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.

Signature…….Date……

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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.

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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-VNTR*Plus* 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. cannettii* 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-aminosalicyclic 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 *rpo*B gene. Therefore, mutations in the gene *rpo*B 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).

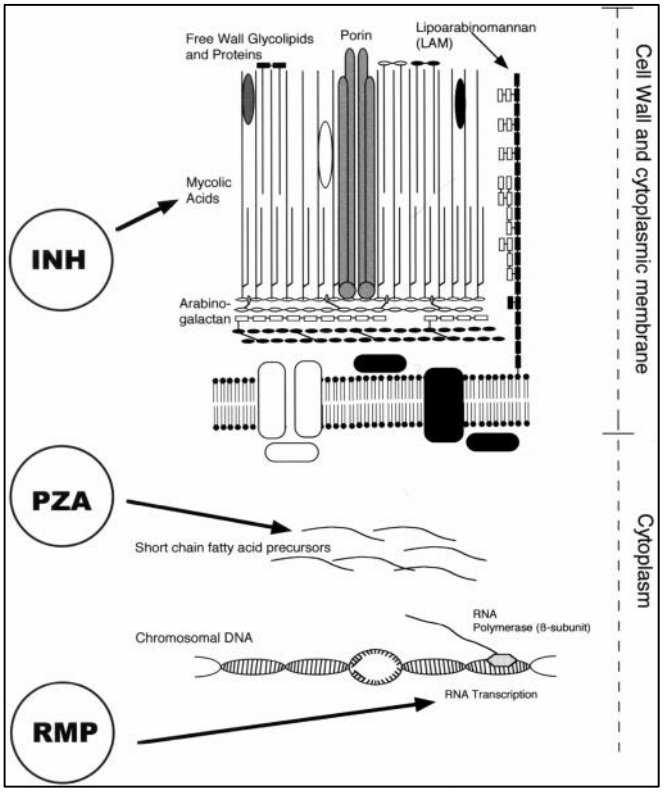

Figure 1:
Shows drug targets in the cell wall, cytoplasmic membrane and cytoplasm of *Mycobacterium tuberculosis* for Isoniazid, Rifampicin and Pyrazinamide. Adapted from (Somoskovi *et al.*, 2001).

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

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-Oceonic (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-Oceonic, 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

(Asiimwe *et al.*, 2009). 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).

