MOLECULAR CHARACTERISATION OF MULTIDRUG RESISTANT TUBERCULOSIS
HUMAN ISOLATES IN KAMPALA

By

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DECLARATION

I Muyombya George William do hereby declare that the work presented in this dissertation is my original work, except where stated by reference and it has never been submitted to this or any other university for academic awards.

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DEDICATION

This work is specifically dedicated to people who invest time and resources to envisage how mere biomolecules command life processes.
ACKNOWLEDGEMENTS

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# LIST OF ABBREVIATIONS AND ACRONYMS

AIDS…………………….Acquired Immunodeficiency Syndrome  
ARVs……………………Antiretroviral drugs  
CAS…………………….Central Asian  
CWRU…………………..Case Western Research Unit  
DNA…………………..Deoxyribonucleic Acid  
DR…………………….Direct Repeats  
DST…………………..Drug susceptibility tests  
EAI…………………..East-African-Indian  
EMB…………………..Ethambutol  
HIV…………………….Human Immunodeficiency Virus  
INH…………………..Isoniazid  
JCRC………………….Joint Clinical Research Centre  
LAM…………………..Latin-American-Mediterranean  
LJ-PM……………………Lowenstein Jansen Proximate Method  
MDR-TB………………Multi-Drug Resistant Tuberculosis  
MGIT…………………..Mycobacteria Growth Indicator Tube  
MIRU………………….Mycobacterial Interspersed Repetitive Units  
MTC…………………..Mycobacterium tuberculosis Complex  
NTRL………………….National Tuberculosis Reference Laboratory  
PAS………………….Para-aminosalicyclic Acid  
PCR…………………..Polymerase Chain Reaction  
PZA…………………..Pyrazinamide  
RD……………………Regions of Difference  
RIF…………………..Rifampicin  
rRNA………………..Ribosomal Ribonucleic Acid  
RUFORUM……………..Regional Universities Forum for Capacity Building in Agriculture  
SIT…………………..Spoligotype International Type  
SM ..........................Streptomycin  
UBOS ........................Uganda Bureau of Statistics  
VNTR………………..Variable Numbers of Tandem Repeats  
WHO…………………..World Health Organization  
ZN…………………..Ziehl-Neelsen
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ABSTRACT

Background

Strains of *Mycobacteria tuberculosis* complex that are resistant to the two first line drugs rifampicin and isoniazid are increasing in Kampala. Archived human multidrug resistant tuberculosis isolates collected from Kampala between 1997 and 2007 were analysed. We used regions of deletion to determine the species involved. Spoligotyping was employed to identify strain types in relation to the international SpolDB4 database. Epidemiological linkages between isolates were also ascertained by comparing the numerical 15-loci MIRU-VNTR outputs to the MIRU-VNTRPlus database to elucidate clustered strains.

The predominant species were *M. tuberculosis* of the “modern” type (98.7%), “ancestral” *M. tuberculosis* (1.03%) and *M. bovis* “classical” (1.03%). A strain diversity of 46.9% was revealed by SpolDB4. Predominant spoligotype strain lineages were T2-Uganda (20.4%) and CAS1-Delhi (12.2%). Spoligotype clusters were split by the 15-loci MIRU-VNTR typing, forming two MIRU-VNTR clusters each having two strains. Predominant MIRU-VNTR strain lineages were Uganda I (29.6%) and CAS1-Dehli (11.2%). Majority (98%) of the strains were unclustered.

These data suggest that MDR-TB strains circulating in Kampala are endemic, genetically diverse and epidemiologically independent.
CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Tuberculosis is a chronic mycobacterial infection in humans and other animals. The disease is caused by seven mycobacteria species namely *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, *M. caprae*, *M. pinnipedii* and *M. canettii* which form the *Mycobacterium tuberculosis* complex (MTC) (Asiimwe et al., 2008). The MTC are genetically related and show high conservation of DNA (Frothingham et al., 1998; Asiimwe et al., 2008). Some species such as *M. tuberculosis*, *M. africanum* and *M. canettii* are specific to human and *M. microti* to rodent while *M. bovis* and its variants are zoonotic. The disease is globally distributed, however, the geographical distribution of MTC differ with some species and strains being specific to a particular human sub-population (Gagneux et al., 2005). Nonetheless, the prevalence is high in urban areas with high population densities (Gandhi et al., 2006). Tuberculosis manifests through persistent cough, constant fatigue, weight loss, loss of appetite, fever, coughing up blood and night sweats (Traore et al., 2007) and has devastated human populations worldwide (Brosch et al., 2002; Dye et al., 2002; Gagneux et al., 2005).

Several control and treatment strategies have been undertaken using drugs to manage the disease. The first effective drugs against tuberculosis were Streptomycin (SM) and Para-aminosalicylic acid (PAS) discovered in 1944 (Salyers et al., 2002). Later, it was observed that a combination of the two drugs was more effective at both achieving cures and preventing acquired drug resistance. When Isoniazid (INH) was added to the treatment regimen in 1952, an efficacious triple therapy was derived. However, treatment
duration of 24 months was required to achieve complete cure. Later ethambutol (EMB), Rifampicin (RIF) and pyrazinamide (PZA) were also introduced. When drugs were administered in appropriate combinations the treatment period reduced to only 6 months (Iseman, 2002). As such, RIF and INH became the major first-line anti-tuberculosis drugs. After introduction of these drugs, the disease was significantly reduced (Iseman, 1993). Consequently, control and treatment programmes were relaxed followed by non compliance to the regimen hence development of drug resistance due to sub-optimal drug administration (Iseman, 1993; Iseman, 1994; Salyers et al., 2002). Strains resistant to RIF and INH with or without resistance to other TB drugs are known as multidrug resistant tuberculosis (MDR-TB). During early 1990s, new cases emerged carrying drug resistant phenotype (Iseman, 1994). Today, disease outbreaks caused by strains resistant to RIF and INH are increasing (Sreevatsa et al., 1997; WHO, 2006; Zignol et al., 2006; Fauci et al., 2008) posing a great challenge to management of the disease globally. Indeed, by the year 2006, over 500,000 individuals globally were estimated to be infected with MDR-TB (WHO, 2006).

In Uganda, treatment of TB is complicated by HIV/AIDS co-infection. Besides, the interaction between the antimycobacterial agents and ARVs complicate the disease control strategies (WHO, 2006). Additionally, congregate tuberculosis wards that characterize health facilities in Kampala may facilitate infection and transmission of TB in general (Umubyeyi et al., 2006). As such, the index patients may be a constant source of MDR-TB primary infections in this setting. Owing to the high population (density) of Kampala comprising of people of different ethnicities and nationalities, the transmission
rate could also be high. Surveys conducted within and around Kampala city have indicated occurrence of drug resistant strains using Drug Susceptibility Tests (DST) (Asiimwe et al., 2008; Temple et al., 2008; Lukoye et al., 2011). However, the diversity of MDR-TB species and strains in Kampala is not known. Besides, molecular fingerprinting methods have never been used to characterize the strains causing human MDR-TB in Uganda. Therefore, the present study characterized archived TB isolates from Joint Clinical Research Center (JCRC) and National Tuberculosis Reference Laboratory (NTRL) Wandegeya, using molecular fingerprinting techniques. Further, the study aimed at ascertaining the diversity and epidemiological linkages of MDR-TB species and strains circulating in Kampala.

