CHARACTERIZATION OF *TUBERCULOSIS* AMONG NEWLY DIAGNOSED *TUBERCULOSIS* AND COMORBID *TUBERCULOSIS*- DIABETES PATIENTS RESIDING IN NAIROBI AND KIAMBU COUNTIES, KENYA

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Characterization of *Tuberculosis* among Newly Diagnosed *Tuberculosis* and Comorbid *Tuberculosis*- Diabetes Patients Residing in Nairobi and Kiambu Counties, Kenya

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DECLARATION

This Thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

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LIST OF ABBREVIATIONS AND ACRONYMS

AI	Artificial Intelligence
BMI	Body Mass Index
BUN	Blood Urea Nitrogen
C	Cure
CART	Classification and regression tree
CAS	Central and Middle East Asia
CI	Confidence interval
D	Died
DM	Diabetes Mellitus
DNA	Deoxyribonucleic acid
DOTS	Direct Observation Therapy short course
DR	Direct repeat
DRa	Direct repeat forward
DRb	Direct repeat reverse
EAI	East Asia Beijing
EDTA	Ethylenediaminetetraacetic Acid
F	Failure
FM	Florescence Microscopy
Glu	Glucose
HbA1c	Hyper glycosylated hemoglobin
HF	Healthy Facility
HIV	Human immunodeficiency virus

HPLC	High performance Liquid Chromatography
IGT	Impaired glucose tolerance
KNTP	Kenya National Tuberculosis Program
LAM	Latin America and Mediterranean
LJ	Lowenstein Jensen media
LMIC	Low and medium income country
MGIT	Mycobacteria growth indicator tube
MOTT	Mycobacteria other than Tuberculosis
MTBC	Mycobacteria Tuberculosis Complex
NaOH	Sodium Hydroxide
NC	Not Complete
NTLD	National Tuberculosis and Lung Disease
OOC	Out of Control (Loss to follow up)
PTB	Pulmonary Tuberculosis
RF	Random Forest
RGB	Random blood glucose
RH	Rifampicin, Isoniazid
RHE	Rifampicin, Isoniazid, Ethambutol
RHZE	Rifampicin, Isoniazid, Pyrazinamide, Ethambutol
ROC	Receiver operating characteristics
RR	Risk ratio
SRHZE	Streptomycin, Rifampicin, Isoniazid, Pyrazinamide, Ethambutol
TB	Tuberculosis

TB/DM	Tuberculosis-Diabetes
TC	Treatment complete
TO	Transfer out
WHO	World health organization
ZN	Ziehl-Neelsen

DEFINITION OF OPERATIONAL TERMS

CART	Classification and Regression Tree is a decision tree commonly used in data mining with creating a model that predicts the value of a target (dependent variable) based on the values of several inputs (independent) that are associated with high exposure, risk or both.
Diabetes Patient	A patient who has high blood glucose (blood sugar), either because insulin production is inadequate or because the body cells do not respond appropriately to insulin
Gestational Diabetes	Pregnant women who have never had diabetes before but who have high blood glucose (sugar) levels during pregnancy
Hyperglycemia:	Is a condition in which an excessive amount of glucose circulates in the blood plasma. An elevated level specifically of the sugar glucose in the blood that often leads to a diagnosis of Diabetes Mellitus
Insulin	A hormone that helps the body to use glucose for energy. The beta cells of the pancreas make insulin when the body cannot produce enough insulin.
Insulin Resistant	The body's inability to respond to and use the insulin it produces, resulting in obesity, hypertension and high levels of fat in the blood.
The Loss to follow	
Up	TB patients who did not start treatment or whose treatment was interrupted for two consecutive months or more and previously referred to as defaulters (out of control)
Machine learning	
Algorithm	Is an application of artificial intelligence (AI) that provides systems with the ability that it can receive data and use statistical analysis to predict an outcome
New TB Patient	TB patient who has never been treated for TB or have taken TB drugs less than a month

Not Evaluated	A TB patient for whom no treatment outcome is assigned. It includes “Transferred out” as another treatment unit as well as cases
Postprandial	blood glucose above the desirable level 1 to 2 hours after a person has eaten
Threshold	Is a value in an input domain below which the model output does not respond to changes in the input
Treatment Complete	TB patient who has completed the treatment course but has no bacteriological results or the treatment outcome
Treatment Failure	TB patient who is sputum smear or culture positive at five months or later after initiation of the Anti TB treatment
Transfer out	TB patient diagnosed and registered at the facility is transferred out to a nearer facility for treatment
Treatment Success	The sum of cured and treatment complete

ABSTRACT

The double burden of diabetes mellitus (DM) and pulmonary tuberculosis (TB) is a global health challenge. Its management differs from that of either disease alone. Tuberculosis treatment regimens (Rifampicin and Isoniazid) interact with oral anti-diabetic drugs resulting in suboptimal glycemic control. Also, the suboptimal control of diabetes predisposes the patient to tuberculosis and poor response to anti-TB treatment. Diabetes Mellitus and Tuberculosis, therefore, interact with each other at multiple levels, each exacerbating the other. The objective of this study are to determine the burden of diabetes mellitus among newly diagnosed tuberculosis patients. The study was carried out in Kiambu and Nairobi counties in Kenya between February 2014 and August 2015. Patients above 15 yeas who tested positive for *Mycobacterium Tuberculosis* complex on sputum smear microscopy at the time of diagnosis were eligible to participate. We obtained clinical and social demographic data from a semi-structured questionnaire by abstracting patients' medical records from the National TB program database at 2, 3, 5, and 6 months of treatment therapy monitoring. The study enrolled 347 patients. We dropped 7/347 patients that had *Mycobacterium Bovis* from the analysis. The remaining 340 patients: 84% were cured, 7% completed therapy, and 9% had unfavourable outcomes, out of which 4% (n= 32) had a microbiologic failure. DM prevalence (HbA1c > 6%) among TB infected patients was 37.2%. The number of cigarettes smoked per day, and the value of the BUN were significant risk factors for developing DM among TB patients (P values = 0.045). Of the seven identified TB strains within the two counties, East Asia, Beijing, Euro America, and Indo Oceanic were dominant, accounting for 92.4% of infections. DM was not a significant factor in increasing the likelihood of PTB patients to cluster according to the genotype of the infecting M. tuberculosis bacillus. The Classification and Regression Tree (CART) identified the three HbAIc cut off levels depicting the U shaped pattern that interacted with both weight and BMI. The entire cohort revealed that 8/11 (73%) of the patient with >2.95%, 111/114 (97%) with HbA1c 2.95-4.55%, and >4.55% containing 189/215 (88%) of patients who experienced microbiological failure. It is important to screen all newly diagnosed tuberculosis patients for diabetes mellitus as they require close monitoring for the control of both diseases, and health policies should be considered to assess the impact of DM. The study findings demonstrate the feasibility and the value of screening TB patients for DM in a predominantly health facility. The findings from this study will also be useful for national scale-up using simple diagnostic technology in place at the start of screening activities and that it needs to be a high priority area for implementation as a routine in all primary caregivers.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Tuberculosis (TB) remains a major global health problem, with an estimated 10.0 million people who got infected with TB globally in 2018; of whom 89% were adults, most of them males (57%). Out of this, there were approximately 1.2 million deaths among HIV uninfected and 251,000 deaths among HIV-positive (WHO, 2019).

Diabetes mellitus (DM) is a chronic metabolic disease accompanied by impairment of immune function. Globally, an estimated 463 million adults between the ages of 20-79 were living with diabetes in 2019, representing 9.3% of the global population from this age group. This is projected to increase to 578.4 million adults living with diabetes in the world by 2030 and 700.2 million by 2045. About 4.2 million people died due to diabetes-related causes in 2018. 79.4% of those living with diabetes come from low- and middle-income countries where the burden of tuberculosis is also high (IDF, 2019). It is, therefore, important to address the public health challenges resulting from DM and TB comorbidity (Kelly E Dooley & Richard E Chaisson, 2009).

High-risk groups undermine the success of TB programs in reducing the disease burden, notwithstanding targeted interventions. Diabetes Mellitus (DM), triples the chances of developing active TB (Christie Y Jeon & Murray, 2008). Moreover, Prediabetes (pre-DM) is also a risk factor for developing TB (Almeida-Junior et al., 2016; Shivakumar et al., 2018) .

Despite those indications, most TB infected individuals are unaware of their DM or pre-DM status. TB screening among DM patients is essential for early diagnosis and better outcomes (Anil Kapur & Anthony D Harries, 2013). There is no data on screening among DM patients within intermediate TB burden countries, and the most appropriate time point for TB screening is still unclear (D. Kumar et al., 2010)

Available DM screening tests such as HbA1c, fasting glucose, and oral glucose tolerance have variable sensitivity with HbA1c detecting more people with DM and pre-DM than others(D. Kumar et al., 2010).

1.2 Tuberculosis and Diabetes Mellitus type 2

Tuberculosis (TB) remains the leading cause of morbidity and mortality in low and medium-income countries, which are similarly experiencing an upsurge in diabetes mellitus (DM) cases (Bailey & Grant, 2011; Rodríguez-Rodríguez, Ruy-Díaz-Reynoso, & Vázquez-López, 2015)). Infectious and chronic disease co-morbidity is often due to mutual risk factors as well as direct interaction (Bailey & Grant, 2011; Rodríguez-Rodríguez et al., 2015) as seen in the double burden of DM and pulmonary TB (A. Kapur & A. D. Harries, 2013; Zheng, Hu, & Gao, 2017). *Tuberculosis* (TB) and Diabetes Mellitus (DM) co-morbidity have been well documented in low and medium-income countries (LMIC), accounting for 95% and 75% of TB, and DM cases, respectively (Zheng et al., 2017). This rising DM epidemic in LMICs already burdened with TB may threaten to reverse some of the gains made by TB control programs (A. Kapur & A. D. Harries, 2013). HIV, malnutrition, alcoholism, smoking, and Diabetes Mellitus (DM) exacerbate this syndemic relationship as a risk factor for TB (Baker et al., 2011).

In 2014, 1.5 million people died from TB, and another 9.6 million are speculated to have fallen sick (WHO, 2015a). Out of this, one million had TB and DM, referred to as TB/DM (Lönnroth *et al.*, 2014). Worldwide, TB/DM co-morbidity burden was estimated to be higher than TB-HIV co-infection in 2010 (Ruslami *et al.*, 2010). The incidence of TB was reported highest among people with impaired immunity, HIV infection, or diabetes (Sen *et al.*, 2009). Prevalence studies from different parts of the world indicate that 12 - 44% of TB disease is associated with DM (Viswanathan *et al.*, 2012).

In Africa, DM prevalence among TB patients also varies significantly between studies ranging from 3.35–16.4% (Workneh *et al.*, 2016), depending on immune status, age (older than 45), overweight, and being male (Kibirige *et al.*, 2013). Despite the collaborative framework for care and control by WHO guidelines on

TB/DM co-morbidity management (WHO 2011), most sub-Sahara African countries still lag in screening all TB patients seeking care for DM (Castellanos-Joya *et al.,* 2014; Harries et *al.,* 2015).

Kenya is among the top 22 countries in the world with the highest burden of TB in the world. In 2016, Kenya had an incidence of 348 cases per 100,000 population among the Pulmonary *Tuberculosis* (PTB) patients and a mortality rate of 22/100,000 (Enos et al., 2018), while the National Tuberculosis, Leprosy, and Lung Program (NLTD) had a point prevalence of 558 per 100,000, according to the 2016 prevalence survey.. Reports indicate higher rates of non-communicable resultant deaths, indicating a shortage of data or underestimation of the disease burden and, consequently, TB/DM co-morbidity worldwide and Kenya (Enos et al., 2018).

1.2.1 Treatment options among newly diagnosed TB and TB/DM Patients

National TB program recommends DM screening among TB patients and verse versa Kenya. There is, however, a shortage of data on the performance of specific TB tests in individuals with DM, specific DM tests among TB patients, and screening and preventive therapy for latent TB infections in individuals with DM. Some of these results from weak data management systems in health facilities.

Studies indicate that TB patients with DM have a lower concentration of TB drugs and a higher risk of drug toxicity than those without (Christie Y Jeon & Murray, 2008). Besides, good glycemic control, which reduces long-term DM complications and could also improve TB treatment outcomes, is hampered by chronic inflammation, drug-drug interactions, suboptimum adherence to drug treatments, and other factors (Alsultan & Peloquin, 2014; Christie Y Jeon & Murray, 2008) . Besides drug treatments for TB and DM, other interventions, such as education, intensive monitoring of TB therapy, and lifestyle interventions, might also be needed, especially for patients with newly diagnosed TB or those who require insulin.

From a health systems point of view, delivery of optimum care and integration of services for tuberculosis and diabetes is a massive challenge in many countries, more specifically limited to the medium-income setting (Alsultan & Peloquin, 2014) .

1.2.2 Prognostic factors among newly diagnosed TB and TBDM Patients

Diabetes Mellitus (DM) screening among TB patients is now widely recommended, especially in setups with high DM prevalence (Alsultan *et al.,* 2014). A case example is India and the Pacific Islands region's TB control programs (Azman *et al.,* 2014). Despite these efforts, the best time and methods to diagnose for DM diagnosis among TB patients remains unclear (Balakrishnan *et al.,* 2012). In one of the observational studies, glycemic control status influenced the radiological manifestations of diabetic pulmonary TB (Bruins *et al.,* 2013). DM prevalence rises with age, but the most efficient age cut-off for screening is also unclear among populations (Barba *et al.,* 2004). Blood glucose concentration measurement at a single time point might lead to a false diagnosis of DM in patients with TB because they could have intermittent hyperglycemia through induction of insulin resistance, mediated by inflammation in adipose tissue, due to TB (Kaona *et al.,* 2004). Repeat testing could identify transient hyperglycemia. Glycated Hemoglobin (HbA_{1c}) concentration assessment is the only diabetes test that shows the average glycaemia over time (Borg *et al.,* 2011). In a single study, HbA_{1c} was more sensitive than fasting blood glucose when used as a screening test for newly diagnosed diabetes in patients with tuberculosis (Borg *et al.,* 2011).

1.2.2.1 Treatment outcome and Risk Factors

TB is the ninth foremost reason of death worldwide and the leading cause of a single infectious agent, ranking above HIV/AIDS (WHO, 2017). Overall, a relatively small proportion (5–15%) of the people infected with Mycobacterium tuberculosis (MTB) will develop TB disease during their lifetime. However, the probability of developing TB disease is much higher among people infected with HIV and also higher among people affected by risk factors such as under-nutrition, diabetes, smoking, and alcohol consumption (WHO, 2017).

Pulmonary TB (PTB), the most common type of TB, has great epidemiological significance due to its extremely contagious nature (Baker et al., 2011) . Those with PTB vary among countries and depend on associated diseases, geographical, social, ethnic, and economic status (Sunnetcioglu et al., 2015) . Diabetes Mellitus (DM) is a

risk factor for TB and although immune deficiency also occurs with diabetes, little is known about the epidemiological or clinical relationship between diabetes and TB (Sulaiman et al., 2013).

The association between these diseases is of greater importance for populations with low socioeconomic status and high prevalence rates of both conditions. As a result of globalization and high migration rates, detecting patients with DM and who are at risk for active TB is a global public health challenge (Schwartzman *et al.*, 2005). Other risk factors for comorbidity of active TB in patients with DM include low body weight, poor glycemic control and nutritional deficiencies (Leung *et al.*, 2007)

1.2.3 Molecular Clustering among newly diagnosed TB and TBDM Patients

Some studies have also evaluated the burden and relationships between Diabetes mellitus (DM) and TB. Socio-epidemiological data, molecular strain typing of TB, together with conventional epidemiologic methods, have previously helped to characterize TB strains and understand the dynamics and patterns of its transmission in different regions and populations (Jagielski *et al.*, 2014). Patients with identical strains of TB are considered to belong to one cluster resulting from the recent transmission and rapid progression in molecular characterization. Unique patterns of strain characterization result mainly from reactivation of latent infection or recent transmission from patients out of the period or area under study (Kamerbeek *et al.*, 1997).

Variation in individual, biological and, social determinants is associated with Pulmonary TB (PTB) case clustering. This includes being male, young, country of birth, a resident of an urban area, alcohol or drug consumption, homelessness, HIV infection or having acid-fast bacilli in sputum smear (Abebe *et al.*, 2011). Despite numerous studies on the association of HIV and increased risk of TB transmission within the sub-Saharan region, few have investigated if DM increases the likelihood of Pulmonary Tuberculosis (PTB) patients to cluster according to the genotype of the infecting *M. tuberculosis* bacillus. There is a dearth of data addressing this question and it remains unclear whether patients with DM and TB are more likely than TB patients without DM to be grouped into molecular clusters defined according to the

genotype of infecting *TB* strain. Specifically, the question is whether there is convincing molecular epidemiological evidence for TB transmission among DM patients and whether *Mycobacterium Tuberculosis* (MTB) lineage impacts the clinical outcome of a patient on treatment.

1.2.4 Predictive of poor treatment outcome among newly diagnosed TB and TBDM Patients

Glycated hemoglobin (HbA_{1c}) is a measure of the beta-N-1-deoxy fructosyl component of hemoglobin. This component is obtained during hemoglobin type A, separation on chromatography. The first to separate, probably considered to be pure Hemoglobin A, while others were designated as HbA_0, HbA_{1a}, HbA_{1b}, and HbA_{1c}, respective of their order of elution or cation exchange chromatography (Miedema, 2005). When blood glucose levels are high, glucose molecules attach to the hemoglobin in red blood cells. The longer hyperglycemia occurs in blood, the more glucose binds to hemoglobin in the red blood cells and the higher the glycated hemoglobin. Once a hemoglobin molecule is glycated, it remains that way. Once there is glycated hemoglobin build up within the red cell, it reflects the increase or the presence of glucose level to which the cell has been exposed during its lifecycle process. Measuring glycated hemoglobin assesses the effectiveness of therapy by monitoring long-term serum glucose regulation or even diagnosis in itself, (Miedema, 2005).

There have subsequently been many more sub fractions as separation techniques/processes are improved. Normal levels of glucose produce a normal amount of glycated hemoglobin. As the average amount of plasma glucose increases, the fraction of glycated hemoglobin increases predictably. This serves as an indicator of long term glycemic control continues to grow, (Peterson *et al.*, 1998). Though HbA_{1c} concentration assessment has been indicated as the preferred test method for DM among TB infected patients, hyperglycemia levels predictive of poor clinical outcomes among patients with both DM and TB, for which clinicians must target for optimal care, is not well defined (Kaona *et al.*, 2004).

It has been shown that experimental, hollow-fibre models of tuberculosis chemotherapy in TB patients have shown that pharmacokinetic variability which directly leads to failure of TB therapy, (Srivastava *et al.,* 2011). Moreover, rifampin is known to exacerbate hyperglycemia in patients starting TB therapy, suggesting that therapy in patients at risk of TB can be optimized and personalized, (Srivastava, Pasipanodya, et al., 2011).

On the other hand, artificial intelligence (AI) algorithms especially machine learning has been able to unravel the relationship between weight and Pharmacokinetics, Pharmacokinetics and TB outcomes and even changes in maturation in drug metabolism, (Jeena *et al.,* 2011). These can be either linear or nonlinear interactions. Such algorithm classification and regression tree (CART), is impervious to conventional statistical assumptions and is more adept at a data-driven pattern recognition selecting predictors and their thresholds based on reproducible models, (Pasipanodya *et al* ., 2011).

1.3 Statement of the Problem

The relationship between Tuberculosis (TB) and diabetes mellitus (DM) are important health challenges and additional problems in the TB program. Tuberculosis is a major cause of morbidity and mortality in Kenya. Some reports indicate that Kenya has a high rising TB disease burden and was ranked 15th out of the 22 high burden countries (WHO, 2016). Several studies have shown there is syndemic interaction between DM and TB and that each disease exacerbates and worsens the clinical outcome for the other (Christie Y Jeon & Murray, 2008). Non-communicable diseases and diabetes in particular already account for a substantial share of the disease burden in the country and are likely to increase further as the country progresses in controlling infectious diseases and thus consequently changing the population age structure.

It has further been shown that although TB is more strongly associated with immune deficiency diseases such as HIV, DM remains a more significant risk factor because at the population level the number of people with diabetes is greater than that of patients with other immune-compromised states (B. I. Restrepo et al., 2007). It is

noteworthy that the convergence of these two diseases may be complicated by the emergence of drug-resistant TB strains, which is causing a major threat to the TB control program. It is therefore of utmost importance to know the prevalence of TB/DM cases to provide proper control and treatment intervention.

American Diabetes association recorded 775,210 DM cases in 2014 with 582,000 cases remaining as undiagnosed (ADA, 2014). Most of these patients present late and may not get the standard of care they need, leading to high morbidity and mortality. Nairobi contributes the highest number of TB on case notification in Kenya. It's a cosmopolitan, interacts with Kiambu, and thus interesting as they share the same environmental status.

The true burden of type 2 Diabetes mellitus among newly diagnosed TB patients in Kenya is unknown and the data is scarce. The relationship between the merging epidemics of *tuberculosis* (TB) and diabetes mellitus (DM) is raising concerns due to their impact on health outcomes in the control of both conditions in the country and globally. The synergistic interaction of co-morbidities Tuberculosis and Diabetes Mellitus may affect treatment outcomes due to the multiplication of the disease burden. The pathways through which Tuberculosis and Diabetes Mellitus interact in the body within populations to allow multiplication of adverse health effects are not characterized. Understanding whether and why DM increases the probability of *Tuberculosis* patients to cluster according to genotype in the country would guide in developing disease control strategies.

1.4 Justification of the study

Diabetes Mellitus triples the risk for active *tuberculosis,* particularly among the impoverished, thereby demonstrating a syndemic interaction. This necessitates Diabetes mellitus screening among newly diagnosed TB patients as they may increase the risk of adverse treatment outcomes in TB patients. Primary and secondary prevention of diabetes will help prevent TB at the population level. Besides, TB preventive therapy could potentially be indicated in people with diabetes who have had recent exposure to TB. There is thus a need for sound public health measures as rising prevalence of DM in TB-endemic areas may adversely affect TB

transmission dynamics and control. As a result of the public health implication of a causal link between TB and DM, there is a clear need for a systematic assessment of the syndemic association of *Tuberculosis* and Diabetes association especially in areas where both diseases are endemic.

The relationship between diabetes and TB among patients has additional challenges in control of TB. In Kenya, TB/DM comorbidity data is scarce despite the way knowledge about the way TB-DM affects each other is important in identifying and theorizing the pathways of disease co-morbidity. Little is known on the social demographic and clinical characteristics of patients. The strains of *Mycobacterium Tuberculosis (MTB)* among these patients is also unknown. There is a need for a systematic assessment of the TB-DM association especially in areas where both diseases are endemic. This will reveal the importance of TB-DM clustering within the population.

In Kenya, TB/DM comorbidity data is scarce. Information derived from this study in identification for the novel public health research will: Contribute to the development of effective preventive strategies for TB transmission. Inform policymakers on the merits and demerits of screening newly diagnosed TB patients before TB treatment at the health facility

1.5 Study Rationale

In 2014, WHO estimated that 422 million people were living with diabetes (WHO, 2016) , 7 million among them developed the disease during that year with 3.9 million deaths attributed to diabetes (Shaw, Sicree, & Zimmet, 2010). It is predicted that the prevalence of diabetes will reach 522 million people by 2030, with 80% of prevalent cases occurring in the developing world (WHO, 2016). Tuberculosis (TB) remains a considerable global public health concern, mainly affecting poor and vulnerable populations (Knut Lönnroth et al., 2010). Every year, more than 9 million people fall ill with this infectious disease, and close to 2 million die from it (Knut Lönnroth et al., 2010). Diabetes, which is increasing globally, is associated with higher risks of TB (Christie Y Jeon & Murray, 2008) and adverse TB treatment outcomes (Baker et al., 2011) . The increase in the number of people with diabetes may further

complicate care and control of TB, especially in the many areas with a high burden of both diseases (Baker et al., 2011). In Kenya, the rising burden of diabetes mellitus (DM) remains largely neglected as the country is still struggling with the infectious disease, TB. It's estimated that in 2014 about 775, 210 DM cases were recorded in Kenya with 582,000 people with DM remaining undiagnosed (ADA, 2014). Therefore the rationale of the study was to assess the association of Diabetes Mellitus (DM) and TB in selected areas of Kenya to formulate appropriate interventions for the two diseases.

1.5 Hypotheses

1. Does Diabetes Mellitus increase the probability of *Tuberculosis* patients to cluster according to the genotype of the infecting *Mycobacterium Tuberculosis?*

1.6 Objectives

1.6.1 General Objectives

To characterize Mycobacterium *Tuberculosis* among newly diagnosed *Tuberculosis and comorbid Tuberculosi*s - diabetes residing in Nairobi and Kiambu counties, Kenya.

1.6.2 Specific Objectives

1. To describe the social demographic and clinical characteristics of patients with TB and TB/DM co-morbidity among the newly diagnosed TB patients
2. To determine prognostic factors among TB and TB/DM comorbidity among newly diagnosed TB patients on short-course regimen
3. To determine the Molecular clustering of Patients with MTB strains cultured from the diabetic and non-diabetic newly diagnosed TB positive cases.
4. To describe hemoglobin $A1_C$ ($HbA1_c$) cutoff thresholds predictive of poor tuberculosis treatment outcome and risk factors by use of a CART

CHAPTER TWO

LITERATURE REVIEW

2.1 Mycobacterium tuberculosis

Tuberculosis (TB) remains one of the most infectious diseases in the world. It is caused by several types of *Mycobacterium* bacteria species, mainly the *Mycobacterium Tuberculosis* Complex (MTBC) (Kumar, 2007). The disease usually affects the lungs (pulmonary tuberculosis (PTB)), but it can also affect other parts of the body (extra pulmonary TB (EPTB). The bacteria is airborne, transmitted through aerosols, and spreads from one person to the next when an infected person coughs, sneezes, or spits.

When people with active pulmonary TB cough, sneeze, speak, sing, or spit, they expel infectious aerosol droplets of 0.5 to 5.0 μm in diameter. A single sneeze can release up to 40,000 droplets. Each one of these droplets may transmit the M*ycobacterium Tuberculosis* bacteria since the infectious dose of tuberculosis is tiny (inhalation of fewer than ten bacteria may cause an infection). Those with prolonged, frequent, or close contact people with TB bacteria are particularly at high risk of becoming infected (Ochei, Obeagu, Emmanuel, Ndidiamaka, & Ifeoma Stella, 2018), with an estimated 22% infection rate. A person with active but untreated tuberculosis may infect 10 -15 or more other people per year (Ahmad, 2011).

Only about 5–10% of individuals infected with *Mycobacterium Tuberculosis* (MTB) progress to active TB disease (WHO, 2015). Houben and Dodd (2016) estimated that by 2014 about 1.7 billion people had latent TB infection or LTBI in the world. This is just under a quarter of the world's population driving the disease as they retain significant risk of disease progression to TB by reactivation of latent TB when immunocompromised (Ronacher *et al.*, 2015).

The leukocytes of the Primary human cells strongly indicate that innate immune responses play a significant role in determining the outcome of TB by helping control bacterial load and through shaping the nature and magnitude of adaptive immune responses (Russell, Cardona, Kim, Allain, & Altare, 2009). The innate antimicrobial pathways that are activated early and throughout infection play a role in limiting disease. Moreover, MTB has evolved multiple strategies to modulate innate immune responses and prevent optimal activation of adaptive immunity. Thus, understanding between innate and adaptive immune cells in human TB is critical for identifying novel targets of immunomodulatory therapies and for elucidating mechanisms of protective immunity (Philips & Ernst, 2012).

Transmission occurs from people with active TB, and those with latent infection not thought to be infectious (S. G. Kumar *et al.,* 2015). The probability of transmission from one person to another depends on several factors such as the number of infectious droplets expelled by the carrier, the effectiveness of ventilation, the duration of exposure, the virulence of the *M. tuberculosis* strain and the level of immunity in the uninfected person, and others as defined by increased transmissibility as well as being associated with higher morbidity and mortality in infected individuals (Ochei *et al.,* 2018).

For several decades, the research community has been working on developing effective preventive measures/strategies for TB control and intervention. It is clear that although the current preventive efforts against the spread of TB have lowered its incidence, TB elimination is still unsolved (Glaziou, Sismanidis, Floyd, & Raviglione, 2014). As a result, the focus of research has now shifted to the previously untargeted risk factors involved in the spread of TB, which includes Diabetes mellitus (DM). Diabetes Mellitus is known to impair the immunity of patients and therefore is an independent risk factor for infections such as TB (Blanca I. Restrepo, 2016).

2.1.1 *Tuberculosis* bacterium and the immune system

When the Tuberculosis bacterium infects a person, the body's immune response is critical to how the disease will progress. This is either helping the body fight the bacterium or, if key molecules become involved, actually exacerbating infection and how the TB bacterium co-opts mechanisms of the immune systems of its advantage. The body fights tuberculosis through the Inflammasome, interleukins, interferon (Wassermann *et al.,* 2015).

When *M. tuberculosis* infects a person, it attacks the lungs' first-response immune cells, the macrophages. The immune response by the macrophages involves a complex of four different proteins called the "inflammasome." The main role of the inflammasome is to prepare certain immunity proteins in the macrophages, which are called "interleukins." When *M. tuberculosis* infects the lungs, interleukins from the macrophages are in the first line of defense (Wassermann *et al.,* 2015).

If this is left uncontrolled, this defense can also cause serious damage to the patient. To prevent this, macrophages also release another group of proteins called "type I interferon." While interferon is important for defending the body against viruses, when it comes to tuberculosis, they help the bacterium, thereby exacerbating the disease. And although the interleukin-inflammation part of the immune response is rather well understood, the part involving Interferon’s has been elusive (Wassermann *et al.,* 2015).

M. tuberculosis carries out a subtle assault on the host’s immune defense. The key is a molecule called cGAS, which is found in the lung's macrophages, and is part of a group of DNA-sensor molecules; cGAS patrols the inside of macrophages, and when it detects unidentified pieces of DNA, such as those released by *M. tuberculosis*, it triggers an immune response from the macrophages (Wassermann *et al.,* 2015).

The tuberculosis bacterium uses a specialized secretion system to release its array of toxic proteins into macrophages. But, it also releases small bits of DNA, which are detected by sensing systems inside the macrophages, namely the inflammasome and cGAS. This causes macrophages to release two types of proteins: interleukin-1,

which fights the bacterium and type I interferon, which ends up helping it. What the bacterium DNA sensor cGAS is essential for is to signal the production of type 1interferon (IFN) induction in response to MTB infection, which reduces the immune response. In other words, the bacterium tricks the macrophages to cut back on their defense against it making it possible to manipulate *M. tuberculosis* in such a way that it can no longer activate the production of interferon's through cGAS, while still keeping the production of interleukin-1 and thus the body's immune response remains intact (Wassermann *et al.,* 2015).

2.2 Epidemiology of Tuberculosis and Diabetes

2.2.1 Historical Perspective of Tuberculosis

Evidence of TB was found in Egyptian mummies dating back to 1550 BC. Hippocrates identified TB and then referred to it as "phthisis pulmonaris" or "consumption" or "white plaques", as a widespread, usually fatal disease, causing high mortality. The syndemic association between TB and DM have been reported in studies dating back to 1000 A.D. (Morton, 1964; Wassermann *et al.,* 2015). Avicenna noted that phthisis (consumption due to wasting patients) often complicated diabetes and that the presence of diabetes resulted in an increased risk of developing TB (Blanca I. Restrepo, 2016). Another description, as seen in the works of Yugimahamuni, a traditional Indian saint showed that the symptoms of DM in TB included obesity, glycosuria, thirst, incontinence, respiratory symptoms, and unconsciousness (Rajalakshmi & Veluchamy, 1999). The introduction of insulin in the 1920s, the discovery of streptomycin in the 1940s, and the subsequent development of other antibiotics substantially lowered case fatality rates for individuals with diabetes mellitus or tuberculosis (Rajalakshmi *et al.,* 1999).

