between countries and it depends on the health care resources and epidemiological situation in each country (Fuentes and Caminero, 2006, Houston and Macallan, 2014, Norbis et al., 2014, Lee, 2015a). As such, the treatment of EPTB remains controversial.

To add, the ability of the blood-brain barrier to limit intracerebral concentrations of anti-TB drugs should be taken into consideration particularly when managing TB meningitis individuals. This is because isoniazid, pyrazinamide, protionamide, and cycloserine penetrate are best known for their well penetration into CSF and the opposite while, ethambutol and p-aminosalicylic acid cannot penetrate the blood-brain barrier. Rifampicin, streptomycin, and kanamycin on the other hand are thought to be able to penetrate the CSF well only in the presence of meningeal inflammation (Nau et al., 2010, Cabrera-Maqueda et al., 2018). Furthermore, the optimal duration of therapy is still up for debate. In general, 6 months of standard anti-TB therapy is recommended for most forms of EPTB while the treatment duration is longer for TB meningitis and bone and joint TB (Duo et al., 2011, Lee, 2015). However, some experts prefer evidence of radiological or pathological evidence of regression of disease occurs otherwise the duration of may be more than 12 months (Lee, 2015).

The diagnostic challenges of EPTB and lack of clear treatment guidelines can results in the emergency of drug resistance. Emergence of drug resistance is a major drawback in the fight to eliminate TB as it severely complicates the situation especially in TBM cases. One study reported that a two-fold increase in mortality was observed recently among the isoniazid drug resistant TBM cases (Verdon et al., 1996, Vinnard et al., 2011). Another study on drug resistance in Korea reported that the overall resistance rate to at least one anti-TB drug was 8.9%, and multidrug resistance rate was 1.8% among, however, this was slightly lower than those in the

entire PTB patients (Cho et al., 2011). Extrapulmonary TB studies on drug resistance are on the rise and notable more studies from different parts of the world are reporting predominance isoniazid resistance among EPTB cases especially in TBM cases (Rock et al., 2006, Duo et al., 2011, Lai et al., 2011, Vinnard et al., 2011).

2.8 MOLECULAR EPIDEMIOLOGY OF *MYCOBACTERIUM TUBERCULOSIS*

Molecular epidemiology studies aim to study the diversity and relatedness of various *M. tuberculosis strains* through genotypic characterization. Molecular characterization of *M. tuberculosis* has been employed for the past few decades to study the diversity and relatedness of various *M. tuberculosis* strains. Molecular epidemiology studies include the identification of transmission chains and outbreaks, classification of *M. tuberculosis* strains into families and lineages to determine their population structure, differentiation of episodes of exogenous re-infection and/or relapse (Yaganehdoost et al., 1999, van Embden et al., 1993, van Rie et al., 1999, Weis et al., 2002, Sola et al., 2001, Stavrum et al., 2009, Sankar et al., 2013). Most recently, molecular characterization of *M. tuberculosis* strains has made it possible to better understand the occurrence of mixed infections and bacterial subpopulations in an individual (Shamputa et al., 2004, Warren et al., 2004, Streit et al., 2015, Ssengooba et al., 2015, Cohen et al., 2016). Lastly, molecular characterization has been key in the identification of the presence of heterogeneity involving drug-resistance mutations in patients' sputum isolates (Post et al., 2004, Kaplan et al., 2003, Cohen et al., 2011).

2.8.1 *Mycobacterium tuberculosis* Lineage and Site of Disease

The bacterial determinants of TB disease as extrapulmonary versus pulmonary are not well documented. Studies directed at understanding both host and pathogen determinants of clinical disease are emerging (Click et al., 2012). Important differences have been documented in experimental models, however, the relevance of different genetic subgroups of *M. tuberculosis* to clinical disease in humans remains a widely speculation. Understanding the relationship Between *Mycobacterium tuberculosis* lineage and clinical site of TB has significant implications for

vaccine development, treatment, and diagnosis (Gagneux et al., 2007). Available studies aimed at establishing the association between certain *M. tuberculosis*

lineages and EPTB and PTB have shown conflicting results and are often limited by their relatively small size and phylogenetic diversity (Click et al., 2012). The East-African Indian lineage or Central Asian lineage has been previous associated with EPTB in a population-based study of 1009 cases in Italy. Additionally, the Beijing family or East Asian lineage has been associated pulmonary rather than extrapulmonary disease in two studies (Lari et al., 2009, Click et al 2012). However, two studies in Cape Town and among Vietnamese adults could not find any association between Euro-American lineage and meningeal tuberculosis (Nicol et., 2005, Caws etal., 2008).

Molecular characterization methods include but not limited to: the IS*6110*-based restriction fragment length polymorphism (IS*6110*-based RFLP), spacer oligonucleotide genotyping known as spoligotyping, mycobacterial interspersed repetitive-unit-variable-number of tandem repeats (MIRU VNTR), and most recently, whole genome sequencing. Other molecular methods include the polymorphic GC rich repetitive sequence analysis (PGRS) and single nucleotide polymorphism (SNP) analysis, however these will be discussed briefly. The IS*6110*-based RFLP is the gold standard for the typing of *M. tuberculosis* strains, however a combination of MIRU VNTR and spoligotypying have been reported to be as accurate as the gold standard method (Oelemann et al., 2007).

2.8.1 IS*6110*-Based Restriction Fragment Length Polymorphism

IS*6110*-based Restriction Fragment Length is the current gold standard that is used for genotypic characterization of *M. tuberculosis* strains. The IS*6110*-RFLP method analyses the distribution and variability in chromosomal positions of the insertion sequence IS*6110* in different *M. tuberculosis* strains. The IS*6110* element sites are distributed randomly throughout the genome of *M. tuberculosis* strains and copy numbers may vary between 0 to 26 copies per strain (Kurepina et al., 1998). Therefore, *M. tuberculosis* strains that are epidemiologically linked are

thought to have identical IS*6110* RFLP banding patterns, whereas strains that are not epidemiologically linked will have different RFLP banding patterns. In terms of transmission, this means individuals with *M. tuberculosis* strains with identical RFLP patterns have had direct contact with each other or contact through a common source (Barnes and Cave, 2003). As a result, identical RFLP patterns are indicative of recent transmission which may signify an outbreak. *Mycobacterium tuberculosis* strains with less than six IS*6110* insertion elements are referred to as "low-copy number" strains and have a limited degree of polymorphism. As a result, other genotyping methods are recommended (Bauer et al., 1999).

Limitations of this method includes a long turnaround time and the tedious nature. IS*6110* is culture depended and a result has a turnaround time estimated around 30 to 40 days and a viable culture is required to generate an accurate genotype (Rhee et., 2000). The IS*6110* is technically more complicated compared to other fingerprinting methods. Furthermore, IS6110 cannot be used to reliably type and discriminate between strains with less than six IS*6110* copies (Gutacker et al., 2006). Other studies have shown that strains that have less than six IS*6110* bands may display identical banding patterns or unique fingerprinting patterns using IS*6110* RFLP whereas other fingerprinting methods indicated that these strains are genotyping different (Rhee et al., 2000, Bauer et al., 1999).

2.8.2 Spoligotyping Assay

Spacer oligonucleotide genotyping known as "spoligotyping" is the most commonly used PCR-based method for genotyping and differentiating between MTBC strains (Kamerbeek et al., 1997, Brudey et al., 2006, Roetzer et al., 2011). Members of the MTBC contain a chromosomal region that has 10 to 50 copies of a 36bp direct repeat (DR) sequence which is punctuated by spacer DNA sequences. These spacer sequences that occur in-between any two DRs are thought to be conserved within strains (van Embden et al., 1993). Therefore, the principle of this method is based on the composition of the DR locus in MTBC strains which contains a variable number of both DRs as well as the presence or absence of various spacer sequences.

Spoligotyping method uses visualization of 43 interspersed spacer sequences existing in the genomic DR locus of MTBC strains. By means of two inversely oriented primers complementary to the sequences of the DRs thus amplifying the DNA sequences located in-between two adjacent DR sequences, the DR region of unknown strain is amplified resulting the detection of the 43 spacers within a mixture of a large number of different size fragments (Kamerbeek et al., 1997; Roetzer et al., 2011). The reverse primer is biotin labelled to ensure all the reverse strands synthesised are biotin labelled and the hybridised products are ultimately visualised using streptavidin peroxidase and enhanced chemiluminescence (Kamerbeek et al., 1997).

The spoligotyping method as compared to the IS*6110* RFLP includes: (i) the benefit that only small amounts of DNA are required for spoligotyping, and (ii) that the results of this typing method can be expressed as positive or negative for each spacer, which are easily conveyed in a digital format. While, this method is simple, robust and powerful, it has a less discriminatory power compared to the IS*6110*-based RFLP, the gold standard. As a result, its application in molecular epidemiology studies should be compensated with a secondary method such MIRU VNTR method (Oelemann et al., 2007; Roetzer et al., 2011; Said et al., 2012).

2.8.3 MIRU VNTR Typing Assay

The genome of members MTBC contains numerous interspersed repeat units (MIRUs) which constitute a variable number of tandem repeat sequences (VNTR) that are scattered throughout the genome. Of the 41 MIRUs previously described, 12 MIRU loci were selected for the genotyping of clinical *M. tuberculosis* strains (Supply et al., 2000). For epidemiology, a 15-locus set was proposed and while for phylogenic studies a 24-locus was set (Supply *et al*., 2006). The MIRU VNTR uses PCR and gel electrophoresis to categorise the number and sizes of the repeats in each of the 12 (or more) independent loci, each of which have a unique repeated sequence (Supply et al., 2000; Barnes and Cave, 2003).

The results obtained from the method are then captured in a twelve-digit format. This allows MIRU link the results into a computer database thus allowing for intra- and inter-laboratory

comparisons of strains. The discriminatory power of MIRU VNTR depends on the number of loci evaluated. Compared to the IS*6110*, MIRU VNTR method is technically simpler to perform than IS*6110* and that it can be performed directly on the cell lysate (Supply et al., 2001). Stutter peaks are common during the genotyping of short tandem repeat sequences and mostly reflect art factual strand slippage of the polymerase during PCR and these are carefully analysed using the GeneMapper which allows to edit samples. Stutter peaks can be easily seen because they appeared as a ladder of much-lower-intensity peaks, corresponding to sizes of PCR fragments that lack one or more repeats (Figure. 2.1). Furthermore, MIRU VNTR can be automated and therefore be used as a high throughput technique for *M. tuberculosis* genotyping (Supply et al., 2001, Said et al., 2012).

