PROFILE OF DRUG RESISTANCE CONFERRING GENE MUTATIONS IN *Mycobacterium tuberculosis* AMONG NEW AND PREVIOUSLY TREATED PULMONARY TUBERCULOSIS CASES FROM KISUMU COUNTY, KENYA

BY

OGUMBO FREDRICK ABONYO

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DECLARATION

This thesis is my original work; it has not been presented for any degree award at any other University or Institution. Acknowledgement has been accorded to any other works touched as referenced in this document. No part of this report may be reproduced without prior permission of the author and/or Maseno University.

Ogumbo Fredrick Abonyo

PHD/PH/00101/17

SIGNATURE..... DATE.....

Supervisors

This work is submitted for examination with our approval as supervisors.

Dr. Bernard Guyah, PhD

Department of Biomedical Science and Technology, Maseno University

SIGNATURE..... DATE.....

Dr. James Nonoh, PhD

Department of Biomedical Science and Technology, Maseno University

SIGNATURE..... DATE.....

Dr. Steve Wandiga, PhD

Centre for Global Health Research, Kenya Medical Research Institute

SIGNATURE..... DATE.....

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DEDICATION

This work is dedicated to men, women, organizations, entities, and governments that are involved in supporting the progress towards World Health Organization's End TB Strategy.

ABSTRACT

Tuberculosis (TB) remains a significant public health concern in Kisumu County, Kenya, with a high burden of drug-resistant cases. Existing studies focus on epidemiological aspects, but lack comprehensive investigation into the genetic basis of drug resistance. This research gap hinders the development of targeted interventions and strategies for effective TB control in Kisumu County. This study aimed to investigate the profile of drug resistance-conferring gene mutations in *Mycobacterium tuberculosis* among new and previously treated pulmonary tuberculosis cases in Kisumu County, Kenya. Specifically, the study aimed to establish drug resistance conferring gene mutations in *M. tuberculosis* from HIV cases; determine gene mutations in *M. tuberculosis* that confer resistance to first and second line TB treatment; evaluate the diagnostic performance of molecular line probe assay in *M. tuberculosis* drug resistance testing among new and previously treated pulmonary TB cases in Kisumu County, Kenya. In this hospital and laboratory based cross- sectional study, sputum samples were collected from 256 TB clinical suspects attending various health facilities in Kisumu County between November 2020 and October 2021. Bacteriological and molecular techniques, including line probe assays, were employed to identify gene mutations and drug resistance patterns. Line probe assay assessed mutations in the genes *rpo*B, *kat*G, *inh*A, *emb*ABC, *pnc*A, *rrs; gyr*A, *gyr*B, and *eis*. Using statistical package for the social sciences v23, data was descriptively analysed into frequencies and proportions. Binary logistic regression was used to model for predictors of drug resistance while cross tabulation was used to describe mutant patterns. Contingency table was used to assess sensitivity, specificity, PPV and NPV. Out of a sample of 256 from TB suspected cases, 145(56.6%) were confirmed cases of which 113(77.9%) retreatment. Greater variability of mutations was exhibited from HIV positive cases compared to HIV negative cases and age was a predictor to isoniazid resistance, rifampicin resistance(RR) and Multidrug resistance (MDR). Low INH resistant strains had alterations in the promoter region of *inh*A gene at codon *-15* indicating amino acid change of *S315T1*. High INH resistant strains showed mutations at *kat*G gene, codon *315*. The study presented that 2 MDR, 4 RR and 4 high INH resistance had the same nucleotide and amino acid changes and higher number of unknown mutations were exhibited in retreatment cases compared to new cases. Using phenotypic drug susceptibility testing as surrogate marker, genotypic test showed a statistical significant relationship with phenotypic method for detection of INH resistance (*p=0.001)*, RIF resistance (*p=0.001*) and MDR (*p=0.001).* Understanding the genetic basis of drug resistance is crucial in guiding appropriate targeted treatment strategies and mitigating the spread of drug resistant strains. The results from the study will also guide policies and TB programs in regional specific anti-TB regimen based on mutation patterns, strengthen TB surveillance, and increase array of TB drug resistance diagnostic options for implementation by County and National governments in Kisumu County, Kenya.

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LIST OF ABBREVIATIONS AND ACRONYMS

Am	Amikacin
CC	Critical concentration
CDC	Centres for Disease Control
CGHR	Centre for Global Health Research
COVID-19	Corona Virus Disease, 2019
DST	Drug Susceptibility Testing
FL-LPA	First line – line probe assay
FM	Fluorescent microscopy
FQ	Fluoroquinolones
INH	Isoniazid
KEMRI	Kenya Medical Research Institute
LPA	Line probe assay
MDR-TB	Multi-drug resistant tuberculosis
X DR- TB	Extensively drug resistant tuberculosis
MGIT ™	Mycobacterial Growth Indicator Tube™
MTB	*Mycobacterium tuberculosis*
MUT	Mutation probe
NTLLD-P	National Tuberculosis Leprosy and Lung Disease Program
NTM	Non-tuberculous mycobacteria
QRDR	Quinolone-resistance determining region
RRDR	Rifampicin- resistance determining region
RIF	Rifampicin
SPSS	Statistical Package for Social Sciences
SLI	Second-line injectable (drug) (i.e., kanamycin, amikacin, capreomycin)
SL-LPA	Second line – line probe assay
TB	Tuberculosis
WHO	World Health Organization
WT	Wild-type

OPERATIONAL TERMS

New case: Newly recorded occurrence of TB in a patient who, in response to direct questioning denies having had any prior anti-tuberculosis treatment

Previously treated case: Newly registered episode of TB in a patient who, in response to direct questioning admits having been treated for TB for at least one month

Drug-susceptibility testing (DST): *In-vitro* testing using either phenotypic methods to determine susceptibility or molecular techniques to detect resistance-conferring gene mutations to a drug

Minimum inhibitory concentration (MIC): The lowest concentration of an anti-microbial agent that prevents growth of more than 99% of a microorganism in a solid medium or broth dilution susceptibility test

Isoniazid-resistant TB: *Mycobacterium tuberculosis* strain which is resistance to isoniazid and susceptibility to rifampicin

RR-TB (Rifampicin-resistant tuberculosis): Is caused by a strain of MTB that do not respond to rifampicin

MDR-TB (Multidrug-resistant tuberculosis): Occasioned by a strain of MTB that do not respond to, at least, isoniazid and rifampicin

XDR-TB (Extensively drug-resistant TB): Form of MDR-TB which is also resistant to two groups of second-line anti-TB agents, making it more difficult to treat.

Heteroresistance of *Mycobacterium tuberculosis* (MTB): Coexistence of both resistant and susceptible strains to anti-tuberculosis (TB) drugs in the same patient

Profile of drug resistance-conferring gene mutations: Pattern of specific genetic mutations or alterations in a pathogen's genes that lead to its resistance against certain drugs or medications.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Tuberculosis (TB) is a communicable disease that is a major cause of ill health and one of the leading causes of death worldwide and until the coronavirus (COVID-19) pandemic, TB was the leading cause of death from a single infectious agent, ranking above HIV/AIDS (WHO, 2022). Globally, the estimated number of deaths from TB increased between 2019 and 2021, reversing years of decline between 2005 and 2019 (WHO, 2022). Tuberculosis is caused by the bacillus *Mycobacterium tuberculosis* (MTB) and the emergence and spread of drug resistant and multidrug resistance strains are a global health concern as the disease remains among the top three infectious disease killers (Khan *et al.*, 2019).

Mycobacterium tuberculosis, the causative agent of TB, continues to evolve and develop resistance to anti-tuberculosis drugs, posing a serious obstacle to effective TB control and treatment efforts worldwide (WHO, 2022). Drug-resistant TB, including multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB), has garnered increasing attention due to its potential to escalate transmission rates and lead to treatment failure (Lee *et al*., 2021).

Tuberculosis is a global health concern, and its management is complicated by the emergence of drug-resistant strains of *Mycobacterium tuberculosis*. Studies have established that drug resistance in *M. tuberculosis* is primarily caused by specific genetic mutations in genes associated with the targets of anti-tuberculosis drugs (Johnson *et al.*, 2018). The most common forms of drug resistance are multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB). The identification and understanding of

these gene mutations have been instrumental in guiding treatment decisions and developing targeted therapies to combat drug-resistant TB (Miotto *et al.*, 2018).

Kenya is among the countries with a high TB burden, and the county of Kisumu has been particularly affected by the disease (MOH, 2021). The emergence and spread of drug-resistant strains of *M. tuberculosis* in this region necessitate continuous surveillance and understanding of the molecular mechanisms underpinning drug resistance. Identifying specific mutations associated with drug resistance is crucial to guide treatment decisions, develop targeted therapies, and implement effective public health strategies.

The study aimed to investigate the profile of drug resistance conferring gene mutations in *Mycobacterium tuberculosis* among two distinct groups of pulmonary TB cases: new cases and previously treated cases. Understanding the differences in mutation patterns between these groups is vital in identifying risk factors and optimizing treatment regimens for both patient categories.

Studying retreatment cases allows for the surveillance of drug-resistant TB strains, enabling healthcare authorities to design appropriate treatment strategies and prevent the spread of drug-resistant TB (Nduba *et al*., 2019). Additionally, research on new and retreatment cases allows for the identification of risk factors associated with treatment failure, relapse, or default, leading to better patient management and targeted interventions (Golub *et al.,* 2007).

Rifampicin is a critical first-line drug used to treat tuberculosis and rifampicin resistance is primarily caused by mutations in the *rpo*B gene, which encodes the RNA polymerase beta subunit. For example, "mutations in the *rpo*B gene have been identified as the primary mechanism of rifampicin resistance in *Mycobacterium tuberculosis*" (Johnson *et al.,* 2018). Isoniazid is another essential first-line drug used in tuberculosis treatment and resistance to isoniazid is often associated with mutations in the *kat*G gene and the *inh*A promoter region.

According to a study by Smith *et al*. (2019), "mutations in the *katG* gene and *inh*A promoter region are commonly found in isoniazid-resistant *M. tuberculosis* strains."

Pyrazinamide is another first-line drug used in short-course tuberculosis treatment and resistance to pyrazinamide is believed to be mainly caused by mutations in the *pnc*A gene, as suggested by the findings of a recent study (Lee *et al*., 2021). Ethambutol is another first-line drug, and its resistance is often associated with mutations in the *emb*B gene. Johnson and colleagues (2020) demonstrated that "mutations in the *emb*B gene play a significant role in ethambutol resistance in *Mycobacterium tuberculosis*."

Fluoroquinolones are second-line drugs used to treat drug-resistant tuberculosis and resistance to fluoroquinolones in *Mycobacterium tuberculosis* is primarily caused by mutations in the *gyr*A and *gyr*B genes, which encode subunits of DNA gyrase, an enzyme essential for DNA replication and transcription (Johnson *et al.*, 2018). Additionally, mutations in the *parC* gene, encoding another subunit of DNA gyrase, can also contribute to fluoroquinolone resistance (Smith & Brown, 2020).

Aminoglycosides, such as kanamycin and amikacin, are important second-line drugs used to treat drug-resistant tuberculosis and resistance to aminoglycosides in *Mycobacterium tuberculosis* is primarily caused by mutations in the *rrs* gene, which encodes the 16S rRNA of the bacterial ribosome (Johnson *et al.,* 2018). Mutations in the *eis* gene have also been associated with aminoglycoside resistance, as the *eis* gene affects the level of resistance to kanamycin and amikacin (Smith & Brown, 2020).

The association between TB and HIV co-infection is well-documented, particularly in high-burden regions like sub-Saharan Africa (Johnson *et al*., 2021). HIV-infected individuals are at a higher risk of developing active TB due to their weakened immune systems. Moreover,

TB-HIV co-infection often leads to worse treatment outcomes, including increased morbidity and mortality (Getahun *et al.,* 2010). While the association between TB and HIV is well-established, the specific gene mutations in *M. tuberculosis* linked to HIV status and their role in drug resistance development among TB patients remain areas of limited research.

The specific association between gene mutations in *M. tuberculosis* and HIV status among new and previously treated pulmonary TB cases is not well-documented (WHO, 2017b). Understanding how HIV status influences the genetic profile of *M. tuberculosis* strains could provide insights into the potential interactions between these two diseases and their implications for treatment outcomes. It is unclear whether certain gene mutations are more prevalent in HIV-infected individuals and how these mutations may impact drug resistance and treatment responses.

The identification and characterization of gene mutations in *M. tuberculosis* that confer drug resistance to first-line TB treatment among new and previously treated pulmonary TB cases are essential to optimize treatment regimens and improve patient outcomes. While some drug resistance-conferring mutations are well-known, there may be variations in mutation patterns among different populations and regions (Cohen *et al.,* 2015). Therefore, understanding the specific mutations responsible for drug resistance in the context of new and previously treated cases in the study area is crucial for tailoring effective treatment strategies.

Similarly, the gene mutations in *M. tuberculosis* that confer drug resistance to second-line TB treatment among TB patients have not been fully characterized (Lee *et al., 2020)*. Second-line drugs are critical for managing MDR-TB and XDR-TB cases, which are particularly challenging to treat (Johnson *et al.,* 2020). Identifying specific gene mutations associated with resistance to these drugs will be vital for guiding treatment decisions and improving treatment outcomes.

The diagnostic performance of molecular line probe assays in *Mycobacterium tuberculosis* drug resistance testing among new and previously treated pulmonary TB cases has not been extensively evaluated in the study area (MOH, 2021). Assessing the accuracy and efficiency of these assays will determine their potential as rapid and reliable tools for detecting drug resistance, which is crucial for early detection and appropriate management of drug-resistant TB cases.

By addressing these knowledge gaps, the study aimed to provide valuable insights into the genetic profile of drug-resistant *M. tuberculosis* strains among new and previously treated pulmonary TB cases, the association with HIV status, and the diagnostic performance of molecular line probe assays. This research has the potential to inform more effective TB management and control efforts in Kisumu County and contribute to the global fight against drug-resistant TB.

1.2 Statement of the Problem

Kisumu County, Kenya, faces a significant burden of tuberculosis (TB), including both new and previously treated cases. The emergence and spread of drug-resistant strains of *Mycobacterium tuberculosis* pose a serious threat to effective TB control efforts in the region. However, there is a lack of comprehensive data on the specific genetic mutations responsible for drug resistance in this local context. This knowledge gap hampers the development of tailored treatment strategies and compromises the management of TB cases. Therefore, the study aimed to investigate and characterize the profile of drug resistance-conferring gene mutations in *M. tuberculosis* among new and previously treated pulmonary TB cases in Kisumu County.

The co-occurrence of tuberculosis (TB) and HIV in Kisumu County presents a significant healthcare challenge. The emergence of drug-resistant strains of *Mycobacterium tuberculosis* among individuals co-infected with HIV and suffering from TB is poorly understood. This knowledge gap necessitated a study to identify and characterize drug-resistant gene mutations in *M. tuberculosis* within this vulnerable population to inform more effective treatment strategies and prevent the further spread of drug-resistant TB.

Despite efforts to control TB in Kisumu County, drug-resistant TB remains a substantial threat to public health. The prevalence and specific genetic mutations responsible for resistance to first-line TB treatment are not well-documented in this region. This information gap necessitated a study to understand the extent and nature of drug resistance in order to tailor treatment regimens, reduce treatment failure rates, and improve patient outcomes.

The emergence of multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) in Kisumu County poses a serious threat to TB control efforts. However, the specific genetic mechanisms behind resistance to second-line TB drugs in this region remain unclear. This lack of data necessitated a study to identify and characterize these gene mutations, providing insights for designing effective treatment strategies and preventing further transmission of drug-resistant TB.

The accuracy and efficiency of drug resistance testing are crucial in combating drug-resistant TB. However, the diagnostic performance of the molecular line probe assay in the context of Kisumu County's healthcare system is not well-established. This knowledge gap needed a diagnostic evaluation study to assess the effectiveness and reliability of this line probe assay in identifying drug-resistant *M. tuberculosis* strains.

1.3. Objectives

1.3.1 Main objective

To investigate the profile of drug resistance conferring gene mutations in *Mycobacterium tuberculosis* among new and previously treated pulmonary tuberculosis cases in Kisumu County, Kenya

1.3.2 Specific objectives

I. To establish drug resistance conferring gene mutations in *M. tuberculosis* from HIV cases among new and previously treated pulmonary TB cases in Kisumu County, Kenya

II. To determine gene mutations in *M. tuberculosis* that confer resistance to first line TB treatment among new and previously treated pulmonary TB cases in Kisumu County, Kenya

III. To determine gene mutations in *M. tuberculosis* that confer resistance to second line TB treatment among new and previously treated pulmonary TB cases in Kisumu County, Kenya

IV. To evaluate the diagnostic performance of molecular line probe assay in *M. tuberculosis* drug resistance testing among new and previously treated pulmonary TB cases in Kisumu County, Kenya

1.3.3 Research questions

I. What are the drug resistance conferring gene mutations in *M. tuberculosis* from HIV cases among new and previously treated pulmonary TB cases in Kisumu County, Kenya?

II. What are the gene mutations in *M. tuberculosis* that confer resistance to first line TB treatment among new and previously treated pulmonary TB cases in Kisumu County, Kenya?

III. What are the gene mutations in *M. tuberculosis* that confer resistance to second line TB treatment among new and previously treated pulmonary TB cases in Kisumu County, Kenya?

IV. What is the diagnostic performance of molecular line probe assay in *M. tuberculosis* drug resistance testing among new and previously treated pulmonary TB in from Kisumu County, Kenya?

1.4 Significance of the study

The first objective of the current study was to establish gene mutations in *Mycobacterium tuberculosis* from HIV cases among new and previously treated pulmonary TB cases in Kisumu County, Kenya. Investigating gene mutations in *M. tuberculosis* among individuals with HIV could provide insights into how these mutations impact the progression and severity of tuberculosis (TB) in the presence of HIV. This knowledge could help clinicians better predict disease outcomes in Kisumu County.

The second objective of the study was to determine gene mutations in *Mycobacterium tuberculosis* that confer drug resistance to first-line TB treatment among new and previously treated pulmonary TB cases in Kisumu County, Kenya. First-line TB drugs, such as isoniazid

and rifampicin, are essential for effective TB treatment. Identifying specific gene mutations responsible for drug resistance will enable the development of targeted treatment regimens and inform the selection of appropriate therapeutic approaches in Kisumu County.

The third objective of the study was to determine gene mutations in *Mycobacterium tuberculosis* that confer drug resistance to second-line TB treatment among new and previously treated pulmonary TB cases in Kisumu County, Kenya. Second-line drugs are crucial for managing MDR-TB and XDR-TB cases. Understanding the genetic basis of drug resistance to these drugs will assist in designing effective treatment protocols and improving patient outcomes.

The fourth objective was to evaluate the diagnostic performance of molecular line probe assays in *Mycobacterium tuberculosis* drug resistance testing among new and previously treated pulmonary TB cases in Kisumu County, Kenya. Molecular line probe assays are rapid diagnostic tools for detecting drug resistance-conferring mutations in *M. tuberculosis.* Assessing their accuracy and efficiency in the context of Kisumu County's TB burden will contribute to their potential integration into routine diagnostic practices.

The results from the current study will be essential in guiding the development of effective treatment protocols, optimizing drug regimens, and formulating targeted public health interventions to curb the spread of drug-resistant TB strains in Kisumu County. Furthermore, the findings will aid in informing local, national and international TB control policies and contribute to the global effort to combat this persistent and evolving infectious disease.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

2.1.1 *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a species of pathogenic bacteria in the family of *Mycobacteriaceae* characterized by possession of hoisted lipid content, most notably waxes (mycolic acids) and is the causative agent for tuberculosis (Tuboly, 1968). These mycolic acids are behind the organism's resistance to decolonization by acid alcohols hence referred to as being acid-fast (Cheesbrough, 2006; Tuboly, 1968). Tuberculosis is mainly spread when people who are sick with MTB expel the bacteria into the air through coughing and it typically affects the lungs (pulmonary TB) but can as well affect other sites (extra pulmonary TB) (WHO, 2017b).