2.2.2 Global Tuberculosis Epidemic

In contrast to DM, tuberculosis (TB) incidence at the global level has been decreasing by an average of 1.5% per year since 2000 (Zheng *et al.,* 2017). However, TB remains a major global health problem causing ill-health among millions of

people each year and ranks ninth among the leading causes of death from infectious diseases, and above HIV worldwide (WHO, 2019).

Africa accounted for 24% of all tuberculosis cases in 2018 (WHO, 2019). Kenya is among the 30 high-burden tuberculosis (TB) countries in the world using incidence per 100,000 population and also 15th among the high burden countries in the world (WHO, 2019). The current estimated epidemiological burden of TB in Kenya stands at 558 (95%CI 455-662) per 100,000 populations (Enos *et al.,* 2018; MoH, 2016). In addition to the TB Challenges, there is an even more unsettling problem of Multidrug-resistant TB (MDR-TB) (Figure 2.2 and 2.3).

The statistics, along with TB through comorbidities such as HIV and DM, jeopardize the global objective of ending TB by 2030 and achieving its elimination by 2050 (WHO, 2015). The Incidence of tuberculosis is greatest among those with conditions that impair immunity, such as HIV infection and diabetes (Corbett *et al.*, 2003). Kenya is among the 14 countries globally that are on all three of the new high TB burden country lists for 2016-2020 (TB, MDR-TB, TB /HIV).

2.2.3 Global Diabetes Epidemic

Globally, an estimated 463 million adults between the ages of 20-79 were living with diabetes in 2019, representing 9.3% of the global population from this age group. This is projected to increase to 578.4 million adults living with diabetes in the world by 2030 and 700.2 million by 2045. About 4.2 million people died due to diabetes-related causes in 2018. 79.4% of those living with diabetes come from low- and middle-income countries where the burden of tuberculosis is also high (IDF, 2019).

Sub-Saharan Africa, like the rest of the world, is experiencing an increasing prevalence of diabetes alongside other non-communicable diseases (Shaw et al., 2010). In 2019, approximately 19 million people were estimated to be living with diabetes in Africa, and this is projected to increase to 29 million by 2030 (Shaw *et al.,* 2010).

In Kenya, the prevalence of diabetes is estimated to be about 3.3% but this may be an underestimation as over 60% of people who are diagnosed with diabetes in the country present to facilities for other unrelated complications but end up diagnosed with diabetes (MoPHS, 2010). This prevalence is projected to rise to 4.5% by 2025 (MoPHS, 2010). It was further reported that Kenya is among six African countries with the highest number of diabetes patients with 1.8 million people living with the disease, with a prevalence of 3.3% (Hall, Thomsen, Henriksen, & Lohse, 2011)

2.2.4 Aetiology of Association of *Tuberculosis* and Diabetes Mellitus

Infectious and chronic disease co-morbidity is often due to mutual risk factors as well as direct interaction (Young, Critchley, Johnstone, & Unwin, 2009). DM has recently shown an alarming increase worldwide and there is growing evidence indicating that DM affects TB disease presentation and treatment (Kelly E. Dooley & Richard E. Chaisson, 2009). Multiple concepts have been put forward to explain the causal connection between diabetes and tuberculosis which attests to the uncertainty in this respect (Yorke et al., 2017).

The dominant manifestation of diabetes mellitus, namely hyperglycemia has been assumed to favor the growth, viability, and propagation of tubercle bacilli which increases the incidence of pulmonary tuberculosis in diabetics (Mboussa et al., 2003; Yorke et al., 2017). The immune disorganization involves the cell-mediated arm of the immune system (Lee et al., 2016). Alterations in cytokines, monocyte-macrophages, and CD4/CD8 T cell populations are due to infection with tubercle bacilli. Cluster differentiation 4 (CD4) and CD8, subsets of the T lymphocyte, play the main role in the modulation of host defenses against Mycobacteria and have a profound influence on the rate of regression of active pulmonary tuberculosis (Lee et al., 2016). Biologically, diabetes creates a more favorable environment for the conversion of TB to active disease. Simply stated, bacteria love sugar, and hyperglycemia provides a rich medium for the growth of the bacilli.

Diabetes also affects immune system metabolism increasing interleukin10 and cytokines and decreasing T-cell lymphocytes in ways that make it more difficult to kill *M. tuberculosis* bacilli. The reduced T-lymphocyte count as well as function and

a low neutrophil count decrease cellular immunity (Meigs, 2010). Diabetics show a reduced T-helper 1 (TH 1) cytokine response level, tumor necrosis factor (TNF-alpha and TNF-beta), interleukin-1, and interleukin-6 production compared to their non-diabetic TB counterparts. The susceptibility of diabetic patients to TB is mainly due to reduced numbers and function of T-lymphocytes, which could be due to macrophage dysfunction in diabetes, or impaired production of reactive oxygen species and phagocytic and chemotactic function (Yorke et al., 2017).

Chemotaxis of monocytes is also impaired in patients with diabetes, a defect that does not improve with insulin. Therefore, DM deteriorates cell-mediated immunity by altering the function and activation of macrophages, monocytes, and lymphocytes. Patients with sustained hyperglycemia seem to be a higher risk of acquiring TB than those with controlled blood sugar levels suggesting that hyperglycemia is an important determinant in this interaction (Blanca I. Restrepo & Schlesinger, 2014). A combination of these dysfunctional processes contributes to an increased risk of TB in diabetes.

The stress response to infection may also play a role in dysglycemia, a situation mediated by the effect of interleukin-1 (IL-1), interleukin-6 (IL-6), and TNF-alpha (Niazi & Kalra, 2012). The immune responses in DM cases showing the alterations in humoral innate immune response mediated through a change in cytokine levels as shown below in Figure 2.1 adapted from (Pal, Ansari, Hameed, & Fatima, 2016). The mechanism involved in the enhancement of TB infection during DM is as presented in Figure 2.1 below.

During MTB infection the innate host immunity activates the macrophages, which causes phagocytosis and activates cytokines.

Under the non-diabetic condition, cytokines become activated leading to reactive oxygen species (ROS) generation subsequently killing MTB.

Under the diabetic condition, due to immunocompromised state, the alteration in cytokine release leads to the survival of MTB due to suppressed immunity.

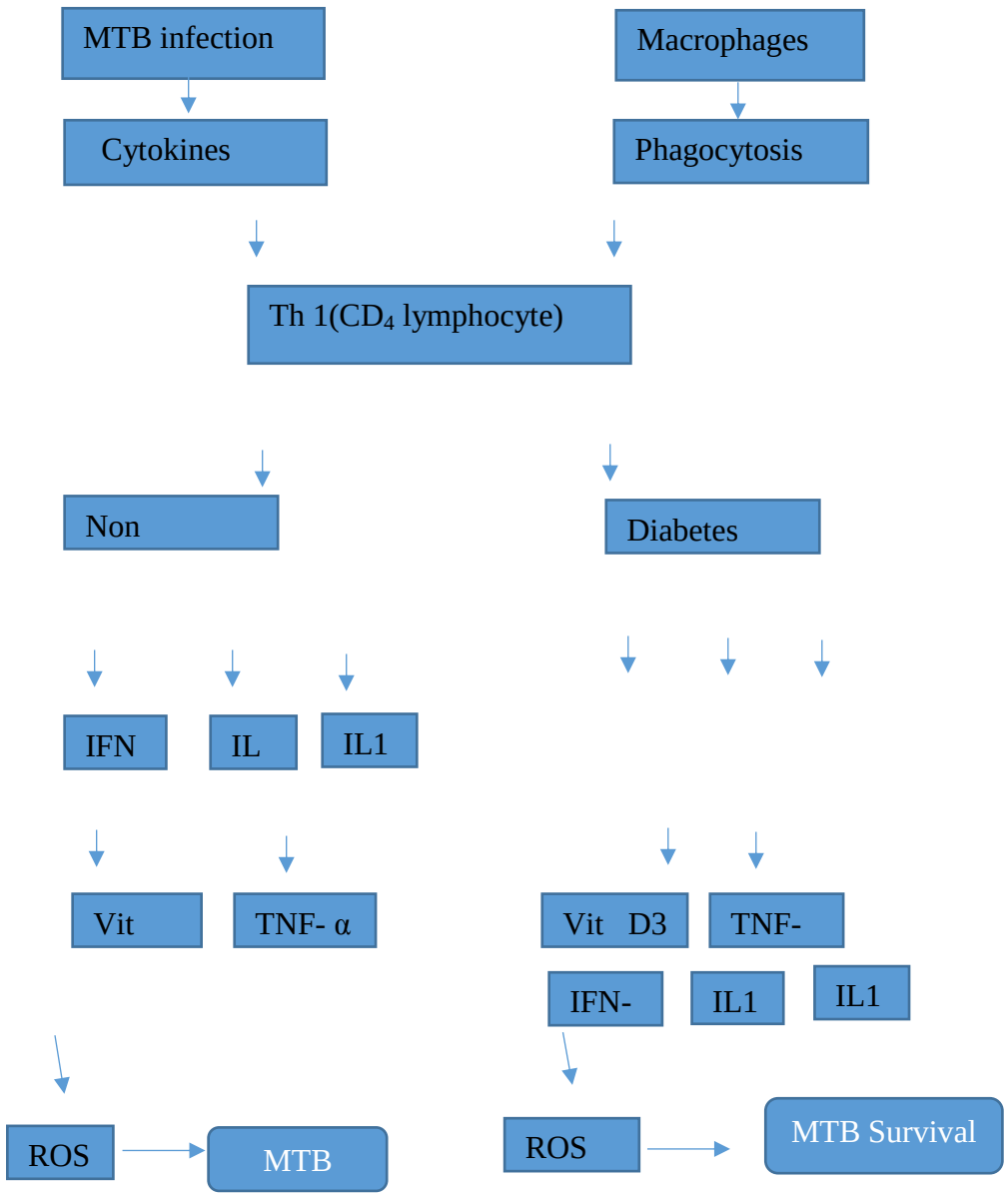

Figure 2.1: Mechanism involving TB infection during DM

Source:(Pal et al., 2016)

Diabetes can also lead to the reactivation of older foci of TB rather than through new contact, and often exhibit lower lobe involvement more commonly than in non-diabetics (Yorke *et al.*, 2017). Various studies have shown that 5-30% of patients with TB have DM as well (Rovina Ruslami *et al.*, 2010). Furthermore, for every 100 people with TB, between five and ten show symptoms of hyperglycemia (Foster *et al.*, 2015).

2.2.5 Tuberculosis as a risk factor for diabetes type 2

The relationship between DM and TB is bi-directional. Tuberculosis may lead to the development of new diabetes cases, and vice versa (Yorke *et al.*, 2017). Studies have shown a high prevalence of diabetes, as well as impaired glucose tolerance, in patients with tuberculosis (Christie Y. Jeon *et al.*, 2010). Impaired glucose tolerance is a significant risk factor for developing DM. In most of these cases, impaired glucose tolerance reverts to normal after successful treatment for TB; however, the increased risk of developing DM persists (Niazi & Kalra, 2012).

Active tuberculosis should be a differential diagnosis in patients with the enlarged pancreas. Tuberculosis (TB) is a known cause of pancreatitis and tuberculosis pancreatitis might reveal itself only after the development of diabetes (Niazi & Kalra, 2012). Even though hyperglycemia is associated with TB, it may be attributed to the severe stress associated with the infection itself, however, the major factor in this process is the hypo-function of the pancreas (Niazi & Kalra, 2012).

On the other hand, it has been shown that testing for DM in previously undiagnosed people before the appropriate treatment for TB may lead to an over-diagnosis of DM (Niazi & Kalra, 2012). Tuberculosis (TB) can lead to infection-related hyperglycemias, which may mimic DM. The hyperglycemia associated with TB often aggravates the glycemic control of diabetics and thus warrants an adjustment in the dose of insulin (Niazi & Kalra, 2012). The dose adjustment should be repeated after the patient has been successfully treated for TB.

2.2.6 Diabetes as a risk factor for Tuberculosis

Diabetes is an independent risk factor for all lower respiratory tract infections and its incidence has been increasing worldwide, especially in developing countries where TB is most prevalent (Zheng *et al.*, 2017). Therefore, the convergence of these two epidemics is most likely to occur in places with the least amount of healthcare resources.

Although a lot of emphases have been put on the interaction between TB and HIV not much emphasis has been in place on TB and DM patients yet those with DM are more than those with HIV (B. I. Restrepo *et al.,* 2007). A meta-analysis demonstrated that diabetes was associated with a relative risk of 3.1 of contracting TB (Christie Y Jeon & Murray, 2008). An Indonesian study has found that multi-drug resistant TB is associated with DM with an odds ratio of 2.1 (Alisjahbana *et al.,* 2007).

Although type 2 DM is more prevalent than type 1 DM, the latter carries a stronger risk of contracting TB than the former (Yorke *et al.,* 2017). Other risk factors for developing TB in people with type 1 DM include low body weight, young age, and poor glycemic control (Yorke *et al.,* 2017). It is not clear whether DM can affect the presentation of TB. However, patients with concomitant TB and DM may have a higher rate of fever and hemoptysis and atypical radiological images. Some studies have reported a higher frequency of cavities in the lungs, while others have reported a lower frequency in the lungs of people with diabetes as compared to non-diabetics with TB (Alisjahbana *et al.,* 2007).

Some studies have also reported a negative effect of DM on the treatment efficacy and prognosis of TB. However, results are conflicting and there is no clear evidence that DM affects the efficacy of treatment of TB (Alisjahbana et al., 2007; Yorke et al., 2017). Diabetes Mellitus also alters the pharmacokinetics of several anti-TB drugs. Since the efficacy of most anti-TB drugs depends on their plasma concentration, it may explain the negative effect of DM on the treatment of TB (Nijland *et al.,* 2006). The altered plasma levels may be due to differences in absorption, distribution, metabolism, and/or excretion in diabetics. Lower plasma levels of anti-TB drugs are associated with resistance to these drugs, which may complicate the course of treatment of TB in people with diabetes (Niazi & Kalra, 2012).

2.2.7 Management of Diabetes Mellitus (DM) Type 2 in Tuberculosis Patients

Management of both TB and DM patients should be administered and initiated immediately it is diagnosed. An optimal glycemic control results in a better patient's

health outcome (TB and TBDM), therefore, vigorous efforts should be made to achieve such control. Insulin therapy should be initiated at the outset, using a basal-bolus regime or premixed insulin (Rodbard *et al.,* 2009). The American Association of Clinical Endocrinologists (AACE) and the American College of Endocrinology (ACE) recommends the use of modern insulin or insulin analogs, as they are more predictable in action and cause less hypoglycemia. The use of traditional human insulin is discouraged (Rodbard *et al.,* 2009). Insulin requirements are high, to begin with, but fall after a few weeks once glucotoxicity is corrected and infection is controlled. Insulin requirements may rise again as appetite returns to normal and caloric intake increases. TB-DM patients should be tested for ketonuria (Niazi & Kalra, 2012). Rapid-acting analogs such as part insulin may obviate the need for admission in patients with ketonuria and are useful for critically ill patients as well (Niazi & Kalra, 2012).

An average patient will need 1.0 U/kg/day of insulin initially, divided as 60% bolus and 40% basal insulin. In a few weeks, the requirement comes down to 0.5 U/kg/day and maybe by two or three equal doses of premixed aspart/lispro(Niazi & Kalra, 2012). In patients with co-existing peripheral neuropathy due to diabetes, it is mandatory to give the patient pyridoxine if Isoniazid is to be used.

Oral hypoglycemic agents are contraindicated in severe tuberculosis but may be used with caution once the disease has settled. Rifampicin is a potent hepatic enzyme-inducer (Niazi & Kalra, 2012). It accelerates the metabolism of several oral hypoglycemic agents, especially sulphonylureas and biguanides, and lowers their plasma levels. Therefore, it may cause hyperglycemia in diabetic patients using these drugs. In non-diabetics, it augments the intestinal absorption of glucose and may simulate the symptoms of diabetes (Niazi & Kalra, 2012). Isoniazid, in contrast to rifampicin, inhibits the metabolism of oral hypoglycemic agents and may lead to an increase in the plasma levels of these drugs. Its main interaction is with Sulphonylureas, the action of which it antagonizes and worsens the glycemic control of diabetics on this medication. It also impairs the release and action of insulin leading to hyperglycemia even in non-diabetics (Lorenzati, Zucco, Miglietta, Lamberti, & Bruno, 2010). Therefore, the dosage of insulin should be adjusted to

these drugs from the patients' prescriptions. Dipeptidyl Protease inhibitors (the gliptins), a comparatively newer class of hypoglycemic agents, have a theoretical possibility of reducing immuno-competence because of their mechanism of action (Madsbad *et al.*, 2011). This effect could worsen the outcome of patients with TB.

Tuberculosis worsens glycemic control and makes control of diabetes difficult (Chiarelli & Capanna, 2004). Maintenance of blood sugar levels at a normal or near-normal level is one of the most fundamental aspects of patient care. Insulin is the preferred agent of choice in Diabetes with TB (of any type) due to its anabolic action apart from it lowering the pill burden, improving appetite, and promoting weight gain. Insulin has clear advantages; though one may shift to oral anti-diabetic agents during the maintained phase, insulin is still preferred, (Chiarelli & Capanna, 2004). It is logical though yet to be validated that the outcomes of TB therapy and relapses/ recurrences are cleared/altered by better glycemic control with insulin (Chiarelli & Capanna, 2004).

A systematic review by Shewade et al. (2017) found that among people with TBDM, there was a dearth of studies with minimal or no risk of bias for the effect of glycemic control and the effect of insulin (with/without OHA), when compared to the Oral hypoglycemic agent (OHAs) only, on TB treatment outcomes. Countries may continue to follow the existing guidelines of DM management among people with TB/DM. This also provides an opportunity for the national TB programs to, systematically record glycemic control and TB treatment outcomes.

2.2.8 Clinical Presentation of combined TB / DM

There is conflicting information about the influence of associated diabetes on the clinical features, radiological manifestations, sputum conversion rates, drug resistance patterns, and treatment outcomes of pulmonary tuberculosis patients (Niazi & Kalra, 2012). Experienced clinicians observe that patients with both diabetes and tuberculosis usually have a prolonged duration of fever and more significant weight loss with the co-existent disease than with diabetes or pulmonary tuberculosis alone (Abdul-Ghani, Tripathy, & DeFronzo, 2006). Several studies showed higher frequencies of certain clinical findings such as lower lung field

lesions, cavities, and acid-fast bacilli (AFB) smear positivity among patients with TB and DM (TBDM) comorbidity (Chang *et al.*, 2011).

2.2.9 Diabetes Mellitus Type 2 and *Tuberculosis* Treatment

In general, the clinical course of a patient with both TB and DM tends to be more severe due to factors like immunosuppression, different pharmacokinetics of anti-TB and DM drugs, and comorbidities that affect the clinical course. Treatment failure occurs in 4.8% of TB/DM patients compared with a failure rate of 1.5% in non-diabetic TB patients (Baker *et al.*, 2011). Furthermore, the TB relapse rate (20%) and multidrug-resistant TB frequency (17.7%) are statistically significantly higher in DM patients compared with non-DM (5.3% and 8.4%, respectively) (Baker *et al.*, 2011). Although the implementation of the Directly Observed Treatment Short-course (DOTs) strategy has been widely accepted in developing countries, there are still many uncertainties related to the optimum treatment strategies in patients with both TB and DM (Baker *et al.*, 2011).

Some of the most important problems are the length of anti-TB treatment, drug-drug interactions (e.g. rifampicin and oral sulphonylurea derivatives), drug-disease interactions (e.g. peripheral neuropathy induced by both isoniazid and DM), adherence to medication being compromised by high pill counts or adverse drug effects, and the lack of DM clinics designed to prevent easy *Mycobacterium tuberculosis* transmission (Sullivan & Amor, 2012).

2.3 Time to Sputum Conversion among smear-positive

Diabetics have up to five times higher risk of developing sputum positive pulmonary TB (Sen, Joshi, & Udwadia, 2009). Sputum smear and culture conversion are important in determining how infectious the patient is and the efficacy of the treatment. Patients, who respond, are likely to become smear and culture-negative during treatment. However, viable bacilli continue to be expelled for some time, during which they may continue to spread infection (Amare, Gelaw, Anagaw, & Gelaw, 2013).

It is expected that 80 to 90% of patients will undergo smear conversion within two to three months of treatment. Several factors have been identified that may delay the time to smear conversion. These include high initial sputum smear acid-fast bacilli (AFB) grade, cavitatory lesion, uncontrolled hyperglycemia/diabetes mellitus, old age, certain ethnic initial treatment with less than four anti-tuberculosis drugs, and non-rifampicin-based treatment regimens (Bouti *et al.*, 2013).

2.3.1 Laboratory Diagnosis of Sputum

Depending on the clinical manifestation of the disease, virtually any specimen type may be processed for the detection of mycobacteria (García de Viedma, Mokrousov, & Rastogi, 2011)). The most common specimen is sputum for smear microscopy in which the acid-fast bacilli are examined routinely in the microscope for TB diagnosis. The sample is normally collected in a leak-proof, sterile container. The sample does not require transport media to preserve the viability due to the status of the *Mycobacterium* organisms in the sample.

2.3.2 Laboratory Diagnosis of Glycated Hemoglobin (HbA1c)

Proteins are frequently glycated during enzymatic reactions when the conditions are favorable. However, hemoglobin glycation occurs by the non-enzymatic reaction between the glucose and the N-terminal end of the β-chain, which forms a Schiff base (Ohtsubo, Chen, Olefsky, & Marth, 2011) (Ohtsubo *et al.*, 2011). The Schiff base is converted during the rearrangement, into Amadori products, of which the best known is HbA1c (Ohtsubo *et al.*, 2011; Sherwani, Khan, Ekhzaimy, Masood, & Sakharkar, 2016). The major sites of hemoglobin glycosylation are β-Val-1, β-Lys-66, and α-Lys-61. The Normal adult hemoglobin consists predominantly of HbA (α2β2), HbA2 (α2δ2), and HbF (α2γ2) in the composition of 97%, 2.5%, and 0.5%, respectively. About 6% of total HbA is termed HbA1, which in turn is made up of HbA1a1, HbA1a2, HbA1b, and HbA1c fractions based on cation exchange, (Sherwani et al., 2016).

2.3.3 Assay of HBA1c from Blood Sample

Glycated hemoglobin A1C (HbA1c) gives an integrated plasma glycaemia for the previous 2-3 months and its measurement is central to the management of diabetic patients. Hemoglobin A1C (HbA1c) is an important index of average glycemic patients with diabetes mellitus recently adopted by the World Health Organization (WHO, 2011) and by the International Expert Committee guidelines as a laboratory tool for the diagnosis of the world's most prevalent metabolic disease (Gillett, 2009).

The HbA1c value expresses the percentage of HbA1c on adult normal hemoglobin (HbA1c) for the Diabetes Control and Complication Trial (DCCT) reference assay definition or the ratio between HbA1c (millimoles per mole) according to the new American Diabetes Association (ADA). This strictly correlates with the average blood glucose levels during the previous 3 months (Hanas, John, & International, 2010). Compared with other markers used for T2D diagnosis and provided that measurements are calibrated to international standards and no condition impeding accurate measurement is present (Hare, Shaw, & Zimmet, 2012). HbA1c usage displays several advantages. Among these, the most important can be considered great pre-analytical stability of the measure, no need for fasting condition, a less intra-individual variability, and the necessity of a single sample.

2.4 Treatment options among TB and TBDM Co-morbidity Patients

Drug development began in the 1940s when streptomycin was discovered but after treating some patients, resistant mutants began to appear (Kerantzas & Jacobs, 2017). The discovery of isoniazid in 1952 brought renewed hope. This potent agent resulted in rapid sterilization of sputum and, when used in conjunction with streptomycin, reduced the rates of drug-induced resistance. In 1954 pyrazinamide was discovered and later ethambutol (1962) and rifampicin (1963), which revolutionized TB treatment, (Kerantzas & Jacobs, 2017).

The current treatment of tuberculosis consists of two phases, which are the aptly named initial or intensive phase and the continuation phase. In the intensive phase, all actively dividing bacteria should be killed as soon as possible. The continuation

phase is meant to eradicate the persisters i.e. presumably slower replicating or dormant bacteria. The recommended initial phase for the treatment of tuberculosis consists of rifampicin 10mg/kg (maximum 600mg), isoniazid 5mg/kg (maximum 300mg), pyrazinamide 15–30mg/kg (maximum 2g), and ethambutol 15–20mg/kg (maximum 1.6g) given daily for 8 weeks (R. Ruslami, Aarnoutse, Alisjahbana, van der Ven, & van Crevel, 2010). This is followed by the continuation phase of isoniazid 5mg/kg (maximum 300mg) and rifampicin 10mg/kg (maximum 600mg). TB drugs are not used singly except in latent TB or chemoprophylaxis (giving medication to people who have no active disease and are currently well This is because the regimens that use only a single drug result in the rapid development of resistance and treatment failure (Wang et al., 2015).

The different drugs in the regimen have different modes of action. Isoniazid is bactericidal against replicating bacteria. Ethambutol is bacteriostatic at low doses but is used in TB treatment at higher, bactericidal doses. Rifampicin is bactericidal and has a sterilizing effect. Pyrazinamide is only weakly bactericidal but is very effective against bacteria located in acidic environments, inside macrophages, or in areas of acute inflammation. To prevent non-adherence to TB drugs and treatment dropouts, the WHO has introduced the Directly Observed Therapy (DOT) strategy (WHO, 2015).

2.4.1 Predictors of Poor Outcomes

Among patients afflicted with both TB and DM, diabetes is reported to be associated with poor TB treatment outcomes (Baker *et al.,* 2011). Diabetes has been associated with increased risk of TB treatment failure or relapse, and diminished 2-month and 6-month culture conversion rates (Kelly E. Dooley & Richard E. Chaisson, 2009). Diabetes mellitus has been associated with increased risk of all-cause mortality in TB patients and, more specifically, with death related to pulmonary TB, (Wang *et al.*, 2015). In Egypt, in one case-control study, diabetes conferred a 9-fold increased risk of treatment failure, and this risk increased with adjustment for adherence (Kelly E. Dooley & Richard E. Chaisson, 2009). Adverse effects of DM on TB treatment

outcomes, i.e. increased risk of death (Chang *et al.,* 2011), treatment failure, default, and relapse were reported in many studies (Baker *et al.,* 2011).

2.5 Molecular Clustering among TB and TBDM co-morbidity

Genotypic analyses of *Mycobacterium tuberculosis* together with conventional epidemiologic methods have helped to further characterize *M. tuberculosis* strains, understanding the dynamics of transmission of TB in different regions and populations (Demay *et al.,* 2012). For patients with identical strains of M. *tuberculosis*, considered to belong to molecular clusters, their disease is due to recent transmission and rapid progression. Unique genetic patterns are likely due to reactivation of latent infection or recent transmission from patients out of the period or area under study (Demay *et al.*, 2012)

A great variety of individual, biological, and social determinants have been associated with the clustering of pulmonary TB (PTB) cases. In highly endemic areas for TB some of these factors include being male, young, having been born in an endemic country, resident of an urban area, alcohol or drug consumption, homelessness, HIV infection, or having acid-fast bacilli in sputum smear (Blanco-Guillot *et al.,* 2017).

International TB control urgently needs a concerted and coordinated response to monitor and assess global TB spread, drug-resistance surveillance, and populations at risk. This is possible through an integrated approach based on the networking of laboratories, TB control programs, and application of new molecular typing techniques to identify, track and report the circulating and emerging clones of tubercle bacilli worldwide (García de Viedma *et al.,* 2011)

Most of the earlier molecular epidemiological studies were performed using IS6110-RFLP, considered a gold-standard due to its reproducibility, discriminatory power, and low cost (Goyal, Saunders, van Embden, Young, & Shaw, 1997). However, this labor-intensive methodology was replaced with alternative PCR-based strategies in the last decade, creating the basis for large-scale, high-throughput genotyping of Mycobacterium tuberculosis, well adapted for epidemiological investigations

(Demay *et al.,* 2012). Integration of new genotyping tools like spoligotyping based on the polymorphism of the direct repeat (DR) locus (Demay *et al.*, 2012).

At the micro-population level, this strategy allows us to determine whether recurrences in a patient are due to reactivation, exogenous reinfection or due to polyclonal infection; and to identify recent transmission versus reactivation, whereas, at the macro population level, it allows to define the global distribution of TB genotypic lineages, to monitor the international spread of high-risk isolates, as well as to explore the evolutionary features of M. tuberculosis, (García de Viedma *et al.,* 2011). Publicly available databases such as SpolDB4 TB fingerprinting data, and have provided a new conceptual framework to study global TB epidemiology, and human demographical and TB co-evolutionary history (Demay *et al.,* 2012). Furthermore, usage of molecular epidemiologic techniques has previously allowed us to show that increased risk of TB among patients with DM is due to both reactivation and recently transmitted infection (Demay *et al.,* 2012)

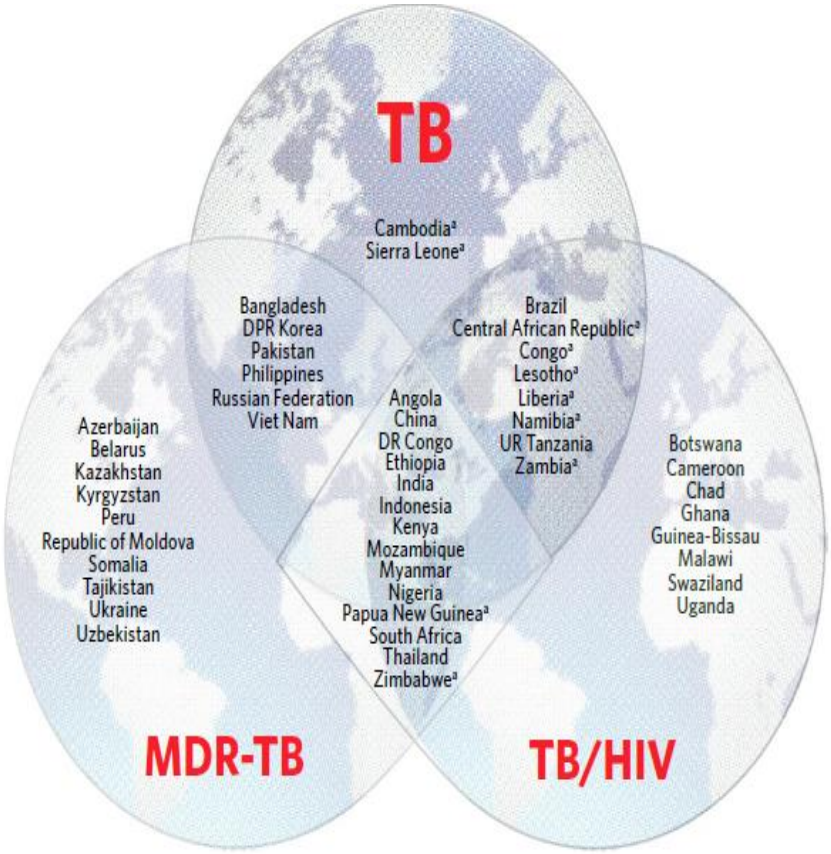

Figure 2.2: WHO revised list of high burden countries

(Source on WHO 2016- 2020: WHO/HTM/TB/2015.29)

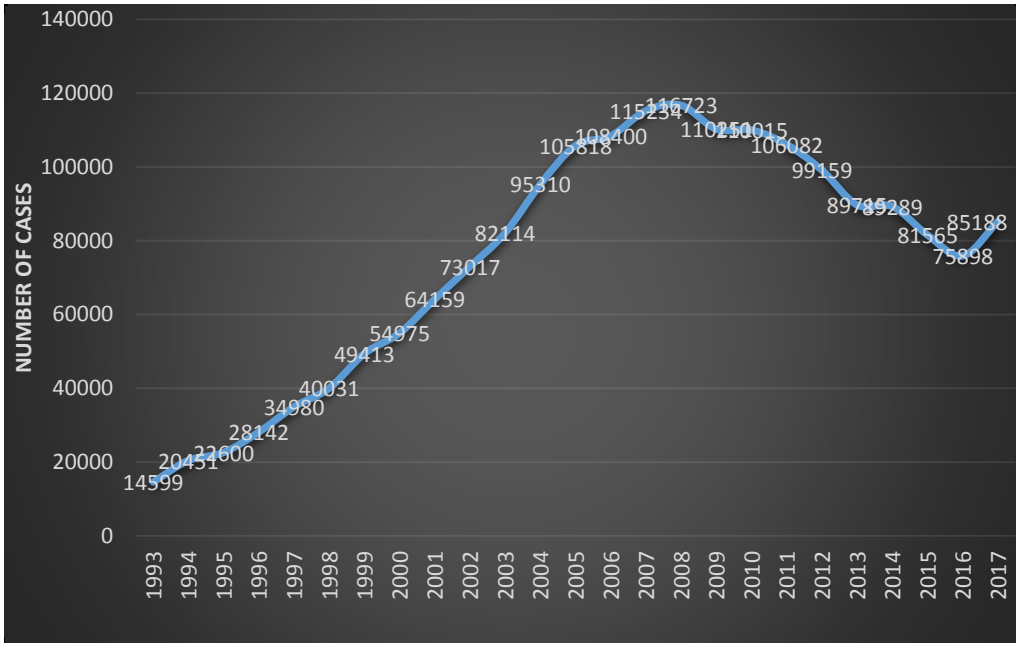

Figure 2.3: TB Case Notification by Year, Kenya

(Source: Republic of Kenya, Ministry of Health 2017 NLTP Annual report).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site

The study was carried out in Nairobi and Kiambu counties in Kenya. Nairobi is the capital and largest city in Kenya and is situated at 1°17′S 36°49′E. It occupies 696 Km^2 with a population of about 3,375,000 people. On the other hand, Kiambu County is adjacent to the northern border of Nairobi County and has a population of 1,623,282 occupying 449.2 km2 (945.6 sq. mi). The county is predominantly rural. These two counties are interconnected in terms of infrastructural development, business activities and movement of people. The efficient communication network between the two counties facilitates the movement of communicable diseases between these regions as if they were one. Additionally, the health providers are largely shared between the two regions to the extent that the quality of care can be said to be similar.