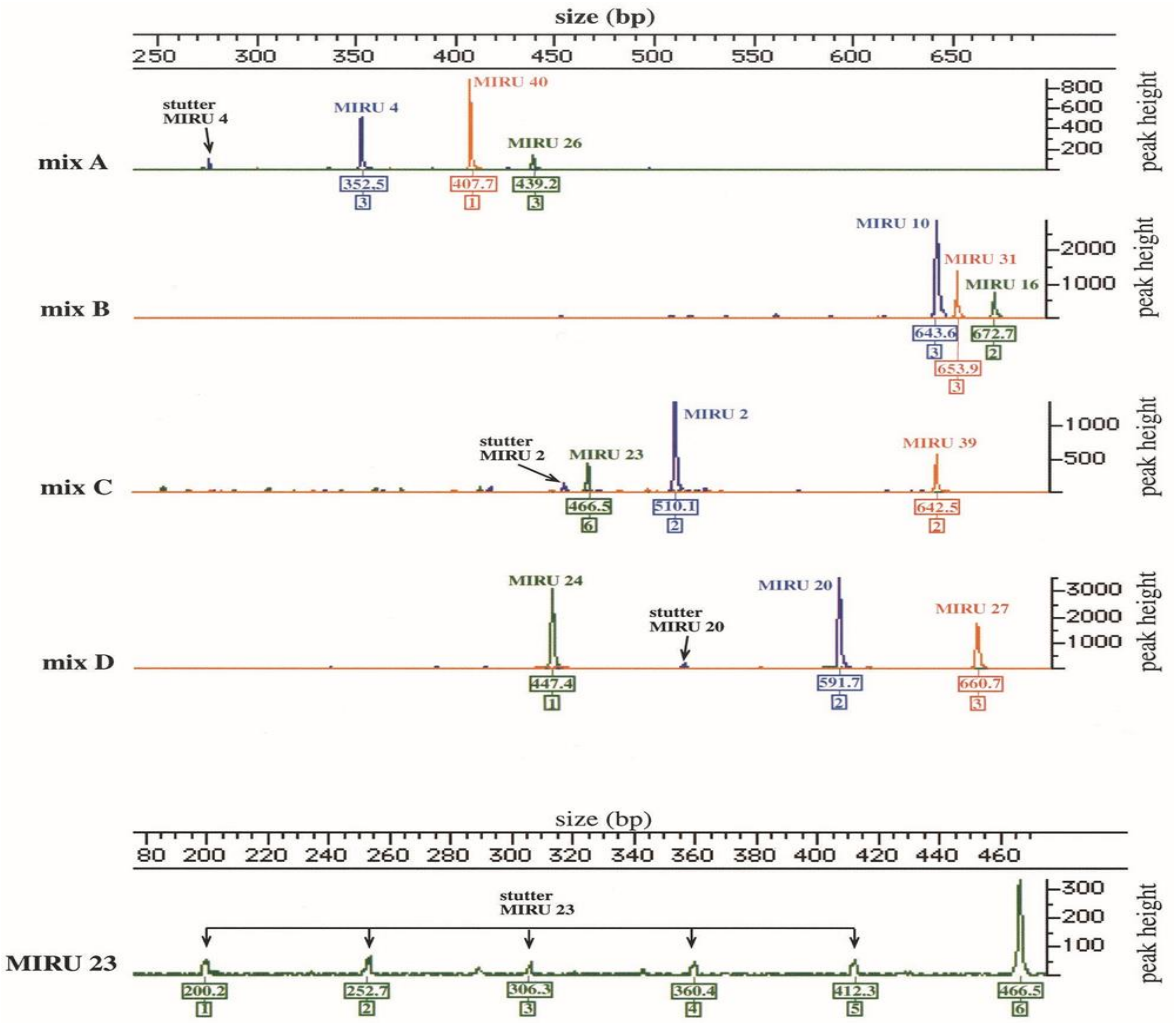

Figure 2.6: Multiplex PCR analysis of the 12 MIRU VNTR loci PCRs were performed on genomic DNA from *M. tuberculosis* H37Rv with a detailed view of locus 23 (bottom). Boxed numbers below are signal peaks, calculated sizes (in base pairs) of the labelled PCR products and the corresponding identified MIRU VNTR alleles. Positions of stutter peaks, easily diagnosed as low-intensity PCR artifacts, are shown (Supply et al., 2001)

2.8.4 Whole Genome Sequencing

Whole genome sequencing (WGS) has higher resolution than traditional typing methods, such as spoligotyping, mycobacterial interspersed repetitive units (MIRUs), including the gold standard (Gardy et al., 2011, Walker et al., 2015, Ssengooba et al., 2016, van Soolingen et al., 2016). In a case of an outbreak investigation, it is recommended that if the traditional method gives a result of 'different strain', WGS is not necessary to answer the clinical question however, if the traditional typing method gives a matched pattern, WGS may be required to confidently distinguish a related strain due to ancestry from a true match (Roetzer et al., 2013, Lee and Behr, 2016). The latter is normally observed during laboratory cross-contamination or relapse. Although, WGS can improve our understanding of the within-host complexity of infections and the dynamic response of these mycobacterial populations under the selective pressure of treatment (Cohen et al., 2016). However, this assay is associated with substantial financial costs and technical infrastructure. Therefore, its usage maybe limited epidemiological studies and drug resistance at reference laboratories.

2.8.5 Polymorphic GC Repetitive genotyping

Polymorphic GC rich repetitive sequence (PGRS) is a Southern blot hybridisation technique used to genotype *M. tuberculosis* strains. This method uses PGRS-specific probes cloned in a plasmid PTBN12 and was first described in 1992 (Ross et al., 1992). Since then, the PGRS has been used to further characterize strains that display identical IS*6110* RFLP patterns particularly strains with IS*6110* low-copy-numbers (Yang et al., 1996, Rhee et al., 2000, Chaves et al., 1996). However, this method is resource intensive and raw data from this method is complex to be entered and standardised on a computer database.

2.8.6 Single –Nucleotide Polymorphisms

Synonymous single-nucleotide polymorphisms (sSNP) and nonsynonymous single nucleotide polymorphisms (nsSNP) are able to provide genetic data that can be used to discriminate different *M. tuberculosis* strains. The nsSNP analysis results in change in amino acid and as a results it's subject to selective pressures such as antibiotics (Mathema et al., 2006). Therefore, nonsynonymous single nucleotide polymorphisms can indicate the nature and spread of drug resistance strains within populations. The sSNP analysis on the other hand do not change the encoding amino acid. The sSNP analysis is useful to study genetic drift and evolutionary relationships amongst different *M. tuberculosis* strains (Mathema et al., 2006).

2.9 GENETIC HETEROGENEITY AND HETERO-RESISITANCE IN *MYCOBACTERIUM TUBERCULOSIS*

Molecular epidemiology studies that documented the phenomenon of mixed infections or superinfection, a scenario in which more than one strains of *M. tuberculosis* are retrieved from an individual host at a single point in time, highlighted the importance of reinfection in tuberculosis epidemics. However, this phenomenon of mixed infections is not limited to *M. tuberculosis* but appears to occur with pathogens of all types. The occurrence of mixed infections and hetero-resistance has been documented in other micro-organisms including *Cryptococcus neoformans*, *Gardnerella vaginalis*, *Helicobacter pylori* and *Pseudomonas aeruginosa*, *Staphylococcus aureus* (Ryffel et al., 1994, Weel et al., 1996, Mondon et al., 1999).

2.9.1 Heterogeneity and Hetero-resistance in *Mycobacterium tuberculosis*

Heterogeneity and hetero-resistance in *Mycobacterium tuberculosis* was formally thought to be hidden. However, in the advent of robust of molecular genotypic tools more epidemiological studies are emerging confirming presence of heterogeneity while hetero-resistance is limited by current available diagnostic tool especially phenotypic hetero-resistance. It is well known that acquisition of resistance reduced growth rate and therefore, biological fitness limits occurrence of heteroresistance especially in liquid culture unless solid culture is used (Rinder, 2001). In

addition, the decontamination step during processing of either liquid or solid culture is known to significantly reduce *M. tuberculosis* diversity in clinical samples (Martin et al., 2010, Cohen et al., 2012).

Heterogeneity of *M. tuberculosis* refers to TB infection caused by at least more than one clonally distinct *M. tuberculosis* strain and this thought be as the result of two mechanisms: 1) the appearance of a new strain that diverged from a pre-existing persistent *M. tuberculosis* clone or through accumulation of mutations ; and 2) within-host heterogeneity of *M. tuberculosis* can arise from subsequent infectious episode caused by a distinct strain that may result in relapse of the original infection, giving rise to a disease with two unique *M. tuberculosis* strains that may have the same or different drug susceptibility profiles (Shamputa et al., 2004, Cohen et al., 2012, McIvor et al., 2017). While within host heterogeneity in PTB is well documented in literature, the scant literature on occurrence of with host heterogeneity in EPTB especially rare, but important cases in patients is having concomitant extrapulmonary and pulmonary TB disease.

Within host heterogeneity of *M. tuberculosis* in patients with PTB studies has been associated with adverse outcomes such as drug resistance, relapses and high mortality rate (Shamputa et al., 2004, Zetola et al., 2014, Ssengooba et al., 2015, Cohen et al., 2016). Different *M. tuberculosis* strains have different virulence factors; hence the presence of multiple *M. tuberculosis* strains may significantly implicate clinical management of patients. The Beijing and Latin-America-Mediterranean (LAM) families are thought to be the most virulent circulating strain among the *M. tuberculosis* families (Stavrum et al., 2009, Cooke et al., 2011). The Beijing family has been closely associated with multi-drug resistance (MDR) globally while the LAM 4 family was implicated in the international spread of MDR TB from Tugela ferry in Kwa-Zulu natal, South Africa (Cookie et al., 2011).

Hetero-resistance has no formal accepted definition. The term heter-oresistance is generally associated with the occurrence of populations of both drug-susceptible and drug-resistant isolates within the same clinical sample (Rinder 2001; McIvor et al., 2017). Based on this definition,

hetero-resistance is thought to arise when *M. tuberculosis* single infection undergoes genetic change through mutation of genes associated with drug resistance. In addition, hetero-resistance may also arise in multiple infections in which one strain is resistant to a particular TB drug, while the other strain is susceptible (McIvor et al., 2017). This definition is based on studies focused solely on heterogeneity of sputum specimens or at least specimens collected from one anatomical site. Moreover, it does not take into considerations that different clinical specimens from different anatomical specimens may present heterogeneous DST results (Gadhi et al., 2006).

2.10 SUMMARY

EPTB is heterogeneous in nature with regards to its clinical presentations and it can potentially affect any organ in the body. It remains unknown whether DST profiles of specimens collected from concomitant PTB and EPTB are the same. The presence of diverse genotypic *M. tuberculosis* strains within a single host often results in conflicting phenotypic and molecular DST results. One case study reported that an MDR strain was isolated from a sputum specimen while a susceptible strain was isolated from a CSF of the same patient (Bernard et al., 2014). Such conflicting laboratory results can cause a dilemma in the management of patients. In addition, pulmonary and extra-pulmonary specimens from the same host can have distinct strains with different phenotypic and molecular characteristics. One genotypic characterization study involving both pulmonary and extra-pulmonary specimens reported that in 3/50 (6%) concomitant patients were heterogeneous in the cohort study (de Viedma et al., 2003). The authors hypothesised that different strains may be involved in the respiratory infection, however after the course of infection some strains may be more adapted to disseminate or have tropism certain body organs while the other strains remain at the pulmonary site, leading to compartmentalization of the infection. Furthermore, the pathogenicity of EPTB is mostly thought to seed from the haematogenous or lymphatic dissemination of *M. tuberculosis* strains from the lung parenchyma to other organs the assumption would be that the genotypic and phenotypic characteristics of the dissemination bacilli should be identical to that of the parent strain in the

lung parenchyma. However, it remains unknown if this assumption stands as molecular epidemiology studies show that a single host can be repeatedly infected with different *M. tuberculosis* strains (van Rie et al., 1999, Lambert et al., 2003).

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CHAPTER THREE:

GENOTYPIC AND PHENOTYPIC DIVERSITY OF *MYCOBACTERIUM TUBERCULOSIS* STRIANS IN PATIENTS WITH CONCOMITANT PULMONARY AND EXTRA-PULMONARY TUBERCULOSIS

In the following chapter, the editorial and referencing style of The Journal of Infectious Diseases was followed.

ABSTRACT

Background: Genetic heterogeneity of *Mycobacterium tuberculosis* (*M. tuberculosis*) in patients with pulmonary tuberculosis (TB) has been associated with adverse outcomes. This holds true for diversity of *M. tuberculosis* genotypes as well as diversity in drug resistance profiles of bacilli occurring at the same time in a single patient. It remains unknown whether heterogeneity of infection also occurs in patients with concomitant extrapulmonary TB (EPTB) and pulmonary TB (PTB). We performed genotypic and phenotypic analysis of *M. tuberculosis* strains isolated from patients with both forms of TB to determine the rate of genotypic concordance and heterogeneity as well as the occurrence of phenotypic and genotypic hetero-resistance.

Materials and Methods: Patients with microbiologically confirmed extrapulmonary and pulmonary TB within a one-month period were identified in the laboratory information system; we included patients with *M. tuberculosis* isolates from blood or bone marrow, cerebrospinal fluid, lymph node and pleural fluid as EPTB specimens. Strains were retrieved retrospectively from the biobank at the National TB Reference Laboratory of the Eswatini Health Laboratory Services in Eswatini (n = 17) and collected prospectively at the Tshwane Academic Division of the National Health Laboratory Services in South Africa (n = 15). Drug susceptibility for isoniazid and rifampicin were determined using the First Line - Line Probe Assay (FL-LPA) and MGIT system. Genotyping of strains was done using spoligotyping and standard 24-loci

mycobacterial interspersed repetitive units (MIRU) variable number of tandem repeats (VNTR) analysis. Dendrograms were generated to determine genetic relatedness.