2.1.2 Transmission and Pathogenesis of *Mycobacterium tuberculosis*

Inhalation and Alveolar Macrophage Infection: Upon inhalation of aerosolized droplets containing *M. tuberculosis*, the bacteria enter the respiratory system and reach the alveoli of the lungs. The first-line of defense against the bacteria is alveolar macrophages, which are specialized immune cells responsible for engulfing and destroying pathogens. However, *M. tuberculosis* can survive and replicate within these macrophages, evading the host immune response (Davis & Ramakrishnan, 2009).

Formation of Granulomas: The immune system responds to the presence of *M. tuberculosis* by recruiting other immune cells, such as T-cells and other macrophages, to the site of infection. This immune response leads to the formation of granulomas, which are organized

structures consisting of a central core of infected macrophages surrounded by immune cells (Flynn & Chan, 2001).

Latent Tuberculosis: In some individuals, the immune response successfully contains the infection, and the bacteria enter a dormant state known as latent tuberculosis infection (LTBI). During this stage, the bacteria remain alive but are held in check by the host's immune system. People with LTBI are asymptomatic and not contagious but have the potential to develop active TB if their immune system weakens (Russell & Flynn, 2010).

Reactivation and Active Tuberculosis: In individuals with weakened immune systems, such as those with HIV infection or other immunosuppressive conditions, the bacteria can reactivate and cause active tuberculosis. The reactivated bacteria can break out of the granulomas and spread to other parts of the lungs or other organs, leading to the development of active TB disease (Mancuso *et al*., 2017).

Dissemination and Transmission: If active TB is not diagnosed and treated promptly, the bacteria can spread from the lungs to other organs, such as the lymph nodes, bones, and brain, through the bloodstream or lymphatic system. Additionally, active TB patients who cough or sneeze can release infectious aerosol droplets into the air, contributing to the transmission of TB to others (Worrell *et al.*, 2018).

2.2 Conceptual framework

2.2.1 Socio-demographic descriptors

The conceptual framework involving age, sex, new TB cases, retreatment cases can provide valuable insights into the development and spread of drug-resistant gene mutations in *Mycobacterium tuberculosis*. Understanding these factors' interactions is essential for effective TB control and the design of appropriate treatment strategies **(Figure 2.1)**.

Age and Sex: Certain gene mutations associated with drug resistance may be more prevalent in specific age groups or genders due to different exposures, immune responses, and treatment histories.

New TB Cases and Retreatment Cases: Retreatment cases are more likely to have drug-resistant strains due to previous treatment exposures, leading to a higher likelihood of encountering specific gene mutations associated with resistance.

2.1.2 HIV status and gene Mutations in *Mycobacterium tuberculosis*

The conceptual framework presented in **Figure 2.1**, illustrates the intricate interplay between HIV infection and drug resistance-conferring gene mutations in *Mycobacterium tuberculosis*, immunocompromised, treatment challenges, and their combined impact on disease progression and treatment outcomes. Understanding these interactions is crucial for developing effective strategies to address the dual burden of HIV and drug-resistant TB in affected populations.

2.2.3 First and Second line anti-tuberculosis drug resistance

The conceptual framework in **Figure 2.1**, highlights the importance of appropriate drug use and early detection of drug-resistant TB. Monitoring for drug resistance and gene mutations can inform treatment decisions, including the use of alternative drugs or combinations to effectively manage drug-resistant TB cases. Understanding the interactions between first-line anti-tuberculosis drugs and gene mutations in *Mycobacterium tuberculosis* is crucial for the development of more effective strategies to combat TB and prevent the spread of drug-resistant strains.

2.2.4 Drug resistance Testing

Conceptual framework in **Figure 2.1**, emphasizes the importance of phenotypic and genotypic drug resistance tests in identifying gene mutations in *M. tuberculosis* that confer resistance. This information guides clinicians in selecting appropriate treatment regimens, improving patient outcomes and implementing effective strategies to control drug-resistant TB.

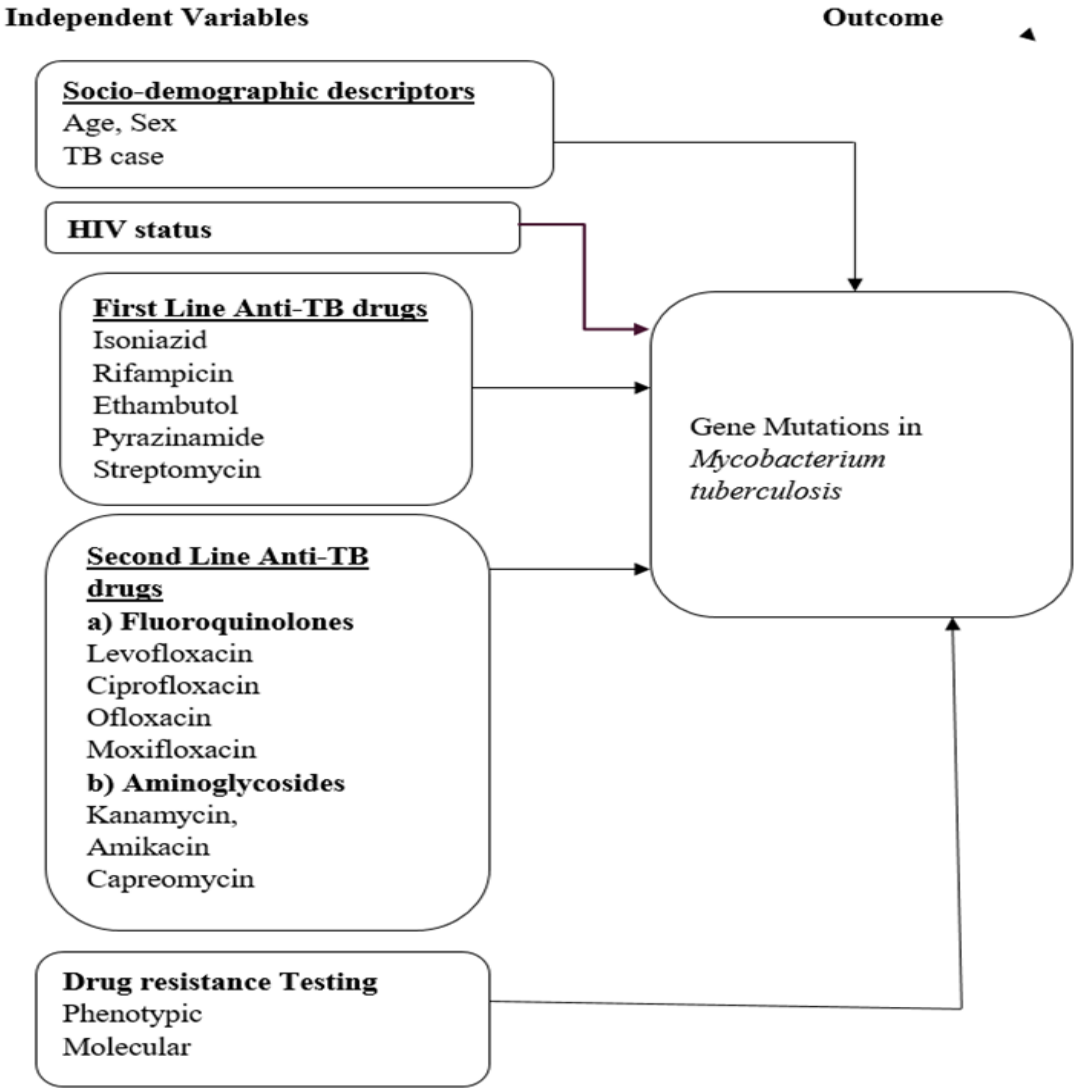

Figure 2.1. Conceptual Framework

2.3 *Mycobacterium tuberculosis* drug resistance

Drug resistant tuberculosis (DR TB) poses a major public health threat globally and it occurs when the TB bacilli undergoes mutations enabling it to survive exposure to anti TB drugs (Zhang &Telenti, 2000), and the commonest forms are Multi-drug resistant (MDR) TB and Rifampicin resistant (RR) TB (Campbell *et al.*, 2011). MDR TB refers to a form of TB where the germ is resistant to both isoniazid and rifampicin while rifampicin resistant (RR) TB refers to resistance to rifampicin only (WHO, 2022).

Globally in 2018 an estimated 3.4% of new TB cases and 18% of previously treated patients had MDR/RR TB and in the same year there were 484,000 MDR/ RR TB cases notified worldwide (WHO, 2018b). Isoniazid mono-resistance is estimated to have occurred in 7.2% of new cases and 11.2% of previously treated cases (WHO, 2020b). Among those with MDR/ RR TB, 20.8% had resistance to fluoroquinolones (pre-XDR TB) and the global treatment success rate (TSR) for MDR/RR TB was 56% (WHO, 2022).

Kenya is among the top 30 high burden countries for TB, MDR TB and TB-HIV and WHO estimated that 1.3% of new TB cases and 4.4% of previously treated TB cases have MDR/ RR TB in 2019 (WHO, 2020a). The number of DR TB cases has increased significantly over the years and multi-drug and rifampicin resistant (MDR/RR) TB contribute 70% of all DR TB cases notified in Kenya. Additionally, Kenya is estimated to have had 2,300 incident MDR/RR TB cases in 2018, however the country notified 485 MDR/RR TB cases, missing the detection of 79% of the estimated incident cases (MOH, 2020b). In Kenya, general cases of DR TB that have never been treated for TB before signify transmission of DR TB, whether diagnosed or not and the proportion of DR TB cases due to transmission has also increased significantly from 17% in 2013 to 46% in 2018 (MOH, 2021). Nevertheless, the effective

diagnosis and treatment of *Mycobacterium tuberculosis* has remained a national as well as regional health challenge for many years which is further complicated with the frequent co-existence of epidemics of TB and human immunodeficiency virus especially in Western Kenya (Emery *et al.*, 2021). This is corroborated by the report from Kenya National Tuberculosis Leprosy and Lung Disease Program that showed Siaya had a case notification rate of 276 per 100,000 and Homabay case notification rate(CNR) of 187 per 100,000 and MDR cases of 3, followed by Kisumu with CNR of 161 per 100,000 and MDR cases of 2 while Migori had a CNR of 131 and MDR cases of 2 (MOH, 2020b).

2.4 *Mycobacterium tuberculosis* drug resistance and HIV

A third of 37 million people living with human immunodeficiency virus (PLHIV) are infected with TB bacillus and Sub-Saharan Africa is the region with the highest burden of coinfection, comprising 71% of global co-infected cases and among 30 high-burden countries with TB and HIV coinfection (WHO, 2022). Human immunodeficiency virus infection has been detected in 70% of patients with TB and drug-resistant tuberculosis is considered a potential obstacle for elimination of TB globally (Singh *et al.*, 2020).

Human immunodeficiency virus coinfection with M/XDR-TB is an impending threat with reports showing adverse clinical outcomes and alarmingly elevated mortality rates among HIV cases co-infected with M/XDR-TB. The report further indicates that co-infection is also accountable for all forms of M/XDR-TB occurrences (Singh *et al.*, 2020). Superior outcomes with decreased mortality have been documented in concomitant treatment encompassing antiretroviral drugs for the HIV constituent and anti-tubercular drugs for the DR-TB component. However, the extend of global affliction of DR-TB and HIV coinfection has not been exactly well-defined, the basic reason for this gap is that HIV infection and anti-TB drug resistance testing are not adequately available for surveillance teams (Abhijeet *et al.*,

2020). Epidemiological studies from different countries have shown discordant associations as there has been heterogeneity in setting, demographic profile, methodology, and analysis of data (Abhijeet *et al.*, 2020). In the fourth WHO–International Union against Tuberculosis and Lung Disease global drug-surveillance report, 24 countries reported data on MDR-TB stratified by HIV status. There was heterogeneous geographic distribution, with the majority confined to high-risk groups, even in countries showing a high prevalence of MDR-TB along with an emerging HIV epidemic only eleven countries, the majority from Eastern European and Central Asian regions, reported strong associations between HIV and drug resistance (UN, 2019).

Several epidemiological reasons indicating that M/XDR-TB may be associated with HIV have been suggested as; rapid progression of disease due to harboring of DR strains, particularly in the immune compromised compared to immunocompetent state; drug mal absorption of anti-TB drugs, such as Randethambutol (E), leading to drug resistance and treatment failure; early reactivation of an infection due to increased vulnerability in an immune compromised state acquired from community or institutional transmission; direct contact with DR-TB cases, suggesting primary or transmitted resistance (WHO, 2017a).

2.5 Gene Mutations in *Mycobacterium tuberculosis* from HIV cases

The existing literature has extensively explored the association between TB and HIV co-infection, highlighting the increased risk of TB in HIV-infected individuals and the impact of HIV on TB disease progression and treatment outcomes (WHO, 2022). Studies have reported that HIV-positive individuals are more likely to develop active TB due to their compromised immune systems (Getahun *et al*., 2010). However, limited research has specifically focused on the association between gene mutations in *M. tuberculosis* and HIV status among TB patients (Johnson *et al*., 2020). This knowledge gap underscores the need for comprehensive

investigations to understand how HIV status influences the genetic profile of *M. tuberculosis* strains and its implications for drug resistance development especially in Kisumu County with higher cases of TB-HIV coinfection.

2.6 Anti -TB drugs

Tuberculosis can be treated effectively by using first line drugs (FLD) isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), Ethambutol (EMB) and streptomycin (SM). However, this first line therapy often fails to cure TB for several reasons and may lead to relapse and the continued spread of the disease which is a contributor to the emergence of drug resistant bacteria (Ahmed *et al.*, 2018). The development of multidrug resistant tuberculosis (MDR-TB) strains, remains a concern, because its management requires the use of second-line anti tuberculosis agents that are difficult to acquire, are much more toxic and expensive than FLDs (WHO, 2018b). Therefore, the recognition and management of drug susceptible strains or single drug resistant isolates is a significant strategy in averting the development of MDR-TB (UN, 2018). In Western Kenya, extensively drug resistant-TB (strains that are resilient to either isoniazid or rifampicin, any fluoroquinolones, and at least one of three second-line anti-tuberculosis injectable agents—*i.e*., capreomycin, kanamycin, and amikacin) have also been reported (WHO, 2020a). Although the development of drug resistant TB strains and consequent treatment failure is a common clinical scenario in TB disease in Western Kenya, and associated with high mortality rates, information on drug resistance as well as both multi-drug and extensively drug resistant tuberculosis currently limited, especially in Kisumu County which is highly associated with tuberculosis modifiable factors compared to the neighbouring counties (MOH, 2020b).

2.6.1 Gene Mutations in *Mycobacterium tuberculosis* and First-Line TB Treatment

The literature provides substantial information on gene mutations in *M. tuberculosis* that confer drug resistance to first-line TB treatment, particularly isoniazid and rifampicin (Johnson *et al.,* 2018). Studies have identified specific genetic markers associated with drug resistance, such as mutations in the *rpoB* gene for rifampicin resistance and the *katG* and *inhA* genes for isoniazid resistance (Miotto *et al.,* 2018). However, most of these studies have been conducted in various geographical regions, and there might be regional variations in mutation patterns. Therefore, determining the gene mutations responsible for first-line drug resistance among new and previously treated pulmonary TB cases in Kisumu County is crucial to tailor appropriate treatment regimens.

2.6.1.1 Isoniazid

Isoniazid (INH) is a frontline antibiotic used in the treatment of tuberculosis (TB) and plays a crucial role in standard TB therapy (Johnson *et al.,* 2015). However, the development of resistance to isoniazid poses a significant challenge to TB control efforts worldwide (Schön *et al*., 2017). Understanding the mechanisms of isoniazid resistance is essential for the design of effective treatment regimens and the management of drug-resistant TB cases.

2.6.1.1.1.1 *KatG* Mutations

The primary mechanism of isoniazid resistance in *Mycobacterium tuberculosis* involves mutations in the *katG* gene, which encodes the enzyme catalase-peroxidase (Johnson *et al.,* 2015). Catalase-peroxidase activates isoniazid through the formation of a toxic metabolite that disrupts mycolic acid synthesis, an essential component of the mycobacterial cell wall (Banerjee *et al.,* 1994). Mutations in the *katG* gene reduce or abolish the catalase-peroxidase activity, leading to the inactivation of isoniazid.

2.6.1.1.1.2 *inhA* Mutations

In addition to *katG* mutations, resistance to isoniazid can also be mediated by mutations in the *inhA* gene, which encodes the enoyl-acyl carrier protein reductase involved in mycolic acid biosynthesis (Banerjee *et al.,* 1994). Mutations in *inhA* result in overexpression of the enzyme, reducing the binding affinity of isoniazid to the target site and rendering the drug less effective (Bergval *et al.,* 2009).

2.6.1.1.1.3 *ahpC* Mutations

Some isoniazid-resistant strains of *M. tuberculosis* have been associated with mutations in the *ahpC* gene, which encodes alkylhydroperoxidase *C*. These mutations may lead to increased expression of the enzyme, reducing the toxic effects of isoniazid's reactive metabolites and conferring resistance (Mishra *et al*., 2013).

2.6.1.1.1.4 Compensatory Mutations

Compensatory mutations in the *rpoB* gene, associated with rifampicin resistance, have been reported to play a role in isoniazid resistance as well. These mutations can mitigate the fitness cost associated with drug resistance and contribute to the survival and spread of isoniazid-resistant strains (Cohen *et al*., 2015).

Isoniazid resistance in *Mycobacterium tuberculosis* primarily arises through mutations in the *katG* and *inhA* genes, which interfere with the activation and target site binding of isoniazid. Additionally, mutations in the *ahpC* gene and compensatory mutations in *rpoB* may also contribute to isoniazid resistance. Understanding these mechanisms is vital for developing effective treatment strategies and surveillance programs to control the emergence and transmission of isoniazid-resistant TB strains.

2.6.1.2 Rifampicin

Rifampicin is a critical first-line antibiotic used in the treatment of tuberculosis (TB) (Johnson *et al*., 2018). However, the emergence of drug-resistant strains of *Mycobacterium*

tuberculosis, particularly rifampicin-resistant strains, poses a significant challenge to TB control efforts worldwide. Understanding the mechanisms of rifampicin resistance is essential for developing effective treatment strategies and combatting drug-resistant TB.

2.6.1.2.1 Target Mutation in the *rpo*B Gene

The most common mechanism of rifampicin resistance in *M. tuberculosis* involves mutations in the *rpoB* gene, which encodes the *β*-subunit of RNA polymerase, the enzyme responsible for transcription in bacteria (Ramaswamy *et al.*, 1998). Rifampicin binds to the *β*-subunit, preventing RNA synthesis and inhibiting bacterial growth. Mutations in the *rpoB* gene, particularly within the Rifampicin Resistance-Determining Region (RRDR), alter the binding site of rifampicin, reducing the drug's efficacy (Telenti *et al*., 1993).

2.6.1.2.2 Compensatory Mutations

Rifampicin resistance can be associated with a fitness cost for the bacterium, leading to reduced growth and reproduction. However, compensatory mutations in other regions of the *rpo*B gene or in other genes of the RNA polymerase complex can occur, restoring bacterial fitness without reversing rifampicin resistance. These compensatory mutations play a crucial role in the survival and spread of rifampicin-resistant strains (Miotto *et al.*, 2018).

2.6.1.2.3 Heteroresistance

Heteroresistance refers to the coexistence of drug-resistant and drug-susceptible bacterial subpopulations within the same clinical isolate. In the case of rifampicin resistance, heteroresistance can arise due to the presence of minor subpopulations with *rpo*B mutations. These mutations may not be detectable through routine diagnostic tests but can lead to treatment failure if the resistant subpopulations expand during treatment (Schön *et al.*, 2017).

