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Nosocomial transmission of *Mycobacterium tuberculosis* in a regional hospital

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Submitted in fulfilment of the requirements for the degree of Masters of Medical Sciences (Medical Microbiology) in the Department of Medical Sciences and Infection Prevention and Control, College of Health Sciences,

University of KwaZulu-Natal

Declaration

I, Lungile Msizi declare as follows:

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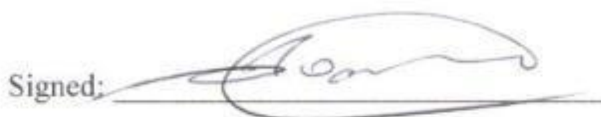
Signed:  Date: 28/06/2017

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List of Abbreviations

TB	Tuberculosis
M. tb	*Mycobacterium tuberculosis*
HIV	Human Immunodeficiency Virus
MDR-TB	Multidrug resistant tuberculosis
XDR-TB	Extensively drug-resistant tuberculosis
WHO	World Health Organization
CDC	Centres for Disease Control and Prevention
SA	South Africa
KZN	KwaZulu-Natal
CoSH	Church of Scotland Hospital
DOTS	Directly Observed Treatment Short-course
INH	Isoniazid
RIF	Rifampicin
STR	Streptomycin
PAS	Para-aminosalicyclic
PZA	Pyrazinamide
ETH	Ethionamide
KM	Kanamycin
OFX	Ofloxacin
TZ	Terizidone
DNA	Deoxyribonucleic acid
DR	Direct repeat
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
PCR	Polymerase Chain Reaction
DST	Drug susceptibility testing
RFLP	Restriction Fragment Length Polymorphism
MIRU-VNTR	Mycobacterial Interspersed Repetitive-Unit-Variable-Number Tandem Repeat
SpolDB4	International Spoligotyping Database
NALC-NaOH	N-acetyl-L-cysteine-sodium hydroxide
MGIT	Mycobacteria Growth Indicator Tube
OADC	Oleic Acid Dextrose Complex
LJ	Lowenstein-Jensen

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Ethical Approval

This study was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal (REF: BF240/12).

ABSTRACT

Background

Nosocomial transmission of drug resistant tuberculosis is well-documented in South Africa. The level of risk varies by patient population and effectiveness of *M.tb* infection control measures. The risk is higher in places where large numbers of infectious *M.tb* patients are being treated, particularly in the absence of other infection control measures such as respiratory protection. Globalization has led to the exchange and circulation of the various strains worldwide, leading to greater global diversity of *M.tb* strains. This ultimately increased the risk of individuals being infected with more than one strain at a time ie; having mixed infection

Methodology

In this study, we investigated the extent of transmission of drug resistant *M. tuberculosis* between patients hospitalized in a specialized TB hospital. The study was carried out at the FOSA TB hospital, Durban, South Africa. Genotyping was performed using IS*6110*-RFLP and spoligotyping.

Results

A total of 52 patients were recruited, from which 28 isolates were successfully cultured and genotyped. Cluster analysis of the isolated genotypes suggests nosocomial transmission of drug resistant strains amongst the in-patients. A majority of the strains found among the patients belonged to the F15/LAM4/KZN family (64%), followed by three clusters of formerly uncharacterised strains which were named Unique I, II and III. Only one of the drug resistant isolates belonged to the Beijing family of strains. Several locations and activities were identified where transmission could potentially have occurred, but this could not be done for individual patients

Conclusion

Implementation of rapid diagnostic testing for drug-resistant TB and redesign of healthcare facilities, to minimize congregate spaces, are critical elements that should be included in TB infection control programs; in addition to administrative, environmental and personal protective measures.

CHAPTER 1: INTRODUCTION

Infections with *Mycobacterium tuberculosis (M.tb)* account for high rates of morbidity and mortality worldwide (Cox *et al.,* 2010). The organism has the ability to persist in the human host in a dormant state for prolonged periods of time in the face of an active immune response. Progression from initial infection to disease is influenced by the balance between bacillary persistence and the immune system impairment (Ulrichs and Kaufmann, 2002, Kaufmann and Schaible, 2005, Mlambo, 2011). The causes of an impaired host response include among others, age, malnutrition, excessive use of steroids, uncontrolled diabetes mellitus, renal failure, underlying malignant disease, chemotherapy as well as human immunodeficiency virus (HIV) infection. All of these factors can result in the reactivation of dormant bacilli, resulting in clinical manifestations of tuberculosis (Mlambo, 2011).

The development of drug resistance among circulating tuberculosis (TB) strains continues to pose a challenge to health systems worldwide. Multidrug resistant (MDR-TB) is defined as, TB caused by *M.tb* resistant to both isoniazid (INH) and rifampicin (RIF), the two most powerful first-line anti-TB drugs (Chakraborty *et al*., 2010, Marahatta, 2010, Magana-Arachchi, 2013). Extensively drug-resistant (XDR-TB) is MDR-TB in which the causative agent is additionally resistant to any fluoroquinolone, and at least one of the three injectable second-line drugs (viz: capreomycin, kanamycin and amikacin) (Chakraborty *et al.*, 2010, Marahatta, 2010, Klopper *et al.,* 2013).

A South African countrywide survey, in 2002, revealed that 1.8% of all new TB patients and 6.7 % of previously treated TB patients had MDR-TB (Weyer *et al.,* 2004). This finding translated to an estimated annual case load of 13 000 MDR-TB cases, which placed South Africa (SA) fourth after China, India and the Russian Federation (WHO, 2008). However, this case load may be an underestimation due to poor case finding and reporting (Cox *et al.,* 2010).

Gandhi *et al*., (2006) reported a prevalence of 14% MDR-TB among study patients, 24% of which was found to have XDR-TB in Tugela Ferry, Umzinyathi District of South Africa. Additionally, they reported that all the XDR-TB infected patients were HIV-seropositive. The mortality in the latter was 98% with a median survival time from sputum collection to death of 16 days (Gandhi *et al*., 2006). It is also worth noting that the majority of these patients had not been previously treated for TB, which implies that they were newly infected with a TB strain with the XDR resistance profile. (Gandhi *et al.,* 2006, Grobusch, 2010). Furthermore, genotyping of the collected isolates showed that 39 of 46 (85%) patients with XDR-TB were infected with the same strain, suggesting possible nosocomial transmission (Gandhi *et al.,* 2006, Grobusch, 2010).

Adding to the challenge of *M.tb* transmission, hospitals across SA routinely discharge patients, if all available treatment options have been exhausted (Kellend, 2014). Premature release of these patients may be due to lack of bed space in designated tuberculosis hospitals, and the scarcity of alternative

long-term residential and palliative care facilities, resulting in community-based spread of the disease (Kellend, 2014).

There have been reports showing the occurrence of polyclonal or "mixed" infections within a single individual (Cohen *et al.,* 2012). The prevalence of mixed infections is not well documented (Mbabazi and Ouifki, 2010). Some studies have shown that it ranges between 2.3% and 75% in patients with active TB (van Rie *et al.,* 1999, Richardson *et al.,* 2002). An individual may have active and latent forms of the disease caused by different strains concurrently (Mbabazi and Ouifki, 2010). This has resulted in a paradigm shift in our understanding of the epidemiology of TB and may have important consequences on the implementation of interventions (e.g. treatment of latent infection), and on the prospect of developing effective preventative or therapeutic vaccines (Cohen *et al.,* 2011).

Infection prevention measures for TB in healthcare facilities are seldom prioritized. This results in unrecognized nosocomial transmission (Escombe *et al.,* 2010). Long length-of-stay, crowded congregate wards, poor infection prevention practices, and high prevalence of HIV infection create the ideal conditions for nosocomial transmission of *M.tb*. (Gandhi *et al.,* 2013). Furthermore, with little being known about the scope of mixed infections despite advances in strain genotyping, this greatly impacts on the interpretation of epidemiological data and subsequent treatment of patients (Shamputa *et al.,* 2006).

The aim of this study was to determine the extent of cross infection amongst patients admitted at the FOSA TB hospital, in KwaZulu-Natal, South Africa and to ascertain possible transmission chains between patients. This hospital is earmarked for the care of patients with MDR- and XDR-TB.

The objectives of the study were:

1. To determine patients' previous history of TB and current symptoms of TB
2. To perform daily mycobacterium culture on sputum specimens from all patients admitted to the designated wards during the 2-week study period followed by specimens collected at month 1 and month 2 post-recruitment
3. To perform susceptibility tests on all *M.tb* isolates
4. To genotype the *M.tb* isolates using IS*6110*-RFLP and spoligotyping
5. To link patient bed proximity and socialisation patterns during hospital stay to subsequent development of TB with a new strain

CHAPTER 2: LITERATURE REVIEW

2.1. Global burden of TB

In 2014 it was estimated that there were 9.6 million incident cases of *M.tb* globally, up from 8.6 million in 2012 (WHO, 2015). The highest number of cases occurred in Asia (58%) and Africa (28%); smaller proportions of new cases occurred in India (23%), Indonesia (10%) and China (10%) regions. The 22 high burden countries (including South Africa), accounted for 80% of all estimated incident cases worldwide (WHO, 2014).

South Africa was found to be one of the countries with the highest burden of *M.tb* infections, where an estimated incidence of 450,000 cases of active TB was reported in 2013 (WHO, 2014). Of a population of approximately 50 million, almost 1% develops active TB each year. WHO estimated that, of the 450,000 incident cases, 270,000 (60%) were co-infected with HIV (WHO, 2014).

The province of KwaZulu-Natal (KZN) in SA, is the epicentre of the HIV and TB co-epidemic (Kasprowicz *et al.,* 2011), and is also the province with the highest prevalence of drug-resistant TB (Shenoi *et al.,* 2009, Cohen *et al.,* 2015). Over 100,000 cases of TB are reported each year from this province alone (WHO, 2014). In 2006, the largest outbreak of XDR-TB was reported from KZN, SA (Gandhi *et al.,* 2006, Cooke *et al.,* 2011, Gandhi *et al.,* 2012, Gandhi *et al.,* 2013).

2.2. The TB bacillus

M.tb, the causative agent of TB in humans, is a non-motile rod-shaped bacterium. It belongs to the order Actinomycetales, family Mycobacteriaceae, and genus *Mycobacterium* (Ducati *et al.,* 2006). It is an acid fast bacillus, classified as an obligate aerobe. The microbe's width and length vary from 0.3 to 0.6 µm and 1-5 µm, respectively. It is characterized by a thick and complex lipid rich cell wall with components which are associated with its pathogenesis (Reed *et al.,* 2004, Zuber *et al.,* 2008) and considerably slow growth (Ducati *et al.,* 2006).

For many years *M.tb* has been described as a strict aerobic, facultative intracellular pathogen with cells of the macrophage lineage as its primary target cell. The organism enters these cells by adhesion to the C1 receptor on the cell surface. The resulting endocytosis is incomplete as it does not result in fusion with a lysosome and therefore killing of the bacterium does not occur. More recently, it was shown that alveolar epithelial cells are also important target cells. In addition, there is substantial evidence that the organism is not a strict aerobe but can also metabolise and multiply under anaerobic conditions (Harakiram P, PhD thesis, UKZN, 2008).

Another point, thought for decennia to be true, is that close contact of long duration is needed for transmission to occur effectively. Close contact, which means within the distance that allows for infected droplet nuclei from a patient to reach an uninfected person, is still essential. However, it has been shown as early as mid-1990s by comparing genotyping results with classic epidemiology that casual contact is sufficient (Wilkinson D, PhD Thesis, University of Natal, 1997). This was followed by several other studies confirming this observation (Dodd *et al,* 2016).

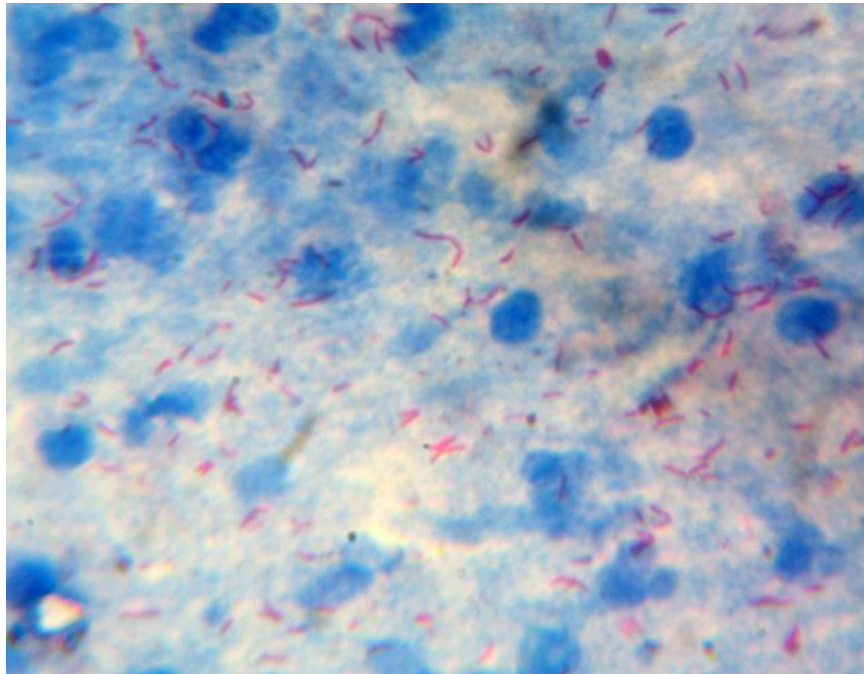

Figure 2.1: ***Mycobacterium tuberculosis*** **revealed with acid-fast Ziehl-Neelsen stain (1000x).**

Resistance development in *M.tb* is largely due to mutational events affecting the genes coding for drug target molecules. The reason for this is that exchange of resistance gene carrying genetic elements like plasmids cannot occur due to the cell wall structure of mycobacteria. In addition, no transduction takes place between mycobacteria and species from other genera since only mycobacteriophages do infect mycobacterial species with limited host range for each of them within the family of Mycobacteriaeceae. Since the chances that more than one mutation occurs in the same bacterial cell, it was thought that the multidrug approach which has been practiced for many years would prevent resistance to multiple drugs to occur. However, since the mid 1990's strains resistant to more than one drug used as firstline treatment of TB have emerged and are now found throughout the world.

2.3. Multidrug resistant tuberculosis

The emergence and spread of MDR-TB continues to threaten TB control globally (Corbett *et al.,* 2003, Alexander and De, 2007, Cox *et al.,* 2010). In 2007, global TB data from 198 countries reported an estimated 0.5 million cases of MDR-TB, with 27 countries accounting for 85% of all cases (Magana-Arachchi, 2013). India, China, the Russian Federation, SA and Bangladesh were ranked the highest in terms of total numbers of MDR-TB cases (WHO, 2009). The global burden of MDR-TB is thought to be much higher than the current estimates (Chakraborty *et al.,* 2010, WHO, 2014).

