GENOTYPIC CHARACTERISTICS AND MOLECULAR MARKERS OF ANTIBIOTIC RESISTANT NONTUBERCULOUS MYCOBACTERIA AND ASSOCIATED CLINICAL OUTCOMES AMONG PATIENTS ATTENDING BUNGOMA COUNTY, KENYA

Ronald Wamalwa

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DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award.

Signature…….. Date…….

Ronald Wamalwa

HML/H/01-55338/2020

CERTIFICATION

The undersigned certify that they have read and hereby recommended for acceptance of Masinde Muliro university of Science and Technology a thesis entitled, "**Genotypic Characteristics and Molecular Markers of Antibiotic Resistant Nontuberculous Mycobacteria and Associated Clinical Outcomes among Patients attending Bungoma County, Kenya."**

Signature…….. Date……

Dr. Nathan Shaviya,

Department of Medical Laboratory Science and Technology

School of Public Health, Biomedical Science and Technology,

Masinde Muliro University of Science and Technology

Signature…….. Date…….

Dr. Bernard Guyah,

Department of Biomedical Sciences and Technology

School of Public Health and Community Development

Maseno University

DEDICATION

I dedicate this work to my dear wife Diana Salome Akhabosa Wanyonyi. God bless you for supporting me right from the beginning to this end.

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ABSTRACT

Free-living, saprophytic non-tuberculous mycobacteria (NTM) are widely distributed and mostly found in sediment, aquatic habitats, biofilms, aerosols, animals, and people. *M. tuberculosis* (MTB) and NTM are both members of the Mycobacterium genus. It could display similar microscopic, radiological, and clinical features as MTB. Studies have reported 33.4% of cases are in North America, 23.8% are in Europe, 20.8% are in Asia, and 7.5% are in Africa. Worldwide, non-tuberculous mycobacterial infection prevalence varies. Currently, NTM infections and the rising incidence of antibiotic resistance (AMR)are the two major global health issues are. A significant prevalence of 33.6% for MTB among HIV patients have been reported in Kenyan counties like Bungoma. This study examined the antibiotic susceptibility profiles and genotypic traits of NTM isolates from people living with HIV (PLWH) at Bungoma County Referral Hospital (BCRH). This study assessed the relationship between AMR molecular markers and clinical outcomes such underweight status, immunosuppression, and viral suppression. A total of 167 suspected TB patients were selected purposively in an analytical cross-sectional study. Smear microscopy and culture were used to determine presence of NTM, while ELISA determined HIV status. The Abbott m2000 system was used to establish viral load and flow cytometry was used to measure CD4+ T cell count, and NTM infection. While NTM isolates' minimum inhibitory concentrations were assessed using microdilution broth, NTM species in positive cultures were identified using the Hain Genotype Mycobacterium CM/AS assay. Continuous data were analysed using the Mann-Whitney U test, while categorical variables were compared using chi-square testing. Using logistic regression and Pearson correlation, the relationship between AMR in NTM isolates and clinical outcomes among PLWH was evaluated. PLWH had a 41.1% prevalence of positive NTM cultures, whereas HIV-negative individuals had a 21.3% prevalence (P = 0.280). The most common species was *M. intracellulare* (42.9%), which was followed by *M. fortuitum* (26.3%). Less often found were *M. lentiflavum*, *M. scrofulaceum*, and *M. abscessus*. Drug-resistant NTM isolates from PLWH and HIV-negative individuals showed resistance to pyrazinamide (15.2% vs. 4.3%, P<0.001), ethambutol (9.1% vs. 0.0%, P < 0.001), and isoniazid (12.1% vs. 8.7%, P < 0.043). HIV-negative individuals had a median CD4+ T cell count of 853 cells/mm³, whereas PLWH had a median of 454 cells/mm³. In 9.6% and 3.2% of PLWH cases, respectively, the presence of the *rpoB* and *katG* genes was associated with AMR. In HIV-negative individuals' the prevalence of mutations in the *inhA* gene were 2.1%, while PLWH had a 2.7% prevalence. PLWH co-infected with *inhA*-carrying NTM had a twofold increased risk of underweight (OR: 2.409, 95% CI: 1.858-7.871; P = 0.040). The *rpoB* and *katG* genes were not associated with outcomes like underweight, viral load, or immunosuppression. The study's findings highlight how common AMR is in NTM isolates from PLWH, which has significant implications for therapeutic treatment. In addition, mutations in the *inhA* gene were associated with a higher risk of underweight status in PLWH. Therefore, there is need of molecular surveillance as well as addressing educational and vocational variables in the management of AMR among PLWH in Bungoma County.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immunodeficiency syndrome
AMR	Antimicrobial resistance
AST	Antimycobacterial Susceptibility Test
ATCC	American type culture collection
ATS	American Thoracic society
BCG	Bacillus Calmette-Guérin
BCRH	Bungoma County referral Hospital
cDNA	Complementary/copy deoxyribonucleic acid
CSLI	Clinical and laboratory standards institute
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme linked-immunosorbent assay
HGT	Horizontal Gene Transfer
IDSA	Infectious Diseases Society of America
ITS	Internal transcribed spacer
L-J	Löwenstein–Jensen medium
LPA	Line probe assay
MAC	Mycobacterium Avium Complex
Mce	Mammalian cell entry
MF	*Mycobacterium fortuitum*
MG	*Mycobacterium gordonae*
MGIT	Mycobacteria growth indicator tube
MI	*Mycobacterium intracellulare*
MICs	Minimum inhibitory concentrations
MK	*Mycobacterium kansasii*
MS	*Mycobacterium simiae*

MTB	*Mycobacterium tuberculosis*
MTBC	Mycobacterium Tuberculosis Complex
MZ	*Mycobacterium szulgai*
NACC	National Aids Control Council
NACOSTI	National commission for science, technology and innovation
NTM	Non-tuberculous mycobacteria
NTMPD	Non-tuberculous mycobacteria pulmonary disease
PD	Pulmonary disease
PGLs	Phenolic glycolipids
PLWH	People living with HIV
PNB	P-nitrobenzoic acid
PP/PPE	Proline-glutamine /proline-proline-glutamine motif protein
RGM	Rapid growing mycobacteria
SGM	Slow growing mycobacteria
SNPs	Single nucleotide polymorphisms
Tat	Twine-arginine translocase
TB	Mycobacterium Tuberculosis
WHO	World Health Organization
WT	Wildtype

OPERALIZATIONAL DEFINITION OF TERMS

Horizontal gene transfer: It refers to the transfer of genetic information between genomes without the involvement of sexual reproduction. The process allows for the replacement of existing genes with incoming DNA or RNA, or the introduction of new genes into a genome. commonly referred to as lateral gene transfer.

Housekeeping genes: These pertain to genes that are consistently expressed and are necessary for sustaining fundamental cellular functions that are vital for the survival of a cell, irrespective of its particular function within the tissue or organism. These are manifested across all cellular types within an organism, both in standard and pathological physiological states.

Internal transcribed spacers: The internal transcribed spacer (ITS) represents a segment of nonfunctional RNA situated between the structural ribosomal RNAs (rRNA) within a shared precursor transcript.

Molecular marker: A DNA sequence exhibiting polymorphism that can be identified through a molecular technique. Molecular markers serve as tools to discern variations or polymorphisms within specific regions of DNA that manifest among individuals within a population. A marker facilitates the precise identification of the gene of interest.

Single nucleotide polymorphisms: A variation at a single position within the DNA sequence among individuals represents the most prevalent form of genetic variation

observed in humans. Each SNP signifies a variation in a singular DNA component, known as a nucleotide.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Park et al. (2019) and Ratnatunga et al. (2020) found that non-tuberculous mycobacteria (NTM) are saprophytic microbes that live freely in the environment. Natural and man-made water sources, soil, biofilms, aerosols, plants, and, of course, people and animals are the most common habitats for these microbes (Larsson et al., 2017). The genus Mycobacterium includes both the tuberculosis-causing and leprae-causing species of mycobacteria, as well as several non-tuberculous mycobacteria. The Mycobacterium tuberculosis Complex (MTBC) includes M. tuberculosis, M. africanum, M. bovis, M. caprae, M. canetti, M. microti, and M. pinnipedii, and research suggests that non-tuberculous mycobacteria are its genetic relatives (Jenkins et al., 2017). As a result, MTBC, M. leprae, and NTM are the three main groups of mycobacteria. Consequently, they share some genetic information.

In addition, phylogenetic studies by Veyrier et al. (2011) and Wang et al. (2015) indicate that MTBC may have evolved into a more dangerous disease by a series of gene deletions and acquisitions. There are over 200 species of non-tuberculous mycobacteria, and there seems to be a lot of geographical diversity in the NTMs found in clinical samples, according to studies from different nations and areas. It seems that the most often identified non-tuberculous mycobacterium in clinical settings is Mycobacterium avium complex (MAC). Variability in NTM isolation across continents, within nations, and between them is defining the increasing body of knowledge about the worldwide epidemiology of NTM illness. According to previous research, the prevalence of NTM infections is 33.4% in North America,

23.8% in Europe, 20.8% in Asia, and 7.5% in Sub-Saharan Africa (Okoi et al., 2017; Varghese & Al-Hajoj, 2020).

Previously, NTM were considered to be non-pathogenic (Lake *et al.,* 2016). However, recent studies have revealed NTM as emerging etiologic factors influencing significantly the burden of disease (Monde *et al.,* 2018). Diseases like lymphadenopathy, mycobacterial pulmonary disease, Buruli ulcer, skin and soft tissue disease have been linked to NTM infections (Ando *et al.,* 2018; Larsson *et al.,* 2017). Of note, is mycobacterial pulmonary disease that contributes the greatest burden especially in immune-compromised individuals (Johnson & Odell, 2014; Larsson *et al.,* 2017). Globally, the prevalence and incidence of non-tuberculous mycobacterial pulmonary disease (NTMPD) is on the increase. For instance, in the past twenty years, studies have revealed an exponential increase of NTMPD in Asia, Western Europe and America (Donohue & Wymer, 2016). Donohue and friends reported an increase in the prevalence of NTMPD from 2.4 cases/ 100,000 in the 1980s to 15.2 cases/ 100,000 in 2013 in US. In addition, similar trends have been observed in Canada, United Kingdom, Denmark and Germany (Andréjak *et al.,* 2010; Brode *et al.,* 2017; Diel *et al.,* 2017; Moore *et al.,* 2010).

In Africa, comparable patterns have been observed in as far as the burden of NTMPD is concerned. Recent studies in sub-Saharan Africa have shown an overall prevalence of 7.5% (Okoi *et al.,* 2017). From the findings, there appears to be a variation of the prevalence of NTMPD across different African countries. For

example, Ghana reported a prevalence of 23%, Nigeria 36.0%, Uganda 9.2%, Tanzania 15.0%, Zambia 25.8% and South Africa 30.2% (Sookan & Coovadia, 2014; Bainomugisa *et al.,* 2015; Bjerrum *et al.,* 2016; Chanda-Kapata *et al.,* 2015; Hoza *et al.,* 2016; Silveira Paro Pedro *et al.,* 2021). In Kenya, studies have reported varied NTM prevalence's depending on different geographical regions and population of study; for instance, a study by Ngayo and colleagues reported a prevalence of 42.4 % individuals suffering from NTMPD (Ngayo *et al.,* 2015). In Western Kenya, Siaya County another study reported a prevalence of 2.6% NTMPD among infants (Kaguthi *et al.,* 2019). These studies signify NTMPD as an emerging disease which is a neglected area of public health importance. Nevertheless, of much concern is the rise of NTMPD burden among immune-compromised individuals especially PLWH (Bjerrum *et al.,* 2016; Donohue, 2018; Ngayo *et al., 2*015; Saxena *et al.,* 2021).

Antimicrobial resistance (AMR) among NTM has developed to be a key public health concern of this century (Bryant *et al.,* 2016; Faverio *et al.,* 2016). AMR is a threat to effective treatment of pathogenic microbes. Therefore, sentinel surveillance for AMR markers in both pathogenic and non-pathogenic microbes is important. NTM being abundant micro-organisms in nature pose a threat of spreading drug resistance traits by their interaction (Munita & Arias, 2016). Previous evidence has revealed the role that NTM have played in escalating antimicrobial resistance (Johansen *et al.,* 2020). However, the mechanisms by which NTM spread AMR are not fully understood. But some of the inherent characteristics in NTM that are believed to be capable of decreasing drug uptake that eventually causes resistance to antibiotics include, their thick, impermeable cell walls, their presence in biofilms and granulomas (Bryant *et al.,* 2016; Luthra *et al.,* 2018). In addition, some NTM

express proteins that specifically target antibiotics that consequently reduce drug efficacy (Nessar *et al.,* 2012). It is also crucial to map genotypic and allelic variations connected to AMR at the molecular level. For instance, plasmids are used by bacteria to reproduce, therefore there could be possibilities that resistant features will be introduced into the genomes of previously vulnerable NTM by plasmids (Morgado *et al.,* 2017; Tagini *et al.,* 2021).

Some of the conditions that cause immune deficiency in people includes cancer, organ transplant, HIV and AIDS and some genetic diseases (Henkle & Winthrop, 2019; Sharma & Upadhyay, 2020). HIV and AIDS is the most prevalent cause of immune deficiency (Agizew *et al.,* 2017; Ngayo *et al.,* 2015). Globally, people are living with HIV and with the advent of antiretroviral therapy, fewer number of patients with AIDS are being recorded (Lapinel *et al.,* 2019). Previous studies have attributed the global spread of NTM to PLWH. However, MBC has been for a long time reported as the most prevalent opportunistic infection in patients with HIV and AIDS (Peters *et al.,* 2019). However, dearth research on the epidemiology of NTM in HIV and AIDS patients has led to underestimation of its prevalence within TB endemic countries such as Kenya (Kaguthi *et al.,* 2019). The situation is further compounded by antibiotic resistance in PLWH patients co-infected with NTM. Although, several NTM species are now recognized as a major infective threat in HIV and AIDS individuals, their in-depth genomic investigation has not been carried out systematically (Yeung *et al.,* 2016).

The limited comprehensive phylogenetic analyses of the entire genus *Mycobacterium* conducted thus far have predominantly relied on the comparison of individual or concatenated housekeeping genes. The 16S rRNA gene has served as the predominant marker, and the structure of the phylogenetic trees derived from its

sequences largely aligns with those produced by the multilocus sequencing method (Wang et al., 2015). In summary, the findings from these studies indicate that the molecular characterization of NTM should be approached within the framework of antimicrobial resistance in patients with HIV and AIDS.

Furthermore, research has demonstrated that phenotypic characterization alone cannot reliably distinguish between MTB and NTM (Raju *et al.,* 2016). Additionally, investigations have shown that NTM are responsible for immune interference in the Bacillus Calmette-Guerin (BCG) vaccine via cross reactive immune responses. This has been linked to the low BCG efficacy in areas where NTM are common. Therefore, molecular characterization was carried out to detect antimicrobial resistance indicators in NTM's, and this can help to decrease the misdiagnosis of MTB and NTM.

Bungoma County has an estimated 30,000 adults living with HIV and AIDs. However, there are variations in HIV prevalence by age, sex, and by type of population (NACC, 2014). The challenge of HIV and AIDS among key populations needs to be tackled in the County. This challenge is compounded by TB and other opportunistic infections. While there is no sufficient data to account for Key population in the County, its proximity to the borders of Busia, Malaba, Uganda to the West, Kisumu, Kakamega and Uasin Gishu Counties calls for intensified examination of the issue (Magomere & Obwoge, 2018). Of the ~30,000 PLWH in Bungoma County, 56% presented with presumptive tuberculosis. NTM are often misdiagnosed as TB and since most NTMs require specialized and focused treatment, as a result, AMR is rampant in NTM isolated from HIV and AIDS

patients. In order to effectively manage the PLWH co-infected with NTM in Bungoma County, establishment of the AMR pattern in NTM isolated PLWH was necessary, because it will lessen NTM mortality and morbidity.

1.2 Statement of the Problem

Non-tuberculous mycobacterium infections present a serious management problem for PLWH. Treatment breakthroughs notwithstanding, antibiotic resistance in NTM isolates complicates therapeutic approaches and raises PLWH morbidity and fatality rates (Tarashi *et al.,* 2022). This problem is made worse by incomplete knowledge of the genotypic traits, patterns of antibiotic susceptibility, and molecular markers linked to NTM resistance. Furthermore, the link between important clinical variables in HIV patients including underweight, immunosuppression, and viral suppression and antimicrobial resistance in NTM isolates is still not fully understood. Thus, in order to improve the management outcomes of NTM infections among PLWH in this area and to inform targeted interventions, a thorough investigation of these features is urgently needed.

Additionally, the clinical similarity between NTM infections and TB presents significant challenges. In regions like Bungoma County, Kenya, where TB is prevalent, NTM infections often exhibit symptoms closely resembling those of TB, such as coughing, fever, weight loss, and respiratory issues (Ochayo *et al.,* 2023). This similarity frequently leads to misdiagnosis, as healthcare providers may initially mistake NTM infections for TB. Such misdiagnosis can have detrimental effects, including delayed treatment and exacerbated illness.

Moreover, accurately distinguishing between NTM and TB requires specialized laboratory tests, like culture and molecular techniques, which may not be readily available in resource-limited settings such as Bungoma County. Consequently, there are often delays in diagnosing NTM infections, further complicating the management of these cases.

This diagnostic challenge not only affects individual patient care but also has broader public health implications. Mismanaged NTM cases can impact TB control efforts, potentially leading to false-positive TB diagnoses and misallocation of resources (Asgharzadeh *et al.,* 2020). Additionally, inappropriate treatment of NTM infections with anti-TB drugs can result in treatment failure and the emergence of drug-resistant strains, further exacerbating the problem. It is important to enhance diagnostic capabilities, raise awareness among healthcare providers about the distinctions between NTM and TB, and implement custom-made management strategies for NTM infections.

1.3 Objectives of the study

1.3.1 General objective

To determine genotypic characteristics and molecular markers of antibiotic resistant NTM isolates and their association with clinical outcomes among HIV-NTM co-infected patients attending BCRH.

1.3.2 Specific objectives

i. To determine the genotypic characteristics of antibiotic resistant NTM isolates from PLWH attending BCRH.

ii. To determine antibiotic susceptibility patterns in NTM isolates from PLWH attending BCRH.

iii. To identify the molecular markers in non-tuberculous mycobacteria isolates causing antibiotic resistance among PLWH patients attending BCRH.

iv. To determine the association between antimicrobial resistance of NTM isolates with clinical outcomes in PLWH in BRCH.

1.4 Research questions

i. What are the genotypic characteristics of antibiotic resistant NTM isolates from PLWH attending BCRH?

ii. What are the antibiotic susceptibility patterns in NTM isolates from PLWH attending BCRH?

iii. What are the molecular markers in NTM isolates causing antibiotic resistance among PLWH patients attending BCRH?

iv. What is the association between antimicrobial resistance of NTM isolates with clinical outcomes in PLWH in BRCH?

1.5 Justification

Previously NTM were regarded as non-pathogenic, hence they have been disregarded (Ratnatunga *et al.,* 2020). There emergence as potential pathogens especially in PLWH, has led to a rise in their prevalence due to indiscriminate diagnosis and treatment. Infection with NTMs presents a considerable public health problem, particularly in those who have HIV. It is vital to understand the genotypic traits, antibiotic susceptibility patterns, molecular markers, and relationships with clinical variables in NTM isolates from PLWH.