2.6.1.2.4 Efflux Pumps

Efflux pumps are another mechanism through which *M. tuberculosis* can develop resistance to rifampicin. These pumps actively transport rifampicin out of the bacterial cell, reducing its

intracellular concentration and effectiveness. Although less common than *rpo*B mutations, efflux pump-mediated resistance has been observed in clinical isolates (Louw *et al.,* 2011).

Rifampicin resistance in *Mycobacterium tuberculosis* is primarily driven by mutations in the *rpoB* gene, particularly within the Rifampicin Resistance-Determining Region (RRDR). These mutations alter the binding site of rifampicin to the RNA polymerase, reducing its inhibitory effect on bacterial growth. Compensatory mutations and heteroresistance play critical roles in the survival and spread of rifampicin-resistant strains, while efflux pumps contribute to resistance in some cases. Understanding these mechanisms is vital for designing effective treatment regimens and surveillance programs to control the emergence and transmission of rifampicin-resistant TB strains.

2.6.1.3 Pyrazinamide

Pyrazinamide (PZA) is a key component of the first-line anti-tuberculosis drug regimen used for the treatment of tuberculosis (TB) (Lee *et al*., 2021). However, the emergence of drug-resistant strains of *Mycobacterium tuberculosis*, particularly pyrazinamide-resistant strains, presents a significant challenge to TB control efforts worldwide. Understanding the mechanisms of pyrazinamide resistance is crucial for developing effective treatment strategies and managing drug-resistant TB cases.

2.6.1.3.1 Mutation in *pncA* Gene

The primary mechanism of pyrazinamide resistance in *M. tuberculosis* is associated with mutations in the *pncA* gene, which encodes the enzyme pyrazinamidase (Shi *et al.,* 2016). Pyrazinamide is a prodrug that requires conversion to its active form, pyrazinoic acid, by pyrazinamidase to exert its anti-mycobacterial activity (Scorpio & Zhang, 1996). Mutations in the *pncA* gene lead to reduced or loss of pyrazinamidase activity, preventing the conversion of pyrazinamide into its active form and conferring resistance (Scorpio & Zhang, 1996).

2.6.1.3.2 Efflux Pumps

Efflux pumps, which actively transport drugs out of bacterial cells, have also been implicated in pyrazinamide resistance in *M. tuberculosis*. These pumps can reduce the intracellular concentration of pyrazinamide, limiting its efficacy against the bacterium (Li *et al*., 2015).

2.6.1.3.3 Altered Pyrazinoic Acid Efflux

In addition to efflux pumps, alterations in the efflux of pyrazinoic acid, the active form of pyrazinamide, have been associated with resistance. Mutations or changes in transporters involved in pyrazinoic acid efflux can decrease the drug's concentration within the bacterial cell, leading to resistance (Wang *et al.*, 2013).

2.6.1.3.4 Bacterial Acidification

Pyrazinamide is most effective at killing *M. tuberculosis* under acidic conditions. Mutations that alter the bacterium's ability to acidify its intracellular environment can reduce the drug's activity, leading to resistance (Zhang *et al.*, 2018).

Pyrazinamide resistance in *Mycobacterium tuberculosis* primarily arises through mutations in the *pncA* gene, which results in reduced or loss of pyrazinamidase activity and prevents the conversion of pyrazinamide into its active form. Efflux pumps, alterations in pyrazinoic acid efflux, and changes in bacterial acidification can also contribute to resistance. Understanding these mechanisms is vital for designing effective treatment regimens and surveillance programs to control the emergence and transmission of pyrazinamide-resistant TB strains.

2.6.1.4 Ethambutol

Ethambutol is an essential first-line antibiotic used in the treatment of tuberculosis (TB) (Johnson *et al*., 2020). However, the development of resistance to ethambutol poses a significant challenge to TB control efforts worldwide. Understanding the mechanisms of

ethambutol resistance is crucial for developing effective treatment strategies and managing drug-resistant TB cases.

2.6.1.4.1 *EmbB* Gene Mutations

The primary mechanism of ethambutol resistance in *Mycobacterium tuberculosis* involves mutations in the *embB* gene, which encodes the arabinosyltransferase enzyme involved in the biosynthesis of arabinogalactan, a crucial component of the mycobacterial cell wall. Ethambutol disrupts arabinogalactan synthesis by binding to the *EmbB* protein, inhibiting its function. Mutations in the *embB* gene alter the binding site of ethambutol, reducing its inhibitory effect and leading to resistance (Hazbon *et al.*, 2006).

2.6.1.4.2 Compensatory Mutations

Similar to rifampicin resistance, ethambutol resistance can be associated with a fitness cost for the bacterium. However, compensatory mutations in other regions of the *embB* gene or in other genes involved in cell wall biosynthesis can occur, restoring bacterial fitness without reversing ethambutol resistance (Pang *et al.*, 2012).

2.6.1.4.3 Efflux Pumps

In addition to mutations in the *embB* gene, efflux pumps have also been implicated in ethambutol resistance. These pumps actively transport ethambutol out of the bacterial cell, reducing its intracellular concentration and effectiveness. While efflux pump-mediated resistance may not be as common as *embB* mutations, it can contribute to ethambutol resistance in some clinical isolates (Jang *et al.*, 2017).

2.6.1.4.4 Changes in Cell Wall Permeability

Ethambutol resistance can also be associated with changes in the mycobacterial cell wall that reduce the permeability of the drug into the bacterial cell. These changes can be influenced by alterations in cell wall structure or composition, limiting ethambutol's access to its target site (Shi *et al.*, 2016).

Ethambutol resistance in *Mycobacterium tuberculosis* primarily arises through mutations in the embB gene, which alter the binding site of the drug and reduce its inhibitory effect on arabinogalactan synthesis. Compensatory mutations and efflux pumps play critical roles in the survival and spread of ethambutol-resistant strains, while changes in cell wall permeability may also contribute to resistance. Understanding these mechanisms is vital for designing effective treatment regimens and surveillance programs to control the emergence and transmission of ethambutol-resistant TB strains.

2.6.1.5 Streptomycin

Streptomycin is one of the first-line antibiotics used in the treatment of tuberculosis (TB) (Gutierrez *et al.*, 1998). However, the emergence of drug-resistant strains of *Mycobacterium tuberculosis*, particularly streptomycin-resistant strains, poses a significant challenge to TB control efforts worldwide. Understanding the mechanisms of streptomycin resistance is crucial for developing effective treatment strategies and managing drug-resistant TB cases.

2.6.1.5.1 *rpsL* Gene Mutations

The primary mechanism of streptomycin resistance in *M. tuberculosis* involves mutations in the *rpsL* gene, which encodes the ribosomal protein S12. Streptomycin binds to the S12 protein and disrupts the accuracy of protein synthesis, leading to bacterial cell death. Mutations in the *rpsL* gene reduce the binding affinity of streptomycin to the ribosome, reducing its inhibitory effect and conferring resistance (Böttger, 1989).

2.6.1.5.2 *rrs* Gene Mutations

Another mechanism of streptomycin resistance involves mutations in the rrs gene, which encodes the 16S ribosomal RNA (rRNA). Streptomycin binds to the 16S rRNA, and mutations in the rrs gene can interfere with the drug's binding, leading to resistance (Gutierrez *et al.*, 1998).

2.6.1.5.3 Efflux Pumps

Efflux pumps, which actively transport drugs out of bacterial cells, have also been implicated in streptomycin resistance in *M. tuberculosis*. These pumps can reduce the intracellular concentration of streptomycin, limiting its efficacy against the bacterium (Ahmed *et al.*, 1994).

2.6.1.5.4 Aminoglycoside-Modifying Enzymes

Some streptomycin-resistant strains of *M. tuberculosis* produce enzymes that modify the drug, rendering it inactive. These enzymes, known as aminoglycoside-modifying enzymes, chemically modify streptomycin, preventing it from binding to its target site and conferring resistance (Lee *et al.*, 2001).

Streptomycin resistance in *Mycobacterium tuberculosis* primarily arises through mutations in the *rpsL* and *rrs* genes, which interfere with the drug's binding to the ribosome and reduce its inhibitory effect on protein synthesis. Efflux pumps and aminoglycoside-modifying enzymes can also contribute to resistance in some clinical isolates. Understanding these mechanisms is vital for designing effective treatment regimens and surveillance programs to control the emergence and transmission of streptomycin-resistant TB strains.

2.6.2 Gene Mutations in *Mycobacterium tuberculosis* and Second-Line TB Treatment

While the literature extensively covers drug resistance to first-line TB treatment, there is relatively limited information on gene mutations in *M. tuberculosis* associated with resistance to second-line TB drugs (Smith *et al.*,2019), and second-line drugs are critical for managing MDR-TB and XDR-TB cases. Identifying specific gene mutations linked to resistance to these drugs is essential for developing targeted and effective treatment strategies. This gap in the literature highlights the need for more research to characterize gene mutations responsible

for second-line drug resistance among new and previously treated pulmonary TB cases in Kisumu County.

2.6.2.1 Fluoroquinolones

Fluoroquinolones, a group of potent antibiotics, are crucial components of second-line treatment for multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) (Maus *et al*., 2005). However, the emergence of drug-resistant strains of *Mycobacterium tuberculosis* to fluoroquinolones poses a significant challenge to TB control efforts globally. Understanding the mechanisms of fluoroquinolone resistance is essential for developing effective treatment strategies and managing drug-resistant TB cases.

2.6.2.1.1 Mutation in the *gyrA* and *gyrB* Genes

The primary mechanism of fluoroquinolone resistance in *M. tuberculosis* involves mutations in the *gyrA* and *gyrB* genes, which encode subunits of DNA gyrase, the enzyme responsible for DNA replication and repair. Fluoroquinolones target DNA gyrase, preventing the proper functioning of the enzyme and causing bacterial cell death. Mutations in the *gyrA* and *gyrB* genes can alter the binding site of fluoroquinolones, reducing their inhibitory effect and conferring resistance (Maus *et al.,* 2005).

2.6.2.1.2 Mutation in the *rrs* Gene

The *rrs* gene, which encodes the 16S rRNA component of the bacterial ribosome, is also involved in fluoroquinolone resistance. Fluoroquinolones can bind to the 16S rRNA, and mutations in the *rrs* gene can interfere with the drug's binding, leading to resistance (Sreevatsan *et al.,* 1997).

2.6.2.1.3 Efflux Pumps:

As in the case of other antibiotics, efflux pumps have been implicated in fluoroquinolone resistance in *M. tuberculosis*. These pumps actively transport fluoroquinolones out of

bacterial cells, reducing the intracellular concentration of the drug and limiting its efficacy (Li *et al.*, 2016).

2.6.2.1.4 DNA Repair Mechanisms

Mycobacterium tuberculosis possesses various DNA repair mechanisms that can counteract the lethal effects of fluoroquinolones. Enhanced DNA repair capacity can contribute to reduced drug susceptibility and contribute to the development of resistance (Kumar *et al.*, 2011).

Fluoroquinolone resistance in *Mycobacterium tuberculosis* primarily arises through mutations in the *gyrA*, *gyrB*, and *rrs* genes, which interfere with the drug's binding to DNA gyrase and the ribosome and reduce its inhibitory effect on bacterial replication. Efflux pumps and DNA repair mechanisms can also contribute to resistance in some clinical isolates. Understanding these mechanisms is vital for designing effective treatment regimens and surveillance programs to control the emergence and transmission of fluoroquinolone-resistant TB strains.

2.6.2.2 Aminoglycosides

Aminoglycosides, including kanamycin, amikacin, and streptomycin, are important second-line antibiotics used in the treatment of drug-resistant tuberculosis (TB) (Smith *et al.*,2029). However, the emergence of drug-resistant strains of *Mycobacterium tuberculosis* to aminoglycosides poses a significant challenge to TB control efforts globally. Understanding the mechanisms of aminoglycoside resistance is crucial for developing effective treatment strategies and managing drug-resistant TB cases.

2.6.2.2.1 Mutation in the *rrs* Gene

The primary mechanism of aminoglycoside resistance in *M. tuberculosis* involves mutations in the rrs gene, which encodes the 16S rRNA component of the bacterial ribosome (Li *et al.*, 2015). Aminoglycosides target the 30S subunit of the ribosome and interfere with protein synthesis, leading to bacterial cell death. Mutations in the *rrs* gene can alter the binding site

of aminoglycosides, reducing their inhibitory effect and conferring resistance (Hillemann *et al.,* 2007).

2.6.2.2.2 Mutation in the *eis* Gene

Some aminoglycoside-resistant strains of *M. tuberculosis* harbor mutations in the eis gene, which encodes an enzyme that acetylates aminoglycosides, modifying the drug and rendering it inactive. Mutations in the eis gene can increase the expression of the enzyme, leading to aminoglycoside inactivation and resistance (Maus *et al*., 2005).

2.6.2.2.3 Efflux Pumps

As in the case of other antibiotics, efflux pumps have been implicated in aminoglycoside resistance in *M. tuberculosis*. These pumps actively transport aminoglycosides out of bacterial cells, reducing the intracellular concentration of the drug and limiting its efficacy (Li *et al.,* 2015).

2.6.2.2.4 Ribosomal Methylation

In some cases, *M. tuberculosis* can acquire resistance to aminoglycosides through ribosomal methylation. This modification is mediated by enzymes encoded by the *rmt* genes, and it can alter the binding site of aminoglycosides on the ribosome, reducing their effectiveness (Liu *et al.,* 2016).

Aminoglycoside resistance in *Mycobacterium tuberculosis* primarily arises through mutations in the rrs and eis genes, which interfere with the drug's binding to the ribosome and increase its inactivation. Efflux pumps and ribosomal methylation can also contribute to resistance in some clinical isolates. Understanding these mechanisms is vital for designing effective treatment regimens and surveillance programs to control the emergence and transmission of aminoglycoside-resistant TB strains.

2.6.3 Diagnostic Performance of Molecular Line Probe Assay in *M. tuberculosis* Drug Resistance Testing

2.6.3.1 Phenotypic TB Drug Resistance Tests

Phenotypic tests assess the actual growth or behavior of *Mycobacterium tuberculosis* in the presence of specific drugs. These tests involve culturing the bacteria and then exposing them to different drugs to observe their response. Some common phenotypic tests for TB drug resistance include: MGIT (Mycobacteria Growth Indicator Tube) test**.** The MGIT test is a type of automated DST that uses a liquid culture medium to detect drug resistance. It monitors bacterial growth and fluorescence to determine susceptibility to TB drugs.

The emergence and spread of drug resistant *Mycobacterium tuberculosis* strains is an obstacle to the control and management of tuberculosis as well as a threat to the World Health Organization's goal of eliminating the disease by 2050 (WHO, 2018a).

Detection of drug resistant strains requires screening for resistance markers to specific antibiotics and utilizing results to design a treatment regimen (Abanda *et al.*, 2017).

There are a few isolated cases of MDR that have been reported in Western Kenya (MOH, 2020a). This is attributed to very low rate of detection, as the diagnostic methods available in the public health facilities are still largely based on sputum slide smear microscopy (Mukati *et al.*, 2019). This technique has low sensitivity and lacks specificity for susceptible tuberculosis mutant strains that commonly confer resistance to the approved MTB regimens (Khan *et al.*, 2019). Phenotypic methods allow the identification of drug resistance regardless of the resistance mechanism and can be performed as direct or indirect tests on solid media or in liquid media while molecular tests detect the genetic determinants of resistance rather than the resistant phenotype (Uplekar *et al.*, 2015). In phenotypic drug susceptibility testing, *Mycobacterium tuberculosis* is detected and/or characterized with respect to its drug

susceptibility based on direct or indirect observations of cellular growth and/or bacterial metabolism in a chosen media (WHO, 2018a). This is done using BD BACTEC^{TM} MGIT^{TM} 960 system (Becton Dickinson Bioscience, Erebodegem, Belgium) which has automated culture-based diagnostic systems for detection of *Mycobacterium tuberculosis* and subsequent phenotypic DST (Smith *et al.*, 2008). Phenotypic DST has been approved by WHO as the gold standard for drug resistance monitoring in MTB, however, the process of culturing is laborious, bioharzadous and time consuming (Eddabra &Mounsef, 2020). Line probe assay which are molecular based methods are an alternative that are faster, can be done directly on sputum samples and requires minimal biological containment.

2.6.3.2 Genotypic TB Drug Resistance Tests

Genotypic tests detect specific genetic mutations in *M. tuberculosis* that are associated with drug resistance. These tests directly analyze the bacterium's DNA to identify mutations related to resistance. Some common genotypic tests for TB drug resistance include: Line Probe Assays (LPAs).

LPAs detect specific gene mutations linked to drug resistance. They use molecular techniques to target genes associated with resistance, such as the *rpoB* gene for rifampicin resistance and the *katG* and *inhA* genes for isoniazid resistance.

Molecular line probe assays are DNA strip-based tests that determine the drug resistance conferring mutations of *Mycobacterium tuberculosis* strain through binding of amplicons to probes targeting the common resistance associated changes to first- and second-line agents and to probes targeting the corresponding wild-type DNA sequence **(Figure 2.2)**.

The GenoType MTBDR*plu*s and GenoType MTBDR*sl* assays (Hain Life science GmbH, Nehren, Germany) are molecular-based test for detecting drug resistant strains of *Mycobacterium tuberculosis.* Two independent systematic reviews concluded that the Geno Type MTBDR*plus* and Genotype MTBDR*sl* assays were highly accurate in diagnosing

MDR-TB when compared to the culture-based proportion method (Ahmed *et al.*, 2018; WHO, 2016b). These two reviews reported pooled sensitivities of 88.7% (Abanda *et al.*, 2017) and 91% (Dantas *et al.*, 2017) and specificities of 99.2% (Abanda *et al.*, 2017) and 99% (Dantas *et al.*, 2017), respectively, for detection of MDR-TB. Although there is evidence of the accuracy of the MTBDR*plus* assay, the prevalence of MDR-TB varies widely globally. This variation in prevalence of MDR-TB might have a significant impact on the predictive value of the MTBDR*plus* and MTBDR*sl* assay (Abanda *et al.*, 2017).

Molecular line probe assays, such as the GenoType MTBDR*plus* assay, have shown promise as rapid and accurate tools for detecting drug resistance in *M. tuberculosis* strains (WHO, 2020). The literature provides evidence of the diagnostic performance of these assays in different settings, demonstrating high sensitivity and specificity for detecting rifampicin and isoniazid resistance (Barnard *et al*., 2012). However, their performance may vary depending on the prevalence of specific mutations in different geographical regions. Therefore, evaluating the diagnostic performance of molecular line probe assays in drug resistance testing among new and previously treated pulmonary TB cases in Kisumu County is crucial to determine their utility in routine diagnostic practices.