South Africa's estimated incidence of TB has risen from 317 to 1000 per 100 000 between 1995 and 2012 (Bantubani *et al.,* 2014). The spread of drug-resistant TB within SA constitutes an additional challenge in control of the disease (Bantubani *et al.,* 2014) and has resulted in a fifth place among countries with the highest global incidence of MDR-TB after China, India, Russian Federation, and Pakistan (Bantubani *et al.,* 2014). The urgency in addressing drug-resistance in a high HIV prevalence setting was underscored by the report of an outbreak of XDR-TB in Tugela Ferry centred in and around the Church of Scotland Hospital (CoSH) in the Msinga sub-District of KZN (Gandhi *et al.,* 2006). This report documented the largest outbreak of XDR-TB with 6% of the study population harbouring XDR-TB strains; additionally, all of the latter were also HIV-sero-positive. This focused global attention, not only to the drug-resistance problem in SA, but also to worldwide resistance (Gandhi *et al.,* 2006, Ioerger *et al.,* 2009). Furthermore, XDR-TB has been reported throughout SA (Karim et al., 2009), in neighbouring sub-Saharan countries and in 58 countries worldwide (Bwanga *et al.,* 2010, WHO, 2010).

In order to determine the extent of the drug resistance, mixed infections, as well as nosocomial infections, molecular biology techniques have been applied to determine the genetic fingerprint of the clinical isolates. Applying such techniques, a study by Pillay and Sturm (2010) revealed that the strains prevalent in Tugela Ferry were of the F15/LAM4/KZN genotype (Pillay and Sturm, 2010). Furthermore, retrospective studies on the evolution of this genotype in the province showed that this *M.tb* strain type has been responsible for cases of MDR-TB since 1994 and XDR-TB cases from 2001 (Pillay and Sturm, 2010). XDR-TB has been reported in all of the country's provinces and has been shown to belong to various strain families (Mlambo *et al.,* 2008, Gandhi *et al.,* 2014).

2.4. Treatment of tuberculosis

The dawn of anti-TB chemotherapy was in 1944 with the discovery of streptomycin (STR), followed by the discovery of para-aminosalicyclic (PAS) in 1946 (Daniel, 2006, Zhang and Yew, 2009). A study done by Fleury (1951), comparing the efficacy of STR and PAS when used alone and in combination, showed that combined therapy was more effective (Wada,

2007). This provided initial evidence for the multidrug approach in the management of TB. The addition of isoniazid (INH) and pyrazinamide (PZA) in 1952 to the treatment regimen improved treatment outcomes with cure rates approximating 100% (MRC, 1950, Jawahar, 2004). The duration of treatment however, was still lengthy (18 to 24 months). With the introduction of rifampicin (RIF) in the early 1960s, the treatment duration of TB was sequentially decreased to the currently recommended duration of 6 months for uncomplicated drug susceptible TB (Wada, 2007).

The internationally recommended TB chemotherapy strategy known as Directly Observed Treatment Short-course (DOTS) is aimed at completely eliminating active and dormant bacilli, and involves two phases of treatment using first-line anti-TB drugs (Narita *et al.,* 2002). The initial (intensive) phase comprises the use of INH, RIF, ethambutol (EMB) and PZA for 2 months, followed by 4 months continuous phase treatment using INH and RIF only (Mitchison, 2005). Despite the effectiveness of anti-TB drugs in the management of the disease, the burden of disease remains a global problem and is exacerbated by the emergence and spread of drug-resistant strains (Mlambo, 2011). While infections with drug-sensitive *M.tb* can be effectively treated with first-line drugs, the treatment of infection with MDR-TB strains remains a challenge with poor treatment outcomes. The long treatment duration can significantly increase the risk of patient non-compliance and increases the cost of treatment significantly (WHO, 2008, Mlambo, 2011). The current recommended standardized regimen for MDR-TB treatment comprises 4 months intensive phase using kanamycin (KM), ofloxacin (OFX), ETH, PZA, EMB or Terizidone (TZ), followed by a continuous phase for 12 to 18 months comprising ETH, OFX, EMB or TZ (Mlambo, 2011).

The emergence of XDR-TB strains of *M.tb* has further complicated the treatment regimens for tuberculosis. Treatment of XDR-TB cases is associated with even poorer outcomes than MDR-TB, especially in patients co-infected with HIV resulting in high mortality (Gandhi *et al.,* 2006, Gandhi *et al.,* 2010).

2.5. Transmission

M.tb is spread from person to person through the air by droplet nuclei which are produced when persons with pulmonary or laryngeal TB cough, sneeze, speak or sing (Frieden *et al.,* 2003). Droplet nuclei can also be generated through sputum induction, during inhalation therapy, and through processing of laboratory specimens (Tang *et al.,* 2006, Selekman, 2006, Mlambo, 2011). Due to their small size, droplet nuclei can remain airborne for minutes to hours after expectoration (Nardell, 2004). When inhaled, droplet nuclei are carried down the bronchial tree and implant in respiratory bronchioles or alveoli in the lungs. Whether or not an inhaled tubercle bacillus establishes an infection

in the lung depends on both bacterial virulence and the strength of the immune system (Frieden *et al.,* 2003).

Transmission of *M.tb* is highest in overcrowded and poorly-ventilated environments such as prisons, clinics and hospitals, which have been reported to have higher incidence rates compared to the general population (Ramtahal, 2011).

2.5.1. Nosocomial transmission

TB has long been recognized as a potential occupational hazard by the medical community. In the mid-1980s, only a few institutions were prepared for the changing epidemiology of the disease, which led to the resurgence of TB (Raviglione *et al.,* 1993). Infection control measures were put in place to reduce transmission, however, as the incidence of TB, and with that the risk of occupational exposure and infection, declined less attention was paid to infection control practices (Menzies *et al.,* 1995). In the 1990s, outbreaks of the disease highlighted the risk of nosocomial transmission of TB and risks for health care workers, highlighting again the importance of effective infection-control measures (Reichman and Hershfield, 2000).

Since then, nosocomial transmission has been well documented (Dooley *et al.,* 1992, Pearson *et al.,* 1992, Gandhi *et al.,* 2006), and factors that facilitate nosocomial transmission of TB have been identified (Table 2.1) (Reichman and Hershfield, 2000). The level of risk varies by patient population and effectiveness of *M.tb* infection control measures. The risk is higher in places where large numbers of infectious *M.tb* patients are being treated, particularly in the absence of other infection control measures such as respiratory protection (CDC, 2005).

Table 2.1: Factors facilitating nosocomial transmission of TB.

1. *Inefficient infection-control procedures*
   - Delay in ordering diagnostic tests
   - Clustering of patients
   - Failure to isolate patients with active pulmonary disease
   - Failure to employ appropriate PPE
2. *Laboratory delays in identification and susceptibility testing of M.tb isolates*
3. *Inadequate isolation facilities and engineering controls*
   - Lack of isolation rooms
   - Recirculation of air from isolation rooms to other parts of the hospital
4. *Delayed initiation of effective anti-TB therapy*

*Adopted from (Reichman and Hershfield, 2000)

In previous outbreaks investigated by the Centers for Disease Control and Prevention (CDC), the lack of adequate procedures to identify patients with possible TB, and the failure to isolate such patients immediately once TB was suspected, was highlighted as a major factor responsible for nosocomial transmission of the disease (McGowan, 1995). Other factors have included fraternization of suspected *M.tb* patients with patients at increased risk for TB infection eg: immune compromised HIV-infected patients. Inadequate laboratory facilities and far from robust rapid diagnostics and susceptibility testing for TB also lead to a delay in initiation of anti-TB therapy, which allows for prolonged infectivity and increased risk of transmission of *M.tb* (Jarvis, 1995).

2.5.2. Prevention of nosocomial transmission of TB

Nosocomial spread of *M.tb* can be prevented by a combination of administrative, infrastructural and engineering controls, as well as personal protective equipment for healthcare workers and patients (Table 2.2).

Table 2.2: Elements involved in nosocomial TB prevention.

Administrative Controls	**Infrastructural & Engineering controls**	**Respiratory protection**
Managerial policies designed to reduce exposure to aerosols and thus reduce the risk of transmission, infection and disease.	Fundamental facilities, practices, or equipment that reduces the concentration of infectious bacilli in the environment.	Protection of personnel working in areas with contaminated air.
Examples: Rapid identification of high-risk patients and removal to a separate area. Rapid screening policies. Isolation of patients with suspected or confirmed TB.	**Examples:** Natural ventilation (e.g. open windows). Negative-pressure isolation facilities.	**Examples:** N95 particulate filter masks.

The implementation of the above-mentioned measures presents major difficulties, especially in resource-limited settings (Whitelaw, 2011, Shenoi et al., 2012). A systematic approach used to assess and analyse the effectiveness of a health care facility's existing control programs, strengthens them and curbs nosocomial transmission of *M.tb* (Pearson et al., 1992, Pillay and Sturm, 2010).

2.6. Dedicated MDR-TB hospitals in SA

With drug resistant TB on the rise, SA has insufficient infrastructure to cope with the ongoing epidemic. In existing dedicated MDR-TB hospitals infrastructure constraints remain an obstacle to safe health service delivery. This is due to the fact that the buildings used for housing TB patients have been built before MDR-TB developed. At that time cross infection could not occur because the treatment regimen a patient was on protected against this. Other key issues include funding and staffing constraints and lack of bed space for the increasing number of infected TB patients. Ideally, infected patients should be housed in isolation rooms to reduce the opportunity of cross-infection. Research has shown that patients housed in multi-bed units are often cross-infected with different strains of TB (Ulrich *et al.,* 2008, Sissolak *et al.,* 2011) resulting in increased treatment costs and an increase in the length of stay.

The treatment policy in SA requires the hospitalization of drug-resistant patients until they have two consecutive negative TB cultures at least a month apart (NDoH, 2007). The increasing infection rate and improved case finding have led to an increasing number of patients who require hospitalization (Sissolak *et al.,* 2011). Meeting the accommodation needs for the patients infected with a drug resistant strain provide a significant challenge which has resulted in existing TB hospitals being used as alternative accommodation to house the increasing number of drug-resistant patients. Most of the existing TB hospitals were designed with large open wards which are suitable for drug-susceptible TB, but are not appropriate for patients with drug-resistant TB (Parsons *et al.,* 2010).

In 2010, the Department of Health began decentralising treatment for drug resistant TB from specialised hospitals to facilities closer to patients living areas. This increased the bed availability for MDR-TB patients and decreased the obstacles for family members to visit patients (NDoH, 2010). Decentralization has allowed for earlier treatment initiation resulting in shorter hospital stays, and earlier release into controlled out-patient treatment facilities closer to their homes (Sinanovic *et al.,* 2015).

There are currently 9 MDR-TB decentralised management units and 17 MDR-TB satellite sites, found in 8 districts (Ugu, eThekwini, Zululand, Harry Gwala, Umkhanyakude, UThungulu, UMgungundlovu and Umzinyathi) in the KwaZulu-Natal Province.

Infection prevention and control (IPC) for TB in SA is the responsibility of the individual healthcare facilities (Sissolak *et al.,* 2011). As mentioned above, TB-IPC is based on a three-level hierarchy of controls, including administrative, environmental, and respiratory protection (Sissolak *et al.,* 2011). Sissolak *et al.,* (2011) discovered a total lack of isolation facilities in public sector hospitals in SA (Sissolak *et al.,* 2011), in 2011. Furthermore, Tshitangano *et al.,* (2013) found that IPC interventions, in SA, were still not aligned to the WHO policy on TB infection control in healthcare facilities. The researchers also reported the lack of TB infection control plans in these hospitals (Tshitangano *et al.,* 2013). This greatly contributes to nosocomial transmission amongst patients in SA (Sissolak *et al.,* 2011, Gandhi *et al.,* 2013). Nurses in Port Elizabeth's Joseph Pearson TB hospital reported that MDR-TB patients were contracting XDR-TB strains even though they were housed in designated wards (Sissolak *et al.,* 2011). TB-IPC needs to be strengthened and regularly monitored in South African healthcare facilities in an attempt to curb the drug-resistant TB epidemic.

2.7. Mixed strain Infections

While it was originally assumed that *M.tb* was a homogenous species, with the advent of genotyping methodologies, different strains were reported from different geographic regions (Mbabazi and Ouifki, 2010). Globalization has led to the exchange and circulation of the various strains worldwide, leading to greater global diversity of *M.tb* strains (Beyers *et al.,* 1996). This ultimately increased the risk of individuals being infected with more than one strain at a time ie; having mixed infection (Mbabazi and Ouifki, 2010).

Genetic heterogeneity of *M.tb* within a host may arise by one of two mechanisms: (i) intra-host diversification following a single infection event, or (ii) sequential acquisition of *M.tb* from different persons resulting in a mixed infection with more than one strain (Cohen *et al.,* 2011). The clinical consequences of intra-host diversity are most obvious when manifesting as subpopulations of bacteria with resistance to anti-TB antibiotics, either reflecting acquired drug resistance or transmitted drug resistance (Cohen *et al.,* 2011). The intra-host competition between strains may influence the clinical outcomes for co-infected patients (van Rie *et al.,* 2005b) because individuals can be simultaneously infected by strains with different phenotypic characteristics (e.g. growth rates, drug resistance) (Cohen *et al.,* 2011).

Using molecular genotyping methods, the frequency of mixed infection was shown to range from 2.8% to 19% in countries like Malawi, China, Uganda, Taiwan, Georgia, Central Asia and SA (Warren *et al.,* 2004, Shamputa *et al.,* 2006, Hanekom *et al.,* 2013), in 2013. As mentioned previously, the prevalence of mixed infections among the various *M.tb* cohorts is not well

documented (Mbabazi and Ouifki, 2010). Some studies have shown that it ranges between 2.3% and 75% in individuals with active TB (van Rie *et al.,* 1999, Richardson *et al.,* 2002). However, its prevalence in the total population is unknown. The difficulty in determining the prevalence of mixed infections is due to the deficiencies associated with current diagnostic tests.

2.7.1. Detection of mixed infections

The diversity present within *M.tb* isolates has generated a new appreciation for this genetically conserved bacterial species (Brown *et al.,* 2010, Cohen *et al.,* 2010). Molecular genotyping applications, such as restriction fragment length polymorphism (RFLP), using the IS*6110* insertion sequence, spacer oligonucleotide (spoligo) typing, as well as mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) typing, have been used to identify transmission chains (Daley *et al.,* 1992, Alland *et al.,* 1994), to classify strains into families and lineages (Allix-Beguec *et al.,* 2008, Comas *et al.,* 2009) and to detect the presence of intra-host genetic heterogeneity (Warren *et al.,* 2004, Shamputa *et al.,* 2006).

Molecular epidemiological studies have been able to identify individuals with repeated episodes of disease due to re-infection (Richardson *et al.,* 2002, Small *et al.,* 1993, Chiang and Riley, 2005). The data confirm previously reported findings, suggesting previous exposure or disease does not confer protection against subsequent re-infection (Canetti *et al.,* 1972, van Rie *et al.,* 2005a, Andrews *et al.,* 2012). This has resulted in a paradigm shift in our understanding of the epidemiology of TB and may have important implications for the implementation of interventions (i.e. treatment of latent infection), and for the prospect of developing effective preventative or therapeutic vaccines (Cohen *et al.,* 2011).

2.8. Genotyping of *Mycobacterium tuberculosis*

Genotyping of *M.tb* has greatly improved the study of TB epidemiology (Zaczek *et al.,* 2013). It is essential in the identification of strains circulating within a population, making it possible to monitor prevalent strains and their potential mutations (Gori *et al.,* 2005). Identification of epidemiologically linked *M.tb* strains helps to reveal the source of infection, to trace the transmission routes of various strains, and to determine the risk factors for *M.tb* transmission in a community (Zaczek *et al.,* 2013). This may assist coordinators of TB-control programs to direct and evaluate their programme activities (Miller *et al.,* 2002). The gold standard for *M.tb* genotyping is RFLP using IS*6110* as a marker, but other molecular typing techniques such as spoligotyping and MIRU-VNTR, could be used as adjuncts in select circumstances (Cohn and O'Brien, 1998).