Bungoma County is among 10 counties in Kenya with a high TB burden in both HIV negative and positive individuals (Ogwang *et al.,* 2021). Approximately 30,000 individuals in this county are PLWH, and nearly 60% of them are potentially coinfected with TB and or NTMs (Abongo *et al.,* 2020; Ochayo *et al.,* 2023). Moreover, characterizing NTMs in order to differentiate them from TB requires specialized laboratory tests, like culture and molecular techniques, which may not be readily available in resource-limited settings such as Bungoma County referral hospital. This therefore increased the burden and like of NTMs, TB and its potential for misdiagnosis. It also contributes to development of drug resistant strains resulting from wrong treatment.

In individuals living with HIV, NTM infections are being more frequently identified as opportunistic infections, resulting in significant illness and death. Studying the genetic traits and susceptibility to antibiotics of NTM isolates can offer valuable information on how to effectively treat NTM infections, therefore decreasing the impact of these illnesses on susceptible individuals. The global increase in antibiotic resistance among NTM isolates is becoming a significant problem (Saxena *et al.,* 2021). Identifying molecular markers linked to antibiotic resistance in NTM isolates from PLWH can assist in the creation of focused treatments and infection control strategies, ultimately enhancing patient outcomes and decreasing the transmission of resistant strains.

It is crucial to understand the correlation between the resistance of NTM isolates to antimicrobial drugs and factors such as being underweight, having a weakened immune system, and suppressing viral activity in people living with HIV. This

understanding is necessary to improve the clinical treatment of these individuals. This knowledge can assist healthcare practitioners in customising treatment regimens and interventions to enhance the health outcomes of PLWH who have NTM infections.

Although, NTM infections in PLWH are becoming increasingly recognised, there is a dearth of thorough research that specifically investigate the genotypic traits, antibiotic susceptibility patterns, molecular markers, and clinical correlations of NTM isolates in this population.

1.6 Significance

The inconclusive diagnosis of NTMPD is a significant public health hazard on a global basis. As a result, NTM has become a disease-causing agent in both those with compromised immune systems and those with healthy immune systems. In Bungoma.

County, there is currently a paucity of knowledge on the distinct differences between MTB and NTM. As a result, there are few insights into how NTM contributes to TB-like symptoms and its prevalence, impact, and other factors. NTMs and MTB are equally likely to cause pulmonary disease (PD), but clinical symptoms, smear microscopy, radiological abnormalities, and even cultures are insufficient to accurately differentiate between them. By genetically defining NTMs and performing susceptibility testing to determine NTMs AMR, this study intends to close this gap. The results of this study are crucial in helping Bungoma County and the entire country effectively identify and treat NTM infections, reducing the burden of disease among people living with HIV (PLWH) there. Furthermore, the

information linking NTM and AMR will enable stakeholders to create guidelines for appropriate diagnostic techniques and powerful antibiotics to combat NTMPD. This information is essential since different mycobacteria species necessitate different management strategies. Successful NTMPD management will ultimately lead to a decline in sickness, mortality, and healthcare costs.

1.7 Scope of the study

The study was done among HIV positive adult patients presenting with presumptive TB in Bungoma County Referral Hospital comprehensive care centers. Purposive sampling technique was used to collect the samples between January, 2023 to April, 2023.

1.7.1 Limitations

The findings from a specific context or population may not be generalizable to other settings or to a larger population. Furthermore, cross-sectional studies provide results at a single point in time and may not establish causation due to limited time and therefore limited in adequately representing the diversity within the population.

1.7.2 Delimitations

This investigation was restricted to BCRH in Bungoma County. And, the analysis was skewed toward PLWH attending comprehensive care units. Additionally, the study's exclusive emphasis was on individuals who showed symptoms and signs similar to tuberculosis.

CHAPTER TWO

LITERATURE REVIEW

2.1: Non-tuberculous Mycobacteria species

Non-tuberculous mycobacteria (NTM) are ubiquitous, free-living saprophytic organisms inhabiting several environmental niches, including aquatic systems, soil, and flora (Ratnatunga *et al.,* 2020). The words "anonymous" and "atypical mycobacteria" have been abandoned (Larsson *et al.,* 2017). Non-tuberculous mycobacteria (NTMs) are classified within the Mycobacterium genus, which includes *M. tuberculosis* (MTB) and *M. leprae*. Research suggests that NTM may be evolutionary precursors to the Mycobacterium tuberculosis complex (MTBC), potentially experiencing genetic alterations that render them more aggressive pathogens analogous to MTBC (Jenkins *et al.*, 2017). Recent research indicates more than 180 species of non-tuberculous mycobacteria (NTM) (Sood and Parrish, Seth-Smith *et al.,* 2019), with certain estimations exceeding 200 (Lipman *et al.*, 2021). NTM are distinguished by their microaerobic development in 6–12% oxygen and lipid-rich cell walls. They demonstrate remarkable resilience, tolerating diverse ambient temperatures, and display resistance to conventional medicines and disinfectants (Ratnatunga *et al.,* 2020).

NTMs are globally prevalent and are increasingly acknowledged as pathogens responsible for infections that are often overlooked, difficult to diagnose, and complex to manage. They are categorized into "slow-growing mycobacteria" (SGM)

and "rapid-growing mycobacteria" (RGM). SGMs necessitate a minimum of seven days for colony formation, whereas RGMs establish colonies in less than seven days (Primm *et al.,* 2004). Species classification utilizing 16S rRNA sequencing has uncovered intricate genetic diversity within the genus (Honda *et al.*, 2018). NTM were initially characterized in the late nineteenth century, shortly following Robert Koch's identification of Mycobacterium tuberculosis as the etiological agent of tuberculosis in 1882 (Johnson & Odell, 2014). Years later, NTMs were identified as causative agents of non-tuberculous mycobacterial pulmonary disease (NTMPD) and linked to biofilm formation, a trait that enhances their resistance to antibiotics and disinfectants (Johnson & Odell, 2014).

Understanding the genotypic features of antibiotic-resistant NTM isolates among PLWH at Bungoma County Referral Hospital (BCRH) is essential for enhancing treatment outcomes. NTMs have complex antibiotic sensitivity profiles and considerable genetic diversity, perhaps affecting their ability to withstand standard therapies. Recent studies have highlighted the significance of genetic markers in NTM antibiotic resistance, as particular gene mutations facilitate resistance mechanisms that pose significant challenges in immunocompromised individuals. Identifying these molecular markers in NTM species isolated from PLWH may facilitate the prediction of antibiotic resistance and enable more effective treatment regimens.

The *Mycobacterium avium* complex (MAC), comprising *M. intracellulare*, *M. kansasii* and *M. xenopi*, is the predominant cause of non-tuberculous mycobacterial infections in humans. Nonetheless, rapidly proliferating species, including *Mycobacterium abscessus*, *M. chelonae*, and *M. fortuitum*, have been associated with

a considerable incidence of NTM lung illness worldwide (Huang *et al.,* 2020; Simons *et al.,* 2011). These animals exhibit unique genetic modifications that improve antibiotic resistance. *M. abscessus* consists of three subspecies (*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii*), each exhibiting distinct resistance profiles, whereas the *M. fortuitum* group encompasses various members such as *M. peregrinum*, *M. senegalense* and *M. houstonense*.

This study examined the correlation between antimicrobial resistance in NTM isolates and clinical outcomes, including underweight status, immunosuppression, and viral suppression among PLWH at BCRH. Evidence indicates that the resistance features of NTMs may affect these clinical situations, with underweight and immunosuppressed patients possibly at an elevated risk for infections caused by drug-resistant NTM strains. This study intended to clarify these relationships to address knowledge gaps and guide methods for treating AMR in high-risk populations, ultimately driving more effective health interventions in areas such as Bungoma County.

2.2 Epidemiology of Non-Tuberculous Mycobacteria

NTM comprise a heterogeneous assemblage of over 200 species (Sharma & Upadhyay, 2020), with the most clinically significant being *M. avium, M. intracellulare, M. kansasii, M.xenopi*, and *M. abscessus*. These organisms can induce many diseases, including lung disease, disseminated disease, and localized lesions. Pulmonary disorders are the predominant category, with up to 94% of NTM-associated patients (Monde *et al.,* 2018). The prevalence and kinds of NTM infections differ worldwide, shaped by factors like geographic region, gender, age,

and certain health problems. Immunomodulatory drugs, rheumatoid arthritis, and thoracic skeletal abnormalities are associated with an elevated risk of NTM infection. Moreover, genetic predispositions and environmental conditions, such as warm and humid climates, increase the likelihood of infection (Borand *et al.*, 2019).

Regional studies reveal significant variations in the prevalence and kinds of NTM. In Kenya, the prevalence of NTM among clinical patients rose from 1.7% during 2007-2009 (Nyamogoba *et al.*, 2012) to 42.4% in 2014-2015, with a substantial proportion being individuals living with HIV (PLWH) (Ngayo *et al.*, 2015). A research in sub-Saharan Africa indicated a 7.5% frequency, with MAC species predominantly impacting males and younger demographics, the median age being 35 (Okoi *et al.*, 2017). Conversely, elevated latitudes in China and Taiwan correlate with increased frequencies of MAC infections, specifically *M. intracellulare* in the northern regions and *M. abscessus* in the southern regions (Yu *et al.*, 2016; Huang *et al.*, 2020). *M. abscessus, M. fortuitum* and *M. intracellulare* are the primary non-tuberculous mycobacteria species extracted from clinical specimens in India (Desikan *et al.*, 2017).

South Korea documented a 62% rise in NTM lung illness from 2002 to 2008, primarily attributed to *M. abscessus* infections (Park *et al.*, 2010). In the United States, *M. abscessus* complex infections were documented as secondary to MAC, accounting for 3-13% of NTMPD cases, with the states at highest risk being Hawai'i, California, New York, and Florida (Adjemian *et al.*, 2012). In geriatric populations, prevalence rates have more than doubled, increasing from 20 cases per 100,000 to 47 cases per 100,000 between 1997 and 2000, attributed to immunological decline and chronic diseases (Mirsaeidi *et al.*, 2014). Genetic

variations in susceptibility to NTM disease have been noted across the Middle East, Europe, and Africa, indicating possible genetic or environmental factors influencing these geographical disparities (Borand *et al.,* 2019).

This research expands upon global findings to fill information gaps about NTM infections in PLWH, with a specific emphasis on antibiotic-resistant NTM isolates from Bungoma County. The research aimed to clarify the genotypic traits and antibiotic resistance profiles of NTM isolates, which are inadequately studied in relation to HIV. This research seeks to uncover molecular indicators of antimicrobial resistance and assess their influence on clinical outcomes, including underweight status, immunosuppression, and viral suppression, which are vital for the health of people living with HIV. Furthermore, comprehending the correlation between antibiotic resistance in NTM isolates and these clinical variables may facilitate targeted treatments, enhancing the management and prevention of NTM infections in areas with elevated HIV incidence. This study's results are anticipated to considerably enhance regional and worldwide understanding of NTM epidemiology, aiding in the formulation of strategies to tackle the distinct issues posed by NTM and antimicrobial resistance in at-risk groups.

2.3 The pathology of Pulmonary NTM infection

Numerous pathogenic disorders, such as lung, skin, bone, joint, and disseminated diseases, can be brought on by NTM (Fedrizzi *et al.,* 2017). However, NTMPD, on the other hand, is the most common clinical infection caused by NTM (Ceyhan *et al.,* 2019). The frequent isolation of NTM bacteria in human samples is regarded as colonization or contamination because NTM is pervasive in the environment. To distinguish the pathogen from the pollutants, the American Thoracic Society

(ATS)/Infectious Diseases Society of America (IDSA) issued guidelines on pulmonary NTM (Ando *et al.,* 2018).

Consequently, the prevalence of infection has been recorded globally ever since the IDSA released its NTM guidelines. According to other studies, NTM are now obligatory pathogens that can infect both immunocompetent and immunosuppressed people and produce NTMPD and NTM extra pulmonary illness (Fedrizzi *et al.,* 2017). These findings are quite concerning to the general public because the pathophysiology of NTM is challenging to treat and necessitates a different regimen than TB.

Ratnatunga et al. emphasize that although NTM disease can present with a wide range of clinical symptoms, the most typical clinical manifestation—and which was the focus of this investigation in BCRH is lung infection (Ratnatunga *et al.,* 2020). Additionally, they classified NTMPD in to three types; fibro-cavitary disease, nodular bronchiectasis disease, and hypersensitivity pneumonitis, each of which has a unique pathology. The range of incubation times makes diagnosis challenging and tracing the source of infection nearly impossible. NTM is now recognized as an emerging disease agent that causes significant morbidity and mortality in both immunological competent and immune compromised populations as a result of an increase in the number of internationally recorded NTM infections (Tan *et al.,* 2018). The MAC species are the most prevalent diseases causing NTM pathogens worldwide, however prevalence varies significantly by age, gender, and geographic location (Prevots & Marras, 2015). And, due to extremely high levels of antibiotic resistance and the disease's increasing prevalence in East Asian nations like Japan, Korea, and Taiwan, MABS are more prevalent in such sides (Simons *et al.,* 2011).

According to Mehrian et al, all the three kinds of NTMPD are observed to be prevalent in PLWH (Mehrian *et al.,* 2019). However, the study also showed that less frequent types exist, including hypersensitivity pneumonitis and the nodular pattern, a tumor that mimics malignancy. Due to the low virulence and slow growth of these organisms, illness symptoms frequently appear gradually and are difficult to diagnose because their incubation periods can range from months to years. One technique of identifying NTM according to IDSA is radiological diagnosis, which can range from widespread disease found in the upper lobes or apical regions of lower lobes to disease that mainly affects the middle lobe and lingula (Fedrizzi *et al.,* 2017). The most severe types of presentation include chronic exhaustion with weight loss and hemoptysis (Ratnatunga *et al.,* 2020). However, Paucity symptomatic forms, such as cough and sputum production, which are particularly related with bronchiectasis are also observed.

2.4 Genetic relationships between NTM and MTB

According to studies, *M. prototuberculosis*, *M. canetti*, and later *M. bovis* shared a common ancestor with the tuberculous mycobacteria (Jenkins *et al.,* 2017). Brosch *et al*, further noted that, multiple deletion events of genes led to the evolution of genus mycobacteria (Brosch *et al.,* 2002). The evolution view is further augmented by Veyrier et al, who and coworkers argue that, genomic data have revealed a wealth of information on the genetic makeup of mycobacteria. This has made it possible to identify a number of genetic changes that have taken place in the mycobacterial genome (Veyrier *et al.,* 2011). According to them, genetic events reveal a more plausible evolutionary scenario, where it was revealed that MTBC descended from NTM via simultaneous genomic deletions and gene acquisition. These genetic occurrences point to a more plausible evolutionary pathway which revealed that *M.*

tuberculosis evolved into a more specialized/professional pathogen from NTM through concomitant genomic deletions and horizontal gene transfer (HGT) of genes (Veyrier *et al.,* 2011).

In addition, Veyrier et al demonstrated relatedness of MTBC and *M. kansasii* which is an NTM species using the phenolic glycolipids (PGLs), which are typical of slow-growing mycobacteria. It was shown that genomic comparison of a sequence of HGT events happening at the PGL's locus also suggested evolution from *M. kansasii* to MTB. A phthiocerol dimycoserate (DIM) backbone, present in all mycobacteria, was used to produce the PGL. The genetic link between MTBC and NTMs may explain why BCG is less effective in regions where NTMs are common, according to his research and the findings of his colleagues (Kaguthi et al., 2019). Both NTM and Mycobacterium tuberculosis elicit cellular immunity through the type-1 cytokine pathway in the immune system. Mycobacterial infections may be hereditary or acquired, and this immune response can be disrupted in a variety of ways (e.g., by HIV or a family history of the disease) (López-Varela et al., 2015).

2.5 Association of Human Immunodeficiency Virus and NTM

Studies carried out globally have shown that, the rate of NTM cases reported among PLWH is increasing. In Kenya, research have revealed, a significant number of PLWH to be co-infected with NTMPD; In 2012, Nyamogoba reported prevalence of 41.8% and 2015 Ngayo reported 22% of PLWH to be co-infected with NTMPD respectively (Ngayo *et al.*, 2015; Nyamogoba *et al.,* 2012). Kenya is ranked among top African countries with high TB cases coupled with high HIV/AIDS burden (Ogwang *et al.*, 2021). Bungoma County has an estimated 30,000 adults living with

HIV/AIDS (NACC, 2014). Since this problem is made worse by the fact that TB and NTM are both opportunistic illnesses, but inconclusive diagnosis of NTM has sadly resulted in the incorrect diagnosis and improper treatment of NTM infections in resource-constrained areas like Bungoma County. Because of this, there is reason to be concerned that an unclear diagnosis based on smear microscopy, clinical symptoms, and/or radiological evidence could result in a false positive for pulmonary TB and/or improper care of NTMPD cases (Okoi *et al.*, 2017). The major paradox is, besides MTB and NTM sharing the genus *Mycobacterium*, both of them cannot de differentiated using basic mycobacteriology techniques like microscopy, clinical history, radiologic imaging and the tuberculin skin test (Okoi *et al.*, 2017). Furthermore, the problem has been compounded by the emergence of NTM as opportunistic infections in the HIV/AIDS patients, and their treatment is not directly analogous to that of TB (Ngayo *et al.*, 2015).

Similar findings of co-infection of PLWH and NTMPD were reported in several other African countries like Zambia and Botswana which had prevalence's of 71.5% and 56% respectively. Equally, investigations from European countries has also reported significant results associating PLWH coinfected with NTM. In USA, 49% of people having NTM infection were PLHIV (Lapinel *et al.*, 2019). 30% of the PLWH coinfected with NTM were severely immune compromised with the median CD4 cell count of 64 cells/mm3. And the majority were male (71%), middle-aged (median age 44 years), and most common NTM in this group of people was MAC, followed by *M.fortuitum.* Coinfection of NTM and PLWH, could be attributed to the fact that, NTM being majorly opportunistic pathogens would easily infect and cause disease in PLWH since immunity of such people is compromised hence predisposing them to diverse co-infections including NTM. More so, NTM are

known to be ubiquitous, free living, environmental organisms, therefore, it's easy to interact with them anywhere (Ratnatunga *et al.*, 2020).

According to (Chang, 2021), the most common NTM species which immunocompromised individuals especially PLWH are at a high risk includes *M. intracellulare,* and *M. avium* complex being frequently reported. Furthermore, it's noted that, typically it occurs in HIV-infected individuals with $CD4^+$ T cell of <50 cells/mm3, and this is significantly lower in people with SGM than those with respiratory isolation of RGM (Lapinel *et al.,* 2019). The faulty T-cell-mediated immunity in HIV infected patients possess an immunologic vulnerability which may predispose them particularly to pulmonary infection with NTM. Similar findings were reported in the 1980s where Horsbugh identified MAC disease as an important pathogen in PLWH (Henkle & Winthrop, 2015). Misdiagnosis of MTMPD jeopardizes proper management of NTM which in turn can result to drug resistance, high morbidity and mortality rates goes up. As Kenya works to prevent and treat tuberculosis, it is crucial that NTM be included in the care of clinical pulmonary TB, particularly among PLWH. Ultimately, it is crucial to properly diagnose, treat, and monitor pulmonary NTM illness since it is a neglected and growing public health concern. Cell-mediated immunity (CD4 T- cells count) is a crucial element of host defense against mycobacteria, is generally disrupted or depleted as the underlying mechanism that increases the risk of NTM illness in immunosuppressed patients. By stimulating the CD4+ T-helper 1 (TH1) pathway, which includes interleukin-12 (IL-12) and interferon gamma (IFN-), mycobacteria infect macrophages. Infected macrophages are then activated by IFN-, which stops the infection. Tumor necrosis factor alpha (TNF-), a proinflammatory cytokine necessary for the development and

maintenance of granulomas that potently restrict bacterial growth, is another important mechanism of regulation.