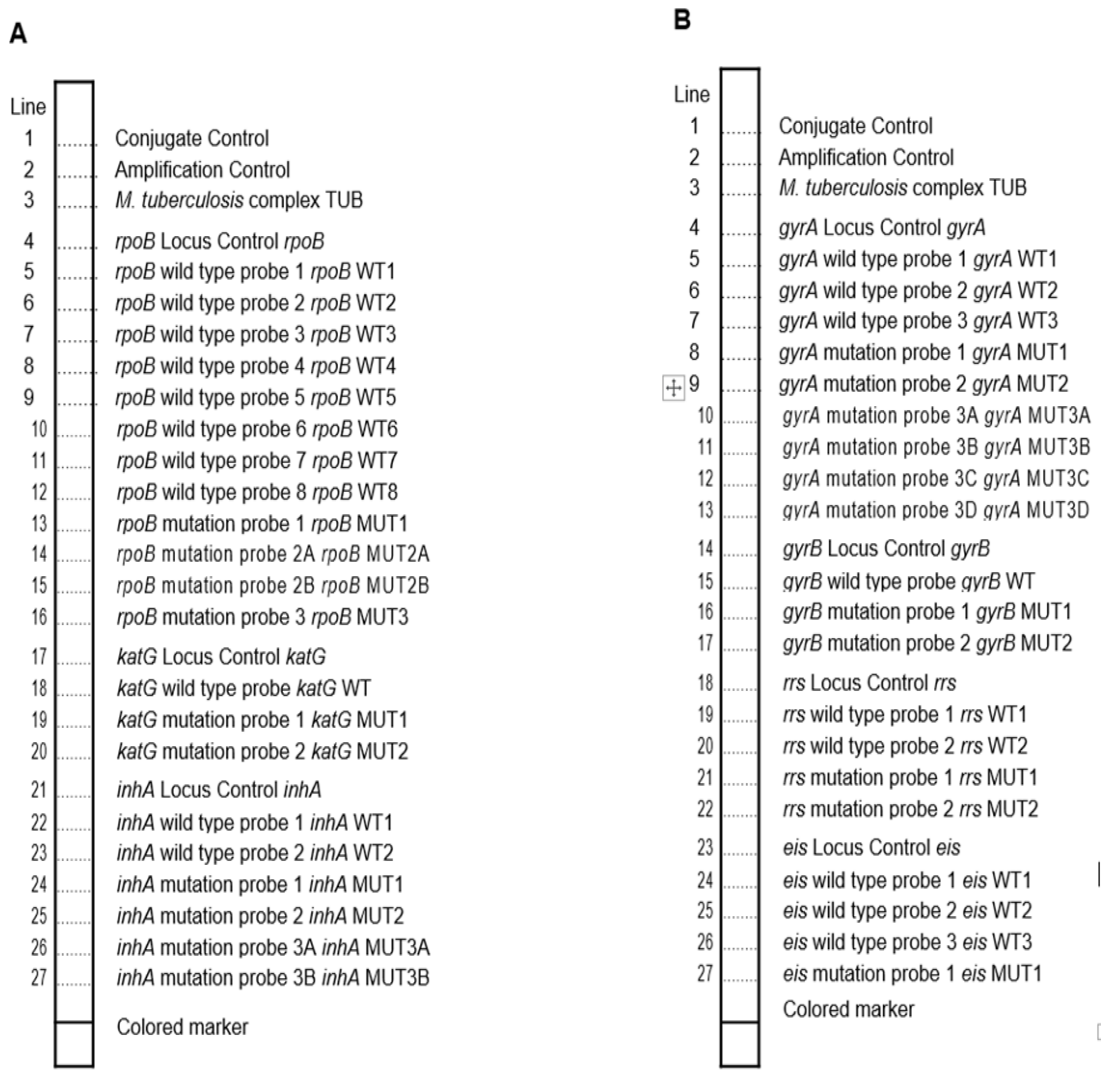

Figure 2.2. Configuration of GenoType MTBDR*plus* V2 (a) and GenoType MTBDR*sl* V2 (b) strips
Source: GenoType MTBDR*plus* and GenoType MTBDR*sl* test (Hain Life science GmbH, Nehren, Germany).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Site

This study was carried out in Kisumu County, Western Kenya which lies between longitudes $33^0$20'E and 35^0 20'E and latitude 0^0 20' South and $0^0$50' South. The County covers approximately 567 km^2 on water and 2086km^2 land area, representing 0.36% of the total land area of Kenya's 580,367km^2 (County Government of Kisumu, 2018).

Kisumu County has a proximate population of 1,153,343; 489,392 and administratively has seven Sub-counties namely: Kisumu West, Kisumu East, Kisumu Central, Muhoroni, Nyakach, Nyando and Seme, and the average HIV prevalence is 17.4% against the national prevalence of 4.3%, and TB-HIV co infection rate of 44% against national rate of 23% (MOH, 2021) **(Figure 3.1)**.

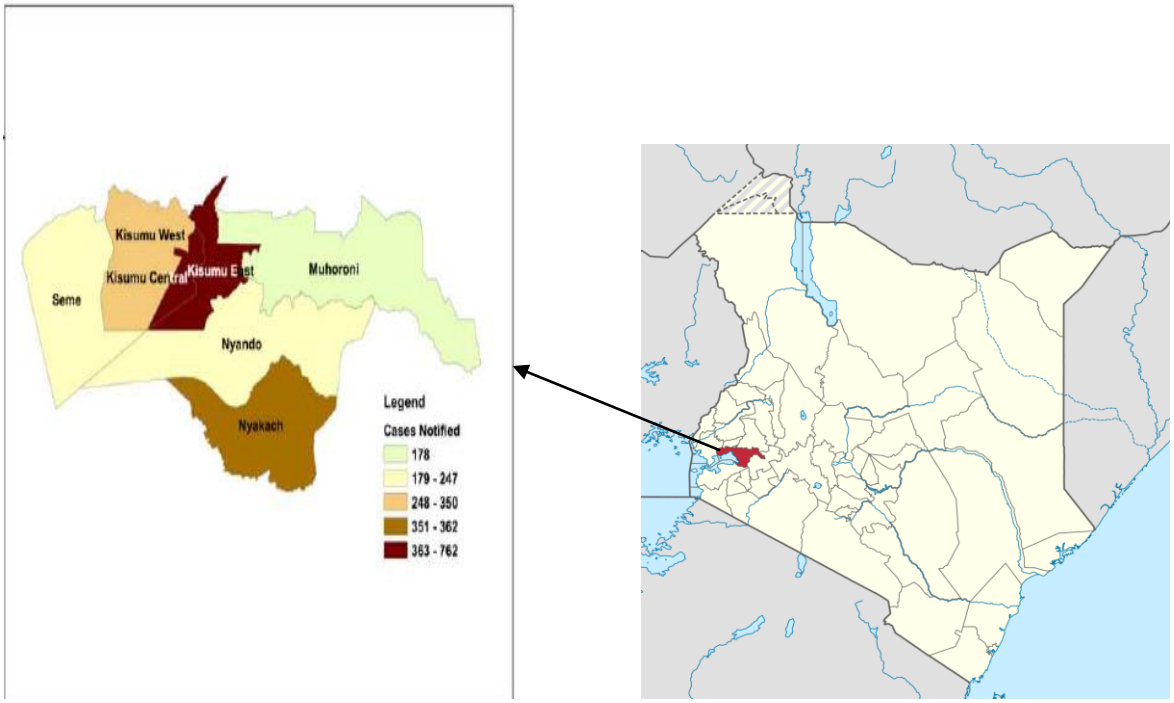

Figure 3.1. Map of Kisumu County showing notified TB cases in 2018 (GOK, 2018)

Source: National Tuberculosis, Leprosy and Lung Disease Program Annual Report 2018

3.2 Study Design

Hospital and laboratory based cross sectional study design was done on tuberculosis patients attending TB clinics and hospital facilities within Kisumu County. This study was conducted between November 2020 and October 2021 to investigate the profile of drug resistance conferring mutations in *Mycobacterium tuberculosis* among new and previously treated pulmonary tuberculosis in Kisumu County, Kenya. The total number of facilities where participants were recruited were 36 **(Table 3.1).**

Table 3.1. Distribution of participants across various Heath facilities

Sub-County	Health Facility	Cases	Percent
Kisumu Central	(JOOTRH)	3	1.2
Kisumu Central	AP Dispensary	4	1.6
Kisumu Central	Kisumu District Hospital	1	0.4
Kisumu Central	Lumumba Health Centre	17	6.6
Kisumu Central	Migosi Health Centre	3	1.2
Kisumu Central	Nyalenda Health Centre	3	1.2
Kisumu West	Chulaimbo Sub-District Hospital	50	19.5
Kisumu West	Kemri Clinic	26	10.2
Kisumu West	Kodiaga Prison Health Centre	4	1.6
Kisumu West	Lwala Kadawa Dispensary	7	2.7
Kisumu West	Maseno Mission Hospital	9	3.5
Kisumu West	Maseno University Medical Clinic	3	1.2
Kisumu West	Nyahera Sub District Hospital	3	1.2
Kisumu West	Ober Kamoth Health Centre	5	2
Kisumu West	Port Florence Hospital	5	2
Kisumu West	Rota Dispensary	5	2
Muhoroni	Chemelil Sugar Health Centre	1	0.4
Muhoroni	Kandege Dispensary	3	1.2
Muhoroni	Kibigori Dispensary	1	0.4
Muhoroni	Mashambani Dispensary	5	2
Muhoroni	Masogo Sub District Hospital	2	0.8
Muhoroni	Muhoroni Sub-District Hospital	6	2.3
Muhoroni	Ogen Dispensary	1	0.4
Nyakach	Katito Health Centre	3	1.2
Nyando	Ahero Sub-District Hospital	23	9
Nyando	Hongo Ogosa Dispensary	1	0.4
Nyando	Magina Dispensary	1	0.4
Nyando	Nyakongo Dispensary	9	3.5
Nyando	Nyangande Dispensary	2	0.8
Nyando	Okana Dispensary	3	1.2
Nyando	Rabuor Health Centre	6	2.3
Seme	Arito Langi Dispensary	4	1.6
Seme	Bodi Health Centre	3	1.2
Seme	Kombewa District Hospital	15	5.9
Seme	Manyuanda Health Centre	13	5.1
Seme	Nduru Kadero Dispensary	6	2.3
	Total	**256**	**100**

3.2.1 Inclusion criteria

1. The patients enrolled in the study had to be clinically presenting as a TB case (as per the Government of Kenya Ministry of Health case definition for suspected TB case).
2. Capable of expectorating sufficient sputum sample for study purposes.
3. Informed consent/assent (or parental permission), after demonstrating their understanding formed the basis for recruitment. For children under age 12 parental consent was sought, and both assent and parental consent sought for those $\geq$ 12 to <18 years of age, assent also required signing of a consent form.

3.2.2 Exclusion criteria

1. New patients who had started TB treatment more than one week before the study were excluded from enrolment. This was because patients who submit sputum samples after starting treatment, and in whom a positive sputum smear was observed, may be more likely to be harbouring drug resistant strains, thus introducing bias and additionally, a notable percentage of cultures would fail to grow in patients on treatment.
2. Patients who were unable to provide adequate sputum specimen for testing.
3. Lack of informed consent/assent.

3.3 Sampling technique

All eligible patients presenting to each health facility in the study area (Kisumu County) within the defined study period were duly consented and enrolled in the study and samples meeting clinical acceptance criteria shipped from the recruiting TB clinic (facility) within Kisumu county and shipped to Kenya Medical Research Institute (KEMRI) microbiology laboratory. The study population included both children and adults of all age groups. At KEMRI microbiology laboratory, Simple random sampling was done for received samples

meeting the laboratory acceptance criteria prior to laboratory analysis. Steps for simple random sampling was as follows: The samples that met the laboratory acceptance criteria were defined as sample size which were 750 samples; then based on the samples size of 256 samples that was determined from WHO guidelines for surveillance and survey in drug resistance TB, the sample size was selected using random number generator.(https://stattrek.com/statistics/random-number-generator#table)(Appendix 3).

3.4 Sample Size

The desired sample size was calculated based on the sampling method recommended by WHO Guidelines for surveillance of drug resistance in tuberculosis (WHO, 2015).

The sample was determined by taking the prevalence of rifampicin resistance of 1.3% in 2019, desired precision of 0.5%, a 95% confidence interval and nonresponse rate of 20%.

The calculated sample size was adjusted by 15–20% to account for expected losses. Losses included patients whose susceptibility testing does not give interpretable results; WHO Guidelines for Drug Resistance Survey (WHO, 2015).

The sample size was determined as follows:

$$n = N * z^2 * (1 – g) / (N – 1) * d^2g + z2 * (1 – g)$$

Where:

N = total number of new sputum smear positive pulmonary patients registered in the selected sentinel sites (Kisumu County) during one year (2019);

z = z-value (from the standard normal distribution) that corresponds to the desired confidence level (narrowing the confidence interval from 95% to 90% will result in some reductions in sample size; if confidence interval =90%, z= 1.65);

d = absolute precision of 0.05

g = previous estimate of proportion of new cases with rifampicin resistance

Where, N=223 per 100,000, d= 0.05%, Z=1.96, g=1.3%

The calculated samples size was **213.**

WHO recommends that calculated sample size be adjusted by 15–20% to account for expected losses (WHO, 2015).

We choose the upper limit of the range to compensate for additional losses due to HIV status variable non response.

20% of 213 = 43

The total sample size was hence 213+43

=256

3.5 Data Collection and Research Procedure

The study clinician or laboratory personnel in the recruiting facility employed clinical case reports forms and laboratory request forms as the tools for collecting data. The tools included information such as sociodemographic of the respondent and laboratory tests (Appedix1a and 1b). Study participants who met the inclusion criteria were explained for the purpose of the study, possible risk and benefits and those who agreed to participate in the study were duly informed, consented and enrolled into the study (Appendix 4 and 5).

3.5.1 Sample collection

Patient who met the minimum inclusion criteria were recruited into the study and then given sputum cups by the clinician or laboratory personnel in the recruiting facility to have their sputum samples taken. A pipette drop from the sample was used to bacteriology confirm the sample for acid fast bacilli at the facility and an aliquot of the sample was then packed in screw cups with double biohazard bags inside a cooler box and transported to Kenya Medical Research Institute (KEMRI) Microbiology Reference Laboratory in Kisian for further confirmatory staining, culturing, and molecular drug resistance testing. Local specimen

shipment was done according to regulation provided by the International Air Transport Association (http://www.iata.org/ ads/issa/htm).

At KEMRI Microbiology reference laboratory, sputum samples together with the lab request forms were received from health facilities within the County and checked for completeness in filling in the laboratory request form, correct sample tube labeling and leakages and those meeting the acceptance criteria were given laboratory study number and refrigerated at -4°C awaiting processing.

During processing, four aliquots of 1.0 ml each were made from the stock sample,1 aliquot was used for florescent microscopy, another for phenotypic DST, Line Probe Assay and the remaining stored at − 80 °C for retesting incases of failure (**Figure 3.2)**.

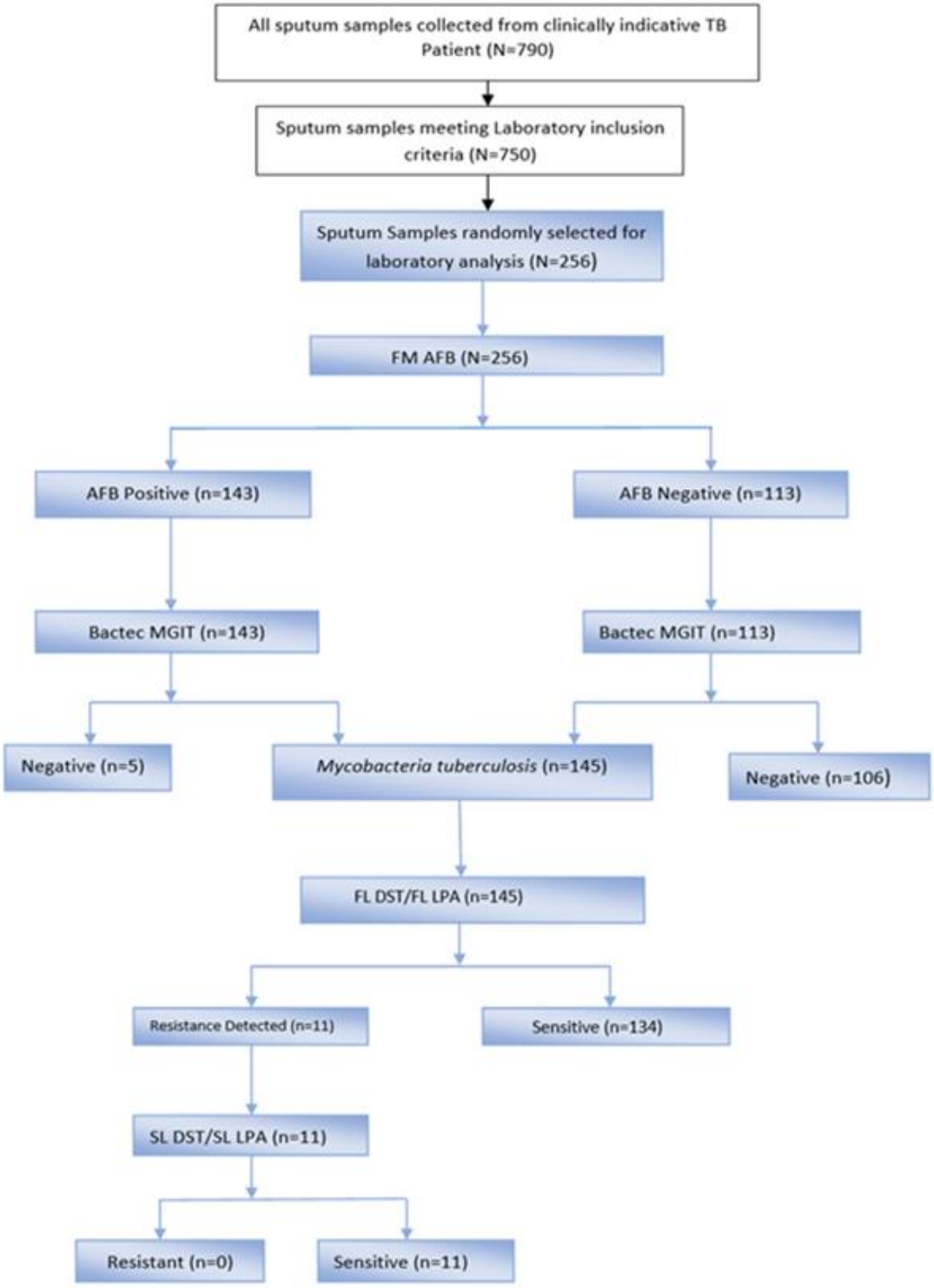

Figure 3.2. Figure showing the flow of samples from the collection to final laboratory analysis

3.5.2 Specimen preparation and Staining procedure for Fluorescent Microscopy

Decontamination of sputum specimen was done to all 256 clinical samples using the *N*-acetyl-l-cysteine-sodium citrate-NaOH (NALC-NaOH) method (WHO, 2015). From each sample, 2ml of the vial was then centrifuged at 3000 g for 15 min to decant, and sediments re-suspended into 3 ml using phosphate buffer solution.

Using a transfer pipette, ~100 µl of specimen was dropped into glass slide, spreading over an area approximately 1 x 2 cm and Air-dried.

The dry slide was then flooded with absolute alcohol for 10 minutes and heat fixed at a temperature between 65°C to 75°C for at least 2 hours. Staining by flooding the slide with Carbolic Auramine and letting it stand during 20 minutes was done and later rinsed in tap water. Discolouration was done by flooding the slide with alcohol solution for 3 minutes. Discolouring solution was later washed off with tap water and counterstained by flooding the slide with methylene Blue 0.3% which was later washed with tap water. The smear was then air dried and examined using the fluorescent microscope (Cheesbrough, 2006).

3.5.3 Phenotypic drug susceptibility testing

Phenotypic drug susceptibility testing was conducted for all 256 clinical suspected samples using BACTEC™ system (Becton Dickinson (BD) Bioscience, Erebodegem, Belgium) at the KEMRI Tuberculosis Microbiology Laboratory. After decantation of sediments to be used for culturing, one vial of mycobacteria growth indicator tube (MGIT) with lyophilized combination of antimicrobials was reconstituted together 15.0 ml of Mycobacteria Growth indicator enhancement. A micropipette was used to transfer, 0.8 ml of the combination to each MGIT tube to be inoculated with negative and positive controls. Using a pipette, 0.5 ml of the sample was added to labelled MGIT tubes and inverted a couple of times to get a uniform reconstitution. The MGIT tubes were later scanned and inserted into the BACTEC

machine (Cheesbrough, 2006). The optimum growth temperature for *M. tuberculosis* was maintained at +37 °C plus or minus -1 °C. Mycobacteria Growth Indicator tubes were incubated up to a maximum of 6 weeks for the negative (no growth) while positive tubes were flagged on as soon as growth was detected. Positive tubes were removed and scanned on the BACTEC instrument.