2.8.1. IS6110-RFLP

IS*6110*-RFLP analysis relies on the determination of the number of copies and location of the IS*6110* elements on the chromosome (McEvoy *et al.,* 2007). This insertion sequence was first recognized by van Embden and colleagues in the 1990s (van Embden *et al.,* 1993). The IS*6110* sequence belongs to the IS3 family and is 1.355-kilo base pairs long. It is found in species within the *M.tb* complex, and in most members of the complex, the sequence is present as multiple copies. The exception is *Mycobacterium bovis* which usually contains one copy (Jagielski *et al.,* 2014). The IS*6110*-RFLP technique is considered as a reference standard for strain differentiation in molecular epidemiological studies, and has been widely used for the identification and investigation of outbreaks, the identification of laboratory cross-contamination, and distinction between re-infection and relapse (McEvoy *et al.,* 2007). The method includes digestion of genomic DNA with *PvuII* restriction enzyme that cleaves the IS*6110* sequence only once, generating DNA fragments that are separated through gel electrophoresis. The separated products are then transferred onto a membrane, and hybridized with a peroxidise-labelled probe complementary to part of the 3'-end of the IS*6110* sequence (Jagielski *et al.,* 2014). The method is highly discriminatory and reproducible. However, the method has several disadvantages that limit its use in routine practice: 1) poor discriminatory power when applied to isolates with a low IS*6110* copy number, 2) labour-intensive, 3) requires weeks of incubation to obtain sufficient quantities of high-quality DNA, and lastly, 4) generates band patterns that may be difficult to share between laboratories (Sougakoff, 2011).

Despite the limitations, IS*6110*-RFLP method remains one of the most commonly used approaches for *M.tb* typing and has long been considered the gold standard technique in molecular epidemiological investigations of TB (Jagielski *et al.,* 2014). Its main advantage is high reproducibility and high discrimination between organisms.

2.8.2. Spoligotyping

Spoligotyping is based on the amplification and detection of the presence, or absence, of non-repetitive sequences called spacers found between direct repeat elements in the clustered regularly interspaced short palindromic repeats (CRISPR) region (van Embden *et al.,* 2000, Kato-Maeda *et al.,* 2011). Regularly interspaced short palindromic repeats (CRISPRs) can be used for both epidemiology and evolutionary analysis of *M.tb* (van Embden *et al.,* 2000). The entire direct repeat locus is amplified by Polymerase Chain Reaction (PCR (Jagielski *et al.,* 2014). The PCR products are then hybridized onto a membrane with 43 covalently bound synthetic oligonucleotides representing the polymorphic spacers identified in *M.tb* H37Rv (spacers 1-19, 22-32, and 37-43) as well as *M. bovis* BCG (spacers 20-21 and 33-36) (Jagielski *et al.,* 2014). The hybridization signals are then detected by chemiluminescence through biotin labelling of the PCR products (one of the primers is biotinylated)

and a streptavidin-peroxidase conjugate system and then visualised by autoradiography (Jagielski *et al.,* 2014). Individual strains are differentiated by the number of the spacers that are missing from the complete 43-spacer set (Kamerbeek *et al.,* 1997). As opposed to IS*6110*-RFLP, spoligotyping is based on PCR amplification which requires small amounts of DNA thus bypassing the need for a large amount of isolates (Sougakoff, 2011). It is a relatively simple, cost effective, and high-throughput method, whose results are accurate, reproducible and are obtained in up to 2 days (Jagielski et al., 2014). Furthermore, given the binary format of the data, the spoligotyping results can easily be interpreted, computerized, and compared between different laboratories using the international spoligotyping database (SpolDB4) which was released in 2006 and is accessible online (Jagielski *et al.,* 2014).

The signatures given by the 43 spacer-spoligotyping patterns have been used to define strain families and a description of the circulating genotypes of tubercle bacilli worldwide (Brudey *et al.,* 2006). As compared with the other molecular methods, the main limitation of spoligotyping is its limited discriminatory power, particularly in some lineages such as the Beijing strains, where nearly all share a single spoligotype (Sougakoff, 2011). The discriminatory power of spoligotyping was reported to be lower than IS*6110*-RFLP (Kremer *et al.,* 1999). The reason for the limited discriminatory capacity of the spoligotyping method is due to the fact that it targets only a single genetic locus, covering less than 0.1% of the *M.tb* complex genome (Jagielski *et al.,* 2014). Nevertheless, spoligotyping can be effectively used for the differentiation of *M.tb* strains with low IS*6110* copy numbers (Jagielski *et al.,* 2014).

2.8.3. MIRU-VNTR

Variable numbers of tandem repeats (VNTR) analysis is a powerful method that can provide adequate discrimination of *M.tb* strains, in terms of both identification of genetic lineages and estimation of transmission (Supply *et al.,* 2000). The method is PCR-based, relies on the analysis of tandem repeats present in 41 genetic elements (called mycobacterial interspersed repetitive units [MIRUs] scattered throughout the *M.tb* genome, with a variable number of copies of the repeat unit in each locus. MIRU-VNTR analysis involves PCR amplification of a specific MIRU locus, followed by determination of the sizes of the amplicons by gel electrophoresis or, after running multiplex PCRs, on an automated, fluorescence-based sequencer (Jagielski *et al.,* 2014). Since the lengths of the repeat units are known, the calculated sizes reflect the numbers of the amplified MIRU copies. The final result is a multi-digit numerical code (MIRU-VNTR code) corresponding to the repeat number at each analysed locus (Jagielski et al., 2014). This coding system allows the results to be readily compared across laboratories worldwide and facilitates the data to be deposited in the global databases via the internet for large-scale epidemiological and population genetic studies (Jagielski *et al.,* 2014).

The MIRU-VNTR method is an efficient and reliable typing system, whose discriminatory capacity approximates, or even exceeds that of IS*6110*-RFLP profiling (Supply *et al.,* 1997). In general, the discriminatory power of MIRU-VNTR analysis increases with the number of loci evaluated. MIRU-VNTR typing based on 12 loci is slightly less discriminatory than IS*6110*-RFLP analysis for *M.tb* isolates with high copy number of IS*6110* but at the same time more discriminatory than the IS*6110*-RFLP if low copy number IS*6110* isolates are investigated (Jagielski *et al.,* 2014). In 2006, a new system employing 24 MIRU-VNTR loci was proposed. Of these 24 loci, 15 (including 6 of the original set of 12) account for 96% of all detected polymorphisms in *M.tb* strains, and the discriminatory power of this new 24-locus MIRU-VNTR typing system is said to be equal to that IS*6110*-RFLP profiling (Oelemann *et al.,* 2007, Allix-Beguec *et al.,* 2008)

A particular advantage of the MIRU-VNTR genotyping, compared to the IS*6110*-RFLP typing, is its portability due to digitalization of the generated patterns and therefore simple intra- and inter-laboratory comparability as well as the amenability to inclusion in web-based databases (Jagielski *et al.,* 2014).

CHAPTER 3: METHODOLOGY

3.1 Study design

3.1.1. Setting

FOSA TB hospital is a dedicated facility for the treatment of MDR and XDR-TB patients for periods ranging from 3 to 18 months. The hospital is a step-down facility (a hospital providing follow-up care following initial management in an acute care hospital). It admits patients from King Dini Zulu hospital, formerly King George V Hospital, which is the acute care facility for patients with drug resistant TB for KZN. The facility has 6 wards (3 male and 3 female wards) with a total of 180 beds and a 70-80% bed occupancy rate. At the time of the study, the hospital had a total of 121 MDR-TB cases and 43 XDR-TB cases. In some of the wards patients with MDR and XDR-TB were housed together due to lack of space.

3.1.2. Ethical Approval

This study was approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (#BF240/12). Informed consent was obtained from the patients during the recruitment process (Annexure 1).

3.1.3. Specimen Collection

Following recruitment, baseline sputum specimens were collected from the patients. Two more sputum specimens were collected at month 1 and month 2 post-recruitment. The collected sputum specimens were transported to the Infection Prevention and Control Department's TB laboratory at the School of Laboratory Medicine, University of KwaZulu-Natal for culture, drug susceptibility testing (DST), and genotyping analysis using both IS*6110*-Restriction Fragment Length Polymorphism (RFLP) and spoligotyping.

3.1.4. Patient Data Collection

Patient demographic information and data for epidemiological linkages, in the form of questionnaires was gathered upon commencement of the study. Additionally, the location of each patient in the ward was recorded daily on a 'bed allocation chart' (Annexure 2 and 3). Duration of exposure was determined retrospectively, as the number of patient days that each individual was in contact with other infected patients. Patient contact was categorized as "close contact" if the patient occupied an adjacent bed and as "intermediate contact" if the patient was in the same ward. A mobility

questionnaire was also administered daily to all patients in an attempt keep track of patients' movements around the ward and their social interactions with other patients.

Patient clinical outcomes and additional data were obtained via the patient files. The information gathered included: age, gender, HIV serology and other blood test results, chest X-rays, patients' previous TB history and current symptoms, DST profile, anti-TB treatment as well as outcomes at the end of the study.

MDR-XDR-TB outcomes were defined as follows (Laserson et al., 2005):

1. **New patient:** a TB treatment naïve patient, or on TB treatment for < 1 month.
2. **Treatment Completed**: a patient has completed treatment according to the country's protocol but does not meet the definition of cure or treatment failure due to lack of bacteriological results (i.e. fewer than five cultures were performed in the final twelve months of therapy).
3. **Death:** a patient who died for any reason during the course of TB treatment.
4. **Transfer out:** a patient who has been transferred to another health care unit and for whom the treatment outcome is unknown.
5. **Relapse:** a patient who has been previously treated for TB and was declared cured or treatment completed at the end of the most recent course of treatment, and is now diagnosed with a recurrent episode of TB (either a true relapse or a new episode of TB caused by re-infection).
6. **Treatment failure:** a patient who has been previously been treated for TB and whose treatment failed at the end of the most recent course of treatment.
7. **Treatment default:** a patient whose TB treatment was interrupted for two or more consecutive months for any reason.
8. **Loss to follow-up:** a patient who has been previously treated for TB and was declared lost to follow-up at the end of the most recent course of treatment.

3.2 Specimen Processing

All specimens were transported to the laboratory within four hours of collection and processed the same day as collection. The decontamination procedure involved using the N-acetyl-L-cysteine/sodium hydroxide (NALC-NaOH) method of (Kent and Kubica, 1985). Direct smear microscopy was performed using Ziehl-Neelsen and fluorescent auramine staining for detection of acid-fast bacilli (AFB).

3.2.1. Decontamination of sputum specimens

The specimens were processed in the routine TB culture laboratory according to protocol. In brief, after digestion and decontamination using the NALC-NaOH method (Kent and Kubica, 1985) (Appendix 1.1), the sputum was cultured in MGIT broth, incubated in a Bactec 940 incubator (BD, USA) and 7H11 Middlebrook agar plates (Appendix 1.6) (Isenberg, 2004a).

3.2.2. Culture in Mycobacteria Growth Indicator Tube (MGIT)

Five hundred micro-litres of decontaminated specimen was added to a MGIT tube containing MGIT broth with growth supplement and MGIT PANTA (Polymixin, Amphotericin, Nalidixic acid, Trimethorprim, Azlocillin) antibiotic mixture (BD BioSciences, Sparks, MD, USA) (Appendix 1.3). The cultures were incubated at 37˚C in a Bactec 940 incubator. H37Rv laboratory strain served as a growth control, and a tube containing un-inoculated broth was used as a negative control.

3.2.3. Culture onto Middlebrook 7H11 agar plates

One hundred micro-litre of each decontaminated specimen was deposited onto the surface of a Middlebrook 7H11 agar plate. A sterile quadloop (Merck, SA) was used to streak the inoculum across the entire plate. Plates were incubated in sealed gas permeable bags for 3-6 weeks at 37°C in a regular incubator. Plates were viewed weekly for 3 weeks for the presence of *M.tb* colonies.

3.2.4. Microscopy

Ziehl-Neelsen staining was used to confirm the presence of acid-fast bacilli on each of the positive MGIT broth. Those containing acid-fast bacilli were then sub-cultured onto Middlebrook 7H11 agar plates to obtain single colonies. Plates were heat sealed in gas permeable bags and incubated for 3-6 weeks at 37°C. Plates were viewed after 3 weeks for the presence of *M.tb* single colonies, which were then inoculated in Middlebrook 7H9 broth (Appendix 1.4) for further growth, followed by inoculation onto Lowenstein-Jensen agar slants.

3.2.5. Culture on Lowenstein-Jensen (LJ) slants

Each isolate was inoculated into Middlebrook 7H9 broth. Tubes were incubated for 3-6 weeks at 37°C until growth was observed. Then the culture was aspirated using a sterile glass pasteur pipette and inoculated onto LJ slants. The LJ slants were inverted a few times to spread the inoculum across the surface of the slant. The LJ slants were incubated with slightly loosened caps for 3-6 weeks at 37°C. These cultures were used to extract DNA for genotyping.

3.3 Identification of culture positive isolates

Identification to species level of the growth on Middlebrook 7H11 agar plates was done using two biochemical tests, the Nitrate (Appendix 1.7.1) and Niacin (Appendix 1.7.2) tests. If both nitrate and niacin tests were positive, this confirmed the culture as being *M.tb*.

3.4 Drug susceptibility testing (1% Modified Proportion Method)

The 1% proportion method calculates the proportion of resistant bacilli present in an isolate and is considered the "gold standard" method for DST (Kim, 2005). Comparing the number of *M.tb* colonies growing on the drug-free control to that on the drug-containing media, enables the calculation of the proportion of drug-resistant colonies for the drug incorporated in the media.

Colonies from a 3-week old culture were suspended in phosphate buffered saline (PBS) (Merck, SA) containing 0.05% Tween-80 (Merck, SA) and 4 sterile glass beads (diameter: 5mm). This suspension was thoroughly vortexed following which the remaining clumps were allowed to settle by leaving the suspension for 15 minutes undisturbed on the bench. The top layer was aspirated and matched to a McFarland standard of 1.0 which is equivalent to $\pm$ 3.0×10^8 CFU/ml by adding PBS. Four 10-fold serial dilutions were done by adding 0.5ml of the suspension of 10^4 colony forming units per ml (cfu/ml) to 4.5 ml PBS. Of this suspension, 100µl was inoculated on quadrants of the Middlebrook 7H10 agar plates (BD, Difco Laboratories, USA) (Appendix 1.5) containing different drugs (Table 3.1) and a drug free control. The drug concentrations were chosen according to the WHO recommended critical concentrations (WHO, 2007). This resulted in a final inoculum of 10^{-3} cfu/quadrant. Plates were allowed to dry for 30 minutes and sealed in gas permeable bags and incubated at 37°C. Susceptibility tests were read after 3 weeks.