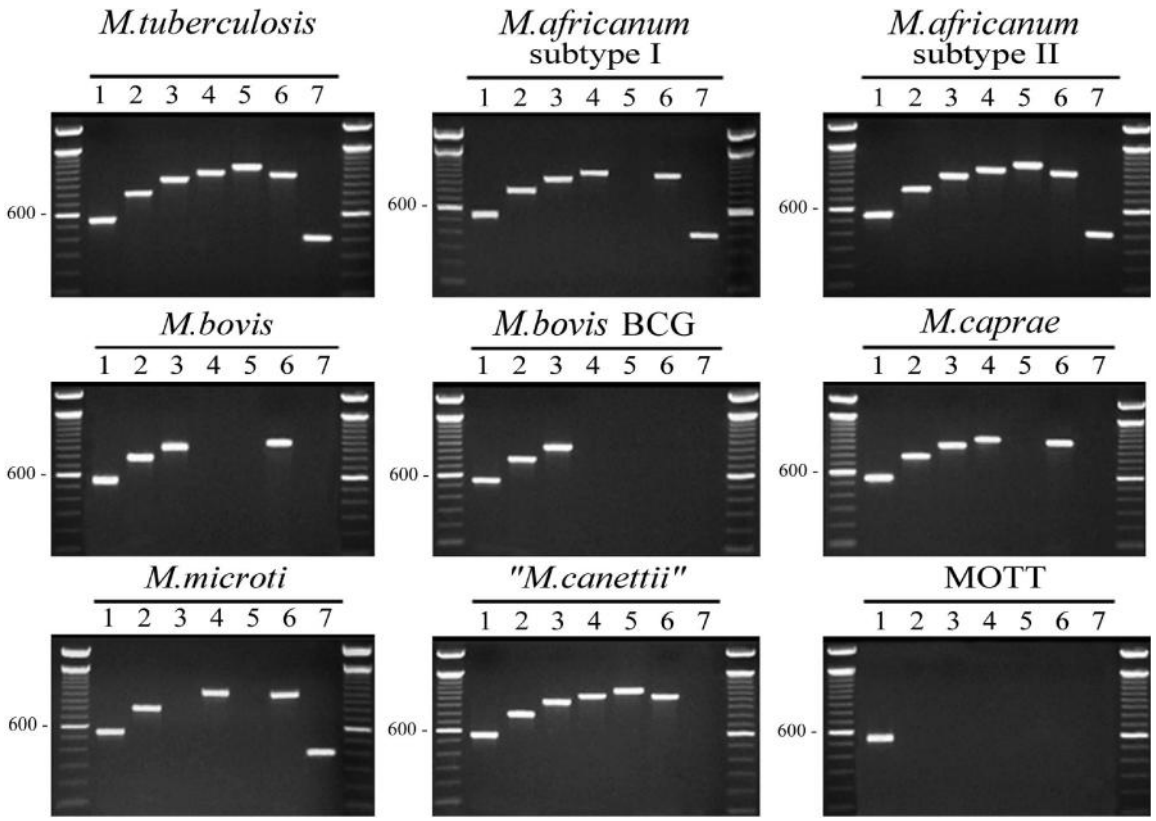

Figure 2:
The MtbC PCR typing panel illustrating typical MtbC PCR panel typing results for a single representative of each MtbC subspecies as well as MOTT. Lanes: 1, *16S rRNA*; 2, Rv0577; 3, IS*1561* ; 4, Rv1510; 5, Rv1970; 6, Rv3877/8; 7, Rv3120. Unlabeled lanes in each panel contain the 100-bp ladder. (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 over 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.

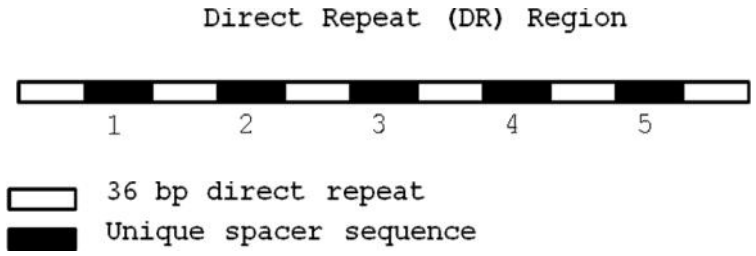

Figure 3:
The structure of the direct repeat (DR) locus in *Mycobacterium tuberculosis.*

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).

