ISONIAZID AND RIFAMPICIN RESISTANCE AND PATIENT TREATMENT RESPONSE IN A TUBERCULOSIS AND HIV-1 CO-ENDEMIC POPULATION IN WESTERN KENYA IN 2012-2014

BY

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DECLARATION

I, hereby, declare that this thesis is the result of my original work and its findings have not been presented for the award of a degree certificate in any institution.

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DEDICATION

To my Dad and Mom- Maurice and Roseline, my dear loving wife- Gladys and Son- Ethan.

ABSTRACT

In 2015, 10.4 million people worldwide had tuberculosis (TB) and 1.4 million deaths occurred, 400 000 of whom were HIV-positive, Sub-Saharan Africa (SSA) accounted for 81% of these cases. In 2015, 480 000 new cases of multi-drug resistant TB (MDR-TB) were detected globally. In Kenya, in 2015, 107 000 people had TB and 36 000 were HIV co-infected. In western Kenya, the prevalence of TB and HIV in 2015 was 39.2% and 15.1%, respectively and in 2008, 10 cases of MDR-TB occurred. Patients with HIV have a deficient immune system and are likely to be TB co-infected. As a result of the pill burden of anti-retroviral and TB therapy, poor adherence may occur. Rifampicin (RIF) and isoniazid (INH) are first-line anti-TB drugs. Resistance to INH is associated with mutations on the *kat* G and *inh* A genes while *rpo* B gene mutations lead to RIF résistance. Multi-drug resistant TB arises after acquisition of either INH or RIF resistance followed by resistance to the companion drug. Patients with resistant TB require admission which increases transmission. Methods for determining drug resistant TB includes drug susceptibility tests (DST), GeneXpert, Line probe assay (LPA) and sequencing. In western Kenya, current data on the distribution of RIF and INH mutations is not available. In addition, the association of drug resistant mutations with HIV and the treatment response of HIV infected and uninfected patients with TB are not known. As such, the objectives of the current study were to determine the proportion of drug resistant *Mycobacterium tuberculosis* in sputum isolates and investigate the association of RIF and INH gene mutations with HIV status and monitor treatment response in a TB/HIV co-endemic population in western Kenya. The present study was longitudinal in which enrollment was done between 2012 and 2014 after the revision of the TB treatment regimen and patients with confirmed drug resistant TB were followed up for one year to establish the TB treatment response as confirmed by sputum smear microscopy. Random sampling of 415 facilities that support routine TB diagnosis in 13 counties in western Kenya was done. Patients with suspected TB symptoms and Ziehl-Neelsen (ZN) positive patients were targeted for enrollment. A total of 1381 new and 18 previously treated TB patients were enrolled. HIV infected patients accounted for 61% of the enrolled participants. Sputum samples were cultured on *Mycobacteria* growth indicator tubes (MGIT), DST and LPA performed to identify drug resistance and specific mutations on the *rpo* B, *kat* G and *inh* A genes. Discordant samples were sequenced. Conversion rate was calculated by finding the percentage of smear negative and positive patients at follow-up and initial visit, respectively. Proportion of mutations as estimated by LPA and DST was as follows: MDR-TB, 0.95%, 1.53%; RIF mono-resistant TB, 0.88%, 0.66%; INH mono-resistant TB, 1.83%, 1.97%, respectively. Regression analysis showed that RIF resistance was associated with HIV status (P = 0.025). Mann-Whitney tests revealed that the conversion time of HIV infected and uninfected patients with TB drug mutations was comparable (P = 0.180). The results of the study showed that INH mono-resistance was common. Detection of INH mono-resistance in TB endemic areas should be scaled-up as well as TB contact investigation studies to increase early detection of resistant strains.

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LIST OF ABBREVIATIONS

ACP - Acyl Carrier Protein

CAP - College of American Pathologists

CDC – Centers for Disease Control and Prevention

DLTLD – Division of Leprosy Tuberculosis and Lung Diseases

DNA – Deoxyribonucleic Acid

DST – Drug Susceptibility Test

EQA - External Quality Assurance

ERC – Ethical Review Committee

HAART – Highly Active Antiretroviral Therapy

HIV – Human Immunodeficiency Virus

INH – Isoniazid

INHMR – Isoniazid Mono Resistant

IUATLD - International Union Against Tuberculosis and Lung Diseases

KEMRI – Kenya Medical Research Institute

LPA – Line Probe Assay

MDR – Multi Drug-Resistant

MGIT – Mycobacteria Growth Indicator Tube

MTB – *Mycobacterium tuberculosis*

NALC – N-Acetyl-L-Cysteine

NaOH – Sodium Hydroxide

NASCOP - National AIDS and STI Control Programme

NHLS - National Health Laboratory Services

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

RIF – Rifampicin

RMR – Rifampicin Mono Resistant

SSA – Sub Saharan Africa

TB – Tuberculosis

USA – United States of America

ZN - Ziehl-Neelsen

DEFINITION OF TERMS

INH mono-resistant TB–Resistance to Isoniazid

Multi-drug resistant TB – A patient with resistance to at least both Isoniazid and Rifampicin.

New TB case –A patient who has never been treated for TB or has taken anti-TB drugs for less than 1month.

Retreatment TB case – A patient who has received 1 month or more of anti-TB drugs in the past.

RIF mono-resistant TB – Resistance to Rifampicin

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CHAPTER ONE: INTRODUCTION

1.1 Background

In 2015, an estimated 10.4 million people worldwide developed tuberculosis (TB) and 1.4 million died from the disease, 400 000 of whom were HIV-positive, Sub-Saharan Africa (SSA) accounted for 81% of these cases (WHO, 2016). In Kenya, in 2015, 107 000 cases of TB were reported and 36 000 people were HIV co-infected (WHO, 2016). In western Kenya, 39.2% adolescents in the age group 12-18 years had suspected TB (Nduba *et al.*, 2015). Tuberculosis remains the most common HIV related infection and the leading cause of death in HIV infected persons (Chetty *et al*., 2014). This is mainly because TB distorts the immune-regulatory networks during HIV infection by causing an increase in cytokine production and therefore T-cell exhaustion and subsequently rapid progression of TB infection (Chetty *et al*., 2014).

Globally, in 2015, there was approximately 480 000 new cases of multi-drug resistant TB (MDR-TB) defined as *Mycobacterium tuberculosis* resistant to both rifampicin (RIF) and isoniazid (INH) confirmed by phenotypic or genotypic methods, with or without resistance to other anti-TB drugs and an additional 100 000 RIF mono-resistant (RMR) TB cases (WHO, 2016). In Kenya, in 2014, the percentage of TB cases with MDR-TB was 2.2% and 14% in new and retreatment TB cases, respectively (https://extranet.who.int/sree/Reports?op=Replet&name=/WHO_HQ_Reports/G2/PROD/EXT/TBCountryProfile&ISO2=KE&outtype=pdf). The most recent national survey of drug resistant TB in Kenya in 1995 reported no cases of MDR-TB (Jezmir *et al*., 2016). The association of

HIV and drug resistance in patients with TB have yielded varying results (Kidenya *et al*., 2014). In East Africa, no study has reported a correlation between MDR-TB and HIV, however, in regions outside East Africa where an association was found, it was as a result of nosocomical outbreaks in institutionalized settings such as hospitals and refugee camps (Kidenya *et al*., 2014). The present study made an attempt to provide data on the association of MDR-TB and HIV in the general western Kenya population.

Drug resistant *Mycobacterium tuberculosis* remains a major concern in the control of TB globally (Villegas *et al*., 2016). Rifampicin (RIF) and isoniazid (INH) are important drugs in first-line anti-TB treatment irrespective of HIV status (Jamieson *et al*., 2014). The prevalence of INH mono-resistance has been reported by numerous studies in ranges from 4–12% for all TB cases with a global average of 8.1% for new TB cases (Villegas *et al*., 2016). There is, however, less evidence for RIF mono-resistance because it is less studied, but prevalence rates of less than 1% in Europe (Villegas *et al*., 2016) and 1.5% in SSA in new TB cases have been previously reported (Lukoye *et al*., 2015). In 2014, 3.5% of patients newly diagnosed with TB globally had MDR-TB (Villegas *et al*., 2016). In Kenya, a phenotypic cross-sectional study in 2015 reported a prevalence rate of 13.6% and 0.5% for INH and RIF mono-resistance, respectively, in new TB cases (Kerubo *et al*., 2016). The same study also reported an MDR-TB prevalence rate of 4.4% in new cases of TB in 2015 (Kerubo *et al*., 2016).

Drug resistance TB can be classified according to the anti-TB drug therapy used into four categories, namely; mono-resistance TB, multi-drug resistance TB, poly resistance TB and extensive drug resistant TB (WHO, 2016). Random *Mycobacterium tuberculosis* mutation rate

that leads to TB drug resistance of 3×10^{-7} to 1×10^{-9} per organism per generation has been previously reported (Sharma *et al*., 2016). This low level of drug resistant TB mutants multiplies as a result of inaccurate or incomplete chemotherapy (Sharma *et al*., 2016). Acquisition of drug resistance by *Mycobacterium tuberculosis* is a stepwise process amongst first line drugs (Meyssonnier *et al*., 2014). Therefore, MDR-TB arises after acquisition of either INH or RIF resistance followed by acquisition of resistance to the companion drug (Meyssonnier *et al*., 2014). Extensive drug resistant TB occurs as a result of resistance to at least INH and RIF as well as further resistance to any fluoroquinolone and a second-line injectable drug such as kanamycin, amikacin or capreomycin (Singhal *et al*., 2016). Patients with drug resistant TB respond poorly to treatment and are at an increased rate of dying (Sharma *et al*., 2016).

In 1952, INH which is a pro-drug that is activated by catalase-peroxidase encoded by *kat* G was introduced as an anti-TB drug (Palomina *et al*., 2014). The active form of INH inhibits the synthesis of mycolic acids through the NADH-dependent enoyl-acyl carrier protein (ACP)-reductase, encoded by *inh* A (Palomina *et al*., 2014). Resistance to INH is associated with mutations on the *kat* G gene and *inh* A gene or its promoter region (Ando *et al*., 2014). The *kat* G S315Tcodon mutation is the most common and it leads to an INH product deficient in forming the INH-NAD adduct needed to exert its antimicrobial activity (Ando *et al*., 2014). This mutation is associated with high-level INH resistance (MIC > 1 µg/mL) and occurs more frequently in MDR strains (Palomina *et al*., 2014). The second most common mutation occurs in the promoter region of *inh* A and resistant strains have a decreased affinity for the INH-NAD adduct (Palomina *et al*., 2014). The most prevalent mutation found is at position −15C/T and is more commonly associated with low level resistance to INH (< 1 µg/mL) (Ando *et al.*, 2014).

Mutations on *kat* G codon 315 occur in 50 to 95% of INH resistant strains whereas 20 to 42% of such strains have mutations in the promoter region of the *inh* A gene (Huyen*et al*., 2013). Studies have demonstrated that *kat* G codon 315 mutations are significantly associated with unfavorable treatment outcome while mutations on the *inh* A gene have been linked to relapses (Huyen *et al*., 2013). Rifampicin received therapeutic approval in 1968; it interacts with the ß subunit of RNA polymerase, encoded by the *rpo* B gene of *Mycobacterium tuberculosis* and interferes with protein synthesis (Coovadia *et al.,* 2013). Most gene mutations occur in an 81 base pair region located between *rpo* B codons 507 and 533 (Coovadia *et al*., 2013). According to previous studies, RIF mono resistant (RMR) TB is a proxy for the identification and subsequent treatment of MDR-TB because a large proportion of RIF resistant strains have INH resistance (Coovadia *et al*., 2013; Rana, 2013). However, a recent culture based study in KwaZulu-Natal province in South Africa cautioned against this measure in areas experiencing increased rates of RIF resistant TB (Coovadia *et al*., 2013). The emergence of MDR-TB complicates treatment because ineffective first-line drugs are replaced with costly second-line drugs and adverse drug reactions from second-line drugs have been reported and also, diagnosis is expensive and complicated (Jamieson *et al*., 2014). A recent retrospective study in Nigeria involving 60 patients who had not adhered to the national TB treatment guidelines showed that 16.7% developed pre-extensively (pre-XDR) drug resistant TB and this proves that inadequate treatment and poor patient compliance selects for drug-resistant isolates (Olusoji *et al*., 2013). Patients with INH mono-resistant TB require long treatment periods than those with INH-susceptible TB (Huyen *et al*., 2013). However, in western Kenya, precise data on the extent of drug resistant TB is sparsely available. Accurate and prompt detection of drug resistant TB is vital for the management of cases and is also an indicator of the quality of TB control in the region. The number of patients

with INH resistant TB in western Kenya is currently unknown. With the large-scale adoption of the GeneXpert testing method in most health facilities in western Kenya, patients with INH mono-resistant TB are likely to be diagnosed as RIF mono-resistant and widespread usage of the GeneXpert tests will lead to lower diagnosis of INH mono-resistance in facilities that do not have additional drug resistant testing methods leading to adverse treatment outcomes. Studies are therefore needed to accurately determine the prevalence of INH mono-resistant TB. In addition, the proportion of patients with specific RIF mutations and MDR-TB in western Kenya is unknown and therefore, implementation of control strategies is a challenge.

Drug resistant TB in HIV infected patients poses a great challenge because it is difficult to diagnose as it requires expensive laboratory facilities and treat since patients require extended hospitalization periods (Mesfin *et al*., 2014). In addition, the magnitude of drug resistant TB is not known precisely because of the lack of prevalence data in most TB endemic countries (Mesfin *et al*., 2014). People living with HIV are at an increased risk of developing mono-resistant and MDR-TB because HIV infected patients have a rapid disease progression and more so, in institutionalized settings where MDR-TB is prevalent (Mesfin *et al*., 2014). This may lead to rapid development of a pool of drug resistant TB patients, or an outbreak (Mesfin *et al*., 2014). Furthermore, people living with HIV may also be more likely to be exposed to MDR-TB patients, due to either frequent admissions in settings with poor infection control or association with peers who may have MDR-TB, including in prison cells (Mesfin *et al*., 2014). Patients with HIV and TB co-infection progress to active disease faster than immune competent people (Mesfin *et al*., 2014). Pill burden on TB and HIV co-infected patients and drug mal-absorption in HIV infected patients, especially RIF and Ethambutol, can also lead to drug resistance and

contributes to treatment failure (Mesfin *et al*., 2014). In western Kenya where HIV is common, drug failure is likely to occur. Studies in high burden TB countries have emphasized the importance of molecular characterization of *rpo* B, *kat* G and *inh* A mutations for the management of drug resistant TB particularly in HIV infected patients (Sitienei *et al*., 2013). Laboratory based studies in the general population will identify specific mutations associated with drug resistant TB in order to investigate the association of *rpo* B, *kat* G and *inh* A gene mutations with HIV status.