However, it is evident that different species have variable levels of virulence and immune response, as shown by species variations in the primary site of infection and the fact that some species almost exclusively cause infection.

2.6 Antimicrobial Resistance of Non-tuberculosis Mycobacteria

Despite NTMs being less widespread pathogens for humans than *M. tuberculosis*, Griffith et al observes that, they are an emerging threat to not only immunocompromised population but also in immunocompetent group (Saxena *et al.*,2021). Owing to the absence of an accurate diagnosis, it is challenging to promptly recognize NTMPD disease through fundamental mycobacteriology, clinical history, radiologic imaging, and the tuberculin skin test (Okoi et al., 2017). Moreover, the identification techniques involving culture and molecular biology necessary for diagnosing non-tuberculous mycobacterial diseases are prohibitively costly for regular clinical application in under-resourced health systems, where such diagnostics are not prioritized. There exists a legitimate concern that inconclusive results derived from smear microscopy or clinical and radiological assessments may result in misdiagnosis of non-tuberculous mycobacterial pulmonary disease and potentially lead to inadequate management of pulmonary cases (Okoi et al., 2017). For instance, *Mycobacterium abscessus* which is one of the NTM species causing NTMPD reported to be multidrug-resistant, and it's emerging as an important global threat to individuals with cystic fibrosis (Bryant *et al.*, 2016). Consequently, multidrug-resistant NTM are potentially emerging to be causing relapse and reinfection (Faverio *et al.*, 2016).

At present, the therapeutic approach for nearly all NTM infections relies on macrolide antibiotics, including clarithromycin or azithromycin (Saxena *et al.,* 2021). However, for Slow Growing group, for example *Mycobacterium avium* (MAC), *Mycobacterium intracellularae* (MI), *M. szulgai* (MZ), *M. kansasii* (MK) and M. *smiae* (MS), their regimen also includes ethambutol and rifampicin (Ceyhan *et al.,* 2019). While for rapid growers Mycobacterium (RGM) e.g. M. *fortuitum, M. chelonae,* and *M. abscessus*, their regimen includes an aminoglycoside and either cefoxitin, imipenem or tigecycline (Brown-Elliott & Woods, 2019; Kwon, Levin, *et al.,* 2019).

The efficacy of these treatments is primarily based on empirical evidence, with durations extending up to 18 months. They tend to be expensive and frequently come with a range of toxicities and side effects. Moreover, a significant impediment lies in the limited susceptibility of NTMs to the majority of antibiotics, including those employed in the treatment of MTB (Wu et al., 2018). This situation is concerning, as it suggests the potential for a significantly elevated rate of drug resistance in NTM, which may result in a scarcity of viable treatment options. Resistance may manifest as either intrinsic or acquired; intrinsic resistance refers to the inherent characteristics of an organism that enable it to withstand a specific drug or endure in a chemically adverse environment (Huh et al., 2019). The intrinsic resistance mechanisms of NTMs to antibiotics encompass their robust, impermeable cell walls and their existence within biofilms and granulomas, which significantly reduce drug uptake. Additionally, there is the expression of proteins that specifically interact with clinically utilized antibacterial agents (Luthra et al., 2018). Conversely, acquired resistance refers to the phenomenon in which a resistant strain develops from a population that was initially susceptible to the drug (Huh et al., 2019). The

occurrences in question are typically associated with the extended antibiotic therapies necessary for the treatment of NTM infections. The resistance that is acquired is notably pronounced in non-tuberculous mycobacteria (NTMs) possessing only a solitary copy of genes that code for widely recognized target proteins, including ribosomes. This circumstance heightens the likelihood of developing protective mutations in response to single-drug therapies (Moon et al., 2016).

There is a scarcity of information regarding treatment protocols for NTM; however, research indicates that antimycobacterial susceptibility testing (AST) is not typically performed prior to initiating treatment for NTM, except in cases of non-responsive disease caused by SGM (M. avium complex, M. kansasii) or infections due to RGM. This practice may lead to the development of drug resistance (Sharma & Upadhyay, 2020). The administration of treatment for NTM is typically sustained for a duration of 12 months following the conversion of sputum cultures. The response to this treatment in cases of NTMPD exhibits variability, influenced by the specific NTM species isolated and the severity of the underlying NTMPD (Sharma & Upadhyay, 2020). The prevalence of pulmonary diseases attributed to non-tuberculous mycobacteria is rising at a concerning pace, to the point where it is exceeding that of tuberculosis in numerous nations (Wu et al., 2018). Moreover, existing chemotherapeutic approaches necessitate extended durations of treatment, and the resultant clinical outcomes frequently fall short of expectations. Consequently, it is imperative to embark on the creation of novel drug models and advancements to expedite the drug discovery process, resulting in more effective anti-NTM medications with reduced toxicity compared to those currently available.

2.7 Genotypic characterization of non-tuberculous mycobacteria

For many years, Mycobacterium tuberculosis and Mycobacterium leprae have been the principal species of the genus Mycobacterium that garner clinical and microbiological attention (Fedrizzi et al., 2017). The remaining members, particularly NTM, have been subject to scrutiny for an extended period. As previously indicated by Brosch et al. (2002), it was posited that several gene deletions suggest that tuberculosis evolved from a common ancestor, specifically M. proto-tuberculosis or M. canetti, and subsequently from M. bovis (Brosch et al., 2002). Nonetheless, a recent study has yielded genomic data that has significantly enhanced our understanding of the genetic characteristics of mycobacteria as a non-tuberculous species. Veyrier et al. (2011) indicate that the genetic events imply a more suitable evolutionary pathway, positing that MTBC has its origins in NTM, as noted by Jenkins et al. (2017).

Nevertheless, numerous NTM species are currently acknowledged as significant infectious threats, and a comprehensive genomic analysis has yet to be conducted in a systematic manner (Yeung et al., 2016). Considering the concurrent genomic deletions and gene acquisition through horizontal gene transfer, alongside the evolution of M. tuberculosis into a more specialized pathogen, it is noteworthy that, despite the advancements in whole genome sequencing technologies, there has been a paucity of efforts directed towards the genetic characterization of NTM species. Consequently, the taxonomic and phylogenetic framework of the genus is still unresolved, and there is a deficiency of genomic data to facilitate the identification of these organisms in clinical contexts (Fedrizzi et al., 2017). For instance, it remains uncertain whether the characterized virulence factors present in M. tuberculosis and the extensively researched NTM species, such as proline-glutamate/proline-proline-

glutamate motif proteins (PE/PPE), the ESX export systems, the mammalian cell entry (Mce) protein family, the Sec-dependent general secretion system, and the Twin-arginine translocase (Tat) export system are prevalent across the numerous NTM species lacking available genomic data (Fedrizzi et al., 2017).

2.8 Phenotypic and molecular identification of NTM

The Mycobacterium tuberculosis complex and non-tuberculous mycobacteria can exhibit similar clinical presentations; however, the treatment regimens for each are markedly distinct (Singh et al., 2013). NTM are environmental organisms that opportunistically induce diseases in animals or humans, and they are being increasingly acknowledged as pathogens in humans. Consequently, accurately diagnosing NTM infection is essential for formulating an effective treatment strategy. Discerning NTM from MTB infection presents a significant challenge in numerous clinical settings, necessitating the prompt and precise identification of the pathogens (Yu et al., 2016). The clinical manifestations frequently exhibit considerable similarities between NTM and MTB infections, significantly complicating the diagnostic and therapeutic processes for diseases caused by MTB and NTM (McGrath & Anderson, 2010). Traditional approaches for the identification of mycobacteria rely on various factors, including colony morphology, pigmentation, growth rate on Lowenstein-Jensen slant medium, and outcomes of biochemical assays such as the niacin test, nitrate reduction test, Tween 80 hydrolysis at 7 and 14 days, urease activity, 5% NaCl tolerance, and arylsulfatase activity at 3 and 14 days (Lee et al., 2010; Singh et al., 2013). Furthermore, traditional techniques such as the smear for ZN staining of AFB

are expedient; however, they fail to distinguish between MTBC and NTM (Singh et al., 2013).

Given that the management of mycobacterial infections is contingent upon the specific species involved, it is imperative to achieve rapid detection and identification of the infecting mycobacterial species. This is essential for administering targeted chemotherapy and enhancing patient management outcomes. The conventional approach involving PNitro benzoic Acid (PNB) /TCH culture requires several weeks for completion and is limited to differentiating NTM from MTB, lacking the capability to classify NTM (Yu et al., 2016). Accurate identification of species is crucial, as non-tuberculous mycobacteria exhibit varying degrees of clinical significance. Moreover, there are significant differences in their growth rates, temperature tolerances, and susceptibilities to drugs (Malama et al., 2014). The diagnosis of NTM disease is intricate and necessitates effective communication among clinicians, radiologists, and microbiologists. Nonetheless, due to the constraints in sensitivity and specificity associated with symptoms, radiological assessments, and direct microscopic examination of clinical samples, culture continues to be regarded as the gold standard in the laboratory diagnosis of non-tuberculous mycobacteria. Culture, while requiring considerable time investment, necessitates the utilization of various media types and incubation temperatures to achieve optimal yield.

The traditional biochemical assays employed for the identification of various mycobacterial species are intricate and require considerable time (Marzouk et al., 2011). As a result, various methodologies have been established for the swift differentiation and identification of distinct Mycobacterium species, facilitating effective disease treatment. In recent years, gene sequencing techniques have been effectively utilized for the swift classification of species. The 16S rRNA gene and ITS sequence function as complementary techniques for the genotyping of species (Dastranj et al., 2018; Roth et al., 1998). Moreover, a range of genetic probes and amplification methodologies for the diagnosis of tuberculosis have been established, with several of these now accessible as commercial kits designed for the direct detection and identification of Mycobacterium tuberculosis complex and non-tuberculous mycobacteria in clinical samples (Lee et al., 2010). Recent methodologies, including high-performance liquid chromatography, chemiluminescent DNA probes, nucleic acid amplification, and sequencing of 16S ribosomal RNA genes, exhibit a level of sophistication that is accompanied by significant costs and the necessity for advanced equipment (Ichiyama et al., 1997). Recently, the World Health Organization initiated a mission aimed at minimizing the duration required for culture, identification, and drug resistance detection to a maximum of 2 days. This endeavor involves the utilization of line probe assays (LPA), such as the GenoType® Mycobacterium common mycobacteria/additional species (CM/AS) assay, a novel commercial kit designed to differentiate and identify various species of non-tuberculous mycobacteria (NTM) from cultures (Singh et al., 2013).

2.9 Anthropometric and demographic characteristics

Anthropometric measurements are noninvasive and readily accessible metrics that serve a diverse array of purposes in both pediatric and adult demographics (Casadei & Kiel, 2022). In adults, body measurements serve as a valuable tool for evaluating health and dietary status, as well as potential future disease risk (Fryar et al., 2016). Similarly, socio-demographic characteristics contribute to understanding the determinants of a disease within a specific study population. For example, in Europe, males exhibit a higher propensity for developing NTM PD compared to females (Prevots & Marras, 2015). A comparable pattern was noted in Central and South America, particularly in Brazil, where 62% of males exhibited a higher likelihood of being infected by NTM PD. In the Middle East and South Asia, specifically Turkey, it was reported that 87.1% of males were more likely to contract NTMPD compared to their female counterparts (Bicmen et al., 2010; de Mello et al., 2013). The heightened incidence of NTM infections among males compared to females may be attributed to a variety of factors, including socioeconomic dynamics. For example, within certain African cultural contexts, males often assume the role of primary breadwinners, which can lead to circumstances where they are compelled to engage in work within detrimental environments. The timely identification of metabolic and developmental issues within pediatric populations is essential for the implementation of effective treatment strategies. Nonetheless, in adults, these assessments can serve to gauge the severity of conditions such as obesity and cognitive impairments, as well as to track patients longitudinally to ascertain their progress following therapeutic interventions. Adult body measurements serve as a valuable tool for assessing contemporary dietary practices, overall health, and potential risks of illness. Adults may utilize these measurements to assess their body composition,

thereby identifying obesity and evaluating their fundamental nutritional status (Casadei & Kiel, 2022).

2.10 Conceptual framework

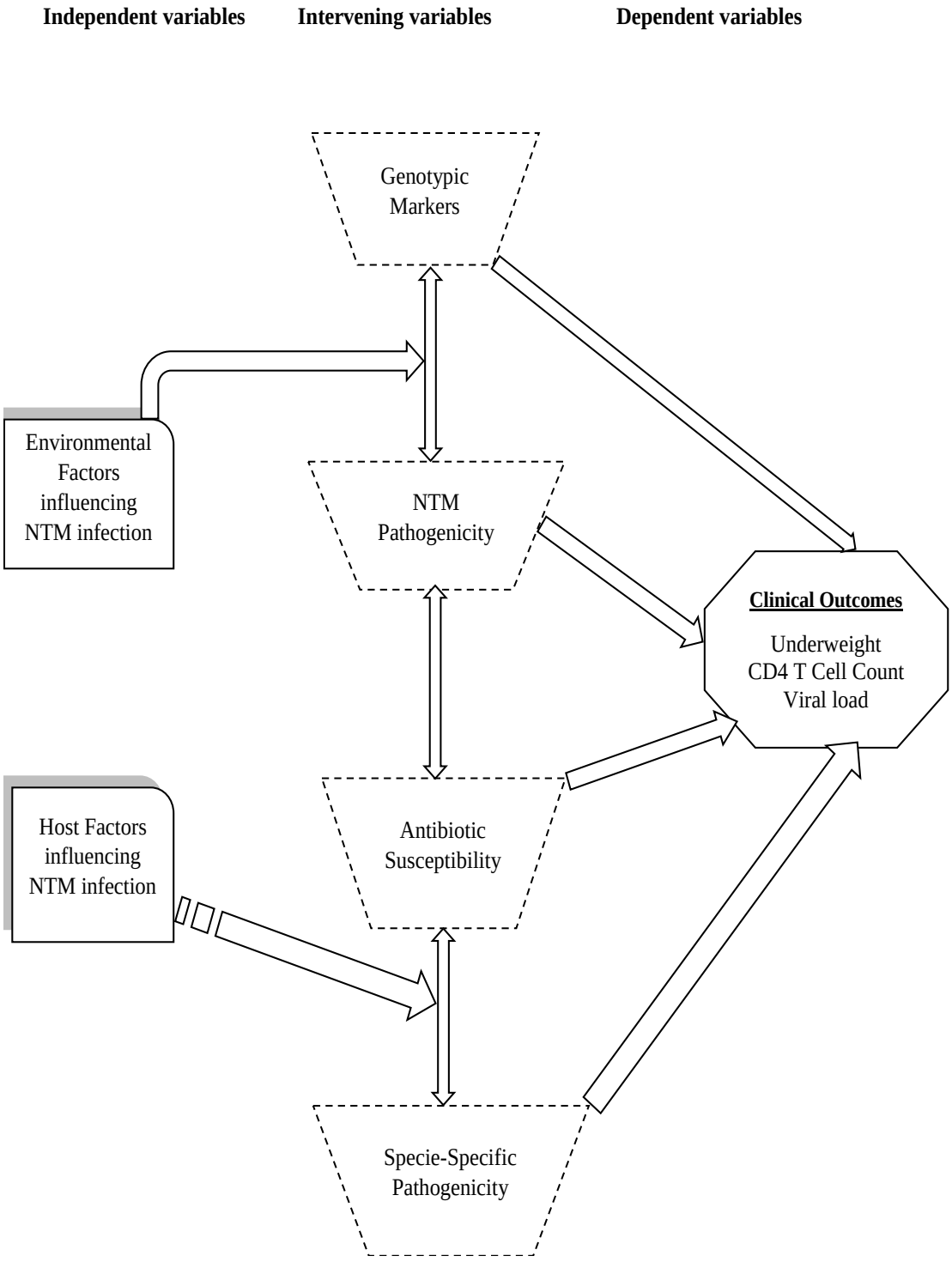


Figure 2.1 Conceptual framework describing the inter-relatedness of the variables

This study's conceptual framework offers a systematic method to comprehend the determinants affecting NTM infection and its consequences. Environmental variables encompass aspects like water and soil pollution, which increase exposure risk. Host variables, such as immunosuppression, socioeconomic status, and co-morbidities, significantly influence individual susceptibility to infection. The environmental and biological circumstances collectively constitute independent variables that enhance susceptibility and predisposition to NTM infection. The framework subsequently presents intervening variables that encapsulate distinct traits and resistance mechanisms inside NTM, influencing the progression and severity of infection. The pathogenicity of NTM, or the organism's intrinsic capacity to induce disease, differs among various species. The species-specific pathogenicity factor recognises that not all NTM species possess identical disease-causing capabilities, potentially affecting infection advancement and therapeutic response. Genetic markers within NTM are regarded, as these genotypic indicators are frequently linked to antibiotic resistance. Antibiotic susceptibility, the response of diverse NTM strains to different treatments, is essential in assessing treatment efficacy and illness outcomes. The intervening variables denote the biological and genetic characteristics within the bacterium that alter the effect of infection on the host. The results of these interactions are depicted in the framework's dependent variables, especially emphasising clinical outcomes that indicate the impacts of both the independent and intervening variables. Essential clinical outcomes encompass indicators such as underweight status, potentially indicative of chronic infection or malnutrition; CD4 T cell count, a metric of immune function particularly pertinent in individuals with HIV; and viral load, which signifies viral activity and may correlate with the severity of infection and treatment response.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study was done among HIV positive patients presenting with presumptive TB in Bungoma County Referral Hospital comprehensive care centers. Bungoma County borders the Republic of Uganda to the West, Teso North and Nambale sub-counties (Busia County) to the South West, Matungu sub-county (Kakamega County) to the South, Kiminini, Saboti (Trans-nzoia County), Lugari sub-counties and Malava sub-county (Kakamega County) to the North East. The population of Bungoma is estimated at 1.7M which constitute 52% female and 48% male, age percentage distribution stands at; 0-14 years 45.9 %, 15-64 years 51.4 % and over 65 years 2.3% with literacy level of 60.5%, poverty level at 52.9% and the County occupies an area of 2,069 km^2 (Gitaka *et al.*, 2018)**.** It lies between 1,200 and 1,800 meters above sea level and experiences mean temperatures of 23 degrees centigrade. Its latitude stands at 0.57 with the longitude of 34.56.

The region undergoes two distinct rainy seasons: the prolonged rains from March to July and the brief rains from August to October, with average annual precipitation ranging from 1200 mm during the shorter rainy period to 1800 mm during the extended rains. Bungoma County is home to a total of 136 health facilities, comprising 11 hospitals, 78 dispensaries, 16 health centers, 27 medical clinics, and 134 community units; however, BRCH remains a notable mention. A significant portion of the land within the county, exceeding 80%, is suitable for cultivation, and the prevailing climatic conditions are conducive to the growth of a diverse array of both food and cash crops. The cultivated crops include maize, finger millet, beans,

sweet potatoes, bananas, sorghum, Irish potatoes, along with an assortment of vegetables. The primary cash crops encompass sugarcane, tobacco, coffee, tea, sunflower, cotton, and palm oil. The primary livestock maintained in the county includes cattle, sheep, goats, and poultry, with a focus on indigenous breeds.