Results: A total of thirty-two patients with concomitant extrapulmonary and pulmonary TB were included in this analysis. The most prevalent lineages were lineage four (Euro-American: n = 40, 63%) followed by Lineage two (East Asian: n = 20, 31%) and lineage three (East African Indian: n = 4, 6.3%). Genotypic analysis showed that 94% (30/32) had *M. tuberculosis* strains with the same spoligo type and MIRU VNTR pattern isolated from each body site. Heterogeneous infection was identified in 4 (12%) patients. Two (6%) patients showed presence of discordant strains: in both patients their extrapulmonary isolates showed presence of T1 strains and while unknown spoligo types were isolated from their pulmonary isolates; MIRU VNTR profiles were also discordant however, drug susceptibility patterns on FL-LPA and MGIT were the same. In another two patients (6%), their extrapulmonary and pulmonary isolates showed presence of the same genotype however, their drug susceptibility patterns showed presence of hetero-resistance.

Conclusions: This study confirms that heterogeneity of infection occurs in patients with concomitant extrapulmonary and pulmonary TB in a setting of high incidence where patients may present with two overlapping infections at the same time. The rate at which the genetic and phenotypic heterogeneity occurs suggests that presence of different genotypes in different samples may be clinically relevant and therefore, microbiological testing of specimens from both body sites in these patients is highly recommended. Our cohort study, moreover add to the scant literature which challenges the old dogmas related to TB immunity, pathogenesis and progression from latent to active disease. In future, more robust or higher-resolution genomic typing tools, such as target Next Generation Sequencing (t-NGS) or metagenomics analysis are recommended to improve our understanding of the complexity of infections and for detection of both minority and major variants within pulmonary and extrapulmonary specimens for better estimation of the extent of *M. tuberculosis* genotypic and phenotypic heterogeneity.

Keywords: *Mycobacterium tuberculosis*, heterogeneity, extrapulmonary tuberculosis, pulmonary tuberculosis, concomitant infection, hetero-resistance, genotyping analysis, genotypes, *M. tuberculosis* lineages, drug susceptibility testing

3.1 BACKGROUND

Tuberculosis (TB) recurrences were previously thought to be caused by reactivation of endogenous infection rather than by a new, exogenous infection. However, characterization of the genotype of *Mycobacterium tuberculosis* (*M. tuberculosis*) by DNA fingerprinting studies have shown that some of these recurrences represent reinfection with a different strain from the first episode as opposed to treatment failures [1, 2]. In high TB burden settings with diverse *M. tuberculosis* genotypes a single host may be repeatedly infected with different *M. tuberculosis* genotypes [3]. To date, the occurrence of extrapulmonary TB (EPTB) is considered to be caused by the same genotype that infect and cause disease at the pulmonary site [4, 5].

Genetic heterogeneity of *M. tuberculosis* genotypic strains causing infection in both HIV infected and HIV un-infected patients and from both high and low TB incidence settings is well documented [6-9]. While heterogeneity of infection in pulmonary TB (PTB) is well documented in literature, it remains unknown whether heterogeneity of infection also occurs within a host concomitant with both extrapulmonary and pulmonary TB. Moreover, the proportions of patients with mixed-strain infections among HIV-infected individuals have been estimated to be as high as 38%, and 17% among those without HIV infection, which could possibly imply that impaired cell-mediated immunity may increase the probability of harboring multiple infections [3]. In addition, genetic heterogeneity of *M. tuberculosis* in patients with PTB has been associated with adverse outcomes such as drug resistance, relapse and increased mortality rate [6, 9, 10]. This holds true for diversity of *M. tuberculosis* genotypes as well as diversity in drug resistance profiles of bacilli occurring at the same time in a single host [8, 9, 11].

Mycobacterium tuberculosis strains involved in heterogeneity infections may consequently have different susceptibility patterns, requiring treatment to be adjusted to the most resistant strain. However, the most resistant strain can be missed if only one body compartment is sampled. One case study reported a patient with simultaneously infection with a multi drug resistant (MDR) strain and a susceptible strain, isolated from a sputum and cerebrospinal fluid (CSF) specimens

respectively. Moreover, genotypic analysis showed different genotypes for the lung MDR and the CSF-susceptible isolates [11]. *Mycobacterium tuberculosis* genotypic strains have different virulence factors; hence the presence of diverse *M. tuberculosis* genotypic strains may have a negative impact in clinical management of affected patients. Animal model studies show that different strains differ in their severity ability to cause disease, and in humans a subset of the Euro-American *M. tuberculosis* lineage was found to be less common in TB meningitis than in pulmonary TB, suggesting an interaction between bacterial genotype and clinical phenotype [12].

Within-host heterogeneity of *M. tuberculosis* refers to TB infection caused by at least more than one clonally distinct genotypes of *M. tuberculosis* strain and this is thought to be a result of one of at least two mechanisms. Firstly, within host heterogeneity can be explained as the appearance of a new strain that diverged from a pre-existing persistent *M. tuberculosis* clone or through accumulation of mutations [3, 5]. Secondly, within-host heterogeneity of *M. tuberculosis* can arise from subsequent infectious episodes caused by a distinct strain that may result in relapse of the original infection, giving rise to a disease with two unique *M. tuberculosis* genotypic strains that may have the same or different drug susceptibility patterns [3, 5].

Characterization of the genotype of *M. tuberculosis* by DNA fingerprinting can show whether a new episode of the disease is caused by infection with the same strain that caused a previous episode or by a different strain [2]. To assess heterogeneity of *M. tuberculosis* infection the IS*6110*-based restriction fragment length polymorphism (IS*6110* RFLP) analysis, spacer oligonucleotide genotyping (spoligotyping) and the mycobacterial interspersed repetitive unit-variable-number of tandem repeats (MIRU VNTR) typing can be used. The IS*6110* RFLP analysis is the gold standard for genotypic characterization of *M. tuberculosis* strains, however available literature shows that this assay is cumbersome and has low discriminatory power among strains with less than six IS*6110* copies [13-16]. The combination of spoligotyping and MIRU VNTR has been shown to be less cumbersome and has same discriminatory power as gold standard [16, 17].

As genetic heterogeneity of *M. tuberculosis* infection affects treatment outcomes, we assessed its occurrence in patients with concomitant extrapulmonary and pulmonary TB. In this study, we performed genotypic and phenotypic analysis of *M. tuberculosis* strains isolated from patients with both forms of TB to determine the rate of genotypic concordance and heterogeneity as well as the occurrence of phenotypic and genotypic hetero-resistance.

3.2 METHODS

3.2.1 Study design and Settings

In this study we included patients with the EPTB as diagnosed by positive culture of *M. tuberculosis* from Cerebrospinal Fluid (CSF), pleural fluid, blood or bone marrow aspirate, and lymph node. These patients were identified from two Laboratory Information Systems (LIS) : 1) Using DISA LIS to retrieve isolates retrospectively from the biobank at the National TB Reference Laboratory (NTRL) of the Eswatini Health Laboratory Services (EHLS), Eswatini (n = 17), and 2) were identified and collected prospectively through the TrackCare LIS at the National Health Laboratory Service, Tshwane Academic Division (NHLS/TAD) in the Department of Medical Microbiology University of Pretoria, South Africa (n = 15) (Table 3.2). The NTRL of Eswatini is the only diagnostic laboratory that receives specimens for microscopy, culture and drug susceptibility testing from all four administrative regions in Eswatini and the NHLS/TAD is a high throughput diagnostic laboratory that receives specimens for microscopy, culture and drug susceptibility testing from surrounding clinics and hospitals in the Gauteng Province, South Africa. Once patients with these four types of EPTB were identified, we searched for availability of concomitant sputum specimens and, if available, the results of microbiological testing (MGIT culture, First Line – Line Probe Assay (FL-LPA) and MGIT drug susceptibility testing [DST] data) was collected. Patients were included in further analysis if *M. tuberculosis* isolates were available for both forms of TB, and if these specimens had been obtained less than 30 days apart and currently not on treatment.

3.2.1 Ethical Consideration

This study was approved by the National Health Research Review Board of Eswatini (13/04/2017) and the Research Ethics Committee of the University of Pretoria (115/2017) (Appendix B and C).

3.2.2 *Mycobacterium tuberculosis* isolates from patients with concomitant infection

Microbiologically confirmed *M. tuberculosis* isolates from 32 individual patients were selected in this analysis as obtained from the following extrapulmonary anatomical sites: CSF (n = 1), pleural fluid (n = 17), bone marrow aspirate (n = 4), and lymph node (n = 10). Patients with concomitant culture-positive pulmonary specimens collected more 30 days apart or were currently on treatment were excluded from the study.

3.2.3 Laboratory Methods

The culture isolates were transported to the Centre for TB (CTB) division of the National Institute for Communicable Diseases (NICD) and for spoligotyping (Ocimum Biosciences, India) and mycobacterial interspersed repetitive units–variable number of tandem repeats [MIRU VNTR] (Genoscreen, Institute Pasteur, Lille, France) typing. This laboratory is WHO collaboration centre and therefore forms part of the WHO TB Supranational Reference Laboratory Network (SRLN).

3.2.3.1 Genomic DNA Extraction

Mycobacterial DNA extraction was performed using the automated NucliSENS easy MAB 2.0 platform with NucliSens magnetic extraction reagents (bioMérieux) according to manufacturer's instructions. This is nucleic extraction method is based on Boom Chemistry using magnetic silica particles [18]. Briefly, 2 mL liquid medium-based Bactec Mycobacteria Growth Indicator Tube (MGIT) (Becton Dickinson, Franklin Lakes, NJ, USA) was aspirated from into 2-mL Eppendorf tubes and centrifuged at 4000 rpm g for 20 minutes. At least 1.8 supernatant was discarded, and

three glass beads were added to the mycobacterial pellets, incubated at 80°C for 20 minutes. The pellets were left at ambient temperature overnight. Five hundred microlitres of the pellet were pipetted into the NucliSENSEsense EasyMAG cartridges (bioMérieux) and the generic protocol with pellet input of 500ul, 50ul magnetic beads and final elution of 100ul. The final elution was stored at −80°C freezer for further analysis.

3.2.3.2 Spoligotyping of *M. tuberculosis*

Spoligotyping was performed using a spoligotyping kit according to the manufacturer's instructions (Ocimun Bio Solutions, India). The oligonucleotides DRa and DRb (Ocimun Bio Solutions, India) were used as primers to amplify the whole DR region by Polymerase Chain Reaction (PCR) (Table 3.1).

Table 3.1: 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

3.2.3.2.1 Amplification of DR sequence:

Ten nano-grams of genomic mycobacterial DNA extracted was used as a target for PCR. A volume of 12.50µL of the following ready mixed reaction was used for the PCR (10X Super ^{Tth} buffer, 2.5M dNTP, 0.5 U of Taq polymerase) (Kapa Biosystems, South Africa), 2.00 µL each of primers DRa and DRb (0.2pmol) and 2µL of template DNA as shown in table 3.2. The amplification of the DR was performed using the spoligopattern thermo-cycle programme as shown in table 3.3. The PCR products were then diluted by adding 20µL to 150µL 2X

SSPE/0.1% SDS (Separation Scientific, South Africa) and heat denatured for 10 minutes at 99ºC and cooled on ice immediately.

Table 3.2: Reagents used in spoligotyping PCR.

Reagent	**1x Reaction**	**M x Reaction**
Master mix	12.50	12.50 x m
milliQ water	6.50	6.50 x m
Dra	2.0	2.0 x m
DRb	2.0	2.0 x m
Total	23.0	23.0 x m

Table 3.3: Spoligo-pattern thermo-cycle programme for PCR.