Time to liquid culture positivity on Mycobacterial growth indicator tubes is inversely correlated with the number of colony forming units on solid media and can therefore be used to determine *Mycobacterium tuberculosis* bacilli burden (Olaru *et al*., 2014). Similarly, smear microscopy using Ziehl-Neelsen (ZN) has been shown to reflect the extent of TB infection with higher smear grades being associated with presence of cavitary lesions (Olaru *et al*., 2014). Smear microscopy provides rapid results, is inexpensive, easy to perform, does not require complex laboratory equipment and is therefore very suitable for low-resource settings (Olaru *et al*., 2014) and can be used to appropriately monitor the effectiveness of treatment regimens in HIV infected and uninfected patients with TB. In western Kenya, the association between ZN smear conversion time of HIV infected and uninfected TB patients with confirmed RIF and INH resistant mutations has not been extensively studied. Information generated is useful in determining the effectiveness of the current guidelines for the treatment of HIV patients with TB drug resistance.

The current study therefore aimed to establish the prevalence of INH and RIF mono-resistant and MDR-TB as well as the association of *rpo* B*, kat* G and *inh* A *Mycobacterium tuberculosis* gene

mutations with HIV status and to determine the time to sputum smear conversion of patients with confirmed drug resistant TB in western Kenya.

1.2 Statement of the problem

In Kenya, in 2015, 107 000 people had TB out of which 36 000 were HIV co-infected; a phenotypic cross-sectional study in Kenya also reported a prevalence rate of 13.6% and 0.5% for INH and RIF mono-resistance, respectively in new TB cases. The same study also reported an MDR-TB prevalence rate of 4.4% in new cases of TB in 2015. However in western Kenya, precise data on the extent of the distribution of RIF and INH mutations in patients with TB is not available. Therefore the management of patients with drug resistant TB is a challenge since these patients are not appropriately diagnosed and treated. Patients with drug resistant TB require longer treatment period of close to 24 months as compared to patients with drug susceptible TB. Therefore, in the absence of accurate diagnosis, they are a constant source of transmission of drug resistant strains. The present study used both phenotypic and genotypic methods of assessing drug resistant. These methods included the following; drug susceptibility tests (DST), GeneXpert MTB/RIF assay, line probe assay (LPA) and gene sequencing methods. Drug susceptibility tests are phenotypic methods used to determine the sensitivity of *Mycobacterium tuberculosis* to first-line drugs. The GeneXpert MTB/RIF assay uses real-time PCR to amplify an *M. tuberculosis*-specific sequence of the *rpo* B gene. The LPA detects *rpo* B, *kat* G and *inh* A gene mutations associated with RIF and INH resistance after DNA extraction, multiplex PCR and sequence hybridization procedure. Gene sequencing identifies drug resistance mutations as well as markers with which transmission can be monitored effectively.

In 2014, according to the National AIDS and STI Control Programme (NASCOP), counties in western Kenya had a HIV prevalence rate of between 3%-19% (Kenya AIDS Response Progress Report, 2016). Patients with HIV infection are at an increased risk of TB infection because they are immune compromised and therefore there is rapid disease progression and reactivation of latent infection. Studies in institutionalized settings such as prisons and refugee camps reported positive association between MDR-TB and HIV infection. There is currently no evidence supporting an association between RIF, INH and MDR-TB and HIV in the general population particularly in western Kenya where HIV is common. Data on the genetic variation of isolates of *Mycobacterium tuberculosis* in western Kenya and frequency of occurrence of *rpo* B, *kat* G and *inh* A mutations determining resistance to INH and RIF especially in HIV positive patients have not yet been reported.

In Kenya, HIV infected and uninfected patients with TB are treated with a 6 months regimen of RIF, INH, Pyrizinamide and Ethambutol for the first 2 months followed by 4 months of RIF or INH. It is however not known if the same treatment regimen should be used for HIV infected and uninfected patients with drug resistant TB. Follow-up studies on TB and HIV co-infected patients with confirmed RIF and INH mutations are urgently needed to monitor the effectiveness of the current treatment regimen. As such the current study determined the association of RIF and INH gene mutations with HIV status and patient treatment response in western Kenya in 2012-2014.

1.3 Objectives

1.3.1 Main Objective

To determine the prevalence of RIF and INH drug resistance and the association of gene mutations with HIV status and treatment response in patients with TB in western Kenya in 2012-2014.

1.3.2 Specific objectives

1. To determine the prevalence of RIF and INH mono-resistant and MDR *Mycobacterium tuberculosis* in western Kenya in 2012-2014.
2. To establish the association of *rpo* B, *kat* G and *inh* A gene mutations with HIV status in western Kenya in 2012-2014.
3. To compare the sputum smear conversion time of HIV infected and uninfected patients with RIF and INH mono-resistant and multi-drug resistant gene mutations in western Kenya in 2012-2014.

1.3.3 Research questions

1. What is the prevalence of RIF and INH mono-resistant and MDR *Mycobacterium tuberculosis* in western Kenya in 2012-2014?
2. What is the association of *rpo* B, *kat* G and *inh* A gene mutations with HIV status in western Kenya in 2012-2014?
3. What are the comparisons between the sputum smear conversion time of HIV infected and uninfected patients with RIF and INH mono-resistant and multi-drug resistant gene mutations in western Kenya in 2012-2014?

1.4 Significance of the study

In western Kenya using Drug Susceptibility Tests, Line Probe Assay and gene sequencing methods to identify cases of drug resistant TB will ensure that patients with drug resistant TB are adequately treated based on prompt and accurate diagnosis. This will reduce the transmission of resistant strains, which cause high mortality to healthy individuals particularly in hospitals. Identifying drug resistant strains in patients receiving treatment also provides adjustments in drug regimens and minimizes re-infection. The level of drug resistance provides an epidemiological indicator that is said to assess the amount of transmission of resistant TB in the community because patients become infectious for a prolonged period (Sethi *et al*., 2013). Therefore, the current study provided data to strengthen research on drug resistant TB in western Kenya to ensure continuous monitoring of the epidemiological profiles of RMR, INH mono-resistant and MDR *Mycobacterium tuberculosis.*

Appropriate intervention strategies such as intensive HIV screening in patients with drug resistant TB will improve clinical care particularly in HIV infected patients. The present study provided information on the association between specific mutations on the *rpo* B, *kat* G, and *inh* A and HIV status. In western Kenya, genotyping of drug resistant *Mycobacterium tuberculosis* establishes levels of mutations to anti-TB drugs, which are indicative of chemotherapeutic failure. In addition, future immunological studies can be done on patients to identify markers of drug resistance to design point of care tests as an alternative to conventional methods.

Sputum smear microscopy has been the primary method for diagnosis of pulmonary TB in resource limited countries (Bawri *et al.,* 2008). However, the usefulness of smear microscopy in determining the conversion time of patients with *rpo* B*, kat* G and *inh* A gene mutation in a HIV endemic population has not been extensively studied. Therefore, in this study, the sputum smear conversion time of HIV infected and uninfected patients with mutations associated with INH and RIF resistance was established in order to monitor the effectiveness of the current treatment regimen recommended for HIV patients with drug resistant TB.

CHAPTER TWO: LITERATURE REVIEW

2.1 Aetiology and epidemiology of TB

In 2015, 10.4 million people worldwide developed tuberculosis (TB) and 1.4 million died from the disease (WHO, 2016). An estimated 2.7 million people had TB in Africa and 107 000 of these cases were reported in Kenya (WHO, 2016).

The global male to female prevalence ratio of TB is 1.85 and this has been shown to increase with age (Boum *et al*., 2014). Likewise in Africa, male patients constitute a majority of TB patients because of the health seeking habit of female patients in suspecting TB infection (Boum *et al*., 2014). This therefore contributes to unreported cases of TB (Boum *et al*., 2014). In addition, more females are HIV infected and most cases of pulmonary TB in HIV infected patients have smear negative results (Boum *et al*., 2014). Most of these studies that show the gender specific nature of TB infection have used microscopy techniques that are less sensitive (Boum *et al*., 2014). In the present study, molecular methods for screening TB were used to confirm these findings.

The incidence of TB is strongly associated with age (Blaser *et al*., 2016). The increased incidence in children less than 5 years is attributable to high transmission and rapid progression of disease as a result of a less developed immune system while in those aged between 30 – 50 years; it is as a result of a previous history of TB treatment (Blaser *et al*., 2016). However, it is not known if similar findings can be obtained particularly after the adoption of anti-retroviral therapy (ART) and case finding programs in children and the elderly (Blaser *et al*., 2016). The

present study provided data on the distribution of age in TB infected patients during wide use of ART in western Kenya for the treatment of HIV infection.

2.2 HIV and Tuberculosis co-burden

Globally, in 2015, 1.2 million Human Immunodeficiency Virus (HIV) infected patients had TB and 400 000 deaths occurred as a result of tuberculosis (TB) and HIV co-infection (WHO, 2016). In Africa, in 2015, 834 000 patients who had TB were HIV infected and 300 000 deaths occurred in TB and HIV co-infected patients (WHO, 2016). In Kenya, in 2015, 36 000 people were TB and HIV co-infected and approximately 7 200 deaths were reported (WHO, 2016). The present study provided the prevalence TB and HIV co-infection in western Kenya in the general population.

2.3 Multi-drug resistant tuberculosis

Multi-drug resistant (MDR) TB, defined as *Mycobacterium tuberculosis* strains with resistance to Isoniazid (INH) and Rifampicin (RIF) has a major impact on the treatment success of TB particularly in Africa where HIV is common (Andrews *et al*., 2007). This is because less effective and more expensive second-line drugs, which have been associated with side effects are used in treating MDR-TB patients (Fenner *et al*., 2012). In 2015, approximately 132 000 cases of MDR-TB were detected and notified globally, 26 929 of these cases were reported in Africa (WHO, 2016). Drug resistance TB is caused by poor treatment adherence enabling selection and growth of resistant strains or infection with a drug resistant strain (Andrews *et al*., 2007). Bacterial factors such as epistatic interactions (where the effect of one gene is modified by one or several other genes) between different strain genetic backgrounds and acquired drug resistance mutations could play a role in this context also contribute to the global emergence and spread of

drug resistant TB (Fenner *et al*., 2012). It has been shown that adherence to long-term therapy is determined by a set of factors such as social and economic factors which include low-income, lack of social support, low-education, financial problems and inability to afford services that have been linked to TB treatment adherence (Muture *et al*., 2011). Older age, the male gender, inadequate knowledge, ignorance on the need for treatment compliance and stigma are among reported patient-related factors that influence TB treatment default (Muture *et al*., 2011). Reported health care related factors for default include poor service provider attitude, negative attitude by TB patients towards the treatment centre, running out of drugs, access to health services and living near the treatment centre (Muture *et al*., 2011). Side effects to drugs, drugs being too strong and feeling better are among therapy related factors that influence TB treatment default while HIV co-morbidity is among the condition-related factors reported (Muture *et al*., 2011).

The distribution of MDR-TB and RIF mono-resistant TB among new and previously treated TB cases in 2013 was 3% and 15%, respectively in Africa (WHO, 2016). Kenya is among the 30 high TB burden countries worldwide, and currently the estimated rate of MDR-TB and RIF mono-resistant TB was 1.3% and 9.4% in new and previously treated TB patients (WHO, 2016). In western Kenya, in 2008, a TB re-treatment surveillance program reported 10 cases of MDR-TB (Park *et al*., 2012). However, the data available on the overall proportions of MDR-TB in western Kenya is not extensive and up-to-date. This therefore poses a major challenge to the management of drug resistant TB. The current study provided data on the proportions of patients with MDR-TB in western Kenya.

2.4 Anti-TB drugs and treatment modalities

Treatment of drug susceptible TB in HIV infected and HIV uninfected patients is based on a 6 months first-line drug regimen of a combination of drugs to prevent the emergence of resistant TB (http://guidelines.health.go.ke:8000/media/TB_Treatment_GUIDELINES_2013.pdf). In the first two months of intensive treatment, four drugs, namely, Isoniazid (INH), Rifampicin (RIF), Pyrazinamide, Ethambutol or Streptomycin are used to rapidly reduce the bacillary load (http://guidelines.health.go.ke:8000/media/TB_Treatment_GUIDELINES_2013.pdf). This is followed by 4 months of continuation therapy of either RIF or INH. Second-line drugs are used to treat patients with resistant TB and therapy consists of an intensive phase of 8 months and a continuous phase of 12 months (http://guidelines.health.go.ke:8000/media/TB_Treatment_GUIDELINES_2013.pdf). During the intensive phase, the following drugs are used, Kanamycin, Prothionamide, Levofloxacin, Cycloserine and Pyrazinamide while Prothionamide, Levofloxacin, Cycloserine and Pyrazinamide are used during the 12 months continuation phase (http://guidelines.health.go.ke:8000/media/TB_Treatment_GUIDELINES_2013.pdf). The effectiveness of the current guidelines has not been done particularly in HIV infected patients. The present study therefore established the treatment response of HIV infected and uninfected patients with drug resistant mutations in western Kenya.