1.2 Problem statement

Tuberculosis is a common disease among patients with compromised immunity especially in HIV/AIDS persons. The co-infection thus complicates treatment and control because of associated drug interactions. The emergence of MDR-TB has aggravated the situation particularly in developing countries including Uganda. Proper identification of the etiology is essential for the development of appropriate control and preventive strategies. Species and/or strain identification of MDR-TB requires the use of molecular techniques. However, these methods have never been utilized to characterize the human MDR-TB strains in Kampala. As such, there is no information on the diversity of strains causing human MDR-TB in Kampala. Moreover, epidemiological linkages of the existing strains within Kampala have never been established. In this study we used RD analysis, spoligotyping and MIRU-VNTR to characterize human MDR-TB isolates archived at JCRC and NTRL.
1.3 Justification

Many sub-Saharan countries are heavily burdened with MDR-TB disease and cases of resistant strains to first line drugs are increasing. However, routine diagnostic tests do not type to strain level and this has complicated management of MDR-TB patients. Previously, no study in Uganda has determined the diversity of species and strains in human MDR-TB isolates. This study determined the diversity of species and strains in MDR-TB human isolates including their epidemiological links using molecular assays. This in turn helped to determine the type of resistance in Kampala and may guide on appropriate measures by the tuberculosis control program.

1.4 Research questions

1. What are the species causing human MDR-TB in Kampala?

2. Do the species isolated in Kampala MDR-TB cases have different strains?

3. Is there epidemiological relatedness among strains causing MDR-TB in Kampala?

1.5 General objective

To establish the diversity and epidemiological linkages of human MDR-TB strains in Kampala.

1.5.1 Specific objectives

i) To determine the human MDR-TB species in the isolates collected in Kampala using Regions of Deletion analysis.

ii) To determine the strains of human MDR-TB species isolated in Kampala using Spoligotyping.

iii) To ascertain epidemiological links between MDR-TB strains in Kampala using MIRU-VNTR analysis.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Tuberculosis disease
Tuberculosis remains today one of the top three killers and most infectious human
disease together with malaria and HIV worldwide (Zignol et al., 2006). Estimates show
that about one third of the global human population is currently infected with the bacilli
that cause tuberculosis (WHO, 2006; Keshavjee et al., 2008). The bacterium belongs to
the genus Mycobacterium, which are aerobic, non-motile, and rod-shaped acid fast
bacteria (Salyers et al., 2002). The human and animal TB causing Mycobacteria are
grouped together within the Mycobacterium tuberculosis complex (MTC) (Bifani et
al., 2001; Gagneux et al., 2005). Members of the MTC are highly related, exhibiting
remarkable nucleotide sequence level homogeneity despite the varying pathogenicity,
geographic range, certain physiological features, epidemiology, and host preference
(Frothingham et al., 1998; Juliano, 2006). The classical species of the MTC include M.
tuberculosis, M. africanum, M. microti, M. bovis, M. bovis bacillus Calmette-Guérin
[BCG], M. caprae, M. pinnipedii and M. canettii (Van Soolingen et al., 1998; Asiimwe et
al., 2008).

2.2 Treatment and control
The current treatment of TB involves use of four standard, or first-line, anti-TB drugs
INH, RIF, PZA, and EMB. Poor adherence to first-line antimycobacterial drugs results in
sub-optimal drug administration which favor selection of multidrug-resistant TB (MDR-
TB) strains. Multi-drug resistant TB (MDR-TB) is a disease form caused by strains
resistant to both INH and RIF, the major anti-tuberculosis drugs (Iseman, 1993; Pillay et al., 2007). Because of the complicated and long regimen, low cure rates compared to drug-susceptible tuberculosis (TB), and the possibility of patients remaining infectious for months or years, MDR-TB is a major public health problem (Keshavjee et al., 2008). Moreover, the treatment of MDR-TB is expensive and difficult due to the prolonged treatment period of at least eighteen months with 'second line' drugs that exhibit enhanced toxicity (WHO, 2006).

2.3 Drug resistance in Mycobacteria tuberculosis complex (MTC)
Drug resistance is a phenotypic expression by a bacterial cell to survive presence of a drug at concentration that normally kills or inhibits its growth (Iseman, 1994). Naturally, *Mycobacteria* are highly resistant to conventional antibiotics like Penicillin and Sulphur. This is attributed to the presence of unique molecules; arabinogalactan and lipoarabinomannan in their cell wall (Iseman, 1994). MTC strains carrying this phenotypic expression are resistant to the first line drugs particularly INH and RIF with or without resistance to other anti-TB drugs cause multidrug resistant tuberculosis (Espinal et al., 2001). MTC resistant strains are significantly challenging treatment and control measures of tuberculosis worldwide (Iseman, 1993; Van Soolingen et al., 1998; Zhang et al., 2005). As such, the global incidence of multidrug resistant tuberculosis is increasing tremendously in countries of Eastern Europe, East Asia and China (Dye et al., 2001; Fauci et al., 2008). Accordingly, recent reports shows that African countries particularly sub-Saharan Africa have high MDR-TB incident cases. In fact, Uganda is ranked 16th for MDR-TB incident cases on the international scale (Amor et al., 2008).
2.4 Development of multi-drug resistant tuberculosis (MDR-TB)
Resistance to drugs can be categorized as either primary drug resistance (bacilli isolated from individuals who have never taken drugs) or acquired or secondary drug resistance (bacilli isolated from patients who have been treated for tuberculosis for at least one month (Weyer et al., 1992). Studies have shown that resistance to drugs is associated with spontaneous mutations in genes encoding for either drug targets or enzymes involved in drug activation (Somoskovi et al., 2001). In M. tuberculosis, drug resistance is attributed to nucleotide substitutions, insertions, or deletions in specific resistance-determining regions of the genetic targets or activating enzymes of anti-TB chemotherapeutic agents (Ormerod et al., 1990). These mutations are exclusively confined to chromosomal DNA and are not linked, thus the probability of a strain developing a spontaneous mutation to both drugs is very low (Baghaei et al., 2009).

Resistance to drugs is traced back to the introduction of chemotherapy in 1944, for the management of tuberculosis cases. Upon its discovery and clinical use in 1943-1945 by W. Selman, Streptomycin was highly effective to all Mycobacteria species and strains (Iseman, 1994). However, after a few years of successful use, refractory cases to SM monotherapy were recorded among the population. Subsequently, p-aminosalicyclic acid, Isonizid, Pyrazinamide and Rifampicin were added to the regimen which significantly reduced emergent cases. The resultant cross protection from co-administration of Isoniazid and Streptomycin completely cured the disease. This was because of the ability of Isoniazid to kill mutants resistant to Streptomycin and vice versa (Iseman, 1994).

Despite the efficacy of the combined treatment with INH and SM, the regimen had to be taken for 18 – 24 months. This lengthy treatment was widely advocated for
and adopted with rigorous adhesion to the regimen, under the support from several professional programmes. By 1960, the threat for the deadly disease was under control. However, the stringent health programmes were relaxed at policy level (drug quality and inadequate patient supervision) and individual level (sub-optimal dosages due to poor drug regimens design, patient compliance). This subsequently, created a selective environment for the survival of drug resistant mutants to first line drugs Isoniazid and Rifampicin (Iseman, 1994; Kanduma et al., 2003; Post et al., 2004; Baghaei et al., 2009). Three decades after treatment success, the disease reappeared with a new face of multidrug resistance (Iseman, 2002; Bifani et al., 2008). Apparently, MDR-TB has spread worldwide by index case patients particularly to people in close contact (Embden et al., 1993; Iseman, 1994; Kamerbeek et al., 1997; WHO, 2006; Zignol et al., 2006; Fauci et al., 2008).