3.2 Study Design

A laboratory-based cross-sectional study was adopted. The main outcome group was HIV positive coinfected with NTM patients presumed to have TB. Cofounders were partly controlled by comparing the findings with HIV negative-NTM positive group.

3.3 Study Population

The study participants were recruited from adults living with HIV-1 and having presumptive TB attending the Bungoma County Referral Hospital Comprehensive Care Clinic.

3.3.1 Inclusion criteria

The study enrolled consenting adult who were HIV-1 but naïve to HAART, as well as those already on 1st HAART regime comprising of TDF+3TC+EFV presenting with TB-like symptoms such as chronic productive cough lasting more than 2 weeks, loss of appetite, fever, fatigue, headache and night sweats. Since some NTM are natural flora, a presumably “healthy control” of HIV negative participants was included matching the main outcome group of HIV-1 positive individuals. The 167 NTM infected participants were proportionately apportioned 94 (HIV negative) and 73 (HIV-1 positive).

3.3.2 Exclusion criteria

Participants without consent to the study were excluded, and HIV-1 patients having other commodities other than TB/ TB like conditions.

3.4 Sampling design

Purposive sampling was employed to choose participants seeking medical care at Bungoma Referral County Hospital in Bungoma County. All HIV-positive people exhibiting tuberculosis-like symptoms, including a persistent productive cough persisting for over two weeks, anorexia, fever, lethargy, cephalalgia, and nocturnal hyperhidrosis, were enrolled in the study.

3.5 Sample Size Determination

Sample size will be determined using Cochran's formula (Cochran, 1977) while considering prevalence 12.8% of NTM among people living with HIV in western Kenya (Achayo *et al.,* 2023)

$$n = \frac{Z^2 p(1-p)}{e^2}$$

$$n = \frac{1.96^2 \times 0.118(1-0.118)}{0.05^2}$$

$$n = \frac{0.4533088 \text{ x} 0.882}{0.0025}$$

$n = 160$

Where n=

sample

size,

Z=constan

t=1.96

P is prevalence=11.8%

e=error margin

10% of the 160 participants were added to cater for non-response, that is 160x0.1=16. Therefore, 176 participants were recruited.

However, 167 participants produced quality sputum that was used, while 9 participants did manage to give quality sputum.

3.6 Anthropometric and demographic characteristics

The subsequent anthropometric measurements were conducted by qualified clinicians in accordance with the established methodologies outlined by (Cameron & Scheepers, 2022). The collection of demographic and anthropometric data, including age, gender, height, weight, and locality of the participant, was conducted utilizing a laboratory request form. All anthropometric measurements were conducted by trained clinicians. The participants' weight was recorded in lightweight clothing to the nearest 0.1 kg, and their height was assessed using a measuring tape to the nearest 1.0 cm while they stood in an upright position. Waist circumference (WC) was assessed with precision to the nearest 0.1 cm at the narrowest point between the iliac crest and the lower rib during minimal respiration. The measurement of hip circumference (HC) was conducted with precision, recorded to the nearest 0.1 cm at the point of maximum girth of the buttocks. The measurement of middle upper arm

circumference (MUAC) was conducted at the midpoint, recorded to the nearest 0.1 cm, between the acromion and olecranon process. A non-stretched measuring tape was utilized while the right arm was allowed to hang in a relaxed position. The calculation of the body mass index (BMI, kg/m2) was performed. BMI was categorized into underweight (<18.50 kg/m^2), normal (≥18.50 ≤ 24.99 kg/m2), overweight (≥25.00 ≤ 30.00 kg/m^2), and obese (≥30.00 kg/m^2).

3.7 Sample collection

Sputum samples were collected in leakproof plastic containers with blue screw top lids tubes having a capacity of 5ml. At least 3 ml of three sputum specimens (spot, early morning, spot) was collected from participants with suspected TB/NTM under the supervision of trained and competent medical staff in 2 falcon tubes ("1" and "2). Three sputum specimens (spot, early morning, spot) were collected from participants. Using a marker pen, sample containers were labelled with a unique code specific to each patient, date of birth, collection date, time and gender was recorded. The patients were instructed to provide three samples on consecutive days, because lesions drain intermittently, samples collected may give contrasting results on different days. The sample in set "1" of the sterile falcon tubes were examined microscopically for Mycobacterium infection after staining specimens using the ZN method. Samples were processed within 7 days of collection in order to minimize loss of viability of the mycobacteria. This was done at BCRH laboratory in Bungoma County. The samples that were Ziehl-Neelsen (ZN) stain positive, their set Samples in "2" of falcon blue tubes were refrigerated at 4°C awaiting transportation in cool boxes to the Mycobacteria Reference Laboratory, at the National Public Health Reference Labs (NPHLs) for analysis. At NPHLs the samples were refrigerated at 4°C till processing. Further analysis at NPHLs were culture, NTM antimicrobial drug susceptibility and molecular procedure. Blood samples were obtained into vacutainer Brand SERILE interior EDTA (K3) tubes from consenting patient to screen for HIV, viral load and CD4+ count.

3.8 Laboratory Procedures

3.8.1 HIV testing

The HIV testing protocol for each patient comprised a dual-step methodology that ensured precise diagnosis and quality assurance. A qualitative immunochromatographic (lateral flow) assay for HIV-1 antibodies was initially conducted utilizing the Alere HIV-1/2 Rapid Test from Japan. This assay, intended to identify HIV-1 antibodies in plasma specimens, commenced with plasma collected from patients via venipuncture, thereafter processed to isolate the plasma for analysis. A designated volume of plasma, usually a drop or several microliters according to the test kit's guidelines, was introduced into the sample well of the testing apparatus. A buffer solution was then introduced to facilitate the sample's migration across the membrane strip via capillary action. As the material traversed the membrane, it interacted with specific antibodies or antigens fixed in the test region, yielding findings in around 15–20 minutes.

The outcomes of this preliminary test were interpreted according to the visibility of colored lines on the membrane. A solitary line in the control region signified a negative result, but two lines—one in the test region and one in the control region denoted a positive result for HIV-1 antibodies. Any outcome displaying a discernible line in the test area was deemed reactive, irrespective of intensity, and necessitated a confirmatory test. The Genscreen HIV-1/2 ELISA from Bio-Rad Laboratories in France was utilized for confirmation. The enzyme-linked immunosorbent assay (ELISA), recognized for its elevated sensitivity and specificity, was performed on plasma samples from patients who yielded positive results in the fast test.

The confirmatory test entailed introducing the plasma sample into wells of a microplate that was coated with HIV antigens. The plate was incubated to facilitate the binding of possible HIV-1 antibodies in the plasma to the antigens on the well

surface, followed by the removal of unbound components to avert any non-specific reactions. A secondary antibody, conjugated to an enzyme, was subsequently introduced to bind to any HIV antibodies present in the sample. Subsequent to a further incubation period, a substrate was introduced, interacting with the enzyme to elicit a color change in wells containing antibodies. The extent of color change was assessed spectrophotometrically and associated with the presence of HIV antibodies, where any color change exceeding the kit's stated cutoff value confirmed the presence of HIV antibodies.

Quality control methods were upheld throughout the operation to guarantee correctness. Each batch of ELISA tests incorporated positive and negative controls, thereby confirming the accuracy and consistency of the assays. All testing procedures were performed under standardized operating settings to avert cross-contamination, with findings evaluated by qualified staff to guarantee adherence to quality standards. This thorough two-step testing methodology first employing fast screening for sensitivity and subsequently utilizing ELISA for specificity yielded dependable HIV diagnoses.

3.8.2 CD4+ T Cell count

The quantification of CD4+ T cell levels was conducted using a BD FACSCalibur flow cytometer (Becton-Dickinson™, Franklin Lakes, USA), facilitating accurate measurement of immune cells in the blood sample. Initially, 5.0 µl of whole blood anticoagulated with EDTA was transferred into a flow cytometry tube. A red blood cell (RBC) lysis buffer was subsequently included to eliminate red cells from the sample, facilitating enhanced visualisation and analysis of white blood cells, including CD4+ T cells. The sample was treated with the lysis buffer for

approximately 5 minutes, resulting in the efficient lysis of red blood cells. Subsequent to the incubation, the sample was rinsed to eliminate any remaining lysis buffer and debris, so assuring a purer preparation for antibody staining. Subsequent to the wash process, fluorescently labelled antibodies were introduced to the cell sample. The antibodies included anti-CD3, anti-CD4, and anti-CD45, each targeting distinct surface markers on immune cells. The anti-CD3 antibody targets a marker found on all T cells, anti-CD4 particularly binds to CD4+ T cells, and anti-CD45 attaches to a common leukocyte antigen, facilitating the differentiation of white blood cells from other cell types.

The cells were treated with these antibodies for 30 minutes to facilitate optimal binding to their corresponding cell surface markers. Subsequent to the incubation period, the material underwent an additional wash to eliminate any unbound antibodies, thereby diminishing background fluorescence and enhancing the precision of the analysis. The processed sample was subsequently fed into the BD FACSCalibur flow cytometer, which employed lasers to activate the fluorescent tags on the antibodies. As cells traversed individually through the laser beam in the flow cytometer, the fluorescence of each cell was detected, recorded, and analysed. The flow cytometer measured the quantity of cells expressing CD4 within the total T cell population, therefore yielding an accurate enumeration of CD4+ T cells in the sample. This approach, by concentrating on cell-specific markers and implementing stringent washing processes, guaranteed accurate and dependable CD4+ T cell counts vital for clinical evaluation.

3.8.3 HIV viral load determination

The HIV viral load was quantified using an automated Abbott m2000 System (Abbott Molecular Inc., Illinois, USA), which ensured accurate measurement of viral

RNA in patient samples. Initially, 200 µl of serum served as the source material for RNA extraction. The extraction technique entailed isolating the viral RNA from plasma components and concentrating it for subsequent analysis. After the RNA extraction, it underwent reverse transcription, a process in which the RNA was transformed into complementary DNA (cDNA). The reverse transcription procedure was crucial since it facilitated the subsequent amplification and measurement of HIV genetic material. Subsequent to the reverse transcription phase, the cDNA was amplified utilising HIV-specific primers. These primers were engineered to target and attach to specific sequences within the HIV genome, facilitating the selective replication of solely HIV-related cDNA. The amplification, accomplished using a polymerase chain reaction (PCR) procedure, significantly enhanced the quantity of HIV-specific genetic material, becoming it detectable and quantifiable. Upon completion of amplification, the Abbott m2000 analyser identified the amplified HIV cDNA via fluorescently labelled HIV probes. These probes generated light signals corresponding to the quantity of HIV cDNA present. The analyser subsequently quantified the strength of the fluorescent signals, translating them into a measurable viral load result. This measurement, quantified as the number of viral copies per millilitre of serum, offered a precise evaluation of the patient's HIV viral load. The automated functionality of the Abbott m2000 System guaranteed elevated sensitivity and consistency during the procedure, providing a dependable method for monitoring viral levels in clinical treatment.

3.8.4 Sputum samples for Ziehl-Neelsen (ZN) staining for microscopy

The three sputum specimens collected from each PLWH with presumptive pulmonary tuberculosis were processed as per the standard protocol (Pingle *et al.,*

2014). N-acetyl-L-cysteine (NALC)-NaOH solution (5% NaOH+ 0.5% NALC) was added to sputum samples to liquefy and decontaminate the mucous sputum.

The mixture underwent centrifugation at a force of 3,000xg for a duration of 18 minutes at a temperature of 4℃. The supernatant was removed, and 1 mL of phosphate-buffered saline was introduced after the sediment had undergone vortexing. The sputum sample was initially applied to a glass slide, followed by fixation through heating at temperatures ranging from 65°C to 75°C within a Class 1 exhaust protective cabinet, until the smeared material was thoroughly dried and adhered to the slide. The stationary smear was subsequently inundated with potent carbol fuchsin and subjected to gentle heating for a duration of 3 to 5 minutes. Subsequently, thorough rinsing with water was conducted, followed by decolourisation for 2–3 minutes using a (3% v/v) acid-alcohol solution. Another rinse with water was performed, after which the slide was treated with fresh acid-alcohol for 3–4 minutes until a faint pink hue was achieved. The specimen was thoroughly rinsed with water, subsequently counterstained with a 1% w/v solution of methylene blue for a duration of 30 seconds, followed by another rinse with water, and then permitted to dry. A dry stained smear was observed under bright field microscopy, utilizing an immersion oil, with the apparatus produced by Olympus, USA. The focus was attained through the application of X10 magnification, while X100 magnification facilitated the examination of the stained specimen. Approximately one hundred fields were scrutinized for each slide. Mycobacteria, including both MTB and NTM, are classified as “acid-fast bacilli” (AFB) positive and appear as red rods when observed under a ZN stain (Grange et al., 1996). A

tuberculosis suspect was deemed ZN smear positive if at least one of the three samples exhibited pink or red rod-shaped bacteria upon microscopic examination.

3.8.5 Growth Characteristics of Non-tuberculous mycobacteria

After decontamination, each processed sputum sample was cultured onto Löwenstein-Jensen medium containing p-nitrobenzoic acid (PNB), 500 mg/litre (LJ-PNB medium) at 37°C for up to 8 weeks. Media was inoculated using disposable plastic loop (Grange *et al.,* 1996). The loops were Withdrawn from the suspension edgewise to avoid large convex drops being transferred. The LJ-PNB media were incubated at 37°C in an internally illuminated incubator. The cultures were inspected at 3, 7, 14 and 21 days, up to a maximum of 42 days. Growth was examined by visual inspection for colonies. Those cultures with growth were confirmed by Ziehl-Neelsen staining. Tubes with evident growth on the LJ-PNB slope medium were examined for pigmentation. Members of the *M. Tuberculosis* complex do not grow on LJ-PNB medium and do not produce yellow to orange pigment in the light or dark (Grange *et al.,* 1996). Isolates that grew in PNB modified L-J medium were considered as NTM. Additionally, Surface morphology, pigment production, the texture; whether smooth or rough, dome shaped or flat shaped, glossy or creamy white were the key cultural characteristics distinguishing the NTM species. Also, Pigmentation was used for differentiation factor among NTMs. Majority of the NTM produce yellow-orange pigmentation on L-J media, either in light (photochromogenic) or both in light and dark (scotochromogenic). Furthermore, incubation period was also used for differentiation; some NTM are RGM (grow

within 3 days) on LJ-PNB medium at 37^0C, while some NTM are SGM (grow after 3 days).

Utilizing the Mycobacterium growth indicator tubes (MGIT), samples were introduced into the tubes containing Middlebrook 7H9 broth and subjected to continuous incubation at 37°C. Their fluorescence was meticulously observed to ascertain whether the tubes yielded positive or negative results. The tubes that exhibited a positive reaction indicated that the sample harbored viable organisms. Culture tubes that exhibited a negative result for a minimum duration of 42 days and displayed no discernible indicators of positivity were extracted from the instrument, classified as negative, and subsequently discarded. The speciation of non-tuberculous mycobacteria was conducted utilizing Hain's genotype mycobacterium CM for the prevalent species, alongside Genotype mycobacterium AS assays for the supplementary species (Singh et al., 2013).

3.8.6 Determination of minimum inhibition concentrations (MICs)

The broth microdilution method was used to determine the minimum inhibitory concentration of the antibiotics for the NTM isolates, and the results were interpreted in accordance with the guidelines provided by the Standard Clinical and Laboratory Standards Institute (CLSI) (Brown-Elliott & Woods, 2019). A commercial radiometric medium made by Johnston Laboratories was utilized in the broth dilution technique, and the BACTEC 460-TB instrument was used to measure the CO_2 released as a result of the growth of NTM isolates in the 7H12B medium. The following concentrations of the different drugs were tested; streptomycin (STR):

≤0.5, 1, 2, 4, 8,16 and ≥64 µg/ml; isoniazid (INH), ≤0.25, 0.5, 1, 2 ,4, 8, and 8≥ µg/ml; rifampicin (RIF), ≤0.12, 0.25, 0.5, 1, 2, 4, 8, and ≥64µg/ml; ethambutol (EMB), ≤0.5, 1, 2, 4, 8 and ≥16 µg/ml. An appropriate amount of antibiotic stock was added to Middle Brook 7H9 broth, which already contained 100 mL of oleic acid/dextrose/catalase (OADC) growth supplement and 2 ml of glycerol, in order to obtain the necessary dilution (Figure 3.1). To make a solution for well injection, growing colonies were extracted from the LJ-PNB medium and used at a concentration of 1.5×10^5 colony-forming units (0.5 McFarland standard).

In 96-well microtiter plates, 100µl of 7H9 medium containing OADC was distributed. Serial concentrations were created for each antibiotic, followed by addition of 100 µl of bacterial suspension to each well. Parafilm and zip lock bags were employed to keep the microplates from drying out during the 2-week incubation period at 37℃ (Brown-Elliott & Woods, 2019). The MIC is the lowest amount of the antibiotic required to fully stop the NTM from growing (Inderlied *et al.,* 1987). And, the susceptibility was determined based on CLSI breakpoint recommendations and published studies (Brown-Elliott & Woods, 2019). As shown in appendix V. The reference strains that were used as part of this analysis included *M. kansasii* ATCC® 12478 for SGM and *M. peregrinum* ATCC® 700686 for RGM (Li *et al.,* 2017). Fast-growing mycobacteria were seen on day 5 of incubation in comparison to the growth of the positive control well; observations were made on days 10 through 14. Retesting the drug susceptibility test was advised if the growth of the positive control well did not improve by day 21 and indicated.



Plate 3 1 Showing catalase test

3.8.7 Molecular identification of Non-tuberculous mycobacteria using line probe assay for NTM

The molecular identification of non-tuberculous mycobacteria (NTM) was conducted via a combination of biochemical assays, DNA extraction, and a line probe assay. Initially, strains that tested negative for the Mycobacterium tuberculosis (MTB) complex were subjected to additional confirmation to classify them as non-tuberculous mycobacteria (NTM). This confirmation entailed multiple stages, including a negative niacin accumulation test that distinguishes MTB complex from NTM. The bacteria were also cultivated on Löwenstein-Jensen (LJ) media supplemented with paranitrobenzoic acid (PNB). The proliferation of mycobacteria in PNB media signified non-tuberculous mycobacteria, as Mycobacterium tuberculosis complex strains generally do not proliferate on this medium. The samples were subjected to a catalase test, yielding a positive result for NTM. A ribosomal RNA-based DNA hybridisation assay (Accuprobe® System, Gen-Probe Inc., San Diego, CA, USA) was employed, with a negative result further validating the material as NTM rather than MTB complex. Following biochemical confirmation, DNA was extracted from the NTM strains with the GenoLyse® kit, VER1.0 (Hain Lifescience, GmBH, Nehren, Germany), in accordance with the manufacturer's guidelines. The extraction procedure entailed disrupting cell walls to

liberate DNA, succeeded by purification to yield DNA appropriate for molecular identification. The isolated DNA was subsequently analysed using a line probe assay (LPA) for the identification of non-tuberculous mycobacteria (NTM). The GenoType® Mycobacterium CM, VER 1.0 (Hain Lifescience, GmBH, Nehren, Germany) was utilised for this objective. In accordance with the manufacturer's specifications, the DNA was amplified via PCR to enhance the quantity of target genetic material, facilitating improved detection.