Figure 3.1 below is the map of Kenya showing the Case notification rate in the two counties. The study was carried out in the following health facilities; Thika and Kiandutu in Kiambu and Rhodes, Riruta, and Kangemi in Nairobi, from Aug. 2014 to Aug. 2015

The Microbial analysis was done at the National Tuberculosis Reference Laboratory within the KNH grounds and the Blood sample experimental procedures were done at the Kenyatta National Hospital in Nairobi Kenya. The Molecular analysis on the harvested DNA from all the newly diagnosed TB patients who were enrolled in the study was done at the Medical Research Council South Africa (SAMRC).

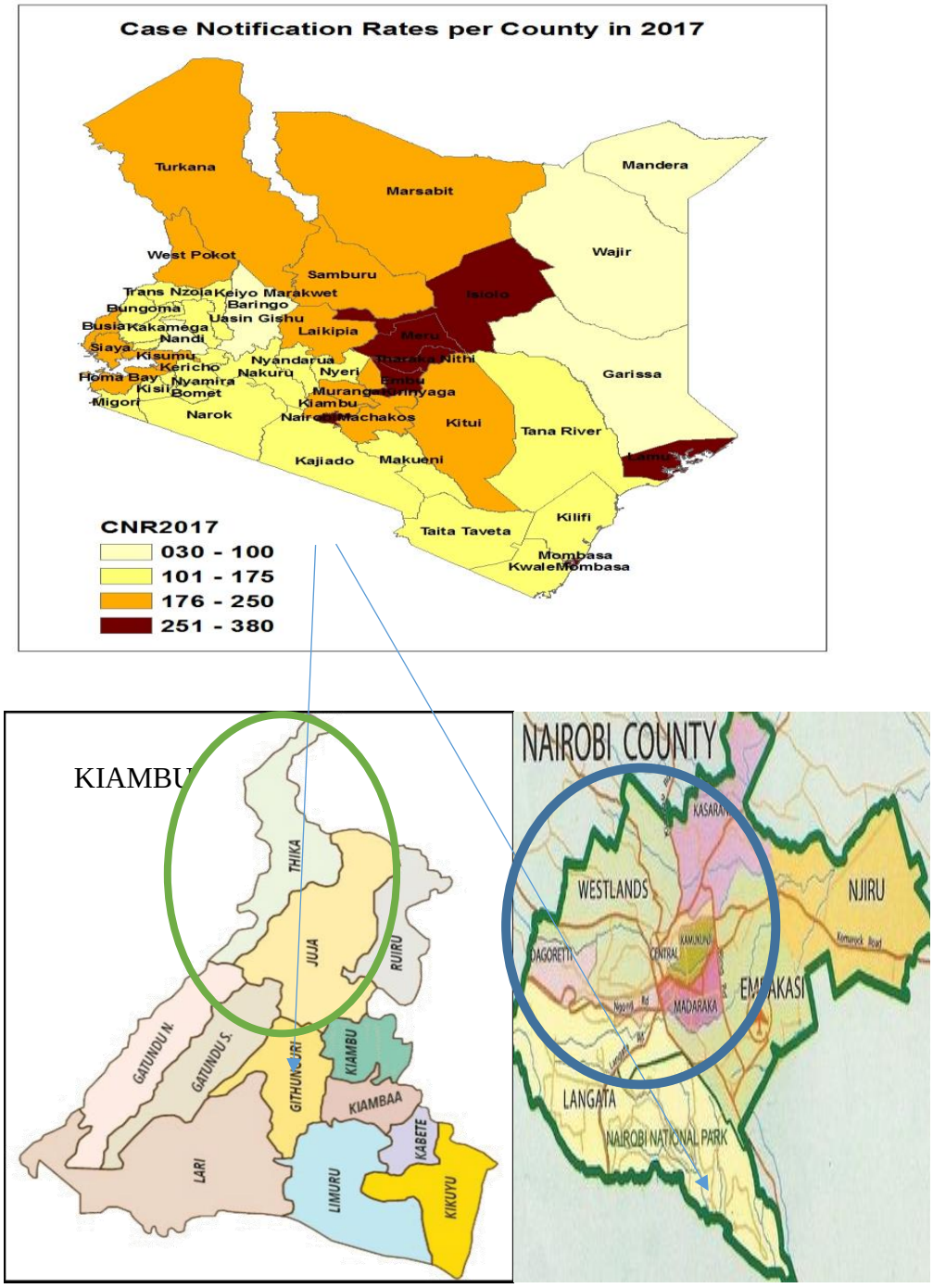

Figure 3.1: Map of Kenya showing Kiambu and Nairobi Counties

(Source: unpublished programmatic management data with the arrows showing the facility in the counties

3.2 Study design

This was a prospective cohort study carried out in the two urban counties of Kenya (Kiambu and Nairobi) between August 2014 and August 2015 for 1 year. Participants above 15 years of age who were newly diagnosed and tested positive for tuberculosis on the initial sputum smear were selected. These were recruited and enrolled after they consented to participate in the study. After the participants signed the consent forms, procedures comprising administration of the structured questionnaire, medical history, and clinical examination were carried out. Participation was voluntary, and the confirmed AFB Smear positive patients were followed for the 2^{nd}, 4^{th} and 6^{th} month for sputum analysis and treatment, adherence and sputum conversion monitoring.

3.3 Target Population

The target population comprised a heterogeneous population of all persons (Male and Female) attending TB Chest clinics and Referral patients, all who were aged 15yrs and above. The Laboratory diagnosis on *Mycobacterium tuberculosis* was based on the Kenya National Tuberculosis Program (KNTP) guideline criteria, for a duration period of this study from August 2014 to August 2015: (1 year.)

3.4 Study Population

The TB Patients enrolled and recruited in the study were initially registered at the health facilities where they were diagnosed. This was done from August 2014 to August 2015. From the 347 study participants 277(80%) were from Nairobi County while Kiambu enrolled 70(20%) participants. The Newly diagnosed TB patients were all screened for Diabetes and registered at the health facilities (Rhodes, Riruta, Kangemi, Thika District hospital and Kiandutu Health centers) for treatment. Participants were identified from the following health facilities: Riruta 103 (30%), Kangemi 115 (33%), Rhodes 59 (17%), Thika hospital 47 (14%) and Kiandutu 23(6%) (Table 3.1).

Table 3.1: Study population enrolled

County	Health Centre	No of participants	percentage
Nairobi	Riruta	103	30
	Kangemi	115	33
	Rhodes	59	17
Sub total		**277**	**80**
Kiambu	Thika county level 5 hospital	47	14
	Kiandutu	23	7
Sub total		**70**	**20**
Grand total		**347**	**100**

Nairobi County contributes 15% of all notified TB patients in Kenya followed by Kiambu County which contributes 5% being 3rd among the high burden in Kenya, (Annual Report, 2014). The 3 health facilities selected from Nairobi (Dagoretti , Westland's and Starehe TB Zones) showed the high burden, same as the 2 health facilities in Kiambu County (Thika East and Thika west TB zones) with Thika level 5 being the highest followed by Kiandutu.

3.4.1 Criteria for inclusion of Study subjects

All Tuberculosis infected positive Patients aged 15 years and above and presenting to the clinic with symptoms suggestive of PTB and who consented were included in the study. The following were considered in recruiting the patients:

1. Newly diagnosed TB confirmed patients
2. Willing to be registered at the health facility
3. Willing to consent voluntarily
4. Willing to receive treatment and be monitored at the Health facility for 6 months till declared treatment outcome as Cured, treatment complete or failure.

3.4.2 Criteria for Exclusion of Study Subjects

1. Pregnant women, as this is a high-risk group due to Gestational Diabetes,
2. Those with Chronic renal failure
3. If patient not consenting to the study
4. Patient with ongoing treatment- TB therapy as Rifampicin
5. Patient with known Diabetes Mellitus on treatment

3.5 Sampling technique and Sample size

3.5.1 Sampling technique

Nairobi and Kiambu Counties were purposively selected. The three and two sub counties health facilities were randomly selected from Nairobi and Kiambu Counties respectively. In each case, facility with highest burden in the year 2014 were selected as outlined in the Study Model shown on Figure 3.2 below.

3.5.2 Determination of Sample size

The sample size was determined using Eng. (2003), formula to determine the number of Diabetes Mellitus patients among the TB infected patients. This study was designed to measure a characteristic in terms of a proportion; (Essentials of Biostatistics in Public Health Lisa Sullivan 3rdedition).

Where: n=sample size,

D = margin of error =0.05

Z = fraction is constant value at the 95% Confidence Interval (1.96)

p=the proportion of diabetics patients among the TB infected patients in the population.

$$n\ (SRS) = \frac{N*Z^2*p(1-p)}{d^2\ (N-1)+Z^2*p*(1-p)} = 347$$

6891*3.8416*0.05(1-0.5)

0.005625(1880) +3.8416*0.5*(1-0.5)

= 347

Design effect 2.0 due to follow up of the TB patients

3.5.3 Demographic information

The following information was obtained from the recruited subjects;

i. History of the Patients including: parameters such as age, sex, drug
ii. history, Patient Knowledge on both disease, distance covered by the patient, level of Education, income, visit of health facility, source of information.
iii. Social demographic information/ risk factors such as HIV status, alcoholism, smoking, prior anti-tuberculosis treatment and delay in cure seeking, BMI (i.e. among the newly TB patients) was recorded and entered on an excel data sheet (appendix 5).

3.5.4 Criteria for Diabetes diagnosis in a clinical setting

Glycated hemoglobin type c (HbA1c) was the test of choice in diagnosis of diabetes as it captures chronic hyperglycemia for the previous 3 months prior to the hospital visit and Random blood glucose was done on the spot. Therefore, it was more robust and reliable measurement for fasting plasma glucose as the blood was drawn early Morning once the participants submitted their sputum sample.

This was considered as a diagnostic tool for diabetes where all the newly diagnosed TB patients were screened for diabetes using the rapid glucose test and HbA1c. Elevated fasting glucose concentration was recorded with reference values (Venous whole blood ≥ 120mg/dl (6.7mmol/l)

3.5.5 Sputum samples

i. All Sputum positive samples from the selected health clinic were confirmed once the samples were taken to the National Reference Tuberculosis laboratory for confirmation.
ii. All patient samples were processed for culture at the National Tuberculosis Reference Laboratory.
iii. All the isolates from the patient had their DNA extracted and analyzed at Medical Research Council in South Africa.

Study Model

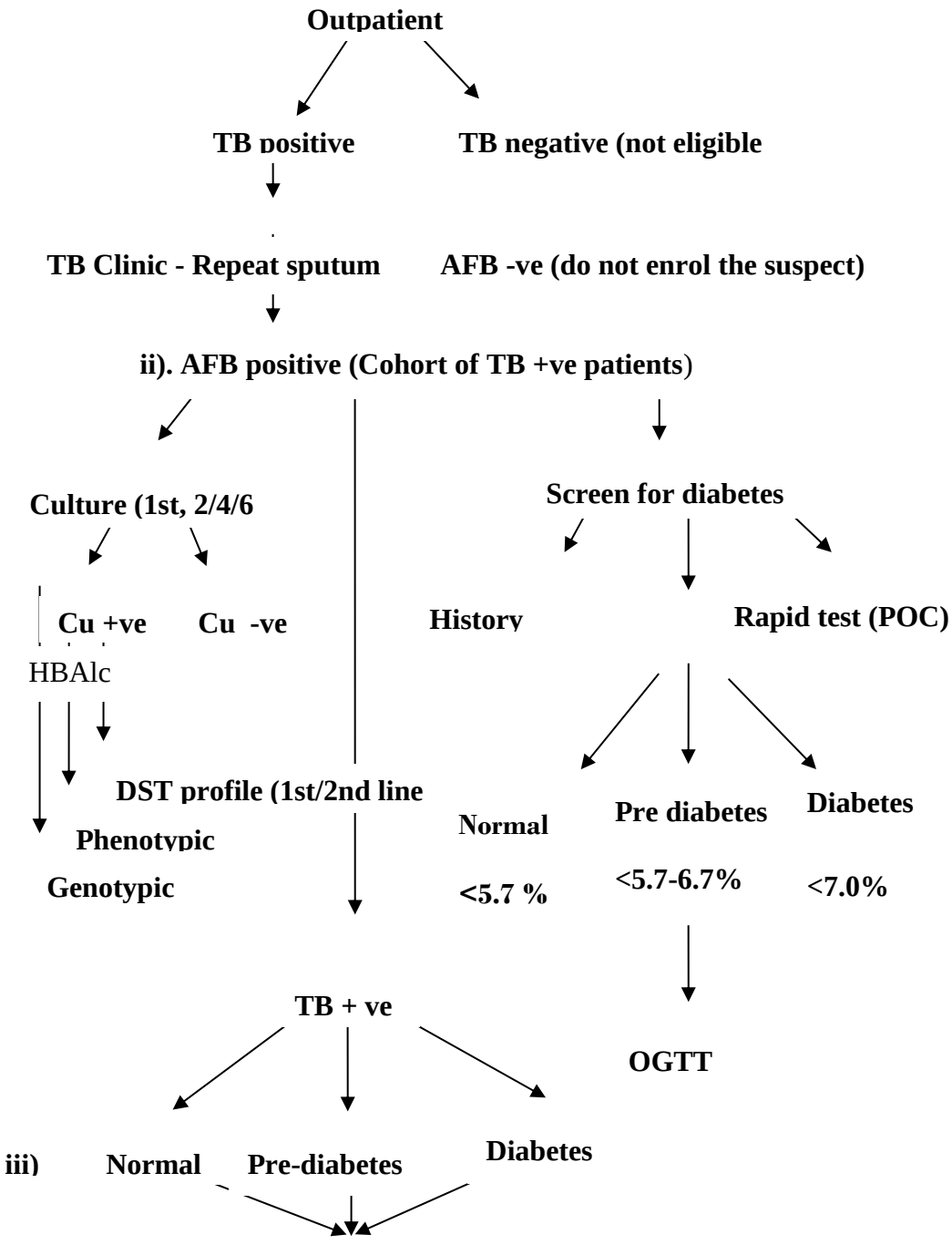

Figure 3.2: Flow chart on the Study Model

3.5.6 Administration of questionnaires

A semi structured questionnaire (Appendix 5) was administered by a trained health care personnel.They collected detailed history from the newly diagnosed TB patient regarding social demographic as outlined in section 3.5.3 above . These included signs and symptoms of Diabetes Mellitus, cigarette smoking, habitual risk like factors e.g. family history (Study patient questionnaire). Type of TB, status of TB treatment and category. Consent was sought and obtained from the participants (their parents or guardian) who volunteered to participate in the study following adequate information related on the objectives and benefits of the study.

3.6 Specimen collection and laboratory Analysis at the selected facilities

3.6.1 Sputum sample collection

At the sampled health facility, the participants were explained to and instructed on how to collect specimens before sputum collection, in order to get quality specimens and not saliva. To prevent transmission, Sputum collection took place in a designated well-ventilated area and not within the laboratory. A spot specimen sample was collected from all eligible participants using a 50 ml screw-cap falcon tube container. All the sputum samples for both morning and on spot were kept in cold chain and taken to the National Reference Tuberculosis laboratory for further analysis

3.6.2 Blood sample collection

Blood samples were drawn from the participants at each health facility and was put into an EDTA. Random blood glucose (RBG) and Glycated hemoglobin type 1a (HbA1c) tests were done using appropriate kits. The procedures were based on the Manufacturer's instruction. Procedures in the laboratory were based on the Standard Operating Procedures, with all the equipment's to ensure measurement traceability for all the samples done at the Kenyatta National Hospital laboratory.

3.7 Laboratory Analysis at NTRL

3.7.1 Microscopy Analysis

A direct smear was done from both the morning and on spot samples and stained with Ziehl Neelson (ZN). This was followed by sputum microscopic examination at (x40 and x100 magnification), oil immersion and reported as either negative or positive AFB according to the WHO guidelines on Smear Microscopy.

3.7.2 Sputum sample Processing

Sputum samples collected in a sterile bottle were processed, digested and decontaminated. The samples were decontaminated with the N-acetyl-L-Cysteine (NALC), Sodium Hydroxide (NaOH 2%) and the reaction was stopped with the addition of 6.8% phosphate buffer, as described by Universidad Cayetano Heredia (CLSI 2008). Briefly, 2ml of sputum sample were placed in 15mls centrifuge tube, capped tightly closed and vortexed for 20 seconds. This reaction was left to stand for 15minutes. The tubes were filled to 14mls with phosphate buffer (PH 6.8) and centrifuged at 3000xg for 15minutes. The supernatant was carefully poured off and the remaining pellet retained at the falcon for the media inoculation as described by Somoskovi *et al.* (2003).

3.7.2 Culture of Processed Sample on Solid Media

Ten microliters (10 µl) of the processed sputa pellets was inoculated on to two slopes of Lowenstein Jensen (LJ) medium. The inoculated isolates were incubated at 37°C and monitored for growth for up to 8 weeks, read weekly and recorded in the lab logbook. Positive LJ was confirmed by use of ZN, as negative cultures were discarded. Lowenstein Jensen (LJ) media allowed for visible MTBC colony growth within 18–24 days (Somoskovi *et al.*, 2003).

3.8. Internal Quality control

A fully susceptible H37Rv American Type Culture Collection 27294 (ATCC 27294), a rifampicin resistant strain American Type Culture Collection 35838 (ATCC 35838)

and isoniazid resistant reference strain of *M. tuberculosis* were used as controls, and served as susceptible and resistant controls respectively. These strains were sub cultured on fresh LJ medium to obtain pure culture. Contamination of the solid medium was monitored separately with 3-5% as an acceptable contamination rate (Kubica *et al.,* 1985). Conversely, contamination rates approaching 0% suggested that decontamination conditions were too harsh and may be adversely affecting mycobacterial recovery (Pfyffer *et al*., 2015).

3.9 Identification of Isolates using Biochemical and Molecular Analysis

3.9.1 TBc antigen identifying kit

This product (MGIT TBc identification kit-BD MGIT Identification test) is a chromatographic immunoassay for a qualitative detection. It's a rapid procedure that identifies MTbc from a culture grown in liquid medium (MGIT). The test line of MTbc is specific to monoclonal antibody and a control line of anti-species antibody. This product detects MPT64, a mycobacterial protein fraction that was secreted from MTBC cells during culture. When samples were added to the test device, MPT64 antigen bound to the anti-MPT64 antibodies which conjugated to form particles on the test strip. The antigen-conjugate complex migrated across the test strip to the reaction area and was captured by a second specific MPT64 antibody applied to the membrane. If MPT64 antigen was present in the sample, a color reaction was produced by the labeled colloidal gold particles and was visualized as a pink to red line which was positive showing the presence of the MTBC. (AFB smear positive MGIT tube by inverting and vortexing and letting to stand for 15 minutes. 2-3 drops of the positive sample on the specimen area of the test plate (SD-Bio line plate from its foil pouch) and time for 15minute. Read the results and interpret the results within 15 minute).

3.9.2 DNA extraction from the isolates

This was done through mechanical method where the bacteria colonies from the solid culture were collected with an inoculating loop and re suspended in 300 µl of molecular water. It was placed in (heat inactivation) boiling water, then to the

heating block at 90^{o}c for 30minutes to denature the *Mycobacterium tuberculosis* bacteria. This was then incubated in the Sonicator (Ultrasonic hot bath) to allow the breaking of the Mycolic cell wall to release the MTB DNA for 15 minutes. The mixture (suspension) was centrifuged for 5minutes at 14000g in the micro centrifuge (Hettich Mikro 200/200R tabletop microliter centrifuge). The harvested (extracted) DNA in the supernatant was stored in the -80^{o}c freezer to await further analysis. The Medical research issued a transport permit to South Africa. This was shipped to South Africa using Courier services (FedEx) under International Air Transport Authority (IATA) regulations.

3.9.3 Spoligotyping DNA Samples

Spoligotyping was done in the South Africa Medical Research Council (SAMRC, Pretoria) as described by Goyal *et al.*, (1997) and Kamerbeek, Schouls, and Kolk (1997). Spoligotyping was performed using the spoligotype kit (Ocimum Bio Solutions Company, Netherlands) according to the manufacturer's instructions. The direct repeats (DR) from the DNA were amplified by ABI 9700 96 well PCR (Bio Systems Thermo Cycler) by use of oligonucleotide primers (DRa: 5' GGT TTT GGG TCT GAC GAC 3' and DRb: 5' CCG AGA GGG GAC GGA AAC 3') derived from the DR sequence. The DRa primer is biotinylated at the 5'-end. Biotinylation exploits polymorphisms in the spacer sequences found in the direct repeats (DR) locus in the chromosomes of MTBC Strains (Kamerbeek *et al.*, 1997).

After the initial denaturation step, subsequent PCR cycles began with a separate denaturation, step that lasted 0.5–2 minutes at 94–98°C. As with the initial template DNA denaturation step, the time and temperature were optimized according to the nature of the DNA template, DNA polymerase, and buffer components. The reaction temperatures were lowered to allow binding of the primers to the target DNA. Often, incubation time of 0.5–2 minutes was done for primer annealing. The optimization of annealing temperatures, gradient thermal cycler blocks are in built in the thermal cycler, where highest and lowest temperature are set across the block so variations in temperature could be assessed across a series of wells or reactions at the same time. The reaction temperature were raised to the optimal temperature of the enzyme for its

maximal activity, which is generally 70–75°C for thermostable DNA polymerases. Amplification of the spacers were done with both primers DRa and DRb which amplified the whole DR region using small amount of DNA. Polymerase Chain Reaction (PCR) were performed on 10 ng purified chromosomal Mycobacterial DNA extracts from the clinical samples as the templates. The following thermo cycling were used: (3minute 96^0C, 1minute 96^oC,1 minute 55^oC , 30 sec 72^oC and 5 minute 72^oC) 20 X (Appendix 7).

3.9. Spoligotype Analysis

All strains in this study were analyzed based on Spoligotyping molecular analysis patterns (Kamerbeek *et al.,* 1997). These were converted into binary and octal code designations for easier analysis and interpretation. SITVIT WEB, an online consultation global database of *Mycobacterium tuberculosis* strains and molecular spoligotyping markers database were used for analysis. Spoligotype patterns were formed by the presence or absence of 43 spacers in the DR locus of *Mycobacterium tuberculosis* during the analysis. The patterns were seen as 43 digital patterns or under a reduced octal form of only 15 numbers. Association of all of them generated an allele profile which was used for the identification and the evolutionary studies of *M. tuberculosis* complex. This was provided by the Institute Pasteur de Guadeloupe (established 1993), an inbuilt excel model file filled with the octal code and the binary pattern. This was then uploaded in search of similar profiles in SITVIT. It has developed a well-known expertise in diagnostics and research; it collaborates with research groups worldwide with special emphasis on global *M. tuberculosis.*

3.9.1 Identification of TB Lineage

The identification of the TB lineage was done by the use of appropriate target sequences, ideally present in all *Mycobacterium species* (MTB complex) and absent in others. These were generated from the isolates in the study populations whose strain types were similar, based on the standard spoligotype assay (SpolDB4). This data was compared with the previously identified strains in a data bank from the spolDB4 for both the octal code and the binary pattern binary as it was represented in the spoligotype shared international types, SITVIT2 web(Demay et al., 2012).

3.9.2 Phylogenetic tree and Mapping

MLVA Compare V1.03 software (Genoscreen; Lille, France), was used to draw the minimum spanning trees (MSTs). The Standard International Type (SIT) or orphan spoligotype number appeared inside each node, and the distance (number of spacers of difference) between the two nodes, was shown on the edge linking these nodes. The phylogenetic tree was colored in relation to various characteristics of MTBC lineages and geographical location as described in SITVIT and the place of isolation. Mapping of all the strains was done from all the samples which were done spoligotyping. Glycosylated hemoglobin measurements and Quality Assurance

3.10.1 Assay of Glycosylated hemoglobin (HbA1c)

Glycated hemoglobin A1C measurement was done on Ethylenediamine-tetra-acetic acid (EDTA) with whole blood samples. The assay was done 4-6 hours after sample collection using cation exchange high performance liquid chromatography (HPLC) as per Nicol and Wilkinson (2008) procedure. The assay values were standardized to the Diabetes Control and Complications Trial - Deflection Check: 0.5, Discreteness Check: 0.1, Sensitivity Check: 0.14, Blank Level Checks -0.1-0.7, carried out at Kenyatta National Hospital lab. The Laboratory procedures were performed as per the manufacturer's Kit instructions. Commercial controls consisting of HbA1c Abnormal Low, Normal and Abnormal High and HbA1c calibrators were included during the assay procedure.

The whole blood sample was centrifuged at 2000rpm for 5minutes. Then 25 µl of the deposited red blood cells was aliquoted into a sample cap Eppendorf microfuge tube using a calibrated pipette. Addition of 500 µl of hemolysin was added to denature the deposited red cells. This was homogenously mixed and the analysis was performed using the appropriate automated analyzer - DIRUI CS 4000 Automated Chemistry Analyze, (Appendix xvi.)

3.10.2 HbA1c Quality Assurance

The test were based on colorimetric, enzymatic method. The concentrations of HbA1c and total hemoglobin were determined separately. Red blood cells are known to survive in the human body for 8-12 weeks before renewal. Therefore, measuring glycated hemoglobin (or HbA1c) can be used to reflect average blood glucose levels over that duration, providing a useful longer-term gauge of blood glucose control. An HbA1c of 48 mil/mol (6.5%) was recommended as the cut-off point for diagnosing diabetes. Quality control was done using A1c-Cellular Vial for the entire HbA1c performance, including the lysing of the red blood cell. (Appendices xvi).

The whole blood sample was centrifuged (spinned) at 2000rpm for 5 min, 25ul of the red cells deposit was aliquoted into a sample cup or Eppendorf microfuge tube using the calibrated pipette. Add 500ul of hemolysin /denaturant or the pretreatment solution to the 25ul of the aliquoted erythrocytes. The mixture was shaken vigorously in a closed Eppendorf tube till it lysed. The homogenously mixture was homogenized (haemolysate) and then the assay was run after 5 minutes using the automated analyzer, (Appendices 16).

3.11 Care and Treatment

New tuberculosis patients were put on a six-month category I regimen comprising 2 months of isoniazid, rifampin, pyrazinamide and ethambutol (2RHZE) followed by four months of isoniazid and rifampin (4RH). Previously treated patients, including those who had failed prior therapy were put on category II regimen which was similar to category I except that, streptomycin was additionally administered for the first two months, while the other four drugs were prolonged by one month and isoniazid, rifampin and ethambutol given for a further five more months. Dosing was per Daily fixed dose combinations formulations as per NTLD and WHO guidelines and were given using directly observed Treatment, short course (DOTs) (WHO, 2014). On the other hand, the Diabetes patients were referred to their health facilities for further testing and treatment.

3.12 Data Collection and Analysis

3. 12.1 Data Collection

Trained health research assistants retrieved clinical records for each of the TB patients from the facility's TB treatment registers. The data collected covered a time period 1 year between Aug. 2014 to Aug. 2015. A questionnaire was used for the prospective study (Appendix 5). These contained questions used to assess risk factors of TB and TB/DM namely age, sex, family history of Diabetes Mellitus. Physical parameters like height, weight, body mass index (BMI) were recorded. The other variables like HIV status, treatment imitation and completion, sputum follow up and treatment outcome information were retrieved from the TB facility treatment registers and the TIBU online reporting platform at the TB program as the patients were monitored during the treatment period and followed for 6 months. This created the study database that contained a record of each patient enrolled.

3.11.2 Data Management

A purpose developed validated data collection form was used for collecting demographic and clinical data. Demographic data included patients' gender, age, weight, residential area, marital status, smoking and alcohol consumption status. Age and weight first recorded as continuous data was then classified into categories. The data collected was ensured to be accurate and complete (data quality). This was essential part of the evidence collected for evaluating and validating the study research results among the study population. The data collected was kept safe, under lock and Key, ensuring no loss through storage (stored in the clouds) and archiving in a good, organized and documented manner; Data was secured against unwanted changes, which were achieved by a controlled access control and a password, to allow others to validate and replicate findings on research results sharing and further analysis

Clinical data collected included clinical presentation, serum biochemistry results, nature of TB case [New, retreatment (failure, default and relapse)], site of TB infection (pulmonary) with confirmed DM, TB and DM medications, results of

sputum culture and treatment outcomes. Disease classification, treatment protocol and treatment outcomes were defined by Diabetes care 2015.

3.12.3 Data analysis

Clinical and social demographic data were obtained from the administered questionnaire and medical records of the National TB program patient data base. The data was entered and analyzed using the statistical package for social sciences (SPSS) version 17.1 statistical software.

Univariate analysis was used to compare each potential risk factor in the TB and TB/DM patients. Pearson chi square test of proportions or fisher exact test as appropriate, was done to identify the factors that were independently associated with the outcome of TB/DM. Multiple logistic regression analysis, Richard–Mandelbrot log-log plots were used to examine the fractal behavior. The one sample t-test and the nonparametric Kruskall-Wallis test were used to test and assess the representativeness of the sample in representing population of patients with diabetes or pre-diabetes mellitus using cut off points of 6.7% and 5.7%, respectively.