2.5 Mechanism of drug resistance in Mycobacterium tuberculosis

Like all other bacteria, Mycobacteria employ several strategies to resist antimycobacterial agents. These can be due to over expression and modification of the drug target, barrier mechanisms, drug inactivating enzymes, inactivation of drug activating enzymes and drug extrusion mechanisms (Iseman, 1994). In TB treatment, antimycobacterial agents may interfere with enzymes involved in cell wall biosynthesis; inhibit protein synthesis or inhibit transcription and DNA replication (Pfyffer, 2000; Somoskovi et al., 2001). Hitherto, members of the MTC have developed resistance to antimycobacterials and increasing cases are being reported (Zignol et al., 2006; Amor et al., 2008; Yuan-Chuan Wang, 2009; Bazira et al., 2010; Lukoye et al., 2011).
TB drugs such as Isoniazid, blocks synthesis of cell-wall mycolic acids, which are the major components of *M. tuberculosis* cell envelope. The drug targets the fatty-acid, enoyl-acyl carrier protein reductase (InhA), the complex of acyl carrier protein (AcpM) and the ketoacyl-ACP synthase (KasA), (Figure 1) (Somoskovi et al., 2001). INH is a pro-drug that requires catalase-peroxidase in the bacteria for activation (Iseman, 1994; Somoskovi et al., 2001). The enzyme catalase-peroxidase is encoded for by catalase-peroxidase gene (katG). In INH resistant strains, the katG is altered, thus, reducing the activation ability of the pro-drug INH (Iseman, 1994; Bifani et al., 2008). Particularly, the point mutation at Ser315Thr reduces the catalase-peroxidase activity by approximately 50%, thus, creating high-level resistance to INH (Pfyffer, 2000; Bifani et al., 2008).

Rifampicin (RIF) provides an early bactericidal effect on metabolically active *M. tuberculosis* and excellent late sterilizing action on semi-dormant organisms undergoing short bursts of metabolic activity (Pfyffer, 2000; Bifani et al., 2008). The drug binds to bacterial RNA polymerase, consequently interfering with RNA synthesis, (Figure 1). The bacterial RNA polymerase is encoded for by rpoB gene. Therefore, mutations in the gene rpoB inhibit binding of the drug to RNA polymerase because of the associated modifications. Pryrazinamide (PZA) targets an enzyme involved in synthesis of short chain fatty acid precursors. It is also a pro-drug that is activated to pyrazinoic acid (POA) by bacterial pyrazinamidase (pZase)/nicotinamidase. This causes cytoplasmic acidification and inhibition of cellular metabolic activities. The mis-sense mutations in the gene encoding for bacterial pyrazinamidase (pZase)/nicotinamidase causes amino
acid substitutions and nucleotide insertions or deletions. Moreover, the mutation causes non-sense mutations in the pncA structural gene or non-sense mutations in putative promoter region results into defective pZase activity. However, M. bovis and M. bovis BCG, are naturally resistant to PZA due to a unique C to G point mutation in codon 169 of pncA (Pfyffer, 2000; Zhang et al., 2005; Somoskovi et al., 2008).

Other drugs like Ethambutol (EMB) inhibit biosynthesis of arabinogalactan the major polysaccharide of the Mycobacterial cell wall. The drug interferes with the polymerization of cell wall arabinan of arabinogalactan and lipoarabinomannan and induces the accumulation of β-D-arabinofuranosyl-P-decaprenol, an intermediate in arabinan biosynthesis. The drug targets an enzyme arabinosyl-transferase. Mutations in

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Figure 1:
Shows drug targets in the cell wall, cytoplasmic membrane and cytoplasm of Mycobacterium tuberculosis for Isoniazid, Rifampicin and Pyrazinamide. Adapted from (Somoskovi et al., 2001).
the *emb*CAB operon, codon 306 of *emb*B and in amino acid residues Asp328, Gly406 and Glu497 are believed to account for the resistant phenotype (Iseman, 1994; Bifani *et al.*, 2008).

Streptomycin (SM) acts at ribosomal protein S12 and 16S rRNA of the 30S sub-unit of the ribosome resulting into bacterial protein synthesis interference, cell membrane damage, respiration inhibition and RNA synthesis stimulation including mis-reading or mis-coding of the genetic code. Mutations in the *rpsL* gene which encodes ribosomal protein S12 and *rrs* gene that encodes 16S rRNA limits the action of the drug (Iseman, 1994; Pfyffer, 2000; Bifani *et al.*, 2008).

### 2.6 Occurrence of MDR-TB strains

Strains of *M. tuberculosis* causing MDR-TB disease are distributed worldwide (Amor *et al.*, 2008; Keshavjee *et al.*, 2008). Several species and strain lineages of *M. tuberculosis* are associated with the disease depending on the geographical location. The strains fall into distinct families and sub-families that form six main phylogenetic lineages which are sub-structured geographically. The phylogenetic lineages are Indo-Oceanic (IO), East Asian (EA), East-African-Indian (EAI), Euro-American, West-African 1 and West-African 2 (Gagneux *et al.*, 2005). The Benjing family of the East Asia lineage forms large clusters and often associated with disease outbreaks globally (Freeman *et al.*, 2005; Filliol *et al.*, 2006; Glynn *et al.*, 2008).

All the six lineages are represented on the African continent. So far, records indicate that MDR-TB strains belonging to Euro-American lineage are predominant in Africa (Gagneux *et al.*, 2005). Other lineages are reported to occur frequently in patients within specific countries. The two West African 1 and 2 lineages are prevalent in the West
African countries (Homolka et al., 2008) while East-African-Indian lineage occur frequently in Central African countries (Niobe-Eyangoh et al., 2004). Three lineages: Indo-Oceanic, East-African-Indian and Euro-American are prevalent in East Africa. In South African countries, the East Asian lineage is prevalent among the MDR-TB patients (Gagneux et al., 2005).

MDR TB cases have been reported in all countries found in the East African region. In the Democratic Republic of Congo (DRC) on the western side, MDR-TB prevalence was recorded as 2.3% (WHO, 2006). A prevalence of 1.2% was reported in Rwanda located on the South western side, with a possibility of drug resistance amplification effect (Umubyeyi et al., 2007). Tanzania in the South it was estimated at 1.1% and predominating strains were of Central Asian Sub-family, Latin American Mediterranean (LAM) and East-African-Indian (EAI) families (Eldholm et al., 2006). From the Eastern side, prevalence of 2.7% has been reported in Kenya. Predominant families were CAS-Kili and LAM11-ZWE, CAS1-Dehli, EAI, LAM9 and T family, Beijing strains were also reported (Githui et al., 2004). In the North, prevalence of MDR-TB cases in southern Sudan stood at 1.9% (WHO, 2006; Otto et al., 2008). Uganda in the centre MDR-TB cases were estimated at 1.3% among new cases and 12.3% in previously treated cases (Asiimwe et al., 2008; Bazira et al., 2010; Lukoye et al., 2011).

2.7 Epidemiology of MDR-TB
Formulation of comprehensive control and management programmes for tuberculosis requires critical understanding of the transmission dynamics of the disease. Importantly it requires understanding the risk factors, transmission direction (epidemiological linkages), infection recurrence and progress
High disease burden in countries is usually related to poor living conditions, social and cultural behavior, and prevalence of other immune-compromising diseases (Zignol et al., 2006). In Uganda, limited resources hinder timely access to TB diagnosis and quality anti-tuberculosis drugs. Urban centers are characterized by poor housing conditions and extensive social mixing resulting into overcrowding that exacerbates tuberculosis transmission. Additionally, the country is faced with high HIV/AIDS prevalence (6.5%), which is aggravated by tuberculosis (WHO, 2006). Existence of risk factors of TB may result into increased cases in Kampala. Consequently, these cases may be dominated by specific strains that are most adapted to the human population and therefore appear clustered (Glynn et al., 2008).