The amplified DNA was subsequently applied to line probe strips, which contained probes specifically designed to bind to sequences present in common mycobacterial species. Every probe on the strip was coated with a DNA sequence that was complementary to the target sequences of diverse NTM species. Upon hybridisation with the sample DNA, distinct binding patterns emerged on the strip, facilitating species-level identification using the observed band patterns. Upon detection of NTM in a sputum sample, a subsequent request was issued to the treatment providers to get three consecutive sputum samples from the patient. This methodology conformed to the American Thoracic Society (ATS) standards to ascertain the presence of a genuine NTM infection. Each subsequent sputum sample underwent identical testing procedures, including smear microscopy, culture, and the line probe assay as previously delineated, to validate and comprehensively characterise the NTM infection. This thorough methodology guaranteed precise identification and suitable clinical follow-up for patients suspected of NTM infections.

3.8.8 GenoType MTBDRplus V.2.0 assay on NTMs

The GenoType MTBDRplus V.2.0 assay was conducted to evaluate the resistance profile of NTM strains, adhering to the methodology provided by Hain Lifescience GmbH. This assay employs DNA strip technology and consists of three primary

steps: DNA extraction, multiplex PCR amplification, and reverse hybridization. The approach commenced with DNA extraction from NTM cultures, involving cell lysis to liberate genetic material, succeeded by a purification step that produced a clean DNA sample devoid of cellular detritus. This DNA sample functioned as the template for subsequent amplification and hybridization processes. Subsequently, the extracted DNA was subjected to multiplex PCR amplification, a method that enables the concurrent amplification of several target DNA regions within a single reaction. The PCR amplification targeted genes linked to resistance in mycobacteria, including the *rpoB* gene for rifampicin resistance and the *katG* and *inhA* genes for isoniazid resistance. Primers targeting these resistance-determining areas were included into the reaction mixture, alongside the DNA template and other reagents. During thermal cycling, the primers hybridized to their corresponding target sequences, enabling DNA polymerase to replicate multiple copies of each target gene. This amplification produced a concentrated quantity of genetic material from the resistance-associated areas, prepared for detection in the subsequent stage. Subsequent to amplification, the procedure transitioned to reverse hybridization. The amplified DNA was denatured, resulting in the separation of double-stranded DNA into single strands, and subsequently applied to a membrane strip with immobilized probes. Each probe on the strip was tailored to either wild-type or mutant gene sequences linked to drug resistance. The sample DNA hybridized with corresponding probes on the strip, binding to specific sites according to the presence or absence of mutations. Following a wash phase to eliminate any unbound DNA, a substrate solution was introduced, which interacted with an enzyme conjugated to the DNA-probe complex, resulting in visible bands on the strip. The strip's band pattern was thereafter matched to the manufacturer's reference sheet for result interpretation. A specific banding

pattern signified the existence or lack of mutations linked to resistance against rifampicin and isoniazid.

3.8.9 LPA interpretation

The interpretation of LPA is illustrated in figure 3.3 below. The susceptibility of non-tuberculous mycobacteria to anti-TB drugs was characterized by the presence of hybridization to all wild-type probes, indicated by the presence of a band, and the absence of hybridization to mutant probes, signified by the absence of a band. The lack of hybridization observed in any wild type and/or the hybridization of any mutant gene signifies a resistance to the corresponding drugs. The hybridization of wild-type and mutant genes suggests the presence of hetero-resistance or a mixed infection.

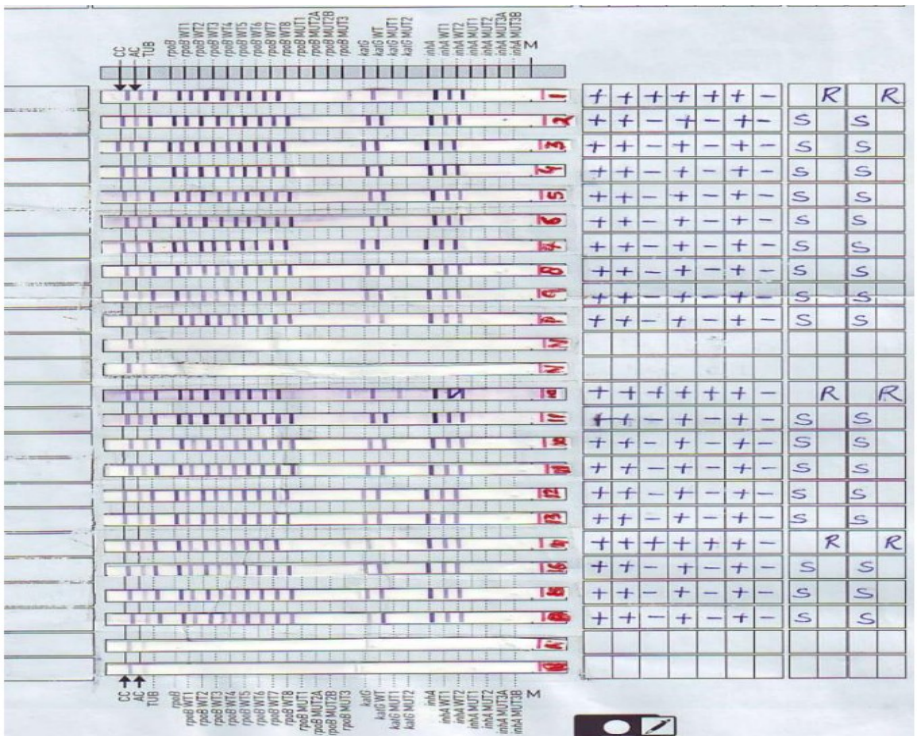


Plate 3 2 Showing resistant (R) and sensitive (S) probe hybridization on the NTM samples

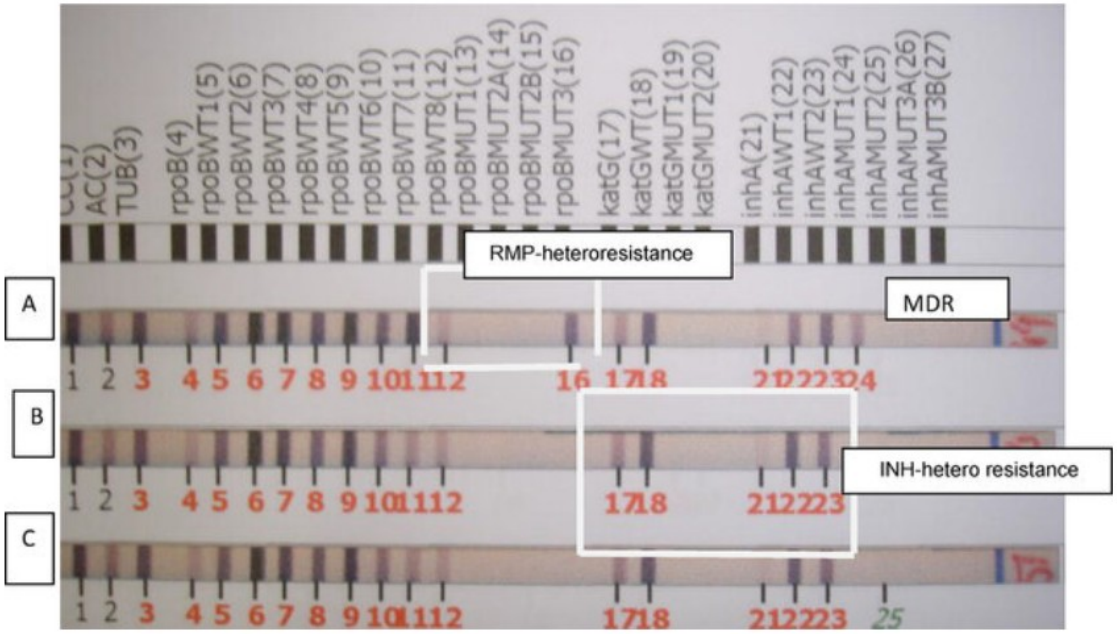


Plate 3 3 Showing the interpretation of LPA following probe hybridization

3.9 Quality Control

The collection of specimens was conducted with meticulous attention to aseptic techniques to mitigate potential sources of contamination, particularly from tap water, given the frequent presence of environmental mycobacteria. No fixative was employed. Systematic safety measures were implemented by gathering samples in sterile, leak-proof, disposable, and appropriately labeled containers that are approved for laboratory use.

Samples should ideally be refrigerated at 4°C if there is a delay of over 1 hour in transportation to the laboratory, aside from the use of transport media (Griffith et al., 2007). N-acetyl-l-cysteine–sodium hydroxide (NALC-NaOH) was employed to digest and decontaminate aerobic gram-negative rods, particularly Pseudomonas aeruginosa (Griffith et al., 2007). The criteria established by the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) were employed to

diagnose pulmonary NTM disease, thereby distinguishing between NTMPD and colonization (Okoi et al., 2017).

3.10 Management and Analysis of Data

The data gathered from participants underwent a coding process to ensure confidentiality was maintained. The results of the tests were meticulously documented within a password-protected Microsoft Excel spreadsheet. The analysis was conducted utilizing IBM SPSS version 23. Proportions of categorical variables, including gender and BMI category, were systematically summarized and presented in tabular form. Continuous variables, such as laboratory measures, age, height, weight, and BMI, were characterized through measures of central tendency. Mann Whitney The U test, or Student's t-test, was employed to analyze the laboratory measures across the clinical groups (HIV (-) versus HIV (+)). A Pearson correlation test and logistic regression were employed to ascertain the relationship between antimicrobial resistance in NTM isolates and clinical outcomes in HIV patients, specifically viral load, CD4+ count, and BMI.

3.11 Ethical Considerations

Approval for ethical considerations was obtained from the MMUST Institutional Ethical Review Committee, under protocol number MMUST/IERC/097/2022. The National Commission for Science, Technology, and Innovation (NACOSTI) has granted authorization for the study under permit number NACOSTI/P/23/22686. Additionally, approval was obtained from the Ethical Research Committee of

Bungoma County Referral Hospital (BCRH) to gather data from clients of BCRH. Furthermore, participants were approached to obtain their consent for the collection of samples pertinent to the study. Participation was entirely voluntary, allowing participants the freedom to withdraw from the study at their discretion. Participants received guidance from a qualified professional.

CHAPTER FOUR

RESULTS

4.1 Demographic characteristics of study participants

A total of 167 participants were recruited to the study at Bungoma County Referral Hospital (BCRH) and they were able to produce sputum. They presented with TB-like symptoms including chronic productive cough lasting more than 2 weeks, loss of appetite, fever, fatigue, headache and night sweats hence satisfied the inclusion criteria. Majority of participants were male 124 (74.3%) compared to female participants 43 (25.7%). Out of the total 167 participants, 73 (43.1%) were HIV positive co-infected with NTM while 95 (56.9%) were HIV negative but infected with NTM. The median age for cases was 41.0 and controls had 40.5 (Table 4.1). Statistically, there was no significance difference (P =0.806) in age both for PLWH coinfected with NTMs and HIV negative but are infected with NTM. However, the body mass index (BMI, kg/m^2) of PLWH coinfected with NTM was significantly different from the HIV negative but infected with NTM group (*P=0.026*). Similarly, the weight of PLWH coinfected with NTMS significantly differed from the HIV negative group with a (*P=0.046*). It was also noted that, majority of the cases were underweight (53.8%) compared to controls (46.2%).

Furthermore, comparable trends were noticed in the categories of normal and overweight. In the normal category, 39.5% of cases were within the normal range compared to 60.5% of control group. While 44.6% of cases were classified as overweight compared to 55.4% in the control category. Height did not differ substantially between cases and controls (P=0.258), same finding was observed for Mid upper arm circumference (MUAC), waist circumference, hip circumference and

bust circumference (Table 4.1). Although, there was no significant difference between PLWH-coinfected with NTM and HIV negative group in terms of the faith that one professes (*P*=0.390), majority of participants were protestants; PLWH-NTM 49 (52.1%) vs the HIV negative-NTM positive 39 (53.4%) followed by Catholics. The least participants were Muslims (table 4.1). Education did not differ significantly between the two groups, but it seems to be a risk factor for NTMs inflections in PLWH (*P*=0.155). Majority of infected participants were those who dropped out of school at primary level (60.3%) compared to those that reached secondary (30.1%) or tertiary level (9.6%) respectively.

The kind of job that one does was a significant risk factor for the infection of NTMs. Those having informal jobs were significantly infected with NTM compared to those with formal jobs (*P*=0.024). Marital status did not seem to influence the infection of NTMs in people living with HIV, although single people and separated people seemed to be mostly affected than married people. The characteristics of the participants are summarized in Table 4.1 below.

Table 4. 1: Anthropometric and demographic characteristics

Characteristics	HIV (-), n=94	HIV (+), n=73	*P*
Gender			
Female	25 (26.6)	18 (24.7)	0.776
Male	69 (73.4)	55 (75.3)	
Age, yrs.	40.5 (24.0)	41.0 (17.0)	0.806
Height, m	1.7 (0.1)	1.7 (0.1)	0.258
Weight, kg	68.1 (24.9)	60.4 (19.3)	**0.046**
BMI, kg/m^2	23.8 (7.3)	19.8 (8.5)	**0.026**
Nutrition status			
Underweight	12 (12.8)	30 (41.1)	
Normal	46 (48.9)	14 (19.2)	0.436[a]
Overweight	36 (38.3	29 (39.7)	
WC, cm	74.0 (9.3)	76.0 (9.5)	0.624
HC, cm	89.5 (9.0)	90.0 (6.3)	0.821
MUAC, cm	25.0 (4.0)	24.0 (2.0)	0.824
BC, cm	84.5 (6.3)	85.0 (7.0)	0.631
Religion			
Catholic Christians	32 (34.0)	21 (28.8)	
Muslim	13 (13.8)	13 (17.8)	0.390
Protestant Christians	49 (52.1)	39 (53.4)	
Education levels			
Primary	58 (61.7)	44 (60.3)	
Secondary	19 (20.2)	22 (30.1)	0.155
Tertiary	17 (17.1)	7 (9.6)	
Occupation			
Informal	65 (69.1)	38 (52.1)	**0.024**
Formal	29 (30.9)	35 (47.9)	
Marital status			
Divorced	14 (14.9)	11 (15.1)	
Married	19 (20.2)	15 (20.5)	
Separated	43 (45.7)	22 (30.1)	0.059
Single	16 (17.0)	25 (34.3)	
Widow	2 (2.1)	0 (0.0)	

Data are presented as medians, interquartile range (IQR) or numbers (n) and percentages (%) of study subjects. HIV-1(+), human immunodeficiency virus type-1; BMI, body mass index; Normal; ≥18.5≤25.0 kg/m^2, Underweight <18.5 kg/m^2, Overweight ≥25.0 kg/m^2. MUAC, mid upper arm circumference, WC, waist circumference, HC, hips circumference, BMI; body mass index; BC, Bust circumference, *P*, Fisher's exact tests; [a]Chi-square test and Mann Whitney U test for continuous data. Bolded are significant P-values.

4.2 Clinical Signs and Symptoms Associated with NTM infections

The findings of this study showed, majority of the participants had clinical signs associated with NTM infections that are similar to MTB. The most common clinical signs were purulent sputum samples (90.4%), chronic cough lasting more than 2 weeks (86.3%) and fatigue (71.3%). Similar characteristics were observed in the HIV negative group with no observable difference. Summary is shown in table 4.2 below.

Table 4. 2: Clinical sign and symptoms of study participants

Characteristics	**HIV (-), n=94**	**HIV (+), n=73**	***P***
Chronic cough≥ 2weeks	71 (75.5)	63 (86.3)	0.786
Purulent sputum	73 (77.7)	66 (90.4)	0.859
Weight Loss	64 (68.1)	59 (80.8)	0.842
Night Sweats	67 (71.3)	61 (83.6)	0.678
Malaise	63 (67.0)	62 (84.9)	0.789
Fatigue	68 (72.3)	59 (80.8)	0.895
Hemoptysis	2 (2.1)	5 (6.8)	0.309
Fever	54 (57.4)	48 (65.8)	0.467

Data are presented as numbers (n) and percentages (%) of study subjects.
HIV-1(+), human immunodeficiency virus type-1; *P*, Fisher's exact tests;
Bolded are significant p values.

4.3 Sputum smear for microscopy

Sputum samples were collected from 73 participants who were HIV positive and 94 HIV negative participants. Among the PLWH, 33/73 (45.2%) were AFB microscopy positive with ZN staining procedure and 40/73 (54.8%) tested negative. Compared to participants who were HIV negative 19/94 (20.2%) were AFB microscopy positive while 75/94 (79.8%) were AFB negative. There was no significant difference between the two groups in terms of the microscopical results (*P=0.868*). However, there was a considerable difference in grading sputum smear results between the two groups (*P=0.025*). Most of the PLWH participants had category 3+

and 2+ grades compared to the HIV negative group 17/33 (51.5%) vs 1/19 (5.3%) respectively. The summary of AFB microscopy results and grading is shown in table 4.3 below.

Table 4. 3 Sputum samples for ZN staining for microscopy from HIV (+) Patients in BCRH

Smear Examination	**HIV (-), n=94**	**HIV (+), n=73**	***P***
AFB (+)	19 (20.2)	33 (45.2)	0.868
AFB (-)	75 (79.8)	40 (54.8)	
Smear grading results	n=19	n=33	
Scanty	8 (42.1)	5 (15.2)	
+	5 (26.3)	3 (9.1)	[a]**0.025**
2+	5 (26.3)	8 (24.2)	
3+	1 (5.3)	17 (51.5)	

Data are presented as medians, interquartile range (IQR) or numbers (n) and percentages (%) of study subjects. HIV-1(+), human immunodeficiency virus type-1; (+) denotes positive while (-) negative. Accordingly, sputum smear is graded as scanty, 1+, 2+, and 3+. Scanty is when the sputum contains 1–9 AFB in 100 fields, grade 1+ for 10–99 AFB in 100 fields, grade 2+ if 1–10 AFB per field, and grade 3+ for more than 10 AFB per field, respectively. *P*, Fisher's exact tests; [a]Chi-square test and Bolded are significant P values

4.4 Sputum sample cultures on LJ-PNB and M960 tube from BCRH

4.4.1 Primary culture of sputum samples on LJ-PNB and M960 tube

The 167 samples collected from the participants in BCRH were inoculated into the M960 system and LJ-PNB medium at 25°C and 37°C, respectively. The prevalence of NTM by culture combining both MGIT 960 and LJ-PNB media method was 43.8 %. The results of culture showed that PLWH had 32/73 (43.8%) growth compared to 23/94 (24.5%) from the HIV negative group (*P=0.286*). Up on using MGT 960, equally 32/73(43.8%). However, the number of contaminated samples were notably less in LJ-PNB media method as opposed to MGIT 960 method; 1/73(1.4) vs 5/73(6.6). similar observation was noted in HIV negative group 2/94(2.1) vs 7/94(7.4) respectively.

Table 4. 4 Primary culture of sputum on LJ-PNB and MGT960 tube for growth of mycobacteria

Characteristics	HIV (-), n=94	HIV (+), n=73	*P*
LJ-PNB media			
Growth Reported	23 (21.3)	32 (41.1)	
No growth Reported	69 (73.4)	40 (54.8)	0.286
Contaminated	2 (2.1)	1 (1.4)	
MGIT 960 system			
Positive	25 (26.6)	32 (43.8)	
Negative	62 (66.0)	36 (49.3)	0.951
Contaminated	7 (7.4)	5 (6.8)	

Data are presented as medians, interquartile range (IQR) or numbers (n) and percentages (%) of study subjects. HIV-1(+), human immunodeficiency virus type-1; (+) denotes positive while (-) negative. L-J media is Löwenstein-Jensen media; *P*, Chi-square test. Bolded are significant *P*-values.