3.5.4 Line Probe Assay

Line probe assays are a family of DNA strip-based tests that determine the drug resistance profile of a MTBC strain through the pattern of binding of amplicons (DNA amplification products) to probes targeting the most common resistance associated mutations to first- and second-line agents and to probes targeting the corresponding wild-type (WT) DNA sequence.

GenoType MTBDR*plus* assay for detection of first line resistance, and GenoType MTBDR*sl* assay for detection of second line (Fluoroquinolones and Aminoglycosides), were performed for 145 *Mycobacterium tuberculosis* confirmed samples by phenotypic method (Cheesbrough, 2006).

The assay assessed mutations in the genes *rpoB, katG, inhA, embABC, pncA, rrs; gyrA, gyrB, rrs* and *eis*. Line probe assay was performed according to the manufacturer's recommendations (Hain Life Science GmbH, Nehren, Germany)(Hain Lifescience, 2015). Using multiplex PCR, GenoType MTBDR*plus* assay was used to target specific mutations in the Rif-resistance determining region(RRD) of the *rpoB* gene (from codon 505 to 533) to detect rifampicin resistance and mutations in the *inhA* promoter (from -16 to – nucleotides upstream) and *katG* (Codon 315) regions for isoniazid resistance (Appendix 2).These genes responsible for first line drug resistance such as *katG, inhA, rpoB* were amplified and the resulting biotin-labelled amplicons were hybridized to DNA probes bound to membrane probes. Briefly, for amplification 35µl of a primer nucleotide mixture, amplification buffer

containing 5µl mM MgCl2, 2.5µl sterile water, 2.5µl (1 unit) Taq DNA polymerase (ROCHE, Mannheim, Germany), and 5 µl of DNA in a final volume of 50µl were used. The amplification procedure entailed denaturation at 15 min at 95°C, followed by 10 cycles consist of 30s at 95°C and 2min; 20 cycles consisting of 20sec at 95°C, 40sec at 53°C, 40sec at 70°C and, 10cycles final extension at 70°C for 8min. Hybridization of the single stranded amplicons to probes followed by addition of conjugate, and substrate to detectable band patterns. The strips were let to dry and interpreted as per the recommendation by the manufacturer (Annex 1).

3.5.5 Interpretation

Each strip of Line Probe assay had 27 reaction zones and these including six controls bands conjugate band, *M. tuberculosis* complex, amplification, *rpoB*, *inhA* and *katG*, eight *rpoB* wild type (WT1–WT8) and 4 mutant probes *(rpoB MUT D516V, rpoB MUT H526Y, rpoB MUTS531 L and rpoB MUT H526D),* one katG wild type, two mutant and two *inhA* wild type and four mutant probes (Annex 1). Either missing wild type band or the presence of mutant band was taken as a symbol of a resistant strain. To provide a consistent result, all six expected control bands appeared correctly. Otherwise, the result was considered invalid **(Fig 3.3)**.

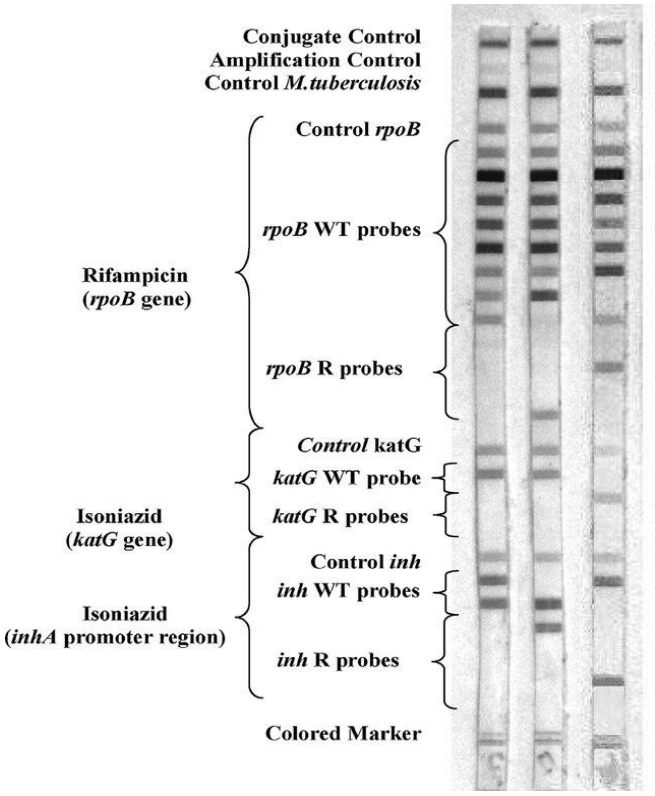

Figure 3.3. GenoType MTBDR*plus* bands

Source: GenoType MTBDR*plus* test (Hain Life science GmbH, Nehren, Germany)

3.6 Data Management

3.6.1 Data storage

This study had both paper and electronic study forms for each patient, linked by a unique study identification number given to each patient. The list linking study ID numbers with specific individuals (face sheet) was stored separately and securely in a different physical location, in a locked Good Clinical Practice (GCP) compliant cabinet at Kenya Medical Research Institute - Centre for Global Health Research (KEMRI-CGHR), Kisumu. The first form contained all demographic and clinical data for each patient while the second form was laboratory request form that contained laboratory test results such as Mycobacteriology data, smear microscopy, phenotypic drug susceptibility testing, molecular line probe assay test results, and dates of all tests. All study form data were kept in locked GCP complaint file

cabinets in the clinics and laboratories at KEMRI-CGHR, Kisumu and electronic files were stored in database with password-protected computers and laboratory test results was communicated directly to the clinicians and collected in the patients' charts and on the laboratory study forms. Only clinical and study staff had access to information collected or generated as part of this study.

3.6.2 Data analysis

Data was collected on both paper and electronic forms and then entered into laboratory information management system database, data quality was ensured through double data entry. The study database had quality check codes built in and was also checked against primary sources from clinicians or laboratory analysts and monitoring of the study site was conducted every 1 month. Statistical Package for the Social Sciences (SPSS) v23 (SPSS Software | IBM) was used for data analysis and it merged clinical and the laboratory databases prior to analysis. Missing at random data was handled through list wise deletion.

To describe general characteristic of the population; Sub county tuberculosis cases profile; Socio demographic and diagnostic characteristics, frequency tabulation was used and this data was presented frequency and proportion.

To establish gene mutations in *Mycobacterium tuberculosis* from HIV cases among new and previously treated pulmonary TB cases in Kisumu County Kenya, Cross tabulation was used to analyse gene mutations across HIV cases.

To predict for HIV status outcome, age, sex, TB cases were modelled with HIV status using binary logistic regression and measure of effect determined through odds ratio. Statistical significance was assessed at $p \leq 0.05$. Cross-tabulation was used to describe mutant probes and amino acid changes among the HIV cases and presented as numbers and percentages.

To determine gene mutations in *Mycobacterium tuberculosis* that confer drug resistance to

first and second line TB treatment, cross tabulation was used to stratify drug resistance among *M. tuberculosis* cases. Predictors of drug resistance drug resistance (age, sex, microscopy) were modelled with dependent variable (drug resistance) using binary logistic regression and measure of effect determined through odds ratio. Odds were increased for the outcome when *OR>1*.

Nucleotide changes, mutation pattern, mutant and wild type probes were described against tuberculosis cases using cross tabulation and presented as percentages and counts.

To assess diagnostic effectiveness of Line probe assay in detection of drug resistance, contingency table was used to assess sensitivity, specificity, PPV and NPV. Using BACTEC MGIT 960 as gold standard, diagnostic accuracy of GenoType MTBDR for detection of first and Second line drug resistance was assessed. Chi-square test of independence was used to determine the accuracy of the MTBDR*plus* assay to detect rifampicin, isoniazid and Multidrug drug resistance. Cross tabulation was additionally useful in assessing the specificity, sensitivity, positive predictive value and negative predictive value with 95% confidence intervals (CI) of the MTBDR*plus* assay for detection, of rifampicin, isoniazid and MDR-TB (Bossuyt *et al.*, 2015).

Sensitivity was described as the proportion of isolates correctly determined as resistant by the MTBDR*plus* assay compared with BACTEC MGIT 960, while specificity as the proportion of tuberculosis isolates that were correctly determined as susceptible by the MTBDR*plus* assay compared with BACTEC MGIT 960. Positive Predictive Value (PPV) was defined as the proportion of resistant isolates determined by the BACTEC MGIT 960 among isolates determined as resistant by the MTBDR*plus* assay while Negative Predictive Value (NPV) as the proportion of susceptible isolates determined by the BACTEC MGIT 960 system among isolates determined as susceptible by the MTBDR*plus* assay.

3.7 Study Assumptions

It was assumed that participants correctly disclosed their HIV status, tuberculosis case history (new or retreatment) particularly from subjects where adequate documentation was not available, and there was no evidence of having taken anti-TB drugs previously for one month or more.

3.8 Ethical Considerations

The study was done as per the requirement of the declaration of Helsinki and the Belmont report (*National Research Act Pub. L. 93-348*; *18 WMA General Assembly, Helsinki, Finland, June 1964).* The major possible potential risk to the patients was the breach of confidentiality. However, after consenting participants for the study, patients were given unique study identification number in order to link microscopy, phenotypic culture, molecular LPA results, and patient data. Linking of identifying information, such as patient name and birthdate, to the study identification number appeared only on a cover sheet of the patient data form. These data forms were kept in a locked GCP-compliant cabinet at Kenya Medical Research Institute-Centre for Global Health Research (KEMRI-CGHR), Kisumu and was only accessible by the investigation team. Sputum samples were labeled with the date of collection and patient's study identification number. After the end of the study, the cover sheet was destroyed, unlinking the study identification number and de-identifying the data. Data entry was performed on site by the local investigators in a password protected computers and only the study investigators and data staff had access to this data.

The benefit to study participants was that their clinicians had quality-assured, rapid, extensive microscopy, and drug resistance results faster than normal routine to decide on treatment. The use of direct LPA which, which was also not a routine practice, shortened the laboratory turnaround time. All participants were under the care of experienced clinicians and were

monitored and treated as per the Kenya Ministry of Health TB guidelines. Any clinical adverse events were reported to the KEMRI-SERU as per established good clinical practice reporting guidelines. Approval was done by Maseno University (Appendix 6), Ethical approval by Kenya Medical Research Institute, Scientific Ethical Review Unit (Appendix 7) and National Commission for Science, Technology & Innovation (Appendix 8). Drug resistance in tuberculosis raises a particular ethical dilemma when survey activities are conducted in settings where there is limited capacity to properly treat patients identified with drug-resistant strains.

CHAPTER FOUR

RESULTS

4.1 Socio-demographic and diagnostic descriptors of the participants

The samples were classified as new TB cases and previously treated cases as per the World Health Organization guidelines for surveillance of drug resistance in tuberculosis.

The study showed that, out of sample size of 256, 168(65.6%) were male while the remaining 88(34.4%) were female. Ages under 18 years were 5(2%), while 18 years and above 251(98%). Mycobacteria culture on BACTEC showed 145(56.6%) Mycobacteria detected, and 111(43.4%) were Mycobacteria not detected. Fluorescent microscopy showed 113(44.1%) negative while 143(55.9%) were positive. The male *Mycobacterium tuberculosis* cases were 112(77.2%), while female cases were 33(22.8%). From the sample, HIV positive cases were 119(46.5%) and negative were 97(37.9%) **(Figure 4.1).**

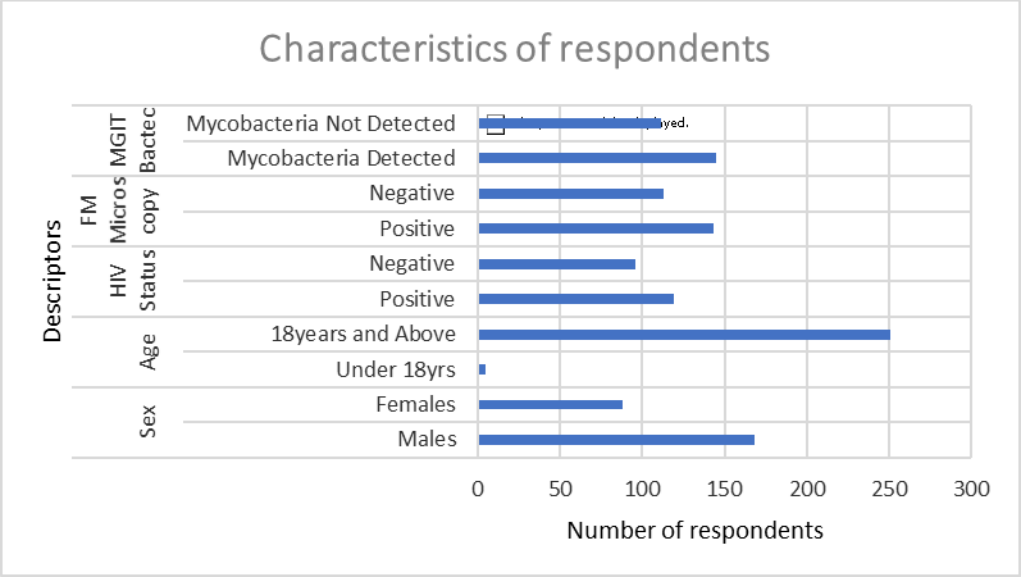

Figure 4.1. Socio-demographic and diagnostic descriptors of participants

4.1.1 Distribution of Study participants across the Sub-Counties

A total of 256 sputum samples obtained from patients with suspected clinical tuberculosis in Kisumu County, Kenya during the 12-month period from November 2020 to October 2021 were included in the study. This time was chosen because at that time there was an increase in Covid-19 cases which resulted into decline in Tuberculosis case detection since patient with respiratory symptoms were not seeking medication for fear of infection. This decline in case detection could mean continued spread of MDR TB in the community unnoticed.

The study showed that Kisumu West Sub-County facilities recruited the highest number of participants 116(45.3%), followed by Nyando 45(17.6%), Seme 41(16.0%), Kisumu Central 31(12.1%), Muhoroni 19(7.4%), Nyakach 3(1.2%) and Kisumu East 1(0.4%) **(Figure 4.2)**.

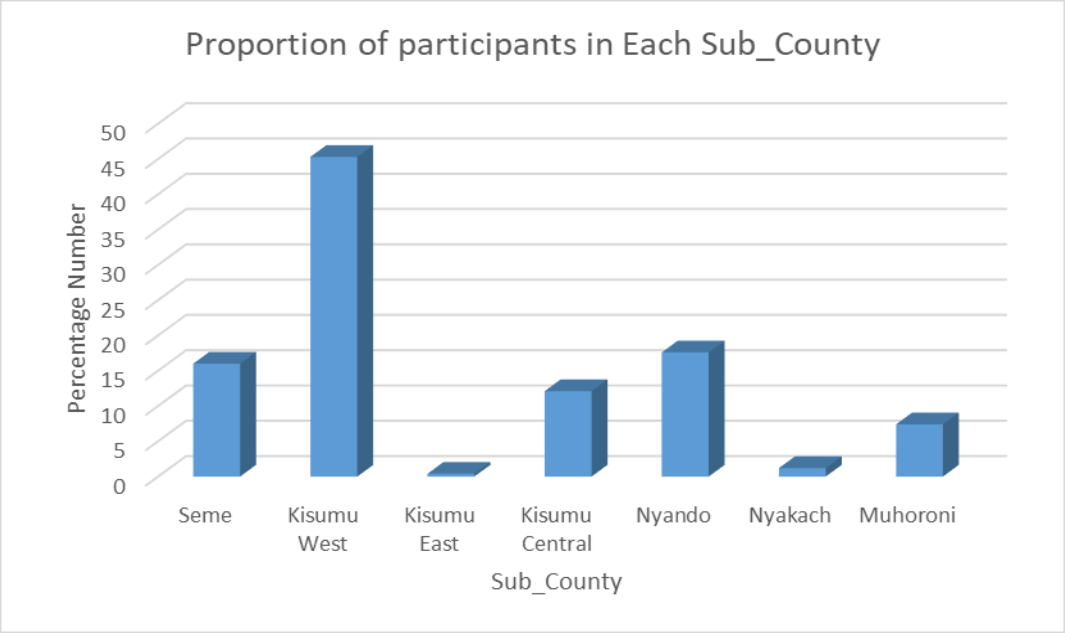

Figure 4.2. Distribution of participants across Sub-Counties

4.1.2 Sub-County tuberculosis cases profile

Out of the sample size of 256, 145(56.6%) were confirmed *Mycobacterium tuberculosis* cases while 111(43.4%) were negative for *M. tuberculosis*. Among the confirmed cases, Kisumu West Sub-County had the highest cases 50(34.5%), while Kisumu East and Nyakach Sub-Counties had 1(0.7%) each **(Figure 4.3)**.

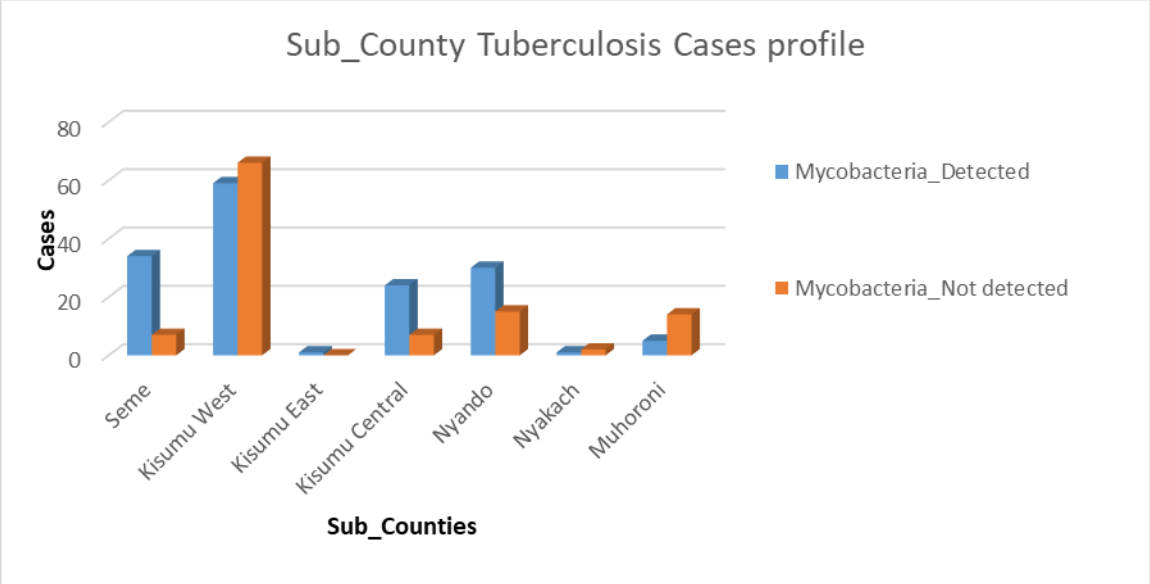

Figure 4.3. Sub-County tuberculosis cases profile

4.1.3 Characteristic of TB confirmed cases

Out of a total of 145 *Mycobacterium tuberculosis* confirmed cases on MGIT BACTEC from tuberculosis suspect cases, 32(22.1%) were from new TB cases and 113(77.9%) retreatment. Males were 112(77.2%) while females were 33(22.8%). Ages under 18 years were 2(1.4%), while 18 years and above 143(98.6%). Additionally, 75(51.7%) were positive for HIV while 46(31.7%) were negative. Non-response for HIV status outcome was 24(16.6%) **(Table 4.1)**.