	Temperature °C	**Time**	**Cycles**
Initial denaturation	94	3 min	1
Denaturation	94	1 min	20
Primer annealing	55	1 min	20
Primer extension	72	30s	20
Final extension	72	7 min	1

3.2.3.2.2 Preparation of spoligotyping membrane containing oligonucleotides:

The amplified products were hybridized to a set of 43 immobilized oligonucleotides, covalently bound to a spoligotyping membrane each corresponding to one of the unique spacer DNA sequences within the DR locus. The membrane (Ocimun Bio Solutions, India) was activated by washing in 250ml 2X SSPE/0.1% SDS at 60ºC for minutes. The membrane was then put on a

supporting cushion using forceps into a mini blotter in such a way that the slots are perpendicular to the line pattern of the applied oligonucleotides and the residual fluid was removed from the mini blotter by aspiration. The mini blotter slot was filled with 140µL of the diluted PCR product and hybridized for 60 minutes at 60ºC on a horizontal surface. After hybridization, the PCR product will be removed by aspiration and membrane removed. The membrane was then washed twice in 250ml 2X SSP/0.5%SDS for 10 minutes at 60ºC, this was followed by placing the membrane in a rolling bottle and 7.5 µL streptavidin POD conjugate (500U/ml) (Roche diagnostics, Germany) and incubated in the solution for 60 minutes at 42ºC in the hybridizer. Following incubation in hybridizer, the membrane was washed twice in 250ml of 2X SSPE for 5 minutes.

The membrane was exposed to chemiluminescence (Separation Scientific, South Africa), and exposed on an X-ray film (Amersham Hyperfilm™ ECL, Separation, South Africa) according to the manufacturer's instructions. The X-ray film was then developed using standard photochemical procedures. Ten millilitres of solution 1 and solution 2 of the ECL detection was mixed in the dark room and used to incubate the membrane at for 1 minute. The membrane was covered with a transparent plastic sheet and exposed in a light sensitive X-ray film; for one minute. This was followed by washing the membrane in a developer solution (AGFA Healthcare W, Belgium) in until black squares appeared. The membrane was rinsed in water then washed again in a fixer solution (AGFA Healthcare W, Belgium) accordance with instructions of the manufacturer. *Mycobacterium tuberculosis* H37Rv and *M. bovis* BCG (Ocimun Bio Solutions, India) were be used as positive controls.

For regenerating the membrane, the membrane was stripped by washing two times for 30 min each time in 1% SDS at 80ºC and then incubated for 15 minutes in 20 mM EDTA (pH 8) for 15 minutes at room temperature. The membrane was then sealed in plastic and stored at 4ºC to avoid dehydration until further use.

3.2.3.3 Standard 24-loci mycobacterial interspersed repetitive units (MIRU) variable number of tandem repeats (VNTR) analysis

Standard 24-loci mycobacterial interspersed repetitive units (MIRU) variable number of tandem repeats (VNTR) analysis (MIRU VNTR 24 loci) was performed as a second-line typing method as described elsewhere [10]. Briefly, analysis was performed by using multiplex PCR, Rox-labeled Map Marker 1000 size standard (BioVentures, Murfreesboro, TN) and ABI 3100 and ABI 3730-XL sequencers. Sizing of the PCR fragments and assignment of the various VNTR alleles were done by using customized GeneScan and Genotyper or Genemapper software packages (PE Applied Biosystems). The PCR mixtures were prepared using 96-well plates and a HotStart- Taq DNA polymerase kit (QIAGEN, Hilden, Germany). Approximately 2 ng of DNA was added to a final volume of 20µl containing 0.08µl of DNA polymerase (0.4 U); 4µl of Q-solution; 0.2 mM concentrations of dATP, dCTP, dGTP, and dTTP (Phar- macia, Uppsala, Sweden); 2µl of PCR buffer; 1.5 to 3.0 mM MgCl2; 0.4M concentrations of each unlabelled oligonucleotide; and 0.04 to 0.4M concentrations of labelled oligonucleotides. Negative controls consisted of the PCR performed on reaction mixtures lacking mycobacterial DNA. The PCRs were carried out by using a Hybaid PCR express cycler (Hybaid, Ashford, Great Britain) as shown in table 3.4.

Table 3.4: MIRU VNTR thermo-cycle programme for PCR

	Temperature °C	**Time**	**Cycles**
Initial denaturation	95	15 min	1
Denaturation	94	1 min	40
Primer annealing	59	1 min	20
Primer extension	72	30s	20
Final extension	72	10 min	1

3.2.4 Data Analysis

The spoligotyping results were entered in an Excel sheet as a binary code representing either a positive or negative hybridization result. Spoligotypes in binary format were entered in the SITVIT2 database (Pasteur Institute of Guadeloupe; http://www.pasteur-guadeloupe.fr /tb/bd_myco.html) which is an updated version of the previously released SpolDB4 database. The MIRU VNTR pattern was analyzed using the MIRU VNTR*plus* database (www.MIRU VNTRplus.org/). The results were entered into the database as numerical codes corresponding to the number of alleles at each locus. A dendrogram was constructed for spoligotyping, MIRU VNTR, and the combination of both methodologies. The dendrogram were constructed using the unweighted-pair group method with arithmetic averages (UPGMA) algorithm (Figure 3.5). Strains that could not be assigned into well-determined designations in both SpolDB4 and its updated SITVIT version, and as such were labelled as ‘‘unknown” [16]. Strains from the concomitant extrapulmonary and pulmonary specimens were considered to be homogeneous when the typing patterns were identical as tested by both spoligotyping and MIRU VNTR and heterogeneous if both the typing patterns were different. We defined MIRU-VNTR patterns that included at least 1 locus with >1 copy number as heterogeneous infections; all other patterns were designated as simple infections (de Viedma et al., 2005). Molecular DST results were considered final if phenotypic DST could not be performed due to either failed DST or contaminated culture or if strains were no longer viable. Strains from extrapulmonary and pulmonary specimens were considered to be molecular and phenotypic homogenous if isoniazid and rifampicin from both FL-LPA DST and MGIT DST patterns were identical. However, if DST patterns of strains isolated from extrapulmonary and pulmonary specimens were different between isoniazid and rifampicin by either FL-LPA DST or MGIT DST, these were interpreted as molecular or phenotypic heterogeneous. A full MGIT DST (included streptomycin and Ethambutol) was considered for all heterogeneous strains either by genotyping or DSTs methods. All isolates showing drug resistance were re-tested for quality assurance. Discordance data was re-checked for any possible transcriptional errors before analysis. Laboratory cross-

contamination was ruled out in all cases after typing isolates processed on the same day and determining that no other isolate shared the typing pattern of the study isolates.

The statistical analysis was predominantly descriptive statistics presenting number with proportions and median with range for demographic variables. Frequency tables of TB genotype and resistance profile were created between extrapulmonary and pulmonary strains.

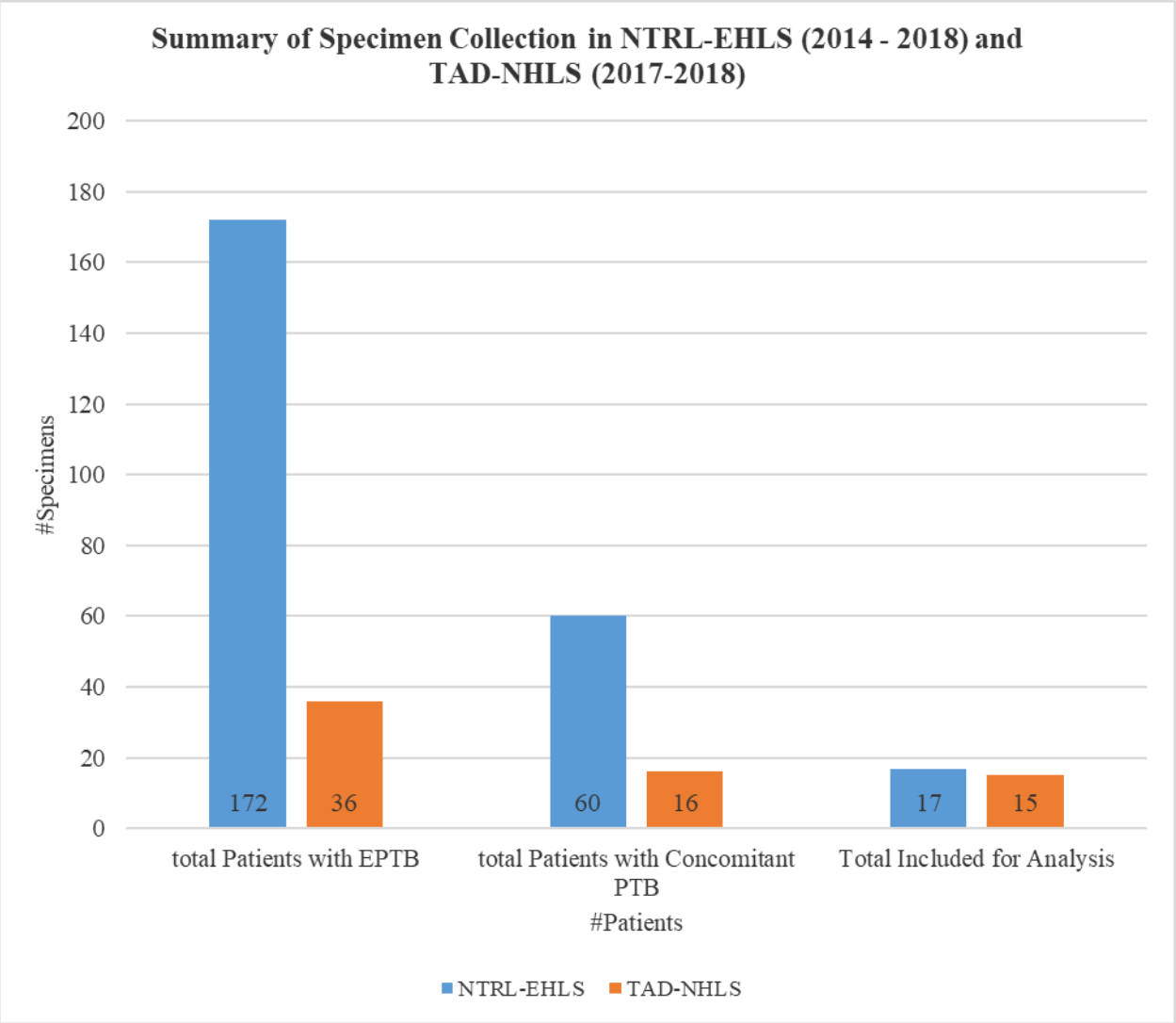
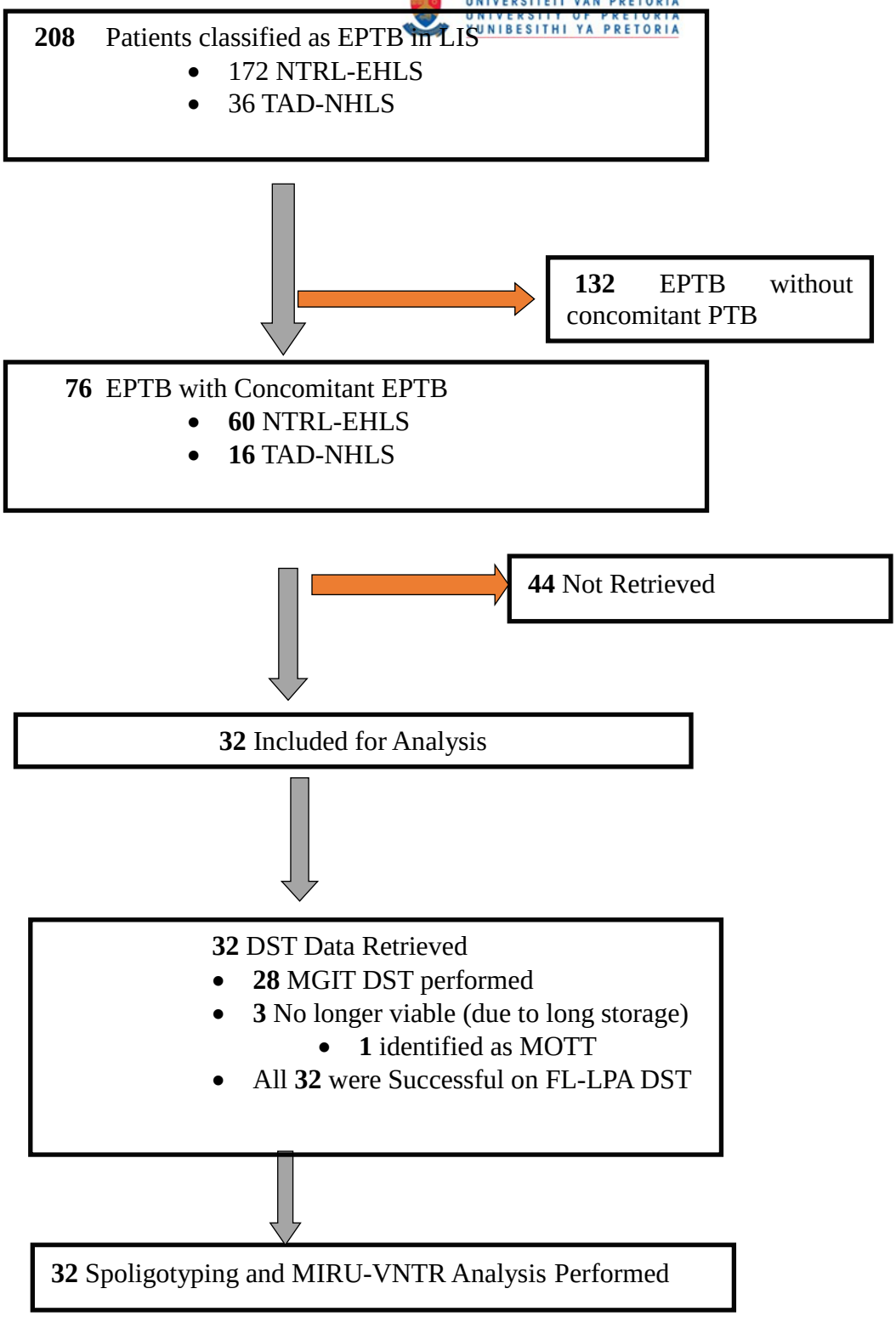