2.8 Detection techniques of Mycobacterium species

Effective management and control of TB disease through vaccination and treatment, particularly for MDR-TB, requires thorough knowledge of pathogen species and/or strains (Asiimwe et al., 2008). Classical diagnostic tests based on growth, biochemical and phenotypic characteristics have limited discriminatory and reproducibility power to type members of the MTC. Therefore, accurate typing of MTC members can only be achieved by employing molecular typing tools which are fast and highly discriminatory.

2.8.1 Region of Deletion (RD) analysis

Region of deletion or difference analysis (RD) is a PCR based genotyping tool that analyses specific deleted regions in the genome of MTC. Amplification success or failure of a given region within the genome precisely differentiates members of the MTC. In the
MTC, there are unidirectional chromosomal region deletions occurring in the genome over generations forming separate species (Gagneux et al., 2005). The deletions at different loci in the genome were exploited to develop a rapid, simple and reliable PCR based typing method for MTC (Huard et al., 2003). The typing panel is composed of several chromosomal regions: 16S rRNA, Rv0577, IS1561, Rv1510, Rv1970, Rv3877/8, and Rv3120 was developed, (Figure 2). The PCR products (amplicons) pattern of the panel, specified by failure or success, differentiates members of the MTC and segregates them from Mycobacteria other than tuberculosis (MOTTS).

![Typing Panel](image)

**Figure 2:**
The Mtbc PCR typing panel illustrating typical Mtbc PCR panel typing results for a single representative of each Mtbc subspecies as well as MOTT. Lanes: 1, 16S rRNA; 2, Rv0577; 3, IS1561; 4, Rv1510; 5, Rv1970; 6, Rv3877/8; 7, Rv3120. Unlabeled lanes in each panel contain the 100-bp ladder. (Adapted from (Huard et al., 2003)).
The RDs have specific tasks on the typing panel. The 16S rRNA is present in all *Mycobacteria* and never deleted, thus, is used to confirm presence of mycobacterial DNA. The Rv0577, a conserved gene in MTC, is used to distinguish MTC from MOTTS. The transposase pseudogene IS1561 (MiD3) is present in all isolates of the MTC except in *M. microti* and hence, it is used for its definition (Huard *et al.*, 2003; Glynn *et al.*, 2008). Rv2073c gene (Region RD9) creates two arms: *M. tuberculosis* arm and *M. bovis* arm. The successful amplification of RD9 region marks the presence of *M. tuberculosis* arm while failure represents presence of the *M. bovis* arm. The amplification and deletion of region TbD1 (mycobacterial specific deletion), represents the ancestral and modern *M. tuberculosis* respectively. Deletion of region RD12 (Rv3120 gene) also known as *M. cannetti* specific deletion, separates *M. cannetti* from *M. tuberculosis* arm (Huard *et al.*, 2003; Gagneux *et al.*, 2005). The Rv1510 [RD4; which is the *M. bovis* (classical) specific deletion], defines *M. bovis* in the sample. The Rv3877 and Rv3878 (RD1) is the specific deletion for *M. bovis* (BCG strain) (Huard *et al.*, 2003; Glynn *et al.*, 2008).

### 2.8.2 Spoligotyping

Spoligotyping involves *in vitro* amplification of the polymorphic direct repeat (DR) locus in the chromosome of MTC members. The polymorphic nature of DR locus arises from its structure of directly repeating short sequences (36 bp) interspersed by 43 unique spacers of variable length (34-41bp) (Figure 2). The amplicons are denatured and subjected to reversed line blot hybridization (Kamerbeek *et al.*, 1997). Presence or absence of spacers in the *in vitro* amplified DNA is determined by hybridization to multiple synthetic spacer oligonucleotides covalently bound to a filter membrane to generate a spoligotype patterns. By comparing spoligotype patterns to the SpolDB4
database, isolates are assigned to strain lineages and dendograms generated to reveal clustered and unclustered isolates. Large clusters may suggest recent or ongoing tuberculosis transmission typical in disease outbreaks, while lack of clustering infers reactivation cases of past infections (Glynn et al., 2008). The method can simultaneously detect and differentiate between members of the MTC (Kamerbeek et al., 1997). Besides, the technique is simple, rapid (performed in a single run) and robust. As such, spoligotyping is recommended for basic molecular investigations for MTC (Streicher et al., 2007). However, the technique tends to overestimate the clustering rate in the archived samples (Fok et al., 2008). Therefore, genotyping methods of superior discriminatory power, such as MIRU-VNTR should be used in conjunction with spoligotyping.

![Direct Repeat (DR) Region](image)

**Figure 3:**
The structure of the direct repeat (DR) locus in *Mycobacterium tuberculosis.*

### 2.8.3 MIRU-VNTR genotyping
The genome of MTC members contains 41 loci with direct tandem repeats of 50-100 base pairs collectively termed as Mycobacterial Interspersed Repetitive Units (MIRUs) located mainly in intergenic regions dispersed throughout the genome (Supply et al., 2000; Supply et al., 2006). The number of repeats per locus varies between strains and this forms the basis of typing with this method (Alonso-Rodríguez et al., 2008). Therefore, the Mycobacterial Interspersed Repetitive Units- Variable Numbers of Tandem Repeats
(MIRU-VNTR) genotyping tool analyses variable numbers of tandem repeats (VNTR) in the minisatellites of the mycobacterium genome (Han et al., 2007). Currently, only 12, 15 and 24 MIRU-VNTR loci of the 41 MIRUs loci of H37Rv are the utilized in genotyping, Figure 3 (Han et al., 2007; Alonso-Rodríguez et al., 2008). Studies have revealed that increasing the number of MIRU loci or combining MIRU-VNTR format with spoligotyping can be used in place of Restriction Fragment Length Polymorphism (RFLP) (Murase et al., 2008). Since lengths of repeat units are known, amplicon sizes reflect numbers of amplified MIRU-VNTR copies after electrophoretic migration. Consequently, the technique has superior discriminatory power to distinguish *M. tuberculosis* than spoligotyping (Allix-Beguec et al., 2008).
Figure 4:  
Genome map of H37Rv showing the distribution of Mycobacterial Interspersed Repetitive Units loci and the positions of the 12 MIRU loci (with dots) used in genotyping. (Adapted from (Supply et al., 2000)).

This genotyping tool is highly reproducible, sensitive, and specific for MTC isolates as well as less technical skill demands. It also combines speed to detection, high discriminatory power and ability to run samples in a high throughput fashion which makes it superior over the other PCR based methods (Han et al., 2007; Allix-Bégue et al.,...
Along this approach, when the 12-loci MIRU-VNTR format was improved to a to15-loci MIRU-VNTR it became very efficient at assigning clusters confirmed by epidemiological data (Glynn et al., 2002). Additionally, this marker is stable and generates portable data since it is in numerical form (Asiimwe et al., 2008; Fok et al., 2008). Therefore standardization across laboratories and research institutions can be achieved and for high resolution clonal identification. Accordingly, generated results can be compared with the existing MIRU-VNTRPlus database to assign lineages (Allix-Bégue et al., 2008).

On evaluation, the 15-loci MIRU-VNTR format shows that, it can identify as many clustered cases as RFLP thus making it the epidemiological tool of choice in many research institutions (Glynn et al., 2002). Additionally, it can be employed in detection of multiple strain infections (Dickman et al., 2010). However, in settings where Beijing strains are predominant, a specifically designed 12-loci MIRU-VNTR format is sufficient (Murase et al., 2008).
CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site
Kampala the capital of Uganda has an estimated human population of 1.66 million (UBOS 2010). Most of the people live in crowded peri-urban slums and/or work in crowded commercial centers within and around the Kampala. Although there are several public and private health facilities in the city, majority carry out only Ziehl-Neelsen (ZN) smears, which cannot differentiate MTC species. As such, most TB suspected cases are usually referred to either National Referral Hospital Mulago or Joint Clinical Research Centre (JCRC) where comprehensive diagnosis of the disease using culture and susceptibility testing are conducted.