Because of its potent bactericidal activity, INH is recommended as a first-line agent for the treatment of TB (Wang *et al*., 2014). INH enters *Mycobacterium tuberculosis* as a pro-drug by passive diffusion and is activated by catalase-peroxidase which is encoded by *kat* G, to generate free radicals that attack multiple targets within the *Mycobacterium tuberculosis* cell (Bardou *et*

al., 1998), mutations on the *kat* G gene lead to high-level INH resistance (>1 µg/ML in 7H10 agar) (Bardou *et al*., 1998). After the introduction of INH, resistant strains which had lost catalase activity were reported by numerous studies (Ramaswamy *et al*., 2003). Most common mutation in *kat* G occur on the Ser315Thr codon (Rattan *et al*., 1998). An enoyl (ACP) reductase, encoded by *inh* A gene, is also an intracellular enzymatic target for activated INH and is involved in the biosynthesis of mycolic acids and *inh* A mutations lead to low-level INH resistance (resistant to 0.2 µg/ML in 7H10 agar) (Marrakchi, *et al.,* 2000). A T > G transversion in resistant strains, at position 280 in the *inh* A gene, results in the ser 94 to ala 94 replacement which alters the binding affinity of ACP and this ultimately results in INH resistance (Rattan *et al*., 1998). Previous studies have also shown that mutations in the upstream region of the *inh* A locus result in increased levels of *inh* A expression, thereby elevating the drug target levels and producing INH resistance (Ramaswamy *et al*., 2003). Studies have reported that high-level INH resistance is associated with MDR-TB and TB transmission whereas low-level INH resistance leads to Streptomycin resistance and patients with INH mono-resistant TB have an increased risk of relapse (Huyen *et al*., 2013). Rifampicin is also recommended for first-line TB treatment and résistance is caused by an alteration of the β-subunit of RNA polymerase, which is encoded by the *rpo* B gene (Minh *et al*., 2012). More than 95% of RIF mono-resistant (RMR) strains are associated with mutations within an 81-base pair region of the *rpo* B gene known as the RIF resistance determinant region (RRDR) (Sreevatsan *et al*., 1998). The RRDR includes *rpo* B codons 507 to 533, encoding 27 amino acids and the most common mutations from amino acid replacement are at *rpo* B codons 516, 526 and 531 (Minh *et al*., 2012) that lead to high-level RIF resistance (Madania *et al*., 2012). Resistance to RIF has been associated with treatment failure (Mukinda *et al*., 2013). A study in Cape Town showed that more than 90% of patients with

RMR-TB are also had INH resistant (Mukinda *et al*., 2013). Therefore, RMR is frequently used as a proxy for MDR-TB diagnosis and most patients with RMR-TB are treated with MDR-TB regimens, even in the absence of INH susceptibility results (Mukinda *et al*., 2013). This also increases the reported cases of patients with MDR-TB (Mukinda *et al*., 2013). However, this should not be adopted in areas with increasing cases of RMR-TB (Coovadia *et al*., 2013). In western Kenya, where the proportion of patients with RMR-TB is unknown, clinicians are discouraged from adopting similar recommendations (Mukinda *et al*., 2013). Recent studies have demonstrated that INH mono-resistant TB did not decline in recent years despite the reduction in TB cases (Wang *et al*., 2014). This represents a growing concern in western Kenya where the proportion of patients with INH mono-resistant TB is currently unknown. As such, the current study determined the proportion of RMR-TB and INH mono-resistant TB patients in western Kenya.

2.5 Sputum smear conversion time of patients with confirmed drug resistant

Drug susceptibility tests (DST) are recommended for routine monitoring of MDR-TB (Sarin *et al*., 2010). However, recent studies have reported more than 90% agreement between DST and smear results in MDR-TB patients being treated for up to a period of 27 months (Gammino *et al*., 2012; Quy *et al*., 2006). This would decrease the need of performing DST in countries with limited culture facilities. Sputum smear microscopy to monitor treatment response is therefore practical in resource limited countries (Bawri *et al*., 2008). A retrospective study using clinical data showed the benefits of monitoring treatment response of HIV infected patients with drug resistant TB (Palacios *et al*., 2012). Follow-up studies on the specific mutations underlying resistance to RIF and INH and the time to sputum conversion, especially in HIV-infected

patients (Palacios *et al*., 2012). In Kenya, the treatment of drug resistant TB is the same irrespective of HIV status

(http://guidelines.health.go.ke:8000/media/TB_Treatment_GUIDELINES_2013.pdf).

The treatment response rate of HIV infected patients with drug resistant TB is currently unknown to justify the continued adoption of this guideline. In the present study, the sputum smear conversion time of TB and HIV co-infected patients with confirmed *rpo* B*, kat* G and *inh* A *Mycobacterium tuberculosis* gene mutations in western Kenya was determined.

2.6 Tuberculosis drug resistance and HIV

Studies in high-burden TB countries have reported that HIV positive patients are more likely to be infected with TB (Lukoye *et al*., 2013b) because patients with HIV have a deficient immune system and this provides a habitat for the development of TB (Lopez-Alvarez *et al*., 2010). A clinical-based study in South Africa also showed that HIV positive patients with TB had poor intestinal TB drug absorption and of treatment failure with standard regimens (Sethi *et al*., 2013). This potentially increased the risk of acquiring mono-resistant and MDR-TB. The emergence of mono-resistant and MDR-TB in poor resource countries complicates the effective management of TB especially in HIV positive patients because it remains undetected and untreated. Since the emergence of MDR-TB strains in the 1990s, the prevalence of drug resistant TB has increased due to HIV (Homolka *et al*., 2008) particularly in western Kenya which had the highest cases of HIV in 2012 (KAIS, 2012). Rapid determination of TB drug resistant mutation profiles in clinical isolates of *Mycobacterium tuberculosis* is important for the early administration of RIF and INH for the prevention and transmission of resistant TB (Poudel *et al*., 2012). In addition, molecular characterization of drug resistant TB in specific genes has the potential for developing

rapid molecular drug susceptibility tests (Poudel *et al*., 2012). Clinical studies have reported conflicting results on the association of HIV and the development of *rpo* B, *kat* G and *inh* A mutations that have been linked with drug resistant TB (Andrews *et al*., 2007). In western Kenya, current molecular based studies are needed to provide evidence to improve treatment outcome on the association of HIV status and gene profiles of drug resistant TB. Therefore, the current study identified previously described mutations present on the *rpo* B, *kat* G and *inh* A that lead to the selection of INH and RIF resistant *Mycobacterium tuberculosis* in HIV infected and un-infected patients.

2.7 Tuberculosis drug resistance testing methods

Control and management of drug resistant TB is based on the rapid detection of resistant strains followed by appropriate and effective treatment (Santos *et al*., 2017). Current molecular methods based on DNA amplification allow identification of genomic mutations and single nucleotide polymorphisms associated with drug resistance, particularly resistance to rifampicin (RIF) and isoniazid (INH) (Santos *et al*., 2017).

The GeneXpert MTB/RIF assay works by detecting *Mycobacterium tuberculosis* and RIF resistance by polymerase chain reaction (PCR) based amplification of the 81-bp *rpo* B gene segment and probing for the mutations that are related to RIF resistance (Pandey *et al*., 2017). The assay is automated and provides test results within 2 hrs (Pandey *et al*., 2017). In addition, the assay reports a 45% increase in TB case detection in HIV infected patients as compared to sputum smear microscopy and can also be used for diagnosis of extra-pulmonary TB from a

range of biological samples, however, concerns about false-positive RIF resistance results have been reported and the GeneXpert assay does not test INH resistance (Pandey *et al*., 2017).

The GenoType MTBDR*plus* assay is a qualitative *in vitro* test for the identification of the *Mycobacterium tuberculosis* complex and its resistance to RIF and/or INH from pulmonary smear positive and negative clinical specimens and cultured isolates (Meaza *et al*., 2017). This assay is based on DNA-strip technology and involves DNA extraction, master mix preparation and addition, multiplex amplification with bio-tinylated primers and detection with reverse hybridization (Meaza *et al*., 2017). The test is useful in the rapid diagnosis of MDR-TB, which is a prerequisite for the appropriate treatment initiation. Results are usually available within 48 hrs (Meaza *et al*., 2017). However, this test has been shown to have lower INH resistance sensitivity in smear negative sputum samples (Meaza *et al*., 2017).

Direct sequencing of *Mycobacterium tuberculosis* provides the advantage of identifying predictive markers for INH and RIF resistance (Jagielski *et al*., 2014). Information on the nature and frequency of RIF and INH resistance mutations is useful in developing new point of care TB diagnostic tests (Jagielski *et al*., 2014). Direct sequencing is however expensive and labor intensive and requires well trained staff (Jagielski *et al*., 2014).

The conventional *Mycobacterium tuberculosis* drug susceptibility test is considered the gold standard technique for identifying resistant strains however, it has a long turn-around time of close to 8 weeks (Pandey *et al*., 2017). Furthermore, it requires more sophisticated and higher bio-safety level laboratory along with the well trained staffs (Pandey *et al*., 2017).

To overcome the challenges associated with relying on one TB drug resistant method, the present study utilized both phenotypic and molecular techniques to accurately identify TB drug resistance. The GeneXpert assay was used as a rapid screening test for identifying patients with TB and RIF resistance, the GenoTypeMTBDR*plus* assay identified RIF and INH resistance in patient samples, drug susceptibility tests were used to determine sensitivity of cultured *Mycobacterium tuberculosis* to RIF and INH and samples with DST and GenoTypeMTBDR*plus* test results were sequenced to confirm or identify novel gene mutations.

In this study, the proportion of drug resistant *Mycobacterium tuberculosis* in sputum isolates and the association of *rpo* B*, kat* G and *inh* A gene mutations with HIV status and sputum smear conversion time of TB and HIV co-infected patients in western Kenya in 2012-2014 was also determined.

CHAPTER THREE: METHODS

3.1 Study site

Samples from 1369 patients were processed from August 2012 to October 2014 from 415 Division of Leprosy, Tuberculosis and Lung Diseases (DLTLD) health facilities in 13 counties in western Kenya (Figure 3.0). The health facilities were supported by DLTLD in implementing TB and HIV activities. Western Kenya is located on latitude 0° 3' 50" S and longitude 35° 10' 51" E and has a population of 5,442,711 (Kenya National Bureau of Statistics, 2009 population census) which was projected to increase by 2.46% by 2015. Infection with TB contributes to ill health and death and creates an economic health burden. In this region, 39.2% adolescents in the age group 12-18 years had TB (Nduba *et al*., 2015) and in a previous national survey, MDR-TB prevalence was between 0.0% - 1.1% (Nyamogoba *et al*., 2013). The prevalence of HIV infection, a risk factor for TB in this region was 15.1% in persons between 15-64 years of age and it represents the highest cases in Kenya and TB is the leading cause of death in people living with HIV(KAIS 2012). In western Kenya, the Academic Model Providing Access to Healthcare (AMPATH), in collaboration with the National Tuberculosis, Leprosy and Lung Disease Unit (NTLDU), provides integrated models of TB and HIV care in health facilities (Owiti *et al*., 2015). Presently AMPATH supports 35 main and multiple satellite clinics within ministry of health facilities in Kenya (Owiti *et al*., 2015). There is also the wide use of anti-tuberculosis drugs for the treatment of TB. Due to the long treatment periods associated with TB, it is expected that some patients might not adhere to prescribed chemotherapy. In addition, resistant TB could also be selected due to drug pressure. According to the Kenya National Bureau of statistics, the number of households in the 13 counties was 2 158 907 in 2013 and this represents

overcrowding which could lead to transmission of TB. In addition, in western Kenya, the presence of slum areas such as Obunga, Manyatta and Nyalenda would increase the transmission of TB (Kerubo *et al*., 2016).

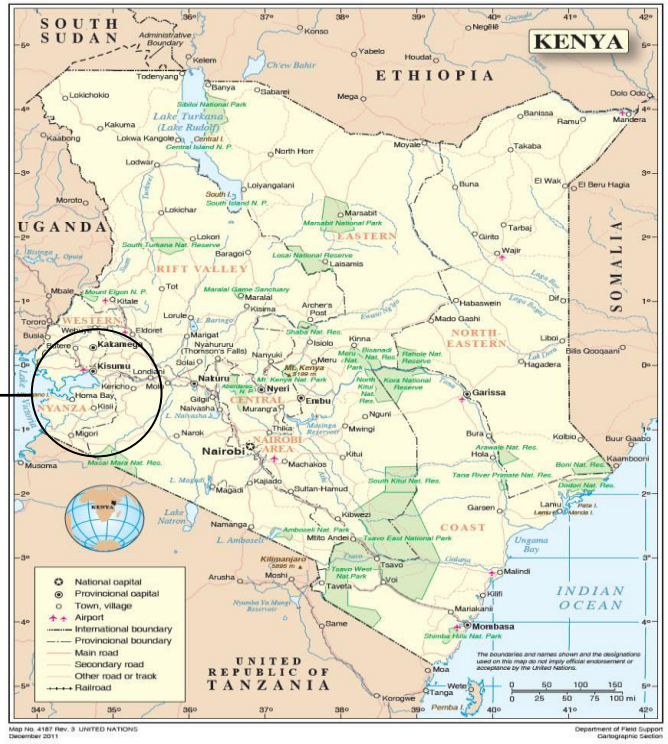

Figure 3.0 Map of the study site

Latitude, 0° 3' 50" S, longitude 35° 10' 51" E

www.googleimages.com

3.2 Study design

The present study was longitudinal in which TB patients were enrolled between 2012 and 2014 after the revision of the 8-months treatment guidelines to 6 months by the Ministry of Health. Patients with drug resistant TB mutations were then followed up for a period of one year to establish the TB treatment response as confirmed by ZN sputum smear microscopy.

3.3 Study population

Age categorization of all study participants was based on arbitrary classification. Sputum samples from eligible patients reporting for TB treatment in 415 health facilities were collected based on the following enrollment criteria;

3.3.1 Inclusion criteria

- Ziehl-Neelsen (ZN) smear positive
- HIV negative or positive
- More than 4 ml of sputum sample
- Evidence of TB treatment history.
- Male or female gender
- Resident of western Kenya.
- More than 5 years of age.
- Signing of informed consent.

3.3.2 Exclusion criteria

- Unwilling to sign consent.
- ZN smear negative sample
- Insufficient sputum sample of less than 4 ml
- Unwilling to test for HIV infection.

3.4 Sample size calculation

The sample size was estimated based on the guidelines for surveillance of drug resistance in TB (http://apps.who.int/iris/bitstream/10665/44206/1/9789241598675_eng.pdf).

$$n = \frac{N \times z^2 \times p \times (1-p)}{d^2(N-1) + z^2 \times p \times (1-p)}$$

where:

n = sample size.

N = total number of new smear positive cases registered during one year in Kenya.

z= Z statistic for a level of confidence.

d = absolute precision.

p = expected proportion of RIF resistance in the target population.

In Kenya, in 2011, 37 085 new smear positive cases were reported (WHO, 2012). Based on laboratory data showing a RIF prevalence of 1.5% in 2011 and using a Z statistic value of 1.96 and an absolute precision prevalence of 1%, the sample size was calculated as follows:

$$n = \frac{37\,085 \times 3.8416 \times 0.015 \times 0.985}{3.7084 + 0.01478}$$

$$n = 565.39$$

Because sampling was done from populations with varying risk factors for TB infection such as HIV, alcoholism and TB transmission, a design effect of 2 was used as previously described (http://apps.who.int/iris/bitstream/10665/44206/1/9789241598675_eng.pdf).

$$n \times \text{design effect}$$

$$565.39 \times 2 = 1131$$

The calculated sample size was then increased by 20% to account for expected patient withdrawal. The current study therefore, targeted 1357 TB patients.