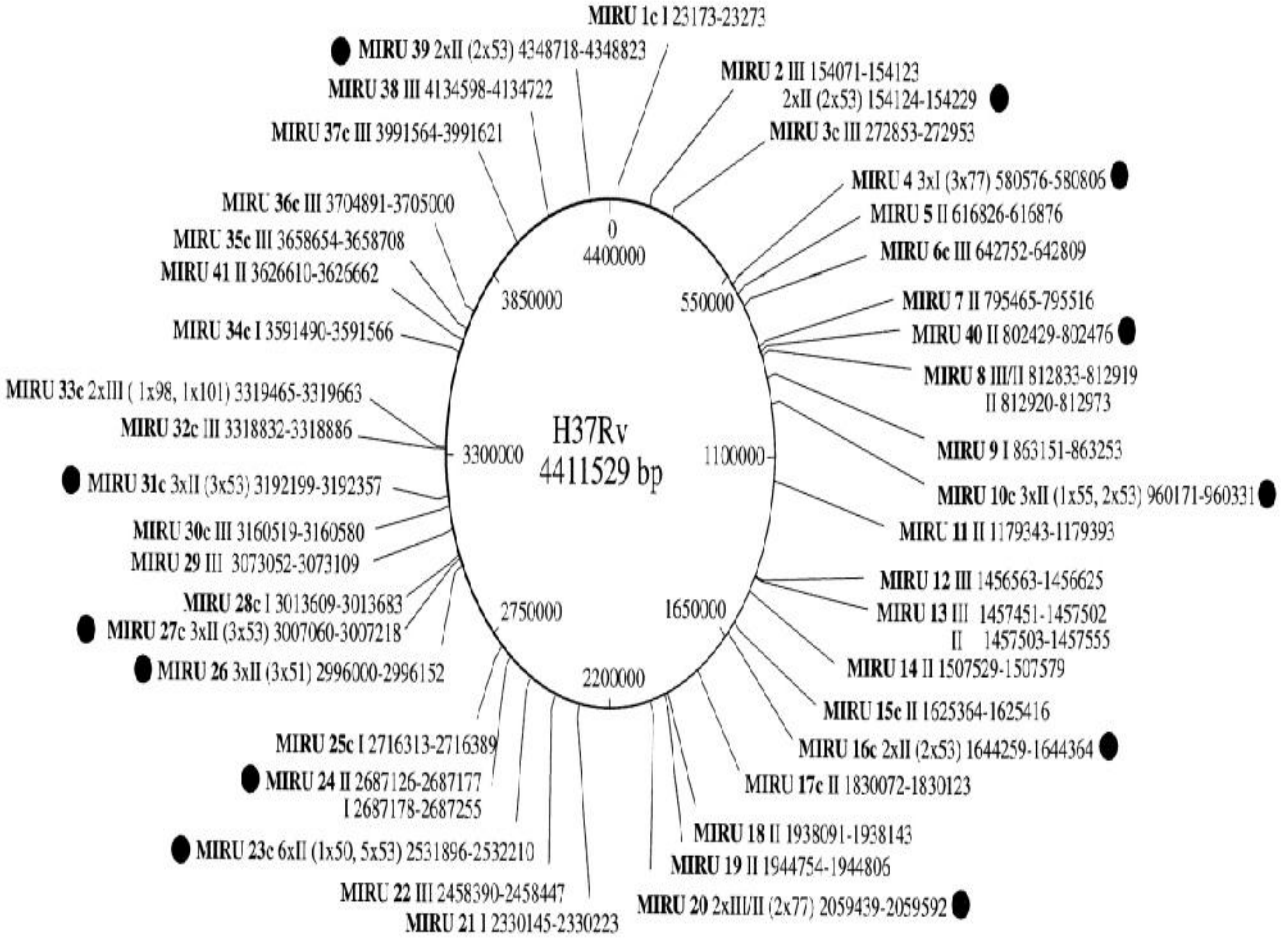

Figure 4:
Genome map of H37Rv showing the distribution of Mycobacterial Interspersed Repetitive Units loci and the positions of the 12 MIRU loci (with dots) used in genotyping. (Adapted from (Supply *et al.*, 2000).

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.*,

2008). 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-VNTR*Plus* 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 IS*6110* and tested for susceptibility to Rifampicin (Rif) and Isoniazid using the BACTEC 460 or the MGIT 960 (Becton-Dicknson 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^{o}C for 30 minutes. The cells were lysed by overnight incubation in a water bath at 37^{o}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^{o}C for 1h in a hybridization oven. Then 100µl of 5M NaCl was added followed by 100µl CTAB/Nacl pre-warmed at 70^{o}C followed by 20 minutes of incubation at 65^{o}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^{o}C for 30 minutes in oven. The dried DNA pellet was eluted in 50µl TE at 37^{o}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 Ta*q* 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 EngineTM systems AL071585, MJ Research Inc, USA. The samples were initially denatured for 5 minutes at 94^{o}C, followed by 35 cycles of 1 minute each at 94^{o}C, 1 minute at 60^{o}C and 1 minute at 72^{o}C and a final extension of 10 minutes at 72^{o}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^{TM} 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)

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

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 (GGTTTTGGGTCTGACGAC, biotinylated) and DRb (CCGAGAGGGGACGGAAAC) 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^{o}C for 10 minutes and cooled immediately on ice. The diluted amplicons were filled carefully into miniblotter slots and hybridized for 1hour at 60^{o}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^{o}C. The membrane was incubated in diluted streptavidin-peroxidase conjugate for 45 minutes at 42^{o}C in a rolling bottle and washed twice with 250µl of pre-warmed 2X SSPE 0.5% SDS for 10 minutes at 42^{o}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^{o}C for 15 minutes, followed by 30 cycles of annealing for 1 minute at 59^{o}C, extension of 1minute at 72^{o}C and a final extension of 10 minutes at 72^{o}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-VNTR*Plus* online database to generate clusters.