Figure 3.1: Cascade of extrapulmonary isolates (lymph nodes, plural fluid, cerebrospinal fluid, bone marrow aspirate) and corresponding pulmonary specimens received at NTRL-EHLS, Eswatini (2014-2018) and TB laboratory, TAD-NHLS, South Africa (2017-2018).



MOTT=Mycobacterium Other Than Tuberculosis

Figure 3.2: Flow chart of retrieval and laboratory processes of extrapulmonary concomitant with pulmonary specimens from the laboratory information system in NTRL-EHLS, Eswatini and TAD-NHLS, South Africa.

Table 3.5: Specimen collected from patients' concomitant with extrapulmonary tuberculosis and pulmonary tuberculosis.

Laboratory	EPTB specimen	Total (%)	PTB Specimen Available Total (%)	Included for Analysis Total (%)
NTRL/EHLS-Eswatini	Pleural Fluid	75 (44)	22 (29)	8 (36)
	Blood/BMA	2 (1.2)	-	-
	CSF	4 (2.3)	1 (25)	1 (100)
	Lymph Node	91 (53)	37 (41)	8 (22)
	Total	172	60	17
	EPTB Specimen	**Total (%)**	**PTB Specimen**	**Included for Analysis**
TAD/NHLS-RSA	Pleural Fluid	16 (36)	9 (56)	9 (100)
	Blood/BMA	9 (36)	4 (44)	4 (100)
	CSF	16 (44)	1 (6.3)	-
	Lymph Node	2 (6)	2 (100)	2 (100)
	Total	36	16	15

BMA=Bone Marrow Aspirate, CSF= Cerebrospinal Fluid, EPTB = Extrapulmonary TB, PTB = Pulmonary TB.

3.4 RESULTS

3.4.1 Study Population

A total of 64 isolates corresponding to 32 patients were analyzed in this study, the difference in collection days between the concomitant specimens showed a median of 5.5 days and a range of 1 to 18 days. The 32 patients recruited were selected from all 4 administrative regions of Eswatini [Hhohho (n = 4), Manzini (n = 9), Shiselweni (n = 3) and Lubombo (n = 1)] and the remaining patients were from Tshwane metropolitan region (n = 15) in South Africa. The demographic and epidemiological data of these patients is summarized in table 3.6. The age of patients ranged from 5 to 61 years (mean 33 years). Gender information and human immunodeficiency virus [HIV] data was available for all cases: males represented 69% of the cases, with a male to female sex-ratio of 2.2 (22/10). At least 97% (31/32) patients were infected with HIV while one patient (3%) was HIV seronegative (Table 3.6). Treatment history and treatment outcome status was available for only 17 TB patients: 82% (14/17) were new cases and 18% (3/17) were previously treated.

Country	Region	n (%)	Average Mean Age	SEX Ratio (M/F)	Treatment History		HIV Status	
					n New Case (%)	n Previous Treated (%)	n Positive (%)	n Negative (%)
Eswatini	Hhohho	4 (14)	37	3/1	4 (100)	0	4 (100)	0
	Manzini	9 (28)	36	100% M	6 (67)	3 (33)	8 (89)	1(11)
	Lubombo	1 (3.1)	NA	100% F	1 (100)	0	1 (100)	0
	Shiselweni	3 (9.4)	29	2/1	3 (100)	0	3 (100)	0
South Africa	Tshwane	15 (47)	30	8/7	-	-	15 (100)	0
	Total	32	33	22/10	-	-	31(97)	1 (3)

Table 3.6: Demographic and epidemiological data of the studied population (n = 32).

M= Male, F=Female

3.4.2 Mycobacterium tuberculosis Lineages Distribution

Based on 24 loci MIRU VNTR and spoligotyping pattern unweighted pair group method with arithmetic mean (UPGMA) phylogenetic tree was generated (Figure 3.5). The most prevalent lineages were Lineage four (Euro-American: *n* =40, 63%) followed by Lineage two (East Asian: *n* = 20, 31%) and Lineage three (East African Indian: *n* = 4, 6.3%). The distribution of lineages between extrapulmonary and pulmonary TB were the same. With regards to anatomical sites the distribution of the lineages patterns was relatively the same with the lineage 4 family being most dominant. Strain distribution by infected anatomical sites and the administrative regions is shown in figures 3.3 and 3.4.

3.4.3 Occurrence of heterogeneity by Genotyping

In 94% (30/ 32) cases available for study, the genotypic characterization of *M. tuberculosis* strains by both spoligotyping and MIRU VNTR patterns were identical. The remaining 6% (2/32) patients both their pulmonary strains isolated from each patient could not be linked to any previously defined spoligo patterns and these were classified as “unknown” while their respective extrapulmonary strains both belonged T1 family. Among the 6% (2/32) of cases (patient 7 and 10) with heterogeneous strains isolated: both patients had T1 strain isolated from their respective extrapulmonary sites (lymph node, and CSF) and while unknown strains were isolated from their respective pulmonary site. In patient 7; a STI 53 T1 (Octal:757774606060731) strain was isolated from the lymph node sample while the corresponding sputum strain isolated was classified as unknown (Octal: 757774606060731). Further genotypic analysis showed different strains in MIRU VNTR patterns (224043102324235152335522 and 234214122824114152532822) at 10 loci (Mtub04, MIRU40, MIRU10, QUB11b, Mtub30, ETR B, MIRU23, Mtub34, Mtub39, QUB26) respectively. In patient 10; 796 STI T1 (Octal:777776777600201) strain was isolated from the CSF specimen while the corresponding gastric aspirate strain isolated was also unknown (Octal: 777776777600201). Further genotypic analysis showed different strains on the MIRU VNTR

patterns (224244342924246251139311 and 222554342843246221359311) were different at 6 loci (QUB11b, ETR A, Mtub29, MIRU26, Mtub34, MIRU31) respectively (Table 3.5).

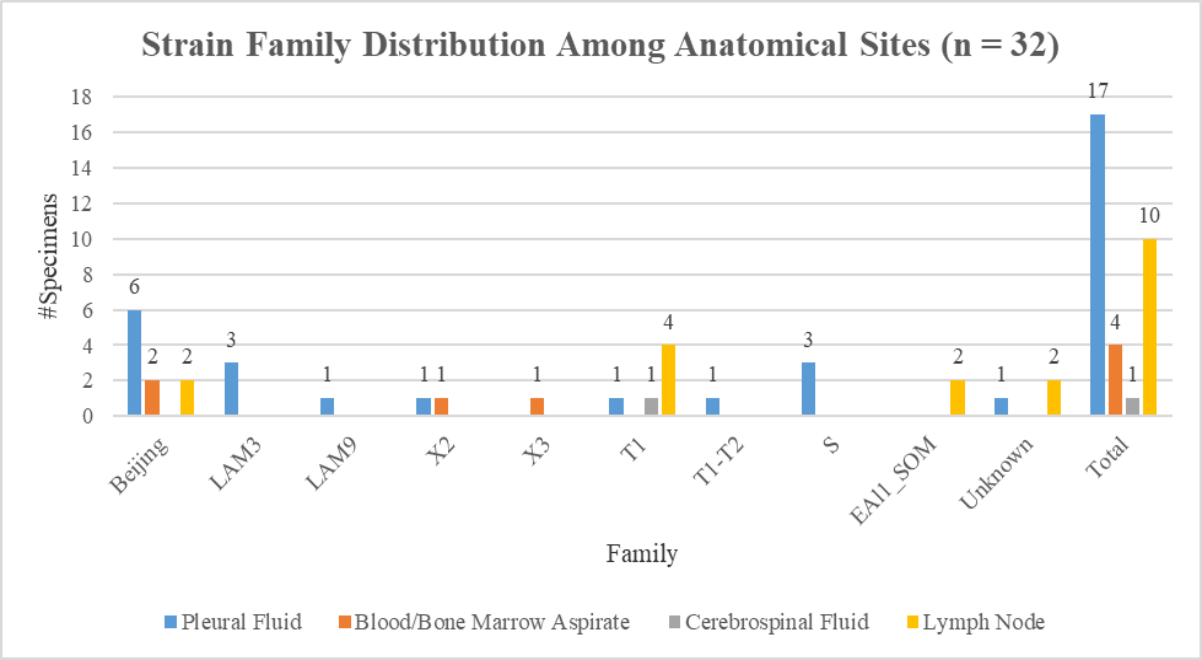


Figure 3.3: *Mycobacterium tuberculosis* distribution in infected anatomical specimens.

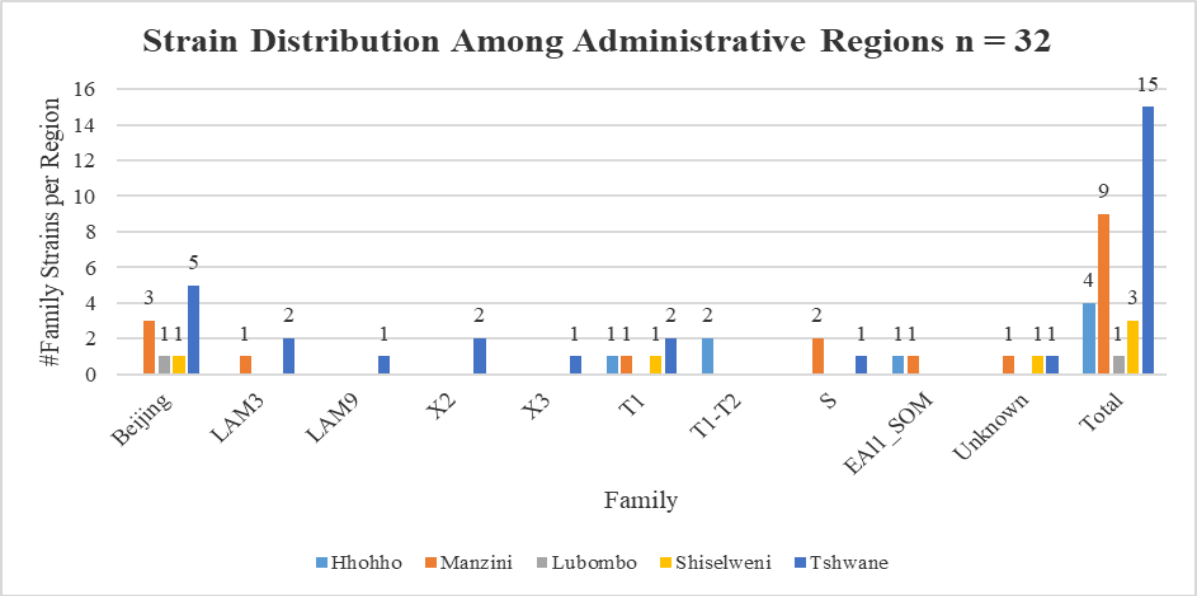


Figure 3.4: Strains distribution comparison against administrative regions catchment area used in the study.