Table 3.1: Drugs and their respective concentrations used in DST determination

Drug*	**Concentration**
Isoniazid	1 µg/ml
Rifampicin	1 µg/ml
Ethambutol	7.5 µg/ml
Streptomycin	2 µg/ml
Ofloxacin	2 µg/ml
Kanamycin	5 µg/ml
Ethionamide	5 µg/ml
Capreomycin	10 µg/ml

*Obtained from Sigma-Aldrich, USA

Any isolate with growth less than 1% in proportion to the drug-free control, was classified as susceptible for that drug while growth of 1% or more was classified as resistant (Reichman and Hershfield, 2000).

3.5 Genotyping of isolates

The Cetyltrimethylammonium bromide-Sodium chloride (CTAB-NaCl) method is an isolation method most commonly used for attaining high purity mycobacterial DNA (Ramtahal, 2011). *M.tb* colonies were scraped off LJ slants using a sterile swab and suspended into 500µl of sterile distilled water. The bacteria were heat killed at 80˚C in a thermomixer comfort (Merck, SA) for 30 minutes following which 70µl of 10% Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA) (Appendix 2.3.4) solution and 50µl of proteinase K (Roche Diagnostics, Manneheim, Germany) (Appendix 2.3.3) of concentration 10mg/ml were added to each tube. The tubes were then incubated at 60˚C for 1 hour. The 5M Sodium chloride (NaCl) (Sigma-Aldrich, USA) (Appendix 2.3.5) and CTAB-NaCl (Sigma-Aldrich, USA) (Appendix 2.3.6) solutions were pre-warmed at 60˚C and 100µl of each solution was added to each tube and incubated at 60˚C for 15 minutes. 700µl of Chloroform (Sigma-Aldrich, USA): Isoamyl alcohol (Sigma-Aldrich, USA) (24:1) (Appendix 2.3.7) solution was then added to each tube and the tubes were centrifuged for 10 minutes at 12000 rpm. The upper aqueous supernatant was transferred to 700µl of Isopropanol (Sigma-Aldrich, USA) and placed in a -20˚C freezer for 30 minutes. Following centrifugation for 10 minutes at 12000 rpm, the isopropanol was discarded and the pellet was washed with 80% ethanol (Merck, SA) (Appendix 2.3.8) and the pellets were left to dry at room temperature for 10 minutes. 55µl of 1xTE buffer (Appendix 2.3.2) was added to each pellet and stored at 4˚C overnight or until further use (Van Soolingen *et al.,* 1991) (Appendix 2.1).

3.5.1 IS6110-Restriction Fragment Length Polymorphism

The IS*6110*-RFLP genotyping technique, using IS*6110* as a genetic marker, is recognized as a reference standard for strain differentiation of *M.tb* and is used in epidemiology studies. The technique has been widely used for the investigation of outbreaks, the identification of laboratory cross-contamination, and distinction between re-infection and relapse (McEvoy et al., 2007).

Genotyping using IS*6110*-RFLP was performed as previously described (Van Embden et al., 1993). The method includes digestion of genomic DNA with *PvuII* restriction enzyme which cleaves the IS*6110* sequence once. It generates DNA fragments that are separated through gel electrophoresis. The fragments are then transferred onto a membrane and hybridized with a horseradish peroxidase-labelled probe complementary to part of the 3'-end of the IS*6110* sequence (Jagielski et al., 2014).

Extraction and isolation of mycobacterial DNA was performed using the Cetyltrimethylammonium bromide-Sodium chloride (CTAB-NaCl) method as previously described (Van Soolingen et al., 1991) (Appendix 2.1).

In order to assess the quality and the concentration of the extracted DNA, gel electrophoresis was performed on a 1% agarose gel (Appendix 2.2). Sterile distilled water was used to dilute the DNA to the acquired concentration (50µg/ml). Of this DNA 22 µL was mixed with 2.5 µL restriction buffer (Roche Diagnostics, Mannheim, Germany) and 1.5 µL of *PvuII* enzyme (1,000 U (10 U/µl)) (Roche Diagnostics, Mannheim, Germany). The restriction reaction mixture was incubated in a water bath at 37°C for 4 hrs. One microliter of the restricted DNA was run with 5 µL DNA loading buffer on a 1 % agarose gel. The gel was initially run at 90 V until the dye front entered the gel then at 36 V overnight prior to being visualised under UV.

The electrophorectically separated fragments were transferred onto a Hybond N^+ membrane (Amersham, Buckinghamshire, UK) by means of vacuum blotting (Appendix 3.2). The membrane was air dried for 5 minutes and thereafter placed into the UV cross-linker and exposed by using a preset setting of 1200 J. The membrane was removed, rolled and placed into a hybridisation bottle. The bottle was then placed in a rotating hybridization oven, set at 42°C, for 24 hours, to ensure maximum coverage of the membrane.

The enhanced chemi- luminescence (ECL) (Amersham, Buckinghamshire, UK) direct system, containing a horse-radish peroxidase labelled IS*6110* probe was used to detect the presence of a nucleotide sequence that is complementary to the probe (target DNA). DNA sequences that have moderate to high sequence similarity to the probe were detected by visualization of the hybridized probe by autoradiography (Appendix 3.3 & 3.4).

The GelCompar (Applied Maths, Kortrijk, Belgium) system was used for RFLP fingerprinting analysis to determine possible clusters.

3.5.2 Spoligotyping

Spoligotyping was performed as previously described (Kamerbeek et al., 1997) and according to the procedures described in the Spoligotyping Manual version 4 (2002).

DNA was isolated using the CTAB-NaCl method (Van Soolingen et al., 1991). Concentration and quality of the DNA was checked by means of gel electrophoresis (3.5.1). This was followed by PCR of the Direct Repeat (DR) region.

Genomic DNA isolated from the *M.tb* cultures was used to amplify the DR region. PCR was done prior to hybridisation and PCR products were stored at 4°C until use. The PCR mastermix contained:

nuclease free water, colourless GoTaq flexi buffer (Promega, Madison, WI, USA), PCR nucleotide mix (Roche Diagnostics, Manneheim, Germany), 20 pmol of each primer DRa (biotinylated) and DRb (Whitehead Scientific, IDT, Iowa) and GoTaq DNA Polymerase (Promega, Madison, WI, USA). The mastermix was aliquoted into PCR tubes and DNA added to each tube. The tubes were centrifuged using a micro-centrifuge (Eppendorf) and placed into the thermocycler (Applied Biosystems) for amplification. The following cycling conditions were used: 3 minutes at 96°C, 1 minute at 55°C and 30 seconds at 72°C for 20 cycles.

Following fixing the immobilized spacer-oligonucleotides that represented spacers of known sequences to the re-usable nylon membrane, the biotin-labelled PCR products were then hybridized to the membrane bound probes. After incubation with streptavidin-peroxidase conjugate, ECL detection was performed (Appendix 4.1).

The presence of spacers was detected using ECL as described in 3.5.1 above.

The patterns were entered in a binary format onto the SITVIT database (Pasteur Institute of Guadeloupe: http://www.pasteur-guadeloupe.fr:8081/SITVIT/DEMO), which is an updated version of the SpolDB4 database. The corresponding shared international type (SIT) was identified for each isolate. The SpolDB4 database was also used to classify isolates into lineages and sub-lineages (Brudey et al., 2006). Isolates which had a unique spoligotyping pattern and were not found on the international database were classified as 'orphans' (Mathuria et al., 2008, Mulenga et al., 2010). Dendrograms were constructed using the Bionumerics version 3.5 software (Applied Maths, Kotrijk, Belgium). The dendrogram demonstrated the spoligotyping patterns, clustering as well as their degree of similarity.

CHAPTER 4: RESULTS

4.1 Patients

The number of patients enrolled in the study was 52. Their main characteristics are summarised in Table 4.1.

Table 4.1: Patient characteristics

Characteristics	n (%)
Males	20 (38)
Females	32 (62)
Total MDR patients on admission	36 (69)
Total XDR patients on admission	16 (31)
New patients	24 (46)
Patients with previous TB history	28 (54)
HIV-1 infected	43 (83)
HIV-1 un-infected	9 (17)

The study population comprised of 20 (38%) males and 32 (62%) females, with an average age of 36 (range: 19-56 years). In all 52 patients the diagnosis on admission was pulmonary tuberculosis with 36 (69%) MDR and 16 (31%) XDR-TB cases. With regards to previous TB treatment history, 24 (46%) patients were classified as new patients (TB treatment naive, or TB treatment for < 1 month), and 28 (54%) were patients with a history of previous TB. Forty three (83%) patients were HIV-1 co-infected while 9 (17%) patients were HIV-1 uninfected.

Of the 52 patients enrolled, 35 patients had a productive cough and only 12 patients had positive cultures at follow-up (Fig.4.1).

4.2 Specimen collection and processing

An initial sputum for microscopy and culture was collected from 35 patients that had a productive cough at baseline collection (Fig. 4.1). Of these, 12 sputum specimens were culture positive at baseline. The aim was to collect specimens at three consecutive time points, 1 month apart. However, this was not achieved (Fig.4.1).

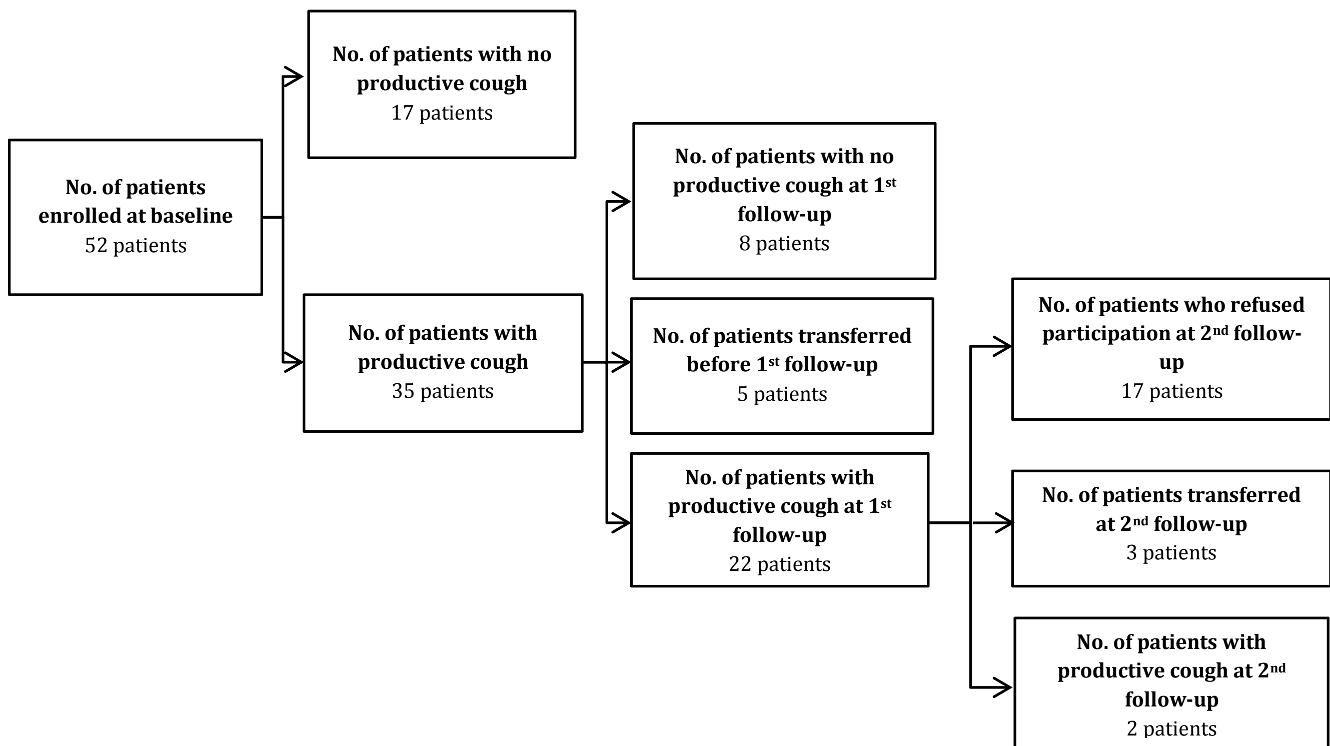

Figure 4.1: Patient flow diagram showing loss of patients at the different sample collection periods.

Thirteen patients were lost at 1st follow-up due to the inability to produce sputum in 8 patients and loss of 5 patients at the planned time of follow-up specimen collection. Twenty-two patients had a productive cough at follow-up, but only 12 culture sputum specimens were culture positive. At 2nd follow-up only two patients agreed to cough, while 3 patients had already been transferred at planned time of follow-up. Seventeen patients of the 22, refused to participate in the study. Therefore, a total of 59 sputa were cultured of which 28 were positive. All cultures were identified as *M.tb* (3.3).

4.2.1 Drug susceptibility test results

4.2.1.1 Antimicrobial susceptibility of isolates

Drug susceptibility profiles were established for all 28 isolates and were compared with the profile in the patient's file as well as between isolates from the same patient at different time points (Table 4.2). The consecutive isolates from patients NT1, NT14, NT27 and NT66 showed differences in their resistance profiles. The majority of the isolates (89%) were XDR, with the exception of three (11%) isolates which were classified as pre-XDR.

Table 4.2: Drug susceptibility profiles of the isolates of patients at the different collection points.

Patient # no.	Diagnosis on admission	Dates	DST results classification								Study Result
			Inh	Rif	Emb	Str	Eth	Ofl	Kan	Cap	
NT1*	MDR	B	R	R	R	R	R	R	S	S	Pre-XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT7	MDR	B	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT14*	MDR	B	R	R	S	S	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT23	XDR	B	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT27*	XDR	B *a*	R	R	R	R	R	R	R	S	Pre-XDR
		B *b*	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT33	XDR	B	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
		2nd	R	R	R	R	R	R	R	R	XDR
NT36	XDR	B	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT41	XDR	B	R	R	R	R	R	R	R	R	XDR
		1st *a*	R	R	R	R	R	R	R	R	XDR
		1st *b*	R	R	R	R	R	R	R	R	XDR
NT55*	MDR	B	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT66*	MDR	B	R	R	R	R	R	R	S	R	Pre-XDR
		1st	R	R	R	R	R	R	R	R	XDR
		2nd	R	R	R	R	R	R	R	R	XDR
NT67	MDR	B	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR
NT75	MDR	B	R	R	R	R	R	R	R	R	XDR
		1st	R	R	R	R	R	R	R	R	XDR

Highlighted in red: Observed DST differences that occurred between sputum collection periods (*)or in different isolates from the same sputum (**) -, B: Baseline, 1st: first follow-up, 2nd: second follow-up.

Key:-

Inh: Isoniazid, **Rif:** Rifampicin, **Emb:** Ethambutol, **Str:** Streptomycin, **Ofl:** Ofloxacin, **Kan:** Kanamycin, **Eth:** Ethionamide, **Cap:** Capreomycin

4.3 Genotyping data

4.3.1 Differentiation of isolates using IS6110 RFLP

Strain cluster analysis was performed on all the isolates using the GelCompar® system (Applied Maths, Kortryk, Belgium). Two or more isolates with fingerprint patterns that do not differ in 2 or more bands were defined as a cluster.

Among the 28 genotyped isolates, 18 (64%) were found to be clustered, or very closely related (Figure 4.2). These isolates belonged to the F15/LAM4/KZN family, which is the dominant XDR strain found in KZN, SA (Pillay and Sturm, 2007). Nine of the other isolates formed three clusters, with each of these clusters displaying a unique fingerprint since these did not have the characteristic fingerprint of one of the known families. These clusters were named Unique I, II and III. The Unique I cluster consisted of 3 (11%) isolates while Unique II had 4 (14%) isolates and Unique III 2 (7%). The remaining isolate belonged to the Beijing family (4%).