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

Group	Locus	Alias	Repeat unit length (bp)	PCR primer pairs (5 to 3)
1	580	MIRU 4; ETR D	77	GCGCGAGAGCCCGAACTGC GCGCAGCAGAAACGCCAGC
	2996	MIRU 26	51	TAGGTCTACCGTCGAAATCTGTGAC CATAGGCGACCAGGCGAATAG
	802	MIRU 40	54	GGGTTGCTGGATGACAACGTGT GGGTGATCTCGGCGAAATCAGATA
	960	MIRU 10	53	GTTCTTGACCAACTGCAGTCGTCC GCCACCTTGGTGATCAGCTACCT
	1644	MIRU 16	53	TCGGTGATCGGGTCCAGTCCAAGTA CCCGTCGTGCAGCCCTGGTAC
	2165	ETR A	75	AAATCGGTCCCATCACCTTCTTAT CGAAGCCTGGGGTGCCCGCGATTT
2	424	Mtub04	51	CTTGGCCGGCATCAAGCGCATTATT GGCAGCAGAGCCCGGGATTCTTC
	577	ETR C	58	CGAGAGTGGCAGTGGCGGTTATCT AATGACTTGAACGCGCAAATTGTGA
	3192	MIRU 31; ETR E	53	ACTGATTGGCTTCATACGGCTTTA GTGCCGACGTGGTCTTGAT
3	2401	Mtub30	58	CTTGAAGCCCCGGTCTCATCTGT ACTTGAACCCCCACGCCCATTAGTA
	3690	Mtub39	58	CGGTGGAGGCGATGAACGTCTTC TAGAGCGGCACGGGGGAAAGCTTAG
	4156	QUB-4156	59	TGACCACGGATTGCTCTAGT GCCGGCGTCCATGTT
	2163b	QUB-11b	69	CGTAAGGGGGATGCGGGAAATAGG CGAAGTGAATGGTGGCAT
	1955	Mtub21	57	AGATCCCAGTTGTCGTCGTC CAACATCGCCTGGTTCTGTA
	4052	OUB-26	11	AACGCTCAGCTGTCGGAT CGGCCGTGCCGGCCAGGTCCTTCCCGAT

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 amplication 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. Agar rose gel images were captured for sizing to assign allele copy numbers and then compared to the MIRU-VNTR*Plus* 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.

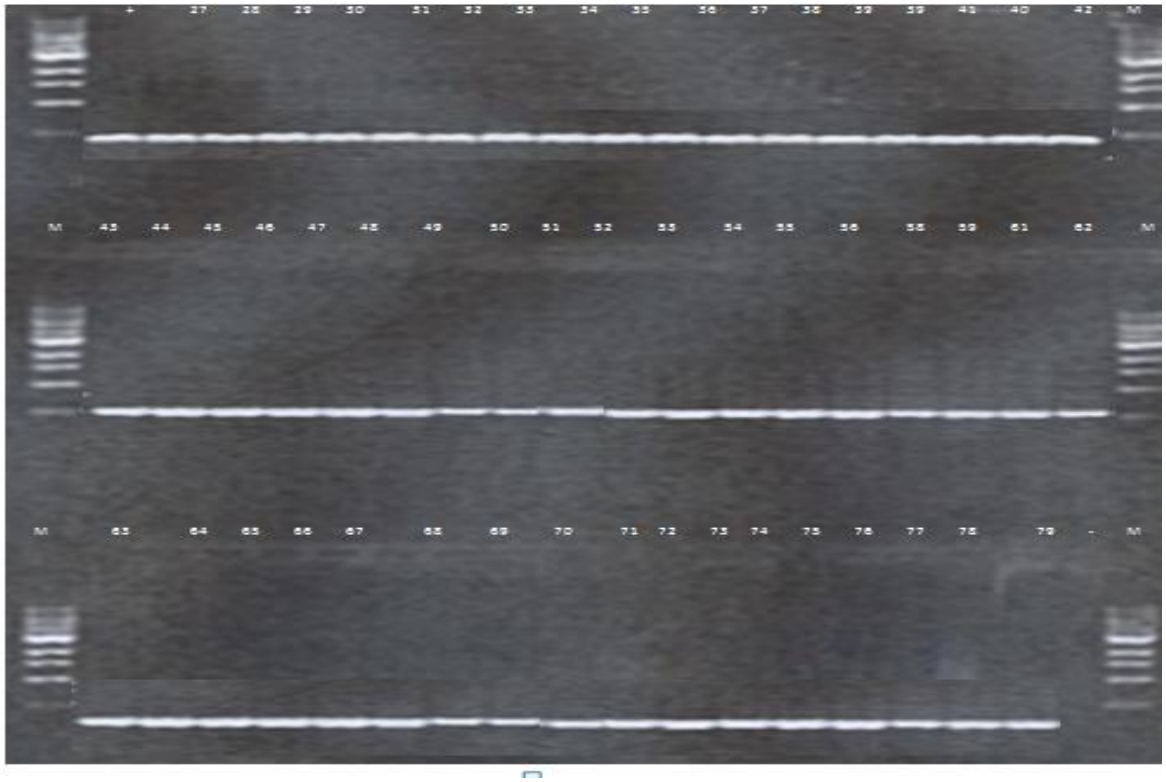

Figure 5:
A representative gel for *16S* rRNA PCR for 52 samples. Extreme ends are 100bp ladders, first lane of first panel positive control (H37Rv), last lane of last panel is negative control (PCR water).