3.4.4 Molecular and Phenotypic Drug Susceptibility Testing

The genotypic drug susceptibility testing (DST) data was available for all 32 cases while phenotypic DST was available for only 88% (28/32) cases. In 12% (4/32) cases phenotyping DST could not be done; 3/32 (9%) the strains were no longer viable due to prolonged storage, and in one case (3%) a Mycobacterium other than tuberculosis (MOTT) was also isolated from one of the corresponding pulmonary specimen therefore, further phenotypic testing could not be done for this case.

3.4.4.1 Genotypic and phenotypic concordance in DSTs

Genotypic (FL-LPA) and phenotypic (MGIT DST) DST data comparison showed that in 94% (30/32) cases the strains isolated were concordant in DST patterns: in 78% (25/32) cases, the strains were susceptible to both isoniazid and rifampicin, in 6% (2/32) cases the strains isolated were resistant to both isoniazid and rifampicin, in 6% (2/32) cases the isolated strains were

isoniazid mono-resistant. However, the remaining 6% (2/32) two cases DST comparison showed that isolates had discordant at molecular and phenotypic levels.

3.4.4.2 Occurrence of heterogeneity by Drug Susceptibility Testing

Drug susceptibility testing was discordant in 6% (2/32) of cases. In patient 14 both extrapulmonary and pulmonary strains isolated were isoniazid mono-resistant on FL-LPA and resistant to both isoniazid and rifampicin on MGIT DST. Additionally, the lymph node specimen was susceptible to streptomycin while the correspond sputum isolate was resistant to streptomycin and ethambutol. For patient 16; both extrapulmonary and pulmonary isolated strains were had similar DST patterns as patient 14 except that full DST consideration showed that these strains were additionally resistant to streptomycin and ethambutol. This means that in the two patients some mutation conferring resistance to rifampicin were missed on FL-LPA but were detected on MGIT DST giving a hetero-resistance rate of 6% (Table 3.9).

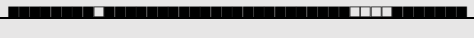
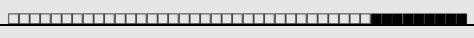
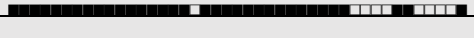
Table 3.7: Genotypic characterization and drug susceptibility profiles of study isolates collected in NTRL/EHLS, Eswatini.

Study	Age/Sex	Specimen Type	Difference Collection Dates	Spoligo Pattern (43)	ST/Family	Lineage	24loci-MIRU VNTR profile	DST FL-LPA R	DST FL-LPA I	DST MGIT R	DST MGIT I
2	43/M	Sputum	10	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■	34/S	4 (Euro-American)	233343312434225142233102	R	S	R	S
		Pleural Fluid		■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■			233343312434225142233102	R	S	*ND	
3	20/F	Lymph Node	0	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■	1/Beijing	2 (East Asian)	24421335274442517135382	S	S	S	S
		Sputum		□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■			24421335274442517135382	S	S	S	S
4	33/M	Pleural Fluid	13	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■	34/S	4 (Euro-American)	233343311834225142233102	R	S	R	S
		Sputum		■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■			233343311834225142233102	R	S	R	S
5	40/M	Sputum	18	■■■□■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□■□□□□	Unknown	4 (Euro-American)	234334342234425153333732	S	S	S	S
		Lymph Node		■■■□■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□■□□□□			234334342234425153333732	S	S	S	S
6	30/M	Pleural Fluid	0	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■	1/Beijing	2 (East Asian)	24423335254442517235382	S	S	*ND	
		Sputum		□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■			24423335254442517235382	S	S	*ND	
7	31/M	Lymph Node	0	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■	53/T1	4 (Euro-American)	22404310232423515233552	S	S	*ND	
		Sputum		■■■■□■■■■■■■□■■□□□■■□□□■■□□□■■■□■■	Unknown		23421412282411415253282	S	S	*ND	
8	35/M	Sputum	3	■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□■■□■■■	73/T2-T3	4 (Euro-American)	23421432143443317322283	S	S	S	S
		Pleural Fluid		■■■■■■■■■■■■□■■■■■■■■■■■■■■■■■■■■□□□■■□■■■			23421432143443317322283	S	S	S	S
9	28/F	Sputum	12	■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■	1129/T1	4 (Euro-American)	233343312834225142233102	R	S	R	S
		Lymph Node		■■■■■■■■□□■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■			233343312834225142233102	R	S	R	S
10	5/F	CSF	4	■■■■■■■■□□■■■■■■□□■■■■■■■■□□■■■■■■■■□□□■■■■■■	796/T1	4 (Euro-American)	22424434292424625113931	S	S	S	S
		Gastric Aspirate		■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□■■	Unknown		22255434284324622135931	S	S	S	S
11	35/M	Sputum	16	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■	1/Beijing	2 (East Asian)	24421335284442517135382	S	S	S	S
		Pleural Fluid		□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■			24421335284442517135382	S	S	S	S
12	29/M	Sputum	0	■■■■■■■■□□■■■■■■□□■■■■■■■■□□■■■■■■■■□□□■■■■■■	33/LAM3	4 (Euro-American)	24424434242422615213172	S	S	S	S
		Pleural Fluid		■■■■■■■■□□■■■■■■□□■■■■■■■■□□■■■■■■■■□□□■■■■■■			24424434242422615213172	S	S	S	S
13	43/M	Sputum	11	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■	1/Beijing	2 (East Asian)	24423335254442517435382	S	S	S	S
		Pleural Fluid		□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■			24423335254442517435382	S	S	S	S
14	39/M	Sputum	1	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□■■■■■■	48/EAI1_SOM	1 (East African-	22263425299324622334721	S	R	R	R

		Lymph Node		[redacted]		Indian)	22263425299324622334721 1	S R R R
		Sputum		[redacted]	Unknown		24424433193442513333383 2	S S S S
15	45M	Lymph Node	8	[redacted]		4 (Euro-American)	24424433193442513333383 2	S S S S
		Lymph Node		[redacted]	48/EAI1_SOM	1 (East African-Indian)	22253324299324622134721 1	S R R R
16	30/M	Sputum	14	[redacted]			22253324299324622134721 1	S R R R
		Lymph Node		[redacted]	926/T1		22424312282423515333552 2	R R R R
17	54/M	Sputum	3	[redacted]		4 (Euro-American)	22424312282423515333552 2	R R R R
		Pleural Fluid		[redacted]	1/Beijing		24423335283442517335382 3	S S S S
18	38/M	Sputum	8	[redacted]		2 (East Asian)	24423335283442517335382 3	S S S S

*ND = Not Done, CSF= Cerebrospinal fluid, R = Rifampicin, I = Isoniazid

Table 3.8: Genotypic characterization and drug susceptibility profiles of study isolates collected in TAD/NHLS, South Africa.

Patient	Age/Sex	Specimen Type	Difference in Collection Dates	Spoligo Pattern (43)	ST/Family	Lineage	24loci-MIRU VNTR profile	DST			
								FL-LPA		MGIT	
								R	I	R	I
1	41/F	Sputum	1		137/X2	4 (Euro-American)	225234242334425153344932	S	S	S	S
		Bone Marrow Aspirate					225334242334425153344932	S	S	S	S
19	35F	Sputum	2		33/LAM3	4 (Euro-American)	244234332424226253131722	S	S	S	S
		Pleural Fluid					2442320124226531102	S	S	S	S
20	45/M	Bone Marrow Aspirate	0		1/Beijing	2 (East Asian)	244233252544425173354823	S	S	S	S
		Sputum					244233252544425173354823	S	S	S	S
21	39F	Pleural Fluid	8		1129/T1	4 (Euro-American)	224343312444225152233 1022	S	S	S	S
		Sputum					224343312444225152233 1022	S	S	S	S
22	28/M	Sputum	0		33/LAM3	4 (Euro-American)	244234342424226153131122	S	S	S	S
		Pleural Fluid					244234342424226153131122	S	S	S	S
23	36/F	Pleural Fluid	2		1/Beijing	1/Beijing	244232352644425172354624	R	R	R	R
		Sputum					244232352644425172354624	R	R	R	R
24	41/M	Sputum	3		1273/X3	4 (Euro-American)	223244332334425133334722	S	S	S	S
		Bone Marrow Aspirate					223244332334425133334722	S	S	*ND	
25	48/F	Sputum	0		30/LAM9	4 (Euro-American)	134254232224126152322522	S	S	S	S
		Pleural Fluid					134364232224126152322522	S	S	S	S
26	61/F	Sputum	1		1/Beijing	2 (East Asian)	244233352544425173343823	S	S	S	S
		Bone Marrow Aspirate					244233352544425173343823	S	S	S	S
27	40/F	Pleural Fluid	0		137/X2	4 (Euro-American)	224223332334425152334 1022	S	S	S	S
		Sputum					224223332334425152334 1022	S	S	S	S
28	42/M	Lymph Node	6		966/T1	4 (Euro-American)	224213222834226142331 0522	S	S	S	S
		Sputum					224213222834226142331 0522	S	S	S	S
29	34/F	Sputum	0		1/Beijing	2 (East Asian)	244213352444425171353823	S	S	S	S
		Lymph Node					244223352444425171353823	S	S	S	S
30	41/M	Pleural Fluid	0		1/Beijing	2 (East Asian)	235213342644425163353933	S	S	S	S
		Sputum					235213342644425163353933	S	S	S	S

31	38/M	Pleural Fluid	0	[redacted]	Unknown	4 (Euro-American)	24423433222422615213172 2	S	S	S	S
		Sputum					24423433222422615213172 2	S	S	S	S
32	23/M	Sputum	2	[redacted]	34/S	4 (Euro-American)	33434321234422514323392 2	S	S	S	S
		Pleural Fluid					33434321234422514323392 2	S	S	S	S

*ND=Not Done, R = Rifampicin, I = Isoniazid

Table 3.9: Spoligo patterns, MIRU VNTR profiles and drug susceptibility profiles of the heterogeneous isolates. Highlighted in red are discordant patterns/profiles.