3.5 Sampling design

Random sampling of 415 health facilities that support routine TB diagnosis and treatment in 13 counties in western Kenya was done. The sample size for patients with TB was set at 1357. Clinicians at the respective health facilities then enrolled patients meeting the eligibility criteria into the study.

3.6 Data collection

Demographic information such as age, gender and HIV status was collected as shown in (Appendix 1) from participating patients after obtaining informed consent (Appendix 2). The health facility name, patient's contact address was also collected. County and treatment history data was available for all patients who participated in the study.

3.7 Sample collection

3.7.1 Sputum

Early morning sputum samples (4-10 ml) were collected from patients at enrollment into 50 ml conical centrifuge tubes and placed on polystyrene racks in cooler boxes containing ice packs. The samples were transported to the KEMRI/CDC TB laboratory in Kisian. This is a referral

laboratory for drug resistant TB testing in western Kenya. The laboratory participates in External Quality Assurance (EQA) for the performance of both the phenotypic and genotypic tests with the National Health Laboratory Services (NHLS) from South Africa and the College of American Pathologists (CAP). Recruited patients were categorized into 'MDR diagnosis' or 'previously treated' cases according to their TB treatment history. Patients with MDR diagnosis had their samples collected for the first time for processing at the laboratory while previously treated cases were patients who had received TB treatment before enrollment into the study.

3.7.2 HIV testing

Patient HIV status was determined using the parallel algorithm for rapid HIV testing after counseling and testing. The Uni-Gold HIV Rapid Test® (Trinity Biotech Plc, Bray, Co Wick low, Ireland) and Abbott Determine® HIV-1/2 test (Abbott Laboratories, Chicago, Illinois, USA) kits were used as per the National AIDS and STI Control Programme (NASCOP) guidelines (http://www.who.int/hiv/topics/vct/policy/KenyaGuidelines_Final2009.pdf).

Briefly, approximately 200 µl of finger prick blood was collected using capillary tubes from the patient and transferred separately onto two HIV test kit strips at the same time. If both kits gave the same results, the test was interpreted as either positive or negative. If the two test results differed, testing was repeated at a stand-alone HIV testing facility after two weeks or patient was referred to a laboratory for ELISA or Western Blot testing for HIV testing.

3.8 Sample processing

3.8.1 Culture of *Mycobacterium tuberculosis*

Sputum specimens were processed using the N-acetyl-L-cysteine(NALC) and sodium hydroxide (NaOH) decontamination and digestion procedure.

(https://www.bd.com/ds/technicalCenter/promotionalFlyers/ssMGIT_Procedure%20_Sheets.pdf)

Processed sputum samples were split into two aliquots of 0.5 ml and one aliquot of 1 ml. The 0.5 ml aliquot (suspended in sterile PBS) was inoculated on the *Mycobacteria* growth indicator tubes (MGIT) tubes containing 0.8ml polymixin B, Amphotericin B, Naladixic acid Trimethoprim, Azlocillin (PANTA) (Becton, Dickinson and company) supplement and incubated at 37°C in the Bactec MGIT 960® instrument (BD Diagnostic systems, Baltimore, Maryland, USA) and monitored weekly for six weeks. Smear microscopy using ZN and the capilia NEO test (Tauns Laboratories, Kamishima, Shizuoka, Japan) were performed on instrument positive MGIT tubes to confirm the presence of *Mycobacterium tuberculosis.* Drug Susceptibility Testing (DST) was done on all instrument positive samples. The 1 ml aliquot was processed according to the Xpert MTB/RIF (Cepheid, Sunnyvale, California, USA) instruction manual. Approximately 2 ml of lysis buffer was added to the sample and mixed. Following incubation at room temperature for 5 min, the suspension was mixed again and then incubated for a further 10 min. Thereafter, 2 ml of the lysate was added to the labeled cartridge and was inserted into a GX16 instrument (Cepheid, Sunnyvale, California, USA). The results were available in approximately 2 hours.

The other aliquot of 0.5 ml was transferred into a vial and stored at -80°C for future DNA extraction: https://www.ghdonline.org/uploads/MTBDRplusV2_0212_304A-02-02.pdf.

All reagents used in sample decontamination and digestion, were processed as indicated in Appendix 3.

3.8.2 Drug Susceptibility Testing

Drug Susceptibility Testing (DST) was done using the BACTEC™ MGIT™ 960 SIRE kit (BD Diagnostic systems, Baltimore, Maryland, USA) for first line anti-tuberculosis drugs on samples that had *M. tuberculosis* after incubation in the Bactec MGIT 960® instrument (BD Diagnostic

systems, Baltimore, Maryland, USA) by adding 0.5ml of inoculum from the instrument positive *M. tuberculosis* MGIT tubes into newly labeled MGIT tubes containing 0.8ml SIRE supplement and 0.1 ml of isoniazid (INH) and rifampicin (RIF) reconstituted to a concentration of 0.1 µg/ml and 1.0 µg/ml, respectively. MGIT tubes were placed on AST carriers and incubated at 37°C in the BACTEC™ MGIT™ 960® instrument for between 4 to 21 days. A growth unit cut-off of 100 was used to score DST results as resistant or susceptible as previously described (https://www.bd.com/ds/technicalCenter/clsi/clsi-960sire.pdf).

3.8.3 DNA extraction and PCR

Extraction of DNA with GenoLyse® kit (Hain Life Sciences, Nehren, Germany) was performed according to the manufacturer's protocol (https://www.ghdonline.org/uploads/MTBDRplusV2_0212_304A-02-02.pdf).

Extraction of DNA was done with the GenoLyse kit (Hain lifesciences). Briefly, approximately, 0.5 ml of digested and decontaminated sample was transferred to a 1.5 ml screw cap tube and centrifuged for 15 min at 10,000*g*. The pellet was re-suspended in 100 µl of an alcalic lysis buffer and incubated for 5 min at 95°C in a water bath. Subsequently, 100 µl of neutralization buffer was added to lysate, vortexed and centrifuged for 5 min at 10, 000*g*. Approximately 5 µl of supernatant consisting of extracted DNA was used for PCR. To determine the concentration of DNA, sample was diluted 1:100 in TE buffer (Sigma-Aldrich, Missouri, USA) and placed in a cuvette in the spectrophotometer (Hitachi, Tokyo, Japan) and absorbance recorded at 260 nm. To determine the purity of DNA, diluted DNA was placed in a spectrophotometer (Hitachi, Tokyo, Japan) and absorbance determined at 280 nm. The ratio of the absorbance at 260nm and 280nm was then calculated.

Approximately, 10 µl of amplification mix A (Hain Lifescience, Nehren, Germany) and 35 µl of amplification mix B (Hain Lifescience, Nehren, Germany) was mixed to constitute a PCR master mix of 45 µl, and 5 µl of extracted DNA from above was added. By multiplex PCR, the *rpo* B*,* *kat* G *and inh* A genes were amplified and the initial amplification conditions were as follows; initial denaturation step of 15 min at 95°C followed by 20 cycles of 30 sec at 95°C and 2 min at 65°C, then 30 cycles of 25 sec for 95°C, 40 sec at 50°C and 40 sec at 70°C, then finally, 1 cycle at 8 min for 70°C. The resulting biotin-labeled amplicons were hybridized to DNA probes bound to membrane strips.

3.8.4 Line Probe Assay

The Genotype MTBDR line probe assay (LPA) (Hain Lifescience, Nehren, Germany) was performed as recommended by the manufacturer (https://www.ghdonline.org/uploads/MTBDRplusV2_0212_304A-02-02.pdf).

Hybridization was detected by addition of a streptavidin/alkaline phosphatase (AP) conjugate and an AP mediated staining reaction. Hybridization was performed using the GTBlot® 48 device (Hain Lifescience, Nehren Germany), hybridization and stringent washing buffers were preheated to 45°C in a water bath. Denaturation buffer, 20 µl, was mixed in a plastic 48-well tray with 20 µl of amplified sample and incubated at room temperature for 5 mins. Subsequently, 1 ml of hybridization buffer was added to each well and mixed. One pre-labeled test strip was added into each well, and the wells were incubated for 30 mins at 45°C. Stringent buffer, 1 ml was added to each strip and incubated for 15 min at 45°C. After the aspiration of the solution, 1 ml rinse buffer was added to each strip and incubated at room temperature for 1 min. The following incubation steps took place at room temperature; removal of rinse buffer, addition of 1

ml of conjugate buffer to each strip, then incubation for 30 min. After incubation, all solutions were removed and the test strips were rinsed twice with rinse buffer for 1 min, followed by a washing step with distilled water for 1 min. Substrate buffer, 1 ml, was added to each strip, and they were incubated for 5 mins. All solutions were removed, and the reaction was stopped by two rinses with distilled water. The test strips were dried and then taped to the MTBDR plus assay worksheet for interpretation.

Results were interpreted as 'sensitive' if all the wild type probes of a gene stained positive, 'mutant' if the mutation bands of the *rpo* B, *kat* G and *inh* A genes stained positive and 'missing wild type', if there was absence of staining for at least one of the rifampicin or isoniazid wild type probes. Thereafter, samples with discordant LPA and DST results were sequenced to confirm the presence of mutations.

3.8.5 *Mycobacterium tuberculosis* drug resistance gene PCR and sequencing

Isolates with LPA and DST discordant results were sequenced as follows: DNA was extracted as explained above, and 1µl of DNA was added to a 24µl total volume master mix for amplification. The PCR master mix recipe consisted of the following; 12 µl quick load Taq 2× master mix (Bio labs, New England, UK), 1.5µl (5µM) *rpo* B, *kat* G or *inh* A forward and reverse oligonucleotide primers as described in Table 3.0 and 8.5 µl molecular grade water (Sigma-Aldrich, St. Louis, Missouri, USA).

The amplification protocol for the respective gene sections consisted of an initial denaturation step of 15 min at 95°C, followed by 50 cycles of 30 sec at 95°C, 30 sec at 60°C, and 30 sec at 72°C, ending with a final extension step of 7 min at 72°C. Gel electrophoresis was done to

visualize DNA bands on 1% agarose powder in 1×TBE buffer (Fisher Scientific, Loughborough, Leicestershire, UK).

DNA fragments of PCR products were purified using QIAquick PCR Purification Kit (QIAGEN, Venlo, Netherlands) according to manufacturer's recommendations. The purified PCR products were sequenced using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc., Foster City, California, USA.). 1 µl of purified PCR product template was added to a 9µl total volume master mix with the following recipe, 2µl BigDye terminator (Applied Biosystems Inc., Foster City, California, USA), 1 µl (5X) BigDye buffer (Applied Biosystems Inc., Foster City, California, USA), 2µl (1.6µM) forward and reverse *rpo* B, *kat* G or *inh* A oligonucleotide primers as described in Table 3.0 and 4µl molecular grade water (Fisher Scientific, Loughborough, Leicestershire, UK). The sequencing protocol consisted of 25 cycles of 10 sec 96°C, 5 sec 50°C and 4 min 60°C. Sequence purification was done utilizing the BigDye® XTerminator™ Purification Kit (Applied Biosystems Inc., Foster City, California, USA) following manufacturer's instructions. Purified sequences were analyzed on an ABI PRISM® 3100 genetic analyzer (Applied Biosystems Inc., Foster City, California, USA). Sequence editing, assembly and analysis were performed using Sequencher® v5.1 software (Gene Corporation, Inc, Suite, Michigan, USA).

Test performance characteristics for the LPA and DST with the gene sequencing as a reference method was calculated using a free online statistical calculator available at http://www.medcalc.org/calc/

Table 3.0: Primer sequences and target gene fragment size

Gene	Primer designation	Oligonucleotide sequence 5'- 3'	Size (bp]
rpo B	rpo B-1f	CTT GCA CGA GGG TCA GAC CA	543
	rpo B-2r	ATC TCG TCG CTA ACC ACG CC	
inh A	inhA-1f	TGC CCA GAA AGG GAT CCG TCA TG	455
	inhA-2r	ATG AGG AAT GCG TCC GCG GA	
kat G	katG-F5	AAC GAC GTC GAA ACA GCG GC	455
	katG-R6	GCG AAC TCG TCG GCC AAT TC	

bp = base pair; *rpo* B = rifampicin resistance conferring gene; *inh* A = Isoniazid resistance conferring gene; *kat* G = Isoniazid resistance conferring gene.

3.7.6 Ziehl-Neelsen microscopy

Ziehl-Neelsen (ZN) microscopy was done on two occasions; at initial visit for drug-resistant TB evaluation and during follow-up visits for monitoring disease progression at respective health facilities. During follow-up visits to the health facilities, approximately 5 ml of sputum sample was collected from each patient. Samples were transported to the referral laboratory for ZN microscopy which was done by staining heat-fixed smears on microscopic slides for 5 minutes with carbolfuchsin (Sigma-Aldrich Co., St. Louis, Missouri, USA), alcohol decolorizing for 3 minutes, followed by counterstaining with malachite green (Sigma-Aldrich Co., St. Louis, Missouri, USA) for 1 minute. Sputum smear microscopy results were interpreted according to the International Union Against Tuberculosis and Lung Diseases (IUATLD) grading system (NLTLD, 2014) as follows, 10 – 99 acid fast bacilli in 100 immersion fields, 1+, 1- 10 acid fast bacilli per field, 50 examined fields, 2+, more than 10 acid fast bacilli per field in 20 examined fields, 3+.The quality of results was ensured by having two independent microscopists read the

slides and an additional third reader to confirm discordant results. Results were entered into an Excel spreadsheet.

3.9 Statistical analysis

The SPSS v17.0 statistical software (Chicago, Illinois, USA) and Microsoft Excel spreadsheet was used in data entry, coding and analysis.

Demographic data which included age, gender, HIV status, health facility, county and TB treatment history was entered on an excel spreadsheet. Data from the GeneXpert MTB/RIF assay was interpreted based on the presence of *Mycobacterium tuberculosis* DNA and RIF resistance as detected or not detected. Continuous variables such as age and categorical variables such as HIV status and gender as well as GeneXpert test results were summarized into frequencies and percentages.