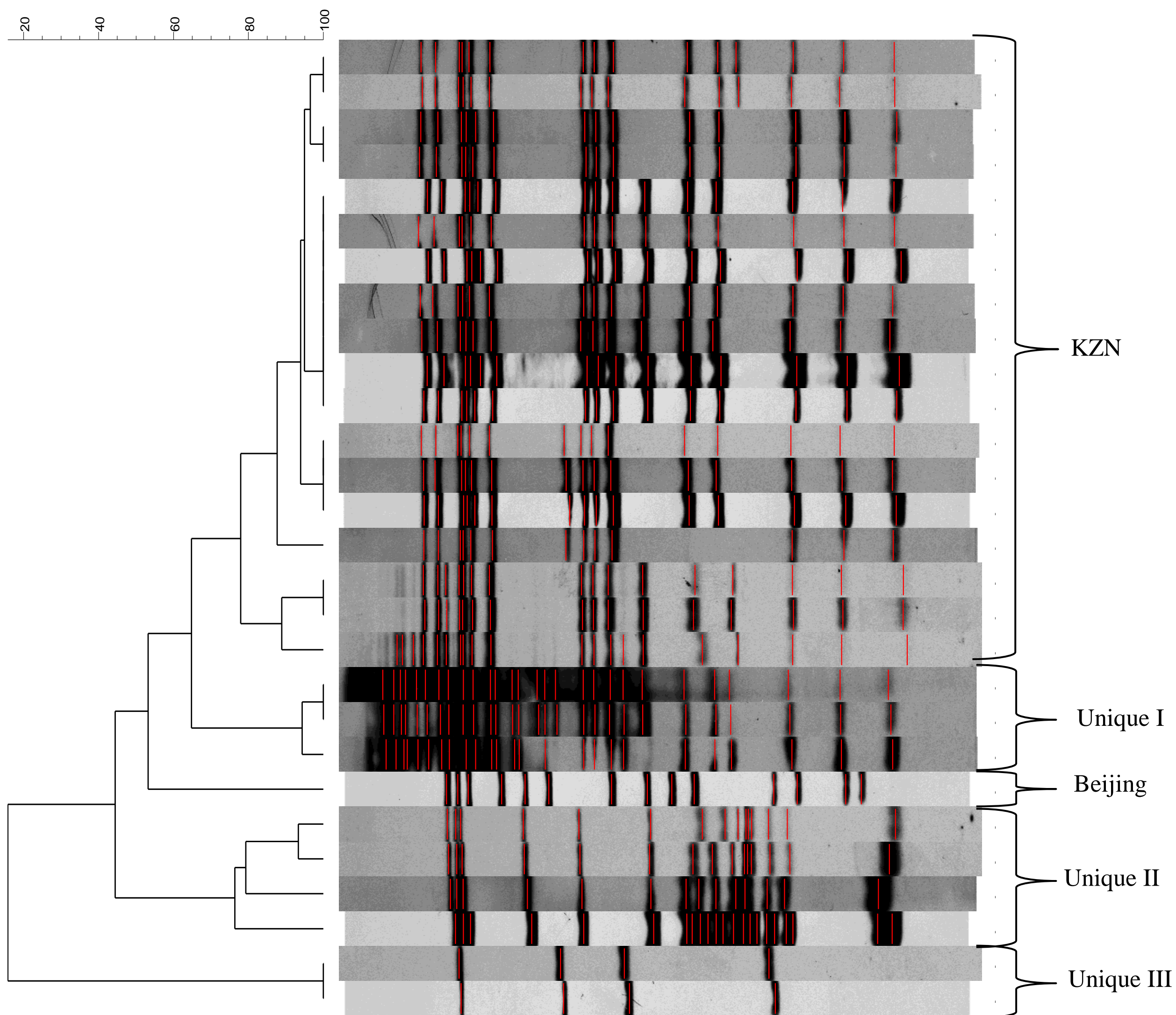

Figure 4.2: IS*6110*-RFLP patterns and dendrogram of the 28 isolates: KZN= F15/LAM/KZN.

4.4. Genotyping of sequential isolates from individual patients

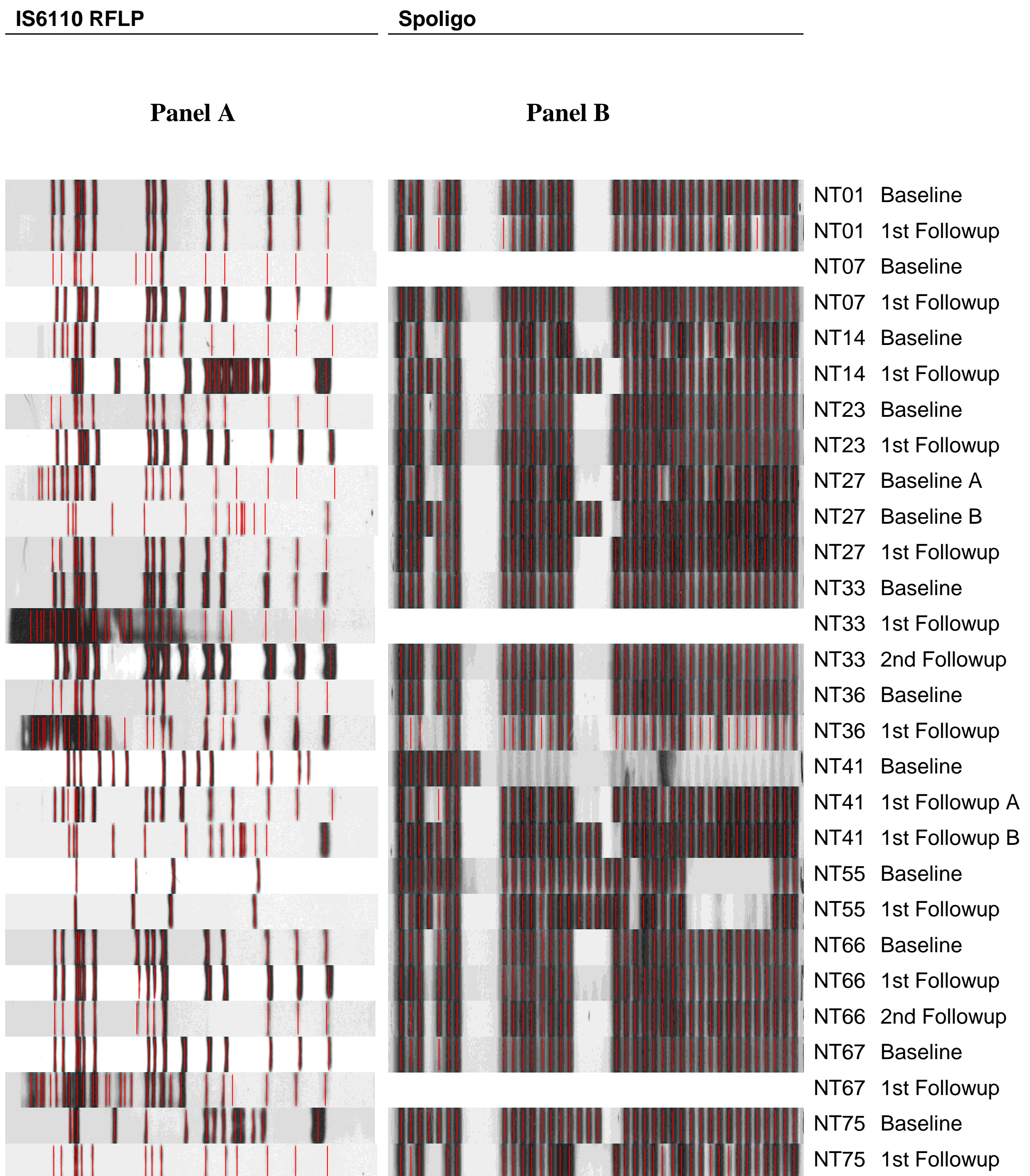

Figure 4.3: Fingerprint patterns of sequential isolates of individual patients. Panel A: IS*6110* RFLP; panel B: spoligotyping.

Figure 4.3 compares the genotyping results of isolates from individual patients, and confirms the similarity between the IS*6110*-RFLP results and the spoligotyping results (panel B). The differences are summarised in table 4.3.

Table 4.3: IS*6110* genotype results in isolates from consecutive sputum specimens from the same patient.

Patient #	Baseline	1st follow up	2nd follow up
NT01	F15/LAM4/KZN	F15/LAM4/KZN	N/A
NT07	F15/LAM4/KZN	F15/LAM4/KZN	N/A
NT14	F15/LAM4/KZN	Unique II	N/A
NT23	F15/LAM4/KZN	F15/LAM4/KZN	N/A
NT27	F15/LAM4/KZN + Unique II	F15/LAM4/KZN	N/A
NT33	F15/LAM4/KZN	Unique I	F15/LAM4/KZN
NT36	F15/LAM4/KZN	Unique I	N/A
NT41	BEIJING	F15/LAM4/KZN + Unique II	N/A
NT55	Unique III	Unique III	N/A
NT66	F15/LAM4/KZN	F15/LAM4/KZN	F15/LAM4/KZN
NT67	F15/LAM4/KZN	Unique I	N/A
NT75	Unique II	F15/LAM4/KZN	N/A

The sputum of 5 (42%) of the 12 patients (NT 01, 07, 23, 55 and 66) showed the same results at the different collection points. Four of the five patients had at each time point isolates that belonged to the F15/LAM4/KZN family while the fifth patient showed the Unique III fingerprint pattern. Two, of the four patients infected with the F15/LAM4/KZN strain (NT01 and NT23), showed the original fingerprint pattern (Pillay and Sturm, 2013), while the other two showed a variations on the original banding pattern.

Seven (58%) of the 12 patients, showed differences in strain fingerprint patterns in sequential isolates. Two of these (NT27 and 41) had two different isolates in the same sputum specimen. NT27 had two strains in the baseline specimen but only one at follow-up while NT41 had one strain in the baseline sputum but two different ones in the follow-up specimen (Fig. 4.4). NT27 showed a F15/LAM4/KZN fingerprint pattern and a Unique II pattern from the specimen at baseline, while NT41 harboured a Beijing isolate in the first specimen but isolates with a F15/LAM4/KZN fingerprint pattern and a Unique II pattern from the same specimen at 1st follow up.

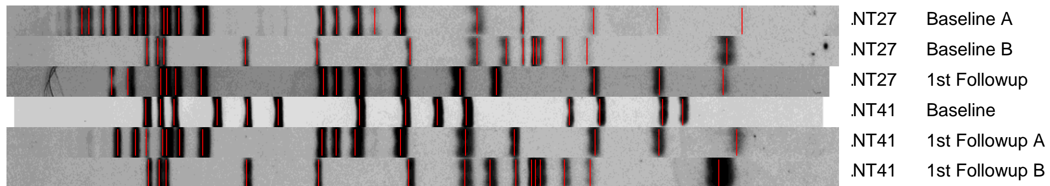

Figure 4.4: Fingerprint patterns showing the difference in genotyping fingerprint from isolates collected at 1st and 2nd time point

4.5. Difference in isolates from patients housed in different hospital wards

Fingerprint patterns of isolates of patients admitted to the same ward were compared to identify possible transmission within the wards.

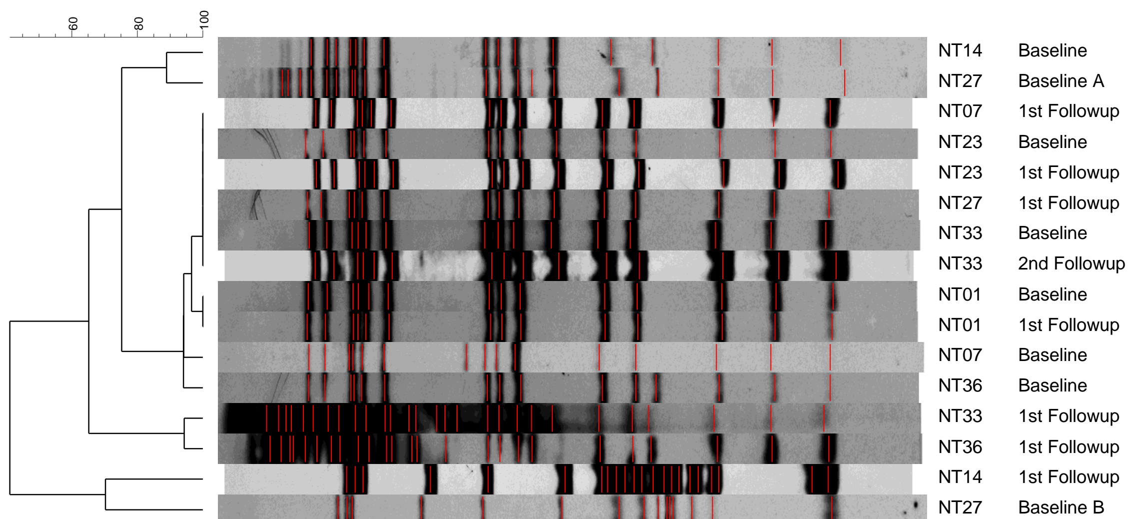

Figure 4.5: IS*6110*-RFLP patterns and dendrogram showing clustering strains among female patients within the same ward.

A total of 16 isolates from 7 female patients were genotyped (Fig. 4.6). Twelve (75%) of the isolates were found to have the F15/LAM4/KZN fingerprint pattern. Two (12.5%) belonged to the Unique I cluster, and the remaining 2 (12.5%) to the Unique II cluster (Fig 4.5).

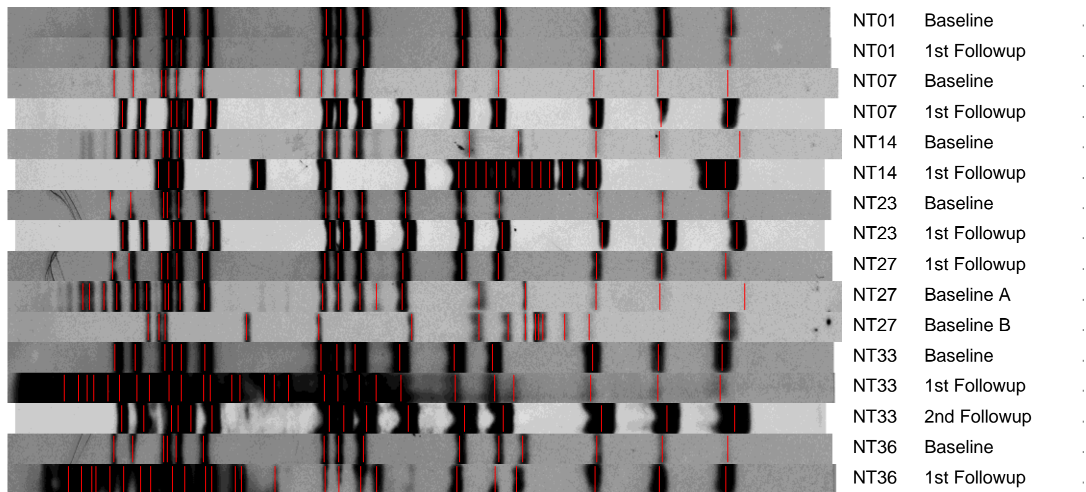

Figure 4.6: Comparison of fingerprint patterns of isolates grown at baseline and follow-up amongst the female patients within the same ward.