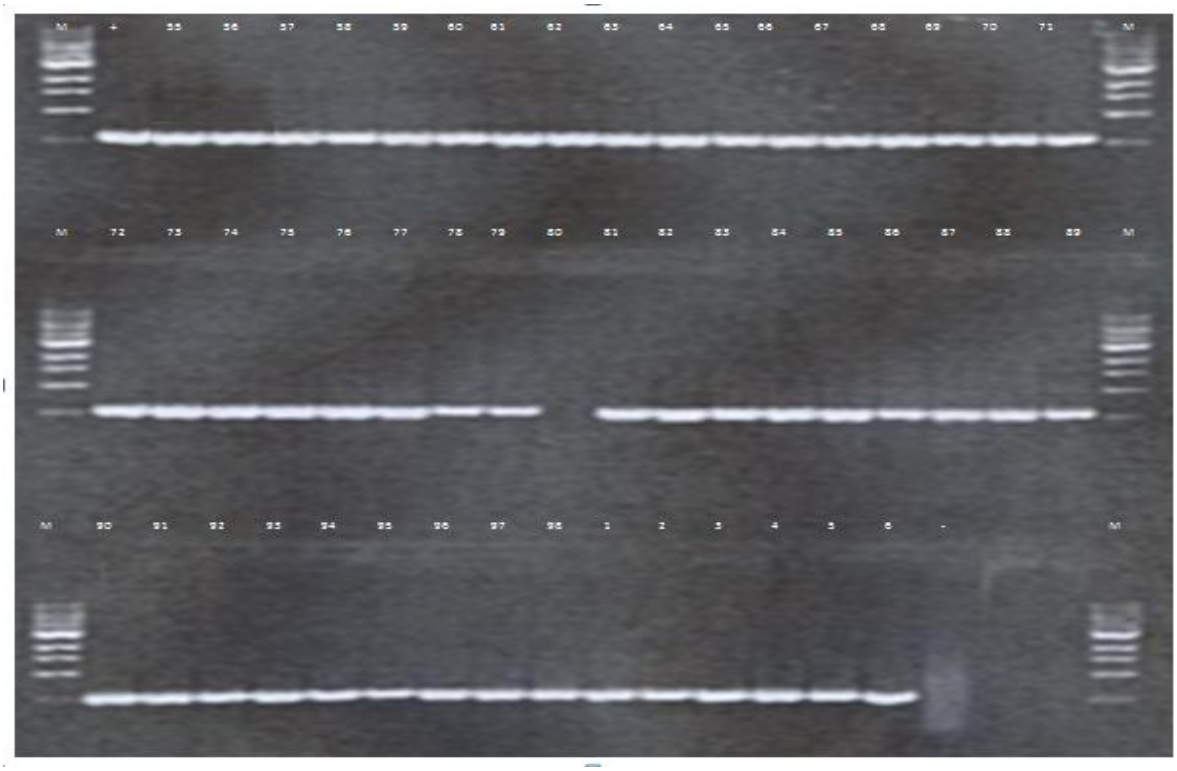

Figure 6:
The RD9 amplicons of the clinical isolates 55-98 and 1-6 and 100bp size maker (M) and the positive control H37Rv (+) and negative control PCR water (-).

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.

M. tuberculosis species	**RD target loci**					
	***16S* rRNA**	**RD9 (Rv2973)**	**TbD1**	**RD12 (Rv3120)**	**RD1 (Rv3877/8)**	**RD4 (Rv1510)**
"Ancestral" *M. tuberculosis* (n= 1)	+	+	+	+	+	+
"Modern " *M. tuberculosis* (n= 96)	+	+	–	+	+	+
M. bovis (classical) (n= 1)	+	–	+	–	+	–

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.