Tuberculosis (TB) treatment outcome was grouped into seven categories (Cured, Died, Failure, Not Complete, Out Of Control, Treatment Complete, and Transfer Out). Diabetic conditions were grouped into two groups (diabetic and non-diabetic), in the two counties considered (Nairobi and Kiambu). Cross tabulation of TB lineage was done with Outcome, diabetic conditions, and counties to compare the proportion of each lineage across each of the categories of the other variables.

Stepwise Multivariate, logic regression analysis (full model method using the same variables used in CART analysis for both development and validation samples with true classification) was then performed. Receiver operating characteristics (ROC) value was then reported in a table format. Relative risk ratio and the Confidence Interval (CI) were also computed given the prospective nature of the study. Confidence intervals for proportions were computed using exact methods approximation.

Forward stepwise approach was used to add covariates to the model. All factors with biological plausibility and P < =0. 2 in the univariate analysis were considered in the multiple regression models. To test for significant interaction terms, Hosmer-Lemeshow was used to test estimate of the goodness of fit of the logistic regression model.

Continuous data was summarized using Median (IQR) while categorical data summarized. The results were presented in tables, figures, cross tabulations of TB lineage across each category of the other variables. The other Descriptive statistics was used to present the data using frequency tables with the corresponding percentages within the parenthesis.

3.13 Ethical Statement

The Study research was approved by the Scientific and ethical review committee from the Kenyatta National Hospital/ University of Nairobi ethical research committee (KNH/UoN-ERC) before implementation (Appendix i). Written consent was obtained from participants who agreed to participate before the study was undertaken in accordance with the principle of Helsinki declaration revised 2000. Confidentiality was maintained and all the participants' information hard copies was put under a lock and key with authorized access. All data collected was stored in the computer while a password was provided for access.

3.13.1 Ethical considerations

The Subjects/ participants were explained the purpose and benefits of the study and their permission was sought to be interviewed, and collect specimens. Participation in the study was totally voluntary. The participants were not forced or persuaded to participate in the study and were free to withdraw in the course of the study if they did not wish to continue. Patients recruited into the study were identified from the selected TB chest clinics in Kiambu and Nairobi counties for specimen collection. Information regarding risk factors and the benefits for the TB/DM infection were informed to the patients by caregivers.

The confidentiality of the information collected during the study was maintained by using code numbers for participants. In addition, the clinical specimens collected during the study period were used for the stated objectives. For those participants who were found to be Diabetic, appropriate treatment was prescribed by physicians and health education given to prevent spread of the infection to others at their initial health facility of diagnosis. The approval of this study was submitted at the Kenyatta National Hospital Ethical research committee (KNH/UoN- ERC) for ethical approval to allow the research to proceed for a period of 1year (2014-2015). Permission was sought from the Nairobi /Kiambu County facilities to access the health facilities and the Patients. The diagnostic findings were communicated to the treating Physicians managing the patients in the enrolled health facilities within Nairobi and Kiambu Counties.

CHAPTER FOUR

RESULTS

4.1 Social demographic and Clinical characteristics of patients with TB and TB/DM comorbidity among the newly diagnosed TB patients

We recruited 347 participants into the study from five selected health facilities from August 2014 to August 2015. Culture results from the 347 specimens showed that seven had *Mycobacteria Bovis,* and were discontinued from the study. We followed the remaining 340 patients who had *M. Tuberculosis* for the outcome at the end of the treatment.

The findings in table 4.1 shows, Socio-demographic characteristics of the patients. Prevalence of DM among TB patients was found to be 37.2% (129/347). The median age of the participants was 31 years (13%) with the age range varying from 15 and 85 years . 84.5% of the patients were less than 40 years with the highest affected group being TB-non diabetic patients who were less than 30 years (104, 47.7%).

The gender analysis showed that there were more males, 71.8% (249), who participated in the study compared to females and most were from the TB-non diabetic group (156, 71.6%). Majority of the participants (78.9%) had completed primary and High School. Also, 67% of the study population was employed, with 31.1% earning more Ksh. 10,000.

Table 4.1: Socio-demographic characteristics of the participants

Variables	Total Participants (n = 347)	TB-Diabetic (n=129)	TB -Not Diabetic (n = 218)
	n (%)	n (%)	n (%)
Age categories			
Median age (IQR)	31 (13)	32 (13)	31 (13)
Under 30	163 (47)	59 (45.74)	104 (47.71)
31-40	130 (37.5)	54 (41.9)	76 (34.9)
41-50	40 (11.5)	9 (7)	31 (14.2)
51-60	11 (3.2)	6 (4.7)	5 (2.3)
Over 60	3 (0.9)	1 (0.8)	2 (0.9)
Gender			
Female	98 (28.2)	36 (27.9)	62 (28.4)
Male	249 (71.8)	93 (72.1)	156 (71.6)
Education level			
No school	15 (4.3)	5 (3.9)	10 (4.6)
Primary	116 (33.4)	37 (28.7)	79 (36.2)
High school	158 (45.5)	61 (47.3)	97 (44.5)
College	58 (16.7)	26 (20.2)	32 (14.7)
Employed			
Yes	233 (67.1)	79 (61.2)	154 (70.6)
No	114 (32.9)	50 (38.8)	64 (29.4)
Income			
<1000	87 (25.1)	29 (22.5)	58 (26.9)
1001-5000	66 (19)	29 (22.5)	37 (17.1)
5001-10000	84 (24.2)	27 (20.9)	57 (26.4)
>10000	108 (31.1)	44 (34.1)	64 (29.6)
Missing data	2(0.6)		

4.1.1. Behavioral characteristics of patients with TB and TBDM

One participant had no data, 54(15.6%) did not respond, 137(39.5%) were not taking alcohol, while 155(44.7%) were taking alcohol. In regards to smoking, 7(2%) were non-response, 99(28.5%) were smoking, while 240(69.2%) were not smoking. We further categorized heavy smokers to be individuals who smoke more than 20 cigarettes per day. 67(67.7%) smokers did not indicate the number of cigarettes smoked per day, 24(24.2%) were classified as non-heavy smokers, while 8(8.1%) were heavy smokers. Further granular data in terms of participant that were TB only and TB-DM are in **table 4.2**.

Table 4.2: Behavioral characteristics of patients with TB and TBDM

Variables	**Total TB patients (347)**	**TB-Diabetic (n=129)**	**TB not Diabetic (n=218)**
	n (%)	n (%)	n (%)
Ever drank alcohol			
Missing data	1 (0.3)	0 (0)	1 (0.5)
NA	54 (15.6)	26 (20.2)	28 (12.8)
No	137 (39.5)	53 (41.1)	84 (38.5)
Yes	155 (44.7)	50 (38.8)	105 (48.2)
Ever smoked			
Missing data	1 (0.3)	0 (0)	1 (0.5)
NA	7 (2)	4 (3.1)	3 (1.4)
No	240 (69.2)	90 (69.8)	150 (68.8)
Yes	99 (28.5)	35 (27.1)	64 (29.4)
No of cigarettes daily*			
Missing data	67 (67.7)	21 (60)	46 (71.9)
<20	24 (24.2)	9 (25.7)	15 (23.4)
>20	8 (8.1)	5 (14.3)	3 (4.7)

4.1.2. Health seeking behaviors of the patients with TB and TBDM

One hundred seven participants (30.8%) reported attending health facilities more than twice per year, and 163 (47%) reported attending once. Two hundred forty-five (70.6%) and one (0.3%) is out of the 30km range. Most of the participants sought care from government facilities (224, 64.6%). The difference between the TB-Diabetic and TB alone can be found in **table 4.3**.

Table 4.3: Health seeking behaviors of the patients with TB and TBDM

Variables	TB (n=347)	TB-Diabetic (n=129)	TB-not Diabetic (n=218)
	n (%)	n (%)	n (%)
Health seeking frequency			
Missing data	1 (0.3)	0 (0)	1 (0.5)
Once a year	163 (47)	63 (48.8)	100 (45.9)
Other	75 (21.6)	30 (23.3)	45 (20.6)
RARE	1 (0.3)	0 (0)	1 (0)
Twice a year more	107 (30.8)	36 (27.9)	71 (32.6)
Distance from the facility			
Missing data	1 (0.3)	0 (0)	1 (0.5)
0–10KM	245 (70.6)	95 (73.6)	150 (68.8)
11–20KM	84 (24.2)	28 (21.7)	56 (25.7)
21–30KM	16 (4.6)	5 (3.9)	11 (5)
>30KM	1 (0.3)	1 (0.8)	0 (0)
Facility			
Missing data	1 (0.3)	0 (0)	1 (0.5)
Government	224 (64.6)	80 (62)	144 (66.1)
Government_NGO mission	4 (1.2)	2 (1.6)	2 (0.9)
Government_other	1 (0.3)	0 (0)	1 (0.5)
Government_ Traditional	2 (0.6)	0 (0)	2 (0.9)
NG0_mission	5 (1.4)	2 (1.6)	3 (1.4)
Private clinic	60 (17.3)	27 (20.9)	33 (15.1)
Private clinic Government	49 (14.1)	18 (14)	31 (14.2)
Private clinic other	1 (0.3)	0 (0)	1 (0.5)
Household members			
<2persons	194 (55.9)	65 (50.4)	129 (59.2)
>2persons	153 (44.1)	64 (49.6)	89 (40.8)

4.1.3. Diet of the patients with TB and TBDM

The participants primarily ate a diet comprising of fats, sugars and meat (107, 30.8%). A further 21.3% indicated their diet consisted of sugars (74) and 17% responded with fats (59). Further results are as shown in **Table 4.4.** below

Table 4.4: Diet of the patients with TB and TBDM

Variables	TB (n = 347)	TB–Diabetic (n=129)	TB–Not Diabetic (n=218)
	(n = %)	(n = %)	(n = %)
Diet			
Fats	59 (17)	27 (20.9)	32 (14.7)
Sugars, Vegetables	4 (1.2)	0 (0)	4 (1.8)
Vegetables, Meat	3 (0.9)	2 (1.6)	1 (0.5)
Vegetables	1 (0.3)	0 (0)	1 (0.5)
Sugars, Vegetables, Meat	2 (0.6)	0 (0)	2 (0.9)
Fats, Meat	1 (0.3)	0 (0)	1 (0.5)
Fats, Sugars	28 (8.1)	13 (10.1)	15 (6.9)
Fats, Sugars, Meat	107 (30.8)	37 (28.7)	70 (32.1)
Fats, Sugars, Vegetables	6 (1.7)	1 (0.8)	5 (2.3)
Fats, Sugars, Vegetables, Meat	32 (9.2)	14 (10.9)	18 (8.3)
Meat	15 (4.3)	6 (4.7)	9 (4.1)
Sugars	74 (21.3)	26 (20.2)	48 (22)
Sugars, Meat	15 (4.3)	3 (2.3)	12 (5.5)

4.1.4. The difference in Demographic and Clinical characteristics in Patients without clinical outcomes ascertained.

Clinical presentation results showed that 87.3% (303) of the 347 patients with TB who enrolled in the study had recorded smear-negative at month 6, with 0.6% still

testing positive (Table 4.5). Overall, the meantime to conversion among those who switched from smear-positive to smear-negative was 3.16 (SD =0.57) months and the median conversion time being 3 (IOR =0) months. The mean time to conversion was slightly higher for the TB/DM patients compared to the TB patients as shown in Table 4.5below.

Table 4.5: The difference in Demographic and clinical characteristics

Variable	Level	All patients n=340 (%)	Outcomes Unknown n=19 (n)	Outcomes Ascertained n=321 (n)	P-value
Demographic	**Characteristics**				
County	Kiambu	56 (17)	4 (21)	52 (16)	0.584
	Nairobi	283 (83)	268 (79)	268 (83)	
	Missing data	1 ()		1 (1)	
Gender	Female	96 (28)	5 (26)	91 (28)	0.848
	Male	244 (72)	14 (74)	230 (72)	
Cigarettes	Ever-smoker	97 (29)	1 (5)	96 (30)	0.021
Age (years)	Mean (SD)	32.11 (8.80)	29 (5.16)	32.29 (8.94)	0.172
Clinical	**Characteristics**				
HIV test	Negative	238 (70)	11 (58)	227 (71)	0.312
	Positive	77 (23)	7 (37)	70 (22)	
	Not Done	25 (7)	1 (5)	24 (7)	
BMI (kg/m^2)	Mean (SD)	19.55 (3.91)	17.96 (2.35)	19.65 (3.97)	0.055
	<18.5	133 (39)	11 (58)	122 (38)	0.156
Underweight					
Ideal	18.5-25	184 (54)	8 (42)	176 (55)	
Overweight	>25	23 (7)	0	23 (7)	
Weight (kg)	Mean (SD)	56 (61)	49.93 (5.61)	54.88 (10.46)	0.023
Blood glucose (mmol/dl)	Mean (SD)	3.61 (1.19)	3.66 (0.69)	3.60 (1.22)	0.548
Glycosylated hemoglobin (%)	Mean (SD)	5.75 (2.22)	6.27 (2.09)	5.72 (2.23)	0.145
Creatinine (mmol/dl)	Mean (SD)	89.39 (20.33)	83.79 (22.78)	89.72 (20.17)	0.087
BUN (mmol/dl)	Mean (SD)	3.76 (1.16)	3.51 (1.09)	3.78 (1.17)	0.378
Treatment	Regimen				
Regimen I	2HRZE/4HR	308 (91)	14 (74)	294 (92)	0.009
Regimen II (retreatment)	2HRZES/1HRZE/ 5HRE	32 (9)	5 (26)	27 (8)	

4.1.5. Clinical presentations of the patients with TB and TBDM comorbidity

A majority of the recruited patients were HIV negative (245, 70.6%), while 77 (22.2 %) were positive among the newly diagnosed TB patients. Most of them were on 2RHZE/4RH (315, 90.8%) and 32 (9.2%) on 2SRHZE/RHZE/5RHE. On treatment outcomes among the enrolled TB patients after 6 months of TB therapy; 292 (84%) attained cure, 6(1.7%) died, and 3 (0.9%) failed treatment. The results are shown on **Table 4.6** below.

Figure 4.6: Clinical presentations of the patients with TB and Comorbidity

Variables	TB (n = 347)	TB-Diabetic (n=129)	TB-Not Diabetic (n = 218)
	n (%)	n (%)	n (%)
HIV status			
ND	25 (7.2)	11 (8.5)	14 (6.4)
Negative	245 (70.6)	91 (70.5)	154 (70.6)
Positive	77 (22.2)	27 (20.9)	50 (22.9)
Regimen			
2RHZE/4RH	315 (90.8)	120 (93)	195 (89.4)
2SRHZE/1RHZE/5RHE	32 (9.2)	9 (7)	23 (10.6)
Smear Conversion Month 6			
Missing data	1 (0.3)	0 (0)	1 (0.5)
ND	51 (14.7)	23 (17.8)	28 (12.8)
Negative	292 (84.1)	104 (80.6)	188 (86.2)
Positive	3 (0.9)	2 (1.6)	1 (0.5)
Treatment Outcome			
C	292 (84.1)	104 (80.6)	188 (86.2)
D	6 (1.7)	3 (2.3)	3 (1.4)
F	3 (0.9)	2 (1.6)	1 (0.5)
NC	4 (1.2)	3 (2.3)	1 (0.5)
OOC	10 (2.9)	5 (3.9)	5 (2.3)
TC	23 (6.6)	7 (5.4)	16 (7.3)
TO	9 (2.6)	5 (3.9)	4 (1.8)
	Median (IQR)	Median (IQR)	Median (IQR)
Glu	3.6 (1.2)	3.7 (2)	3.5 (1)
Blood Urea Nitrogen (BUN)	3.7 (1.4)	3.9 (1.3)	3.6 (1.5)
Creatinine	87 (26)	86 (28.5)	88 (25.15)
Weight	54 (12)	55 (12.2)	54 (12)
Height	1.68 (0.13)	1.68 (0.11)	1.67 (0.14)
BMI	19.06 (3.96)	19.12 (3.67)	19.05 (4.02)

(C=Cured; D=Died; F=Failure; NC=Not Complete; OOC=Out of Control (Loss to follow up); TC= Treatment Complete; TO=Transfer Out)

4.2 Prognostic factors among TB and TB/DM comorbidity among patients on the short-course regimen.

Univariate analysis was used to compare each potential risk factor in the TB and TB/DM patients by the Pearson chi-square test of independent association. Further, multiple logistic regression was used to identify the independent factors that are associated with the outcome of TB/DM. Of the factors shown, none proved to be statistically significant at both bi- and multivariate levels as below Table 4.7.

Table 4.7: Univariate analysis for Potential risk factors

Variable	TB - DB (n=129)	TB (n = 218)		
	n (%)	n (%)	OR (95%CI)	AOR (95%CI)
Age categories				
Under 30	59 (45.74)	104 (47.71)	Ref	
31-40	54 (41.9)	76 (34.9)	1.39 (0.85-2.29)	1.62 (0.91-2.87)
41-50	9 (7)	31 (14.2)	0.65 (0.31-1.37)	0.78 (0.35-1.77)
Employed				
Yes	79 (61.2)	154 (70.6)	0.64 (0.39-1.03)	0.74 (0.42-1.32)
No	50 (38.8)	64 (29.4)		
Ever drank alcohol				
No	53 (41.1)	84 (38.5)	Ref	
Yes	50 (38.8)	105 (48.2)	0.66 (0.40-1.11)	0.62 (0.32-1.20)
Ever smoked				
Yes	21 (60)	46 (71.9)	0.73 (0.43-1.24)	1.06 (0.51-2.17)
No	9 (25.7)	15 (23.4)	Ref	Ref
Household members				
<2persons	65 (50.4)	129 (59.2)		
>2persons	64 (49.6)	89 (40.8)	1.36 (0.86-2.17)	1.09 (0.64-1.85)
Regimen				
2RHZE/4RH	120 (93)	195 (89.4)		
2SRHZE/1RHZE/5RHE	9 (7)	23 (10.6)	0.64 (0.27-1.53)	0.50 (0.18-1.39)
Median (IQR) BUN	3.9 (1.3)	3.6 (1.5)	1.05 (0.87-1.28)	1.13 (0.90-1.41)

***(TB and TBDM Patients** *only variables significant at p-value <0.2 in the univariate analysis are listed +based on the number of patients who ever smoked)*

Table 4.8: below shows that logistic regressions indicated that the number of cigarettes smoked per day and the value of the BUN were significant risk factors of developing DM among TB patients (results in Table 4.8)**.** Those patients taking <20 cigarettes a day are less likely to develop DM compared to those that take >20 cigarettes a day (P values = 0.045). A unit increase in BUN increases the odds of diabetes by 1.211 times. The rest of the variables included in the univariate analysis were not significant risk factors for developing DM. None of the risk factors were significant in the multiple logistics regression, as shown below for DM in TB patients.

Table 4.8: Logistic regression analysis of risk factors*

Variable	OR	p-value	95% C.I	
			Lower	Upper
Blood Urea Nitrogen (BUN)	1.211	0.044	1.005	1.46
No of cigarettes >20	0.304	0.045	0.095	0.972

(Diabetes in TB patients *****only significant risk factors are listed)

Univariate chi-square test indicating that the regimens, employment status, ever taken alcohol, the number of cigarettes smoked per day, age categories and the number of household members were associated with having or not having DM among TB patients at p-value <0.2

Table 4.9 below shows the conversion time of smear from smear-positive to smear-negative among the TB and TBDM comorbidity patients

Table 4.9: Comparison of smear conversion time among patients

	N	Mean	Median	Std. Deviation	Std. Error Mean	P-value
Diabetic	108	3.2037	3.0	.65223	.06276	0.365
Non-diabetic	197	3.1421	3.0	.51518	.03671	

4.3 Molecular Clustering of Patients with MTB strains cultured from the Diabetic and Non-Diabetic Newly diagnosed TB positive cases

Figure 4.1 below shows the Restriction enzymes that were used to digest DNA molecules before using agarose gel electrophoresis to visualize the presence and quality of the fragments on the patients' samples. Plate 4.1 indicates agarose gel electrophoresis DNA being analyzed before spoligotype analysis to look for the presence and quality of the PCR.

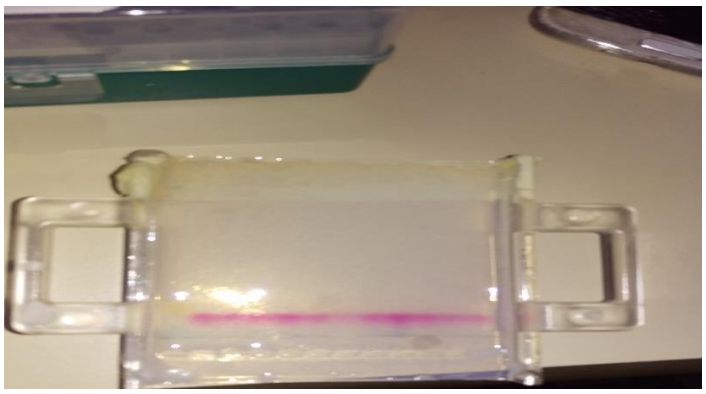

Plate 4.1: Preparation of TB DNA in Gel electrophoresis:

Plate 4.2 below shows spacer oligos of the MTB Strains. A typical spoligotyping result of Negative control, *M. tuberculosis* H37Rv, *M. Bovis* BCG P3, and 38 different clinical isolates. A membrane with 43 spacer oligonucleotides was used (vertical lines). The spacer oligonucleotides were derived from the spacers of *M. Bovis* BCG P3, M. tuberculosis H37Rv as controls, and other isolates for identification. Spoligotyping data from *Mycobacterium tuberculosis* clinical isolates from Nairobi and Kiambu counties. Isolates presented only once (orphan isolates) are designated according to their country of origin. Spoligotype International Types (SIT) geographical distribution among the patients in this study below shows that a total of 282 isolates matched a pre-existing shared-type in the SITVIT2 database with 249 strains identified while 33 isolates were unique (orphan strains) and were not distinguished from the SIT, which brings the number of unclustered isolates in this study to 33/282

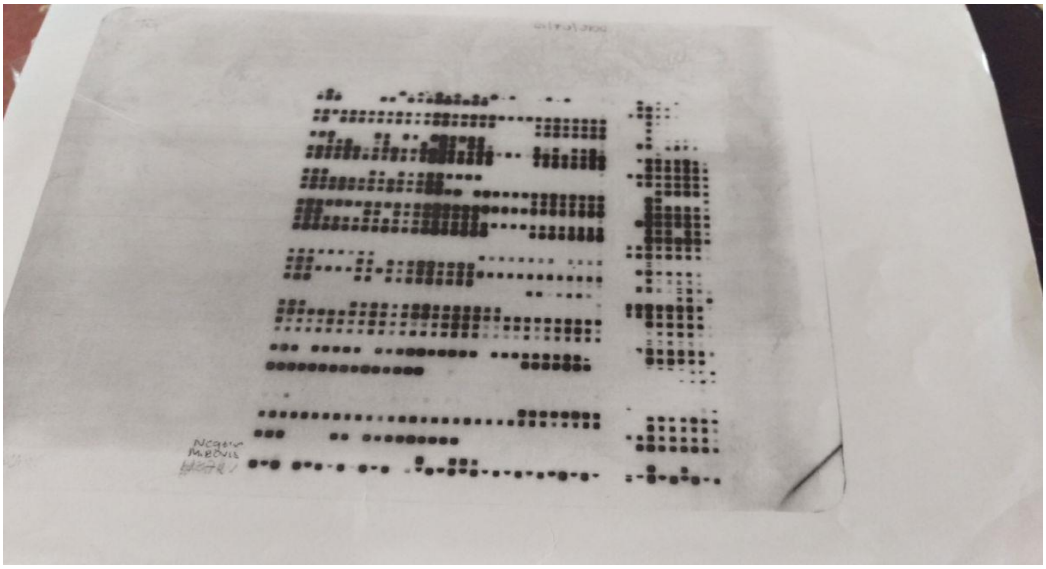

Plate 4. 2: Spoligotyping patterns on a film

Plate 4.2 above also shows a sample set of the spoligo data as it had been converted to octal and binary for analysis of *Mycobacterium Tuberculosis.* This was entered in the global database lineage ever to have been investigated in the data bank. These were identified, and the population structure was reconstructed for the two Counties for evolutionary among the recognized globally at SpolDB4.

From the identified lineages, the spatial distribution in the two counties of the strain haplotypes and allelic diversity, as well as the localization of the ancestral, were shown in the historical designated lineage known globally and their geographical zone. 7 major spoligotype based families were identified: Beijing clade (n=55 as East Asia Beijing), Latin American and Mediterranean (LAM) (n=80 as Euro America), a European family X clade (n=4 as Euro America), Central and Middle Eastern Asia (CAS) (n=63 as Indo oceanic), U clade (n=7 as M bovis), a default family T clade (n=1 as M Africanum), Menu clade(n=3 as West African 1 and n=4 as West African 2), and Orphans (n= 157) as shown below Table 4.10

Table 4.10: Spoligotype International types (SIT) geographical distribution.

NO.	Strain	Spoligotype43	SIT	Year	Country
1	KENO1	701200000000000	Orphan	2016	KENYA
2	KENO2	777777777760771	53	2016	KENYA
3	KENO3	777777777760771	53	2016	KENYA
4	KENO4	000000000000771	269	2016	KENYA
5	KENO5	000000000003771	1	2016	KENYA
6	KENO6	777777606060771	59	2016	KENYA
7	KENO7	777777777760771	53	2016	KENYA
8	KENO8	777777777760751	612	2016	KENYA
9	KENO9	000000000000000	2669	2016	KENYA
10	KENO10	701245400001771	Orphan	2016	KENYA
11	KENO11	000000000001561	Orphan	2016	KENYA
12	KENO12	774037777760700	31	2016	KENYA
13	KENO17	711343703370771	Orphan	2016	KENYA
14	KENO18	000000000000000	2669	2016	KENYA
15	KENO19	000000000000000	2669	2016	KENYA
16	KENO20	777737777760771	37	2016	KENYA

Figure 4.1 below shows the Bio geographical structure of MTB lineage Identified: RA x ML maximum likelihood phylogenetic tree was constructed for all the 282 (81%) DNA and spoligotype color-coded. Radial phylogram representation of the best-scoring maximum likelihood phylogenetic tree constructed using RA x ML software. Samples are color-coded by spoligotype strain showing a clear correlation of SNP and spoligotype clustering. The main lineage and sub-lineage defining nodes were initially identified from the tree, based on the spoligotypes in each clade.

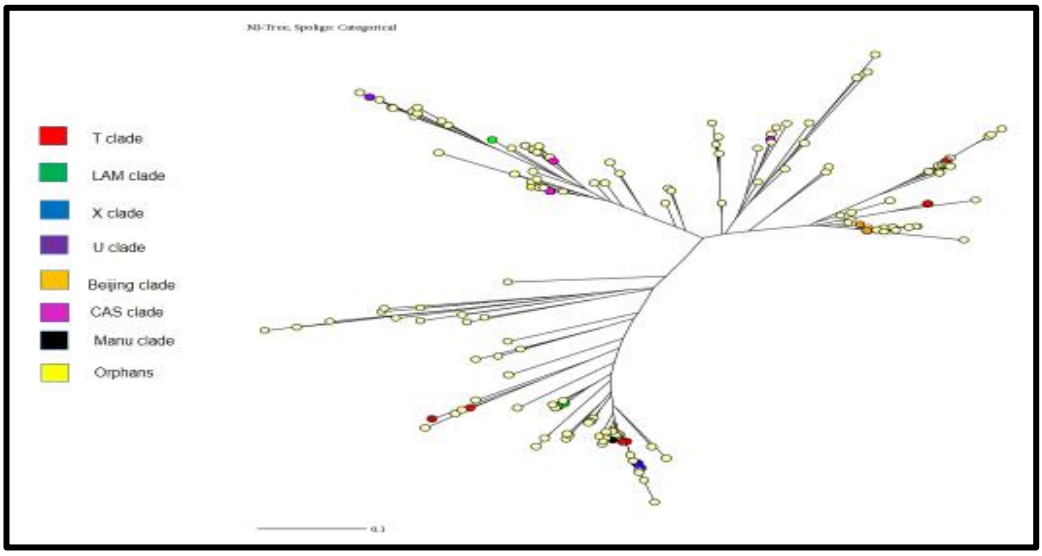

Figure 4. 1: Bio geographical structure of MTB lineage identified

Table 4.11 below shows the difference between the MTB lineages (prevalence of different circulating lineages) within the two counties with the treatment outcome among the patients who participated in the study. This shows the distribution of phylogenetic lineages versus treatment outcomes out of which most patients were cured (217, 87.15%).

Table 4.11: Difference in various clinical outcomes among different TB lineages

	Outcome							
Lineage	C	D	F	NC	OOC	TC	TO	Total
East Asia Beijing	55 (90.2)	0 (0.0)	1 (1.6)	0 (0.0)	2 (3.3)	2 (3.3)	1 (1.6)	61 (24.5)
Euro America	84 (85.7)	0 (0.0)	0 (0.0)	4 (4.1)	2 (2.0)	6 (6.1)	2 (2.0)	98 (39.4)
Indo oceanic	63 (88.7)	1 (1.4)	1 (1.4)	0 (0.0)	1 (1.4)	3 (4.2)	2 (2.8)	71 (28.5)
M Africanum	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.4)
M bovis	7 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (10.0)
West Africa 1	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	4 (1.6)
West Africa 2	4 (57.1)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	1 (14.3)	7 (2.8)
Total	217	2	2	4	6	12	6	249

C-Completed, D- Died, F – Failure, NC- Not Complete, OOC – Out of Control, TC– Treatment Complete, TO- Transfer Out. The results presented in tables using frequencies with corresponding percentages within parenthesis. Treatment failures were reported in East Asia Beijing and Indo oceanic

Table 4.12 below shows the circulating MTB lineages among both the Tuberculosis and Tuberculosis Diabetes Mellitus patients among the participating patients in the study in the two counties. The most common lineages among both the diabetic and non-diabetic patients were the East Asia Beijing, Euro America and Indo Oceanic.

Table 4.6: Difference in TB Lineage among diabetes and non-diabetes patients

	Diabetes condition		
Lineage	Yes	No	Total
East Asia Beijing	46 (75.4)	15 (24.6)	61 (24.5)
Euro America	62 (63.3)	36 (36.7)	98 (39.4)
Indo oceanic	54 (76.1)	17 (23.9)	71 (28.5)
M Africanum	0 (0.0)	1 (100.0)	1 (0.4)
M bovis	4 (57.1)	3 (42.9)	7 (2.8)
West Africa 1	3 (75.0)	1 (25.0)	4 (1.6)
West Africa 2	4 (57.1)	3 (42.9)	7 (2.8)
Total	**173**	**76**	**249**

Table 4.13: below shows the prevalence of the circulating MTB strains and lineage in both Kiambu and Nairobi counties in Kenya. Only 1 (100%) M Africanum lineage was found out of the two counties, and most patients had the Euro America lineage (98, 39.4%).

Table 4.13: Difference in TB lineage among diabetics and non-diabetics

	Country		
Lineage	Kiambu	Nairobi	Total
East Asia Beijing	13 (21.3)	48 (78.7)	61 (24.5)
Euro America	10 (10.2)	88 (89.8)	98 (39.4)
Indo oceanic	12 (16.9)	59 (83.1)	71 (28.5)
M Africanum	0 (0.0)	1 (100.0)	1 (0.4)
M bovis	1 (14.3)	6 (85.7)	7 (2.8)
West Africa 1	2 (50.0)	2 (50.0)	4 (1.6)
West Africa 2	2 (28.6)	5 (71.4)	7 (2.8)
Total	40	209	249

The tables below show the MTB lineage and the treatment outcome among all patients who, after screening, were identified as Normal, Prediabetes, and as having diabetes mellitus (4.14, 4.45, and 4.16).