NT01 and NT23 were the only patients whose isolates did not show any differences in fingerprint patterns at the different collection points. They both showed the original F15/LAM4/KZN fingerprint pattern.

The other five (71%) patients showed a difference at baseline and follow up. Both isolates from patient NT07 belonged to the F15/LAM4/KZN family but differed from each other. The isolate at baseline from NT14 also showed a F15/LAM4/KZN fingerprint pattern (with an extra band), and a Unique II fingerprint pattern at follow up. NT27 also showed differences in sequential isolates as mentioned above (Fig 4.4). NT33 had identical F15/LAM4/KZN fingerprint pattern at baseline and 2nd follow up. However, the isolate at 1st follow belonged to the Unique II cluster (Fig 4.5). The sequence of changing fingerprint patterns in this patient's isolates suggests mixed infection at least from the 1st follow-up date. NT36 showed the F15/LAM4/KZN fingerprint pattern at baseline and the Unique I fingerprint pattern (Fig 4.5).

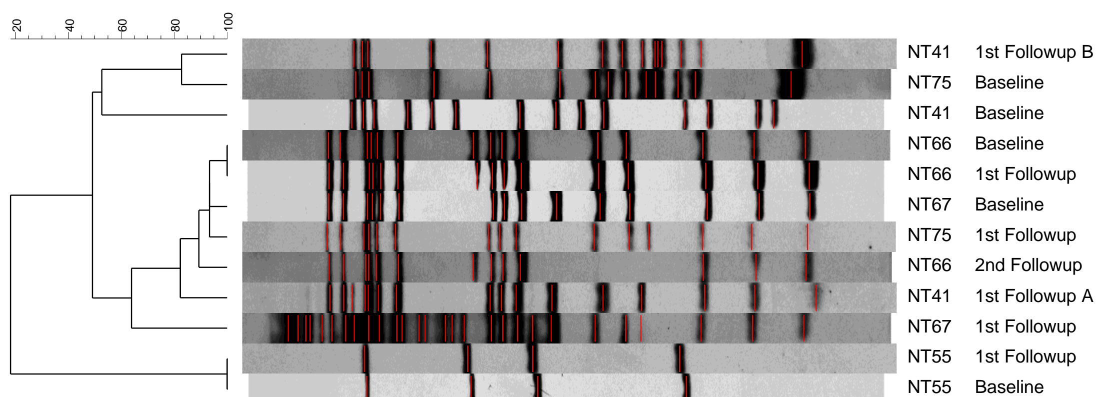

Figure 4.7: IS*6110*-RFLP patterns and dendrogram showing clustering strains among male patients in the same ward.

The genotyping results of 12 isolates from 5 male patients, all admitted to the same ward, are shown in Fig 4.7. Eight (67%) of these isolates were found to belong to the F15/LAM4/KZN family. Two (18%) sequential isolates from the same patient clustered together depicting the Unique III fingerprint pattern (NT55). The isolate of NT67 at 1st follow up showed the Unique I fingerprint pattern. The remaining NT 41 baseline isolate had the Beijing fingerprint pattern.

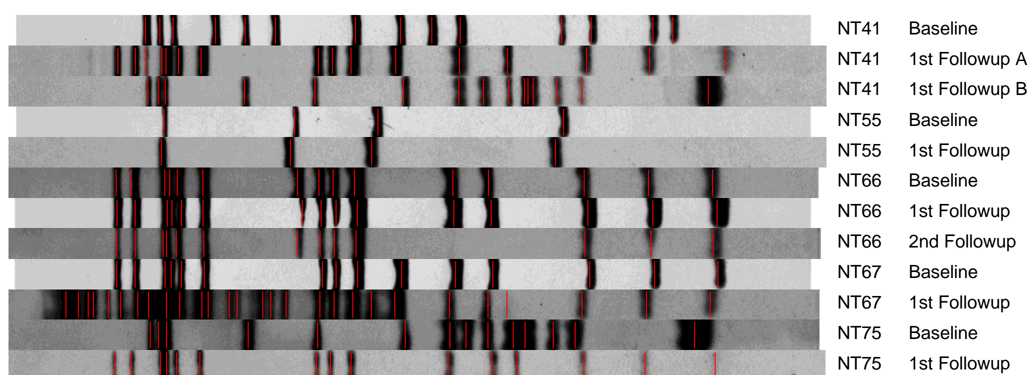

Figure 4.8: Comparison of fingerprint patterns of isolates grown at baseline and follow-up amongst male patients within the same ward.

Isolates grown at baseline and follow-up from sputum of patients NT41, NT66, NT67 and NT75 showed differences in the fingerprint patterns at the different collection points. As previously mentioned, NT41 had one isolate with the Beijing fingerprint pattern at baseline and two isolates with fingerprints different from the baseline isolate at follow up (Fig 4.4 and Fig 4.8). NT55 harboured the Unique III strain at the different collection points. NT66 showed identical fingerprints, which belonged to the F15/LAM4/KZN family, at baseline and 1st follow up. At 2nd follow up NT66 showed a fingerprint that was closely related to the F15/LAM4/KZN fingerprint with slight variations (Fig 4.7). The NT67 baseline isolate also depicted the F15/LAM4/KZN fingerprint, while the 1st follow up isolate showed the Unique I fingerprint (Fig 4.7). Isolates retrieved from sputum of patient NT75 at baseline and follow-up differed from each other with a Unique II fingerprint pattern at baseline and a F15/LAM4/KZN fingerprint pattern at follow up.

CHAPTER 5: DISCUSSION

General

The main finding of this study is that of the 12 patients from whom positive follow-up cultures were available, 7 harboured according to genotype, a different isolate on follow-up. Most of these follow-up specimens were collected 1 month from enrolment. Four of these isolates also differed in resistance profile with a more resistant isolate on follow-up. All these patients were on treatment for either MDR or XDR TB. One patient's follow-up isolate was resistant to 2 first-line drugs which were obviously not part of the patient's treatment. The other patients were XDR patients with follow-up isolates showing resistance to additional XDR defining drugs. These drugs had not been shown effective in these patients or had been replaced by alternatives. Therefore, no protection was provided by the current treatment regimens. Risk factors that possibly contributed to the acquisition of new strains included HIV infection as well as malnutrition due to prolonged TB.

The follow-up sputum specimens did not contain more than one isolate. One would expect in some patients mixed infections with the original and the newly acquired strain. Two different strains were found in one of the specimens on enrolment, confirming the technical capability to detect this. It is likely that the number of patients was too small for this to be found.

It cannot be excluded that the follow-up isolates in some of the patients were actually present but missed in the specimen collected on enrolment. Cross-contamination during specimen processing is unlikely since the dates of processing were different and aseptic procedures were strictly adhered to.

The attempt to identify spacial factors like housed in the same ward or distances between beds were not successful due to the mobility of the patients and the high levels of social contact.

5.1. Nosocomial transmission

We planned to collect sputum specimens at three consecutive time points, 1 month apart from all 52 patients that were enrolled in the study. This was not achieved due to the inability to produce sputum in 17 patients, resulting in 35 sputum specimens being collected at baseline collection (Fig. 4.1). Thirteen patients were lost at 1st follow-up due to the inability to produce sputum in 8 patients and loss of 5 patients at the planned time of follow-up. At 2nd follow-up only two patients agreed to continue with the study due to loss of 3 patients who were transferred at the planned time of specimen collection and refusal to continue with the study from 17 patients. This resulted in a total

of 59 sputum cultures being collected over the collection periods, of which 28 were culture positive by the end of the study.

Sixty nine percent (69%) and thirty one percent (31%) of the study cohort were initially diagnosed with MDR-TB and XDR-TB, respectively. It is worth noting that the initial diagnosis of MDR and XDR-TB were gathered from the patient files.

Drug susceptibility testing is essential for developing effective MDR and XDR-TB treatment regimens (Shah *et al.,* 2011). However, with laboratory facilities not being available in most high incidence settings, DST is often restricted to high-risk patients (Shah *et al.,* 2011, Mei *et al.,* 2014). In 2005, Ghandi, *et.al.,* (2006) reported a large XDR-TB epidemic in Tugela Ferry, South Africa (Gandhi *et al.,* 2006) where the XDR-TB isolates from that study, were found to be resistant to all 6 drugs tested routinely in KZN (Isoniazid, Rifampicin, Ofloxacin, Kanamycin, Ethambutol and Streptomycin) (Shah *et al.,* 2011). In 2007, increasing resistance was noted in the same area after continuous, routine drug resistance surveillance was implemented for all TB suspects. More than 90% of the XDR-TB isolates were resistant to all first and second-line drugs routinely tested in KZN, SA (Gandhi *et al.,* 2010, Shah *et al.,* 2011).

In the subsequent Shah, *et.al.,* (2011) study, DST was expanded to include capreomycin and ethionamide, which were routinely prescribed for MDR and XDR-TB infected patients. The study confirmed that the XDR-TB isolates were resistant to all first-line and second-line drugs, including capreomycin and ethionamide (Shah *et al.,* 2011). These findings resonate with the findings from our study as the majority of the culture positive isolates (89%) were found to be resistant to all the above mentioned drugs as well (Table 4.2). Three isolates (11%) showed slight changes in their resistance profiles and were found to be pre-XDR.

Among the 28 genotyped isolates, 18 (72%) were found to belong to one cluster, or were very closely related (Fig 4.2). The isolates belonged to the F15/LAM4/KZN family, which is in keeping with earlier reports that this strain family is the predominant XDR strain found in KZN, SA. Six (24%) of the other isolates also clustered, but did not belong to any of the families dominant in KZN.

In this study, female patients were separated according to resistance profile, in different rooms, separating XDR-infected and MDR-infected patients; whereas the male patients, with both MDR and XDR-TB, were housed together in the same wards. It is highly likely that this has contributed to the transmission of multiple strains of XDR-TB, rather than a single transmission event as typically seen in outbreaks (Chihota *et al.,* 2011, Gandhi *et al.,* 2013, Dheda *et al.,* 2014).

At the start of the study a TB risk assessment was conducted at the FOSA TB hospital to assess the TB-IPC controls in place, the housing facilities and staff knowledge on TB transmission. The hospital was found to have an IPC plan in place, including an IPC team and an IPC committee but still had several challenges. Firstly, there was no triage system in place for TB patients at FOSA TB hospital at the time of the assessment. The problem was found to be that patients who were referred from King George V hospital arrived at the FOSA TB hospital as MDR-TB patient and the diagnosis of XDR could only be made 8-10 weeks later, contributing to the difficulty of implementing a triage system aiming at separating XDR from MDR cases on arrival. Secondly, the onsite laboratory facility could only perform microscopic examination of sputum specimens. Because there was no radiology facility on site, patients needed to be transported for chest X-ray examination, potentially spreading the infection to patients from other hospitals that were also referred to that radiology department. Lastly, MDR-TB and XDR-TB patients were all admitted together in the same wards as placement of patients was dependent on bed availability. The assessment of the wards revealed that the bed spacing was less than 1 m thus increasing the risk of transmission between the patients. Some of the wards had windows that did not allow for cross-ventilation, and closeness of surrounding buildings prevented the wards from maximizing the use of natural ventilation. The above-mentioned challenges could have contributed to drug-resistant TB transmission amongst the patients. This is supported by the study of Ghandi *et.al*., (2013) where they concluded that lack of appropriate infrastructure allows for multiple generations of drug-resistant strains to be transmitted over time (Gandhi *et al.,* 2013). Furthermore, both female and male patients had daily interactions with each other i.e.; doing laundry, braiding each other's hair, or watching television together. The above-mentioned setting also could have contributed to drug-resistant TB strain transmission occurring in and outside the wards.

Basu *et al.,* (2007) and Sissolak *et al.,* (2011) also found that South African hospitals did not have the capacity for safe airborne isolation and lacked isolation facilities (Basu et al., 2007, Sissolak et al., 2011). Furthermore, Tshitangano *et al.,* (2013) found that IPC interventions in SA were still not aligned to the WHO policy on TB infection control in healthcare facilities due to lack of TB infection control plans in hospitals (Tshitangano *et al.,* 2013). This greatly contributes to nosocomial transmission amongst TB patients in SA (Basu *et al.,* 2007, Sissolak *et al.,* 2011, Gandhi *et al.,* 2013).

The risk of nosocomial transmission is amplified in settings where HIV infection is common among patients (Bantubani *et al.,* 2014). 43 (83%) out of the 52 recruited patients in our study were found to be HIV-1 co-infected (Table 4.1). All of these were on ARV treatment. Majority of the HIV-1 infected patients were smear negative. HIV-infected patients are known to have a low bacillary count in their sputa as confirmed by Mendelson (2007). A study conducted at the Church of Scotland Hospital (CoSH) in KZN highlighted the possibility of large, highly lethal outbreaks

occurring when immuno-suppressed patients in open wards are exposed to other patients with unrecognized, or inadequately treated, highly drug resistant strains of *M.tb* (Bantubani *et al.,* 2014).

5.2. Mixed strain infections

The frequent isolation of several different *M.tb* strain lineages in a single disease episode, often with differing drug susceptibilities, has been highlighted by advances made in molecular genotyping and recent epidemiological studies (Richardson *et al.,* 2002, Cohen *et al.,* 2011, Cohen *et al.,* 2012, Gandhi *et al.,* 2014). These mixed strain infections were previously considered as a rare event, but now their occurrence has been shown with increasing frequency (Lazzarini *et al.,* 2012). This has important implications on drug susceptibility testing and the treatment of patients. Genetic heterogeneity of *M.tb* within a host may arise due to either, i) within-host diversification following a single infection event, or ii) re-infection resulting in a mixed infection with more than one strain (Garcia de Viedma *et al.,* 2004, Cohen *et al.,* 2011). Mixed strains can differ in their resistance and virulence patterns, regardless of the mechanism through which these infections occurred (Moodley *et al.,* 2015). Differences in resistance pattern often results from the dominant strain surviving the drugs used, thus propagating the most virulent and most resistant strain (Lazzarini *et al.,* 2012).

In this study, molecular genotyping was carried out using IS*6110*-RFLP, which is recognized as a gold standard, and spoligotyping was used as supplementary method for the confirmation of IS*6110*-RFLP results. Strain cluster analysis showed the dominant strain within the cohort to be from the F15/LAM4/KZN family.

The F15/LAM4/KZN strain is a member of the Euro-American strain family, and has been dominating among MDR and XDR-TB cases in KZN since 1994 (Pillay and Sturm ,2007) It is prominent in this region and has been linked to nosocomial transmission and high mortality rates (Gandhi *et al.,* 2006, Pillay and Sturm, 2007, Ioerger *et al.,* 2009, Gandhi *et al.,* 2010, Gandhi *et al.,* 2013).

Only one patient from the cohort was found to be infected with the Beijing strain (NT41). This strain was identified at baseline. The Beijing strain has been found in MDR and XDR-TB cases in South Africa's Western Cape and Eastern Cape provinces, but has been commonly found among drug-susceptible TB in KZN (Gandhi *et al.,* 2014).