Patient	Age	History/HIV Status	Region	Specimen Type	Difference in Collection Dates	Spoligopatterns	ST/Family	24loci-MIRU VNTR profile	DST	
									FL-LPA I R	MGIT S I R E
7	31/M	new case/ Positive	Manzini	Lymph Node	0	[redacted]	53/T1	224043102324235152335522	S S	ND
				Sputum			Unknown	234214122824114152532822	S S	ND
10	5/F	new case/ Positive	Shiselweni	Cerebrospinal Fluid	4	[redacted]	796/T1	224244342924246251139311	S S	S S S S
				Gastric Aspirate			Uknown	222554342843246221359311	S S	S S S S
14	39/M	new case/ Positive	Manzini	Sputum	1	[redacted]	48/EAI1_SOM	222634252993246223347211	R S	R R R R
				Lymph Node			48/EAI1_SOM	222634252993246223347211	R* S	S R R R
16	30/M	new case/ Positive	Hhohho	Lymph Node	14	[redacted]	48/EAI1_SOM	222533242993246221347211	R * S	R R R R
				Sputum			48/EAI1_SOM	222533242993246221347211	R * S	R R R R

ND=Not Done S = Streptomycin, I = Isoniazid, R = Rifampicin, E = Ethambutol, R* = *Kat G mutation*

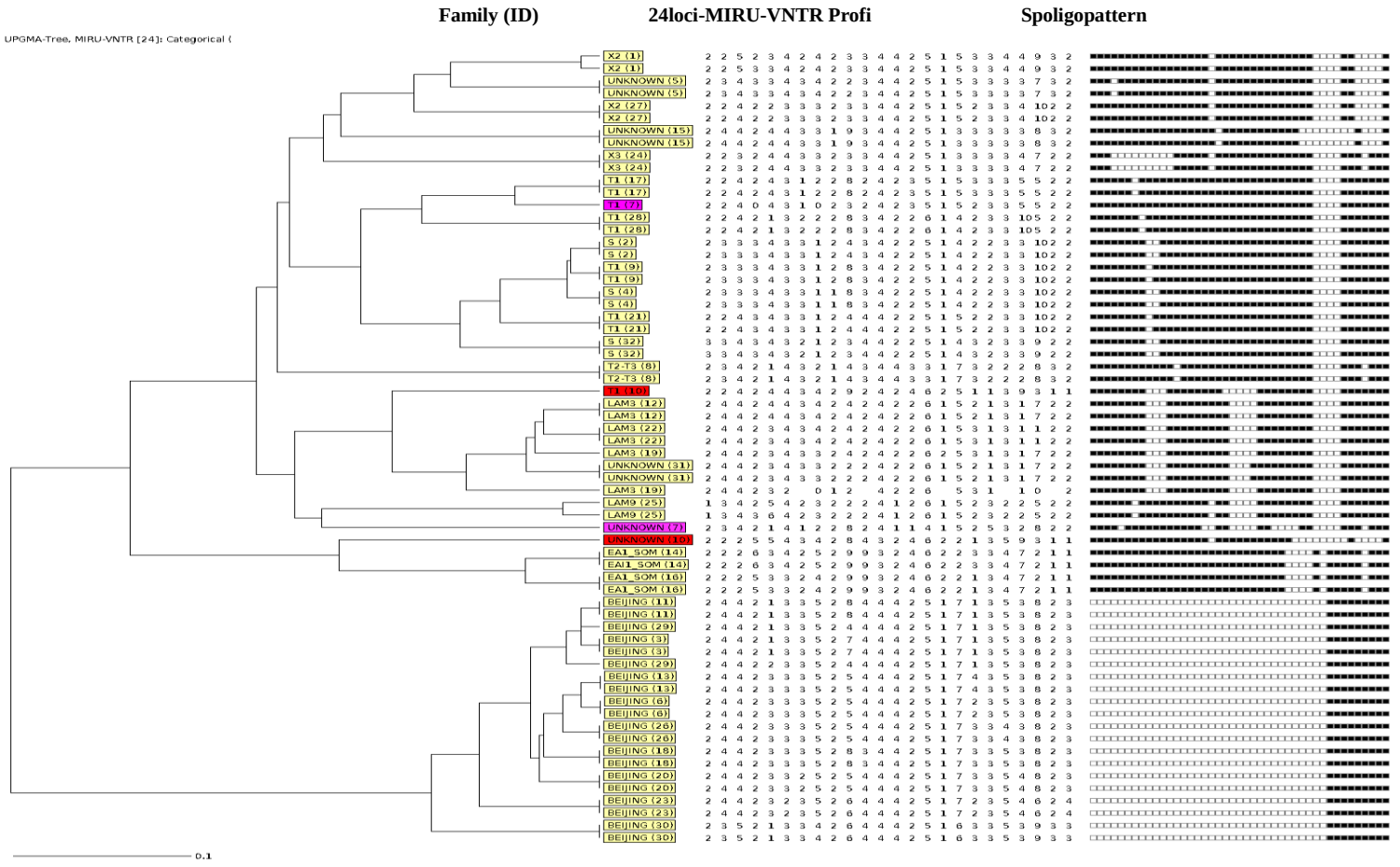


Figure 3.5: UPGMA-dendrogram representing MIRU VNTR and spoligotype pattern of 64 isolates from the 32 concomitant patients. Highlighted in red and purple are patients with discordant spoligopatterns and MIRU VNTR patterns.

3.5 DISCUSSION

Within-host heterogeneity is thought to result from either the appearance of a new strain that diverged from a pre-existing persistent *M. tuberculosis* clone resulting in accumulation of mutations, which results in clonal heterogeneity or simultaneous infection with multiple strains or superinfection of an uncured *M. tuberculosis*, which results in a polyclonal (mixed) infection [3, 5]. The heterogeneity that arose usually yields a different disease with two or more unique *M. tuberculosis* strains that may have the same or different drug susceptibility profiles which is known as hetero-resistance [5, 8]. Genetic heterogeneity of *M. tuberculosis* in patients with PTB has been associated with adverse outcomes such as conflicting drug susceptibility (DST) results, drug resistance, relapse and increased mortality rate. This holds true for diversity of *M. tuberculosis* genotypes as well as diversity in antimicrobial resistance profiles of bacilli occurring within a single host. It is unknown whether heterogeneity of infection also occurs in patients with combined extrapulmonary and pulmonary TB. In this study, we confirm occurrence of genetic heterogeneity and hetero-resistance in patients that are concomitant with extrapulmonary and pulmonary TB in setting of high incidence where patients may present with two overlapping infections. At least 97% (31/32) were HIV seropositive, a high risk factor of overexposure to TB especially among concomitant patients with pulmonary and extrapulmonary disease while one cases (3%) was HIV seronegative (Table 3.6). The one HIV seronegative cases is important as it further validates the quality of our data. However, these findings cannot be generalized to settings of lower HIV prevalence.

In our study, the overall genotypic and phenotypic heterogeneity occurred at a rate of 12%. Heterogeneity due to different *M. tuberculosis* genotypes and heterogeneity to DST equally contributed to this rate with at 6% (2/32) each. With regards to genotypic heterogeneity, the two patients (patient 7 and 10) with dual infection discordant strains: both had T1 strains and unknown pulmonary spoligo types; MIRU VNTR patterns were also discordant but resistance profile on LPA and MGIT were identical (Table 3.9). In our study, it is more likely there is a coexistence of two strains within the same host is due to the overlapping of two independent

infections, a pre-existing infection and superinfection by a new strain. The coexistence of two strains has been found in circumstances of high re-exposure to TB prevalent settings [6, 7, 9, 10].

In patient 7 is a 31 years old man with no history of TB treatment, both the pulmonary (sputum) and the extrapulmonary (lymph node) specimens were collected on the same day STI 53 T1 strain was isolated from the lymph node specimen while the corresponding sputum strain isolated was unknown. The MIRU VNTR patterns were discordant in 10 loci (Mtub04, MIRU40, MIRU10, QUB11b, Mtub30, ETR B, MIRU23, Mtub34, Mtub39, QUB26). Since both specimens were collected on the same date, and that there is no history of TB treatment confirms that this patient might have been infected with two different genotypic strains due to the overlapping of two independent infections. Patient 10 is a 5-year-old female with no previous history of TB treatment, the pulmonary (gastric aspirate) was first collected and then the cerebrospinal fluid (CSF) was collected 4 days later. From the CSF a T1 STI 796 strain was isolated from the CSF specimen while the corresponding gastric aspirate strain isolated was unknown. The MIRU VNTR patterns were discordant in 6 loci respective (QUB11b, ETR A, Mtub29, MIRU26, Mtub34, MIRU31). The close difference in collection dates, no history of TB treatment confirms that this patient was mostly likely infected with two different genotypic strains due to the overlapping of two independent infections. Moreover, the age and HIV seropositive status of this patient makes her to be more susceptible to overlapping infections. One similar study among HIV-infected individuals with concurrent sputum and blood *M. tuberculosis* found that at least half of these individuals were infected with more than one *M. tuberculosis* genotype strain [7]. This could possibly imply that impaired cell-mediated immunity might increase the probability of harbouring multiple infections consider the age and the HIV status of patient 10.

There have been virtually few studies aimed at heterogeneity of *M. tuberculosis* in concomitant patients with pulmonary and extrapulmonary TB especially in sub-Saharan Africa. A study conducted in Uganda among cconcurrent sputum and blood individuals with advanced HIV infection reported that 51% of their studied population had discordant genotypic among *M.*

tuberculosis genotypes. This study is discordant to our study and probably present the highest heterogeneity among documented studies in literature. This discordance in these findings could be due to the fact this study solely focused on mycobactermia. Extrapulmonary TB is thought to occur as result of haematogenous dissemination from the lung parenchyma to high vascular organs such [20, 21]. Therefore, the seeding of *M. tuberculosis* strains in these high vascular organs such as the meninges or the bone marrow may not be the same in the blood. The sample size in our study may also contribute to the lower proportion in our study. However, our findings from this study are in concordant with study of de Viedma *et al* (2003). This study reported a homogeneous rate of 94% and heterogeneity rate of 6% which is similar to our findings except that in our study the heterogeneity is also attributed to by hetero-resistance [22].

Hetero-resistance is defined as the to the occurrence of populations of both drug-susceptible and drug-resistant isolates within the same clinical sample and in the context of this study may be defined as occurrence of populations of both drug-susceptible and drug-resistant isolates within the same patient, but from different anatomical sites [5, 11]. In our study hetero-resistance occurred at a rate of 6% from 2/32 concomitant patients (patient 14 and 16) infected by the EAI1_SOM strains. This rate is concordant with two studies. A rate of DST heterogeneity rate of 8.2% and 7% were reported by Shamputa et al (2004) and Zetola et al (2014) respectively. However, the sample size of these studies were larger as compared to our cohort study and these studies aimed to access occurrence of heterogeneity among different patients on treatment while our study confirm occurrence of within host heterogeneity among patients with concomitant pulmonary and extrapulmonary TB. The rate at which heterogeneity occurs suggests that presence of different genotypes in different samples may be clinically relevant and therefore, microbiological testing of specimens from both body sites in these patients is highly recommended.

In both patients, similar genotypic strains were isolated from the lymph nodes and corresponding sputa specimens (Table 3.9). It is worth noting that, in spite of the high genetic similarity between the infecting EAI1_SOM strains, phenotypic differences were observed in their

susceptibilities to isoniazid at molecular level. This finding is remarkable from a therapeutic point of view because both strains were phenotypically MDR on MGIT DST while isoniazid mono-resistant at genotypic level. This might mean that these patients were infected with different EAI1_SOM with a subpopulation of strains that are isoniazid mono-resistance and another subpopulation resistant to both isoniazid and rifampicin. However, the subpopulation resistant to both principal first anti-TB drugs were detected only by the phenotypic DST. The FL-LPA (GenoType MTBDR plus version 2) targets specific mutations in the rifampicin resistance determining region (RRDR) of the *rpo*B gene (from codon 505 to 533) to detect rifampicin resistance. Therefore, any *rpo*B mutations not found within RRDR region could be missed [23, 24]. Such conflict DST result present a serious challenge in the management of patients since phenotypic DST results may not be available at treatment initiation complicating the management of the affected patients. Once these results are available the patient treatment would need to be readjusted from the current regimen and may hinder treatment success and resulting in unfavourable treatment outcomes such as relapse, emergence of drug resistance, treatment failure and increased mortality rate.

All the heterogeneous cases identified are from Eswatini and none Tshwane, South Africa. These differences could be attributed by several factors. Firstly, even though Eswatini and South Africa are neighbouring countries the TB epidemiology and circulating *M. tuberculosis* strains in Eswatini and South Africa may not be similar. The occurrence of hetero-resistance among cases from Eswatini is of particular interest since the last TB drug resistance survey of 2009/2010 reported that 30% of the MDR strains in an Eswatini study harboured the Ile491Phe mutation in the *rpo*B gene [25-27]. Isoniazid mono-resistance has been strongly associated with Ile491Phe mutation in the *rpo*B gene which confers lower resistance to RIF. This mutation has been associated with poor rifampicin-based treatment outcomes, but is missed by commercial molecular assays (Xpert MTB/RIF and FL- LPA) or scored as susceptible by phenotypic drug susceptibility testing. Even though in our study, the hetero-resistance strains were both isoniazid mono-resistant on FL- LPA and were clear MDR cases on MGIT DSTs, it is possible that heterogeneous infection with this mutation could have influenced the outcome of our findings.