To determine the prevalence of patients with RIF and INH mutations, data for the DST was interpreted as either sensitive or resistant to RIF and INH anti-TB drugs. For the LPA test, results were interpreted as either wild type or mutant on the RIF and INH resistant gene codons being analyzed. MDR-TB was defined as an isolate with both RIF and INH resistance as confirmed by LPA and DST, RIF mono resistant TB was defined as an isolate with only RIF resistance as confirmed by LPA and DST while INH mono resistant TB was defined as an isolate with only INH resistance as confirmed by LPA and DST. Gene sequencing data was interpreted based on the amino acid substitution on the *rpo* B, *kat* G and *inh* A gene codons. The test results were then summarized into frequencies and percentages. Test performance characteristics for the LPA

and DST with the gene sequencing as a reference method was calculated using a free online statistical calculator available at http://www.medcalc.org/calc/

To determine the association of *rpo* B, *kat* G and *inh* A gene mutations with HIV status, Multivariate analysis were used to determine the association of the variables age, gender and HIV status on the drug resistant mutations associated with RIF and INH resistance and subsequent Post hoc Univariate analysis using the Tukey Honest Significant Difference (HSD) test and Games Howell test were used to determine which variable had an effect on the drug resistant mutations. A logistic regression analysis was conducted to determine the potential association of HIV status and RMR-TB, INHMR-TB and MDR-TB mutations. Odds Ratio (OR) from the binary logistic regression analysis was then used to evaluate the association between RIF, INH and MDR-TB mutations and HIV status.

To determine the time to sputum smear conversion of HIV infected and uninfected patients with drug resistant mutations, the proportion of smear converted-patients was calculated by dividing the number of patients who had a negative smear during follow-up and the number of smear positive patients with confirmed drug-resistant TB at the first visit to the health facility and multiplying by 100. Mann-Whitney *U* test was used to compare the median sputum conversion time in HIV infected and uninfected patients.

A *P*-value of ≤ 0.05 was considered statistically significant.

3.10 Ethical considerations

Ethical approval was obtained from the Ethical Review Committee (ERC) of the Kenya Medical Research Institute (KEMRI) Nairobi (SSC number 2854, Appendix 4).

3.10.1 Risks and risk management

There were small risks which could occur to a participant as a result of taking part in this study. A participant may have felt concerned when they were asked questions about their HIV status. In this case the participant was free to answer or not answer any of these questions. It was their choice. A participant may have felt some anxiety about waiting for the results of the testing.

3.10.2 Physical risk

Minimal risk experienced when venous blood is collected.

3.10.3 Informed consent

Written informed consent was obtained from all potential participants.

3.10.4 Consenting process and documentation of informed consent

Once an eligible participant was identified, the patient was informed about the study. The information included the following; who was conducting the study, the purpose of the study, the study procedures, the risks and benefits, the assurance of confidentiality, their rights and that participation is voluntary. Having understood all this information, the eligible patient was allowed to ask questions for clarity. After that, they were requested to participate and if they agreed they were requested to read and understand the informed consent form before signing.

The study participants were requested to allow the isolates obtained from their sputum and blood samples to be stored for future research in KEMRI laboratory. No identifying information such as patient name and telephone number was stored and the information could not be linked to the participant. The investigator assigned this form once the participant has signed. All signed forms were kept under lock and key at the KEMRI/CGHR TB laboratory.

3.10.5 Provision for privacy and confidentiality protections

All efforts were put in place to ensure that ethical considerations and confidentiality of all eligible patients was secured. All eligible patients were provided with information about the study including benefits, risks, procedures, their rights and confidentiality. Informed consent was sought from each patient before the extra sputum sample was obtained.

3.10.6 Data security

All data collected from this study was confidential and not shared with anyone outside study team except if it concerned the patient's health and medication. Storage of study data followed the government regulations for the storage of confidential patient information. The final electronic database did not contain names or addresses of patients. The data was owned by KEMRI. Data case report forms were kept in a secure locked cabinet at KEMRI/CGHR TB laboratory. Electronic databases were password protected and only authorized persons had access. Until the database was finalized only the data manager had editing rights all other investigators had viewing rights.

3.10.7 Anticipated benefits

There were benefits to participants who chose to take part in this study. If they were diagnosed with TB they werereferred to TB clinic for further management. There were also benefits to the community.

3.10.8 Treatment

All recruited patients in this study were notified of their results and wherever necessary, they were referred to TB clinics for further management.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic and HIV-1 status of the TB patients

As described in table 4.1, clinical data was available for 1369 patients (Table 4.1). Majority of the patients were aged between 25-44 years, 801 (58.5%), males, 808 (59%), than females had TB and 835 (61%) of the patients were HIV co-infected (Table 4.1). Patients reporting for TB retreatment were less (n=18, 1.3%) than the patients seeking treatment for TB for the first time (n=1357, 98.7 %), (Table 4.1). All the TB cases reported in all 13 counties were new cases of TB with the exception of Migori County where out of the 75 TB positive patients, only two had been previously treated for TB (Table 4.1). Likewise in Migori County only one case out of the 7 patients with RIF resistance had previously been treated for TB.

The counties of Homabay, Kisumu, Kisii, Migori and Siaya had the highest new cases of TB, 225 (16.4%), 374, (27.3%), 180, (13.2%) 159, (11.6%), 314, (22.9%), respectively, Table 4.2. Only 602 (45.1%) of the samples were *Mycobacterium tuberculosis* positive as determined by the Xpert MTB/RIF® test. Rifampicin resistance was also detected in 47 (7.8%) MTB positive samples (Table 4.2).

Table 4.1: Demographic and HIV-1 status of the TB patients recruited to participate in the study in western Kenya

Parameter	Category (n,%)	Total
Age (years)	2-14 (38, 2.8) 15-24 (177, 12.9) 25-44 (801, 58.5) 45-64 (281, 20.5) 65+ (72, 5.3)	1369
Gender	Male (808, 59.0) Female (561, 41.0)	1369
HIV Status	Infected (835, 61.0) Uninfected (534, 39.0)	1369

n,%, number and percentage

Table 4.2: History of TB treatment and Xpert MTB/RIF assay results of recruited patients

	TB treatment history, n, (%)		Xpert MTB/RIF test, n, (%)	
County	New cases 1351, (98.7)	Retreatment 18, (1.3)	MTB positive, 618, (45.1)	RIF resistance, 47, (7.6)[a]
Busia	3, (0.2)	-	3, (0.2)	-
Elgeyo Marakwet	4, (0.3)	-	3, (0.2)	-
Homabay	225, (16.4)	3, (0.2)	91, (6.6)	5, (0.8)
Kakamega	30, (2.2)	-	16, (1.2)	-
Kericho	11, (0.8)	-	10, (0.7)	-
Kisii	180, (13.2)	6, (0.4)	70, (5.1)	5, (0.8)
Kisumu	374, (27.3)	-	170, (12.4)	11, (1.8)
Migori	159, (11.6)	3, (0.2)	75, (5.5)	7, (1.1)
Nandi	7, (0.5)	-	3, (0.2)	-
Nyamira	29, (2.3)	3, (0.2)	16, (1.2)	4, (0.6)
Siaya	314, (22.9)	3, (0.2)	150, (11.0)	15, (2.4)
Trans Nzoia	5, (0.4)	-	5, (0.4)	-
Uasin Gishu	10, (0.7)	-	6, (0.4)	-

MTB = *Mycobacterium tuberculosis*

RIF = Rifampicin

[a] RIF resistance percentage calculated from the MTB positive cases

No cases were identified in areas without numbers

4.2 Proportion of patients with Isoniazid (INH), Rifampicin (RIF) and multi-drug resistant (MDR) tuberculosis (TB) determined by Line Probe Assay (LPA) and Drug Susceptibility (DST) methods

Using both the LPA and DST methods to identify the prevalence of drug resistant TB in patient isolates, a total of 62 (4.53%) isolates had RIF, INH and multi-drug resistant TB as indicated in table 4.3. Overall, 24 samples had discordant results and are shown in Table 4.3 and 38 samples had concordant results. The LPA and conventional DST methods reported 13 (0.95%) and 21 (1.53%) samples with MDR-TB; 25 (1.83%) and 27 (1.97%) samples with INH mono-resistant TB; 12 (0.88%) and 9 (0.66%) samples with RIF mono-resistant (RMR) TB, respectively, Table, 3.

The overall performance characteristics for the identification of drug resistant mutations of the LPA as compared to DST were as follows; Sensitivity; RIF 66% (49% to 80%) and INH 78% (62% to 89%, 95% CI), Specificity; RIF 88% (68% to 97%) and INH 82% (60% to 95%, 95% CI). The specificity of the LPA as compared to genetic sequencing in identifying drug resistant mutations was 100% (54% - 100%, 95% CI).

Table 4.3: Patients with RIF and INH resistance by the DST and LPA method

		Conventional DST				Total
		RIF^R INH^R	RIF^S INH^R	RIF^R INH^S	RIF^S INH^S	
LPA	RIF^R INH^R	**12 (0.88)**	1 (0.07)	-	-	13 (0.95)
	RIF^S INH^R	4 (0.29)	**18 (1.31)**	1 (0.07)	2 (0.15)	25 (1.83)
	RIF^R INH^S	1 (0.07)	-	**8 (0.58)**	3 (0.22)	12 (0.88)
	RIF^S INH^S	4 (0.29)	8 (0.58)	-	--	12 (0.88)
Total		21 (1.53)	27 (1.97)	9 (0.66)	5 (0.37)	62 (4.53)

RIF = Rifampicin
INH = Isoniazid
RIF^R INH^R = MDR-TB, RIF resistant and INH resistant
RIF^R INH^S = RIF mono-resistant and INH sensitive
RIF^S INH^R = INH mono-resistant and RIF sensitive
RIF^S INH^S = Sensitive to RIF and INH
Concordant test results are shown in bold.

No samples tested had results in the areas with no data

4.3 *rpo* B, *kat* G and *inh* A gene mutations in HIV infected and uninfected patients

In summary, 25 (66%) HIV positive and 13 (34%) HIV negative patients had RIF and INH resistance conferring mutations (Table 4.3).

Using LPA and DST drug resistant testing methods, 38 (61.29%) samples had concordant results and the LPA method was used to identify *rpo* B, *kat* G and *inh* A gene mutations in this samples. The 24 (38.71%) discordant samples were sequenced to confirm the presence of *rpo* B, *kat* G and *inh* A gene mutations (Appendix 5 and 6). Gene mutations in 8 (12.90%) samples were confirmed. Therefore, using LPA and genetic sequencing, mutations were successfully identified in 46 (3.36%) isolates. However, HIV status data was only available for 38 isolates (Table 4.4). The *rpo* B S531L and the *kat* G S315T1 were the most common MDR-TB mutations in HIV negative patients, 3 (8%). The H526Y rifampicin mutation was also common in the HIV positive patients, 3 (8%). The *kat* G S315T1, 7 (18%) was the most common INH mono-resistant mutation in HIV positive patients.

Table 4.4: Rifampicin and Isoniazid gene mutations in HIV infected and uninfected patients

rpo B		*kat* G		*inh* A		HIV Status n, (%)	
Codon	Nucleotide change	Codon	Nucleotide change	Region	Nucleotide change	**Positive 25, (66)**	**Negative 13, (34)**
D516F	GAC-TTC	S315T1	AGC-ACC	-	-	-	1, (3)
D516V	GAC-GTC	S315T1	AGC-ACC	-	-	-	1, (3)
S531L	TCG-TTG	S315T1	AGC-ACC	-	-	2, (5)	3, (8)
S531L	TCG-TTG	-	-	-15	C - T	1, (3)	-
Missing wild type *	-	S315T1	AGC-ACC	-	-	1, (3)	2, (5)
D516V	GAC-GTC	-	-	-	-	1, (3)	-
H526D	CAC-GAC	-	-	-	-	2, (5)	-
H526R	CAC-CGC	-	-	-	-	-	1, (3)
H526Y	CAC-CCC	-	-	-	-	3, (8)	-
H526Y/H526D	CAC-CCC/CAC-GAC	-	-	-	-	1, (3)	-
H526Y/H526D/S531L	CAC-CCC/CAC-GAC/TCG-TTG	-	-	-	-	1, (3)	-
S531L	TCG-TTG	-	-	-	-	1, (3)	-
-	-	S315T1	AGC-ACC	-	-	7, (18)	2, (5)
-	-	S315T2	AGC-ACA	-	-	-	1, (3)
		S315N	AGC-AAC	-	-	1, (3)	-
-	-	-	-	-15	C – T	4, (11)	2, (5)

*Missing wild type = absence of staining of the *rpo* B wild type probe
Amino acid abbreviations: S, Ser; T, Thr; R, Arg; L, Leu; V, Val; H, His; D, Asp; Y, Tyr; F, Phe, N, Asn.
Nucleotide abbreviations: A, adenine; C, cytosine; G, guanine, T, thymidine.
Areas with no data in the table represent undetected gene mutations on the respective genes

4.4 Association of Rifampicin and Isoniazid mono-resistant and Multi-drug resistance gene mutations on age, gender and HIV status

Multivariate analysis indicated that the drug resistant mutations differed significantly in respect to a combination of the variables, HIV status, age and gender ($\lambda = 0.634$, F (6, 64) = 2.735, P = 0.02), Table 4.5. Subsequent univariate analysis showed that age [F (2, 33.422, $P = 0.044$)] and HIV status [F (2, 34) = 3.827, $P = 0.032$)] had a significant effect on RIF and INH resistance conferring gene mutations, Table 4.5.

Tukey *post hoc* analyses showed age had a potential association on INHMR and MDR gene mutations ($P = 0.044$), Table 4.6. Games-Howell *post-hoc* analyses also showed that HIV had a significant effect on RMR and INHMR gene mutations ($P = 0.023$), Table 4.6.

Table 4.5: Regression analysis depicting the association of RIF and INH mutations on age, gender and HIV status

	Multivariate analysis				Univariate analysis		
	λ value[a]	*F*	*P* value[b]	Partial eta squared	*F*	*P*-value	Partial eta squared
Age					3.422	**0.044**	0.168
Gender	0.634	2.735	**0.02**	0.204	1.201	0.313	0.066
HIV Status					3.827	**0.032**	0.184

[a] Wilks' Lambda test statistic
[b] Wilks' Lambda test statistic

Table 4.6: Post-hoc tests showing the association between RIF and INH mutations and age, gender and HIV status

	Genotype		*P* value [a]		Genotype		*P* value [a]		Genotype		*P* value [b]
Age	RMR	INHMR	0.618	**Gender**	RMR	INHMR	0.289	**HIV**	RMR	INHMR	**0.023**
		MDR	0.274			MDR	0.575			MDR	0.239
	INHMR	RMR	0.618		INHMR	RMR	0.289		INHMR	RMR	**0.023**
		MDR	**0.04**			MDR	0.777			MDR	0.376
	MDR	RMR	0.274		MDR	RMR	0.575		MDR	RMR	0.239
		INHMR	**0.04**			INHMR	0.777			INHMR	0.376

[a] Tukey HSD statistic

[b] Games-Howell statistic

RMR = Rifampicin mono-resistant

INHMR = Isoniazid mono-resistant

MDR = Multi drug resistant

HIV = Human immunodeficiency virus

4.5 Association of Rifampicin and Isoniazid mono-resistant and Multi-drug resistant gene mutations with HIV status

Binary logistic regression analysis revealed that patients with RMR-TB mutations were potentially associated with HIV status (P = 0.025), Table 4.7.