3.2 Samples collection
In total, 98 isolates archived at National Tuberculosis and Leprosy Reference Laboratory (NTRL) and JCRC were studied. The mycobacteria in the isolates were indentified and confirmed as MDR-TB using the ca8pillia TB Neo (TAUNS Corporation, Japan) and LJ-PM for NTRL and PCR for IS6110 and tested for susceptibility to Rifampicin (Rif) and Isoniazid using the BACTEC 460 or the MGIT 960 (Becton-Dickinson Microb Systems) for JCRC, respectively. The MDR-TB isolates were stored in glycerol at -70°C. Of the 98 isolates, 26 specimens archived at NTRL were collected from retreatment TB patients, aged 19-56 years, attending Mulago National Referral Hospital during 2008. In addition, 72 isolates from TB patients recruited in several research projects at different health facilities in Kampala between 1997 and 2006 were archived at JCRC. The demographic information of the patients indicated that all patients were residents of Kampala.
3.3 Sample handling and DNA extraction
Upon retrieval, all the isolates were re-cultured at JCRC on 7H10 Agar at 37°C for 3 to 4 weeks. The cultures were harvested under a biosafety level 3 safety cabinet into falcon tubes containing 15ml of absolute ethanol. The cells were heat-killed by incubating the falcon tubes at room temperature for 2h, then at 80°C for 30 minutes. The cells were lysed by overnight incubation in a water bath at 37°C in 400µl TE buffer and 50µl of lysozyme (10mg/ml). For DNA purification, 75µl of SDS/Proteinase K was added to cell lysates and incubated at 65°C for 1h in a hybridization oven. Then 100µl of 5M NaCl was added followed by 100µl CTAB/NaCl pre-warmed at 70°C followed by 20 minutes of incubation at 65°C. An equal volume of chloroform-isoamyl alcohol was added and the preparation thoroughly mixed and centrifuged at 1300rpm for 15 minutes. The supernatant was transferred to a new tube, 500µl of ice-cold absolute isopropanol added to precipitate the DNA and centrifuged again at 1300rpm for 20 minutes and incubated overnight at -20°C after discarding the supernatant. The resultant DNA pellet was washed by 1ml ice cold 70% alcohol, centrifuged at 1300rpm for 15 minutes dried at 37°C for 30 minutes in oven. The dried DNA pellet was eluted in 50µl TE at 37°C for 2h.

3.4 Determination of MDR-TB species
To determine the species in the isolates, Region of Deletion (RD) analysis was used. A reaction mixture of 10µl consisting of 8µl PCR water, 1µl master mix, 0.5µl forward and reverse primers, 0.1µl Taq and 1µl DNA template was prepared for each sample. Besides, M. tuberculosis H37Rv and M. bovis (BCG) and PCR water were used as positive controls and a negative control, respectively. The primer sets adopted from (Huard et al., 2003), (Table 1) were used, with 16S rRNA locus run first, followed by Rv2073c (RD9), TbD1, Rv3120 (RD12), Rv3877/8 (RD1) and Rv1540 (RD4) loci. The
PCR reaction mixtures were run in the thermocycler, model PTC DNA Engine™ systems AL071585, MJ Research Inc, USA. The samples were initially denatured for 5 minutes at 94°C, followed by 35 cycles of 1 minute each at 94°C, 1 minute at 60°C and 1 minute at 72°C and a final extension of 10 minutes at 72°C. The amplification programme was changed depending on the product size by adjusting the annealing temperature and number of cycles. The amplicons and 100bp ladder were visualized by agarose gel electrophoresis and ethidium bromide staining on a 1% agarose gel. Gel images were captured with a BioDoc-It™ Imaging system Upland CA USA. All negative and unexpected positive PCR results were repeated and confirmed at least once again.

**Table 1:** Primer sets used in amplification of the six regions of difference (RD) in the present study including expected amplicon size in base pairs (bp)

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer name</th>
<th>Nucleotide sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>16SRNAF</td>
<td>5’ ACG GTG GGT ACT AGG TGT GGG TTT C 3’</td>
<td>543</td>
</tr>
<tr>
<td></td>
<td>16SRNAR</td>
<td>5’ TCT GCG ATT ACT AGC GAC TCC GAC TTC A 3’</td>
<td></td>
</tr>
<tr>
<td>Rv1540 (RD4)</td>
<td>Rv1540F</td>
<td>5’ GTG CGC TCC ACC CAA ATA GTT GC 3’</td>
<td>1033</td>
</tr>
<tr>
<td></td>
<td>Rv1540R</td>
<td>5’ TCT GCG ATT ACT AGC GAC TCC GAC TTC A 3’</td>
<td></td>
</tr>
<tr>
<td>Rv3877/8 (RD1)</td>
<td>Rv3877/8F</td>
<td>5’ CGA CGG GTC TGA CGG CCA AAC TCA TC 3’</td>
<td>999</td>
</tr>
<tr>
<td></td>
<td>Rv3877/8R</td>
<td>5’ CTT GCT CGG TGG CCG GTT TTT CAG C 3’</td>
<td></td>
</tr>
<tr>
<td>Rv3120 (RD 12)</td>
<td>Rv3120F</td>
<td>5’ GTC GGC GAT AGA CCA TGA GTC CGT CTC CAT 3’</td>
<td>404</td>
</tr>
<tr>
<td></td>
<td>Rv3120R</td>
<td>5’ GCC AA AGT GGG CGG ATG CCA GAA TAG T 3’</td>
<td></td>
</tr>
<tr>
<td>Rv2073c (RD 9)</td>
<td>Rv2073CF</td>
<td>5’ TCG CCG CTG CCA GAT GAG TC 3’</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>Rv2073cR</td>
<td>5’ TTT GGG AGC CGC CGG TGG TGA TGA 3’</td>
<td></td>
</tr>
<tr>
<td>TbD1</td>
<td></td>
<td>5’ TCG CCG CTG CCA GAT GAG TC 3’</td>
<td>500</td>
</tr>
</tbody>
</table>
3.5 Characterizing and determining the clustering rates the MDR-TB isolates

Spoligotyping technique was used to determine the strains of the species identified in the isolates. In the present study, DNA sequences within the direct repeat (DR) loci were amplified using DRa (GGTTTGGTCTGACGAC, biotinylated) and DRb (CCGAGAGGGACGGAAAC) primer sets. The amplification mixture for each sample consisted of 7µl PCR water, 1µl primer DRa and primer DRb, 1µl dNTP mixture, 0.1µl and 1µl of DNA template. H37Rv and M. bovis BCG were used as positive controls while PCR water as a negative control. In the thermocycler, samples were initially denatured at 96°C for 5minutes followed by 1 minute at the same temperature, annealing of 1 minute at 55°C and extension of 30seconds at 72°C. The cycle was repeated 26 times with a final extension of 5 minutes at 72°C and the amplicons were stored at -20°C prior to hybridization. The amplicons were diluted with 150µl of 2X SSPE 0.1% SDS, heat denatured at 100°C for 10 minutes and cooled immediately on ice. The diluted amplicons were filled carefully into miniblotter slots and hybridized for 1hour at 60°C on a horizontal surface. After hybridization, the membrane was washed twice in 250ml of pre-warmed 2X SSPE 0.5% SDS for 10 minutes at 60°C. The membrane was incubated in diluted streptavidin-peroxidase conjugate for 45 minutes at 42°C in a rolling bottle and washed twice with 250µl of pre-warmed 2X SSPE 0.5% SDS for 10 minutes at 42°C. After incubation, the membrane was rinsed twice with 250ml of 2X SSPE for 5minutes at room temperature.