The extent of the transmission of this mutation in South Africa is not yet ascertained. Secondly, the sample collection methods used in Eswatini was retrospective while in South Africa the sample were collected prospectively. Thawing and freezing of isolates could possible change the genetic makeup of strains as some of these isolates were up to four years old [28, 29].

3.6 LIMITATIONS

The study has several limitations. Firstly, sample collection in NTRL-EHLS was retrospective while in Tshwane a prospective sample collection method was used. As a result, we missed a significant number of cases, at least 44/60 cases identified on LIS from Eswatini could not be retrieved from the biobank Figure 3.2. Secondly, all laboratory testing conducted were culture based, as a result we might have missed quite a significant number of strains due to the decontamination process and the MGIT culture only allows growth of only the most dominant strains compared to solid media that allows detection of multiple strains including minority strains. The reduction in diversity of mycobacterial population in a sample after culture especially liquid culture is well documented [3, 30, 31]. This might have limited our ability to detect mixed infections by MIRU VNTR and hetero-resistance by the FL-LPA. Lastly, the study was not powered enough to allow statistical analysis as the sample size was small. Studies with a larger sample size and more robust or higher-resolution genomic typing tools, such as targeted Next Generation Sequencing (t-NGS) or metagenomics analysis are would facilitate improve our understanding of the complexity of infections and for detection of both minority and major variants within pulmonary and extrapulmonary specimens for better estimation of the extent of *M. tuberculosis* genotypic and phenotypic heterogeneity [26, 32-34].

However, despite the above mentioned limitations this is a really unique cohort that provides insights in the pathogenesis of extrapulmonary TB. Firstly, it confirms occurrence of heterogeneity of infection in patients with concomitant pulmonary and extrapulmonary TB in a setting of high incidence. Secondly, the rate at which heterogeneity occurs suggests that presence of different genotypes in different samples may be clinically relevant and therefore,

microbiological testing of specimens from both body sites in these patients is highly recommended. Lastly, this study raises questions on the pathogenicity of extrapulmonary TB taking into account that the occurrence extrapulmonary TB is considered to be to be caused by the same strain that infects the pulmonary site.

3.7 CONCLUSIONS

This study confirms that heterogeneity of infection occurs in patients with concomitant pulmonary and extrapulmonary TB in a setting of high incidence where patients may present with two overlapping infections in time. The rate at which heterogeneity occurs suggests that presence of different genotypes in different samples may be clinically relevant and therefore, microbiological testing of specimens from both body sites in these patients is highly recommended. Our cohort study, moreover add to the scant literature which challenges the old dogmas related to TB immunity, pathogenesis and progression from latent to active disease. Moreover, our findings raise questions on the timing of multiple infections and the mechanism of reactivation of both infections simultaneously and most importantly the occurrence of heterogeneity is thought to be higher in patients in whom the immune system is impaired. In future more robust or higher-resolution genomic typing tools, such as target whole-genome sequencing or metagenomics analysis would improve our understanding of the complexity of infections and for detection of both minority and major variants within pulmonary and extrapulmonary specimens for better estimation of the extent of *M. tuberculosis* heterogeneity and hetero-resistance.

3.8 ACKNOWLEDGEMENTS

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CHAPTER FOUR: CONCLUDING REMARKS

Tuberculosis (TB) is thought to be caused by reactivation of endogenous infection rather than by a new, exogenous infection. However, characterization of the genotype of *Mycobacterium tuberculosis* (*M. tuberculosis*) by DNA fingerprinting studies have shown that some of these recurrences are not treatment failures but rather represent reinfection with a different strain from the first episode (Lambert et al., 2003, van Rie et al., 1999). In high TB burden settings with diverse *M. tuberculosis* genotypes a single host can be repeatedly infected with different *M. tuberculosis* genotypes (Cohen et al., 2012, McIvor et al., 2017). To date, the occurrence of EPTB is considered to be to be caused by the same genotype that infects and cause disease at the pulmonary site (Zumla et al., 2013, Houston and Macallan, 2014, Norbis et al., 2014). In this study, we were particularly interested to see whether *M. tuberculosis* heterogeneity and hetero-resistance occurs among pulmonary and extrapulmonary concomitant patients in high TB incidence settings.

Overall, this study has confirmed that heterogeneity of infection occurs in patients with concomitant pulmonary and extrapulmonary TB in a setting of high incidence where patients may present with two overlapping infections in time. Two patients of the 32 patient had discordant strains: both had extrapulmonary T1 strains and unknown pulmonary spoligo types; MIRU VNTR patterns were also discordant. The findings translate to a concordance rate of 94% and discordance rate of 6%. In addition, there were two patients (6%) with different FL-LPA/MGIT results but had the same genotype strains from pulmonary and extrapulmonary sites. This translates to a heterogeneous infection rate of 12% (4/32). The rate at which heterogeneity occurs suggests that presence of different genotypes in different samples may be clinically relevant and therefore, microbiological testing of specimens from both body sites in these patients is highly recommended. Our cohort study, moreover add to the scant literature which challenges the old dogmas related to TB immunity, pathogenesis and progression from latent to active disease. Moreover, our findings raise questions on the timing of multiple infections and the mechanism of reactivation of both infections simultaneously and most importantly the

occurrence of heterogeneity is thought to be higher in patients in whom the immune system is impaired.

4.1 FUTURE STUDIES

All laboratory testing conducted were culture based, as a result we might have missed quite a significant number of strains due to the decontamination process and the MGIT culture only allows growth of only the most dominant strains compared to solid media that allows detection of different strains. Specimen decontamination steps are optimized to avoid overgrowth by other organisms may not be ideal for facilitating the survival of these minority variants. As a results decontamination processes substantially decrease diversity of Mycobacterial population in a specimen (Martin et al., 2010). The reduction in diversity of mycobacterial population in a sample after culture especially liquid culture is well documented (Rinder, 2001, Cohen et al., 2012, McIvor et al., 2017). In addition, this might have limited our ability to detect mixed infections by MIRU VNTR and hetero-resistance by the FL-LPA. Future studies, should consider solid media methods the yield is generally thought to be better among solid culture media methods as it allows multiple individual clones to be isolated from different colonies analysed separately for evidence of heterogeneity.

Lastly, the study was not powered enough to allow statistical analysis as the sample size was small. In future, prospective studies with a larger sample size and more robust or higher-resolution genomic typing tools should be considered for further studies to estimate evidence of heterogeneity (Das et al., 2013, Lee and Behr, 2016, Ssengooba et al., 2016, Makhado et al., 2018). *Mycobacterium tuberculosis* isolates may also exhibit differences in their abilities to grow and divide in different types of media that is solid versus liquid medium and difference are even documented among solid media methods (Lowenstein-Jensen [LJ] versus Middlebrook medium) (Martin et al., 2010; Cohen et al., 2012; McIvor et al., 2017). Therefore, targeted next generation sequencing is culture independent and would facilitate detection of both minority and major

variants within pulmonary and extrapulmonary samples allowing better estimation of the extent of genetic and phenotypic heterogeneity.

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Appendix A: DETAILED METHODS

NucliSENS easyMAG technology: NucliSENS easyMAG technology is based on the Boom technique, utilizes magnetic silica particles, and processes 24 samples simultaneously. This technology allows for the dynamic capture of nucleic acids and the washing of silica without loss of material.

1.1 Sample lysis: Boom lysis buffer contains 5 M guanidinium thiocyanate, a powerful dissociating and denaturing agent, plus tensio-active compounds. Direct lysis is the most frequently used technique; the sample volume should not exceed 1 mL to respect the ratio between sample volume and lysis buffer volume (1 volume for 2 volumes, respectively). External lysis saves time and enhances the safety of sample manipulation and transport.

1.2 DNA adsorption: Adsorption of nucleic acids occurs in lysis buffer at neutral pH levels. The volume of the magnetic silica suspension can vary between 50 lL and 140 lL (as recommended by the manufacturer). Preliminary experiments showed that 50 lL of silica is sufficient for samples with cell numbers inferior to 104. However, 140 L of silica suspension is required for high-density samples, such as blood and solid tissues. All assays described in the current report were performed using 140 lL of silica suspension. Silica was added manually and homogenized thoroughly.

1.3 Washing: The initial washing step was performed using a buffer containing guanidinium thiocyanate to maintain high stringency, and the final steps were performed at a neutral pH level with high salt concentrations to retain the nucleic acids adsorbed on the silica matrix.

1.4 Elution: The elution step was performed at 70 °C in a volume that can vary between 25lL and 110 lL at pH >8.0. The choice of elution volume essentially depends on the expected DNA quantity, the possible presence of inhibitors, and the volume required for amplification. The elution volume was 50 lL with an exception for the stool samples (elution volume 100 L). The nucleic acids were stored at -80 °C.

First Line Probe Assay

The Genotype® MTBDRplus line-probe assay (version 2.0) kit was performed to determine rifampin and isoniazid susceptibility profiles according to the manufacturer's instructions (Hain LifeSciences, Nehren, Germany). Master mix was prepared in a clean room to prevent contamination of molecular laboratory. In addition, room, 5 µl of DNA extracts were added to the corresponding PCR tubes, 5 µl of DNA extract from H37Rv quality control strain to the positive control tubes and 5 µl of distilled water the negative control tube. After addition, the mixture with the polymerase chain reaction (PCR) tube placed in to PCR machine for amplification. After completion of PCR process, the amplicon was detected with a series of procedures by adding different reagents to the strip. The strips were formed colour bands after addition of the final substrate reagent. Membrane strips were attached to the evaluation sheet, read, and interpreted manually.

MGIT Drug Susceptibility Testing

We performed MGIT DST by inoculating two MGIT tubes with the test culture. A known concentration of a test drug was added to one of the MGIT tubes, and growth was compared with the MGIT tube without the drug (growth control). Growth was monitored by the BACTEC 960 instrument which automatically interprets results as susceptible or resistant. One H37RV sensitivity strain was run per batch of DST set for quality control purposes.

Appendix B: Ethical clearance from National Health Research Review Board of Eswatini.

Research Protocol clearance certificate

Type of review	Expedited	X		Full Board			
Name of Organization	**STUDENT**						
Title of study	**Genotypic and phenotypic diversity of *Mycobacterium tuberculosis* strains in patients with concomitant pulmonary and extra-pulmonary tuberculosis**						
Protocol version	**1.0**						
Nature of protocol	New	x		Amendment			
List of study sites	**MBABANE GOVERNMENT HOSPITAL**						
Name of Principal Investigator	**BUSIZWE SIBANDZE**						
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	**13/04/2017**		End date	**13/04/2018**		
Signature of Chairperson							
Date of signing	**13/04/2017**						
Secretariat Contact Details	Name of contact officers		**Ms Simangele Masuku**				
	Email address	kaluamasi@gmail.com					
	Telephone no.	**(00268) 24040865/24044905**					

MINISTRY OF HEALTH
13 APR 2017
HEALTH RESEARCH REVIEW BOARD
SWAZILAND

Appendix C: Ethical clearance from the Research Ethics Committee of the University of Pretoria.

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

1/06/2017

Approval Certificate
New Application

Ethics Reference No.: 115/2017

Title: Genotypic and phenotypic diversity of Mycobacterium tuberculosis strains in patients with concomitant pulmonary and extra-pulmonary tuberculosis

Dear Mr Doctor Sibandze

The **New Application** as supported by documents specified in your cover letter dated 23/04/2017 for your research received on the 3/05/2017, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 31/05/2017.

Please note the following about your ethics approval:
- Ethics Approval is valid for 1 year
- Please remember to use your protocol number (**115/2017**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of **6 monthly written Progress Reports**, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers; MBChB; MMed (Int); MPharMed,PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

☎ 012 356 3084 deepeka.behari@up.ac.za / fhsethics@up.ac.za http://www.up.ac.za/healthethics
✉ Private Bag X323, Arcadia, 0007 - Tswelopele Building, Level 4, Room 60, Gezina, Pretoria