Table 4.14: Strain Characterization and the various Normal DM outcomes

	DM=<7 (Normal)				
	Outcome				
TB Lineage 2	Cured	Treatment	Failed/Died	Unknown	Total
East Asian(Beijing)	44(89.79)	2(4.08)	1(2.04)	2(4.08)	49(100)
Euro -American	58(84.05)	5(7.24)	0(0)	6(8.69)	69(100)
Indo- Oceanic	54(93.1)	1(1.72)	1(1.72)	2(3.44)	58(100)
M. African	5(71.42)	0(0)	1(14.28)	1(14.28)	7(100)
M. bovis	5(100)	0(0)	0(0)	0(0)	5(100)
Orphan strain	26(78.78)	3(9.09)	2(6.06)	2(6.06)	33(100)
Spoligotyping not done	31(73.8)	6(14.28)	2(4.76)	3(7.14)	42(100)
Total	223(84.79)	17(6.46)	7(2.66)	16(6.08)	263(100)
Pearson chi2(18) =20.0866 Pr = 0.328					

(*The pr value of 0.328 is > 0.05. Indicating no relationship between the lineage and the clinical outcome in the table above)*

Table 4.7: Strain Characterization and the various Prediabetes clinical outcomes

	DM=7 -10 (Pre Diabetes)				
			Outcome Failed/		
TB Lineage 2	Cured	Treatment	Died	Unknown	Total
East Asian(Beijing)	5(100)	0(0)	0(0)	0(0)	5(100)
Euro -American	19(90.47)	1(4.76)	0(0)	1(4.76)	21(100)
Indo- Oceanic	9(81.81)	1(9.09)	1(9.09)	0(0)	11(100)
M. African	2(50)	2(50)	0(0)	1(25)	4(100)
M. bovis	1(100)	0(0)	0(0)	0(0)	1(100)
Orphan strain (unknown)	5(83.33)	1(16.66)	0(0)	0(0)	6(100)
Spoligotyping not done	9(69.23)	1(7.69)	1(7.69)	2(15.38)	13(100)
Total	50(81.96)	5(8.19)	2(3.27)	4(6.55)	61(100)
Pearson chi2 =12.2426 Pr = 0.834					

Table 4.8: Strain Characterization with Diabetes clinical outcomes

DM=>10 (Diabetes Mellitus)				
			Outcome	
TB Lineage 2	Cured	Treatment	Unknown	Total
East Asian(Beijing)	5(83.33)	0(0)	1(16.66)	6(100)
Euro -American	7(87.5)	0(0)	1(12.5)	8(100)
Indo- Oceanic	0(0)	1(50)	1(50)	2(100)
M. African	1(100)	0(0)	0(0)	1(100)
M. bovis	1(100)	0(0)	0(0)	1(100)
Orphan strain	2(100)	0(0)	0(0)	2(100)
(unknown)				
Spoligotyping not done	3(100)	0(0)	0(0)	3(100)
Total	19(82.6)	1(4.34)	3(13.04)	23(100)
Pearson's Chi2 (12) = 15.5015 Pr = 0.215				

Tables 4.17 and 4.18 below show the summary of all the samples which were done DNA finger typing (spoligotyping analysis) with both favorable and unfavorable outcomes among both TB and TB/DM

Table 4.9: Summary of the results obtained using SpolDB; (A)

		Total	DNA Fingerprints			
Variable	Level	N=340 (%)	Not done n=58 (%)	Done, fully Characterized n=241 (%)	Orphan strains n =41 (%)	P-value
Outcomes	favorable	308 (90)	50 (86)	221 (92)	37 (90)	0.761
	Unfavorable	13 (4)	3 (5)	8 (3)	2 (5)	
	Unknown (D/LD/TO)	19 (6)	5 (9)	12 (5)	2 (5)	
Time-to-negative smears*	Baseline (positive)	335/336 (99)	58 (100)	236/237 (100)	41 (100)	0.811
	2/3-months (positive)	32/307 (10)	5/49 (10)	23/220 (10)	4/38 (11)	0.998
	5-months	4/290 (1)	1/44 (2)	2/212 (1)	1/34 (3)	0.559
	6-months	3/290 (1)	1/44 (2)	1/212 (0)	1/34 (3)	0.283
Diabetes Mellitus (DM)	Non-DM	258 (76)	42 (72)	183 (76)	33 (80)	0.798
	Pre-DM	60 (18)	13 (22)	41 (17)	6 (15)	
	DM	22 (6)	3 (5)	7 (7)	2 (5)	
HIV test result	Positive	77 (23)	12 (21)	56 (23)	9 (22)	0.941
	Negative	238 (70)	41 (70)	169 (70)	28 (68)	
	Not Done	25 (7)	5 (9)	16 (7)	4 (10)	
TB regimen	2HRZE/4HR	308 (91)	52 (90)	217 (90)	39 (95)	0.568
	2SHRZE /1HRZE/ 5HRE	32 (9)	6 (10)	24 (10)	2 (5)	
County	K	56 (17)	7 (12	39 (16)	10 (24)	*0.037*
	N	283 (83)	51 (88)	202 (84)	30 (73)	
	Missing	1 (0)	0	0	1 (2)	
Gender	Female	96 (28)	8 (14)	79 (33)	9 (22)	*0.010*
	Male	244 (72)	50 (86)	162 (67)	32 (78)	

Table 4.10: Summary of the results obtained using SpolDB; (Section B)

		Total	DNA Fingerprints			
Variable	Level	N=340 (%)	Not done n=58 (%)	Done, fully Characterized n=241 (%)	Orphan strains n =41 (%)	P-value
Ever smoked	Yes	97 (29)	20 (35)	66 (27)	11 (27)	0.838
Age (years)	(mean [SD])	32.11 (8.80)	32.15 (9.13)	32.18 (9.04)	31.60 (6.81)	0.979
Weight (KG)	(mean [SD])	54.61 (10.32)	54.61 (11.47)	54.86 (10.34)	53.14 (8.39)	0.615
BMI (KG/m^2)	(mean [SD])	19.55 (3.91)	19.37 (3.56)	19.71 (4.11)	18.93 (3.23)	0.456
Blood glucose	(mean [SD])	3.61 (1.19)	3.81 (1.23)	3.59 (1.18)	3.39 (1.20)	0.220
HbA1c (%)	(mean [SD])	5.75 (2.22)	5.64 (2.42)	5.82 (2.18)	5.54 (2.21)	0.699
BUN	(mean [SD])	3.76 (1.17)	4.1 (1.36)	3.68 (1.08)	3.73 (.32)	0.172
Creatinine	(mean [SD])	89.39 (20.33)	96.56 (20.37)	87.99 (19.92)	87.46 (21)	*0.012*

Figure 4.2 below shows the clinical outcomes from the two counties, among the Diabetes Mellitus, Prediabetes, and normal blood glucose. While the last graph TB lineage and their clinical outcomes as unknown, unfavorable and favorable among the study subjects

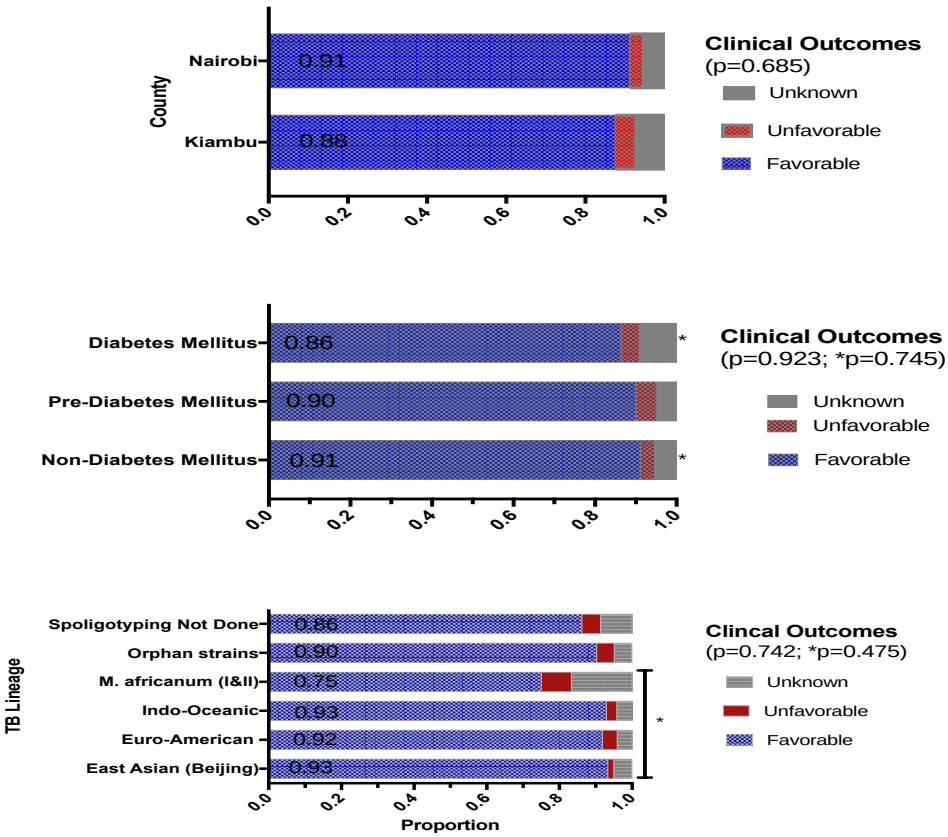

Figure 4.2: Significance of the County, DM Status and TB lineage on clinical outcome

4.4 Hemoglobin A1C (HbA1C) cut-off thresholds predictive of tuberculosis treatment outcomes and risk factors by use of CART

Figure 4.3 below shows the distribution of glycosylated hemoglobin (HbA_{1C}), blood glucose, and weight by observed outcomes. The mean HbA_{1C} levels (**Figure 6.4A**), and blood glucose levels (**Figure 6.4D**) were not significantly different between treatment complete, microbiologic cure, microbiologic failure, transfer out and out of control outcomes. Treatment complete and microbiologic cure outcomes were combined to form composite Favorable (A) outcomes, while the others were combined to form Unfavorable (B) outcomes. The figure shows that the mean and median HbA1c (interrupted arrow) in enrolled whose mean DM (full arrow) patients were significantly lower than those of the hypothetical population

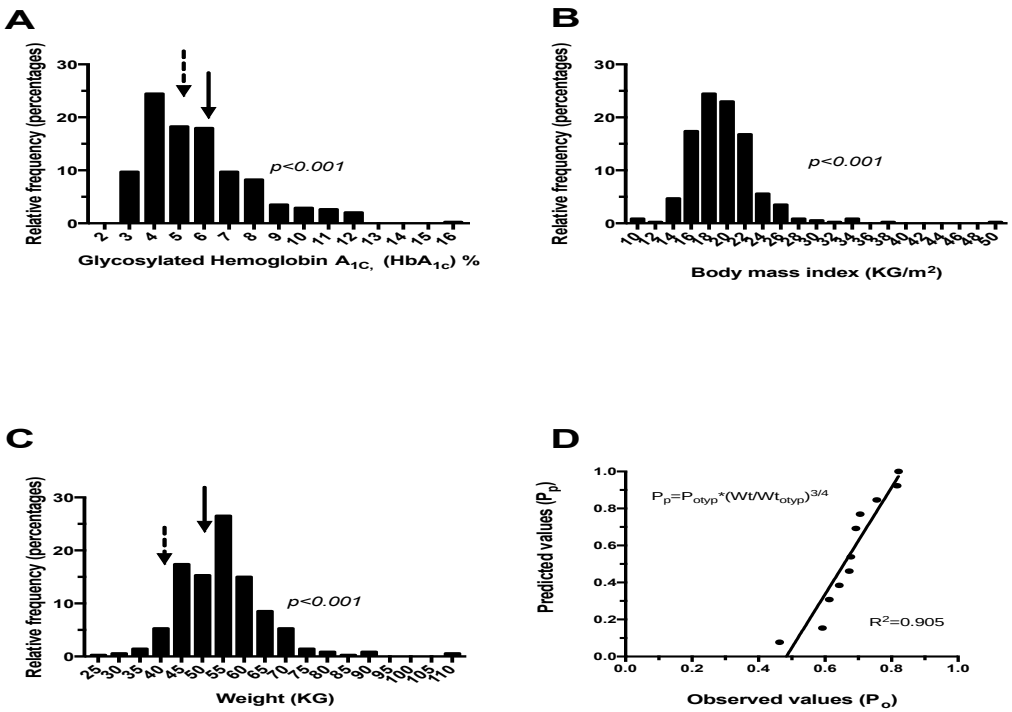

Figure 4.3: Distribution of HbA1c, BMI, and weight

Figure 4.4 below shows that both BMI and weight were very low for these patients given their mean age and height, as indicated in **Figures 4.4: B and C.** The low weight was due to under nutrition from tuberculosis disease. The BMI ranged between 10.36kg/m^2 and 49kg/m^2 and significantly correlated with weight, r=0.71. The mean HbA_{1C} levels and blood glucose levels (**Figure 4.4: D**) were not significantly different between treatment complete, microbiologic cure, microbiologic failure, transfer out and out of control outcomes. Treatment complete and microbiologic cure outcomes were combined to form composite Favorable (A) outcomes, while the others were combined to form Unfavorable (B) outcomes. We then used the HbA_{1C} variable, the blood glucose, and the weight variable to compare favorable and unfavorable outcomes in Figures 4.4: C, E, and H, respectively. The median (47Kg, range 39 to 56Kg) weight of 9 patients with a transfer out outcomes was significantly lower than those with other outcomes, and as a result, patients with unfavorable outcomes also had significantly lower weight than those with favorable outcomes (Figure 4.4: H). The cumulative probability for favorable outcomes with a

change in HbA_{1C} is in Figure 4.4: C, with blood glucose in Figure 4.4F and weight in Figure 4.4: A. The arrows in Figure 4.4: C indicates cut-off thresholds used to define diabetes mellitus in the general population**.**

Figure 4.4 shows that there was no correlation between blood glucose and HbA_{1C} across the clinical outcomes: Spearman's rho=0.035, p=0.515. None of the enrolled patients reported history or received prior treatment for DM. The weight had a borderline significance for transfers (**Figures 4.4: G**). In general, patients who weigh less had unfavorable clinical outcomes compared to their heavier counterparts at p= 0.011 (**Figures 4.4: H).** Cumulative probability for favorable outcomes or microbiological cure decreased wit increase in HbA1c; and, the nonlinear treads are even more apparent before the 6.5% HbA1c thresholds are reached as indicated in **Figure 4.4: C**

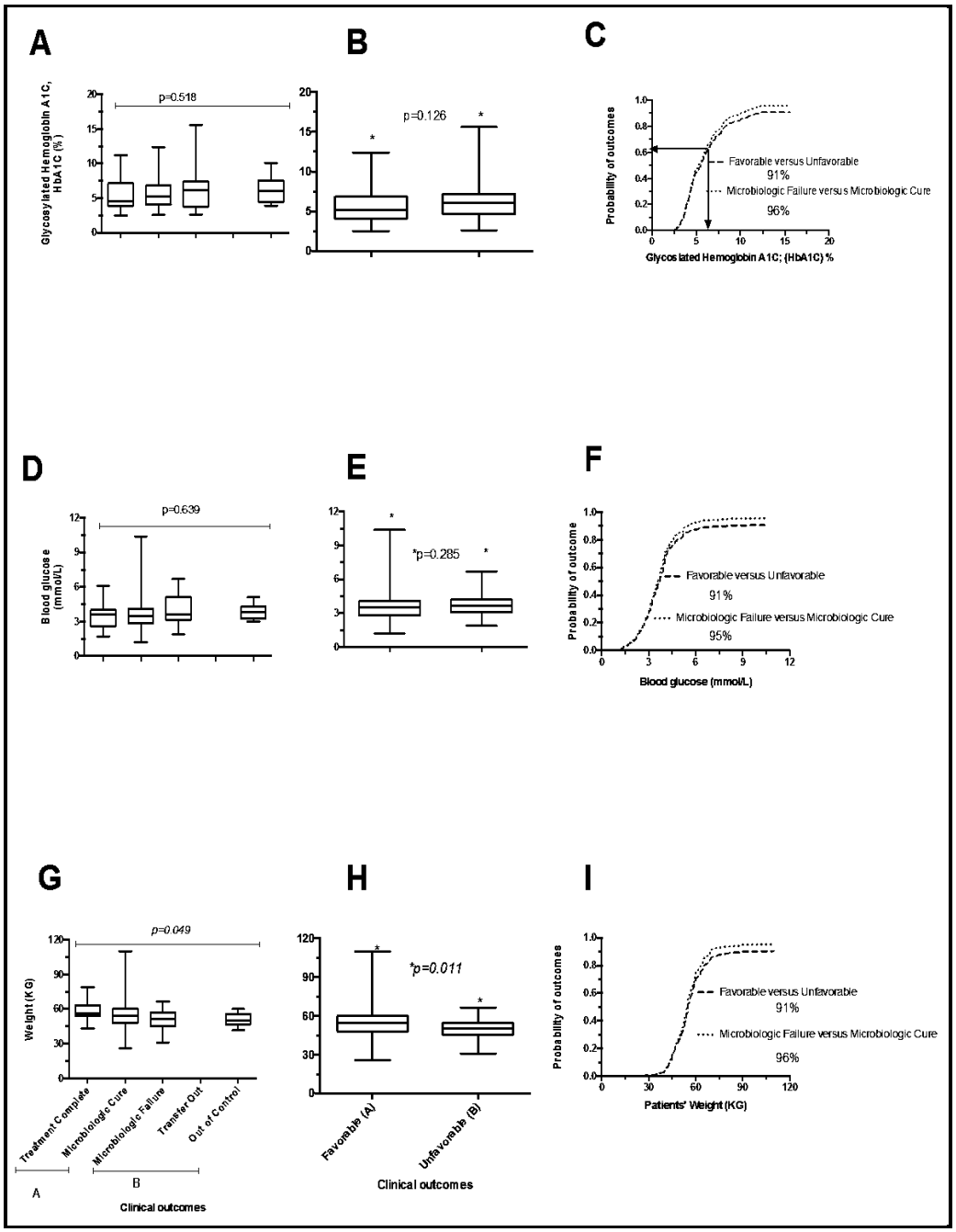

Figure 4.4: Distribution o HbA1c, blood glucose, and weight

Figure 4.5: below shows, the **Hemoglobin A1C** outcome with the nutritional status among the enrolled participants from both counties.

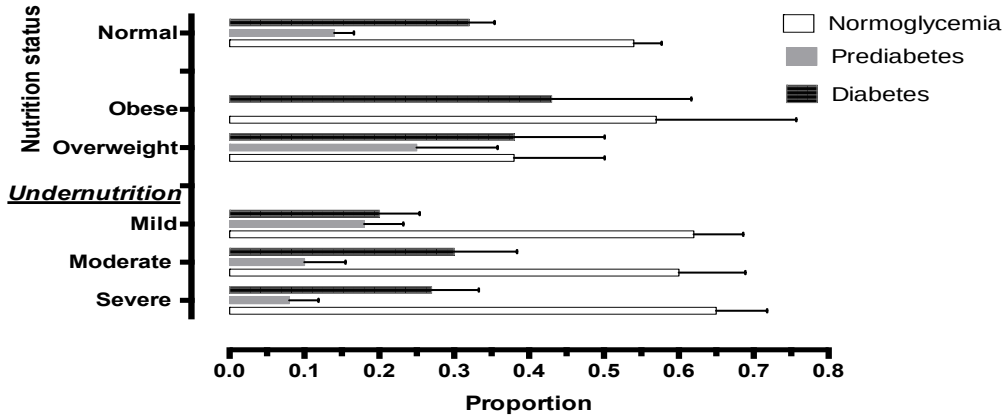

Figure 4.5: BMI status and the HbA1c outcomes

Figure 4.6: (A–D) shows the significant differential effect of weight on outcomes when stratified by treatment regimen and HbA_{1C}. As shown, among 308 patients who received the standard tuberculosis therapy regimen, 121/136 (89%) with weight <53.25KG compared to 162/172 (94%) with weight >53.25KG were less likely to have favorable outcomes. The relative risk was 0.71 (95% CI, 0.53-1.07), and the number needed treat was 6. (**Figure 4.6 A).** Similarly, it shows among the 215 patients with HbA1C>4.55%, 75/92 (82%) with weight <53.25KG versus 114/123 (93%) with weight >53.25KG, had favorable outcomes. The relative risk was 0.61 (95% CI, 0.45-0.88), and the number needed to treat was 4 **(Figure 4.6 D).**

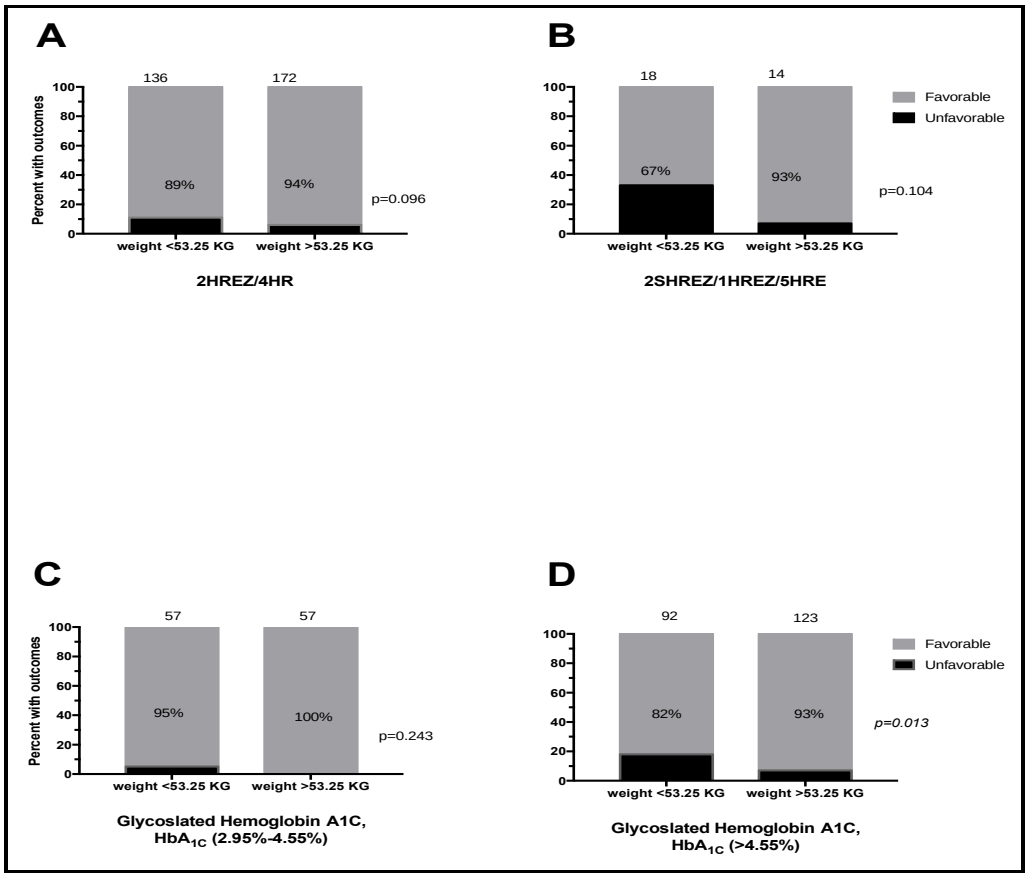

Figure 4.6: Frequency distribution of HBA1c and weight

Table 4.19: below shows that the probability of unfavorable clinical outcomes versus weight (kg), which was carefully described by ¾ power (R^2=0.905).This was in consistent with the effect of reduction in antibiotic concentration as seen below after analysis; The ROC score for each train sample was >0.95 and misclassification rates <0.02 indicating that the model described the data well. On the other hand, ROC scores on test samples after cross validation was >0.65 for each of the model examined which is reassuring in prediction on future similar sample. We depict the pruned back optimal tree for the favorable versus microbiologic failure outcome that examined 321 patients in Table 4.20 below;

Table 4.11: Ranking variables important of Clinical Outcomes based on boosted CART

Model I		**Model II**	
N= 340		**N=321**	
Favorable n=308 (91%)		**Favorable n=308 (96%)**	
Unfavorable n=32 (9%)		**Microbiologic failure n=13 (4%)**	
Ranked Variable	Relative Score	Ranked Variable	Relative Score
1. HBA_{1C}	100	1. HBA_{1C}	100.00
2. Regimen	79.38	2. Regimen	72.61
3. Creatinine	70.09	3. Age	69.19
4. Body mass index	63.93	4. Weight	55.39
5. Blood urea nitrogen	62.47	5. Random blood glucose	49.87
6. Weight	62.63	6. Body mass index	48.74
7. Age	61.63	7. BUN	48.18
8. Random blood glucose	55.62	8. HIV positive results	46.51
9. HIV positive result	39.21	9. Ever smoker	39.69
10. Male gender	34.48	10. Creatinine	37.69
Model properties	ROC_{learn}=0.96±0.01 ROC_{test} = 0.65±0.06	Model properties	ROC_{learn}=0.97±0.01 ROC_{test} = 0.56±0.07

To further examine the nonlinear relationship between weight and other covariates of clinical outcomes, up to 200 boosted classification and regression trees (bCART) were run in the Tree Net software for each binary outcome. One way to interpret the classification tree was by tracking the 13 patients who failed therapy. Ten (77%) out of 13 patients who experienced microbiologic failure had HbA_{1C}>4.55%, and 8

(62%) of these had weight <53.25kg. Thus three HbA_{1C} cut-off levels depicting U-shaped patterns that interacted with both weight and BMI were identified by CART. When we applied the model to the entire cohort, 8/11 (73%) of patients with HbA_{1C} <2.95%, 111/114 (97%) with $HbA_{1C,}$ 2.95% ⇔ 4.55% and 189/215 (88%) of patients with HbA_{1c} >4.55% experienced microbiologic failure. The model confirms that HbA_{1c} thresholds were also statistically significant compared to treatment outcomes, as observed at the end of treatment. The other known risk factors for either tuberculosis or diabetes mellitus were not associated with these thresholds in Figure 4.6:

Figure 4.7: below also shows the simulation on how to interpret the classification tree by tracking the 13 patients who failed therapy. Ten (77%) out of 13 patients who experienced microbiologic failure had HbA1C>4.55%, while 8 (62%) of these had weight <53.25kg. CART identified the three HbA1C cut-off levels depicting U-shaped pattern that interacted with both weight and BMI.

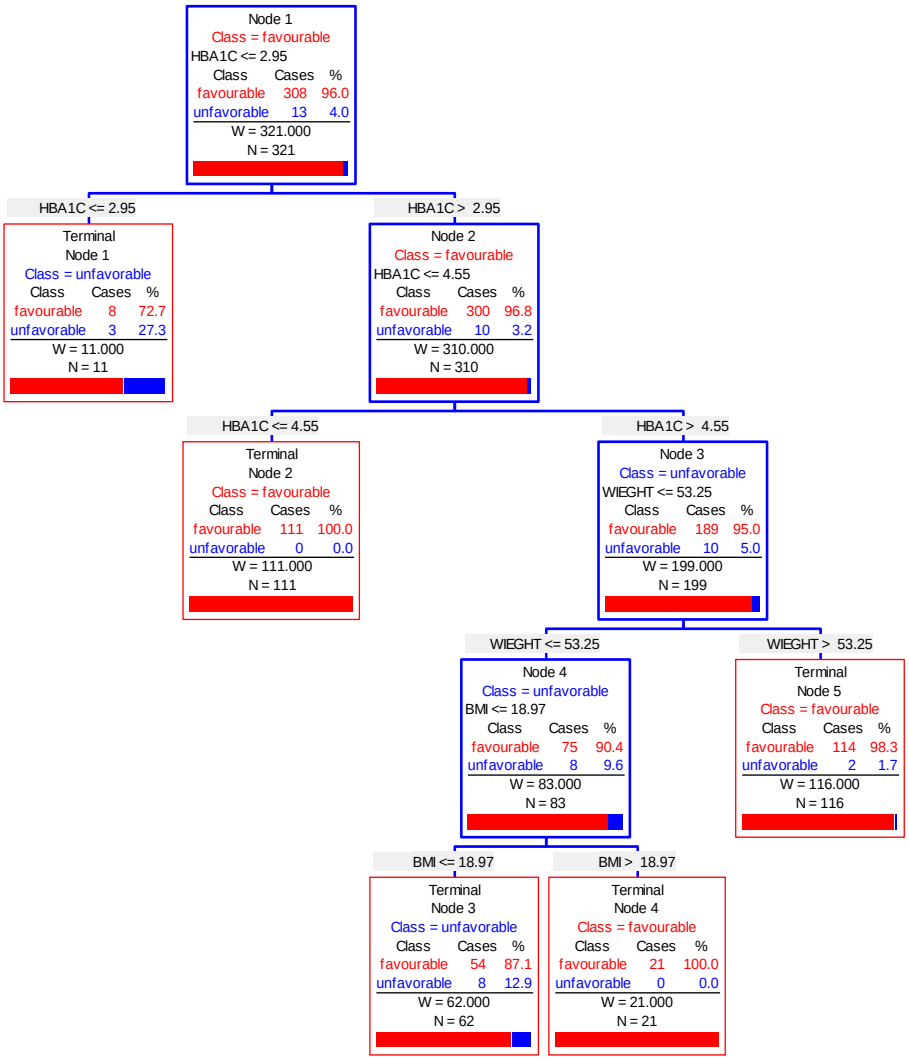

Figure 4.7: Classification and Regression Tree HbA1c cut-off depicting U shaped curve for both weight and BMI

The distribution of demographic and clinical characteristics was almost similar between patients whose outcomes were ascertained compared with those with an unknown outcome, as seen below. Tables 4.20 and 4.21 below confirm that these HbA_{1c} thresholds were also statistically significant for outcomes observed at the end of treatment. The other known risk factors for either tuberculosis or diabetes mellitus were not associated with these thresholds**.**

Table 4.12: HbA1C thresholds levels predict clinical outcomes in Patients treated for Mycobacterium Tuberculosis (TB).

Variable	Total N=340 (%)	HbA_{1c} (%) Levels			P-value
Clinical Outcomes		2.95-4.55 n=114 (%)	<2.95 n=11 (%)	>4.55 n=215	
At end of treatment					
Microbiologic failure	13 (4)	0	3 (27)	10 (5)	<0.001
Microbiologic cure	285 (84)	102 (89)	6 (55)	177 (82)	
Treatment complete	23 (7)	9 (8)	2 (18)	12 (6)	
Unknown (TO/OOC)	19 (6)	3 (3)	0	16 (7)	
Favorable outcome	308 (91)	111 (97)	8 (73)	189 (88)	0.002
Unfavorable outcome	32 (9)	3 (3)	3 (27)	26 (12)	
Modifiable factors					
Weight (mean[SD]) kg	54.61 (10.32)	53.64 (9.29)	50.82 (9.82)	55.32 (10.80)	0.173
BMI (mean[SD]) KG/m^2	19.55 (3.91)	19.35 (4.24)	18.36 (3.34)	19.72 (3.75)	0.417
Blood glucose (mean[SD]) mmol/L	3.61 (1.19)	3.52 (0.95)	3.22 (0.63)	3.67 (1.32)	0.301
Regimen I (2HRZE)	308 (91)	99 (87)	10 (91)	199 (93)	0.24
Regimen II (2SHRZE/1HRZE/5HRE)	32 (9)	15 (13)	1 (9	16 (7)	
Other factors					
Female	96 (28)	30 (26)	3 (27)	63 (29)	0.847
Male	244 (72)	84 (74)	8 (73)	152 (71)	
HIV test –positive	77 (23)	26 (23)	4 (36)	47 (22)	0.518
-negative	238 (70)	82 (72)	7 (64)	149 (69)	
-not done	25 (7)	6 (5)	0	19 (9)	

Table 4.21: Multivariate logistic regression analyses of associations between favorable outcomes at the end of Therapy and HbA1c levels and weight at baseline

Variable	Level	Odds ratio (95% CI)	P-value
$HbA1_C$ (%)	2.95%-4.55%	Referent	
	<2.95%	0.06 (0.01-0.39)	0.003
	>4.55%	0.18 (0.05-0.61)	0.006
Weight (Kg)	>53.25	Referent	
	<53.25	0.36 (0.16-0.76)	0.009
Constant		24.93 (7.74-80.30)	<0.001

Conclusion; this chapter has a comprehensive report of the study findings and results including: socio demographic and behavioral characteristics of the participants; as well as their dietary patterns; association of behavioral characteristics with risk of developing DM among TB patients; treatments outcomes, which were further analyzed MTB lineages to show 7 major spoligotype based families. East Asia Beijing, Euro America and Indo Oceanic lineages were the most prevalent among the TBDM and non TBDM patients. The Euro America lineage was the most common and was particularly found in Nairobi.