Moodley, *et.al.,* (2015) showed that 166 (6.3%), out of 2617 patients, displayed discordant susceptibility profiles (Moodley *et al.,* 2015). These results were based on phenotypic differences

in isolates from different specimens, collected from the same patients on the same day (Moodley *et al.,* 2015). The authors argue that the differences found amongst the different specimens collected on the same day are a reflection of multiple strain infections as opposed to mutational events (Moodley et al., 2015). Even though the DST results from this study showed that majority of the patients' isolates were resistant to all tested antimicrobial drugs, the genotyping results clearly show that the patients harboured mixed infections of TB.

Both female and male patients had daily interactions with each other i.e.; doing laundry, braiding each other's hair, or watching television together. Furthermore, the bed spacing within the wards was found to be less than 1 m increasing the chances of transmission between the infected patients. The above-mentioned setting could have contributed to the observed nosocomial transmission of drug-resistant TB strains.

This study found a high percentage (67%) of patients to be infected with another strain on follow-up as compare to the initial one. The observation that most of these follow-up isolates belonged to the same cluster indicates that this is likely the result of nosocomial transmission. Other explanations for a change in genotype patterns are multiple strain infections where different isolates are cultured at different time points as well as laboratory contamination of the specimens during the decontamination process. While the first possibility cannot be excluded, it is highly unlikely due to the clustering of the follow-up isolates. Laboratory contamination is highly unlikely since the culture protocol includes known negative controls with all batches.

The super-infections took place in patients on treatment for the infection with the initial isolate. This is not surprising since these treatment regimens did not cover the newly acquired strains.

CHAPTER 6: CONCLUSION

Nosocomial transmission of tuberculosis is one of the major challenges in the management of the TB epidemic in SA and globally. The occurrence of mixed infections in TB patients further exacerbates the problem which challenges the infection control strategies in place at healthcare facilities. The success of infection control programs depends on the ability to identify and separate patients timeously in their respective drug-resistance categories. This is not always possible when resources are scarce and HIV co-infection is prevalent. Implementation of rapid diagnostic testing for drug-resistant TB and redesign of healthcare facilities, to minimize congregate spaces, are critical elements of infection control programs in addition to administrative, environmental and personal protective measures.

The findings in this study are subject to a few limitations. Firstly, our findings are likely a minimal estimate of nosocomial transmission, since the majority of the patient cohort was lost to follow-up (Fig. 4.1), and some isolates were lost due to contamination. It is likely that we would have found more epidemiologic links and additional XDR-TB transmission among already infected patients. Secondly, our genotyping data was also incomplete because of the loss of some isolates to contamination; therefore the size of the clusters is likely an underestimate which could have resulted in unidentified links in the transmission network. A larger, more comprehensive investigation as well stringent follow up measures over a prolonged period would give better insight in the magnitude of the problem.

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APPENDICES

Appendix 1

1.1. N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) decontamination method

Decontamination and concentration of sputum specimens using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method of (Kent and Kubica, 1985) is recommended as a gentle but effective digesting and decontaminating agent (Sharma et al., 2012).

2g of N-Acetyl-L-Cysteine (NALC) (Sigma Aldrich, USA) was mixed with 100ml of 4% sodium hydroxide (NaOH) (Acechem, SA) (Appendix 1.4.1) and 100ml of sodium citrate (Acechem, SA) (Appendix 1.4.2). The NALC-NaOH citrate reagent was shaken well to ensure uniform mixing of the solution. 3-5ml of the sputum specimens were decanted into 50ml polytubes. An equal volume of the NALC-NaOH reagent was added to each of the tubes and vortexed briefly (10-15 seconds), until liquefaction of the samples. After 15 minutes, the volume was brought to 50ml by adding phosphate buffer (pH 6.8) (Appendix 1.4.3) to each of the tubes. Tubes were inverted briefly to ensure adequate mixing of solutions. The tubes were then centrifuged for 20 minutes at 3000 x g and the supernatant discarded.

1.2. ***Decontamination Solutions***

1.2.1. 4% NaOH

80g NaOH (4%) pellets (Acechem, SA) were added to 1000ml distilled water in a 2000ml conical flask and allowed to dissolve and cool. NaOH is caustic and becomes quite hot in initial solution. Using a glass funnel, the mixture was gently poured into a 2000ml volumetric flask and volume made up to 2000ml. The solution was aliquoted into 200ml plastic bottles and autoclaved at 120°C for 15 minutes, and then stored at 4°C.

1.2.2. Sodium citrate

58g Tri-sodium citrate $2H_20$ (Acechem, SA) was mixed with 2000ml distilled water. The solution was aliquoted into 200ml Duran (Schott) bottles and autoclaved at 120°C for 15 minutes, and the stored at 4°C.

1.2. 3. Phosphate buffer pH 6.8 0.067M

<u>Stock Alkaline Buffer</u>

Na_2HPO_4 (anhydrous) (Acechem,SA) – 94.7g

Distilled water – 10L

Stock Acid Buffer

KH_2PO_4 (Acechem, SA) – 90.7g

Distilled water – 10L

94.7g of Na_2HPO_4 (anhydrous) powder and 90.7g of KH_2PO_4 powder were added into two separate 2L flasks. Both flasks were filled to the 2L mark with distilled water and shaken to dissolve. The two mixtures were then added to 16L of distilled water in a clean large bucket and mixed well. The pH was adjusted to 6.8 and aliquoted into 1L Duran (Schott) bottles, which were autoclaved at 120°C for 15 minutes and stored at 4°C.

1.3. *Culture in Mycobacteria Growth Indicator Tube (MGIT)*

The materials provided by the manufacturer for culture in MGIT tubes (BD BioSciences, Sparks, MD, USA) included BBL Mycobacteria Growth Indicator Tube (MGIT) and BACTEC™MGIT 960 supplement kit, containing BACTEC MGIT Growth Supplement and BBL MGIT™PANTA (Polymixin, Amphotericin, Nalidixic acid, Trimethorprim, Azlocillin) antibiotic mixture (BD BioSciences, Sparks, MD, USA). The BACTEC MGIT growth supplement was added to a lyophilized vial of the BBL MGIT™PANTA antibiotic mixture. Eight hundred microlitres of the growth supplement and PANTA mixture was then added to each MGIT tube, followed by 500µl of the specimen.

1.4. *Middlebrook 7H9 broth*

9.4g of Middlebrook 7H9 base (BD, Difco laboratories, USA) powder, 4ml of (99.5%) glycerol (Sigma-Aldrich, USA) and 2g of Casitone (BD, Difco Laboratories, USA) were added in 1800ml of distilled water and mixed well. The mixture was autoclaved at 121°C for 15 minutes and cooled in a water bath to 56°C. 200ml of oleic acid-albumin-dextrose-catalase (OADC) (BD, USA), and 2ml of the following antibiotics were aseptically added:

Drug*	**Concentration**	**Catalogue #**
Polymixin B	200.000 IU/L	245114
Amphotericin B	20mg/L	245114
Carbenicillin	100mg/L	245114
Trimethoprim	20mg/L	245114

***Manufactured by Sigma-Aldrich, USA**

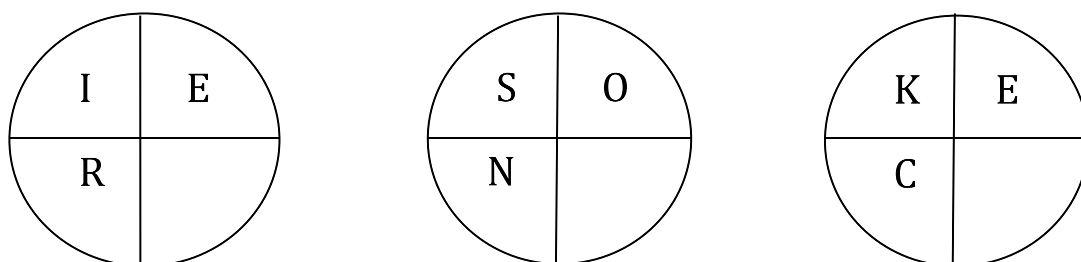
The mixture was then dispensed into 5ml amounts in sterile free standing tubes, followed by storage at 4°C.

1.4.1. Middlebrook 7H9 Broth Buffer

5ml of Middlebrook 7H9 broth (0.2% Glycerol, 10% OADC) was added to 45ml of phosphate buffer pH 6.8 (0.067M) (Appendix 1.3.).

1.5. Middlebrook 7H10 agar plates

7.6g of Middlebrook 7H10 (BD, Difco Laboratories, USA) base, 2ml of glycerol and 0.4g of Casitone were added to 360ml of distilled water and mixed well. The mixture was autoclaved at 121°C for 15 minutes and cooled in a water bath to 56°C. 40ml of OADC and 1 vial of the specified anti-TB drug were then aseptically added and mixed well. 5ml of the resultant mixture was then aliquoted into pre-labelled 900mm diameter disposable quadrant petri dishes (Romachem, Durban) and allowed to set, followed by storage at 4°C.



Abbreviations: I = Isoniazid, E = Ethambutol, R = Rifampicin, S = Streptomycin, O = Ofloxacin, N = Niacinamide, K = Kanamycin, E = Ethionamide, C = Capreomycin

Anti-TB drug concentrations:

Drug*	**Concentration**
Isoniazid	1.0µg/ml
Rifampicin	1.0µg/ml
Ethambutol	7.5µg/ml
Streptomycin	2.0µg/ml
Kanamycin	5.0µg/ml
Ofloxacin	2.0µg/ml
Ethionamide	5.0µg/ml
Capreomycin	10.0 µg/ml

***Manufactured by Sigma-Aldrich, USA**

The empty quadrant in all plates was used for the control (no drug was added).

1.6. Middlebrook 7H11 agar plates

42g of Middlebrook 7H11 base (BD, Difco laboratories, USA) powder, 10ml of glycerol, 10ml of L-Asparagine monohydrate (BD, Difco Laboratories, USA) and 2g of Casitone were added to 1800ml of distilled water and mixed well. The mixture was autoclaved at 121°C for 15 minutes and cooled in a water bath to 56°C. 200ml of OADC and add 2ml of PACT were aseptically added and mixed well. 12ml of the resultant mixture was dispensed into 650mm diameter disposable petri dishes (Romachem, Durban) and allowed to set, followed by storage at 4°C.

1.7. *Culture Confirmation Tests*

1.7.1. Nitrate Test

Sterile tubes were labelled with isolate numbers and 500µl of nitrate broth was aliquoted into each tube. Using a sterile quadloop, a loopful of culture was scraped off the Middlebrook 7H11 agar plate (1% glycerol, 10% OADC) and suspended into the tubes containing nitrate broth. Tubes were incubated at 37°C for 2 hours. Following incubation, 1 drop of concentrated hydrochloric acid (HCl) (Merck, SA), followed by 2 drops of 0.2% sulfanilimide (Sigma-Aldrich, USA) (nitrile 1) solution, and 2 drops of 0.1% N-(naphthyl)-ethylenediamine dihydrochloride (Sigma-Aldrich, USA) (nitrite 2) solution was added to all tubes. Tubes were observed for a colour change, any shade of pink indicated a positive result (Isenberg, 2004a). H37Rv laboratory strain was used as a positive control and a tube containing no culture was used as a negative control.

1.7.2. Niacin Test

A drop of cyanogen bromide (Sigma-Aldrich, USA) was added onto a lawn of confluent growth followed by a drop of aniline (Sigma-Aldrich, USA) on top of the cyanogen bromide. Plates were left for 5 minutes and thereafter observed for the presence of a colour change, a shade of yellow indicated a positive result (Isenberg, 2004a). H37Rv was used as a positive control and un-inoculated culture medium as a negative control.

Appendix 2

2.1. *DNA Isolation*

MTB colonies were scraped off LJ slants using a sterile swab and suspended into 2ml micro-centrifuge tubes (Eppendorf) containing 500µl of sterile distilled water. The bacteria were heat killed at 80˚C in a thermomixer comfort (Merck, SA) for 30 minutes following which 70µl of 10% Sodium Dodecyl Sulfate (SDS) (Sigma-Aldrich, USA) (Appendix 2.3.4) solution and 50µl of proteinase K (Roche Diagnostics, Manneheim, Germany) (Appendix 2.3.3) of concentration 10mg/ml were added to each tube. The tubes were then incubated in a thermomixer comfort (Merck, SA) at 60˚C for 1 hour. The 5M Sodium chloride (NaCl) (Sigma-Aldrich, USA) (Appendix 2.3.5) and CTAB-NaCl (Sigma-Aldrich, USA) (Appendix 2.3.6) solutions were pre-warmed at 60˚C and 100µl of each solution was added to each tube an mixed thoroughly by inverting the tubes a few times.

The tubes were then incubated at 60˚C for 15 minutes and 700µl of Chloroform (Sigma-Aldrich, USA): Isoamyl alcohol (Sigma-Aldrich, USA) (24:1) (Appendix 2.3.7) solution was added to each tube and inverted several times until the contents turned milky. Tubes were centrifuged for 10 minutes at 12000 rpm. The upper aqueous supernatant was transferred to 2ml micro-centrifuge tubes (Eppendorf) which contained 700µl of Isopropanol (Sigma-Aldrich, USA). Tubes were inverted several times and placed in a -20˚C freezer for 30 minutes, followed by centrifugation for 10 minutes at room temperature at 12000 rpm. The isopropanol was discarded and the pellet was washed with 80% ethanol (Merck, SA) (Appendix 2.3.8) and centrifuged for 10 minutes at 12000 rpm. The ethanol was discarded and the pellets were left to dry for 10 minutes at room temperature. 55µl of 1xTE buffer (Appendix 2.3.2) was added to each pellet and stored at 4˚C overnight or until further use. A 5µl volume of each DNA sample was electrophoresed on a 1% agarose gel to estimate DNA concentration.

2.2. *Gel Electrophoresis*

A 1% agarose gel (Appendix 2.3.9) was made using 1xTris-Borate-EDTA (TBE) buffer (Appendix 2.3.11). The agarose (Lonza, USA) was dissolved in 1xTBE buffer by heating in a microwave. The solution was allowed to cool and poured into a casting tray, which was taped up around the edges, with a 20 well comb. The agarose solution was allowed to set at room temperature. Once the gel had solidified the tape and comb were removed and the casting tray, containing the gel, was placed into an electrophoresis tank (Hoefer) containing 1xTBE buffer. A 3-5µl volume of sample loading dye (Appendix 2.3.12) was added to a piece of parafilm for each DNA sample and 2µl of each

DNA sample was mixed with the dye and loaded into a well of the gel. Electrophoresis was performed at 100 volts (V) for 30-45 minutes.

2.3. *DNA Extraction Solutions*

2.3.1. 10X TE Buffer

1,21g of trizma base was dissolved in 80ml of autoclaved distilled water. The pH was adjusted to 8 using concentrated hydrochloric acid (HCl) (Merck, SA), followed by the addition of EDTA. The solution was then made up to a final volume of 100ml using autoclaved distilled water.

2.3.2. 1X TE Buffer

10ml of 10xTE Buffer was dissolved in 90ml of distilled water and stored at room temperature.

2.3.3. Proteinase K

482µl of PCR grade water (Roche Diagnostics, USA) was added to 518µl of PCR grade recombinant proteinase K and stored at 4°C.

2.3.4. 10% Sodium Dodecyl Sulfate (SDS)

1g of SDS powder was dissolved in 10ml of distilled water.

2.3.5. 5M Sodium Chloride (NaCl)

14.6g of NaCl powder was dissolved in 50ml of autoclaved distilled water.