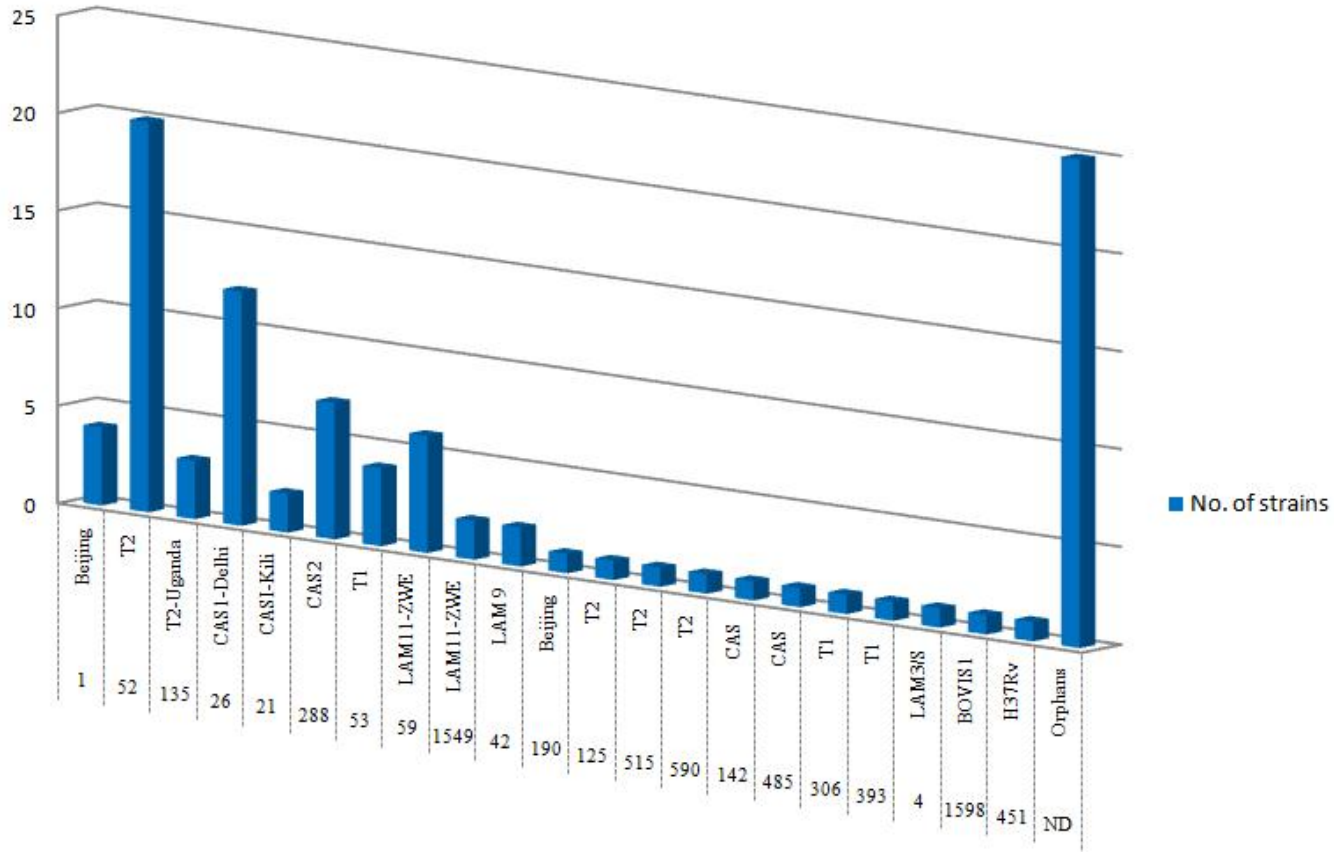

Figure 7:
Spoligotype lineages and families of 98 human MDR-TB isolated from Kampala.

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.