CHAPTER FIVE

DISCUSSION

5.1 The social demographic and Clinical characteristics of patients with TB and TB/DM comorbidity among the newly diagnosed TB patients

This study had three main findings; (i). The prevalence of DM (HBA1C > 6%) among TB infected patients was 37.2%. (ii). Patients regimens, employment status, alcohol intake, smoking, age, and household size were some of the factors associated with DM among TB patients at p-value <0.200 and (iii). The number of cigarettes smoked per day, and the value of the BUN were significant risk factors of developing DM among TB patients as indicated in Tables 4.8 and 4.9: which indicates that patients taking less than 20 cigarettes a day are less likely to develop DM compared to those that take more than 20 cigarettes a day (p-value = 0.045). A unit increase in BUN increases the odds of diabetes by 1.211 times. Though the meantime to conversion was slightly higher for the TB/DM patients compared to the TB patients, the difference was not statistically significant (p=0.365) as indicated in table 4.9.

The study findings do not vary significantly from other studies from studies conducted in other parts of the world where TB is endemic which have shown a higher prevalence of DM among patients infected with tuberculosis. In India, a population-based study conducted in six large cities from different regions estimated an age-standardized prevalence of type 2 diabetes among TB patients to be 39.1%. Similarly, cross-sectional studies estimated DM prevalence among TB patients to be 15.6%, 18.27%, and 38.6%, respectively with a prevalence of 15.8% in rural areas of Puducherry.

In the current study, the prevalence of DM in TB patients was 37.2%. Thus, the prevalence of DM in TB patients in this study is much higher than the prevalence seen in the general population, which ranges from 5.5% to 18.3 % (Christie Y Jeon & Murray, 2008). The higher prevalence is attributable to the fact that DM is known to impair mediated immunity that increases susceptibility to develop TB disease and increases the risk of relapse. A study in India by (Pande *et al.* 2018) found the

prevalence of diabetes in TB patients to be 25.3%, which was higher when compared to the prevalence of diabetes of 7.3% in the general population. In the Kerala, India, higher prevalence studies reported 44% though it had used a different diagnostic criterion, i.e., measurement of HbA1c >6.5% to diagnose diabetes (Balakrishnan et al., 2012; Safraj, Anish, Vijayakumar, Kutty, & Soman, 2012; Shastri, Naik, Shet, Rewari, & De Costa, 2013).

Another study in India found that 25.3% of TB patients had diabetes, and another 24.5% had pre-diabetes. Out of 25.3%, 9% newly detected, and 16% were diabetic. Moreover, the study revealed that men with TB are more likely to have diabetes than women. We did not observe the gender difference among pre-diabetic subjects. Nearly half of the subjects, who had TB and diabetes, had an infectious form of pulmonary TB. This particular finding and the growing number of people with diabetes in the community denote the possibility of a potential impact on TB control and TB treatment outcomes as well (Viswanathan et al., 2012).

The observed TBDM comorbidity prevalence in this study is higher compared to the findings of the previous systematic review conducted in 2010 (Christie Y. Jeon et al., 2010). It might be related to the increasing number of studies addressing TBDM comorbidity in the last six years. Also, there is a variation in the number of new DM patients identified by screening in different studies.

It might further be attributable to the differences in screening methods used and the prevalence of DM in the general population of the respective countries. However, the large proportion of newly identified DM patients suggests the identification of previously undiagnosed DM patients and highlights that screening TB patients for DM in the TB clinic is an essential public health intervention.

The WHO-IUALTD collaborative framework suggests that the type of screening and diagnostic tests for DM in TB patients should be adapted to the context of local health systems and the availability of resources (WHO, 2015). Using similar diagnostic cut-off, studies from China and Indonesia have demonstrated a lower prevalence (Alisjahbana et al., 2006; J. Gao, Zheng, & Fu, 2013; L. Gao, Zhou, Li, & Jin, 2010).

A study by Jain et al. reported a prevalence of impaired glucose tolerance (IGT) of 16.98%, and they had used oral glucose tolerance test to diagnose IGT, (Ghani *et al.,* 2006;Meisinger *et al.,* 2010)

5.2 Prognostic factors among TB and TB/DM comorbidity among patients on a short-course regimen

Patients regimens, employment status, alcohol intake, smoking, age, and household size were associated with DM among TB patients at p-value <0.200. Other studies found family history to be a significant factor in predicting DM among TB patients.

In Tamil Nadu, India, increasing age, body mass index (BMI), positive family history of diabetes, sedentary occupation, and presence of pulmonary TB were the factors significantly associated with diabetes among TB patients. Age, central obesity, low income, and smoking were the risk factors associated with pre-diabetes among TB patients (Viswanathan et al., 2012).

Similar to this study, cigarette smoking was associated with DM among TB patients (Ariyothai et al., 2004; Jali et al., 2013; Nissapatorn, Kuppusamy, Josephine, Jamaiah, & Rohela, 2006). In these studies, the average duration of smoking among smokers was 15.1 ± 12.9 years, while two-thirds of males consumed alcohol, with an average daily consumption of 295 ± 75.9 ml per day. We found out that there was a significant association between alcohol consumption and the prevalence of diabetes among TB patients. We attributed this to the high alcohol intake in the area. Other studies have also indicated age, family history of diabetes, and consumption of alcohol as having a significant association with DM.

This study did not have any significant association between BMI and Diabetes. Other studies have reported similar results (de Wit, van Straten, van Herten, Penninx, & Cuijpers, 2009; Logue et al., 2013; Pierce, Kuh, & Hardy, 2012). Fewer studies have reported that patients with TB and DM are significantly underweight and have more weight loss (de Wit et al., 2009; Logue et al., 2013) . Alisjahbana et al. (2007) Alisjahbana *et al.* reported a significantly higher median BMI in TB-DM patients when compared to non-diabetic TB patients.

The study could not establish a significant association of diabetes with sputum positivity conversion despite most of the studies indicating the same (Ariyothai et al., 2004; Jali et al., 2013; Nissapatorn et al., 2006). Other studies have shown a delay in sputum conversion observed in TB patients with diabetes (Kelly E. Dooley & Richard E. Chaisson, 2009; Blanca I. Restrepo, 2016). A retrospective study conducted in Texas also showed similar findings of more sputum smear-positive cases in subjects with diabetes and TB compared to non-diabetic subjects (B. I. Restrepo et al., 2007).

5.3 The Molecular clustering of patients with Mycobacterium tuberculosis strains cultured from the Diabetic and non-Diabetic newly diagnosed TB positive cases.

In countries like Kenya with a high burden of TB, studies determining the population structure of strains in different geographical areas are essential to monitoring the transmission cycle. Information regarding MTB strains circulating in Nairobi and Kiambu counties, situated at the center of Kenya with a massive influx of people across the country, is lacking. The only data available are from a previous study, including few isolates, thus not representative of MTB strains in Nairobi and Kiambu counties.

Findings from this study have identified and documented seven (7) different lineages with East Asia Beijing, Euro American, and Indo oceanic being the most dominant within the two counties. DM and counties were not significant factors in increasing the likelihood of PTB patients to cluster according to the genotype of the infecting *M. tuberculosis* bacillus. The difference in TB lineages, DM, and County of the patient was independent of the various clinical outcomes observed. Further, the study has demonstrated that patients with TB/DM are likely to have infections caused by the same bacteria as for TB patients.

The study findings showed that within the two counties, the most dominant strains were Euro American (39.4%), Indo oceanic (28.5%), and East Asia Beijing (24.5%). It indicates that the modern strains are the most circulating in the two counties and not ancestral lineages. The three clades were found to be the most prevalent

genotypes in East Africa, similar to other studies (Ahmed, Ehtesham, & Hasnain, 2009; Gutierrez et al., 2006; Thomas et al., 2011). The East Asia Beijing, Euro American, and Indo oceanic lineages occurred in all the countries within the region, but the highest frequencies of the Indo oceanic lineage were in Kenya, depicting it as the dominant strain within the two counties. This study's clustering rate of 33% was similar to that observed in the Ethiopian studies. (Abebe, Bjune, Ameni, Biffa, & Abebe, 2011; Tessema et al., 2001; Zhao et al., 2013). However, 125/374 (33.42%) isolates were not described before and regarded as orphans. It is higher compared to the Ghanaian study, that had 16 % (Asante-Poku et al., 2015). The divergent evolutionary trend of orphan cluster suggests that the cluster may be involved in the evolution of adaptive traits, and thus are not particularly candidate clusters for identifying lineage-specific adaptions.

The study findings documented that Diabetes Mellitus and the counties were not significant factors in increasing the likelihood of Pulmonary *Tuberculosi*s patients to cluster them according to the genotype of the infecting *Mycobacterium tuberculosis* bacillus. It indicates a non-clonal transmission with diverse strains contributing to TB dynamics (Alexander & De, 2007; Barniol et al., 2009; Trauer, Denholm, & McBryde, 2014). Also, there remains a need to type current isolates to get a clear understanding of the genotypic population structure of TB strains and its transmission dynamics. The dense clustering among the three strains might also be due to the new transmission and rapid progression (Zhao *et al.,* 2013 Trauer *et al.*,2014; Barniol *et al.,* 2009;). The few unique genetic patterns observed were likely due to low reactivation of latent infection or almost non-existence recent transmission from patients out of the period or area under study (Ahmad, 2011; Barniol *et al.,* 2009; Trauer *et al.,* 2014). There is no relationship between DM and clustering of the cases also points to no convincing molecular epidemiological evidence of higher TB transmission among DM patients as in other settings (Thomas *et al.,* 2011; Zhao *et al.,* 2013). These settings have observed no association between DM and molecular clustering of PTB in the community at a pooled estimate of 0.84 (CI 95% 0.40±1.72) (Tessema et al., 2001; Zhao et al., 2013).

The findings from this study did not indicate any relationship between the TB lineages, DM, and County of the patient to the various clinical outcomes observed. Other studies have shown poorer outcomes among patients with TB and DM (Magee et al., 2013). Public health impact of the comorbidity is more significant in regions of low and middle income, where 84% of patients with DM live, many of whom are unaware of their condition (Ahmad, 2011; Magee et al., 2013). We attribute a lack of association between the DM and poor clinical outcomes in this study to the high economic status of the two counties. It also indicates that the two counties almost have similar health systems and economic status.

5.4 Hemoglobin $A1_C$ ($HbA1_C$), cut-off thresholds predictive of poor tuberculosis treatment outcome and associated risk factors by use of CART

The study findings show that there was no significant difference in demographic and clinical characteristics between patients whose outcomes were ascertained compared with those with an unknown outcome, with exception to weight, smoking, and regimen I TB therapy. There was no correlation between blood glucose and HbA1C levels at baseline: Spearman's rho=0.035, p=0.515, while patients who weigh less had unfavorable clinical outcomes compared to their heavier counterparts at p= 0.011. Cumulative probability for favorable outcomes or microbiologic cure decreased with an increase in HbA1C.

The nonlinear trends were even more apparent before reaching the 6.5% HbA1C thresholds, as indicated. In this population, the distribution of HbA1C was not normal. Both its mean and median values of 5.76% and 5.28%, respectively, were significantly less than the threshold of 6.5% used to define DM, based on parametric and non-parametric sample tests, $p<0.001$. However, the mean difference of 0.74% (95% CI: 0.50-0.98) was within the 5.7% cut-off used to define pre-DM. It implies that we derived the study sample from a population of patients at risk of DM or pre-DM.

The low weight witnessed in this population was due to under nutrition from tuberculosis disease. The BMI of the study participants ranged from 10.36kg/m^2 to 49kg/m^2 with a significant correlation to weight, r=0.71. The relationship between

weight and predicted clinical outcomes were best described by ¾ fractal geometry law with R^2=0.905, indicative of a fractal with 0.75 dimensions.

We determined the typical weight for the sample as 53.25kg. The study findings suggest that complex nonlinear pharmacokinetics relationships and variability mediated by changes in patients' weight were driving outcomes as noted by Srivastava, Pasipanodya, Meek, Leff, and Gumbo (2011). 121/136 (89%) who had their weight less than 53.25Kg had favorable outcomes compared to 162/172 (94%), who had their weight more than 53.25Kg. The relative risk of weighing higher than 53.25Kg was 0.71 (95% CI, 0.53-1.07). Similarly, of the 215 patients with HbA1C, more than 4.55%, 75/92 (82%) had favorable outcomes with weight less than 53.25Kg compared to 114/123 (93%) with weight more than 53.25Kg. The relative risk of weighing above 53.25 Kg was 0.61 (95% CI, 0.45-0.88). The study findings are comparable to the Mexican study on pharmacokinetic variability of different anti-tuberculosis drug and impact of inadequate dosing that found the relative risk of weighing more than 59.05Kg was 0.74 (95% CI, 0.56-1.08) (Munoz-Torrico et al., 2017).

The ROC score for each sample was more than 0.95, and misclassification rates less than 0.02 indicating that the model used during analysis described the data well. On the other hand, ROC scores on test samples after cross-validation was more than 0.65 for each of the model examined, which is reassuring in prediction on future similar samples. Simulation on how to interpret the classification tree indicated that ten (77%) out of 13 patients who experienced microbiologic failure had $HbA1_C$ more than 4.55%, while 8 (62%) of these had weight less than 53.25kg. The three HbA1C cut-off levels are depicting a U-shaped pattern that interacted with both weight and BMI identified by CART. Glycated Hemoglobin A1c (HbA1c) thresholds were also statistically significant for outcomes observed at the end of treatment; the other known risk factors for either tuberculosis or diabetes mellitus were not associated with these thresholds.

The study findings demonstrated the complex and nonlinear effects of weight on clinical outcomes. The effect of weight probably is mediated via pharmacokinetic

variability of the different anti-tuberculosis drugs and the impact of inadequate dosing (Pasipanodya & Gumbo, 2011). In this study, results showed that when a change in weight is adjusted for, the odds ratio for a favorable outcome increasingly gets worse with each unit increase in HbA1C above 4.95%. The odds ratio was 0.18 (0.05-0.61) and remained robust even when the outcomes were restricted to treatment failure. Indeed this is similar to currently used criteria to distinguish individuals at higher risk for premature mortality from microvascular (retinopathy) or macro vascular (heart attacks) complications based on mostly on observational studies which have established association between those diabetes-related complications and glycosylated hemoglobin (HbA1c) with /out general poor glycemic control (Chen & Levy, 2016; Selvin et al., 2010).

Higher HBA1C thresholds were predictive of unfavorable outcomes in treated *tuberculosis* patients. Several studies, including meta-analyses, have shown that patients with diabetes mellitus and tuberculosis have worse outcomes for either disease, but there are no international guidelines on joint management and control of both diseases (Kaona, Tuba, Siziya, & Sikaona, 2004). One recent Mexican study revealed that, among diabetes mellitus patients who received standard tuberculosis treatment, 81% of recurrences and 77% of relapses, were infections caused by the same *Mycobacterium tuberculosis* isolate based on IS6110-band restriction fragment length polymorphism and spoligotyping fingerprint analyses (Niemann et al., 2000). The proportion of treatment failure in this study was 4.0 (95% CI, 2.2-6.8), similar to those seen in the more extensive Mexican study (Munoz-Torrico et al., 2017). Indeed, the proportion with tuberculosis recurrence will be significantly larger if the lower threshold is used.

Attempt to show the critical role of machine learning algorithms in answering non-specific but pertinent decision-making questions commonly asked in the clinic indicated how the output from CART could be used to generate risk ratios and adjusted odds ratio from multivariate logistic regression for comparison with existing literature. Some example is random forest (RF) that is not consistent if splits are performed to purity (Nicodemus, Malley, Strobl, & Ziegler, 2010; Touw et al., 2013). Therefore, some impurity within the machine-generated tree is required for

consistency. Comparatively, the performance of CART during simulation returned consistency well above most machine- learning approaches for classification and probability estimation.

5.5 Study Strengths

1. Since this study only included patients with no prior history of DM, we were able to assess impact of glucose levels within the previous 3 months prior to TB treatment
2. Standard tuberculosis treatment on patients at risk for Diabetes Mellitus in a routine program setting at the health facility. Thus, the findings contain practical lessons informative to both clinicians and programs in similar settings
3. This study, provides a window of opportunity for clinicians and researchers to carry out further study and follow the patients on early screening among newly diagnosed Mycobacterium Tuberculosis for DM as we may have underestimated the prevalence of DM and also cross transmission through contacts.
4. The study used objective (patient treatment records and records from TIBU register) to define treatment outcome and the data allowed control of various risk factors.
5. The use of qualitative findings to explain some of the differences found in the peri urban and the urban set up is an additional strength to this study

5.6 Study limitations

The study was carried out in one region. Similar studies should be carried out in other regions to gather more evidence.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

1. The prevalence of DM among newly diagnosed TB patients is 37.2% in the study counties. Screening strategies for DM should be implemented in other routine programs for all patients.
2. Patients regimens, employment status, Alcohol intake, smoking, age are associated with DM among the TB patients
3. Diabetes Mellitus is not significant factor in increasing the Molecular clustering among PTB Patients
4. Using fractal geometry relationships to drug PK, low weight has profound influence on failure of Anti-TB treatment among patients at risk for DM

6.2 Recommendation

1. Tuberculosis (TB) and Diabetes Mellitus (DM) programs should be integrated for early detection and treatment
2. TB Preventive measures should be implemented to reduce development of TB in DM patients
3. Glucose level in TB/DM patients should be controlled to ensure good treatment .outcome

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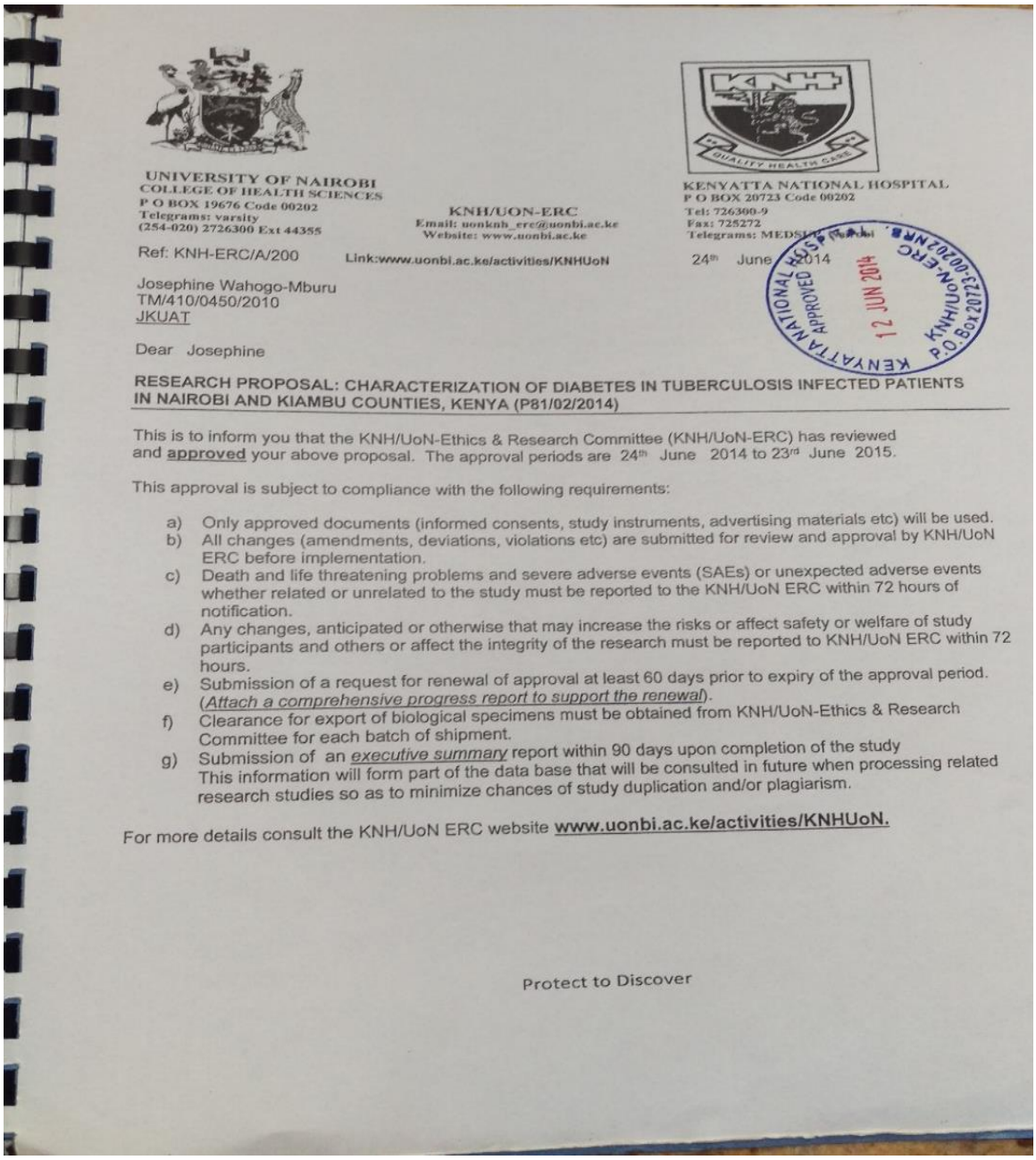
APPENDICES

Appendix I: Ethical Approval from KNH/UON-ERC



UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
(254-020) 2726300 Ext 44355

KNH/UON-ERC
Email: uonknh_erc@uonbi.ac.ke
Website: www.uonbi.ac.ke

KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSU

Ref: KNH-ERC/A/200 Link:www.uonbi.ac.ke/activities/KNHUoN

24th June 2014

KENYATTA NATIONAL HOSPITAL APPROVED 12 JUN 2014 P.O. Box 20723-00202 KNH/UoN-ERC

Josephine Wahogo-Mburu
TM/410/0450/2010
JKUAT

Dear Josephine

RESEARCH PROPOSAL: CHARACTERIZATION OF DIABETES IN TUBERCULOSIS INFECTED PATIENTS IN NAIROBI AND KIAMBU COUNTIES, KENYA (P81/02/2014)

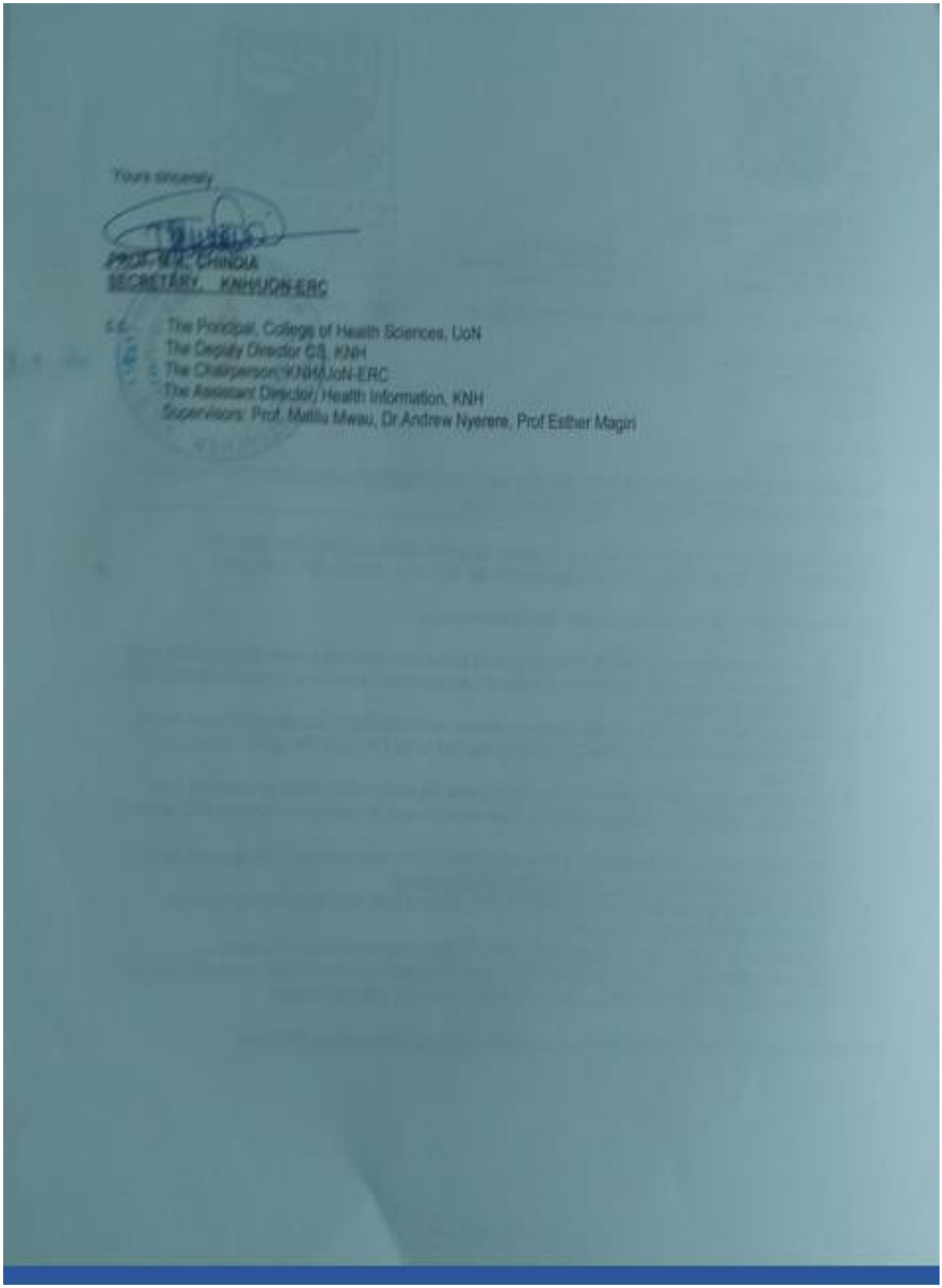
This is to inform you that the KNH/UoN-Ethics & Research Committee (KNH/UoN-ERC) has reviewed and **approved** your above proposal. The approval periods are 24th June 2014 to 23rd June 2015.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc) are submitted for review and approval by KNH/UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH/UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH/UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH/UoN-Ethics & Research Committee for each batch of shipment.
g) Submission of an *executive summary* report within 90 days upon completion of the study This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH/UoN ERC website **www.uonbi.ac.ke/activities/KNHUoN.**

Protect to Discover

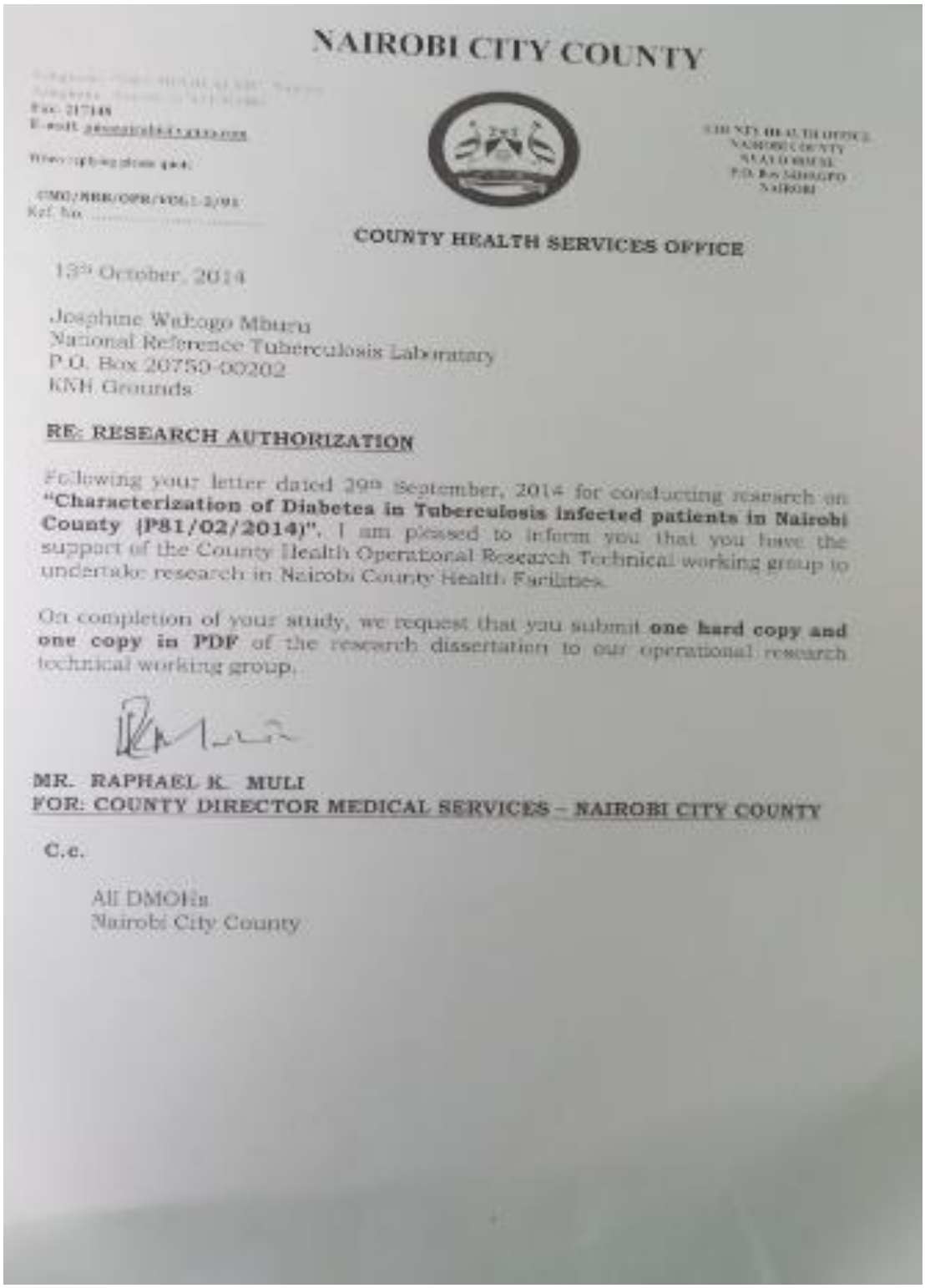


Yours sincerely

PROF. A.N. CHINDIA
SECRETARY, KNH/UON-ERC

c.c. The Principal, College of Health Sciences, UoN
The Deputy Director CS, KNH
The Chairperson, KNH/UoN-ERC
The Assistant Director, Health Information, KNH
Supervisors: Prof. Matilu Mwau, Dr Andrew Nyerere, Prof Esther Magiri

Appendix II: Nairobi County Ethical Clearance:



NAIROBI CITY COUNTY

COUNTY HEALTH SERVICES OFFICE

13th October, 2014

Josphine Wakogo Mburu
National Reference Tuberculosis Laboratary
P.O. Box 20750-00202
KNH Grounds

RE: RESEARCH AUTHORIZATION

Following your letter dated 29th September, 2014 for conducting research on **"Characterization of Diabetes in Tuberculosis infected patients in Nairobi County (P81/02/2014)"**, I am pleased to inform you that you have the support of the County Health Operational Research Technical working group to undertake research in Nairobi County Health Facilities.