4.4.2 Subculture of MGT960 contaminated tubes onto LJ-PNB and Fresh MGIT 960 tubes

The 12 contaminated samples from MGIT 960 tubes were reprocessed to attempt and get valid results. New MGIT 960 tubes were used, 2 samples turned positive.

4.5 Cultural characterization of Non-Tuberculous Mycobacteria in HIV patients

4.5.1 Cultural characterization of Non-Tuberculous Mycobacteria species

After primary culture and subculture from MGT960 tube, a total of 59/167 (35.3) samples had growth on LJ-PNB media 33/73 (45.2%) from PLWH and 26/94 (24.5%) from HIV negative participants respectively. While 108/167 (64.7%) samples had no growth. The 59 positive samples were culturally differentiated to NTM species based on: Surface morphology, pigment production, the texture, whether smooth or rough, dome shaped or flat shaped, glossy or creamy white. Majority of the NTM produce yellow-orange pigmentation on LJ-PNB media, either in light (photochromogenic) or both in light and dark (scotochromogenic) as opposed to MTBC that do not produce pigments. Incubation period and temperature

were also the among the distinguishing factors that were considered; some NTM are RGM (grow within 3 days) on LJ-PNB at 37 ^{0}C, while some NTM are SGM (grow after 3 days). The summary of cultural characteristic is presented in table 4.5.

Table 4. 5 Characterization of NTM isolates grown on LJ-PNB media at 25°C and 37°C

Colonial morphology	**LJ-PNB at 25°C**	**LJ-PNB >3 days (37°C) RGM**	**LJ-PNB <3 days (37°C) SGM**	**Pigment production**	**Isolates**
Smooth, uniform dome shaped and creamy white-yellow	+	-	+	Yellow pigment	***M. intracellulare***
Smooth, uniform and glossy appearance	+	+	-	No pigment produced	***M. fortuitum***
-smooth, moist round creamy white -in light- yellow	+	-	+	Yellow pigment	***M. kansasii***
Glossy, uniform dome shaped colonies appearing yellow orange	+	-	+	Yellow-orange in presence of light/dark-Scotochromogenic	***M. scrofulaceum***
Glossy, uniform orange shaped colonies appearing orange	+	-	+	orange in presence of light/dark - Scotochromogenic	***M. gordonae***
Glossy, flat shaped colonies appearing yellow orange	+	-	+	Yellow pigment when exposed to in light-photochromogens	***M. simiae***
Smooth, uniform dome shaped and creamy white	+	-	+	Yellow-orange pigment	***M. avium***
Rough irregular, dry and corded appearance	+	+		No production of pigments	***M. abscessus***
Glossy, uniform flat shaped yellow colonies	+	-	+	Yellow pigment when exposed to light-photochromog	***M. lentiflavum***

				ens	

Data presented as incubation period; time taken before the growth is noticed, presence of pigmentation and smooth colony morphology and colonies growing within 3 days or after. (+) denotes growth reported, (-) no growth reported. L-J-PNB Löwenstein-Jensen medium containing p-nitrobenzoic acid 500 mg/litre.

4.5.2 NTM species isolated from sputum samples from both HIV (+) and HIV by cultural characteristics

Out of 167 samples, 59 samples that grew in PNB modified L-J medium were considered as NTM since, MTBC growth was inhibited by the presence of PNB in the media. The GenoType Mycobacterium CM/AS was used to speciate isolated NTM from liquid (BACTEC MGIT 960, Becton Dickinson, USA) or LJ-PNB culture media. the most common NTM identified were *M. intracellularae* 24/59(40.7%), followed by *M. fortuitum* 15/59 (25.4%) and *M. avium* 7/59(11.9%) respectively. Other species identified were, *M. kansasii* 7/59 (11.9%), *M. gordanae* 2/59 (3.4%). The species that had one isolate each were: *M. simiae, M. abscessus, M. scrofulacaeum* and *M. lentiflavum* (table 4.6)

Table 4. 6 NTM species isolated from sputum in HIV +/- participants in BCRH

NTM culture positive	n (%)
M. intracellularae	24 (40.7)
M. fortuitum	15 (25.4)
M. avium	7 (11.9)
M. kansasii	7 (11.9)
M. gordanae	2 (3.4)
M. simiae	1 (1.7)
M. abscessus	1 (1.7)
M. scrofulaceum	1 (1.7)
M. lentiflavum	1 (1.7)
Total	59 (100)

Data are presented as numbers (n) and percentages (%) of non-tuberculous mycobacterium

4.6 Susceptibility of slow growing NTM (SGM) to antimicrobial drugs determined by broth microdilution method

The susceptibility tests of all NTM isolates were done on broth microdilutions, and minimum inhibition concentrations was determined according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (appendix V). The MICs for rapid growing mycobacteria- *M. fortuitum* and *M. abscessus* were determined within 3 days of incubation at 37°C (table 4.7), while slow growing mycobacteria- *M. intracellularae, M. avium* among others (Table 4.8) were determined between 3-7 days. The MIC was determined as the lowest concentration of the drug that resulted in no visible bacterial growth after the incubation period. The susceptibility was determined based on CLSI breakpoint recommendations (Appendix V).

Table 4. 7 Susceptibility of rapid growing NTM (RGM) to antimicrobial drugs determined by broth microdilution method

M. fortuitum (n=15), n (%)														
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**	**>64**
Rifampicin	3(20.0)			5(33.3)	6(40.0)	0	0	0	0	1[a] (6.7)				
Isoniazid		7(46.7)			5(33.3)	2(13.3)	1(6.7)	0	0	0				
Ethambutol			2(13.3)			2(13.3)	8(53.3)	2(13.3)	1[b] (6.7)			0		
Streptomycin			0			5(33.3)	3(20.0)	6(40.0)	0		0		1[a] (6.7)	0
Pyrazinamide			3(20.0)			3(20.0)	5(33.3)	1(6.7)	1(6.7)		0		0	2[b] (13.3
M. abscessus (n=1), n (%)														
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**	**>64**
Rifampicin	0	-	-	0	1(100)	0	0	0	0	0	-	-	-	-
Isoniazid	-	0	-	-	0	0	0	1(100)	0	0	-	-	-	-
Ethambutol	-	-	0	-	-	0	0	1(100)	0	-	-	0	-	-
Streptomycin	-	-	0	-	-	1(100)	0	0	0	-	0	-	0	0
Pyrazinamide	-	-	0	-	-	0	0	0	0	-	0	-	1(100)	0

MIC-Minimum inhibitory concentrations of drugs for the isolates, n (%), percentage of the isolates that were inhibited from growth as per the concentration, [a] drug resistant from the HIV negative participants infected by NTM, [b] drug resistant isolate from PLWH coinfected with NTM.

Table 4. 8 Susceptibility of slow growing NTM (SGM) to antimicrobial drugs determined by broth microdilution method.

M. intracellulare (n=24), n (%)													
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**
Rifampicin	4(16.7)			2(8.3)	14(58.3)	1[a](4.2)	0	3[b](12.5)	0	0			
Isoniazid		2(8.3)			15(62.5)	6(25.0)	0	0	2[a] (8.3)	0			
Ethambutol			2(8.3)			8(33.3)	12(50.0)	0	0			2[b] (8.3)	
Streptomycin			3(12.5)			5(20.8)	4(16.7)	8(33.3)	0		0		4[b] (16.7)
Pyrazinamide			4(16.7)			5(20.8)	10(41.7)	2(8.3)	0		0		0
M. avium (n=7), n (%)													
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**
Rifampicin	1(14.3)			2(28.6)	3(42.9)	0	1[a](14.3)	0	0	0			
Isoniazid		1(14.3)			3(42.9)	2(28.6)	0	0	1[b](28.6)	0			
Ethambutol			1(14.3)			3(42.9)	3(42.9)	0	0			0	
Streptomycin			0			0	1(14.3)	5(71.4)	1[b] (14.3)		0		0
Pyrazinamide			0			3(42.9)	0	2(28.6)	0		1(14.3)		1(14.3)
M. kansasii (n=7), n (%)													
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**
Rifampicin	2(28.6)			2(28.6)	3(42.9)	0	0	0	0	0			
Isoniazid		3(42.8)			4(57.2)	0	0	0	0	0			
Ethambutol			3(42.8)			0	3(42.8)	0	1[b](14.4)			0	
Streptomycin			0			0	4(57.2)	3(42.8)	0		0		0
Pyrazinamide			0			0	2(28.4)	1(14.4)	0		4(57.2)		0
M. gordanae (n=2), n (%)													
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**
Rifampicin	0			0	1(50.0)	0	0	0	1[b](50.0)	0			
Isoniazid		0			2(100.0)	0	0	0	0	0			
Ethambutol			1(50.0)			0	1(50.0)	0	0			0	
Streptomycin			0			0	0	2(100.0)	0		0		0
Pyrazinamide			0			0	1(50.0)	1(50.0)	0		0		0
M. simiae (n=1), n (%)													
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**
Rifampicin	0			0	1(100.0)	0	0	0	0	0			
Isoniazid		0			0	0	0	0	1[a](100)	0			
Ethambutol			0			0	1(100.0)	0	0			0	
Streptomycin			0			0	0	1(100.0)	0		0		0
Pyrazinamide			0			1(100.0)	0	0	0		0		0
M. scrofulaceum (n=1), n (%)													
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**
Rifampicin	0			1(100.0)	0	0	0	0	0	0			

Isoniazid		1(100)			0	0	0	0		0			
Ethambutol			0			1(100.0)	0	0	0			0	
Streptomycin			0			0	1(100.0)	0	0		0		0
Pyrazinamide			0			0	1(100.0)	0	0		0		0
M. lentiflavum (n=1), n (%)													
MIC	**≤0.12**	**≤0.2**	**≤0.5**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**>8**	**16**	**>16**	**32**
Rifampicin	0			0	0	1(100.0)	0	0	0	0			
Isoniazid		0			1(100)	0	0	0		0			
Ethambutol			1(100.0)			0	0	0	0			0	
Streptomycin			0			0	1(100.0)	0	0		0		0
Pyrazinamide			1(100.0)			0	0	0	0		0		0

MIC-Minimum inhibitory concentrations of drugs for the isolates, n (%), percentage of the isolates that were inhibited from growth as per the concentration, [a] drug resistant from the HIV negative participants infected by NTM, [b] drug resistant isolate from PLWH coinfected with NTM.

4.7 Summary of minimum inhibition concentrations (MICs) for NTM isolates

Figure 4.1 summarizes MICs for NTM isolates. Generally, Ethambutol was the most sensitive drug across the NTM species. However, the sensitivity was significantly different between the PWLH co-infected with NTM and those who are HIV negative but have NTM infection (*P*=0.043). Same trend was observed with Isoniazid and Pyrazinamide drugs respectively. Resistance was significantly noted in Streptomycin (15.2%) and Pyrazinamide (15.2) and Isoniazid 12.1%) respectively.

Figure 4.1 Antimycobacterial susceptibility testing of non-tuberculous mycobacteria

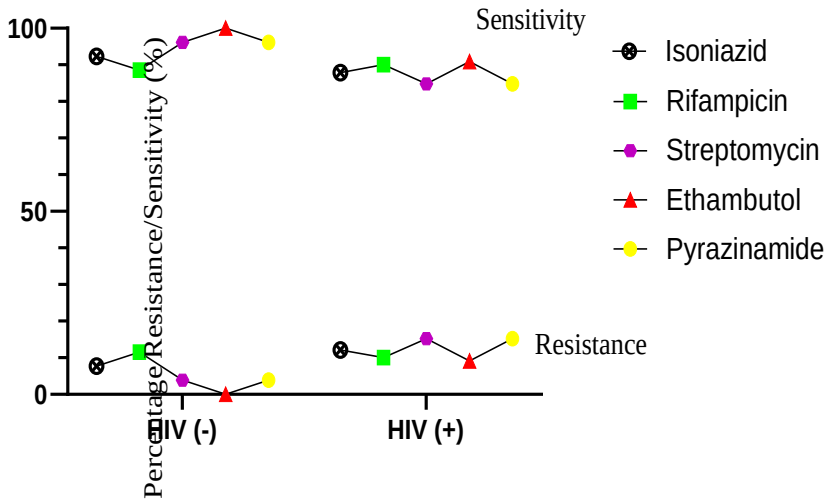


Figure 4.1 Antimycobacterial susceptibility testing of non-tuberculous mycobacteria

4.8 Determination of levels of CD4+ T cells and Viral load

The levels of CD4+ T cells was determined using a BD FACSCalibur flow cytometer. Out of the total 167 participants, 73 (43.7%) were HIV positive co-infected with NTM while 94 (56.3%) were HIV negative with NTM infection. CD4+ T cells count levels differed significantly between the two groups, PLWH-NTM had a median CD4+ T cells

of 454 cells/mm³ compared to 851 cells/mm³ for HIV negative but having NTM infection group (*P*<0.0001), Majority of participants who were HIV negative 80 (85.1%) had CD4+ cell count of more than 500 cells/mm³ compared to HIV positive 33(45.2%) with *P* < 0.0001. The viral load (VL) of 52/73 (71.2%) participants had fewer than 1000 copies/mL, while 21 participants had VL more than 1000 copies/mL. Figures 4.2 and 4.3 give a summary of CD4+ T cell count levels and viral load.

Figure 4.2: Showing CD4+ T cells

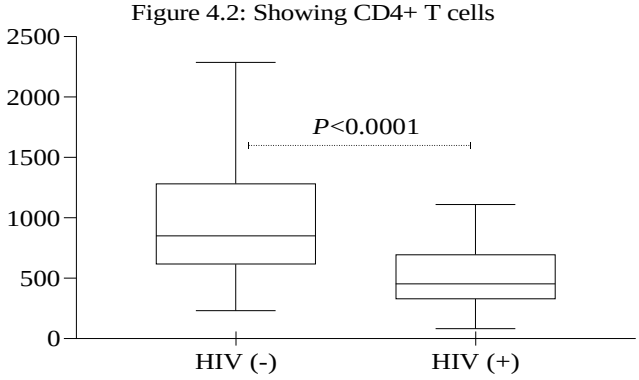


Figure 4 2 Showing CD+ T cells

Figure 4.3 Immune status and viral load

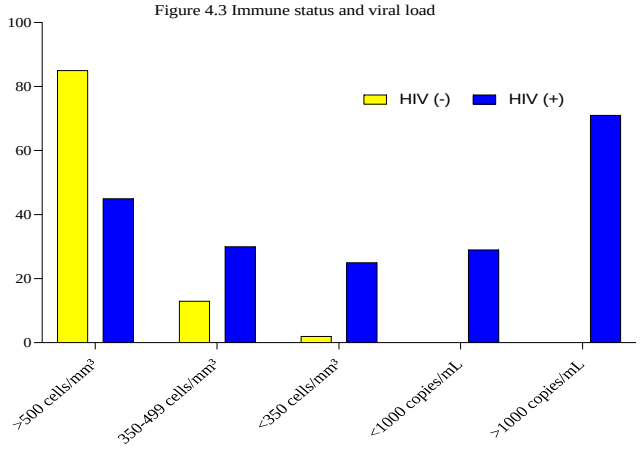


Figure 4 3 Immune status and viral load

4.8.1 Molecular markers in NTM isolates causing antibiotic resistance among HIV patients

The figure 4.4 summarizes findings of the line probe assay. The assay revealed sensitive and resistant markers at different loci that had previously been described in MTB for *rpoβ, katG* and *inhA* genes. Ninenty one (91) of the 94 HIV negative individuals had the wildtype genotype *rpoβ* gene while 3 (3.2%) were mutant hence resistant. Among the HIV positive clinical group, 66 (90.4%) were wildtype hence sensitive while 7 (9.6%) were mutant. There was no significant difference in the sensitivity or resistance between the clinical groups (P=0.106). Assessment of the *katG* locus also revealed similar results to those of the *katG* gene. Similarly, *inhA* locus, 94 (100%) of the samples were sensitive (wildtype) for the HIV negative participants. On the other hand, 71 (97.3%) were sensitive while 2 (2.7%) were resistant in the HIV positive group. The genotype distribution was similar (P=0.304) between the groups.

Figure 4.4 Molecular markers in NTM isolates causing antibiotic resistance among HIV patients

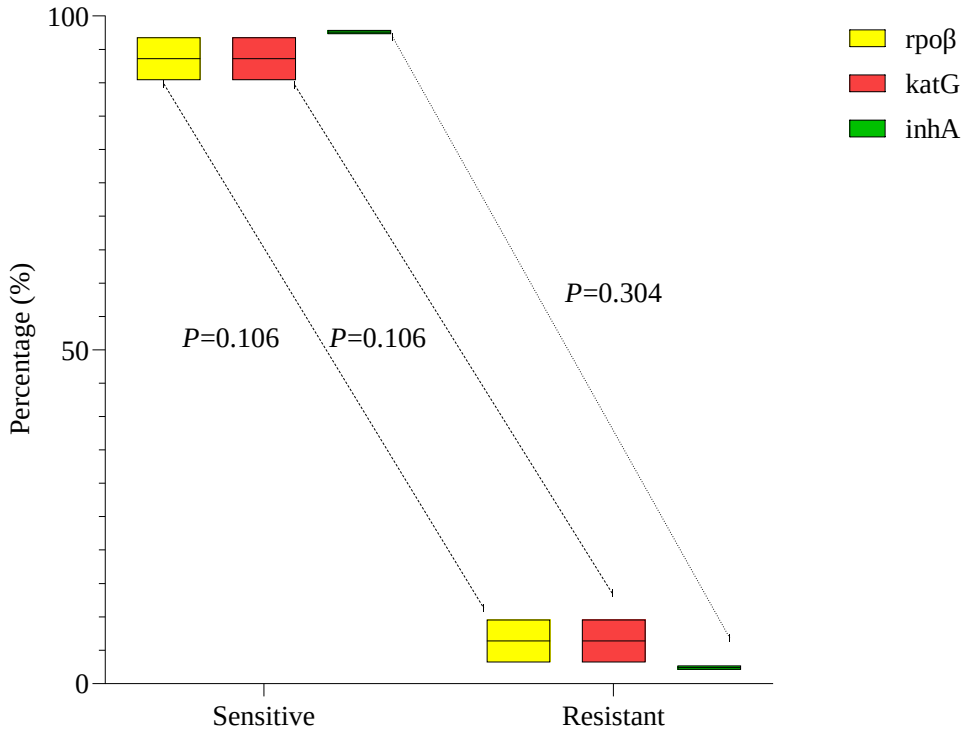


Figure 4 4 Molecular markers in NTM isolates causing antibiotic resistance

4.8.2 Single nucleotide polymorphism identification by Line Probe Assays in NTM

Data on the single nucleotide polymorphisms among NTM infected individuals is summarized in table 4.9 below. The 3 missense mutations coding for resistance at the *rpoβ* locus were revealed to be D516V among the HIV negative individuals, while 4 similar mutations were characterized in the HIV positive individuals. In addition, 2 H526Y and 1 H526D were found to occur in the *rpoβ* locus among the HIV positive. In the *katG* gene, 3 and 7 individuals presented with mutations at codon 315 in HIV negative and positive individuals respectively. The single nucleotide mutations were

specifically S315T. Only 2 mutations were described in the *inhA* locus C15T among the HIV positive participants.