Table 4.1. Table showing descriptors of TB Confirmed cases

Descriptors	**Categories**	**No.(%) n=145**
HIV Status	Negative	46(31.7)
	Positive	75(51.7)
TB Case	New cases	32(22.1)
	Retreatment	113(77.9)
Age	Under 18yrs	2(1.4)
	Above 18yrs	143(98.6)
Sex	Female	33(22.8)
	Males	112(77.2)

4.1.4 Sub-County drug resistance profile

From a sample of 145 tuberculosis confirmed cases on phenotypic culture, rifampicin resistance was highest in Seme Sub-County 4(2.8%) and isoniazid 3(2.1%); followed by Kisumu Central rifampicin 3(2.1%) and isoniazid 3(2. 1%), Nyando had rifampicin 2(1.4%) and isoniazid 2(1.4), Kisumu West showed rifampicin 1(0.7%) and isoniazid 3(2.0%). No resistance to both isoniazid and rifampicin were experienced in Muhoroni, Kisumu East and Nyakach Sub-Counties. Out of the 4(2.8%) phenotypic MDR cases, 3(2.1%) were from Seme, while 1 (0.7%) was from Kisumu Central Sub-County (**Figure 4.4)**.

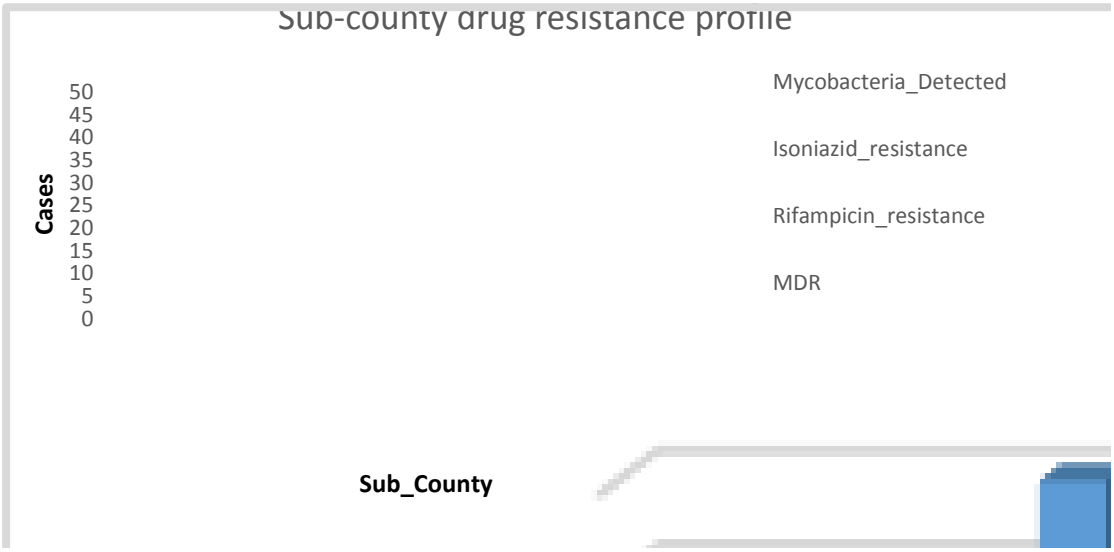

Figure 4.4. Sub-County drug resistance profile

4.2 Gene Mutations in *Mycobacterium tuberculosis* among HIV cases

4.2.1 Factors associated with HIV status outcome

Understanding the complex interplay between age, sex, TB case, with HIV status highlights the need for targeted interventions to address the specific risk factors and challenges faced by different subpopulations, ultimately contributing to more effective public health strategies for TB and HIV prevention and management.

A binary logistic regression was used to model the effect of age, sex, and tuberculosis case on the likelihood of HIV positive case. Tuberculosis case *(p=0.031)* was statistically significant. However, age *(p=0.971)* and sex *(p=0.178)* were not statistically significant. Retreatment cases for TB had higher odds for HIV positivity compared to new cases **(Table 4.2)**.

Table 4.2. Binary logistic regression table of predictors of HIV

	n=75	Sig.	OR	95% C.I.	
Frequency of HIV Positive Cases (%)				Lower	Upper
Sex	Male 53 (71)	0.178	1.918	0.814	1.035
	Female 22 (29)		1.00		
TB Case	New TB case 10 (13)		1.00		
	Retreatment 65 (87)	0.031*	1.250**	1.015	1.540
Age	Below 18yrs 2(2)		1.00		
	18yrs & Above 73(98)	0.971	1.001	0.968	1.034

*$p \leq 0.05$: Statistically significant relationship
**Odds Ratio> 1: Odds are increased for an outcome

4.2.2 Mutant and Wild Type gene probes among HIV cases

The study showed that the mutant probes among the 75(51.7%) HIV positive cases were *inhA MUT1* 1(1.3%), *katG MUT1* 4(5.3%), *rpoB MUT2A* 3(4.0%), *rpoB MUT3* 1(1.3%), and *rpoB MUT3/katG MUT1* 1(1.3%). Among the 46(31.7%) HIV-negative cases, there were 3 mutant probes: *inhA MUT1* 1(2.2%), *katG MUT1* 1(2.2%), and *rpoB MUT2A* 1 (2.2%). In HIV positive cases, wild type gene deletion was found in the probes *katG WT* 3(4.0%), *rpoB WT7* and *katG WT* 1(1.3%). Among the HIV-negative cases, *inhA WT1* 1 (2.2%), *inhA WT1/inhAWT2 1*(2.2%), and *katG WT 1*(2.2%) had wild-type gene deletions **(Figure 4.5)**.

The higher number of unknown mutations and wild-type gene deletions in HIV-positive cases could suggest that HIV infection is associated with increased genomic instability. The virus might induce or exacerbate genetic mutations and deletions, potentially due to its interaction with host cellular machinery or the immune response.

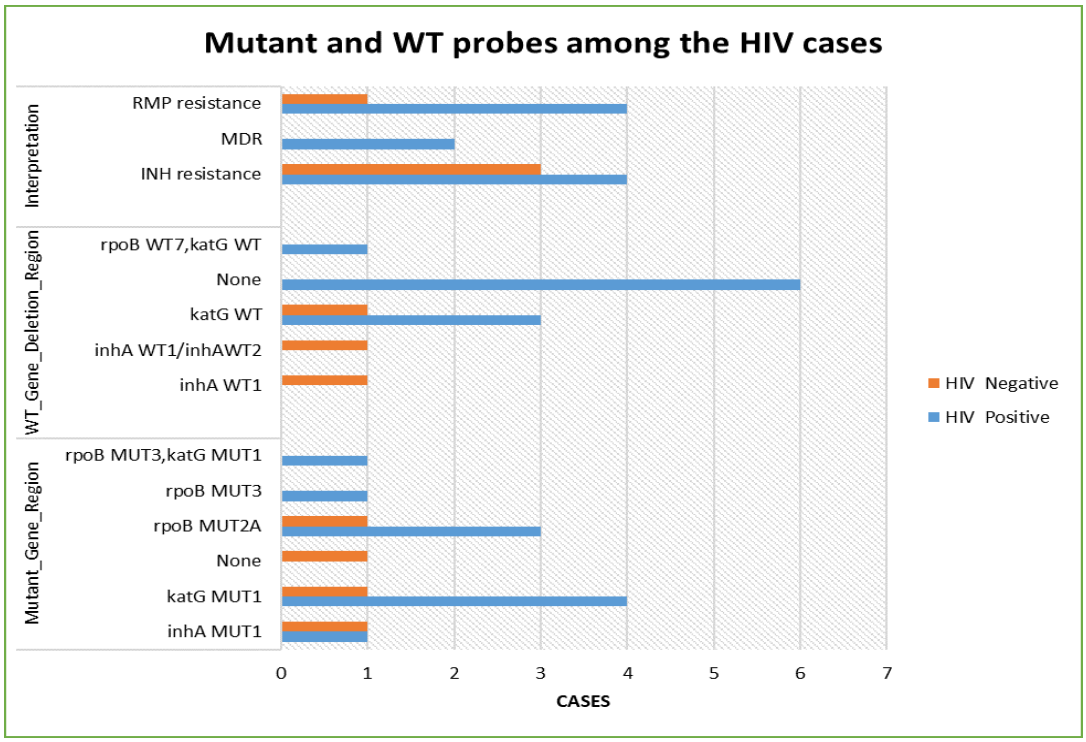

Figure 4.5. Mutant and Wild Type gene probes among HIV Cases

4.2.3 Amino acid change among HIV status outcome

The HIV positive cases had amino acid alterations in the genes *inhA* resulting to amino acid change of *C15T* 1 (1.3%), *katG* resulting to amino acid changes of *H526R, S315T1* 1 (1.3%), *rpoB* resulting to amino acid change of *H526Y* 3 (4.0%), *S315T1* 3 (4.0%), *S531L* 1(1.3%), and *S531L, S315T1* 1 (1.3%). Genes *inhA* resulting to amino acid change at *C15T* 2 (5.6%), *rpoB* gene resulting to change of *H526Y* 1(2.8%), and *S315T1* 1 (2.8%) were the HIV-negative cases. For molecular drug resistance testing, there were 7 (5.8%), INH resistance cases of which 4 (5.3%) came from HIV positive cases and 3 (8.3%) from HIV negative cases. In addition, 4 of the 5 (4.1%) cases of rifampicin resistance came from HIV positive people, while 1 came from HIV negative cases. HIV-positive participants contributed the two (2.7%) MDR cases **(Figure 4.6)**.

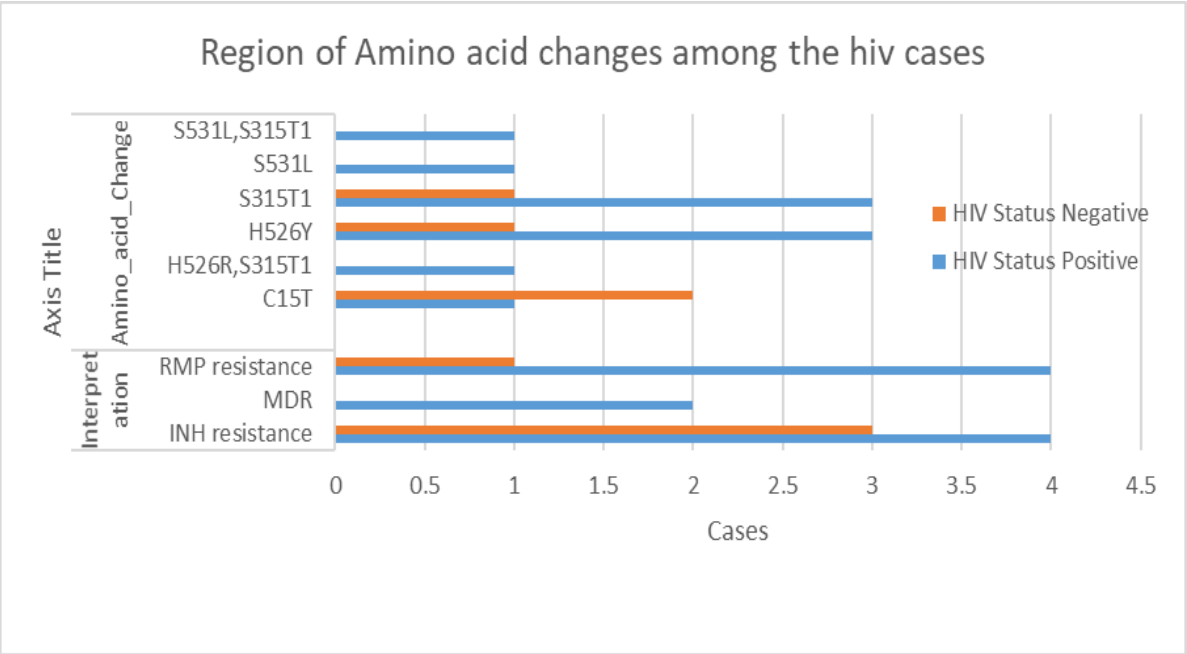

Figure 4.6. Region of amino acid change among the HIV cases

4.3 Gene Mutations in Mycobacterium tuberculosis against First-Line TB Treatment

4.3.1 Factors associated with isoniazid resistance

A binary logistic regression was carried out to assess the effect of age and sex on the likelihood of developing resistance to isoniazid. Sex *(p=0.040)* was statistically significant and Males had higher odds for isoniazid resistance compared to females *(OR=1.25).* However, Age *(p=0.453)* was not statistically significant (**Table 4.3)**.

Table 4.3. Binary logistic regression table of predictors of isoniazid drug resistance

		n=11	Sig.	OR	95% C.I.	
		Frequency of INH Resistance (%)			Lower	Upper
Age	Below 18 yrs.	1(9)		1.00		
	18yrs and Above	10(91)	0.453	1.086	0.876	1.347
Sex	Male	6(54.5)	0.04*	1.250**	1.015	1.539
	Female	4(45.5)		1.00		

*$p \leq 0.05$: Statistically significant relationship
**Odds Ratio> 1: Odds are increased for an outcome

4.3.2 Factors associated with rifampicin resistance

A binary logistic regression was carried out to model the effect of age and sex on the likelihood of developing resistance to rifampicin. Age *(p=0.229),* was not statistically significant and the odds of males developing resistance was 1.37 compared to females. Sex *(p=0.011)* was statistically significant **(Table 4.4)**.

Table 4.4. Binary logistic regression table for predictors of rifampicin resistance

		n=10	Sig.	OR	95% C.I	
		Frequency of RIF Resistance (%)			Lower	Upper
Age	Below 18yrs	1(10)		1.00		
	18yrs and Above	9(90)	0.229	1.150	0.916	1.444
Sex	Males	6(60)	0.011*	1.367**	1.097	1.703
	Females	4(40)		1.00		

*$p \leq 0.05$: Statistically significant relationship
**Odds Ratio> 1: Odds are increased for an outcome

4.3.3 Factors associated with multi-drug resistance

A binary logistic regression was carried out to model the effect of age and sex on the likelihood of developing multi drug resistance. Age *(p=0.537)* was not statistically significant while sex *(p=0.049)* was statistically significant. The odds of males developing multi drug resistance was 1.35 compared to females **(Table 4.5)**.

Table 4.5. Binary logistic regression table for predictors of MDR

		n=4	Sig.	OR	95% C.I.	
		Frequency of MDR (%)			Lower	Upper
Sex	Male	3(75)	0.049*	1.350**	1.015	1.540
	Female	1(25)		1.00		
Age	18yrs and below	0(0)		1.00		
	18 yrs. and Above	4 (100)	0.537	0.940	0.771	1.145

*$p \leq 0.05$: Statistically significant relationship
**Odds Ratio> 1: Odds are increased for an outcome

4.3.4 Nucleotide changes detected by MUT probes among tuberculosis cases

Highest rifampicin resistance was experienced in genes *rpoB*, which had mutant probes *rpoB MUT2A* in the codons *526 to 529*. This mutation resulted into the change of amino acid *H526Y*, changing the nucleotide from *CAC> TAC*. Additional rifampicin resistance was associated with probe *rpoB MUT3* in the codons *530 to 533*, this resulted into the change of amino acid *S531L*, changing the nucleotide from *TCG>TTG*. Multidrug resistance showed mutations in the genes *rpoB* and *katG*. Gene *rpoB* mutations was detected by mutant probes *rpoB MUT3, katG MUT1* codons *530 to 533*. These mutations resulted into amino acid changes *S531L, S315T1* resulting in specific nucleotide changes *TCG>TTG, AGC>ACC* and *CAC> TAC, AGC>ACC*. *katG* gene was detected by mutant probe *katG MUT1*, in codons *526 to 529,315* resulting into changes in amino acid *H526R, S315T1* and nucleotide changes from *CAC> TAC,AGC>ACC*. High level isoniazid resistance was expressed through mutation in the gene *inhA*, which was detected by the mutant probe *inhA MUT1*, in codon *-15*, resulting to amino acid change *C15T*. Low level Isoniazid resistance was shown by gene *katG*, which was detected by mutant probe *katG MUT1*, codon *315*, resulting to change in amino acid *S315T1* and nucleotide change from *AGC>ACC* in the amino acid *S315T1* **(Table 4.6)**. No drug resistance conferring mutations in genes (*emb*ABC, *pnc*A, *rrs* were detected against other first line drugs: ethambutol, pyrazinamide and streptomycin.

Table 4.6. Specific nucleotide changes detected by MUT probes

	Mutant gene	*Mutant Probe*	*n=14*	*codons*	*Amino_acid_ Change*	*Nucleotide Change*
RMP resistance	*rpoB*	*rpoB MUT2A*	4	*526 to 529*	*H526Y*	*CAC> TAC***
	rpoB	*rpoB MUT3*	1	*530 to 533*	*S531L*	*TCG>TTG*
MDR	*rpoB*	*rpoB MUT3,katG MUT1*	1	*530 to 533*	*S531L,S315T1*	*TCG>TTG, AGC>ACC****
	katG	*katG MUT1*	1	*526 to 529,315*	*H526R,S315T1*	*CAC> TAC,AGC>ACC****
INH resistance	*inhA*	*inhA MUT1*	3	*-15*	*C15T*	
	katG	*katG MUT1*	4	*315*	*S315T1*	*AGC>ACC**

Two MDR***, 4 rifampicin resistant** and 4 high isoniazid resistance* had the showed the same mutations resulting to same nucleotide changes.

4.3.5 Mutation pattern by TB cases

Out of a total of 256 samples, 145 had tuberculosis confirmation, with 32 (22.1%) coming from new cases and 113 (77.9%) from retreatments. Molecular Isoniazid resistance totaled 7(4.8%), of which 2 (6.3%) were new cases and 5 (4.4%) were retreatment. There were 2 (1.4%) Molecular MDR instances, all of which were from retreatment cases. There were 5 (3.4%) rifampicin-resistant patients, 1 (3.1%) new case, and 4 (3.5%) retreatment cases.

The study showed that gene *inhA* had amino acid changes *C15T* 1(3.1%), *rpoB* gene at *H526Y* 1 (3.1%), and *katG* at *S315T* 1 (3.1%) were the amino acids that changed among the New Tb Cases. While retreatment showed changes in genes *inhA* at *C15T* 1 (0.9%), *katG* at *H526R/S315T1* 1 (0.9%), *rpo*B gene at *H526Y* 3 (2.7%), *katG* gene at *S315T1* 3 (2.7%), and *rpoB* genes at *S531L* 1 (0.9%), and *S531L/S315T1* 1 (0. 9%) **(Figure 4.7)**.

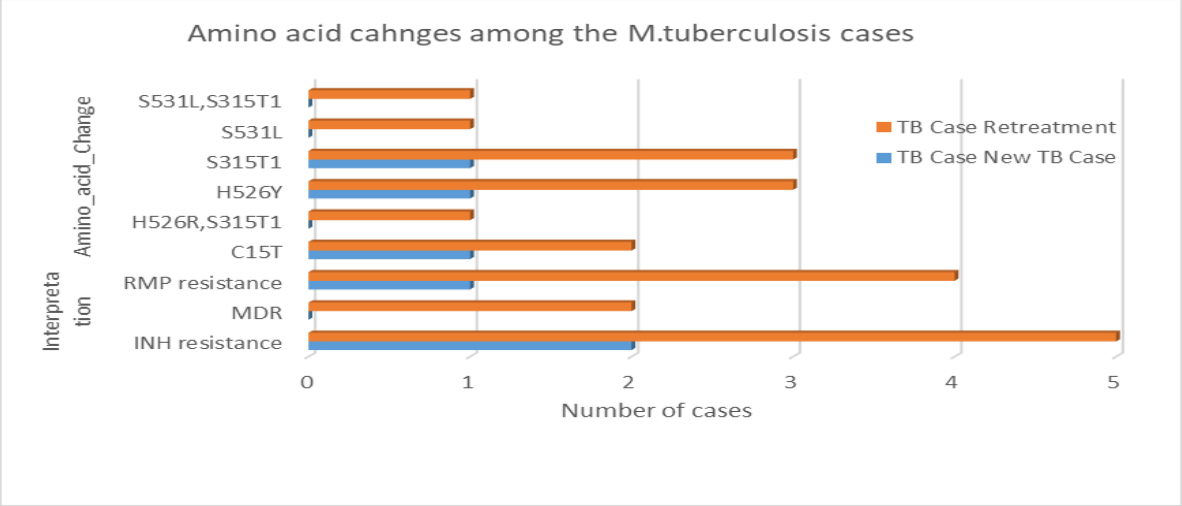

Figure 4.7. Amino acid Mutation pattern by TB cases

4.3.6 Mutant and wild type probes by TB cases

In retreatment cases, mutant probes were *inhA MUT1* 1 (0.9%), *katG MUT1* 4 (3.5%), *rpoB MUT2A* 3 (2.7%), *rpoB MUT3* 1(0.9%), and *rpoB MUT3*/*katG MUT1* 1 (0.9%). Mutant probes in new TB cases were *inhA MUT1* 1 (3.1%), *katG MUT1* 1 (3.1%), and *rpoB InhA WT1* 1 (3.1%) and *katG WT* 1 (3.1%) were the wild type gene probes used in the new TB cases, while *inhA WT1/inhAWT2* 1 (0.9%), *katG WT* 3 (2.7%), and *rpoB WT7*/*katG WT* 1 (0.9%) were used in the retreatment cases **(Figure 4.8)**.