Table 4.7: Binary logistic analysis indicating the association of RIF and INH mutations with HIV status

	HIV Status, n, (%)				
Genotype	Positive, 25, (66)	Negative, 13, (34)	*P* value[a]	OR (95% CI)	95% CI
RMR	9, 23.76	1, 2.60	**0.025**	15.75	1.102 - 5.557
INHMR	12, 31.68	5, 13.10	0.081	4.2	0.8380 - 4.497
MDR	4, 10.56	7, 18.30	0.372	0.571	0.1800 - 0.9072

[a] *P* - value, Wald test. RMR, Rifampicin mono-resistant. INHMR, Isoniazid mono-resistant. MDR, Multi-drug resistant. OR, Odds ratio, 95% CI, 95% Confidence Interval.

4.6 Sputum conversion rate of patients with Rifampicin and Isoniazid mono-resistant and Multi-drug resistant gene mutations

Follow-up smear results for 16 patients were available as indicated in Table 4.8. All the patients with *rpo* B H526Y and *rpo* B S531L RIF conferring gene mutations had smear converted at month 11 and 8, respectively (Table 4.8).

Table 4.8: Sputum conversion rate of patients with Rifampicin and Isoniazid resistant mutations

	Amino acid modifications	Initial ZN smear	Follow-up (Months)	Sputum conversion (%)
RMR	**H526Y**	1+	NEG, (11)	100
	S531L	3+	NEG, (8)	
INHMR	**S315N**	3+	3+, (8)	60
	S315T1	3+	1+, (8)	
	S315T1	3+	NEG, (4)	
	S315T1	1+	NEG, (5)	
	C-15T[a]	1+	NEG, (2)	
MDR	S531L and S315T1	3+	NEG, (5)	67
	D516F and S315T1	3+	NEG, (2)	
	S531L and S315T1	1+	NEG, (9)	
	S531L and C-15T[a]	1+	NEG, (1)	
	S531L and S315T1	2+	1+, (2)	
	S531L and S315T1	1+	NEG, (3)	
	Missing wt and S315T1	1+	NEG, (4)	
	D516V and S315T1	2+	1+, (2)	
	Missing wt and S315T1	3+	2+, (2)	

NEG; Negative, wt; wild type, MDR; Multi drug-resistant, RMR; rifampicin mono resistant; INHMR; Isoniazid mono resistant. HIV co-infected codon mutations are shown in bold.
Amino acid abbreviations: S, Ser; T, Thr; R, Arg; L, Leu; V, Val; H, His; D, Asp; Y, Tyr; F, Phe.
[a]Cytosine (C) to Thymidine (T) position -15 nucleotide substitution for the *inh* A gene regulatory region.
Sputum smear microscopy results were interpreted according to the International Union Against Tuberculosis and Lung Diseases (IUATLD) grading system (NLTLD, 2014) as follows, 10 – 99 acid fast bacilli in 100 immersion fields, 1+, 1- 10 acid fast bacilli per field, 50 examined fields, 2+, more than 10 acid fast bacilli per field in 20 examined fields, 3+.

4.7 Sputum conversion time of patients with Rifampicin and Isoniazid mono-resistant and Multi-drug resistant gene mutations

A total of 6 and 5 HIV-infected and HIV uninfected patients, respectively, had smear converted as shown in Figure 4.0. The median smear conversion time was higher 6.5 months in HIV positive patients and 3 months in HIV negative patients, but this was not statistically different ($P = 0.180$) (Figure 4.0).

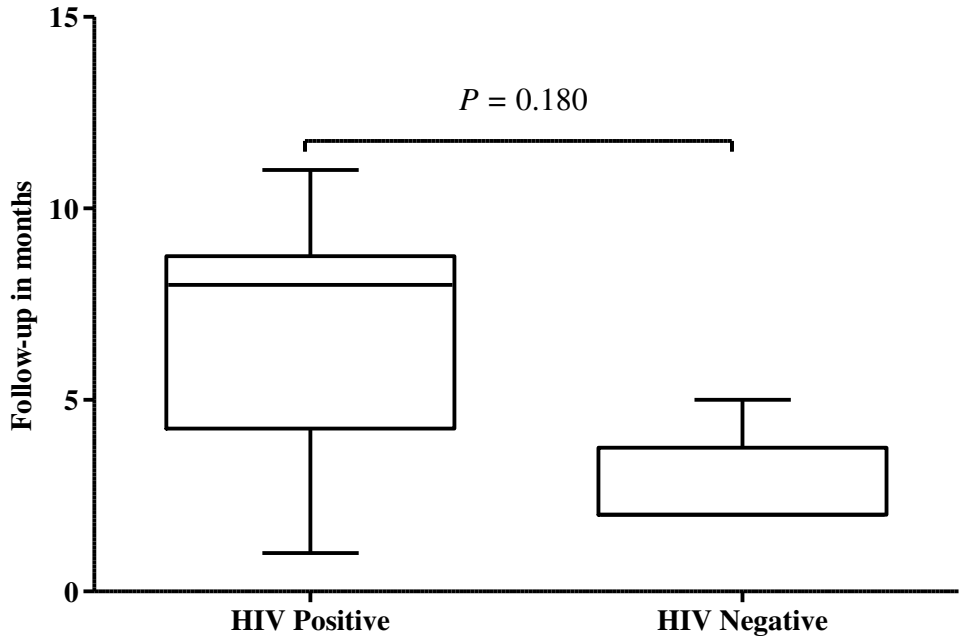

Figure 4.0: Smear conversion of HIV infected and uninfected patients with RIF and INH mutations.
Data are represented in box plots for HIV-infected (n = 6) and HIV uninfected (n = 5) groups. The boxes represent the interquartile ranges; the lines through the boxes are the medians, while the whiskers show the 10^{th} and the 90^{th} percentiles. The median conversion time in months of HIV-infected and uninfected patient with RMR, INHMR and MDR-TB gene mutations was not statistically significant.

CHAPTER FIVE: DISCUSSION

In the current study, the proportion of drug resistant *Mycobacterium tuberculosis* in sputum isolates and the association of RIF and INH gene mutations with HIV status and patient treatment response in western Kenya were determined using LPA, DST and gene sequencing.

In this study, there was a gradual peak of TB cases from the age category 0 -14 through 25 - 44 (Table 4.1). Past studies have shown that age-specific TB notification rate around age 25 years is determined by the protective effect of previous latent infection and the fast progression in previously treated patients (Blaser *et al*., 2016). However, in the present study, most of the patients recruited were newly diagnosed with TB and this observation is attributable to the high prevalence of HIV of between 6% - 10%in the age group 25 – 44 years (KAIS., 2012).

More men (59%) than women patients had TB. Gender bias of TB may be related to health seeking behavior or to steps within the health systems in suspecting and diagnosing TB (Borgdorff *et al.,* 2000). Men are more likely to smoke and nicotine reduces the phagocytic ability of alveolar macrophages this therefore increases susceptibility to TB infection (Narasimhan *et al.,* 2013). Alcoholism is also more common in men and this has been found to alter the signaling molecules responsible for cytokine production and therefore resulting to a weakened immune system (Narasimhan *et al.,* 2013).

The prevalence of TB and HIV co-infection in the current study was 61% (Table 4.1), however, a previous study in western Kenya reported a slightly lower prevalence of 41.8% (Nduba *et al*., 2015). The differences in the TB and HIV co-infection prevalence reported could be attributed to different TB diagnostic methods with varying limits of TB detection and patient study inclusion

methods that were used. In agreement with other studies, which showed that HIV infection is a risk factor of TB (Nyamogoba *et al*., 2013), the current study found that HIV infected patients were more likely to have TB, 61% (Table 4.1). This is because HIV infected patients have a deficient immune system and this provides a habitat for the development of TB.

Overall, majority of sputum samples received at the central laboratory from 415 health facilities in 13 counties in western Kenya were from patients who were receiving TB treatment for the first time (Table 4.2). In 2015, the counties of Homabay, Kisii, Kisumu, Migori and Siaya accounted for 13.6%, 2.9%, 12.4%, 7.2% and 10.8%, respectively, of the total new HIV infection cases in Kenya (Kenya AIDS Response Progress Report, 2016). The high cases of TB reported are therefore expected since HIV is a known risk factor for TB. In addition, these counties had more than 90% of the study participants.

5.1 The proportion of RIF and INH mono-resistant and MDR *Mycobacterium tuberculosis* in western Kenya in 2012-2014.

Conventional DST and LPA methods identified 62 cases of drug resistant TB where 38 samples had concordant results while 24 samples had discordant results (Table 4.3). Overall, the present study reported a higher prevalence of INH resistance compared to RMR as determined by both LPA and DST (Table 4.3). This finding was consistent with a previous clinical trial by the British Medical Research Council where 5% of the participants had INH resistance as compared to 0.02% who had RMR (Ridzon *et al*., 1998). A national survey study in Uganda, also reported INH and RMR resistance prevalence rates of 5% and1.9%, respectively (Lukoye *et al*., 2013a). These findings highlight the importance of determining INH susceptibility, which is not provided by the increasing use of the GeneXpert test, which may miss 10 – 15% of INH resistance (Schaaf

et al., 2016). In a study in Ethiopia, the sensitivity of LPA was low in direct smear negative samples (Meaza *et al*., 2017). This emphasizes the need for having additional drug resistant tests in endemic regions. In the present study 1.53% and 0.95% prevalence of MDR-TB was reported by the DST and LPA method, respectively, (Table 4.3), a previous study in Uganda reported a slightly higher MDR-TB prevalence of 1.4% (Lukoye *et al*., 2013a). A study in Nigeria reported a MDR-TB prevalence of 16% (Ani *et al*., 2009). This was due to inefficient drug resistant TB control program, poor patient adherence practices and diagnostic practices (Ani *et al*., 2009). The low prevalence of MDR-TB in the study area could be attributed to the longer treatment regimen guidelines recommended by the Ministry of Public Health and Sanitation of a minimum of 18 months after culture conversion. Active case finding of persons living with MDR-TB patients which results to an early case detection and provision of fluoroquinolone-based preventive therapy has been shown to drastically reduce cases of MDR-TB (Schaaf *et al*., 2016).

In the current study, the sensitivity for detecting RMR and INH resistance using LPA was lower (89% and 70%, respectively) than what was reported by other studies. A recent LPA multi-site validation study in India also reported a sensitivity of 72% for INH resistance (Raizada *et al*., 2014).This variation in performance characteristics of LPA has also been confirmed by previous studies and is depended on factors such as geographic and genetic distribution of drug resistant strains, bacillary load and routine diagnostic algorithms (Barnard *et al*., 2012; Ling, Zwerling, & Pai, 2008).

5.2 The association of RIF and INH mono-resistant and multi-drug resistant gene mutations with HIV status in western Kenya in 2012-2014

Genetic sequencing was used to confirm the presence of mutations in samples that had discordant results. Only eight samples of the discordant samples were confirmed to be having

gene mutations associated with RIF and INH resistance. In western Kenya, the impact and management of RMR, INH and MDR-TB specific mutations have not been studied extensively. Consistent with previous studies (Baker, Brown, Maiden, & Drobniewski, 2004; Bottger *et al*., 2005; Brossier *et al*., 2006), the current study found that MDR isolates had the *rpo* B S531L and the *kat* G S315T as the most frequent combination of mutations in both HIV positive and negative patients, 5% and 8%, respectively (Table 4.4). Studies have shown that MDR isolates with the *kat* G S315T mutation are associated with increased levels of INH resistance during treatment (Cattamanchi *et al*., 2009). In HIV positive patients, the *rpo* B H526Y mutation was common, 8%. Several studies have shown an increased rate of drug resistant TB among HIV infected individuals (Fenner*et al*., 2012). A previous study in a HIV endemic region in South Africa reported 22.6% and 5.6% prevalence rate of MDR-TB and INH resistance, respectively (Hesseling *et al*., 2012). In areas with increased cases of HIV transmission, such as western Kenya, which accounts for high rates of new HIV infections annually (Kenya AIDS Response Progress Report, 2016), such findings have major implications in the management of drug resistant TB in HIV endemic regions, particularly contact investigation studies that aim to minimize transmission rates.

Multivariate analysis showed that age, gender and HIV status was associated TB drug resistance genotypes (P = 0.02), (Table 4.5). Univariate analysis confirmed that age (P = 0.044) and HIV status (P = 0.032) independently was associated with TB resistance genotypes (Table 4.5). Tukey *post-hoc* analysis suggested that age significantly affected the INHMR and MDR-TB genotypes (P = 0.04) and Games-Howell analysis suggested that HIV status had a significant effect on RMR and INHMR-TB genotypes (P = 0.023), (Table 4.6). A study from Tokyo, Japan also found that patients with MDR-TB were significantly more likely to be younger, for age

range of 21-40 years (Gupta *et al*., 2014). Similarly, a study in East Gojjam Zone in North West Ethiopia reported that the age group 25-34 years was associated with drug resistant TB (Adane, Ameni, Bekele, Abebe, & Aseffa, 2015). From the present study, the finding that age is associated with drug resistant TB could be due to risky behavior such as alcoholism and health-seeking behavior of young TB patients that can interfere with treatment. In addition, youths in the age group 18 – 35 years of age are more at risk population of HIV.

A previous study in Netherlands found MDR-TB to be significantly associated with HIV burden and therefore contributes to high transmission rates, infection and re-infection, which leads to the spread of resistant bacilli (Gupta *et al*., 2014). Using binary logistic regression, the current study demonstrated that RMR-TB was associated with HIV status (P = 0.025) (Table 4.7). In a retrospective TB drug resistance cohort study in California, patients with RMR-TB were seven times more likely than drug-susceptible TB patients to be co-infected with HIV (Prach *et al*., 2013). The strong association of RMR-TB with HIV status could be attributable to poor TB drug absorption and immune-suppression due to HIV, which leads to high disease burden (Prach *et al*., 2013). Previous studies have found that the association of RMR-TB with HIV status could be due to the use of Rifabutin in the treatment of *Mycobacterium avium*. (Ridzon *et al*., 1998), however, in the present study, prior use of RIF derivatives as risk factors for RMR-TB was not assessed. Furthermore, in the present study, there was no association between INHMR and MDR-TB with HIV status (P = 0.081, P = 0.472) (Table 4.7). Similar phenotypic studies in Ethiopia and Uganda reported no association between HIV status and drug resistance to first-line TB drugs (Adane *et al*., 2015; Lukoye *et al*., 2013a). This therefore suggests that patients with INHMR and MDR-TB are not likely to be HIV infected.