The membrane was re-incubated for 1 minute in 20ml of ECL detection liquid before being sealed in transparent plastic sheet and exposed the chemiluminescent film for 15 minutes in darkroom to detect the hybridizing DNA. The film was developed using
Kodak GBX developer to generate spoligotypes. The individual spoligotypes were entered into the international spoligotyping database of the Pasteur Institute of Guadeloupe to assign strain lineages. To determine the clustering rates, the spoligotype patterns were compressed and dendrograms generated using Bionumerics software version 5.0 (Applied Maths, Kortrijk, Belgium). Lineages with at least 2 strains were considered to form clusters. Clusters with ten or more strains were considered major and the rest minor.

3.6 MIRU-VNTR typing of MDR-TB isolates for epidemiological link
The MIRU-VNTR genotyping was used to establish the direction of transmission of the strains (epidemiological linkages). This technique groups strains to indicate that these are same strains transmitting between patients. In this study, the 15 MIRU-VNTR loci typing panel: 580, 2996, 802, 960, 1644, 3192, 424, 577, 2165, 2401, 3690, 4156, 2164b, 1955 and 4052, adopted from (Supply et al., 2006; Han et al., 2007) was used to type the isolates. Depending on amplification conditions, the 15 MIRU-VNTR loci were put into three groups (Table 2). In this study, the thermocycler was set and run at an initial denaturation temperature of 95°C for 15 minutes, followed by 30 cycles of annealing for 1 minute at 59°C, extension of 1 minute at 72°C and a final extension of 10 minutes at 72°C. Mycobacterium tuberculosis H37Rv strain DNA was used as a positive control for all the reactions and sterile water a negative control.

The amplicons were resolved on 2.5% agarose gels using a 100-bp DNA ladder as a size marker. Gel images were captured with a BioDoc-It™ Imaging system Upland CA USA and sizing of MIRU-VNTR bands to assign allele copy numbers was based on the
reference table in the MIRU protocol (Han et al., 2007). The copy numbers for each of
the 15 loci of the individual samples were entered into Excel and analyzed by MIRU-
VNTRPlus online database to generate clusters.

Table 2: Primer sets used in this study for the MIRU-VNTR 15-loci Panel

<table>
<thead>
<tr>
<th>Group</th>
<th>Locus</th>
<th>Alias</th>
<th>Repeat unit length (bp)</th>
<th>PCR primer pairs (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>580</td>
<td>MIRU 4; ETR D</td>
<td>77</td>
<td>GCGCGAGAGCCCGAAGCTGC GCGCAGAGAACCAGCCAGC</td>
<td></td>
</tr>
<tr>
<td>2996</td>
<td>MIRU 26</td>
<td>51</td>
<td>TAGGCTTCTACGCGAAATCTCTGAC CATAGGCGAGAGCGGTAAG</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>802</td>
<td>MIRU 40</td>
<td>54</td>
<td>GGGTTTCTGGAGATCAACGGTG GGGGTATCCTCGGAAATCATCG</td>
</tr>
<tr>
<td>960</td>
<td>MIRU 10</td>
<td>53</td>
<td>GTTCTTGGACAAACTGCTGTCGC GCCACCCTGGTAGATCAGCTCCT</td>
<td></td>
</tr>
<tr>
<td>1644</td>
<td>MIRU 16</td>
<td>53</td>
<td>TCGGTGATCGGATCGTGCTGAGA CCGGTGCGCTAGCCCTGGTAC</td>
<td></td>
</tr>
<tr>
<td>2165</td>
<td>ETR A</td>
<td>75</td>
<td>AAATCGGTCTGATCAGCCCTGTTAT CCGACCCTGGGCTGGCCGGAAT</td>
<td></td>
</tr>
<tr>
<td>424</td>
<td>Mtub04</td>
<td>51</td>
<td>CTGAGGCGGTCATTAGTAGT TAGGAGCCAGCGAGGCTCTT</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>577</td>
<td>ETR C</td>
<td>58</td>
<td>CGAGATGCTGTGGCGGCGGATCTAG AATGACTTGAACGCCGAAGATTGA</td>
</tr>
<tr>
<td>3</td>
<td>3192</td>
<td>MIRU 31; ETR E</td>
<td>53</td>
<td>ACTGATTGGTCTGATACGGGTCTTTA GTGCCGACGCTTGCTGAG</td>
</tr>
<tr>
<td>2401</td>
<td>Mtub30</td>
<td>58</td>
<td>CTGAGGCGGTCATTAGTAGT ACTGGAACCCCCAGGGGCTTATGAGA</td>
<td></td>
</tr>
<tr>
<td>3690</td>
<td>Mtub39</td>
<td>58</td>
<td>CGGATGAGCCAGGAGAAGCTGCTCTT</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4156</td>
<td>QUB-4156</td>
<td>59</td>
<td>TGACACCGATGTCCGCTGAGT GCGGCGGTCATGTT</td>
</tr>
<tr>
<td>2163b</td>
<td>QUB-11b</td>
<td>69</td>
<td>CGTAAGGGGGGATGCGGGGAAATAGG CGAATGATAGTGGCAT</td>
<td></td>
</tr>
<tr>
<td>1955</td>
<td>Mtub21</td>
<td>57</td>
<td>AGATCCAGTTGCTGCTGTC CACATCGCTGCTGCTG</td>
<td></td>
</tr>
<tr>
<td>4052</td>
<td>QUB-26</td>
<td>11</td>
<td>AACGCTACGTGTGCGGT GCGCCGTCGTGGCGGAGGTTGTCGTCGAGTAGT</td>
<td></td>
</tr>
</tbody>
</table>

3.7 Data analysis
Regions of deletion (RD) analysis amplicons were visualized on a bromide stained gel.
Presence of the target loci was indicated by amplification success, while its deletion by
amplification failure.
Individual spoligotypes generated from spoligotyping data were condensed and entered into the international spoligotyping database of the Pasteur Institute of Guadeloupe to assign respective strains and lineages. Clustering rate was determined by compressing spoligotype patterns to generate a dendrogram using Bionumerics software version 5.0 (Applied Maths, Kortrijk, Belgium).

MIRU-VNTR amplicons were resolved on 2.5% agarose gels together with a 100-bp ladder. Agarose gel images were captured for sizing to assign allele copy numbers and then compared to the MIRU-VNTRPlus database to generate clusters using BioNumerics V.50

3.8 Ethical considerations
The archive samples analyzed in this study were part of several of studies conducted in Uganda together with foreign collaborating research institutions that got clearance from the Research and Ethics Committee of the Makerere University School of Medicine. Permission to use archived samples for future studies was also obtained.
CHAPTER FOUR

4.0 RESULTS

4.1 MDR-TB species in the isolates
Using the Region of Deletion (RD) analysis, all 98 isolates amplified for 16S rRNA gene (Fig. 4), the genus specific conserved region for Mycobacteria species. Additionally, 97 of 98 isolates amplified for RD9 region while in only one isolate it was deleted (Fig. 5). This means 97 isolates belonged to the M. tuberculosis arm while the other isolate was in the M. africanum → M. bovis arm.

Figure 5:
A representative gel for 16S rRNA PCR for 52 samples. Extreme ends are 100bp ladders, first lane of first panel positive control (H37Rv), last lane of last panel is negative control (PCR water).
Further RD analysis of *M. tuberculosis* arm revealed that 96 of the 97 isolates were deleted for the *M. tuberculosis* specific deletion (TbD1). The other isolate amplified for TbD1 indicating the presence of “ancestral type” *M. tuberculosis*, which was confirmed by the amplification at the RD 12 locus. The single isolate bearing RD9 deletion (*M. bovis* arm) failed to amplify at the *M. bovis* specific deletion RD4 locus, indicating the presence of *M. bovis*. Repeated using spoligotyping confirmed the isolate as *M. bovis* by its typical signature. Overall, two *Mycobacteria* species: *M. tuberculosis* and *M. bovis* (classical) were recorded (Table 3).
Table 3: The *Mycobacteria* MDR-TB species occurring in the 98 isolates; + indicates amplification of the region; – indicates deletion of the region.