On completion of your study, we request that you submit **one hard copy and one copy in PDF** of the research dissertation to our operational research technical working group.

MR. RAPHAEL K. MULI
FOR: COUNTY DIRECTOR MEDICAL SERVICES – NAIROBI CITY COUNTY

C.c.

All DMOHs
Nairobi City County

Appendix III: Kiambu County Ethical Clearance

MINISTRY OF HEALTH
KIAMBU COUNTY

Ref No.: KIAMBU COUNTY RESEARCH AUTHORITY/130 Date: 9th October, 2014

TO THE CONCERNED HEALTH FACILITY HEADS AND MANAGER,
COUNTY OF KIAMBU

RE: CLEARANCE TO CONDUCT RESEARCH IN THE COUNTY

Kindly note that we have received a request by Ms. Josephine Wahogo – Mbaro of ITROMID, Jomo Kenyatta University, intending to carry out research on the Characterization of Diabetes in Tuberculosis infected Patients in Nairobi and Kiambu Counties, Kenya. The research proposal has been reviewed by a competent scientific and ethical review board, KNH/UON-ERC as proposal No. KNH-ERC/A/209 and was approved on 12 June 2014. The study will run between 1st October 2014 and 1st October 2015 and any extension must be approved by a competent scientific and ethical review board.

The research requires close collaboration with your institution so that she can constitute a cohort of TB patients on whom she will carry out a diagnosis of diabetes and review of serial sputum as outlined in her proposal. Importantly, the work requires access to TB clinic and the laboratory. It has important implications in the provision of chronic care to patients with tuberculosis and thus attracts support from the County Health Research and Development Unit.

However, it is incumbent upon the institution where she is carrying out research to ensure that she adheres to the norms of conducting research in the institution. This note also accords her the duty to provide a feedback on her research to the county and health institutions where the research will be conducted at the conclusion of her research.

Dr M. N. Ndirangu MBChB, MphH, CRD
County Health Research & Development Unit,
KIAMBU COUNTY

CC
Chief Officer – Health Services,
KIAMBU COUNTY

Appendix IV: Informed Consent

I…….confirm that, I have been explained to the nature of the screening exercise and I confirm that I fully understand the nature of the study. I have also had the opportunity to ask questions about the study and I feel that I have received satisfactory answers to my questions. I am aware that in order to be accepted in the study I will have to undergo a Random blood glucose test after being Smear positive AFB to be enrolled and recruited for the study. I understand that my consent is entirely voluntary and that it does not mean that I have to join the study against my Willingness

---

Participant's Name: **Signature:**

DD/MM/YYYY:

_____Time:_____

Person Obtaining Consent:

I have explained the nature of the study, benefits and foreseen small Risks, which could

Occur or happen during the screening process to the Participant.

Name **_____**

Signature **_____**

Dd/mm/yyyy _____ Time_____

Principal investigator : Josephine Wahogo Mburu +254-722-784-603

Research Team : Josephine Wahogo Mburu +254-722-784-603

Prof. Ester Magiri JKUAT

Dr. Andrew Nyerere JKUAT

Prof. Matilu Mwau (JKUAT/ KEMRI)

Lead Clinical Team : Dr. Mwita Riro + 254 721 107 995

Kiambu Hospital

Appendix V: Study Patients Questionnaire English version

1. How old are you?

- Under 30
- 31–40
- 41–50
- Over 50

2. What is your gender?

- Male
- Female

3. What is the highest level of education you have completed?

- No formal schooling
- primary
- High school
- College
- Higher education (professional or post-graduate)

4. Do you currently have paid employment?

- Yes
- No

4b. If yes how much is your monthly income in Kenya shillings?

- < 500 ksh
- > 500- 1000
- >1001 - 5000
- >5001- 10000
- >10000

4c. How much do you spend per day?

5. How many people do you leave with (House hold members?)

- <2 persons
- > 2persons

5b. If more than 2 how many people per room?

5c. what is the size of the house that you leave in?

5d. what is your common (main) diet? and the BMI

- **Fats**
- **Sugars**
- **Meat**

6. How far do you live from the nearest health clinic or hospital?

- 0–10 KM
- 11–20 KM
- 21–30 KM
- More than 30 KM

7. Have you ever smoked cigarettes or any kind of Tobacco?

- Yes
- No

7b. if yes, how many cigarettes /Tobacco did /do you smoke per day on average?

- <less than 20 (pack) per day
- > than 20 (pack) per day on average

8. Have you ever or do you drink Alcohol? In Months.

- If yes, how long did you drink
- If yes, are you a current drinker

8b. if yes, how many days per week/ how much per day?

(One drink is equals to one beer or one glass of wine or one shot of hard liquor or mixed drink).

9. Where do you usually go if you are sick, or for treatment or general health problem?

- Private clinic
- Government clinic or hospital

- Traditional healer
- Clinic run by an nongovernmental organization or church
- Other:

10. How often do you generally seek health care at a clinic or hospital?

- Twice a year or more
- Once per year
- Other:

11. Where did you first learn about tuberculosis or Diabetes?

- Newspapers and magazines
- Radio
- TV
- Billboards
- Brochures, posters and other printed materials
- Health workers
- Family, friends, neighbors' and colleagues

- Religious leaders
- Teachers
- Other (please explain):

12. In your opinion, how serious is TB/DM disease.

- Very serious
- Somewhat serious
- Not very serious

13. How serious a problem do you think TB/DM is?

- Very serious
- Somewhat serious
- Not very serious

14. What are the signs and symptoms of TB/DM?

- Rash
- Cough
- Cough that lasts longer than 3 weeks
- Coughing up blood
- Severe headache
- Nausea
- Weight loss
- Fever

- Fever without clear cause that lasts more than 7 days
- Chest pain
- Shortness of breath
- Ongoing fatigue
- Do not know
- Other:

15. How can a person get TB? (Please check all that are mentioned.)

- Through handshakes
- Through the air when a person with TB coughs or sneezes
- Through sharing dishes
- Through eating from the same plate
- Through touching items in public places (doorknobs, handles in transportation, etc.)
- Do not know
- Other (please explain)

16. Can TB be cured?

- Yes
- No

17. How can someone with TB be cured? (Check all that are mentioned.)

- Herbal remedies
- Home rest without medicine
- Praying
- Specific drugs given by health center
- DOTS
- Do not know
- Other:

18. How expensive do you think TB diagnosis and treatment is in this country?

- It is free of charge
- It is reasonably priced
- It is somewhat/moderately expensive
- It is very expensive
- Interviewer: If respondent gives monetary amount, note the amount here

19. Do you know people who have/had TB?

- Yes
- No

20. Which statement is closest to your feeling about people with TB disease?

- I feel compassion and desire to help.
- I feel compassion but I tend to stay away from these people.
- It is their problem and I cannot get TB.
- I fear them because they may infect me.
- I have no particular feeling.
- Other (please explain):

21. In your community how is a person who has TB usually regarded/treated?

- Most people reject him or her
- Most people are friendly, but they generally try to avoid him or her
- The community mostly supports and helps him or her
- Other (please explain):

22. Do you think that Diabetes positive people should be concerned about TB?

- Yes > go to 20a
- No > go to 20b

23 a) Why?

- Person with Diabetes is more likely to develop TB
- Do not know
- Other:

23 b) Why not?

- Person with Diabetes is not more likely than
- Person without Diabetes to develop TB
- Do not know
- Other:

24. **Do you feel well informed about DM?**

- Yes
- No

25. Do you wish you could get more information about DM?

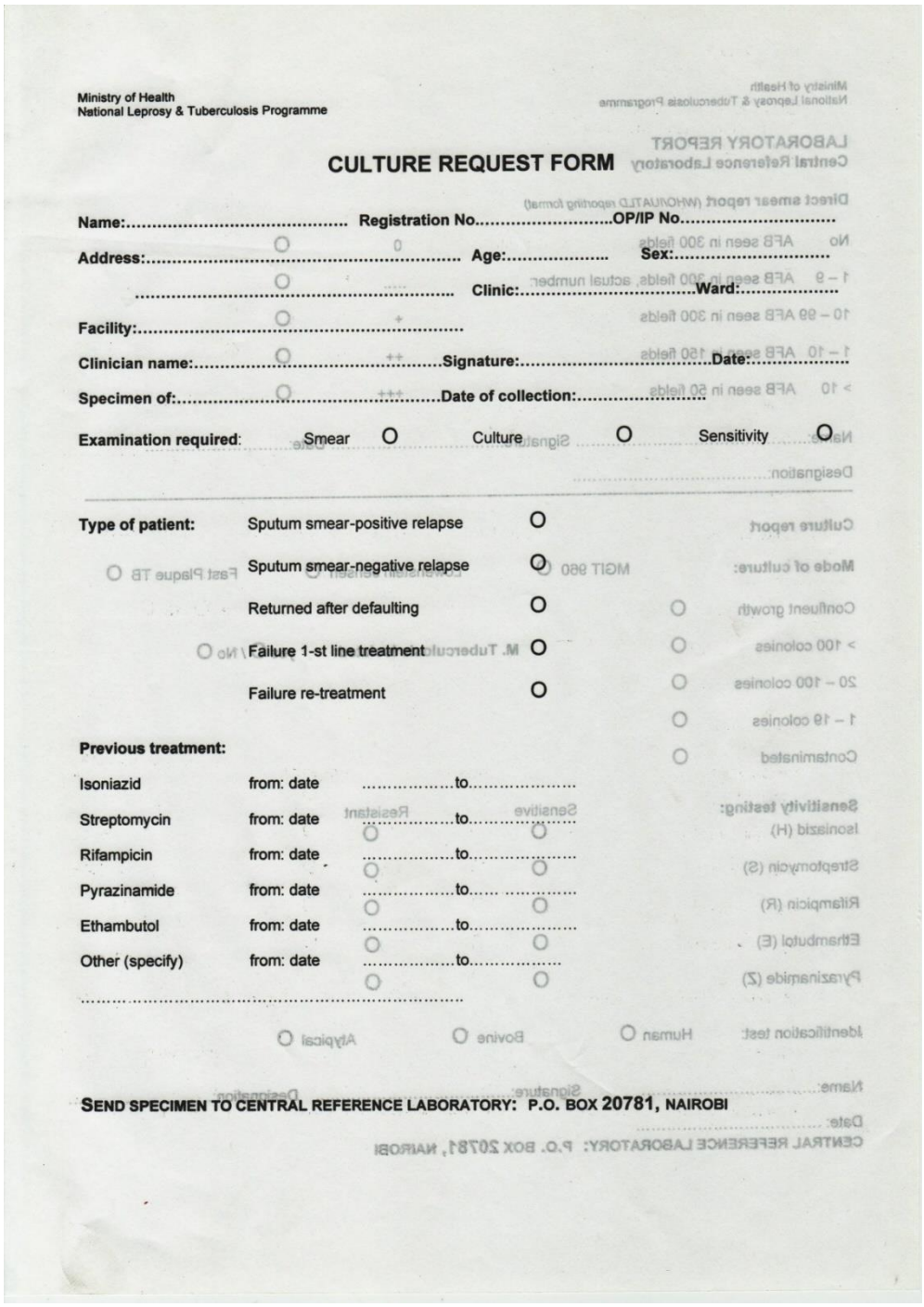
- Yes
- No

26. What are the sources of information that you think can most effectively reach people like you with information on TB/DM? (Please choose the three most effective sources.)

- Newspapers and magazines
- Radio
- TV
- Billboards
- Brochures, posters and other printed materials
- Health workers
- Family, friends, neighbors and colleagues
- Religious leaders
- Teachers
- Other (please explain):

27. What worries you the most when you think about TB/DM

Appendix VI: Laboratory Sputum Culture request reform



Ministry of Health
National Leprosy & Tuberculosis Programme

CULTURE REQUEST FORM

Name:..... **Registration No**.....**OP/IP No**.....

Address:..... **Age:**..... **Sex:**.....

..... **Clinic:**.....**Ward:**.....

Facility:.....

Clinician name:.....**Signature:**.....**Date:**.....

Specimen of:.....**Date of collection:**.....

Examination required: Smear O Culture O Sensitivity O

Type of patient:

Sputum smear-positive relapse	O
Sputum smear-negative relapse	O
Returned after defaulting	O
Failure 1-st line treatment	O
Failure re-treatment	O

Previous treatment:

Isoniazid	from: date	.....to.....	
Streptomycin	from: date	.....to.....	O O
Rifampicin	from: date	.....to.....	O O
Pyrazinamide	from: date	.....to.....	O O
Ethambutol	from: date	.....to.....	O O
Other (specify)	from: date	.....to.....	O O

.....

SEND SPECIMEN TO CENTRAL REFERENCE LABORATORY: P.O. BOX 20781, NAIROBI

AppendixVII: Spoligotyping KITS for DNA Molecular typing

Kit contents (IM9701)

This kit for spoligotyping consists: **four vials, one membrane and one manual**

1) Two vials containing the positive controls:

a) ***Positive control 1.*** (*M. tuberculosis* strain H37Rv)

b) ***Positive control 2.*** (*M. bovis* BCG P3)

Both positive controls are 10ng/µl. For PCR amplification 2µl of each positive control should be added to the PCR reaction mixture (see section 3.1). The volume in each vial is 25µl and should, therefore, be sufficient for approximately 12 PCR reactions.

2) Two vials containing the primers for PCR amplification:

a) ***Primer DRa*** (biotinylated)

b) ***Primer DRb***

The primers are lyophilized. Prepare both primers for PCR amplification both primers should be dissolved in 1.0 ml MQ water. Then split in two separate vials of 0.5 ml each and add 0.6 MQ water to each vial.

3). One spoligotyping Membrane

The spoligo membrane is in 20 mm EDTA. Avoid dehydration at any time, because this will rapidly decrease the quality of the membrane (see section 3.3, item 3.). Store the membrane at + 4°C for optimal storage condition.

4). One Manual

Procedure: (Invitro amplification of spacer DNA by PCR)

Principle Amplification of the spacers is accomplished by using the primers DRa and DRb, which enable to amplify the whole DR region. Only a very small amount of template DNA is required. Typically the PCR is performed on 10 ng purified chromosomal Mycobacterial DNA but, with minor adaptations, DNA extracts from clinical samples or lysed bacteria (from freezer or Lowenstein) can also serve as template. The PCR products are labeled with biotin, because primer DRa is biotinylated.

Procedure

1) Dilute the DNA samples to the required concentration. Always include chromosomal DNA of M. tuberculosis strain H37Rv and M. bovis BCG P3 as positive controls. Use water as a negative control.

2) Prepare the reaction mixture: [a)]

x µl template DNA (20 ng)

4 µl primer DRa (20 pmol) [b)]

4 µl primer DRb (20 pmol) [c)]

4 µl dNTP-mixture (2.5 mM each dNTP, final conc. 0.2 mM each dNTP)

5 µl 10x concentrated Super Tth buffer

0.1 µl Super Tth polymerase (5 units/µl)

33-x µl MQ water (to a final volume of 50 µl)

3) Add one drop of mineral oil to the tubes to prevent evaporation of the PCR-mix during the amplification. 4) Place the tubes in a PCR-apparatus for amplification, and perform the following temperature cycling: [d,e,f)]

3 min 96°C

0 min 96°C]

1 min 55°C } 20x

30 sec 72°C]

5 min 72°C

Procedure :(Hybridization with PCR product and detection)

1) All buffers should be prewarmed before use. Prepare the following buffers from concentrated stocks, using demineralized water for dilution (quantities for one membrane):
250 ml 2xSSPE/0.1% SDS, 60ºC,
250 ml 2xSSPE/0.5% SDS, 60ºC,
250 ml 2xSSPE/0.5% SDS, 42ºC.
250 ml 2xSSPE, room temperature.
2) Add 20 µl of the PCR products to 150 µl 2xSSPE/0.1% SDS.

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

4) Wash the membrane for 5 min at 60°C in 250 ml 2xSSPE/0.1% SDS.

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

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

7) Fill the slots with the diluted PCR product (avoid air bubbles!) and hybridize for 60 min at 60ºC on a horizontal surface (no shaking!). Avoid contamination of neighbouring slots. [b)] (about 120ul)

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

9) Wash the membrane twice in 250 ml 2xSSPE/0.5% SDS for 10 min at 60°C x2

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

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

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

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

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

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

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

Stop the reaction with water (H_2O)

Remarks

a) Do not reuse the support cushions.

b) If less than 45 samples are applied to the miniblotter, fill one neighbouring slot with 2XSSPE/0.1%SDS to prevent cross-flow.
c) Use a dedicated plastic container. Do not use this container for other purposes, since some reagents decrease the intensity of the Spoligopatterns.
d) If the result is unsatisfactory, you can try to improve this. Black spots (background) possibly occur due to contamination during filter handling (e. g. touched with fingers). Start again from step 8. Blank areas in the spoligopatterns possibly indicate that the membrane was not completely soaked with ECL detection liquid. Start again from

Regeneration of the membrane

Purpose

The hybridized PCR product is dissociated from the membrane in order to regenerate the membrane for the next hybridization. A membrane can be regenerated for at least 10 times.

Procedure

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

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

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

Additional reagents (not supplied)

20xSSPE 0.2 M Na2HPO4*2H2O 35.6 g/l

3.6 M NaCl 210.24 g/l

20 mM EDTA 7.4 g/l

The pH should be 7.4. Autoclave.

Store at room temperature for no longer than one year.

2xSSPE

Dilute 20xSSPE ten times with demineralized water.

10% SDS

10 g SDS / 100 ml demineralized water.

2xSSPE/0.1%SDS

Add 100 ml 20xSSPE and 10 ml 10% SDS to 890 ml demineralized water.

2xSSPE/0.5%SDS

Add 100 ml 20xSSPE and 50 ml 10% SDS to 850 ml demineralized water.

Appendix VIII: Preparation of P-nitro benzoic acid (PNB)

Preparation of Lowenstein Jensen PNB Containing Media	NTRL-A-28
	er: 1.0
	:
	TRL staff
	Q.A. Officer
	Head Laboratory

Content

1. **Scope**

This SOP describes the procedure of Lowenstein Jensen PNB containing media for use on drug susceptibility testing on solid media.

2. **Purpose**

Culture medium for performing test for susceptibility to PNB (p-nitro benzoic acid) in strains of Mycobacterium tuberculosis complex.

3. **Materials**

Lowenstein Jensen medium-250ml.

P-nitro benzoic acid-500mg.

4%NaOH (sterile)-5ml.

1M phosphoric acid-2-3ml.

Sterile Distilled water-10ml.

4. Procedure

1. Prepare a batch of Lowenstein Jensen medium.

2. Prepare p-nitro benzoic acid stock solution by dissolving the 500mg PNP in 5ml of 4%NaOH.

Titrate to neutral pH with 1M Phosphoric acid. Add sterile distilled water to make the total volume 10ml (50mg/ml stock solution).

3. Add 2.5ml of PNB stock solution to 250ml of Lowenstein Jensen medium (conc. 500ug/ml)

4. Dispense

5. Label with PNB and expiry date

6. Slope and inspissate with a horizontal surface.

5. Appearance

Apple-green butt

6. Quality Control

Quality control of media – color, texture, humidity, homogeneity and sterility – is carried out as indicated for plain egg-based media (NTRL-A-20).

Sensitivity (capacity to detect resistant strains) and specificity (capacity to detect sensitive strains) of drug-containing media should be tested using reference strains. The procedure is described in NTRL-A-23 (DST using the proportion method).

7. Storage

Store at 4^0c for 3 months.

8. References

Jenkins PA, Pattyn SR Portaels F. (1982) Diagnostic Bacteriology. In “The biology of the Mycobacteria” (Ratledge C and Stanford J, eds) pp.462, Academic Press, London

Appendix IX: Preparation of LJ medium

1. Scope

This SOP describes the preparation of egg-based media suitable for the culture of M. tuberculosis.

2. Principle

A variety of solid and liquid culture media have been developed for the isolation of M. tuberculosis on culture media. Egg-based media are widely used since they can be prepared locally and offer several other advantages – low cost, stability of the chemicals needed, long shelf-life, and characteristic morphology and luxurious growth of tubercle bacilli. However, it may be as much as 8 weeks before cultures become positive, particularly if specimens contain few bacilli.

Egg mass of whole eggs is mixed with ingredients needed for growth of tubercle bacilli. Coagulation yields a solid medium. Malachite green in the medium helps to minimize the growth of contaminants.

3. Samples

NA

4. Materials

Balance (sensitivity 0.01)

Inspissator and racks to hold slopes in correct position

Appropriate sterile glassware and sufficient sterile screw-capped tubes for media dispensing

Material for homogenization of egg mass (5-mm glass beads for manual shaking, a plate with magnetic stirrer, or a sterile low-speed blender with sterile glass/stainless steel bowl)

Funnel with sterile gauze, for filtration of homogenized egg preparation

Reagents and solutions

Best-quality chemicals of certified purity and freshly distilled, sterilized water should be used for the preparation of media.

Ethanol, 70%

TB Medium. (LJ Base)

Hens' eggs

Hens' eggs should be fresh (no more than 7 days old), bought from a farmer who does not use feedstuffs containing antibiotics. The eggs should be medium-sized (for balanced proportions of egg white and egg yolk); about 23 eggs will be needed per liter of egg mass.

5. Quality control

Color

Tubes from the same batch of media showing different shades of green may be the result of poor homogenization or of the presence of material residues in the tubes. A very dark shade of green can be caused by an excess of malachite green or by a very low (acidic) ph. Yellowish coloring of the media can indicate poor-quality malachite green or very high (alkaline) ph. Discoloration of the coagulated medium may also be due to excessive temperature.

Texture

If the medium is liquid or disintegrates when tubes are tapped on the hand (one or two tubes are randomly selected from the batch), the inspissation temperature may have been too low. Tubes containing media of poor texture are not suitable for culture inoculation.

Humidity

The bottom of the tube should show some condensation water. However, an excess of water indicates either that tubes have been tightly capped too soon after inspissation or that the composition of the medium is not standard. The medium should not roll when tubes are moved.

Homogeneity

If bubbles appear in the medium during inspissation, it is possible that the medium has been subjected to excessive temperature and its quality has deteriorated. The presence of clumps in the medium indicates poor homogenization.

Sterility

The batch is tested for sterility. No bacterial growth should be present after 1-day incubation at 37 °C.

Sensitivity

Check sensitivity (ability to yield good growth of tubercle bacilli) of the batch of prepared media. Refer to the SOP for specimen processing for culture, section

6. Procedure

1. Clean and disinfect the work area.
2. Weigh 37.5grams of TB Medium Lowenstein Jensen base.
3. Dissolve in 600mls distilled water.
4. If desired add 12mls glycerin.
5. Put the magnet rod inside
6. Autoclave at 121^0c for 15 minutes keep it hot.
7. Clean the eggs carefully with plain soap and water, soak them in 70% ethanol for 15 minutes and let dry.
8. Crack each egg into a small sterile recipient in order to check its freshness.
9. Transfer the whole egg into t the blender
10. Add sufficient eggs for the volume needed
11. Homogenize the egg mass.
12. Add the salt solution. (1litre of eggs add to 600mls)
13. Mix all components gently (to avoid bubbles) to homogeneity.
14. Filter through sterile cotton gauze.
15. Dispense in 6–8 ml volumes into sterile universal bottles or 16 x 125 mm screw-capped tubes.
16. Place the tubes on the racks so as to achieve appropriate slopes and inspissate at 85 °C for 60 minutes.
17. Take the tubes out of the Inspissator. Once cooled, label to identify the batch and the date of preparation. Tighten screw-caps (otherwise condensation will produce excess water).
18. Keep the tubes in an upright position in the refrigerator, up to 4 weeks.
19. Record batch data into media preparation log.

7. Reading and Interpretation

N/A

8. Reporting

N/A

9. Procedural notes

1. Because of the risk of infection, if glass tubes break, thick-walled, shock-resistant glass tubes or vials should be used.
2. Pre-prepared salt solution mixtures are commercially available and have the advantage that their quality is guaranteed by a certified manufacturer. Mixtures that include dried egg powder should not be used because of their unreliable quality.

Storage

To avoid the accumulation of expired media, estimate the number of tubes to be prepared to cover culturing needs for about 2 months of work. The media should be stored in a clean, cool and humid place, preferably in a refrigerator at 4 °C, for no more than 3 months. Labels on the tubes should indicate:

I. name of medium;
II. name of the producer (laboratory identification);
III. batch number or date of production;
IV. expiry date (the last day the medium can be used for inoculation);
V. Storage conditions (light protection, temperature 4–8 °C).

Shipping

Tubes can be shipped at ambient temperature provided that they are protected from sun and light exposure, overheating and desiccation.

Glycerol in the LJ medium favors the growth of M. tuberculosis, while LJ medium without glycerol but containing pyruvate encourages the growth of M. bovis and may be used in areas of high M. bovis incidence as a second slant for specimens.

Reference

Guidance for countries on the preparation and implementation of TB laboratory standard operating procedures (gli, USAID, TB/CTA)

Appendix X: Preparation of ZN Stain.

1. Scope

This SOP describes the Ziehl–Neelsen (ZN) staining technique for the detection of Acid-Fast Bacilli (AFB) by microscopy. The ZN staining technique is used with ordinary (bright-field) microscopes.

2. Principle

The property of acid-fastness is based on the presence of mycolic acids in the cell wall of mycobacteria. Primary stain (fuchsin) binds to cell-wall mycolic acids. Intense decolourization (strong acid or acid/alcohol) does not release the primary stain from the cell wall and the mycobacteria retain the red color of fuchsin – hence acid-fastness. Counterstaining (with methylene blue) provides a contrasting background.

While mycobacteria are AFB, very few other bacteria possess the property of acid-fastness, and then only weakly (e.g. *Nocardia*). AFB`s found in respiratory specimens of patients from countries with high TB prevalence are almost always TB bacilli. Non-TB mycobacteria are more commonly found in countries where TB prevalence is low. In high-burden countries, however, some patients suspected of having MDR-TB may actually have disease caused by non-TB mycobacteria. AFB found in extra-pulmonary specimens, particularly gastric washings, stool or urine, should never be automatically assumed to represent TB bacilli.

2. Specimens

1. Smears from sediments of decontaminated specimens
2. Direct smears from specimens
3. Specimen Other Than Sputum (SOTS)

4. **Materials**

Bright light Microscope

Reagents and solutions;

1. Carbol fuchsin staining solution, 1%
2. Acid decolorizing solution, 25% H_2SO_4
3. Methylene blue counterstaining solution, 0.1%

5. Quality Control

1. Include positive and negative controls with each day's reading. Read control slides before patient smears; this will also help with focusing and to check proper functioning of the instrument.
2. If results are unacceptable (as described above), re-stain smears of that day together with new controls, paying attention to correct technique; if these controls are also unacceptable, prepare new staining solutions and repeat the staining

Internal QC indicators

Monitor laboratory performance by monthly counts – plotted on a graph – of:

Smear positive culture positive

6. Procedure

Staining method

Place the slides, smear upwards, on the staining rack over a sink, about 1 cm apart.

Place a new filter paper in a small funnel, keep it over the first slide and fill it up with carbol-fuchsin staining solution.

Let the solution filter through the paper, covering each slide completely.

Prepare the torch by dipping its cotton wool end in burning spirit; light it.

Heat all the slides, keeping the torch a little below them and moving it continuously back and forth along the line until steam rises. This is repeated twice at intervals of 3–5 minutes. Do not allow the staining solution to dry on the slides – add fresh solution as required. Contact time of the staining solution with the smear should total at least 10 minutes.

Using forceps tilt each slide to drain off the stain solution. Rinse the slides well with distilled water or clean tap water from a beaker (not directly from the tap).

Pour the acid solution over the smears, covering them completely, and allow to act for 3 minutes.

Using forceps tilt each slide to drain off the acid solution. Gently rinse each slide again with distilled water or clean tap water from a beaker (not directly from the tap).

If necessary, repeat the acid treatment and rinsing until all macroscopically visible stain has been washed away.

Flood smears with methylene blue solution for 1 minute.

Using forceps tilt each slide to drain off the methylene blue solution. Rinse the slides well with distilled water or clean tap water from a beaker (not directly from the tap).

Using forceps, take each slide from the rack and let the water drain off. Stand the slide on edge on the drying rack and allow to air dry.

Reading and interpretation

1. Place a stained slide on the stage, smear upwards.
2. Focus the image with the right eye by looking into the right eyepiece and adjusting with the fine focus knob.
3. While looking into the eyepieces, slowly turn the coarse focus knob to separate the objective lens and the stage. The smear should come into focus within a few turns. Then turn the fine focus knob until the smear is seen most clearly.
4. Place a drop of immersion oil on the left edge of the smear; do not touch the slide with the oil applicator but allow the drop of oil to fall freely onto it. Then rotate the 100x objective into place. Scan the stained smear systematically from left to right side, covering one length (100–150 high-power microscopic fields, depending on the length of the smear – 2 or 3 cm). This is the minimum that must be scanned before reporting a negative result; the process should take about 5 minutes. Count AFB in positive smears for quantification. Always search for useful areas, i.e. those containing mucoid threads and pus cells; do this by moving up or down when arriving at an almost empty area, until another useful zone has been found, then continue moving to the right.
5. Acid-fast bacilli appear bright red against the background material counterstained in blue. Report as positive for AFB when the background is bluish and at least one red AFB is seen in a well de-stained smear (even if the AFB may be mycobacteria other than tubercle bacilli). Tubercle bacilli are quite variable in shape, from very short

fragments to elongated types. The typical appearance is usually of rather long and slender, slightly curved rods. They may be uniformly stained or with one or many gaps, or even granular. They occur singly or in small groups, and rarely in large clumps.

6. Place the slide smear-down on a piece of absorbent paper (e.g. folded toilet paper) to soak up the oil; do not move the slide once it is on the absorbent paper.
7. Store the slides in a slide box in order of the numbers of the laboratory register for 8weeks or until the culture results are read. Do not write results on the slides.

Recording

Finding	Recording
No AFB found in at least 100 fields	negative
1–9 AFB per 100 fields	exact figure/100
10–99 AFB per 100 fields	+
1–10 AFB per field (count at least 50 fields)	++
More than 10 AFB per field (count at least 20 fields)	+++

8. Reporting

1. Results must be reported in the appropriate register of TB laboratory examination, work sheets. Use red ink for positive results.
2. Enter smear results on the LIMS and reports must be provided as soon as possible.
3. For a negative result report: "o."
4. For a positive result: report quantification of AFB seen. (It should not be assumed that AFB are tubercle bacilli.)
5. Never report "No TB" (or equivalent wording).

Procedural notes

Note: The stained smear should show a light blue color. A dark blue color usually indicates that the smear is too thick or that the methylene blue staining time was too long; this will hide the red AFB in the background.

- It is a professional error to focus directly with X100 objective.

Among the possible reasons for false-positive results are:

1. Re-use of containers or positive slides;
2. Contaminated stain prepared with water containing environmental mycobacteria;
3. Use of scratched slides;
4. AFB floated off one slide and became attached to another during the staining procedure because there was no space between adjacent slides;
5. If Inadequate decolourization;
6. Lack of experience, confusion with artefacts (especially if stains are not or poorly filtered);
7. Microscope (lamp) in poor condition or poorly adjusted: interpreting glitter as AFB;
8. If Poor quality of staining solutions.

Among the possible reasons for false-negative results are:

1. Poor quality of specimen;
2. Not taking proper portion of specimen for smear preparation;
3. Due to Excessive decolourization;
4. Poorly prepared staining solution;
5. Too little time staining with carbol fuchsin;
6. Over-staining with methylene blue;
7. Overheating during fixing;
8. Reading less than one length;
9. Slide exposed to daylight for too long; too long interval between staining and reading, particularly if slides were poorly stained or not kept in the dark

References

Guidance for countries on the preparation and implementation of TB laboratory standard operating procedures (gli, USAID, TB/CTA)

Appendix XI: Preparation of Reagents used to process the Culture.