Table 4. 9 Single nucleotide polymorphism identification by Line Probe Assays in NTM

Genes	**HIV (-)** (AA change)	**HIV (+)** (AA change)	Nucleotide change
Rpoβ	D516V (3)	D516V (4)	gac>gtc
	H526Y (0)	H526Y (2)	cac>tac
	H526D (0)	H526D (1)	cac>tag
	S531L (0)	S531L (0)	tcg>ttg
katG	S315T (3)	S315T (7)	agc>acc
inhA	C15T (0)	C15T (2)	tcg>acg

Data presented as codon mapping on LPA. Data is showing missense mutation leading to a change in specific locus of the genes.

4.9 Association of antimicrobial resistance markers in NTM isolates with underweight, immunosuppression and viral suppression in HIV patients

Individuals who were HIV positive co-infected with NTM and had *inhA* drug resistance gene against NTM were two times likely to be underweight OR, 2.409; 95 % Cl, (1.858-7.871); *P*=0.040) compared to those who were HIV negative but infected with NTM. However, they were neither associated with immunosuppression OR, 1.445; 95%Cl (0.147-14.228); *P*=0.752 nor linked to viral load. Similarly, HIV positive individuals co-infected with NTM that had drug resistant *katG* genes were likely to be underweight OR, 2.776; 95%Cl, (0.665-11.580); *P*=0.161, but did not have notable relationship with immunosuppression OR, 0.505; 95%Cl, (0.103-2.462); *P*=0.398 and viral load OR, 1.011; 95%Cl (0.180-5.668); *P*=0.990 respectively. Furthermore, these results also revealed that, same individuals who were HIV positive co-infected with NTM and have

rpoβ drug resistance gene were not significantly related with underweight, immunosuppression and viral load respectively (table 4.10).

Table 4. 10 Association of antimicrobial resistance in NTM isolates with underweight, immunosuppression and viral suppression in HIV patients

Characteristics	OR	95%CI	*P*
inhA			
BMI			
≤18.5 kg/m^2	2.409	1.858-7.871	**0.040**
>18.5 kg/m^2	Ref	-	
CD4 count			
≤500 cells/mm^3	1.445	0.147-14.228	0.752
>500 cells/mm^3	Ref	-	
Viral load			
≤1000 copies/mL	-	-	-
>1000 copies /mL			
katG			
BMI			
≤18.5 kg/m^2	2.776	0.665-11.580	0.161
>18.5 kg/m^2	Ref	-	
CD4 count			
≤500 cells/mm^3	0.505	0.103-2.462	0.398
>500 cells/mm^3	Ref	-	
Viral load			
≤1000 copies /mL	1.011	0.180-5.668	0.990
>1000 copies /mL	Ref	-	
rpoβ			
BMI			
≤18.5 kg/m^2	2.776	0.665-11.580	0.161
>18.5 kg/m^2	Ref	-	
CD4 count			
≤500 cells/mm^3	0.505	0.103-2.462	0.398
>500 cells/mm^3	Ref	-	
Viral load			
≤1000 copies/mL	1.011	0.180-5.668	0.990
>1000 copies /mL	Ref	-	

Data are presented as OR, odds ratio, 95% confidence interval (CI). HIV-1[+], human immunodeficiency virus type-1; *P*, *P*, logistic regression analysis; Bolded are significant *P*-values.

CHAPTER FIVE

DISCUSSION

5.1 Socio-demographic and clinical characteristics of participants

The present study finding showed that, majority of those infected with NTM were male 124 (74.3%) compared to female 43 (25.7%). This finding is correlated with studies done globally. In Europe, males are more likely to develop NTM-PD than women (Prevots & Marras, 2015). Similar finding was noticed in Central and South America-Brazil, where 62% males were more likely to be infected by NTM-PD, equally Middle East and South Asia -Turkey, reported that a higher percentage of male (87.1%) were likely to get NTM-PD than female counterparts (Bicmen *et al.,* 2010; de Mello *et al.,* 2013). Increased frequencies of NTM infection in males than in females could be related to several factors including socioeconomic activities; for instance, in African culture males are the probable primary household bread winners, and in some situations, they are forced to work in unhealthy environments. This finding is confirmed by a study done by Ngayo et al who found out that, patients who raised livestock had a higher risk of contracting NTM than patients who did not (OR 1.6, 95% CI 1.06 to 2.6), same case was noticed in those who were farmers (OR 1.6, 95% CI 0.9 to 2.7) (Ngayo *et al.,* 2015). Such activities could be among the predisposing factors that exposes men to an environment that harbors NTM compared to women.

NTM-PD infection occurs through the aerosolization or aspiration of water and or soil particulates, (Donohue, 2018), so such activities are more likely to expose men to NTM infections. Additionally, some studies in Europe have attributed higher NTM pulmonary disease in male to some predisposing factors like smoking history and other

comorbidities like increased occurrence of chronic obstructive pulmonary disease (COPD) (Donohue & Wymer, 2016).

However, contrasting findings have previously been reported in other studies where NTM are more common in women than men. A study in Beijing, China attributes to more female infection to the fact that, older women could have predisposing factors related to the functionality of immune system being weak (Huang *et al.,* 2020). This was supported by other studies in US, South Korea, Kenya and Japan respectively (Lee *et al.,* 2019; Marras *et al.,* 2017; Morimoto *et al.,* 2014; Ngayo *et al.,* 2015). Additionally, Chan and Iseman observed that abnormal expression of adipokines, sex hormones, and/or TGF-β may predispose slender, older women to NTM infection (Chan & Iseman, 2010) hence making more women to be prone to NTM infection than men.

The present study revealed the median age of PLWH-NTM as 41.0 and 40.5 for those were HIV negative infected with NTM, and there was no notable age difference between the groups. The results are in consistent with the study done in Sub-Saharan Africa that reported median age of 35years ITR 16-80) (Okoi *et al.,* 2017). However, some previous studies have reported contrasting findings. Study by Prevots et al in US, noted that people above the age of 60 years experienced a greater increase in NTM infection than people below the age of 60 (Prevots *et al.,* 2010). Similar results from another study in US was observed by Adjemian et al where mean age of NTM infected participants was 62.7 years while Santos et al in Portugal, reported a mean age of 64± 15.9 (Adjemian *et al.,* 2017; Park *et al.,* 2020; Santos *et al.,* 2022). However, a noteworthy study by Mwangi et al in Kenya, found out that the NTM infection was more common in a younger population of between 21-35 years (Mwangi *et al.,* 2023). In the current

investigation, the majority of the PLWH who were also coinfected with NTMs had a significantly lower BMI than the HIV negative group (*P*=0.026). Further, compared to HIV negative, which was largely within the normal weight range, the majority of the PLWH were notably underweight (*P*=0.046). A number of previous researchers corroborates with the present study suggesting that, patients with NTM-PD tend to have lower body weight and or lower body mass index (BMI). For instance, in USA, Kartilija *et al* concurs with these findings in that, individuals who were having NTM-PD had notably lower BMI (22.06 vs. 23.98 kg/m^2) than those in the control group (Kartalija *et al.,* 2013).

Similar observation was made by Kim et al in another study in USA; the body morphology of Pulmonary NTM infected patients were distinctively slimmer with decreased BMI (21.1 vs. 28.2) and in Japan patients with Pulmonary NTM had BMI lower than 18.5 kg/m^2 (Hayashi *et al.,* 2012; Kim *et al.,* 2008). The same trend was reported in a recent study in South Korea, where incidence rate of NTM-PD was declining with increase in BMI and was highest in the underweight group (Song *et al.,* 2021). However, in a Swedish study finding shows, BMI can sometimes be age-related, thus, when one grows, BMI increases with age, but eventually plateau after 75–79 years of age (Gavriilidou *et al.,* 2015). This is in concurrence with an Italian study which also noted a similar inverted U-trend; Weight and height considerably declined with age in both sexes, although knee height did not (Perissinotto *et al.,* 2002). As a result, older people had slimmer frames than younger subjects of both sexes had in the elderly. This trend of inverted BMI may also be associated to sarcopenia and sedentary lifestyles, as well as the potential for selective attrition of people with very high BMI-related illnesses

like stroke (Gavriilidou *et al.,* 2015). Furthermore, Portillo and Marera argues that, fatal cases of lung disease including TB and NTM were more likely to have low BMI, decreased serum albumin, decreased peripheral lymphocyte count, and negative tuberculin reaction (Portillo & Morera, 2012). Consequently, the findings hypothesize that poor nutrition may contribute to weakened cell-mediated immunity, which is a significant pathogenetic factor for MAC illness. However, due to limited clarity, it is unclear if low BMI is a symptom of the disease or a risk factor for the development of PNTM disease.

Unlike weight, height did not differ considerably between the two groups (p=0.258). However, height just like weight, is not an express predictor for NTM infection because, ageing entails changes in nutritional and physiological condition including a loss of height and body weight (Dey *et al.,* 1999). Further, Perissinotto et al argues that, the oldest people have basal body heights comparable to those of the youngest people, but the height decline with age mostly due to spinal deformity and intervertebral disc thinning (Perissinotto *et al.,* 2002). Other measurements including Mid upper arm circumference (MUAC), waist circumference, Hip circumference and BUST, did not show any considerable variabilities between the PLWH coinfected with NTM and those who were HIV negative but infected with NTM.

Another noteworthy anthropometric measurement in this study, is overweight (BMI 25 to < 30 kg/m^2), which was clustered together with obese (BMI ⩾ 30 kg/m^2). Though the HIV positive coinfected with NTM participants and those who were HIV negative but had NTM infection didn't differ significantly (*p=0.436*). This finding is concordance to a previous Chinese study which reported obesity and overweight to be linked with a

considerably decreased risk of both clinically active and culture-confirmed tuberculosis (Leung *et al.,* 2007). In particular, a 10% decrease in risk of active tuberculosis was noted for each unit increase in BMI over 18.5 kg/m^2. Although notwithstanding the fact that, adiposity under the skin and in the midsection is linked to cardiovascular disorders (Gavriilidou *et al.,* 2015). Similarly, Perez-Guzman et al also observed that, patients with pulmonary TB had faster sputum culture sterilization rates when eating a diet high in cholesterol than those in the control group (Pérez-Guzmán *et al.,* 2005). However, inasmuch as the finding of this study corroborates the previous studies on relationship between Anthropometric measurements and Pulmonary TB, there is still limited information about association between individuals with NTM-PD and anthropometric measurements.

There was no notable difference in religion between participants who were HIV positive coinfected with NTM and those who were HIV negative and had NTM infection (p=0.390). Though, the majority comprised of Protestant Christians and Catholic Christians respectively. The least was Muslims. Although, there is limited information about religion being a risk factor of PNTM infections. But the only related finding to this study, is a study in Ethiopia which found out that, Orthodox Christians made up the majority of the population of TB cases with 77.3%, followed by Muslims with 13.6% (Sinshaw *et al.,* 2019). Education did not differ significantly between the two groups (p=0.155). However, majority of PLWH co-infected with NTM were school dropout at primary level (60.3%), followed by those who reached secondary (30.1%) respectively. Similar trend was noticed in China where, level of education associated with the existence of NTM lung illness (Zhao *et al.,* 2022). Though was observed in PTB

infection, Sinshaw et al, also noted that people in lower education levels (Below grade 7 [55%]) are likely to be infected by pulmonary diseases such as PTB and NTM due to poor hygiene and poor nutrition (Sinshaw *et al.,* 2019). In contrast, a study conducted in Kenya by Ngayo et al, found that patients with tertiary education (higher education) were likely to develop NTM (PR 2.2, 95% CI 0.9 to 5.5) (Ngayo *et al.*, 2015b). This discrepancy with the current study may be as a result of different study participants, geographic coverage, and timing. However, generally a lower education level may be associated with a worse occupational and residential environment, which may be a risk factor for PNTM illness. Similar to this, patients with low education levels may also have lower incomes and less favorable living situations that could favor infections including pulmonary diseases. Nonetheless, education level in and of itself, is not a significant risk factor for NTM lung disease.

Occupation was a significant risk factor for the infection of NTMs, those having informal jobs were significantly affected compared to those with formal jobs (P=0.024). Similar findings to this study was Ethiopian study which reported that, the most common occupation among the participants infected by PTB and PNTM was housewife, 21.1%, followed by daily laborer 18.4% (Sinshaw *et al.*, 2019). The region of origin was a significant risk factor for NTM infection in Bungoma County. Poor feeding practices due to high poverty level of 52.9% of the population in these areas could be linked to an increased risk of NTM disease (Gitaka *et al.*, 2018; Portillo & Morera, 2012). People exposed to dust at work have been found to be at an increased risk for developing lung mycobacterial infections that are acquired in the environment (Tiwari *et al.,* 2007). Due to both cattle husbandry and planting, Bungoma County's majority of areas are dusty,

which may help to explain why these areas have high prevalence rates. In this study, owning livestock and having contact with animals significantly increased the chance of contracting NTM. Additionally, NTMs are ubiquitous, free living which are mostly found in natural and municipal water, aerosols, animals and humans (Larsson *et al.,* 2017; S. C. Park *et al.,* 2019; Ratnatunga *et al.,* 2020). Therefore, close or physical contact, poor disease control practices in both animals and humans, and the fact that humans and their agronomic animals are literally surrounded by NTM, disease transmission between animals and humans is made easier in developing countries.

Marital status did not seem to influence the infection of NTMs in people living with HIV, although participants who were single, were the most commonly affected group. There is paucity of authorities that have documented this demography in relation to PNTM infection, unlike their "cousin" PTB infections. For instance, some studies that have reported contrasting reports though in relation to TB infections include: A study by Ngayo et al in Kenya, reported 77.6% of the participants who had PTB were married women compared to unmarried (Ngayo *et al.,* 2015b). Similar to finding by Ngayo et al, is a study in Ethiopia which observed that 52.3% participants were married whereas 38.1% were single (Sinshaw *et al.,* 2019).

The results of this investigation revealed no statistically significant differences in the clinical signs and symptoms between the PWLH coinfected with NTM(PLWH-NTM) and those who were HIV-negative but infected with NTM (HIV [-] NTM [+]). Nevertheless, the majority of the PLWH demonstrated clinical indications connected to NTM infections when compared to the HIV negative, despite the absence of statistical significance. Purulent sputum was the most prevalent symptom in the PLWH, occurring

90.4% of the time, followed by a persistent cough that lasted longer than two weeks (86.3%) malaise (84.9%), night sweats (83.6%), weight loss and weariness (80.8% each), chest discomfort (65.8%), and hemoptysis (6.8%). In contrast, HIV negative participants had lower frequency of the clinical signs. Purulent sputum was more common in the HIV [-] NTM [+] with (77.7%), as were chronic coughs lasting longer than two weeks (75.5%). These results are in line with a study from Iran that looked into lung disease brought on by *M. simiae* (Baghaei *et al.*, 2012).

Despite HIV infection and clinical sign grading, they reported that patients with *M. simiae* infection, a species of NTM, frequently experienced clinical indicators including a chronic cough, purulent sputum, and weight loss. Another study done in Zambia found that the most common clinical symptoms of NTM infection were cough (52.98%), night fever lasting longer than two weeks (35.10%), chest pain (71.07%), weight loss (35.64%), and HIV positivity (54%) (Chanda-Kapata *et al.*, 2015). Although Chanda-Kapata's findings are far less significant than this study, these symptoms are generally typical of NTM infections, suggesting a connection. It is important to note that NTM infections can have diverse clinical manifestations and can affect various organ systems, including the lungs, skin, and lymph nodes, hence can be chronic and progressive, causing significant morbidity and mortality if left untreated (Ratnatunga *et al.*, 2020). Accordingly, accurate diagnosis and effective management of NTM infections are essential for enhancing patient outcomes. Immune status notwithstanding, majority of patients with infection from a variety of NTM species frequently displayed clinical symptoms as a persistent cough, purulent sputum, and weight loss chest pain among other symptoms. These findings imply that NTM are increasingly and frequently

infecting people who even appear immunologically competent, despite the HIV epidemic being one of the factors for increased NTM infection. This is troubling because the pathophysiology of NTM is difficult to cure (Ratnatunga *et al.,* 2020).

Results from Chanda-Kapata's study in Zambia augment this viewpoint, arguing that inasmuch as it is harder for prospective patients to manage NTM/HIV infection and the associated side effects due to the rising prevalence of NTM among HIV positive people. The HIV negative population also carries a significant burden that cannot be ignored, even though the burden of NTM was greater among HIV positive people (Chanda-Kapata *et al.,* 2015). To further comprehend the clinical traits and therapeutic approaches for various NTM species, more research is required.

5.2 Phenotypic and genotypic, characteristics of antibiotic resistant NTM isolates from participants

Phenotypic characteristics of NTM were identified using ZN Smear microscopy and culture methods. By combining the BACTEC MGIT 960 and Löwenstein-Jensen techniques, a higher percentage of mycobacteria isolates are discovered especially NTM (95.5%). This is according to previous investigations (Sorlozano, 2009). However, this study used LJ-PNB as a backup and MGIT 9460 as the method for culture, this is because, WHO presently regards BACTEC MGIT 960 as the gold standard for culture, and it is frequently used for both primary isolation and drug susceptibility testing (DST) of Mycobacteria. Although, it is known to have high contamination rates hence reduces mycobacteria recovery rate (Subramanyam *et al.,* 2020).

According to this study, the overall prevalence of NTM by AFB microscopy among PLWH in Bungoma County was 45.2% compared to 20.2% NTM infection in non-HIV group. The NTM-HIV co-infection smear positive microscopy rate is marginally lower than the 46.4% rate reported by Nyamogoba *et al*. (Nyamogoba *et al.*, 2012). This slight insignificant decline of 1.2% in regard to this study and that of Nyamogoba et al between 2012 to 2023 could be attributed to the lack of established or acknowledged criteria to accurately define and report NTM. More so, it could also be related to less attention being paid to NTM infections, as well as lack of awareness among public health professionals.

While employing smear microscopy as a diagnostic approach in this investigation, although majority of smear positives were observed in PLWH coinfected with NTM than HIV negative but have NTM, but there was no discernible difference in the number of positive results in the two groups (33/73 [45.2%] vs. 19/94 [20.2%], [p=0.868]). However, there was a significant difference in the two groups' grading sputum smear results (*p=0.025*). To determine the infectiousness of the patients and gauge the severity of the disease, mycobacterial load is measured using the grades of smear positivity (scanty, 1+, 2+, and 3+). Smear, however, is less sensitive and demands high bacilli counts (Najjingo *et al.*,2019). In this study, majority of HIV [+] NTM [+] had 3+ and 2+ compared to the HIV [-] NTM [+]. Conversely, many participants in control group had scanty and 1+ AFB respectively (control 13/19[68.4%]vs cases 8/33[24.3]). There is little information associating NTM-PD clinical data with mycobacterial burden in the samples, but it is hypothesized that a higher mycobacterial burden will be found in patients with severe disease (Danho *et al.*, 2022). Presumably, the findings can be in be

concordance with Kissa *et al* who suggested that patients with higher sputum AFB grades (2+/3+) are more likely than those with lower grades (Scanty/1+) to transfer the illness to contacts and develop active mycobacterial disease (Kassa *et al.,* 2021). And also, same patients with high sputum AFB grades represent a risk factor for the transmission of NTM in the community as well as being a potential hub for the emergence of drug-resistant of mycobacteria. Some studies have observed that, dispute the ambiguity of smear microscopy to differentiate between MTB and NTM, it is a standard part of the diagnostic and monitoring criteria for NTM pulmonary disease in a resource limited environment especially in developing countries to submit sputum for acid fast bacilli smear and culture analysis (Edwards *et al.,* 2022; Hopewell *et al.,* 2006).