2.3.6. CTAB-NaCl Solution

4.1g of NaCl and 10g of CTAB were dissolved in 100ml of distilled water. The solution was heated to 65°C, until powders were completely dissolved.

2.3.7. Chloroform: Isoamyl alcohol (24:1)

1ml of isoamyl alcohol was added to 24ml of chloroform and mixed well.

2.3.8. 80% ethanol

80ml of ethanol were added to 20ml of distilled water.

2.3.9. 1% agarose gel

1.4g of agarose powder was added to a flask containing 140ml 1xTBE buffer. The mixture was boiled using a microwave, until the powder dissolved.

2.3.10. 10X TBE Buffer

108g of trizma base, 55g of boric acid (Sigma-Aldrich, USA) and 9.3g of EDTA were dissolved in 800ml of autoclaved distilled water. After dissolution the volume was made up to 1000ml with autoclaved distilled water.

2.3.11. 1X TBE Buffer

100ml of 10xTBE buffer were added to 900ml of autoclaved distilled water and stored at room temperature.

2.3.12. Loading Dye – 1% Double Dye

1g of bromophenol blue (Sigma-Aldrich, USA) and 1g of xylene cyanole (Sigma-Aldrich, USA) were added to 100ml of autoclaved distilled water. This is the 1% double dye.

<u>50ml of Loading Dye from 1% Double Dye Stock:</u>
5ml of 10xTBE buffer, 25ml of glycerol, and 5ml of 1% double dye were added to a 50ml tube and mixed well with 15ml of autoclaved distilled water.

Appendix 3

IS*6110* is an insertion element that is found exclusively within the *Mycobacterium tuberculosis* complex (MTBC). A benefit of this exclusivity is that IS*6110* has become an important diagnostic tool in the differentiation of MTBC species from other mycobacteria. The element's presence in multiple copies, and at differing locations in the genome, has provided an excellent method by which strains can be genotyped; and because of these characteristics, IS*6110* has been used extensively for epidemiological studies (Coros et al., 2008).

3.1. *Restriction*

Restriction digestion is the process of cutting DNA molecules into smaller pieces with special enzymes called restriction endonucleases. *Pvu*II restriction endonuclease was used for restriction digestion, which recognizes the double-stranded DNA sequence 5'-CAGCTG-3' and cleaves after the G-3' end. *Proteus vulgaris* is the genomic source for *Pvu*II. It is the most commonly used restriction enzyme and it creates blunt ends which can be ligated into a blunt cloning site easily (Yaich et al., 1992).

3.2. *Southern Blotting*

The membrane was cut to the size of 15x20cm ad placed into a glass container containing distilled water. This was followed by a 5 minute washing step of the membrane in 10xSSC solution (Appendix 3.5.4) on an orbital shaker at room temperature. The vacuum blotting unit (Vacugene) was set up by placing the porous support (Amersham, Buckinghamshire, UK) onto the blotting unit correctly, followed by placing the membrane on top. The plastic mask (Amersham, Buckinghamshire, UK) was then aligned accordingly over the edges of the membrane. The gel was removed from the electrophoresis tank and gently slid onto the membrane. The vacuum pump was started and once it reached 55cm bar a 1:100 HCl solution (Appendix 3.5.8) was poured over the gel and the vacuum applied for 20 minutes. The HCl solution was removed by tilting the blotting unit and aspirated using a suction device. The next solution called soak 1 (Appendix 3.5.1) was poured over the gel and the vacuum applied again for 20 minutes. The aspiration step was repeated as previously and the soak 2 solution (Appendix 3.5.2) was poured over the gel followed by application of the vacuum for 20 minutes and aspiration. 10xSSC was poured over the gel, the vacuum was increased to 65cm bar and applied for 1½ hours. At all times the gel was completely covered with the appropriate solution used. On completion of blotting, the last solution was removed by aspiration and the gel removed and discarded.

3.3. *Hybridization*

The membrane was pre-hybridised using 25-30ml of hybridisation buffer (Amersham, Buckinghamshire, UK) (Appendix 3.5.7), rotating for 30 minutes in a hybridisation oven (Stuart) set at 42°C. In a micro-centrifuge tube 10µl of the probe was mixed with 5µl of sterile distilled water, boiled for 5 minutes and placed on ice for 10 minutes. Equal volumes of DNA labelling reagent (Amersham, Buckinghamshire, UK) and gluteraldehyde (Amersham, Buckinghamshire, UK) were added to the probe mixture and incubated in a water bath at 37°C for 10-15 minutes. Hybridisation buffer covering the membrane was mixed with the probe and this mixture was then poured back into the hybridisation bottle containing the membrane. The hybridisation took place overnight at 42°C in a hybridisation oven on a bottle rotisserie.

The hybridisation buffer was discarded the next day and the membrane was rinsed with primary wash buffer (Appendix 3.5.5) which was discarded. Another volume of primary wash buffer was added to the membrane followed by incubation for 30 minutes at 42°C. The membrane was rinsed with 2xSSC solution (Appendix 3.5.6) and transferred to a glass container. Two 5 minute washes with 2xSSC were applied at room temperature on an orbital shaker (Stuart). The final solution was discarded and the membrane was transferred to a plastic container.

3.4. *Detection*

This was performed in the darkroom. For detection of the hybridised DNA, the membrane was submerged for 2 minutes in 8ml of ECL detection solution (Amersham, Buckinghamshire, UK). The membrane was drained and wrapped in cling wrap and wiped with tissue to remove bubbles. It was then placed into a hypercassette (Amersham, Buckinghamshire, UK), followed by exposure of a hyperfilm (Amersham, Buckinghamshire, UK) to it for 20 minutes. The hyperfilm was removed and placed into developer (Agfa-Gevaert, Belgium) until all bands appeared and then placed into fixer (Afga-Gevaert, Belgium) until the background was clear. It was then rinsed with tap water and hung to dry.

3.5. *IS6110 Restriction Fragment Length Polymorphism (RFLP) Solutions*

3.5.1. Soak 1

20g of NaOH and 87.66g of NaCl were added to 800ml of autoclaved distilled water. After dissolution the volume was made up to 1000ml with autoclaved distilled water.

3.5.2. Soak 2

62.6g of trizma base, 87.67g of NaCl, and 40ml of HCl were added to 800ml of autoclaved distilled water. The pH was adjusted to 7.2 using concentrated HCl, and then the volume was made up to 1000ml with autoclaved distilled water.

3.5.3. 20X SSC

175g of NaCl and 88g of sodium citrate was added to 800ml of autoclaved distilled water. The pH was adjusted to 7.0 using concentrated HCl, and then the volume was made up to 1000ml with autoclaved distilled water.

3.5.4. 10X SSC

500ml of 20xSSC was added to 500ml of autoclaved distilled water and stored at room temperature.

3.5.5. Primary Wash Buffer

360g of urea (Sigam-Aldrich, USA), 25ml of 20xSSC, and 4g of SDS were added to 600ml of autoclaved distilled water. After dissolution the volume was made up to 1000ml with autoclaved distilled water.

3.5.6. Secondary Wash Buffer (2X SSC)

20ml of 20xSSC was added to 400ml of autoclaved distilled water and stored at room temperature.

3.5.7. Hybridization Buffer

30ml of 5M NaCl, and 15g of blocking reagent (Amersham, UK) were added to 270ml of hybridization buffer (Amersham, UK). The mixture was heated at 60°C, with continuous stirring, to enable blocking agent to dissolve. The hybridization buffer was then aliquoted into 50ml falcon tubes and stored at -20°C.

3.5.8. 1:100 HCl

5ml of concentrated HCl were added to 495ml of autoclaved distilled water.

Appendix 4

4.1. *Hybridisation*

For hybridisation, 20µl of each PCR product was diluted in 150µl 2xSSPE 0.1% SDS (Appendix 4.2.4). The diluted PCR products were heat-denatured for 10 minutes at 99°C and thereafter cooled on ice. The membrane (Ocimum Biosolutions, India) was washed for 5 minutes at 60°C in 250ml of 2xSSPE 0.1% SDS. The membrane and a support cushion (Ocimum Biosoultions, India) were placed onto the mini-blotter, so that the slots were perpendicular to the applied oligonucleotides present on the membrane. The residual fluid in the slots of the mini-blotter was removed by aspiration. The diluted PCR products were pipette into the slots in such a way that the mini-blotter channels were perpendicular to the row of the oligonucleotides. Hybridisation was done for 1 hour at 60°C. Samples were removed by aspiration and plastic forceps were used to remove the membrane from the mini-blotter and to place this into a plastic container.

The membrane was washed twice using 250ml of 2xSSPE 0.5% SDS (Appendix 4.2.5) for 10 minutes at 60°C. The membrane was allowed to cool in a plastic container to prevent inactivation of the peroxidise that followed. Streptavidin-peroxidase conjugate (Roche Diagnostics, Mannheim, Germany) was added to 20ml of 2xSSPE 0.5% SDS. The membrane was incubated in this solution for 55 minutes at 42°C in its plastic container. The membrane was washed twice using 250ml of 2xSSPE 0.5% SDS for 10 minutes at 42°C and thereafter washed twice using 250ml of 2xSSPE (Appendix 4.2.6) for 5 minutes at room temperature.

4.2. *Spoligoytping Solutions*

4.2.1. 20xSSPE Solution

35.6g of 0.2M $Na_2HPO_4.2H_2O$ (Sigma-Aldrich, USA), 210.24g of 3.6M NaCl, and 20mM EDTA were added to 800ml of distilled water. The pH was adjusted to 7.4 using 4M NaOH and the final volume made up to 1000ml using autoclaved distilled water.

4.2.2. 0.5M EDTA

93.05g of EDTA powder was added to 450ml of distilled water. The pH was adjusted to 8 using NaOH and the final volume made up to 500ml using autoclaved distilled water.

4.2.3. 10% SDS solution

10g of SDS was dissolved in 100 ml of distilled water.

4.2.4. 2xSSPE 0.1% SDS

26ml of 20xSSPE and 2.6ml of 10% SDS were added to 231.4ml of distilled water.

4.2.5. 2xSSPE 0.5% SDS

102ml of 20xSSPE and 51ml of 10% SDS were added to 867ml of distilled water.

4.2.6. 2xSSPE

50ml of 20xSSPE was added to 450ml of distilled water.

4.2.7. 1% SDS

40ml of 10% SDS was added to 360ml of distilled water.

4.2.8. 20mM EDTA

2ml of 0.5M EDTA was added to 48ml of distilled water.

Annexure 1

Consent form

We are doing research on the hospital acquired transmission of TB within ward 2 (female ward) and wards 3 and 4 (male wards) at the FOSA TB Hospital. FOSA TB hospital is a dedicated facility that houses MDR/XDR TB patients. Infection with one strain of *Mycobacterium tuberculosis* does not protect against infection with a new strain from another contact, so the study aims to study the degree of transmission of TB within the designated wards.

We have explained the study to you and would appreciate your participation. The study as we had mentioned requires us to take sputum specimens from you upon commencement of the study, at 6 weeks and after 3 months. You have a choice to participate or decline participation in this study. If you decline participation, the provision of health care will not be any different as compared to those that agree to participate.

If you agree to participate please sign this form.

I, ______________________________________ (Name of Patient) understand the nature of the research project and agree to be part of this study. I also understand that it is my right to withdraw from this study at any time, even after signing this document.

Signature of Patient: _________________________

Witnesses: _________________________ (Name & Signature)

If you have any queries at any time you may contact the following:

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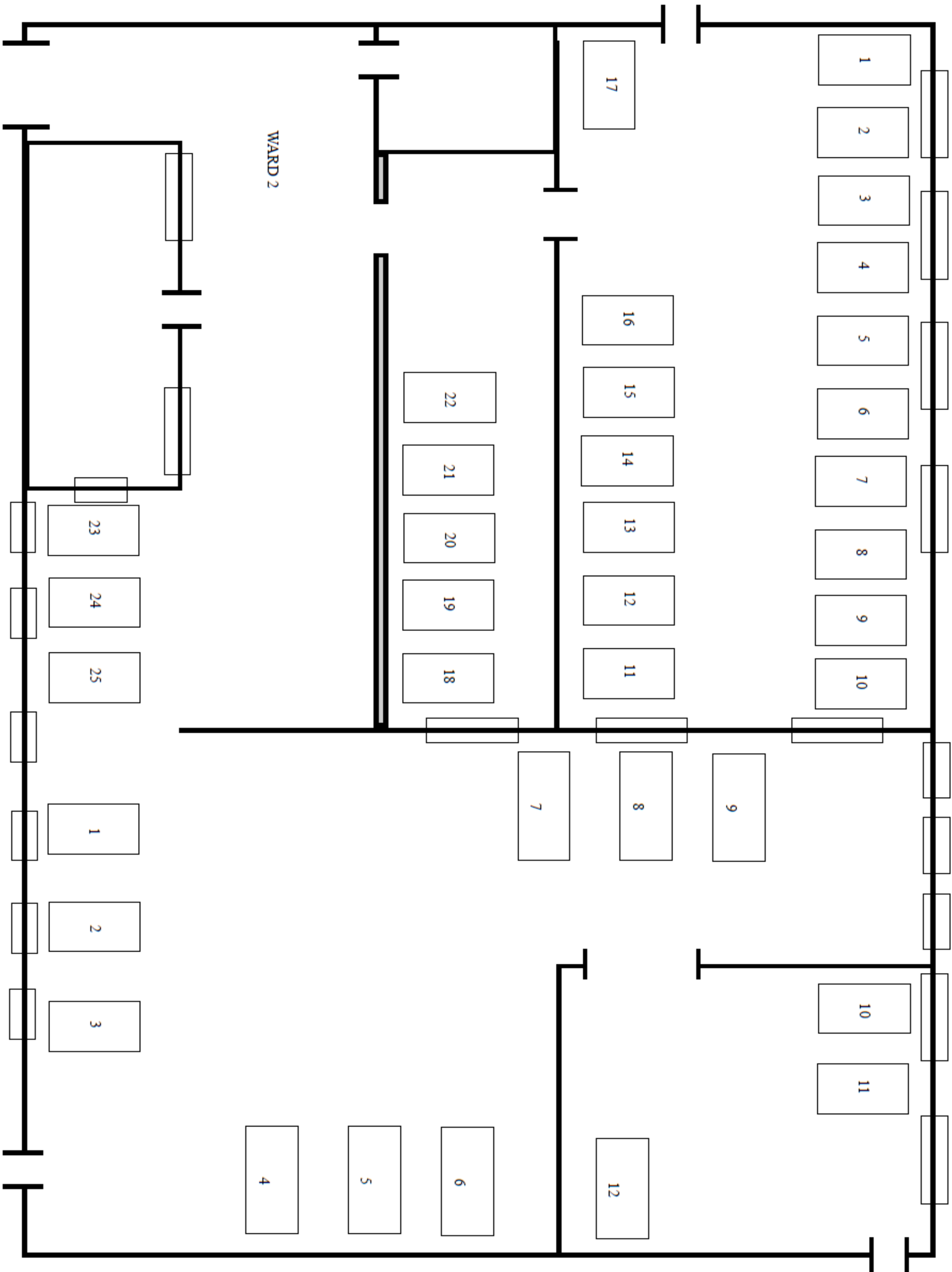
Annexure 2

Ward 2 floor plan (Female ward)



Annexure 3

Ward 3 and 4 floor plan (Male ward)