<table>
<thead>
<tr>
<th><em>M. tuberculosis</em> species</th>
<th>16S rRNA (Rv2973)</th>
<th>RD9 (Rv3120)</th>
<th>TD1 (Rv3877/8)</th>
<th>RD4 (Rv1510)</th>
</tr>
</thead>
<tbody>
<tr>
<td>“Ancestral” <em>M. tuberculosis</em> (n= 1)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>“Modern” <em>M. tuberculosis</em> (n= 96)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>M. bovis</em> (classical) (n= 1)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

4.2 MDR-TB spoligotype clusters, strain lineages and sub-lineages

Of the 98, isolates 71(72.4%) were part of 14 spoligotype clusters with 2-20 strains each. Two clusters, T2-Uganda (SIT 52) and CAS1 (SIT 26) were the largest and regarded as major clusters since they had 20 and 12 strains respectively. The other clusters were regarded minor since they had 2-7 strains each. Strain T2 (SIT 52) formed the biggest cluster constituting 27.4% of all clustered strains, followed by CAS1 (SIT 26) and CAS2 (SIT 288) with 9.8% and 8.4% respectively. Minor clusters constituted 27.5% of all the isolates, see Figures 6 and 7. Beijing strains (SIT 1) predominantly found in countries of South East Asia, formed a cluster of four isolates in the present study. Twenty five isolates (25.5%) were not defined in the SpolDB4 database (orphans) of which 9 (39%) formed four clusters of 2-3 strains each. Among the unclustered isolates, Bovis1 strain (SIT 1598) was a surprising finding in this study. Generally, out of the 98 isolates 46 strains were identified of which 21 were defined in the SpolDB4 database and 25 orphans. A clustering rate of 72.4% involving 14 clusters was recorded and the predominant strain was T2-Uganda strain.
Figure 7: Spoligotype lineages and families of 98 human MDR-TB isolated from Kampala.

All strain lineages are endemic in Uganda with the exception of five Beijing strains. The most predominant strain lineages were T2 and CASI-Dehli forming large clusters of 20 and 12 strains respectively. ND; these are strains which were not defined in the SpolDB4 database.
Figure 8:
Spoligotype patterns of the 98 MDR-TB isolates, major clusters are shown in brackets and a few minor clusters as well as the only *M. bovis* strain recorded.
4.3 MDR-TB transmission patterns and clustering rate with MIRU-VNTR typing
Out of the ninety eight isolates, we recorded thirty five strains distributed among the nine sub-family lineages and the rest were non-existent in the database (Figure 8.) Among the nine sub-family lineages, Uganda II (29.6%) and Uganda I (7.1%) of the Uganda family were the most predominant. The CAS–Delhi sub-family lineage of the Central Asian Strains family occurred in 11.2% of the isolates whereas the Latin America (LAM) family lineages were recorded in 9.1% of the isolates. Regarding strain clusters, two clusters were recorded. Uganda I and CAS–Delhi sub-families were involved and each cluster had two strains, dendrogram (Fig. 9). The overall clustering rate was 4.2% with MIRU-VNTR typing. Accordingly, the 68 remaining strains together with the twenty six orphans did not form clusters.

Figure 9: The MDR-TB strains and frequency of occurrence isolated from patients in Kampala
4.4 Isolates with multiple infections
MIRU-VNTR analysis further revealed multiple infections in five isolates (mw 03, mw 06, mw 13, mw 31 and mw 89) by the presence of two allelic copies at MIRU loci 2165 and 4052. Of these five isolates, four belonged to Dehli/CAS lineage. The strain in mw-89 belongs to the Haarlem family.
Figure 10: The MIRU-VNTR/Plus dendrogram of the MDR-TB strains in archived TB isolates in Kampala, encircled are the 'twin' clusters.
CHAPTER FIVE

5.0 DISCUSSION
Resistance to standard anti-tuberculosis drugs is a major challenge facing control and treatment programmes worldwide (Romero et al., 2006; Zignol et al., 2006). Large epidemics have been associated with *M. tuberculosis* complex (MTC) drug resistant strains (Sreevatsa et al., 1997; Van Soolingen et al., 1998; Niobe-Eyangoh et al., 2004). Accurate identification of the species and strains is critical in patient management. However, routine diagnostic procedures, commonly used in Uganda, do not differentiate between MTC species and strains. As such, little information is available on the etiology of MDR-TB in the country, despite the increasing cases (Temple et al., 2008; Bazira et al., 2011). Therefore, this study determined the MDR-TB species and strains and ascertained their epidemiological linkages in Kampala with the aid of molecular genotyping assays.

All the isolates examined revealed the presence of 16S rRNA region, the conserved region within the *Mycobacteria* species, and is used for taxonomical purposes (Brosch et al., 2002; Huard et al., 2003). As revealed by Regions of Deletion (RD), two species namely, *Mycobacterium tuberculosis* and *M. bovis* are the main etiology of MDR-TB in Kampala. The present results confirm findings of previous studies (Asiimwe et al., 2008). Furthermore, amplification of RD9 region in 99% of the isolates indicates that majority of the MDR-TB isolates belong to the *M. tuberculosis* arm which constitutes exclusively human pathogens (Brosch et al., 2002). Earlier studies have also reported predominance of MTC species and strains belonging to the *M. tuberculosis arm* over *M. bovis* arm in this setting (Asiimwe et al., 2008; Lukoye et al., 2010; Bwanga et al., 2011). Further, the
deletion of *M. tuberculosis* specific deletion (TbD1) in the *M. tuberculosis* arm suggests the presence of “modern” type *M. tuberculosis*. The “modern” type also occurred in 96% of the isolates, hence the main contributor of MDR-TB species circulating in Kampala. The high frequency of “modern” type TB in the present study is similar to the findings of (Asiimwe et al., 2008). This reflects that these species are mutating to better adapt, probably in response to drugs. Indeed tuberculosis outbreaks, not necessarily MDR-TB have been associated with isolates that belong to “modern” strains of tuberculosis (Jansons et al., 2008; Asiimwe et al., 2008; Bazira et al., 2011). Besides, *M. tuberculosis* and *M. bovis* were detected in the isolates examined. The *M. bovis* (BCG strain) specific gene deletion RD1 region was also amplified but the RD4 region failed, indicating presence of an *M. bovis* strain among the samples. Although, *M. bovis* has been associated majorly with animal TB, human isolates have been reported in Kampala (Asiimwe et al., 2008; Bazira et al., 2011).

Lack of deletions in all tested regions of isolate mw 39 qualifies it to the “ancestral” *M. tuberculosis* sub-species. Phylogeny grouping by SpolDB4 placed it in the EAI 5 family which is regarded oldest and endemic to East African- Indian region (Gagneux et al., 2005). Isolate mw 80 had most deletions including the *M. bovis* ‘classical’ specific deletion, RD 4. These findings clearly show involvement of both “ancestral” and “modern” MTC sub-species in MDR-TB disease outbreak in Kampala with the later being the most predominant. Globally, reports indicate that *M. tuberculosis* specific lineages predominate and transmit well in particular human populations (Gagneux et al., 2005).
A total of forty six distinct spoligotypes were observed out of the total ninety eight isolates analyzed giving a diversity of 46.9%. This differs from low diversity rates of 25% observed in Harare, Zimbabwe (Easterbrook et al., 2004), but compares well with 52 % observed in Dar es Salaam, Tanzania (Vegard et al., 2006). However, it was low when compared with a recent study in Uganda which recorded 63.2% from Mbarara District, a rural setting in western Uganda (Bazira et al., 2010). The observed diversity of 46.9% in this study may be attributed to the sampling strategy which considered only archived samples collected in a span of ten years. It is also worth noting that Kampala the Capital of Uganda has been a centre business resulting into extensive social mixing with people of different races for a long period of time (Asiimwe et al., 2008). It is likely that it was during this time when different strain lineages were introduced into this setting resulting into the observed diversity.