5.3 The sputum smear conversion time of patients with RIF and INH mono-resistant and multi-drug resistant gene mutation profiles and in western Kenya in 2012-2014

Sputum smear microscopy is the primary method for monitoring treatment response in resource-constrained countries (Sitienei *et al*., 2015). In this study, the sputum smear conversion time in TB and HIV co-infected patients with confirmed mutations on RIF and INH conferring genes was investigated. Calculation of sputum conversion time is an important measure for determining the progress of treatment (Atif *et al*., 2014).

Previous studies have shown that the *rpo* B H526Y and the *kat* G S315T mutations are associated with higher minimum inhibitory concentrations for RIF and therefore poor drug response (Jamieson *et al*., 2014). These mutations were identified in two HIV co-infected patients who had a 100% smear conversion rate at 11 months and 8 months, respectively (Table 4.8). This finding emphasizes the importance of prolonged RIF TB therapy for improved treatment outcome (Charles *et al*., 2014; Menzies *et al*., 2009). However, previous studies have shown that smear microscopy is less sensitive in HIV-positive patients with MDR-TB and cure should be confirmed after obtaining at least 5 negative sputum culture results during 12 months of treatment (Gammino *et al*., 2012).

Patients with INH mono resistance have unfavorable treatment outcome and relapse (Huyen *et al*., 2013). Therefore, it is expected that smear conversion time will be prolonged. In the present study, the sputum conversion rate for patients with INH mono-resistant was 60% (Table 4.8). The S315T1 mutation that has been associated with high level INH resistance was present in 4 out of 5 HIV co-infected patients, however, most patients with this mutation had smear

converted at the time of follow-up. Patients with the *kat* G S315N mutation had a 3+ bacterial load even after 8 months of treatment (Table 4.8). This finding conflicts with previous studies that recommended replacing the 8 months WHO treatment regimen to a shorter treatment period of 6 months in new TB patients (Huyen *et al*., 2013; Telenti *et al*., 1997). Therefore, treatment of INH mono-resistant TB should be prolonged for an improved treatment outcome. Previous studies have showed that *kat* G S315T1 mutations have been strongly associated with Streptomycin resistance (Huyen *et al*., 2013; Telenti *et al*., 1997). The smear conversion time could therefore be influenced by Streptomycin and INH resistance occurring simultaneously. The *inh* A C-15T promoter mutation was only present in one patient and the 2 month smear conversion rate was expected since this mutation has been strongly associated with low level INH resistance (Huyen *et al*., 2013). This finding concurs with previous studies in areas with increased cases of INH mono-resistance that found that the *inh* A mutation was rare as compared to the S315T1 mutation (Huyen *et al*., 2013).

Patients with the *rpo* B S531L and *kat* G S315T1 and *rpo* B S531L and *inh* A C-15T MDR-TB mutations had smear converted at 9 and 1 months, respectively (Table 4.8). Only three MDR-TB patients had a bacterial load of between 2+ and 1+ at follow-up (Table 4.8) and this observation is expected because patients had only been treated for two months at the time of follow-up (Table 4.8). In the present study, the sputum smear conversion rate for patients with MDR-TB was 67% (Table 4.8). A previous culture-based study using smear microscopy to monitor the treatment outcome of MDR-TB in patients also found cure rates of 66% in retreatment cases (Palacios *et al*., 2012). However, studies in countries with high cases of drug-resistant TB have

reported that MDR-TB patients on treatment with first-line therapy had a high rate of TB recurrence and died within 4 years (Gammino *et al*., 2012).

Overall, HIV positive patients required longer periods of time to smear-convert. A previous culture study in the USA demonstrated a 9 months conversion time, however, since genotyping of sputum samples was not performed, it was not known if this particular finding was as a result of acquired resistance, re-infection, mixed infections or laboratory cross-contamination (Gammino*et al*., 2012). In addition, the study involved TB patients not exposed to HIV infection (Gammino *et al*., 2012). Even though a short smear conversion median time (3 months) in HIV negative patients as compared to HIV positive patients was reported in the current study (Fig 4.0), previous research has shown that microscopy particularly in HIV positive patients failed to precisely detect bacilli in culture positive sputum samples suggesting that smear monitoring alone of drug resistant TB should be used with great caution (Palacios *et al*., 2012). HIV infection has been associated with poor drug efficacy and this could be the likely reason for the long smear conversion time, however, a previous study documented that HIV positive patients with MDR-TB who were on early Highly Active Antiretroviral Therapy (HAART) had improved treatment outcomes as compared to a control group from the pre-HAART era (Palacios *et al*., 2012).

5.4 Study limitations

The present study had several limitations, the exact time at which the study patients had started treatment for drug-resistant TB was not established and adherence to anti-TB drugs as well as the combined effects of anti-retroviral therapy was not evaluated. The immune status of the study

participants was also not established. The study area had few cases of drug-resistant TB. The contribution of other first-line TB drugs such as Streptomycin, Ethambutol and Pyrazinamide to treatment outcome was also not assessed. Documented risk factors for TB such as malnutrition, alcoholism and diabetes was also not studied.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary of findings

The number of confirmed cases with drug resistant TB was 46 (2.76%). Overall, the present study reported a higher prevalence of INH resistance compared to RMR as determined by both LPA and DST. Patients with RMR TB were more likely to be HIV infected. MDR isolates had the *rpo* B S531L and the *kat* G S315T as the most frequent combination of mutations in both HIV infected and uninfected patients, 5% and 8%, respectively. The median smear conversion time for HIV infected and uninfected patients was comparable. Findings from the current study have been published and presented at conferences (Appendix 7 and 8).

6.2 Conclusions

1. In western Kenya, in 2012-2014, the proportion of patients with INH-mono resistant TB was high as compared to patients with RMR-TB.
2. In western Kenya, in 2012-2014, there was an association between RIF mono-resistant *Mycobacterium tuberculosis* gene mutation with HIV infection. There was no association between INH mono-resistant and MDR-TB *Mycobacterium tuberculosis* gene mutations with HIV status.
3. In western Kenya in 2012-2014, the sputum smear conversion time of 6.5 and 3 months in HIV infected and uninfected patients with RIF and INH mono-resistant and multi-drug resistant gene mutation profiles was comparable.

6.3 Recommendations from the current study

1. Molecular screening techniques that identify mono-resistant TB, particularly INH mono-resistant TB should be scaled up in western Kenya in order to accurately identify and treat patients with resistant TB.
2. Tuberculosis contact investigation studies should be adopted for patients with drug resistant TB to enhance early identification and prevent transmission of resistant strains.
3. In western Kenya, HIV infected patients with RIF and INH resistance conferring gene mutations should be put on a longer TB treatment regimen as compared to HIV uninfected patients.

6.4 Recommendations for future research

1. In western Kenya, the contribution of other first-line anti-TB drugs such as Ethambutol and Streptomycin to drug resistant TB needs to be further evaluated by future research studies.
2. In western Kenya, the effect of anti-retroviral therapy on TB patients co-infected with HIV needs to be further evaluated to address concerns such as drug-drug interactions, overlapping drug toxicities, immune reconstitution syndrome, and pill burden.
3. In western Kenya, the DLTLD needs to revise the current anti-TB drug regimens for HIV infected patients with drug resistant TB.

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LIST OF APPENDICES

Appendix 1: Data extraction form

Participants Initials_____
Patient unique No _____

A: Socio-demographic information

A1 Gender: Male ☐ Female ☐
A2 Age:_____
A3 Residence----------
A4 Substance use
A4a Alcohol Yes No
A4b Smoking history Yes No

B: TB symptoms profile:

Are you currently experiencing any of the following **(Circle the appropriate)**

B1Coughing Yes ☐ No ☐
☐ <2 weeks ☐ 2-4 weeks ☐ > 4 weeks
B2 Do you have a productive cough? Yes No
B3 Do you have fever? Yes No
B4 Do you have chest pains? Yes No
B5 Do you have night sweats? Yes No
B6 Have you lost weight? Yes No
B7 Do you have blood in your sputum? Yes No
B8 Are you feeling weak? Yes No

C: TB history

C1 Contact with TB infected person Yes ☐ No ☐

C2 If yes ☐ Person living with ☐ in social places

C3 History of TB infection ☐ ☐

If yes when……..

C4 Currently on TB treatment Yes ☐ No ☐

C5 Have you ever been treated for tuberculosis previously? Yes ☐ No ☐

C6 Did you complete TB treatment Yes ☐ No ☐

If no why……..

Appendix 2: Consent to participate in the study

Research Purpose

The overall objective of the proposed study will be to identify cases of mono-resistant TB and MDR-TB from enrolled patients in health facilities in western Kenya and analyze *Mycobacterium tuberculosis (M. tuberculosis)* mutations associated with resistance to Rifampicin (RIF) and Isoniazid (INH) using the Genotype Mycobacterium® molecular line probe assay (LPA) kit (Hain Lifesciences) and gene sequencing.

Risks:

Minimal risk will be felt when venous blood will be collected. There are small risks which could occur/happen to a participant as a result of taking part in this study. A participant may feel concerned when questions are asked about their HIV status. In this case the participants are free to answer or not answer any of these questions. It is their choice. A participant may also feel some anxiety about waiting for the results of the testing.

Benefits:

Diagnosis and treatment of TB will be offered

Alternative Procedure

The alternative option is not to participate in the study.

Confidentiality:

Once we identify an eligible participant, the patient will be informed about the study. The information will include: who is conducting the study, the purpose of the study, the study procedures, the risks and benefits, the assurance of confidentiality, their rights and that participation is voluntary. Having understood all this information, the eligible patient will be allowed to ask questions for clarity. After that they will be requested to participate and if they agree they will be requested to read and understand the informed consent form before signing. The study participants will also be requested to allow the isolates obtained from their sputum and blood samples to be stored for future research in KEMRI laboratory. No identifying information such as patient name and telephone number will be stored and the information will not be able to be linked to the participant. The investigator will sign this form once the participant has signed. All signed forms will be kept under lock and key at the KEMRI/CGHR TB laboratory office.

Your name and address will be written down only on the front page of the study form. The rest of the form will only have a study number. The list of names and study numbers will be locked away and stored separately from the study forms. We will not share the tests results with anyone other than the clinic, doctors and nurses caring for your child. Your name or your child's name will never be used in any speech or paper written about this study. Your child's confidentiality will be protected to the extent permitted by law.

Compensation and Cost:

You will not be paid to participate in this study. All of the tests done as part of this study will be paid for. **We will also arrange for you to get to the hospital to get the tests done, including providing you with reimbursement for transportation of up to Ksh 500 for any visit that is required only for this study.**

Voluntary participation:

If you do not want to be in this research study, you will still get normal care and treatment. If you agree to be in this research study, you can change your mind and you can stop being in the study at any time. Your decision to participate will not affect the services that you receive at this health facility**.**

Questions

If you have any questions about the study and/or if there is anything that is still not clear to you, please ask any of the people listed below:

Principal Investigator: Clement Shiluli

Telephone: +254-723 474 168

Treatment and Research

Information regarding the research being conducted in this study can be obtained from Dr. Kevin Cain at KEMRICDC or other study team members by calling 057-202-2959/02/83.

If you have questions about this consent process or your rights as a subject, or if you feel you have been harmed by participating in this study, you may contact: The secretary, KEMRI Ethics Review Committee, PO BOX 54840-00200, Nairobi; Telephone Number 020-2722541, 0722205901, 0733400003: Email address ERCadmin@kemri.org who oversees this study being conducted at this research site.

If you have questions about this study, you should first discuss them with your doctor or the ethics committee (contact details as provided on this form). After you have consulted your doctor or the ethics committee, and if they have not provided you with answers to your satisfaction, you should call/write to the National Council of Science and Technology at: P.O.Box30623-00100 Nairobi or call +254- 02 2241349 / 02 310571/ 02 2219420.

Your consent:

When you sign below, you agree to be in this study. You can change your mind at any time. You will still get normal care and treatment whether or not you are in this study. If you have any questions and/or if there is anything that is still not clear to you, please ask your doctor or nurse or one of the study staff people. Please do not sign this form until you get answers for all of your questions.

If you agree to be in this study, please sign here:

Name of Participant*(please print)*
_____ Signature/Thumbprint of Participant_____ Date_____
[ ] Witness used (Independent/Impartial person) (*tick only if applicable*)
_____ Name of the Witness *(Print)* Signature of Witness_____Date_____
Name of staff consenting *(print)* Signature of staff consenting: _____Date_____

Please indicate below if you do or do not give consent for storage of specimens and DNA for future research. These specimens may be stored for up to ten years following completion of the study.

_____ **I do** give consent for storage of processed sputum and DNA for future research

_____**I do not** give consent for storage of processed sputum and DNA for future research

Appendix 3: Laboratory Standard Operating Procedures

6.1. Preparation of decontamination reagent

6.1.1.1. Sodium hydroxide (NaOH), 4.0% (solution 1)

6.1.1.2. Weigh sodium hydroxide pellets (analytical grade) 40 g in a weighing boat using a weighing scale/balance

6.1.1.3. Measure 1,000 ml of distilled water and pour into a 2 litre volumetric flask

6.1.1.4. Dissolve NaOH pellets in the distilled water

6.1.1.5. Gentle heating and magnetic stirring may be applied to help with the dissolution.

6.1.2. Sodium citrate, 2.90% (solution 2)

6.1.2.1. Weigh 29g (tri)sodium citrate·$2H_2O$, analytical grade

6.1.2.2. Measure 1,000 ml of distilled water and pour into a 2 litre volumetric flask

6.1.2.3. Dissolve in the distilled water by heating at 60°C to 80°C (optional).

6.1.2.4. Gentle heating and magnetic stirring may be applied to help with the dissolution.

6.1.3. 4.0%NALC+2.90%NaOH solution freshly prepared

6.1.3.1. Mix equal volumes of solutions 1 and 2 and aliquot in glass bottles before use.

6.1.3.2. Sterilize by autoclaving at 121 °C for 20 minutes.

6.1.4. Add 0.25 g *N*-acetyl *L*-cysteine (NALC) to 50ml falcon tube just before use.

6.2. Preparation Phosphate buffer PH 6.8

6.2.1. Formula for phosphate buffer per 500ml purified water.

6.2.1.1. Weigh 9.47g Di-sodium phosphate (Na_2HPO_4)

perature for up to 6 months

6.5. Labeling Reagent Containers

6.5.1. Label all shipping cartons containing reagents for the laboratory Section with the foll information when received from the supplier (this should be carried out by the receivi room staff if the supplies are kept in storage):

6.5.1.1. Date Received

6.5.1.2. Label all individual reagent containers received in the laboratory with the foll information:

6.5.1.2.1. Date Received

6.5.1.2.2. Date Opened

6.5.1.2.3. Expiration Date

6.5.2. Label all prepared reagent containers with the following information each

6.5.2.1. Name of Reagent (with concentration if appropriate)

6.5.2.2. Date Prepared

6.5.2.3. Date of Expiration

6.5.2.4. Initials of Preparer

6.5.2.5. Open date

6.5.2.6. Batch number

6.5.2.7. NOTE- For batch number, the first four digits stands for the year and changes every new year and the last two digits changes whenever fresh reagents are prepared.