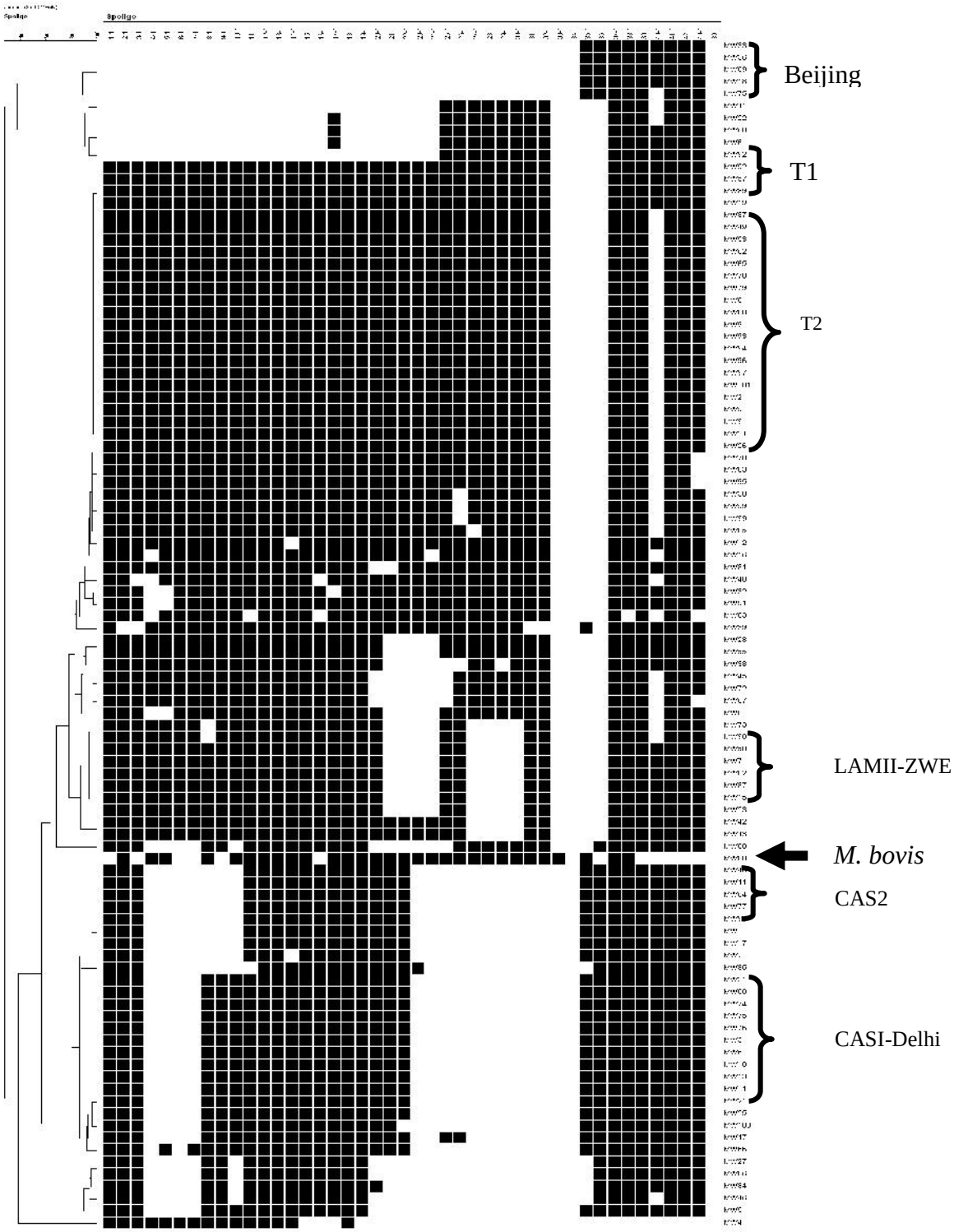

Figure 8:
Spoligotype patterns of the 98 MDR-TB isolates, major clusters are shown in brackets and a few minor clusters as well as the only *M. bovis* strain recorded.

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 1 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.

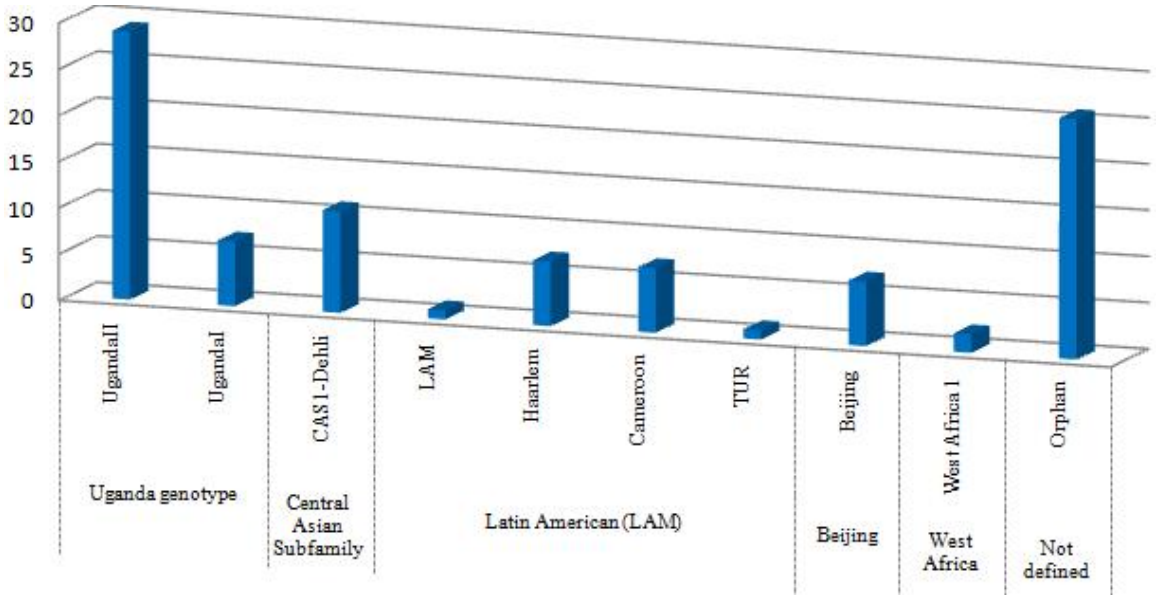

Figure 9: The MDR-TB strains and frequency of occurrence isolated from patients in Kampala

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.