1. Scope

This SOP describes the preparation of all reagents used during specimen processing for culture and identification of Mycobacterium tuberculosis.

2. Principle

Reagents should be prepared in small aliquots to avoid reuse of stock solutions to minimise cross contamination.

3. Samples

NA

4. Materials

Balance (sensitivity 0.01 g), spoons or spatulas, weighing paper

Autoclave

Dispenser device

Dark bottles

Refrigerator

5. Quality control

Use high-grade reagents, sterile distilled water and a balance that is regularly checked for sensitivity.

New reagent batches used for decontamination procedures can be checked by processing a control tube containing all reagents except specimen, centrifuging it and inoculating a culture medium tube/vial with some of the liquid left in the tube. Growth of organisms would lead to the reagent batch being discarded.

6. Procedure

Reagents and solutions

A. Phosphate buffer, 0.067 mol/litre, pH 6.8

- Stock solution A: disodium phosphate, 0.067 mol/litre

Dissolve 9.47 g of anhydrous Na_2HPO_4 in 1 litre of distilled water.

- Stock solution B: mono-potassium phosphate, 0.067 mol/litre

Dissolve 9.07 g of KH_2PO_4 in 1 litre of distilled water

- Mix 50 ml of solution A and 50 ml of solution B.
- Aliquot in volumes required for adding to a single centrifugation tube (e.g. 50-ml amounts if 50-ml centrifuge tubes are used), discarding the extra volume. Sterilize by autoclaving at 121 ºC for 20 minutes
- Left-over volumes of buffer can then be pooled and sterilized again for further use.

B. Reagents for the NALC–NaOH method

1. <u>Sodium hydroxide (NaOH), 4%</u>

Sodium hydroxide pellets (analytical grade) 4 g

Distilled water 100 ml

Dissolve NaOH in the distilled water. Sterilize by autoclaving at 121 ºC for 20 minutes.

2. <u>Tri-sodium citrate, 2.94%</u>

Tri-sodium citrate·$2H_2O$, analytical grade 2.94 g

HOC (COONa) (CH_2COONa) $2{\cdot}2H_2O$

($C_6H_5Na_3O_7.2H_2O$, MW 294.10)

Distilled water 100 ml

Dissolve tri-sodium citrate in the distilled water. Sterilize by autoclaving at 121 ºC for 20 minutes.

NALC–NaOH solution freshly prepared

Mix equal volumes of solutions 1 and 2. Add 0.5 g N-acetyl L-cysteine (NALC) just before use.

Aliquot in 4-ml amounts.

Malachite green solution, 2%,

Malachite green dye 2 g

Sterile distilled water 100 ml

Using aseptic techniques, dissolve the dye in sterile distilled water: place the mixture in the incubator at 37 °C for 1–2 hours or heat in a water-bath at 37°C. Store in dark bottles. This solution is not stable long-term: if precipitation occurs, discard and prepare a fresh solution

C. Other reagents

1. Saline solution

Sodium chloride (NaCl)	0.9 g
Distilled water	100 ml

Sterilize by autoclaving at 121 °C for 20 minutes.

2. Blood agar plates

Nutrient substrate (heart extract and peptones)	20.0g
Sodium chloride	5.0g
Agar	15.0g
Distilled water	1000 ml

- Autoclave at 121 °C for 15 minutes.
- Cool to 45–50 °C and add 5–8 % sheep blood.
- Check that pH is 6.8 ± 0.2 at 25 °C.

- After adding the blood, heat the culture medium for 10 minutes at 80 °C with gentle swirling until it turns brownish (chocolate colour).
- Poured blood plates can be stored for a maximum of 3 months at 4 °C.

3. Mineral Salt solution

Prepare the salt solution by dissolving the components in distilled water (as indicated in the table). Autoclave at 121 °C for 30 minutes in a screw-capped bottle.

Mono-potassium dihydrophosphate (KH_2PO_4), anhydrous	2.4g
Magnesium sulphate ($MgSO_4.7H_20$)	0.24g
Magnesium citrate	0.6g
L-Asparagine	3.6g
Distilled water up to	600ml
Glycerol (ml) or Pyruvate (g)	12ml 0r 7.2g

4. Skim milk 10%

Prepare skim milk powder at 10% in sterile distilled water.

Autoclave at 115 °C for 10 minutes.

5. McFarland turbidity standard No.1

The McFarland No.1 standard is the most widely used turbidity standard for the visual comparison of bacterial suspensions. The density of the resulting barium sulphate precipitate is used as a proxy to approximate the colony count of bacterial suspensions. McFarland No.1 is approximately equivalent to 3 x 10^8 CFU/ml.

Sulfuric acid (H_2SO_4), 1%

H_2SO_4	1 ml
Distilled water	99 ml

Always add acid to water.

Barium chloride ($BaCl_2$), 1%

$BaCl_2$	1 g
Distilled water	100 ml

0.5 McFarland preparation

Barium chloride solution, 1%	0.05 ml
Sulfuric acid, 1%	9.95 ml

Label the suspension and indicate the last day of use (1 month after preparation).

7. Reading and Interpretation

N/A

8. Reporting

All QC results must be recorded in the QC logs

9. Procedural Notes

A nutrient agar base, with the composition given above, is commercially available. Once the bottle is opened, the base may be used up to its expiry date if stored dry, tightly closed and protected from light at 15–25 °C.

Appendix XII: Preparation of Smear slide for AFB staining

1. Scope

This SOP describes the smear preparation technique for AFB staining from direct specimens (sputum, SOTS) and from culture specimens.

2. Principle

Microscopic diagnosis of TB relies heavily on the preparation of the smear especially in the use of direct sputum samples. Though at the CRL we do not use direct sputum samples for microscopy, the principle remains the same.

4. Specimens

a. Smears from sediments of decontaminated specimens (sputum and SOTS)
b. Positive LJ cultures
c. Positive liquid cultures

5. Materials

a. Disposable plastic wire loops with an inner diameter of 3mm
b. Frost-end microscope slides
c. Lead pencil
d. Sterile Pasteur pipette
e. Small bin lined with an autoclavable bag
f. Mycobacteriocidal disinfectant
g. Biosafety cabinet
h. Reagents and solutions: N/A

6. Safety

3. All smears should be prepared in the biosafety cabinet
4. Protective equipment (masks and gloves) should be worn during the procedure

7. Quality Control

5. A newsprint can usually be read if the thickness of the smear is correct
6. Ensure the number on the smear matches that of the specimen. Only one container should be open at a time

8. Procedure

a. From decontaminated specimens

- *Using a lead pencil, label the slides with the corresponding lab numbers*

- *Carefully open the appropriate falcon tube to avoid production of aerosols*
- *Using a disposable plastic loop place a loopful on the slide*
- *Spread the specimen evenly on the slide to make a smear of 2x1 cm in length and width*
- *Air-dry the smear in the biosafety cabinet*
- *Heat fix the smear in the biosafety cabinet or leave it in the BSC overnight*
- *The smear is now ready for staining*

b. From positive LJ slopes

- *Using a lead pencil, label the slides with the corresponding lab numbers*
- *Place a drop of sterile distilled water or buffer on the slide*
- *Using a disposable plastic loop pick out a few colonies and emulsify them on the drop of sterile distilled water. Spread the smear to a size of 2x1cm in length and width*
- *Air-dry the smear in the biosafety cabinet*
- *Heat fix the smear in the biosafety cabinet or leave it in the BSC overnight*
- *The smear is now ready for staining*

c. From liquid culture

- *Using a lead pencil, label the slides with the corresponding lab numbers*
- *Using a sterile Pasteur pipette, place a drop of the broth on the slide*
- *Using a disposable plastic loop pick out a few colonies and emulsify them on the drop of sterile distilled water. Spread the smear to a size of 2x1cm in length and width*
- *Air-dry the smear in the biosafety cabinet*
- *Heat fix the smear in the biosafety cabinet or leave it in the BSC overnight*
- *The smear is now ready for staining*

Procedural notes

Note: All of the above procedures should be carried out in the biosafety cabinet.

Sources of error

- Mix-up of samples due to improper numbering
- Transfer of bacilli from one sample to the other due to improper sterilization of loops.

References

Laboratory Services in Tuberculosis control Part II: (WHO) Microscopy pg. 23

Appendix XIII: Specimen collection, Packaging and Transportation

Scope

This SOP describes methods of specimen collection, packaging and transportation.

Principle

Quality of culture results depends on quality of specimen collected, transported and received

Safety and environment

Do not collect sputum samples in the laboratory, clinic examination rooms or in the patient/client waiting area. Always instruct patient to go outside in open air away from other persons to minimize risk of transmission and infection of others.

The shipment of diagnostics samples such is of potential public health risk and must be done in compliance with shipment of biological specimen regulations of the country IATA. Compliance to transportation standards is the responsibility of the shipper.

Sample

The primary sample (also called specimen) is sputum that is submitted for examination to obtain a diagnosis. The primary sample recommended for the diagnosis of tuberculosis is sputum

Materials

Sputum container plastic, wide mouthed, screw-capped, leak-proof, with a capacity of 50ml and made of translucent material in order to observe specimen volume and quality without opening the container.

- Absorbent material (cotton wool)
- Zip-lock bag
- Safety box
- Request forms

- Cool box

Procedure for sputum collection

Mycobacterium has the capacity to affect all organs of the body but most cases of tuberculosis in Kenya are still pulmonary, requiring sputum specimen for diagnosis.

Patient preparation

No special preparation of patient is required but prior to collection of the sputum, the patient must be advised to rinse his/her mouth thoroughly with water (preferably sterile to exclude Non tuberculosis mycobacterium) before coughing out sputum for laboratory examination.

Sample collection timing

The recommended sputum collection procedure is that there will be 2 spot sputum for microscopy and culture.

Specimen/sample containers

NTRL advises the use of large mouthed screw-capped leak-proof conical shaped, 50 ml graduated disposable plastic containers (e.g. 50ml Falcon tubes from Becton Dickson [BD]). These falcon tubes are large enough to allow processing of sputum in the same containers to increase recovery of bacilli as well as minimize sputum transfer to other processing containers, re-enforcing safety to reference laboratory staff. Other types of containers can only be used with the approval of NTRL otherwise NTRL reserves the right to reject samples in unapproved containers.

Procedure

1. Good specimens are to be obtained; patients must be instructed in how to produce sputum. Give each patient a new sputum container. If two samples are required, give two separate containers. Sputum containers or mugs should never be re-used.
2. Give the patient instructions and demonstrate how she or he can produce and collect good sputum; do up to three deep inhalations and exhalations and on the third exhalation or when a strong cough reflex arises, accompany it with a cough from deep inside the chest as much as possible (do demonstrative actions for the patient).
3. Demonstrate to the patient how to open and close the sputum container so as there are no leaks or smearing on the exterior of the container.
4. The patient should spit the sputum into a 50ml sputum container to avoid spills or soiling of the outer side of the container, and then close the lid tightly.

5. Ideally, a sputum specimen should be a minimum of 5ml in volume, although smaller quantities are acceptable if the quality is satisfactory.
6. Emphasize the need for the patient to supply the most useful specimen, the normally thick, yellowish (sometimes blood-streaked), purulent exudative material brought up from the lungs after a deep, productive cough.
7. Emphasize that saliva is not sputum and that it may be misleading in diagnosis and pose a greater danger to the patient instead. However, if the only sample the patient can produce is salivary, submit it to the laboratory; it can still yield useful results.
8. Encourage the patient to bring the collected specimen back to the MFS as quickly as possible. Once the sample is collected the clinician should place it in a cool box with cold icepacks and ensure that the cold chain is maintained until it's received at NTRL.
9. Fill in a laboratory request form for each patient that should accompany the samples to NTRL. Any sample without a Laboratory request form will be rejected.

Safety precaution

DO NOT COLLECT SPUTUM SAMPLES IN THE LABORTORY, CLINIC EXAMINATION ROOMS OR IN THE PATIENT/CLIENT WAITING AREA. ALWAYS INSTRUCT PATIENT TO GO OUTSIDE IN OPEN AIR AWAY FROM OTHER PERSONS TO MINIMISE RISK OF TRANSMISSION AND INFECTION OF OTHERS

1. Procedural notes

Identification of the Primary Sample (Labeling)

Label/identify the sample container with at least a patient's name and date of collection. Note that patient's name may be replaced by a unique numerical study or clinic Patient Identification Number (PIN). These two parameters must at all times be 100% identical to the ones written on the laboratory request form accordingly, the specimen may otherwise be rejected.

Laboratory Request Form and Completion

The laboratory through the National Leprosy Tuberculosis and Lung Disease Unit (NTLD - U) has its own laboratory request form and will continue to provide them to health units that refer specimens to the laboratory. In the event that an organization/clinic wishes to design its own request form, the laboratory advises that the facility consults the NTRL management for optimal designs.

The laboratory test request forms for referring samples to the laboratory is designed in such a way as to capture the following information (see Appendix 1):

1. The name, sex, age of the patient from whom the sample was collected.
2. Address and mobile phone number of the patient/Guardian.
3. The Health facility, Cluster or research organization requesting examinations. Clinical history of the patient (on chemotherapy or not etc).
4. District and Province
5. Patient category.
6. Reason for Request.
7. Laboratory procedure(s) being requested for.
8. Type of sample collected
9. Date and time of collection of sample.
10. Clinician name, signature and Telephone contact
11. A section for laboratory use only:

a. For recording initials of person receiving specimen, time and date.
b. Macroscopic quality of the specimen (e.g. purulent, muco-purulent, salivary or muco-salivary).
c. A provision for inserting reference laboratory number.

Completing the laboratory request form

1. The laboratory request form must be completed fully at all times. It is the duty of the MFS in-charges or Survey Coordinator to ensure that personnel under their supervision fully complete the request forms. It is important to note that all information requested for on laboratory request forms is vital for laboratory or to the person/organization requesting tests or both.
2. Each specimen sent to the laboratory must have a request form otherwise it will be rejected
3. Fill in the request form using a blue or black pen only. ***DO NOT USE RED PENS.***
4. Fill the request form clearly in block letters avoiding abbreviations. When filling the form, emphasis is made on the following parts.

DATE

This is the date the request form is filled and it should be the date when the sample is collected.

PATIENT IDENTIFICATION

10. **Name**: *Write the Patients three names. AVOID writing one name*
11. **Sex**: *Write Male or Female*
12. **Age (years):** *Indicate the age of patient in years. AVOID Adult*

13. **Patient ID**: *Unique number assigned to each patient at the Health unit. NOTE: For patients under screening record Not applicable (N/A) and not new.*

14. **Contact Tel No**: *For the Patient*
15. **Village:** *The Village where the Patient resides.*
16. **Location**: *The Location where the Patient resides.*
17. **District of Residence**: *The district where the Patient resides.*

HEALTHY FACILITY DETAILS:

Name: Write full names of the Health Unit or Hospital.

District: Write the full District name where the Health Centre or Hospital is located.
Province: Write the name of the Province where the Health Centre is located

EXAMINATION REQUESTED: (include name of the requester) Microscopy, Culture and Drug susceptibility testing
Complete the request form as fully as possible.
Make sure that the patient names, date of collection recorded on the specimen container match with those on the request form, otherwise specimen **will be rejected.**

1. In the event that the sample and the request form are in partial disagreement but there is a single common identifier, the specimen will probationary be accepted but the request form will be returned to the person requesting tests together with another laboratory form headed "Laboratory Request Form Reconciliation (LRFR) form"
2. The person who completed the request form must complete the LRFR form for results of such samples to be released as a sign of accepting to own the discrepancy and the repercussions that might result from it
3. The LRFR form will also be sent for completion if vital information was left out during completion of the laboratory request form.
4. In the event that the LRFR form is not completed and returned to the laboratory, results of such samples will be disregarded and never released.

5. In cases where the laboratory request form and the specimen identification/label are totally different, the specimen will be rejected totally and discarded. The request form will be labeled "REJECTED", a copy sent back to the clinic accompanied by a sample rejection form (Appendix that explains the reason(s) for rejection.
6. Sign and date as a person requesting for the laboratory investigations.

Sample Packaging

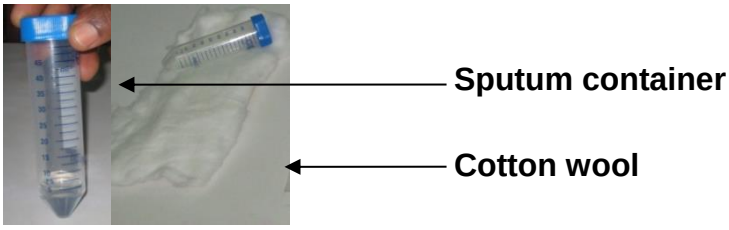
Safety precautions

The shipment of diagnostics samples is of potential public health risk and must be done in compliance with shipment of biological specimen regulations of the country.

Samples must be packed using the Triple Packaging system as detailed on chapter four of the ILRN guideline. Compliance to transportation standards is the responsibility of the sender.

Samples must be packed using the "Triple Packaging system"

1. The health worker fills out the patient's details in a laboratory request Form
2. Health worker wraps the container in cotton wool in sufficient quantity to absorb the entire contents in case of leakage



3. **Secondary packaging** – the cotton-wrapped sputum container is placed inside the zip lock bag and the bag zipped up.

4. **Tertiary packaging**: The Ziplock bag and its contents together with the request form are placed in an approved cool box or other appropriate container.

The sputum is now considered safe to move to the laboratory as soon

as possible for further processing

7. References

Guidance for countries on the preparation and implementation of TB laboratory standard operating procedures (gli, USAID, TB/CTA)

Appendix XIV: SOP on sample processing for MTB culture.

Scope

This SOP describes methods of specimen processing and other laboratory procedures for purposes of culturing *Mycobacterium tuberculosis* culture on solid or liquid media.

Principle

Culture examination detects fewer bacilli than microscopy and increases the number of TB cases found by 20–50%, depending on local incidence. Culture methods provide definitive diagnosis by establishing the viability and identity of the organisms and allow the detection of drug resistance.

The mucolytic agent *N*-acetyl *L*-cysteine (NALC) enables the decontaminating agent, sodium hydroxide, to be used at a lower final concentration. Sodium citrate is included to bind the heavy metal ions that might be present in the specimen and that could inactivate NALC. The method is suitable for inoculation in liquid media.

Specimens for isolation of tubercle bacilli contain associated bacterial and/or fungal flora which have to be eliminated before the specimen is inoculated onto culture media.

Samples

Refer to SOP “Sample collection and transport for culture procedure" for checking the quality of specimens. The following specimens should not be processed:

- dried swabs;
- specimens in broken containers;
- Specimens collected more than 7 days previously.

Materials

BSC, class I or II, annually certified

Refrigerated Centrifuge with safety shield, a minimum RCF of 3000*g*,

Centrifuge tubes, preferably 50-ml capacity, screw-capped thick walled centrifuge tubes, resistant to RCF of >3000*g* RCF.

Vortex mixer

5. Quality Control

1. NALC–NaOH solution must be well reconstituted

2. Decontamination time is critical and must not be left beyond the required time hence it is recommended to process in a batch of 8-12 samples

3. Samples must be inoculated within 60 minutes after decontamination

6. Procedure

Reagents and solutions

Reagents

i. NALC-NaOH solution, freshly prepared for daily use only:
ii. mix equal volumes of Sodium hydroxide (NaOH) solution, 4% and Tri-sodium citrate·$2H_2O$ solution, 2.9% (
iii. Add 0.5 g NALC per 100 ml of NALC–NaOH solution just before use.
iv. Phosphate buffer, 0.067 mol/liter, pH 6.8

Test procedure

1. Mark the volume of sputum on the centrifuge tube (at least 2 ml, not more than 5 ml). Add an equal volume of the NALC–NaOH solution and tighten the screw-cap.
2. Then Vortex for not more than 20 seconds.
3. Keep at 20–25 °C for 15 minutes for decontamination.
4. Fill the tube to the 45-ml mark on the tube with phosphate buffer.
5. Centrifuge at 3000*g* for 15 minutes.
6. Carefully pour off the supernatant through a funnel into a discard can containing 5% phenol or other mycobacterial disinfectant.
7. Resuspend the deposit in approximately 1 ml phosphate buffer.

8. Inoculate deposit on two slopes of egg-based medium labelled with the ID number. Use a sterile disposable loop (two loopful) or sterile Pasteur pipette to inoculate each slope. Smear one drop on two slides, marked with the ID number, for microscopic examination.

7. Reading and Interpretation: N/A

8. Reporting: N/A

9. Procedural Notes

Note: *DLTLD recommend that saliva be processed but that an additional specimen be requested and processed.*

Decontamination with NALC-NaOH method is recommended. Other decontamination methods have not been tested in conjunction with the BBL MGIT medium.Digestant/decontaminant solution may have harmful effect on Mycobacteria.

References

Guidance for countries on the preparation and implementation of TB laboratory standard operating procedures (gli, USAID, TB)

Appendix XV: SOP on Culture reading

Scope

This SOP describes methods of specimen processing and other laboratory procedures for purposes of culturing *Mycobacterium tuberculosis* on solid media.

Principles

Culture examination detects fewer bacilli than microscopy and increases the number of TB cases found by 20–50%, depending on local incidence. Culture methods provide definitive diagnosis by establishing the viability and identity of the organisms and allow the detection of drug resistance.

Materials

Incubator

Specimen

Specimen concentrate from decontaminated sample (refer to SOP specimen decontamination)

Quality control

Record the temperature of the incubator daily with a thermometer accurate to ±1 °C. The temperature should not fluctuate beyond the 35–37 °C range. When an incubation chamber is used, place thermometers at different and easily visible places to ascertain temperature uniformity. Maximum–minimum thermometers provide more information on temperature variation.

Whenever the temperature exceeds 38 °C or falls below 35 °C, the event should be entered in the laboratory registry. Excessive temperature will invalidate culture-negative reports for the specimens that were incubated during the occurrence. New specimens should be obtained from these patients for retesting.

When using automated systems for liquid cultures, refer to the manufacturer's manual.

Procedure

Incubation of cultures

Incubate LJ slopes at 36 ±1 °C.

LJ slopes should be incubated with screw-caps loose, for at least 1 week check for contamination and then caps are tightened to minimize evaporation and drying of the media.

Reading and interpretation

Reading and interpretation using solid media

Check colony formation every week, preferably twice within the first week, to allow rapid detection of contamination and a timely request for another specimen if necessary.

Contaminated cultures and rapidly growing mycobacteria (colonies apparent in less than 7 days) are removed.

M. tuberculosis colonies should be well developed within 3–4 weeks. Report results immediately after detection and identification (refer to SOP "Identification of M. tuberculosis".

Cultures should be kept for up to 8 weeks before being reported as negative.

Laboratory register

Record in the laboratory register:

1. the date of detection of growth of positive cultures;
2. negative and contaminated tubes – individually entered at the end of the recommended incubation time

3. Enter results in laboratory information management system (LIMS)

Reporting

Results should be reported in accordance with qualitative and quantitative criteria.

Fill out an individual form for each positive patient for whom diagnostic specimens were submitted (see form).

Procedural notes

Culture procedures for tuberculosis bacteriology are notoriously time-consuming, often taking weeks or months to complete. For this reason, interim reports should be issued. The following schedule is recommended:

1. If the cultures have been contaminated, a report should be sent out *immediately* and a repeat specimen requested

2. If cultures are positive and growth has been identified as *M. tuberculosis*, perform drug susceptibility testing.

3. At *eight weeks* a final report should be issued containing all the data previously reported so that earlier interim reports can be destroyed and only the final report retained in the patients' file

Guidance for countries on the preparation and implementation of TB laboratory standard operating procedures (gli, USAID, TB/CTA)

Appendix XVI: Kenyatta National Hospital (Glycated Hemoglobin HBA1c).

(ANALYZER: DIRUI CS 4000 AUTOMATED CHEMISTRY ANALYZER)

1.1 Equipment for the Analysis

1.1.1 DIRUI CS 4000 Clinical Chemistry analyzer

The machine, above was used for sample analyses. DIRUI CS 4000 is a discrete, random access clinical analyzer capable of performing a wide range of chemical tests in a single run.

1.1.2 Reagent Preparation

The only additional preparation required was to reconstitute calibrators and controls since the reagents acquired commercially were ready for use and were just put into the reagent cups pre-programmed in a refrigerated analyzer compartment. Where reconstitution was necessary, the reagent kit inserts were utilized. The reagent cups were bar-coded to permit identification by the machine bar code reader of reagent one and two as these were two reagent tests.

1.1.3 Calibration of The Test Parameters

A multisera calibrator or single analyte standard material commercially acquired were used for the purpose of determining the relationship between measured absorbance's to known concentrations of the given analyte/s contained in calibrator solutions (e.g. Serum Assayed Multisera Calibrator). Calibration factors were recorded by the analyzer data manager and displayable on the screen when prompted once the expected relationship was achieved.

1.1.4 Quality Control Materials

Commercially acquired multi-sera normal level and pathological level materials were used for quality control of the analytical work during the study period. The IQC multi-sera were supplied in lyophilized form and were reconstituted as per the Manufacturer's preparation method. For internal quality control assessment, the

prepared IQC multi-sera were analyzed at the time samples for the study were being analyzed.

5.0 Equipment and Reagent

5.1 Equipment

5.1.1 EDTA vacutainer or Heparinized Vacutainer for specimen collection or Eppendorf tubes.

5.1.2 Adjustable pipette capable of measuring 100 - 1000 µl (1.0 ml) for

Hemolysin aliquoting.

5.1.3 Adjustable pipette capable of measuring 10 - 100 µl (1.0 ml)

for erythrocyte aliquoting

5.1.4 *The Automated analyzer (Dirui CS 4000) use for the test.*

5.1.5 Centrifuge for specimen preparation.

5.2 Reagents

R1	Tris buffer	2.7 mol/l
R2	Peroxidase	1500U/L
Fructosyl peptide Oxidase		1500 U/L
Pre-treatment Solution	Hemolysin	5 g / L

Calibrator concentration is specific to the lot number and is on the container label.

Quality Control Concentration is specific to the lot number and is on the container label.

6.0 METHODOLOGY

6.1.1 Background

Hemoglobin (Hb) consists of four protein chains with four haem portions, and is the red pigment located in the red blood cells (Erythrocytes).Its main function is to transport oxygen and Carbon dioxide in blood .Each Hb molecule is able to bind four oxygen molecules. Hb consists of a variety of sub fractions and derivatives. Among this heterogeneous group of hemoglobin's , HbA1C is one of the glycated hemoglobin's , a sub fraction formed by the attachment of various sugars to the Hb molecule.HbA1C is formed in two steps by the non-enzymatic reaction of glucose with the N- terminal amino groups of the Beta –chain of normal adult hemoglobin (HbA) .The first step is reversible and yields labileHbA1c.This slowly rearranges in the second reaction step to yield stable HbA1c.In the erythrocytes , the relative amount of stable HbA converted to stable HbA1c increases with the average concentration of glucose in the blood. The conversion of stable HbA1c is limited by the erythrocytes' lifespan of approximately 100 to 120 days. As a result, HbA1c reflects the average blood glucose level during the preceding 2 to 3 months.HbA1c is thus suitable for monitoring long term blood glucose control in individuals with diabetes mellitus. More recent glucose levels have a greater influence on the HbA1c level. The approximate relationship between HbA1c and mean blood glucose value during the preceding 2 to 3 months has been analyzed by several studies.

6.1.2 TEST OR ASSAY PRINCIPLE (HbA1C Enzymatic Assay Method)

In the first reaction, the concentration of hemoglobin is measured at an absorbance of fixed wavelength, and simultaneously the fructosyl dipeptides are generated from the N – terminal amino groups of the beta-chain of HbA1c by the reaction of protease. In the second reaction, the reaction of Fructosyl peptide oxidase (FPOX) with fructosyl dipeptides, the generated hydrogen peroxide allows, 10-carboxymethylaminocarbony) 3-7-bis(dimethyl amino) phenothiazine sodium salt to develop a color in the presence of peroxidase. The change in absorbance is measured for HbA1c determination. The combined assay results for hemoglobin and HbA1c are used to calculate and express HbA1c (%).

6.2 Test procedure

6.3 Results dispatch and archiving.

6.3.1 For results outcome and interpretation (Refer to appendix 1)

6.3.2 The Health information personnel dispatches the validated results directly to the patient who shall sign for the collection.

6.4 Quality Control

6.4.1 Commercial controls are available for scheduled IQC.

6.5 Possible interferences

6.5.1 Use of expired reagents

6.5.2 Use of specimen haemolysed during phlebotomy

6.6 Calculation of the results

Refer to appendix 1

6.7 Biological reference intervals

Refer to appendix 1

6.8 Reportable intervals

Refer to appendix 1

6.9 Critical values

It is used as a monitoring test so critical values are not significant.

6.10 Potential source of variation

Failure to observe the expiry dates of reagents or sample integrity.

7.0 References

7.1 ISO 15189: 2012(E) Standard.

7.2 Junge w , Wilke B , et al. (2003) Determination of reference intervals in adults for Hemoglobin A1C (HbA1c).Poster presentation 18th International Diabetes Federation Congress .

7.3 CLSI.Evaluation Of Precision Performance Of Quantitative Measurement Methods;Approved Guideline – Second Edition.CLSI document EPs – A2[ISBN 1-56238 -542-9.CLSI,940 West ValleyRoad,SSuite1400,Wayne,Pa19087 USA ,2008.

Appendix XVII: Appendices

8.1 Appendix 1: Test procedure

8.2 Appendix 2: Staff training record

1.2 Specimen Preparation and assaying

1.2.1 Centrifuge or spin the whole blood at 2000 revolutions per minute (2,000 RPM) for 5 minutes.

3.2.2 Aliquot 25µl of the deposited red blood cells into a sample cup or Eppendorf microfuge tube, using the calibrated pipette in use.

3.2.3 Add 500µ l of the hemolysin / denaturant or pretreatment solution to the 25 µl of the aliquoted erythrocytes.

3.2.4 Shake the mixture vigorously in a closed Eppendorf microfuge tube or vacutainer till lysis is achieved.

3.2.5 Homogeneously mix the resultant haemolysate gently and then run the assay after 5 minutes using the appropriate automated analyzer.

3.2.6 Whole blood is stable for 3 days at 15 - 25 0c or 7 days at 2 - 70c.(Haemolysate is stable for 8 hours and 24 hours respectively) at the same temperatures quoted.

3.3 Result Validation, Interpretation and Indication for Repeat Testing

Responsible Staff: Laboratory In - charge, Testing Personnel

3.3.1 Refer to reporting of results SOP (KNH / LAB MED - BIOCHEM /SYP/ 017F5)

3.4 Transcription and release of ready results

Responsible Staff: Laboratory In – charge, Testing Personnel

3.4.1 Validate / verify test results and in appropriate reporting units.

3.4.2 Sign or initial the relevant column of the request/printed report form.

3.4.3 Result entry or matching with request forms can be done by trainees or competent staff whilst authorization is only done by competent technical staff

3.4.4 Immediately take the completed request/ result form to reception for dispatch either to specific patients or into pigeon holes.

3.4.5 Misplaced Results

3.4.5.1 Ask the client at the reception, for the attendance card and date when test was performed or the receipt.

3.4.5.2 Search in the Laboratory register for the log in number previously.

. 3.4.5.3 Search results in the History mode using the Laboratory number and reprint once confirmed as correct.

3.4.5.4 A competent technical staff verifies the results and signs or initials the reprint copy.

3.4.5.5 Write a remark to the effect that results were initially lost on the comment column of the request form and dispatch.

3.5 Page Reference Ranges

Sample Type	Reference range	Si Units
Whole Blood	According to IFCC 2.9 ----- 4.2	%
Whole Blood	According to NGSP / DCCT 4.8 ---- 5.9	%
Whole Blood	***According to JCCLS 4.3 ------ 5.8***	***%***