When the spoligotypes were compared with international database to assign lineages it was observed that T2-Uganda, CAS1-Delhi, CAS2, LAMII-ZWE constituting 20.4%, 12%, 7% and 6% respectively were the predominant strain lineages in Kampala. The population of MDR-TB in Kampala differs from that of Sudan (CASI-Delhi 49%, Beinjing 3%), Kenya (CAS 35.6%, LAM 22%), Tanzania (CAS 37%, LAM 22%, EAI 17%, CASI-Dar 8%), Mozambique (T 11%, CAS 11%, LAM 37%, EAI 29.7%, X 13%, S 8%, Beinjing 7%) and Cameroon where one family; the Cameroon family contributes over 40% of all cases (Niobe-Eyangoh et al., 2004). The population of MDR-TB in Kampala was comparable to what has been described in previous studies in Uganda. A study conducted in one division of Kampala city shows CAS 1.7%, CASI-Kili 3.5%,
CASI-Delhi 2.6% LAM9 2.6%, LAM3/S 1.7%, LAMII-ZWE 1.5% (Asiimwe et al., 2008) and another from a rural setting, M. tuberculosis stricto 59.2%, Uganda genotype 5.6%, LAM 6.4%, Cameroon 4%, Ghana 2.4%, CASI- Delhi 7.2% (Bazira et al., 2011). The increasing MDR-TB strains circulating in Kampala are dominated by endemic strains, suggesting a local outbreak. This is in agreement with previous reports which show that particular strains transmit well in particular human populations (Ganeaux et al., 2005; Bazira et al., 2011). Additionally, it indicates effective tuberculosis control and management strategies in Kampala and Uganda at large, nevertheless adherence to treatment regimen is inadequate.

This study reports five Beijing strains (5.1%), four of which were clustered (SIT-1) and the other (SIT-190) unclustered. This compares well with recent studies in Dar es Salaam (Vegard et al., 2006) and Sudan (Sharaf et al., 2011) where 3% of the isolates were Beijing strains in each case. Increased cases were reported recently in Nairobi Kenya, 12% of the 536 isolates studied were Beijing strains (Ogaro et al., 2011). Elsewhere, Beijing strains predominate for example in Mozambique 31% (Viegas et al., 2009), China 31.9% (Phyu et al., 2008) and Russia 96.6% (Olga et al., 2006). Moreover, these strains are associated with rapid spread in the population and development of drug resistance hence MDR-TB disease outbreaks (Glynn et al., 2005). The small number of Beijing MDR-TB strains reported in this study may be attributed to sampling strategy since we used archived samples collected over a span of ten years whereas the other studies used consecutively sampled isolates both susceptible and resistant combined. Additionally, Beijing strains are predominant in countries of South East Asia and Russia (Glynn et al., 2005) it is likely that they are not well adapted to the human host in Uganda
(Ganeaux et al., 2005). However, Beijing strains from Kenya which serves as the main commercial route to the East and Central African region can easily spread to Uganda which is already burdened with HIV infection that synergizes progression to disease in TB infected individuals (Andrews et al., 2008).

One isolate which was assigned BOVIS1 (SIT-1598) by SpolDB4, was a surprising finding in this study. This is comparable to the two human and three cattle MDR M. bovis isolates in Spain (Romero et al., 2006). A recent study in cattle rearing communities of South Western Uganda did not record M. bovis human isolate among the seventy five isolates analyzed (Bazira et al., 2011). Although one study reported presence of M. bovis among human isolates in Kampala (Asiimwe et al., 2008), to the best of my knowledge this is the first report of MDR M. bovis human isolate in Uganda. Additionally, I have established the first ever MDR-TB database in Uganda comprising of ninety eight isolates.

I observed a high clustering rate of 72.4% involving fourteen spoligotype clusters three of which have 7-20 isolates and the rest having 2-6 isolates in the present study. This was higher in comparison to 33% in Mozambique (Viegas et al., 2010) and 35% in Sudan (Sharaf et al., 2010). But it was comparable to 61% in Dar es Salaam (Vegard et al, 2007) and 84.1% in Harare (Easterbrook et al., 2004). High spoligotype clustering rates are associated with recent and high transmission of tuberculosis (Toungoussova, 2003; Glynn et al., 2008; Temple et al., 2008). Surprisingly, when the same isolates were analyzed with MIRU-VNTRPlus, a clustering rate of 2% involving Ugandan and CAS-Delhi strains, two isolates each was observed. This may be explained by the superior
discriminatory power of 15-loci MIRU-VNTR which can split spoligotype clusters (Han et al., 2007; Allix-Beguec et al., 2008; Alonso-Rodriguez et al., 2008). Additionally, spoligotyping overestimates clustering rate for isolates collected over a long period of time (Fok et al., 2008). Furthermore, MIRU-VNTR typing is based on multilocus sequence in comparison to spoligotyping that utilizes variability in a single DR locus therefore prone to homoplasmy (Inaki et al., 2011). The observed low clustering rates in this study infer epidemiological independence of circulating MDR-TB strains in Kampala. Probably, they are reactivation cases of past remote infections. Consequently, the high clustering rate by spoligotyping which imply recent or ongoing transmission is an over exaggeration. These findings underscore the value of employing more than one molecular tool in molecular epidemiological investigations.

Five isolates were assigned a Beijing genotype by SpolDB4. A cluster of four isolates was formed while one isolate remain unclustered. When MIRU-VNTR results were compared to MIRU-VNTRPlus database, the isolates appeared unclustered on the dendogram. Three isolates gave concordant data, one isolate CAS-Delhi and the other was unidentified. Additionally, four more isolates mw21 previously assigned CAS-Delhi, mw17, mw59 and mw66 were assigned Beijing by MIRU-VNTRPlus database. This might be explained by the superior discriminatory power of MIRU-VNTR typing compared to spoligotyping (Han et al., 2007; Allix-Beguec et al., 2008; Alonso-Rodriguez et al., 2008).

Prevalence of multiple infections was recorded at 5.1% in the present study. In comparison to a recent study in Kampala City, where multiple infections were estimated at 7.1% (Dickman et al., 2010), our study recorded a lower prevalence. Low prevalence
of multiple infections in the present study may be attributed to numerous disease episodes characteristic of MDR-TB patients, yet multiple strains are easily detected on the first episode.
CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion
The main contributor of MDR-TB epidemic in Kampala is “modern” *M. tuberculosis* strains. The MDR-TB epidemic in Kampala is predominated by strain lineages endemic to this setting particularly T2-Uganda and CASI-Delhi genotypes. The transmission rate of MDR-TB in Kampala is low, however presence of Beijing strains is important from the epidemiological point of view.

6.2 Recommendations
Adherence to treatment regimen should be strengthened by the National tuberculosis control program to stop on-going acquired resistance of tuberculosis. A similar country wide study should be conducted to gain insight on the diversity of MTC strains causing MDR-TB in Uganda and monitor trends of acquired resistance to antimycobacterial agents.
REFERENCES