6.6. Rotation Of Reagents

6.6.1. Guidelines

6.6.1.1. Use the oldest dated stock reagents first and replace with newer dated stock reagents as necessary.

6.6.1.2. Rotate all stock reagents according to the specified shelf life. Shelf lives may vary among reagents.

6.6.1.3. Refer to the analysis methods for reagent shelf lives. Use the oldest dated working reagents first and replace with newer dated working reagents as necessary.

6.6.1.4. Rotate all working reagents within the specified shelf life.

6.6.1.5. If there is no expiration date supplied by the manufacturer or no recommended shelf life stated in the method use the following guidelines for rotation of reagents:

6.6.1.5.1. Shelf life of solid reagent: 10 years

6.6.1.5.2. Shelf life of liquid reagents: 5 years

6.7. Reagent Preparation Instructions

6.7.1. Prepare reagents according to the instructions given in the methods/SOPs/ manufacturers manuals.

6.7.2. If a new bottle of reagent is opened and used with a previously opened reagent the working solution must carry the expiration date of the oldest reagent used.

6.7.3. All reagent preparations are documented in the reagent preparation log unless the reagent is made and used for one day and then discarded.

6.8. Disposal Of Used And Expired Reagents

6.8.1. Refer to the MSDS for information about chemicals being used. Some reagent need to be treated as hazardous or treated prior to disposal.

6.8.2. Review the Laboratory Safety Manual and the Hazardous Waste Management

6.8.3. Label the reagent and store in an amber bottle at RT (up to 12 months)

QUALITY CONTROL

Appendix 4: Study approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **December 16, 2014**

TO: **LIKHOVOLE CLEMENT SHILULI,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. STEPHEN MUNGA,**
THE DIRECTOR, CGHR,
KISUMU

FORWARDED
DIRECTOR
CENTRE FOR GLOBAL HEALTH RESEARCH

Dear Sir,

RE: **SSC PROTOCOL NO. 2854 (*RESUBMITTED-INITIAL SUBMISSION*): GENE MUTATIONS ASSOCIATED WITH *MYCOBACTERIUM TUBERCULOSIS (M. TUBERCULOSIS)* RESISTANCE TO ISONIAZID AND RIFAMPICIN IN A HIV-1 ENDEMIC POPULATION IN WESTERN KENYA-(*VERSION 01 DATED 13TH NOVEMBER, 2014*)**

Reference is made to your letter dated 28th November, 2014. The Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study protocol on December 1, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised at the 232nd meeting of the KEMRI ERC on 21st October, 2014 have been adequately addressed.

The study is granted approval for implementation effective this **16th December, 2014**. Please note that authorization to conduct this study will automatically expire on **15th December, 2015.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to SERU by **November 3, 2015**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of SERU. You are also required to submit any proposed changes to this protocol to SERU prior to initiation and advise SERU when the study is completed or discontinued.

You may embark on the study.

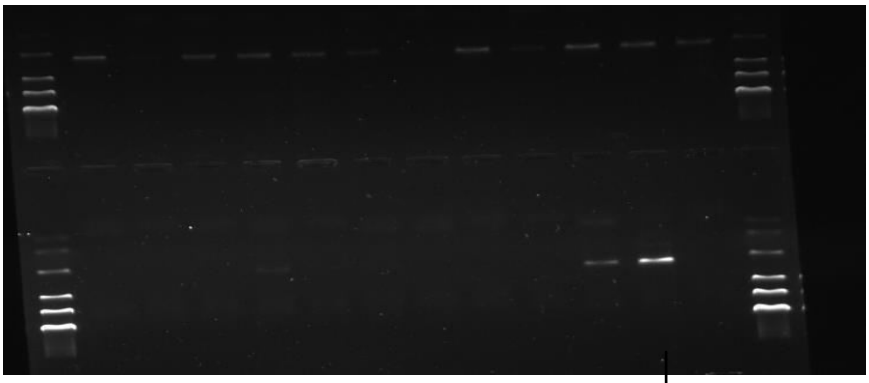
Yours faithfully,

EAB

PROF. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

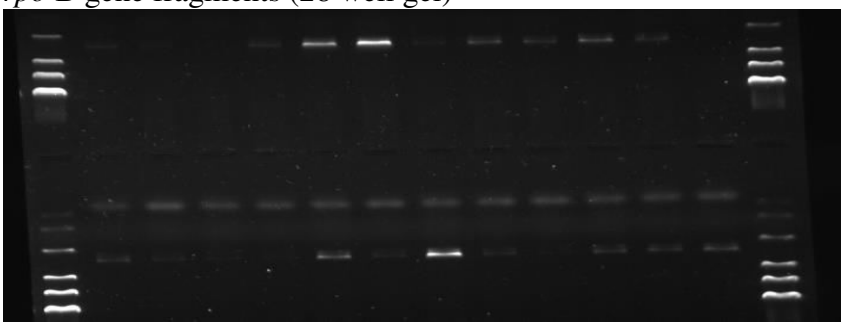
In Search of Better Health

Appendix 5: Gel images of amplified DNA revealing gene fragment size



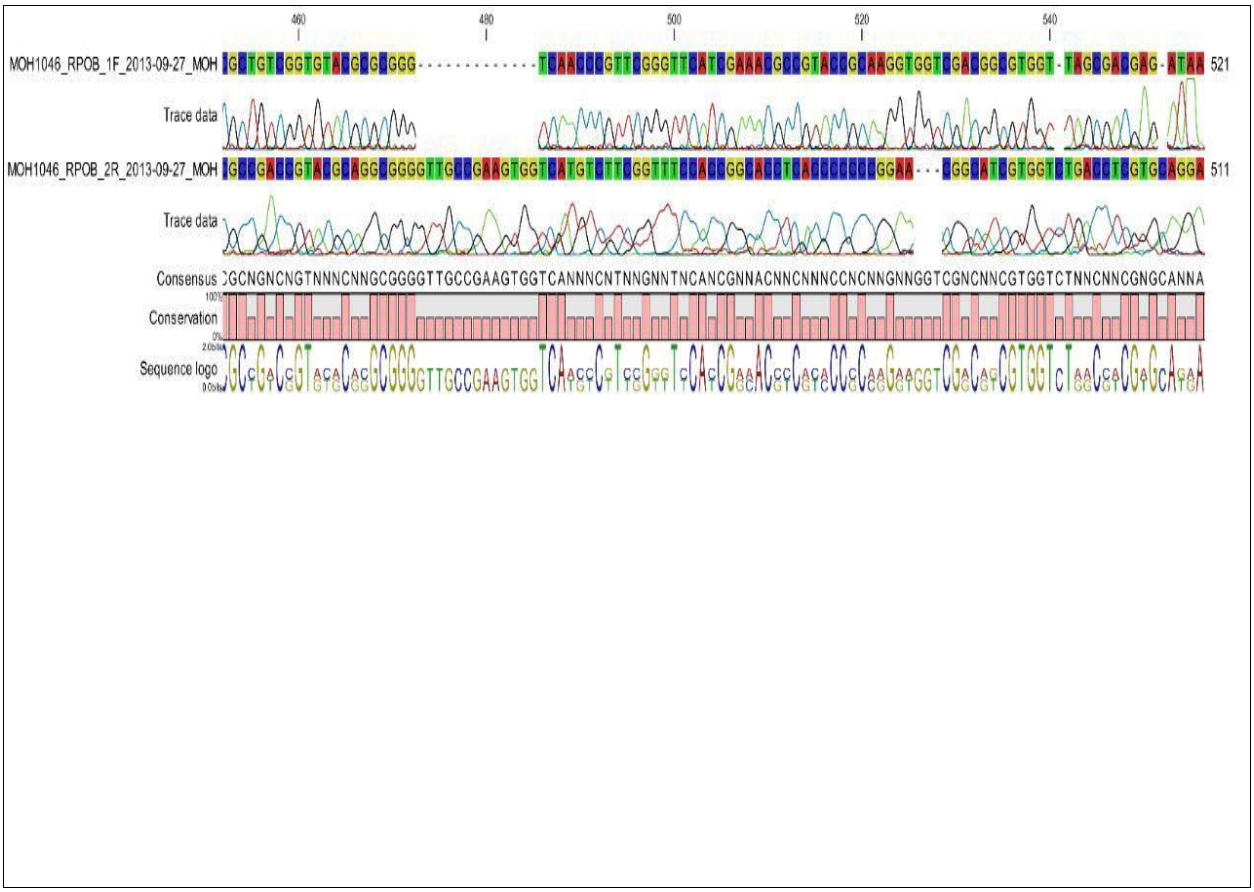
Inh A gene fragment

rpo B gene fragments (28 well gel)



rpo B gene fragments

Appendix 6: Sequence read out images showing regions with nucleic acid substitution



Appendix 7: Abstract for conference proceedings

1. The 2nd African International Biotechnology and Biomedical Conference (AIBBC), September17th – 19th 2015, held at the African Population and Health Research Center (APHRC) Nairobi, Kenya.

Mycobacterium tuberculosis resistance to Isoniazid and Rifampicin in a HIV-1 endemic population in western Kenya in 2012-2014.

Clement Shiluli[1,2], Collins Ouma[2], , John M. Vulule[1], Jeremiah Khayumbi[1], Wilfred Murithi[1], Susan Musau[1] and Albert Okumu[1]

1Kenya Medical Research Institute, Centre for Global Health Research, Kisumu, Kenya, 2School of Public Health and Community Development, Department of Biomedical Sciences and Technology, Maseno University, Kenya

In 2015, 10.4 million people worldwide developed tuberculosis (TB) and 1.4 million died from the disease, 400 000 of whom were HIV-positive, Sub-Saharan Africa (SSA) accounted for 81% of these cases. In Kenya, in 2014, TB cases with multi-drug resistant (MDR-TB) were 2.2% and 14% in new and retreatment cases, respectively. Chemotherapy with effective anti-tuberculosis drugs is used for the treatment of TB. However, cases of mono-resistant TB and MDR-TB are a major threat to the management of TB. The spread of mono-resistant and MDR-TB has been enhanced by delays in the identification of resistant strains. However, resistance gene patterns and the extent and distribution of mono-resistant TB and MDR-TB, particularly in western Kenya where HIV is common is unknown. As such, the objectives of the current study was to determine the proportion of drug resistant *Mycobacterium tuberculosis* in sputum isolates in western Kenya in 2012-2014.Patients with a suspected TB history were referred by clinicians to the health facilities for TB and HIV diagnosis. HIV testing was done using the Unigold and Abbott Determine kits. Early morning sputum samples were collected and cultured on *Mycobacteria* growth indicator tubes (MGIT) and incubated at 37°C. The Xpert MTB/RIF assay was used as a rapid TB diagnostic method. Drug susceptibility testing (DST) using the SIRE® kit was done on Ziehl-Neelsen(ZN) smear positive MGIT tubes and line probe assay (LPA) performed to identify specific mutations on the *rpo* B, *kat* G and *inh* A genes. Mutations on discordant samples were confirmed by the BigDye® Terminator v3.1 Cycle Sequencing Kit. ZN microscopy was done on sputum samples with confirmed mutations during second visits to monitor treatment by determining the sputum smear conversion rate. The prevalence of TB in the study area was 44.5%. The proportion of MDR-TB, RIF mono-resistant(RMR) TB and INH mono-resistant (INHMR) TB as estimated by LPA and DST was as follows: MDR-TB 0.78%, 1.26%; RMR-TB 0.72%, 0.54%; INH mono-resistant TB 1.50%, 1.62%; respectively. The study showed that the *rpo* B H526Y and the *kat G* S315T1 mutations were common in HIV positive patients (8% and 18%, respectively) and that the *kat G* S3I5T1 and *kat G* S531L was the most common mutation in MDR-TB strains in both HIV positive and negative patients (5% and 8% respectively).Research findings showed that there is a potential association between RMR-TB and HIV-1 status in western Kenya.

2. The eighth European and Developing Countries Clinical Trials Partnership (EDCTP) forum, 6th – 9th November, Lusaka, Zambia.

Mycobacterium tuberculosis resistance to Isoniazid and Rifampicin in a HIV-1 endemic population in western Kenya in 2012-2014.

Clement Shiluli[1,2], Collins Ouma[2], , John M. Vulule[1], Jeremiah Khayumbi[1], Wilfred Murithi[1], Susan Musau[1] and Albert Okumu[1]

1Kenya Medical Research Institute, Centre for Global Health Research, Kisumu, Kenya, 2School of Public Health and Community Development, Department of Biomedical Sciences and Technology, Maseno University, Kenya

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Appendix 8: List of published manuscripts

1. Shiluli, C., Ouma, C., Vulule, J., Khayumbi, J., Murithi, W., Musau, S., Okumu, A. (2016). *Mycobacterium tuberculosis* resistance to Isoniazid and Rifampicin in a HIV-1 endemic population in western Kenya in 2012-2014. J. Med. Sci. Clnl. Res. JMSCR Vol.04.Issue12. Page 14605-14612.
2. Shiluli, C., Ouma, C., Vulule, J., Khayumbi, J., Murithi, W., Musau, S., Okumu, A. (2016). Sputum smear conversion time of HIV infected and uninfected patients with rifampicin and isoniazid *Mycobacterium tuberculosis* gene Mutations in Western Kenya. J. Med. Sci. Clnl. Res. JMSCR Vol.04.Issue12. Page 14463-14470.