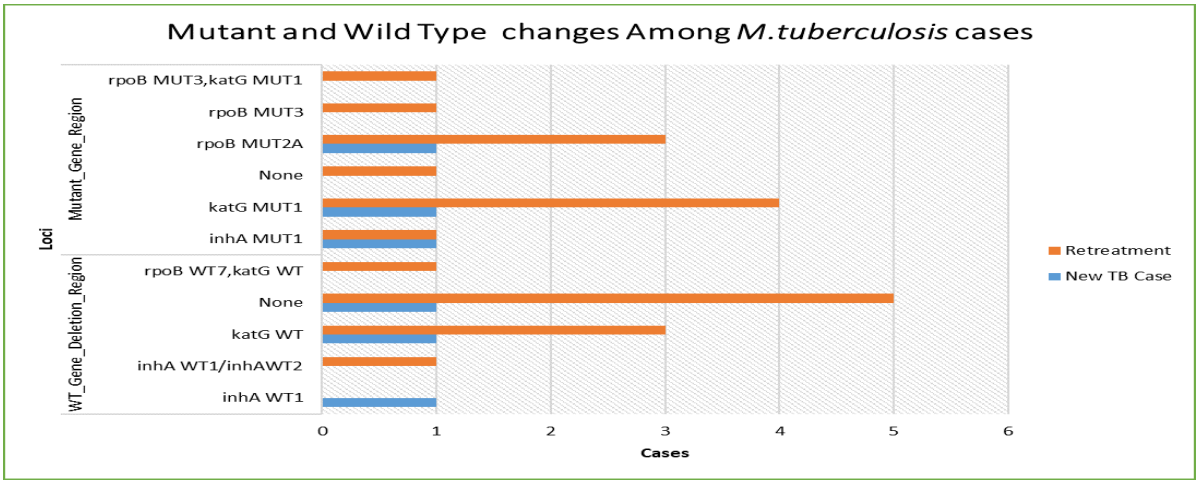

Figure 4.8. Mutant and wild type probes by TB cases

4.4 Gene Mutations in *Mycobacterium tuberculosis* against Second-Line TB treatment

The study found no evidence of gene mutations in *Mycobacterium tuberculosis* that are typically associated with resistance to second-line TB treatment among the 145 tuberculosis confirmed samples. The samples analyzed did not exhibit any of the known mutations in genes such as *gyrA*, *gyrB*, *tyl*A, *rrs*, or *eis*, which are commonly associated with fluoroquinolone and aminoglycoside resistance **(Table 4.7)**.

Table 4.7. Mutant genes against second line anti-tuberculosis agents

Second line Anti-TB agent	*Gene targeted*	*Mutant gene*
Fluoroquinolones Levofloxacin Ciprofloxacin Ofloxacin Moxifloxacin	*gyr*A,*gyr*B	*none*
Aminoglycosides Kanamycin, Amikacin Capreomycin	*eis,rrs, tyl*A	*none*

4.5 Validation of Line Probe assay in detection of drug resistance conferring mutations

4.5.1 Molecular line probe assay for isoniazid resistance using Phenotypic isoniazid as gold standard

Out of a sample size of 256, MGIT BACTEC showed 145 (56.6%) Mycobacteria detected while 111 (43.4%) showed mycobacteria not detected. From sample of 145 Mycobacteria Tuberculosis Complex, DST isoniazid resistance was 11 (7.6%) isolates, while no resistance detected was 134 (92.4%). LPA isoniazid resistance detected was 9 (6.2%) while No resistance detected was 136 (93. 8%). The sensitivity of line prove assay for detection of isoniazid resistance was 81.8% while the specificity was 100%. The positive predicative

value of LPA for isoniazid resistance was 100% while the Negative predictive value was 98.5% **(Table 4.8)**.

4.5.2 Molecular line probe assay for rifampicin resistance using phenotypic rifampicin as gold standard

For culture DST, rifampicin resistant was 10 (6.9%), while resistance not detected was 135 (93.1%), out 145 that showed mycobacteria detected on MGIT BACTEC. LPA rifampicin resistance was 7 (4.8%). The Sensitivity for LPA in detection of rifampicin resistance was 70% while specificity was 100%. The positive predicative value of LPA for rifampicin resistance was 100% while the Negative predictive value was 97.8% **(Table 4.8)**.

4.5.3 Molecular line probe assay MDR using Phenotypic MDR as gold standard

For culture DST, Multidrug resistance(MDR) detected was 4 (2.8%), while MDR not detected was 141 (97. 2%), out 145 that showed mycobacteria detected on MGIT BACTEC. LPA MDR was 2 (1.4%). The Sensitivity for LPA in detection of MDR was 98.2% while specificity was 100%. The positive predicative value for MDR detection was 100% while the Negative predictive value was 99.4% **(Table 4.8)**.

4.5.4 Diagnostic performance of GenoType MTBDR*plus* assay in detecting resistance

Using BACTEC as the gold standard, Chi-square for LPA and DST for isoniazid resistance detection, ($\chi 2 = 116.89$, $df=1$, $p=0.001$), rifampicin resistance detection, ($\chi 2 = 99.29$, $df=1$, $p=0.001$), and MDR ($\chi 2 = 71.49$, $df=1$, $p=0.001$) 95% CI. Geno Type MTBDR*plus* assay and MGIT BACTEC were significantly correlated for detection of RIF, INH and MDR tuberculosis at $p<0.001$ **(Table 4.8)**.

Table 4.8. Diagnostic performance of GenoType MTBDR*plus* assay in detecting resistance in clinical isolates of *Mycobacterium tuberculosis*

		RIF n=145	INH n=145	MDR n=145
Test Assay MTBDR*plus*	*Resistant*	7	9	2
	Resistant Not Detected	138	136	133
	Sensitivity(95%CI)	70	81.80	98.20
	Specificity(95%C)	100	100	100
	PPV (95% CI)	100	100	100
	NPV(95%CI)	97.80	98.50	99.40
	Pearson Chi-Square(χ2)	99.29	116.89	71.49
	p-value (95%CI)	*0.001**	*0.001**	*0.001**

*$p \leq 0.05$: Statistically significant relationship

CHAPTER FIVE
DISCUSSION

A total of 256 sputum samples obtained from clinically suspected TB cases in Kisumu County, Kenya during the 12-month period from November 2020 to October 2021 were included in the study. Out of the sample size, 145 (56.6%) were confirmed *Mycobacterium tuberculosis* cases while 111(43.4%) were negative for *M. tuberculosis*. Among the confirmed cases, Kisumu West Sub-County had the highest cases 50 (34.5%), while Kisumu East and Nyakach Sub-Counties had 1(0.7%) each.

From 145 confirmed cases, there were more males 112 (77.2%) compared to females 33 (22.8%), which is in agreement with the WHO report that relatively more males than females are exposed to TB which may be attributed to the difference between the two sex groups in biological, societal role and access to health facilities (WHO, 2020a, 2022).

Among TB confirmed cases, majority of participants were aged 18 years and above 143(98.6%), while the remaining 2(1.4%), were under 18 years old. In a study that was conducted in India which is one of the high burden TB countries, it was found that 17.2% of samples were from new cases, and 82.8% were from previously treated samples (Ahmed *et al.*, 2018). These findings are consistent with the current study that found out that majority of TB cases were retreatment 113(77.9%) while new TB cases were 32(22.1%). Additionally, the study showed that males were more likely to develop isoniazid (OR=1.25) and rifampicin resistance(OR=1.37) compared to females.

5.1 Gene Mutations in *Mycobacterium tuberculosis* among HIV cases

According to a study that was conducted among drug naïve patients in Nairobi Kenya, HIV increased the incidence of TB and the risk of TB infection by 16 to 27 times in PLHIV than in the general population (Ogari *et al.*, 2019). Singh *et al.*(2020) also estimated that PLHIV,

especially with lower CD4 count, showed increased risk of developing active TB compared with those who are HIV negative. Cases of RIF, INH and MDR among the HIV positive instances were higher for both phenotypic and molecular assays compared to HIV negative cases and all the molecular MDR cases were from HIV positive clients which might be an indicator of poor treatment outcomes. These findings are consistent with findings that showed that HIV infection modifies the fitness of drug-resistant strains (Eldholm *et al.*, 2016; Khan *et al.*, 2019; Ssengooba *et al.*, 2017). These observations could be attributed to with increased genomic instability among the HIV positive. The virus might be inducing genetic mutations and deletions. In other studies conducted in regions of HIV prevalence, there was a growing evidence to suggest that infection with more than one strain occurred (Cohen *et al.*, 2016; Hanekom *et al.*, 2013).

Greater variability and high frequency of unidentified mutations and wild-type gene deletions that was observed in HIV-positive individuals may indicate that the virus also causes more genomic instability. Due to the virus's interaction with the immune system or the host's cellular machinery, genetic mutations and deletions may be brought on by the virus or made worse by it. Similar unfavourable outcomes were found in a systematic review in sub Saharan Africa among HIV-infected versus HIV uninfected tuberculosis patients (Edessa *et al.*, 2020).

5.2 Gene Mutations in *M. tuberculosis* associated with First-Line Anti-TB Treatment

The study showed that phenotypic and genotypic drug resistance for INH and RIF was higher among the retreatment cases compared to the new cases, and all the 4 phenotypic MDR were in retreatment cases and molecular MDR were 2(1.4%). This finding was consistent with a study conducted in Pakistan one of the high burden countries, which indicated that the retreated cases have elevated rates of infection with MDR strain and they can acquire resistance to RIF or INH during the treatment course (Saba *et al.*, 2020). Low MDR resistance in Kisumu County was consistent with other studies in high burden countries

which encountered the same low levels (1.8%) for MDR and Ethiopia with 1.1% (Seyoum *et al.*, 2014) as well as India recording the same percentage (Ombura *et al.*, 2016).

The study showed that 2 molecular MDR, 4 RR and 4 low isoniazid resistance showed the same nucleotide and amino acid changes. High INH resistant strains had mutations in the promoter region of *inhA* gene at codon *-15* with amino acid change of *S315T1*, a similar high prevalence (85%) of *S531L* mutation in rifampicin resistant isolates was reported in a study at Cameroon (Abanda *et al.*, 2017), while low INH resistant strains had mutations in the *katG* gene at codon *315.* The *rpoB* gene displayed mutations at codons *530 to 533* with amino acid changes *of S531L and S315T1*, while *katG* had mutations at codon *526 to 529* and *315* with amino acid changes of *H526R* and *S315T1*. These alterations have been described and are in concordance with previous published studies for clinical isolates from other parts of the world, which reflect a global pattern (Eddabra &Mounsef, 2020).

The incidence of a high occurrence of *S531L* mutation globally might be due to low fitness cost which influences its strong selection transmissibility (Gagneux, 2009). In a different systematic review conducted in Morocco, it was found that mutations in codons *516* (8.26%), *526* (8.05%), and *531*(70.33%) are the most associated mutations with rifampicin resistance (Eddabra &Mounsef, 2020).

In the current study, isoniazid resistance shown by high mutations in the *inhA* promoter showing low level resistance and in the *katG* showing high level isoniazid resistance. A number of publications suggest that resistance of *Mycobacterium tuberculosis* to INH had mutation at codon *315* (Fantahun *et al.*, 2013). Highest proportion of rifampicin resistance was experienced in probes *rpoB MUT2A* which had mutations in the codons *526 to 529*. This mutation resulted into the change of amino acid *H526Y*, changing the nucleotide from *CAC> TAC*. Additional rifampicin resistance was associated with gene *rpoB* detected by probes

rpoB MUT3 which had mutations in the codons *530 to 533*, this resulted into the change of amino acid *S531L*, changing the nucleotide from *TCG>TTG*.

Multidrug resistance showed mutations in the genes *rpoB,* which was detected by probes *rpoB MUT3, katG MUT1*, in codons *530* to *533,526* to *529* and codon *315*. A significant number of reports imply that resistance of *M. tuberculosis* to INH show mutation at codon *315* (Abhijeet *et al.*, 2020). In the present study, the RIF resistant mutants had the modification at the *S531L*; which is the most often documented resistance mutation in numerous countries (Ogari *et al.*, 2019).

Studies show that *katG* is the most common region targeted with a bulk of mutations occurring in codon *315* in 30–90% of INH resistant strains, in the current study, low level isoniazid resistance was exhibited in the gene loci *inhA MUT1* and high level Isoniazid resistance was shown in loci *katG MUT1*. There was no nucleotide change for amino acid *C15T* whereas there was nucleotide change from AGC>ACC in the amino acid *S315T1*.

The study showed that 2 MDR, 4 rifampicin resistant and 4 low isoniazid resistance had the same nucleotide and Amino acid changes, this might be an indication of local drug resistance transmission. Greater variability observed in amino acid changes in retreatment cases compared to new cases might be a suggestion that such mutations might be acquired during treatment courses by repeated administration of the same anti-TB drugs.

This study found no drug resistance conferring mutations against other first line agents, (pyrazinamide, Ethambutol, streptomycin). This is consistent with the study findings in South Western Uganda that showed low or no resistance against pyrazinamide, Ethambutol, and streptomycin in treatment naïve patients with pulmonary TB (Orikiriza *et al.*, 2015).

5.3 Gene Mutations in *M. tuberculosis* associated with Second-Line Anti-TB Treatment

No drug resistance conferring mutations were detected against second line anti-tuberculosis a gents' fluoroquinolones and aminoglycosides. Similar finding were reported in a study done among drug naïve patients in Nairobi Kenya that, no resistance to fluoroquinolones (FQ) or kanamycin (KAN) was observed (Ogari *et al.*, 2019). This study's detailed findings revealed an absence of known gene mutations in *Mycobacterium tuberculosis* associated with resistance to second-line TB treatment in the studied MDR-TB patients. The results highlight the complexity of drug resistance in TB and emphasize the need for further research to explore alternative resistance mechanisms and improve diagnostic strategies to optimize treatment outcomes for patients with MDR-TB.

5.4 Diagnostic performance of LPA in *M. tuberculosis* drug resistance testing

Findings from WHO shows that detection of multidrug resistance and or rifampicin resistance requires bacteriological confirmation of *M. tuberculosis* and testing for drug resistance using Nucleic acid amplification technologies, and culture methods (Sharma *et al.*, 2014; WHO, 2015).

The current study reports the findings of the accuracy of the Genotype MTBDR*plus* assay for detection of drug resistance in new and previously treated PTB patients in Kisumu County. A number of studies show that GenoType MTBDR*plus* V2.0 shows good accuracy for the detection of MDR isolates in smear-positive specimens (sensitivity between 83.3 and 96.4%, and specificity between 98.6 and 100%) (Gupta *et al.*, 2015; Smith *et al.*, 2008). This is consistent with the current study which showed a statistically significant relationship between molecular line probe assay and phenotypic MGIT culture for detection of isoniazid resistance<0.05.

The study found astrong evidence of of relationship between moleculat Line probe assay and Phenotypic MGIT culture for detection of rifampicin resistance $p<0.05$; sensitivity 70%;

specificity100%; positive predicative 100% and Negative predictive value was 97.8. The high specificity of the MTBDR*plus* assay in detecting INH resistance, RIF resistance and MDR MTBC isolates is consistent with the specificity in a number of previous studies (Ahmed *et al.*, 2018; Bai *et al.*, 2016; WHO, 2016a).

In the current study, a number of RIF-resistant isolates failed to hybridize with one or two of the wild type (WT) probes and did not hybridize with any of the probes representing known mutations. These results could be an indication of a new previously unreported mutation. The WT probes with no hybridization were mostly *WT2, WT3, WT4* and *WT8*. This type of result could be likely linked to the failure of the mutant to hybridize with the mutation probe as a result of the presence of a rare or new mutation (Abanda *et al.*, 2017; Bai *et al.*, 2016). The MTBDR*plus* assay was not able to detect the INH resistance in the two isolates and RIF resistance from three isolates which were detected by MGIT 960 system. This could be due to mutations which have not been included in the strips or it could also be due to unidentified mutation.

5.5 Limitations of the study

There was limitations on the generalization of LPA results. Although LPA can detect the mutations that are most frequently identified in resistant strains, some mutations that confer resistance are outside the regions covered by the test and therefore resistance cannot be completely excluded (inferred) even in the presence of all WT probes. LPA is less efficient than conventional culture-based DST in finding resistance in samples harboring both drug-susceptible and -resistant bacteria (heteroresistance) (WHO, 2016b).

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY OF FINDINGS

1. The study found a higher number of unknown mutations and wild type gene deletion regions among the HIV positive group compared to the HIV negative cases.

2. Greater variability was observed in amino acid changes in genes *rpoB, katG* and *inhA* from retreatment cases compared to new cases and two molecular MDR cases detected from different facilities showed the same nucleotide change *AGC to ACC*.

3. No drug resistance conferring mutations were detected in genes *gyrA, gyrB, rrs* and *eis* against second line ant tuberculosis drugs.

4. The study demonstrated that molecular line probe assay was consistent with phenotypic drug resistance testing for detection of gene mutations associated with Isoniazid, rifampicin and multi-drug resistance

6.2 CONCLUSION

1. The higher number of unknown mutations and wild-type gene deletions in HIV-positive cases could suggest that HIV infection is associated with increased genomic instability. The virus might induce or exacerbate genetic mutations and deletions, potentially due to its interaction with host cellular machinery or the immune response.
2. Greater variability that was observed in amino acid changes in genes *rpoB, katG* and *inhA* from retreatment cases compared to new cases may be an indication that such mutations might be acquired during treatment courses by repeated administration of the same anti-TB drugs in Kisumu County.

3. The absence of known gene mutations in *Mycobacterium tuberculosis* associated with second-line TB treatment may indicate the presence of alternative mechanisms of drug resistance, beyond the well-known mutations in the tested genes, might be contributing to drug resistance in these MDR-TB patients.

4. The study further demonstrated that molecular line probe assay was consistent for detection of gene mutations associated with drug resistance *Mycobacterium tuberculosis.* The concordance with conventional phenotypic DST methods and rapid turnaround time make line probe assays useful tests for the diagnosis and management of tuberculosis drug resistance in HIV endemic regions like Kisumu County.

6.3 RECOMMENDATIONS

6.3.1 Recommendations from the current study

1. More data is needed to stratify gene mutations in *Mycobacterium tuberculosis* and HIV status in order to provide solid evidence for clinical decision making among tuberculosis patients co-infected with HIV.

2. Continuous sentinel surveillance systems on tuberculosis drug resistance should be established in Kisumu County to routinely monitor the development of drug resistance conferring gene mutations against first line anti-tuberculosis agents.

3. Second line drug resistance monitoring should be integrated by the Ministry of Health as a routine diagnostic algorithm and not in response to cases of failures to first line and this will help in earlier identifications of XDR *Mycobacterium tuberculosis* strains.

4. Molecular line probe assay should be adopted as a routine diagnostic assay for monitoring and detection of *Mycobacterium tuberculosis* drug resistance in Kisumu County in the context of improving policies and systems in the County's path towards achieving and sustaining universal health coverage.