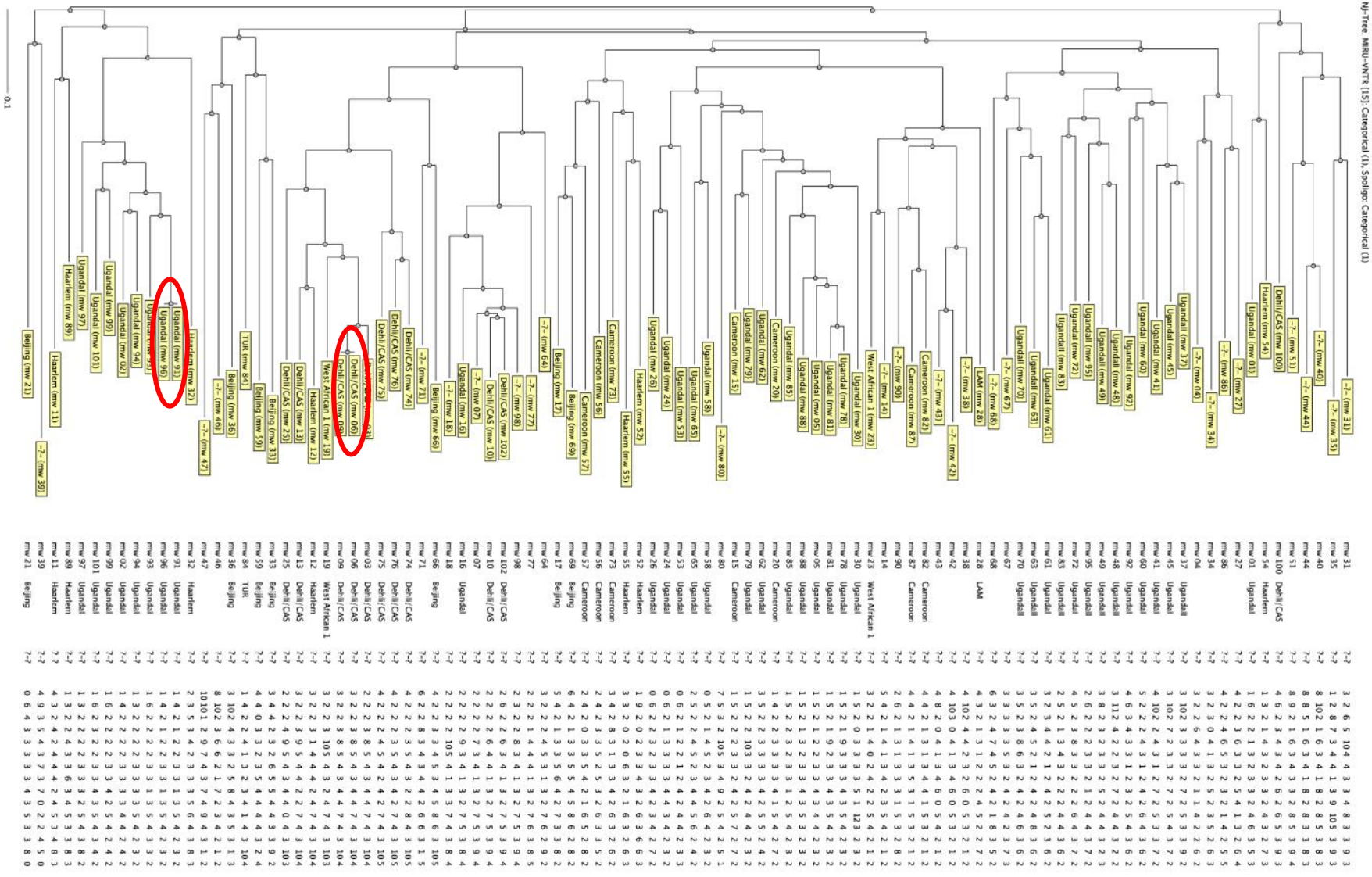

Figure 10: The MIRU-VNTR*Plus* dendrogram of the MDR-TB strains in archived TB isolates in Kampala; encircled ate the "twin" clusters

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 synergies 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-VNTR*Plus,* a clustering rate of 2% involving UgandaI 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-Rodríguez *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-VNTR*Plus* 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-VNTR*Plus* 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.

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