6.3.2 Recommendations for future studies

1. Further investigation into the mechanisms, clinical significance, and potential applications of genetic alterations could contribute to the understanding of HIV pathogenesis and inform novel therapeutic approaches.
2. To help reduce TB drug resistance to the levels targeted in the WHO's End TB strategy, development of new drugs, drug regimens to treat TB disease and the discovery of long-acting safe drug formulations (including for treating latent infections of MDR-TB and XDR-TB), is urgent.
3. This study's unexpected finding of no gene mutations associated with resistance to second-line TB treatment highlights the complexity of drug resistance in *Mycobacterium tuberculosis*. Further research is warranted to explore alternative resistance mechanisms and to develop more accurate and comprehensive diagnostic tools to guide the management of MDR-TB patients effectively.
4. Attempts to detect new mutation genes circulating in Kisumu County via sequencing-based approaches may also crease the array of diagnostic technologies.

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APPENDICES

Appendix 1a: CASE REPORT FORM

Date form completed __ __ / __ __ / __ __ **Name of person completing form**_____

DD MM YY

Social Demographic Information:

Study site/Hospital/Health Centre/Dispensary/Clinic_____

Sub County:

☐ **Seme** ☐ **Kisumu West** ☐ **Kisumu Central** ☐ **Kisumu East**
☐ **Muhoroni** ☐ **Nyando** ☐ **Nyakach**

Age_____

Sex: ☐ **Male** ☐ **Female**

Patient Category

☐ **New TB case**
☐ **Retreatment (relapse or recurrence)**
☐ **Return after default**
☐ **Failure**
☐ **MDR TB Contact**

HIV Sero Status
☐ **Positive** ☐**Negative** ☐ **Not Known**

Appendix 1b: LABORATORY REQUEST FORM

Patient Unique ID number _____

A. Mycobacterial Smear microscopy At the KEMRI TB LABORATORY

Date specimen collected: __ __ / __ __ / __ __

DD MM YY

Specimen ID number (if applicable) _____

Quality of specimen: ☐ **Salivary** ☐ **Mucoid** ☐ **Tenacious** ☐ **Not applicable**

Result of AFB smear:

☐ **Negative** ☐ **Not done**

☐ **Scanty (record actual count) _____**

☐ **1+** ☐ **2+** ☐ **3+**

Result of identification:

☐ **Mycobacterium tuberculosis**

☐ **Mycobacterium other than tuberculosis (Specify)_____**

☐ **Not done**

BACTEC MGIT Culture

Result of tuberculosis testing:

☐ **MTBC identified**

☐ **Negative**

☐ **Contaminated**

☐ **Not done**

E. DST First line

Streptomycin

☐ **Sensitive** ☐ **Resistant**

Rifampicin

☐ **Sensitive** ☐ **Resistant**

Ethambutol

☐ **Sensitive** ☐ **Resistant**

Isoniazid

☐ **Sensitive** ☐ **Resistant**

Pyrazinamide

☐ **Sensitive** ☐ **Resistant**

F. DST Second Line

a. Fluoroquinolone

Levofloxacin

☐ **Sensitive** ☐ **Resistant**

Ofloxacin

☐ **Sensitive** ☐ **Resistant**

Ciprofloxacin

☐ **Sensitive** ☐ **Resistant**

Moxifloxacin

☐ **Sensitive** ☐ **Resistant**

b. Aminoglycosides

Kanamycin
☐ **Sensitive** ☐ **Resistant**

Amikacin
☐ **Sensitive** ☐**Resistant**

Capreomycin
☐ **Sensitive** ☐ **Resistant**

Viomycin
☐ **Sensitive** ☐**Resistant**

G. Line Probe Assay

Result:

1)FL-LPA (GenoType MTBDR*plus)*

☐ Wild type

☐ Mutation in *rpoB*
☐ Mutation in *inhA*
☐ Mutation in *katG*
☐ Other mutation
(Specify)_____

2) SL-LPA (GenoType MTBDR*sl)*

a) Fluoroquinolones

☐ Mutation in *gyrA*
☐ Mutation in *gyyB*

b) Aminoglycosides

☐ Mutation in *rrs*
☐ Mutation in *eis*
☐ Other mutation
(Specify)_____

Appendix 2: Specific nucleotide changes detected by MUT probes

	MUT probe	AA change	Nucleotide change
rpoB MUT probes	MUT1	D516V (D435V)	gac>gtc
	MUT2A	H526Y (H445Y)	cac> tac
	MUT2B	H526D (H445D)	cac> gac
	MUT3	S531L (S450L)	tcg>ttg
	MUT probe	**AA change**	**Nucleotide change**
katG MUT probes	MUT1	S315T	agc>acc
	MUT2	S315T	agc>aca
	MUT probe	**AA change**	**Nucleotide change**
gyrA MUT probes	MUT1	A90V	gcg>gtg
	MUT2	S91P	tcg>ccg
	MUT3A	D94A	gac>gcc
	MUT3B	D94N	gac>aac
	MUT3B	D94Y	gac>tac
	MUT3C	D94G	gac>ggc
	MUT3D	D94H	gac>cac
	MUT probe	**AA change**	**Nucleotide change**
gyrB MUT probes	MUT1	N538D (N499D)	aac>gac
	MUT2	E540V (E501V)	gaa>gta

Appendix 3: Random sample generator Table

1 Set of **256 Unique** Numbers
Range: From **1** to **750**– **Sorted from Least to Greatest**

Set #1

3, 7, 8, 11, 13, 14, 17, 18, 19, 25, 26, 28, 31, 35, 36, 39, 43, 50, 51, 54, 59, 60, 62, 63, 76, 83, 84, 85, 95, 101, 104, 109, 111, 114, 115, 117, 118, 124, 129, 138, 139, 147, 148, 149, 154, 158, 160, 164, 165, 168, 170, 171, 173, 175, 176, 178, 183, 191, 192, 200, 201, 205, 210, 211, 214, 221, 223, 225, 228, 229, 231, 233, 243, 244, 248, 250, 252, 254, 255, 258, 260, 264, 266, 267, 268, 269, 273, 274, 280, 282, 285, 288, 293, 296, 299, 300, 301, 304, 305, 306, 316, 319, 321, 322, 324, 328, 330, 342, 343, 351, 355, 360, 361, 369, 372, 373, 376, 378, 380, 381, 382, 383, 384, 392, 396, 400, 403, 412, 413, 415, 417, 418, 419, 422, 423, 424, 425, 429, 436, 440, 441, 443, 445, 453, 456, 457, 458, 460, 462, 464, 465, 466, 471, 472, 474, 476, 477, 478, 482, 484, 489, 491, 492, 494, 501, 504, 507, 514, 516, 518, 519, 524, 525, 527, 532, 535, 540, 541, 542, 544, 545, 548, 549, 553, 554, 558, 560, 562, 564, 565, 568, 570, 571, 572, 575, 580, 582, 585, 589, 591, 594, 597, 600, 601, 604, 607, 609, 617, 618, 620, 623, 624, 625, 628, 629, 632, 634, 636, 643, 645, 647, 654, 661, 663, 666, 667, 670, 672, 673, 675, 676, 679, 682, 684, 687, 689, 697, 701, 702, 709, 710, 712, 714, 718, 720, 721, 722, 724, 727, 730, 732, 734, 738, 740, 743, 748

Appendix 4: Informed consent/Assent

Research Purpose

The purpose of this study is to describe the profile of drug resistance conferring mutations in *Mycobacterium tuberculosis* among new and previously treated pulmonary tuberculosis cases in Kisumu County, Kenya

Introduction:

We are asking for your consent to participate in a research to determine the profile of drug resistance conferring mutations among new and previously treated pulmonary Tuberculosis cases from Kisumu County, Western Kenya.
The Kenya Medical Research Institute (KEMRI) and Maseno University are collaborating do this study which will help the National and County governments' bridge gaps in TB control programs. This study will provide information on TB drug resistance patterns in Kisumu County which is an essential aspect in Tuberculosis control and reduction of continued drug resistance.

Information on Research

This study will collect sputum samples from patients attending various TB clinics within Kisumu County. Additionally, clinicians will inquire about the patient's pervious exposure to anti TB drugs and any current regimen if any. Additionally, the study will collect social demographic characteristics as well as anti-tuberculosis drug resistance tests. You will be given the results of these tests as a routine procedure from your TB clinics. The results of this research will help the Kenyan Ministry of Health better understand TB diagnosis and diagnosis of drug resistance.

Procedures:

If you agree to participate in the study, you will be asked to provide at least two sputum specimens for TB testing after examining you by the clinician and he agrees that this is appropriate for you. The first sample will be tested at the health facility for the presence of TB bacilli and the other sample shipped to KEMRI-TB reference for further microscopy and Molecular drug resistance testing. With your permission, we will collect your social demographic, clinical and laboratory data tests.
We will store leftover sputum specimens that we collect from you in a freezer in the laboratory so that they may be used only for retesting not any other purposes. The specimen will therefore be destroyed immediately after retesting. However, you may decide to disallow your leftover specimen to be stored for future retesting.

Risks:
There will be below minimum risk to participate in this study. Sputum collection done for this study is normally used to test for TB and stored samples will not have your name on them.

Benefits:

You will benefit from this study by receiving your TB test result and drug resistance test results faster than the normal routine care. This will help in control and early detection Tb drug resistance. Additionally, you may benefit indirectly from the information we get from the study and this might also help other patients in the future.

Confidentiality:
Your name and address will be written down only on the front page of the clinical form to help link you with your lab results for treatment options. 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 you. Your name will never be used in any speech or paper written about this study. Your confidentiality will be protected to the extent permitted by law.

Voluntary participation:
If you do not want to be in this research study, you will still get normal care and treatment. Even If you agree to be in this research study, you can still 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**.**

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: Fredrick Ogumbo, Telephone: +245725849376 or The Secretary, KEMRI Ethics Review Unit, P.O. Box 54840-00200, Nairobi; Telephone numbers: 020-2722541, 0722205901, 0733400003; email address: erc@kemri.org

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 clinician 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 study participant *(print)*
_____ Signature/Thumbprint of participant_____ Date_____
_____ Name of the Witness *(print)* Signature of Witness_____Date_____

_____Nam
e of staff consenting *(print)*
Signature of staff consenting:
_____Date_____

Storage of specimens for later Retesting.

_____I do give permission for specimens to be stored in the laboratory for later retesting on Tuberculosis drug resistance. I understand that I will not be asked later to consent again for this type of testing.

_____ I do not give permission for specimens to be stored for future TB-related testing.

Appendix 5 : Fomu ya idhini

Kusudi la Utafiti

Madhumuni ya utafiti huu ni kuamua upinzani wa dawa unaosababisha mabadiliko ya kifua kikuu cha Mycobacterium kutoka kwa wagonjwa wa kifua kikuu katika Kaunti ya Kisumu, Magharibi mwa Kenya.

Utangulizi:

Tunaomba idhini yako ya kushiriki katika utafiti ili kuamua upinzani wa dawa unaosababisha mabadiliko ya kifua kikuu kutoka kwa wagonjwa wa kifua kikuu katika Kaunti ya Kisumu, Magharibi mwa Kenya. Taasisi ya Utafiti wa Matibabu ya Kenya (KEMRI) na Chuo Kikuu cha Maseno vinashirikiana kufanya utafiti huu ambao utasaidia serikali za Kitaifa na Kaunti kuziba mapungufu katika mipango ya kudhibiti Kifua Kikuu. Utafiti huu utatoa habari juu ya mifumo ya kupinga dawa za Kifua Kikuu katika Kaunti ya Kisumu ambayo ni jambo muhimu katika Udhibiti wa Kifua kikuu na kupunguza upinzani unaoendelea wa dawa.

Habari juu ya Utafiti

Utafiti huu utakusanya sampuli za sputum kutoka kwa wagonjwa wanaohudhuria kliniki anuwai za TB ndani ya Kaunti ya Kisumu. Kwa kuongezea, waganga watauliza juu ya udhihirishaji wa mgonjwa kwa dawa za anti za TB na aina yoyote ya sasa ikiwa ipo. Kwa kuongezea, utafiti huo utakusanya sifa za kijamii za watu pamoja na vipimo vya kupinga dawa ya ugonjwa wa kifua kikuu. Utapewa matokeo ya vipimo hivi kama utaratibu wa kawaida kutoka kliniki zako za Kifua kikuu. Matokeo ya utafiti huu yatasaidia Wizara ya Afya ya Kenya kuelewa vizuri utambuzi wa TB na utambuzi wa upinzani wa dawa.

Taratibu:

Ikiwa unakubali kushiriki katika utafiti, utaulizwa kutoa mifano ya sputum angalau mbili za upimaji wa Kifua Kikuu baada ya kukuchunguza na kliniki na anakubali kwamba hii inafaa kwako. Sampuli ya kwanza itapimwa katika kituo cha afya kwa uwepo wa bacilli ya kifua kikuu na mfano mwingine umepelekwa kwa kumbukumbu ya KEMRI-TB kwa uchunguzi zaidi wa upimaji wa dawa na Masi. Kwa idhini yako, tutakusanya vipimo vya data ya idadi ya watu, kliniki na maabara.
Tutahifadhi vielelezo vya Kikohozi vilivyobaki ambavyo tunakusanya kutoka kwako katika freezer kwenye maabara ili waweze kutumiwa tu kwa kutuliza tena madhumuni mengine yoyote. Walakini unaweza kuamua kuruhusu mfano wako wa iliyobaki uhifadhiwe kwa ujaribu tena au unaweza kutokubali.

Hatari:
Kutakuwa na hatari ya chini kwa kushiriki katika utafiti huu. Mkusanyiko wa sputum uliofanywa kwa utafiti huu kawaida hutumiwa kupima TB na sampuli zilizohifadhiwa hazitakuwa na jina lako juu yao.

Faida:
Utafaidika na utafiti huu kwa kupokea matokeo yako ya mtihani wa Kifua kikuu na matokeo ya upimaji wa dawa haraka kuliko huduma ya kawaida. Hii itasaidia kudhibiti na kugundua mapema upinzani wa dawa ya Tb. Kwa kuongeza unaweza kufaidika moja kwa moja kutoka kwa habari tunayopata kutoka kwa somo na hii inaweza kusaidia wagonjwa wengine siku zijazo.

Usiri:
Jina na anwani yako itaandikwa tu kwenye ukurasa wa mbele wa fomu ya kliniki ili kukusaidia kukuunganisha na matokeo yako ya maabara kwa chaguzi za matibabu. Fomu iliyobaki itakuwa na nambari ya kusoma tu. Orodha ya majina na nambari za masomo zitafungiwa mbali na kuhifadhiwa kando na fomu za kusoma. Hatutashiriki matokeo ya majaribio na mtu yeyote isipokuwa kliniki, madaktari na wauguzi wanaokujali. Jina lako halitatumika katika hotuba yoyote au karatasi iliyoandikwa juu ya utafiti huu. Usiri wako utalindwa kwa kiwango kinachoruhusiwa na sheria.

Kushiriki kwa hiari:
Ikiwa hutaki kuwa katika utafiti huu wa uchunguzi, bado utapata utunzaji wa kawaida na matibabu. Ikiwa unakubali kuwa katika utafiti huu, unaweza kubadilisha akili yako na unaweza kuacha kuwa katika masomo wakati wowote. Uamuzi wako wa kushiriki hautaathiri huduma unazopokea katika kituo hiki cha afya.

Ikiwa una maswali yoyote juu ya utafiti na / au ikiwa kuna chochote ambacho bado hakijafahamika, tafadhali muulize yeyote kati ya watu waliotajwa hapo chini:

Mpelelezi mkuu: Fredrick Ogumbo, Simu: +245725849376 au Katibu, Kitengo cha Maadili cha KEMRI, P.O. Box 54840-00200, Nairobi; Nambari za simu: 020-2722541, 0722205901, 0733400003; anwani ya barua pepe: erc@kemri.org

Idhini yako:
Unaposaini hapa chini, unakubali kuwa kwenye utafiti huu. Unaweza kubadilisha akili yako wakati wowote. Bado utapata utunzaji wa kawaida na matibabu ikiwa uko kwenye utafiti huu au la. Ikiwa una maswali yoyote na / au ikiwa kuna chochote ambacho bado hakijafahamika kwako, tafadhali muulize daktari wako au muuguzi au mmoja wa wafanyikazi wa masomo. Tafadhali usisaini fomu hii hadi utapata majibu ya maswali yako yote.

Ikiwa unakubali kuwa katika utafiti huu, tafadhali saini hapa:

_____ Jina la mshiriki wa masomo *(chapisha)*
_____ Sahihi_____ Tarehe_____
_____ Jina la Shahidi *(Chapisha)* Sahihi ya Shahidi_____Tarehe_____
Jina la Mshiriki
Sahihi ya Mshiriki: _____Tarehe_____

Hifadhi ya vielelezo vya majaribio ya baadaye.

_____ _ Natoa ruhusa kwa vielelezo kuhifadhiwa katika maabara kwa upimaji wa baadaye wa upungufu wa dawa ya Kifua kikuu. Ninaelewa kuwa sitaulizwa baadaye kubali tena kwa aina hii ya majaribio.

_____ mimi sipeani ruhusa ya viashiria kuhifadhiwa kwa upimaji unaohusiana na Kifua Kikuu.

Appendix 6: Approval from Maseno University School of graduate Studies

MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES
Office of the Dean

Our Ref: PHD/PH/00101/2017

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 17th May, 2021

TO WHOM IT MAY CONCERN

RE: PROPOSAL APPROVAL FOR FREDRICK OGUMBO — PHD/PH/00101/2017

The above named is registered in the Doctor of Philosophy programme in the School of Public Health and Community Development, Maseno University. This is to confirm that his research proposal tilted **"Profile of Drug-Resistant-Conferring Mutations among New and Previously Treated Pulmonary Tuberculosis CASES FROM Kisumu County, Kenya."** has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof: J. O. Agure
DEAN, SCHOOL OF GRADUATE STUDIES

16 MAY 2021

Appendix 7: Ethical approval from KEMRI Scientific Ethical Review Unit

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/RES/7/3/1 **October 19, 2020**

TO: **FREDRICK OGUMBO,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DEPUTY DIRECTOR, CGHR,**
KISUMU.

Dear Sir,

RE: **KEMRI/SERU/CGHR/002-02-330/4079 *(RESUBMISSION II OF INITIAL SUBMISSION)*: PROFILE OF DRUG-RESISTANT-CONFERRING MUTATIONS AMONG NEW AND PREVIOUSLY TREATED PULMONARY TUBERCULOSIS CASES FROM KISUMU COUNTY, WESTERN KENYA**

Reference is made to your letter dated October 5, 2020. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on October 5, 2020.

This is to inform you that the Committee notes that the following issues raised during the 302nd Committee A meeting of the KEMRI Scientific Ethics Review Unit (SERU) held on **August 11, 2020** have been adequately addressed.

Consequently, the study is **granted approval** for implementation effective this day, **October 19, 2020** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **October 18, 2021**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **September 6, 2021.**

Please note that only approved documents including (informed consents, study instruments, Material Transfer Agreement) will be used. You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

Prior to commencing your study, you will be expected to obtain a research license from National Commission for Science, Technology and Innovation (NACOSTI) https://oris.nacosti.go.ke also obtain other clearances needed.
Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

In Search of Better Health

Appendix 8: NACOSTI Research Permit

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: 164842

Date of Issue: 09/June/2021

RESEARCH LICENSE

This is to Certify that Dr.. Fredrick Abonyo Ogumbo of Maseno University, has been licensed to conduct research in Kisumu on the topic: PROFILE OF DRUG RESISTANT CONFERRING MUTATIONS AMONG NEW AND PREVIOUSLY TREATED PULMONARY TUBERCULOSIS CASES FROM KISUMU COUNTY, KENYA for the period ending : 09/June/2022.

License No: NACOSTI/P/21/10981

164842

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

ANNEXURES

ANNEX 1: GenoType MTBDRplus v2 assay