Additionally, smear microscopy is still imperative in diagnosis because, it gives semi-quantitative evaluation of microbial burden. Study by Kissa et al further noted that, patients with concomitant disorders or a history of chronic TB were nearly twice as likely to have high sputum smear grades; these pathological alterations were similarly associated with high sputum smear grades (Kassa *et al.,* 2021). Equally, it was theorized that PLWH were more likely to have high sputum smear grades of NTM due to co-infection with HIV that renders their immune system weak.

This study's prevalence of NTM coinfection with PLWH by MGIT 960 culture method was 43.8%. Several studies have previously reported lower prevalence of NTM diagnosis by culture method. Report from Northeastern Tanzania observed prevalence of 30.6%. (Hoza *et al.,* 2016). In Southeast Asia; Thailand and Vietnam 30% of PLWH were reported to be co-infected with NTM-PD (McCarthy *et al.,* 2012). While in Ghana,

a much lower prevalence of 38 (8.0%) out of the 473 subjects living with HIV/AIDS had NTM positive cultures (Bjerrum *et al.,* 2016). Further, in a recent study in China reported 16.6% of PLWH to have NTM infection (Hu *et al.,* 2022). Additionally, 2.2% NTM by MGIT culture was found in a cross-sectional investigation of 639 HIV-positive people with probable tuberculosis who were enrolled from Brazil, Peru, Botswana, and South Africa (Luetkemeyer *et al.,* 2020). In spite of the fact that the treatment for NTM disease is typically not directly comparable to the treatment for TB (Griffith *et al.,* 2007). Therefore, according to the findings of the current investigation, the increasing instances of HIV/AIDS linked NTM mycobacterioses could be mistaken as TB and put on anti-TB chemotherapy, and this will pose a serious danger of misdiagnosis, mistreatments, high morbidity and mortality rates and more importantly, this will potentially create NTM multidrug resistance. This study reported 26.6% NTM culture positive in the participants who were HIV negative. This was much higher compared to a study done in Brazil that observed 4.10% of NTM culture positive from a sample size of 14,394 subjects (Puga *et al.,* 2018). Similarly, report from Cambodia registered a lower prevalence of 10.8% NTM by culture in HIV negative participants compared to this study report (Bonnet *et al.*, 2017). Notably, compared to the current analysis, studies in different regions of Zambia-Katete (78%) and esheke (65%) respectively reported higher prevalence by culture method of NTM infection in HIV negative individuals (Buijtels *et al.*, 2010).

The increasing frequency of PLWH co-infected with NTM poses a challenge in terms of managing patient treatment and its accompanying adverse effects. Even while the burden of NTM was greater among HIV positive people, the HIV negative population

also carries a sizable burden that cannot be disregarded. This view is shared by Chanda Kapata and friends who argues that, although studies show that NTM is much higher in PLWH, equally prevalence of NTM in HIV negative patients is noteworthy (Chanda-Kapata *et al.,* 2015; Jain *et al.,* 2014). Furthermore, difference in prevalence between this study and other studies might be alluded to several factors including, the geographic differences, the type of population in terms of lifestyle, and also, the season may be responsible for the difference in prevalence of NTM infection between this study and other studies. For instance, areas where a lot of farming; both livestock and planting are done, the population around such areas are likely to be infected by NTM because of its ubiquity (Buijtels *et al.,* 2010). These findings might suggest that mycobacteria are distributed differently in Africa than they are in the USA and Europe.

After primary culture and subculture from MGT960 tube, a total of 59/167 samples had growth on L-J media. Using cultural characteristics, the colonies that demonstrated growth on LJ-PNB media and were thought to be mycobacteria were identified (Grange *et al.,* 1996; Raju *et al.,* 2016). Majority of NTM developed yellow-orange pigmentation on LJ-PNB medium.

In this study, the most common NTM species identified was *M. intracellularae* (40.7%). This finding is in concordance with Jenkins et al, who observed that, the slow growing Mycobacterium avium complex (MAC) especially *M. intracellularae*, is the main source of human infection that commonly causes NTM-PD (Jenkins *et al.,* 2017; Sharma & Upadhyay, 2020). In United States, MAC reported higher isolation rate of almost 80% of all NTM-LD cases in the U.S than these findings (Honda *et al.,* 2018). However, the researcher didn't specify which species of MAC was predominant. Contrary to this

study was findings from India where, a lower prevalence of 38.4% of *M. intracellulare* isolates from clinical samples was reported (Desikan *et al.,* 2017). A similar trend was observed in Beijing, China, where Huang et al reported 71 (31.8%) out of 223 clinical samples to be *M. intracellularae* (Huang *et al.,* 2020). Additionally, Okoi *et al*, reported an overall prevalence of MAC, which included *M. intracellularae* as 13.5%. This is according to a Systematic Review and Meta Analyses study in sub-Saharan Africa (Okoi *et al.,* 2017). This overall finding of *M. intracellularae* in sub Saharan Africa is comparatively lower than the current finding. Equally, a study by Nyamogoba *et al* in Kenya, though found out that, the most common speciated NTM was *M. intracellularae* at 20% (Nyamogoba *et al.,* 2012), but the frequency was lower than the current study results. However, in another study in Kenya by Ngayo et al, three years after Nyamogoba et al, also reported that *M. intracellularae* (47/89[52.8%]) was the most commonly isolated NTM species associated with pulmonary disease (Ngayo *et al.,* 2015). And notably, the prevalence was higher than the current study results. Therefore, according to several previous studies, M. *intracellularae*, is the most prevalent NTM pathogens worldwide that is linked to NTM-PD, however the prevalence varies widely by age, gender, and geographic location. The use of immunomodulatory medications, rheumatoid arthritis, and thoracic skeletal deformities are other factors that affect NTM infections (Borand *et al.,* 2019). Further, A heritable genetic propensity to disease susceptibility is suggested by the clustering of illnesses within families, and warm, humid settings with high atmospheric vapor pressure also increase population risk (Borand *et al.,* 2019). *M. intracellularae*, is in group of MAC which are SGM including *M. avium, M. chimaera, M. colombiense, M. marseillense, M. arosiense, M. timonense,* and *M. bouchedurhonense.*

Humans can contract a number of illnesses from *M. intracellulare* as evidenced from the previous studies elucidated above. Those who are immunosuppressed or who have underlying lung problems are more likely to develop respiratory and disseminated infections. Several variables, such as geographic location, population density, healthcare infrastructure, and the frequency of risk factors for NTM infections, can affect the geographical spread of *M. intracellulare*. However, specific places tend to have higher rates of NTM infections, particularly those caused by *M. intracellulare.* Apparently, North America, especially the United States and Canada have reported the highest frequency of *M. intracellularae*. It has also been established that the Southeast of the United States has a greater prevalence of MAC infections. Similarly, some studies have revealed that NTM infections are relatively common in Asia particularly, China, Japan, and South Korea, though not as high as in United States. The prevalence of *M. intracellularae* may vary by geography as it has also been found in other European nations. A somewhat high incidence has also been reported throughout Africa, particularly in South Africa, Zambia, and Kenya, however in not as high as US but more less as in Asia. Additionally, the high prevalence of *M. intracellularae* could also be attributed to the level of environmental NTM exposure, for example increased exposure to NTM sources, such as contaminated soil or water supplies. It's crucial to remember that the distribution and prevalence of NTM infections might alter over time as a result of a variety of circumstances, such as adjustments in medical procedures, variations in migratory trends, and alterations in the environment. Further, differences in surveillance methods and medical professionals' awareness may have an impact on the reported prevalence of NTM infections.

In this study, *M. fortuitum* was identified in 15 out of 59 samples, accounting for 25.4% of the NTM isolates. It was the second most common NTM species isolated in this study.

This finding is consistent with previous studies that have reported the presence of *M. fortuitum* in clinical isolates of NTM. In contrast to this study finding is a study in India which reported much lower prevalence of 3.8% of NTM isolates to be *M. fortuitum* (Desikan *et al.,* 2017). Similarly, study in China by Huang et al also recorded lower frequency of 6.7% M. *fortuitum* to be causing NTM-PD (Huang *et al.,* 2020). Equally, two studies in Kenya by Nyamogoba et al (6.7%) and Ngayo et al (4.5%) and one in Zambia (13%) reported lower frequencies of *M. fortuitum* than these study results (Monde *et al.,* 2018; Ngayo *et al.,* 2015; Nyamogoba *et al.,* 2012). However, some studies recorded higher prevalence of *M. fortuitum* than this study. In one investigation into nontuberculous mycobacterial skin infections, 29 instances were recorded, including infections brought on by *M. fortuitum*(Lee *et al.,* 2010).

According to this study's findings, immunocompromised patients frequently experience many skin lesions and deep tissue infections. This shows that, as in this investigation, the immunological status of the patient may influence the clinical presentation and severity of *M. fortuitum* infections. In contrast to this finding, Lee at el study observed a greater prevalence of *M. fortuitum*, accounting for 9(31.0%) out of 29 cases. This discrepancy in prevalence might be explained by variances in the study population, the research area, or the study's particular therapeutic setting. In addition, another study Korea, reported higher *M. fortuitum* results compared to this finding was study by Yu et al. In their literature review, they discovered that treatments including acupuncture, filler

injections, and other medical procedures frequently caused skin injuries that led to *M. fortuitum* infections of 46% of the NTM isolates (Yu *et al.,* 2013).

This suggests that the prevalence of *M. fortuitum* infections may be influenced by specific risk factors and sources of infection. In conclusion, the research population, geographic location, and certain risk factors connected to *M. fortuitum* infections are some of the examples of the variables that may have an impact on the prevalence rates of *M. fortuitum*. Perhaps further investigation is required to reveal factors associated with the spread of this NTM species. With 11.9 % of the NTM isolates in this investigation, *M. avium* was the third most often isolated NTM species. This result is in concurrence with other research that showed *M. avium* infections were common in NTM infections. The epidemiology of NTM infections during an 11-year period was reported in an Italian study from Tuscany (Rindi & Garzelli, 2016). In 41.5% of NTM patients, they discovered *M. avium,* which was one of the most prevalent NTM species.

This prevalence rate is higher than it was in this study. Similar trend was realized in Asia, where research in Beijing China reported 30 (13.5%) out of 223 NTM isolates to be *M. avium* (Huang *et al.,* 2020). This suggests that the prevalence of *M. avium* infections may vary depending on the location. Another study examined the epidemiology of NTM infections throughout Europe. According to them, *M. avium* and *M. intracellulare* were the most prevalent species, with prevalence rates in Italy, the United Kingdom, and the Netherlands reaching between 40 and 60 percent (Rindi & Garzelli, 2016). This is in line with the results of this investigation, in which *M. avium* was found to be one of the common NTM species, although these studies recorded higher frequencies of *M. avium* isolates than in this investigation. However, a study

done in Egypt by Gaballah et al, noted lower frequency of *M. avium* (MAC=6.6%) compared to the current study (Gaballah *et al.,* 2022). This can be attributed to factors like geography, nature of the population, level of awareness about NTM and the availability of techniques capable speciating NTM. A study in US by Ovrutsky et al, uniquely looked into the co-presence of NTM and free-living amoebae in hospital water networks (Ovrutsky *et al.,* 2013). They discovered that *M. avium* was isolated from the cleaning facility's faucet, indicating the possible significance of water sources in *M. avium* infection transmission.

This implies that environmental factors like water may have a role in the occurrence of *M. avium* infections. As a result, *M. avium* ranked third among the NTM species in this analysis, is in line with findings from other investigations by Rindi & Garzelli. Nevertheless, depending on the region and particular therapeutic setting, there can be changes in the prevalence of *M. avium* infections. Additionally, another study has revealed that *M. avium* is one of the most common type of NTM that causes infectious illnesses, particularly in people with impaired immune systems (Busatto *et al.,* 2019). The slow-growing M. *avium* has the potential to be an opportunistic intracellular pathogen that can survive inside of macrophages and evade the host's immune system, Glycopeptidolipids (GPLs), which are found in cell walls, may have an impact on this mechanism (Nunes-Costa *et al.,* 2016).The most frequent location of infection for *M. avium* is the respiratory tract, and the usual pulmonary disease presents as nodular/bronchiectatic disease or as fibro-cavitary disease when it arises as a later consequence.

M. kansasii isolates were present in this investigation at a rate of 11.9 %. This result is in line with earlier research that found different prevalence rates for *M. kansasii* infections. Unlike in the current study, some studies have reported higher frequency of *M. kansasii*. According to a study done in Israel by Matveychuk et al, *M. kansasii* accounted for 15% of all NTM infections that were isolated, and was the second most often isolated NTM species (Matveychuk *et al.*, 2012). Similar trend was reported in China, where a study by Pang et al, reported a frequency of 12.3% of NTM to be M. *kansasii* (Pang *et al.*, 2017). Additionally, a study done in another Asian country, South Korea, *M. kansasii* accounted for 11.5% of the reported extrapulmonary NTM (Lee *et al.*, 2021). Equally, parts of Europe have also reported observable high prevalence of *M. kansasii* especially in older individuals. Findings by Bakula et al showed that, most frequent NTM in Poland was *M. kansasii*, which was found in 178/486 (36.6%) individuals (Bakuła, Kościuch, *et al.*, 2018). Furthermore, in Europe, in a research conducted in France between 1991 and 1995 on *M kansasii* in a high-HIV prevalence suburb of Paris, one-third of patients tested positive for HIV and all had a history of pulmonary illness within the previous two years (Shitrit *et al.*, 2006).

The association of HIV positive cases being co-infected with *M. kansasii* was also observed by Bloch et al, where 69.3% cases of *M. kansasii* had HIV (Bloch *et al.*, 1998). Lower socioeconomic status indicators were prevalent in the patients, hence HIV-positive individuals were more likely to have *M. kansasii* infection. Although regardless of HIV status, the majority of patients with *M. kansasii* infection exhibit clinical and radiologic evidence of infection. This finding is consistent with what was discovered in the current study, where the majority of those who had NTM were PLWH. In addition, the study found that patients with underlying lung conditions such chronic

obstructive pulmonary disease (COPD) were more likely to develop *M. kansasii* infections. The variation in *M. kansasii* infections could be linked to a number of explanations. These include differences in the patient demographics, geographical locations, immune status and diagnostic techniques. It is also important to note that some risk factors, such as advanced age and underlying lung illness, are more frequently linked to *M. kansasii* infections. *M. kansasii* infections can appear clinically as pulmonary disease, lymphadenitis, or disseminated disease. The most typical presentation is pulmonary illness, which has symptoms similar to tuberculosis, including coughing up sputum and hemoptysis. This research advances the understanding of *M. kansasii* infections' frequency in the patient population. While other researchers have indicated greater rates, the prevalence rate seen in this study is commensurate with several earlier ones.

In this research, it was found out that 3.6% (n=2) of the samples with growth belonged to *M. gordanae*. This predominance of *M. gordanae* is in line with other research that found this species in clinical isolates. In slight divergence with this finding is result from study done in Egypt which lower frequency of 3.3% of NTM isolates compared to this study had *M. gordanae* (Gaballah *et al.,* 2022). However, findings made in a study conducted in Saudi Arabia, recorded a higher frequency of 5.3% of the isolates to be *M. gordanae* compared to these results. (Varghese *et al.,* 2013). These results imply that *M. gordanae* is a species that should be considered while diagnosing and treating mycobacterial illnesses. A comparison *M. gordanae* prevalence in this study to recent prior studies, there are some similarities and differences. This discrepancy might be explained by variances in the study population, study area, and sampling techniques. Conversely, *M. chelonae* and *M. fortuitum* were determined to be the most prevalent

species, according to a study conducted in Brazil compared to *M. gordanae* which was not one of the prominent species isolated *(Matos et al.,* 2004). The distribution of different mycobacterial species may vary regionally, which could explain this disparity in prevalence. In contrast to other species including *M. abscessus, M. fortuitum,* and *M. avium* complex, the prevalence of *M. gordanae* reported in this study and earlier studies is noteworthy for being relatively low. For instance, *M. abscessus* and *M. fortuitum* were the most prevalent species in the study carried out in Saudi Arabia, accounting for 26.3% and 19.0%, respectively, of the isolates. To further comprehend the clinical significance and epidemiology of *M. gordanae* in mycobacterial infections, more research is required.

The other NTM species with one isolate each included *M. simiae,* 1 (1.8%), *M. abscessus* 1(1.8%), *M. scrofulacaeum,* and *M. lentiflavum*. According to research by Roux et al. in France, *M. abscessus* and *M. avium* complex were isolated among the NTM species from cystic fibrosis (CF) patients (Roux *et al.,* 2009). Compared to this investigation, these findings had a higher isolation rate for *M. abscessus* complex of 40 (38.5%) out of 104 NTM. Further, same study has noted that, Western Europe is recognized to have a higher prevalence of *M. abscessus* complex, which includes *M. abscessus*, *M. massiliense*, and *M. bolletii*. It can be alluded to the fact that different locations and nations have differing prevalence's of NTM in CF patients. For instance, a comprehensive review and meta-analysis carried out in mainland China found that the fraction of NTM in Mycobacterium isolates was 11.57%, while the crude isolation rate for NTM among probable TB cases was 4.66–5.78%. Hence *M. abscessus* and MAC were the two most prevalent clinical NTM species in China (Zhou *et al.,* 2020). Notably,

there has been a decline in NTM prevalence in China. And over the course of the investigation, *M. intracellulare* displaced *M. abscessus* as the dominating species. The difference in NTM prevalence can be associated with the geographic diversity of various species demonstrated and how economic and environmental factors affects their distribution hence suggesting that there are still significant elements that hadn't been discovered.

The low prevalence rate of *M. simiae*, and *M. lentiflavum* is comparable to earlier study conducted in French CF facilities, where isolation rates of *M. simiae* and *M. lentiflavum* each were 1 (1%) out of 104 NTM isolates (Roux *et al.*, 2009). It is clear that *M. simiae* and *M. lentiflavum* were relatively less prevalent when compared to other NTM species around the world in terms of prevalence. In a systematic review and metanalysis on *M. simiae* pulmonary disease in Iran, it was revealed that one of the most prevalent NTM bacteria causing lung disease in various nations around the world is *M. simiae* (Nasiri *et al.,* 2018). They found that the cumulative prevalence of *M. simiae* lung disease in NTM patients was 25.0% (95% confidence interval, 16.8-33.2). This is much higher than the results of this investigation. Similarly, even a much higher prevalence of *M.simiae* than what Nasiri et al reported was noted by Dezhkhi et al in a different region in Iran (Dezhkhi *et al.*, 2021). They observed that 92 (54.4%) of the 169 NTM strains were recognized as *M. simiae* isolates. It is significant to remember that NTM species prevalence might differ not just between nations, but also within different regions of the same nation. Environmental circumstances, medical procedures, and patient demographics are only a few examples of the variables that may have an impact on these geographical variances. Therefore, it suggests that lung disease caused by *M. simiae* is demanding more attention as it becomes a growing public concern.

M. lentiflavum has been regarded for a long time as an uncommon NTM that causes lymphadenitis. However, samples from patients with lymphadenitis in Spain revealed 28 cases of NTM, of which 23 were caused by *M. lentiflavum* (82.14%) (Miqueleiz-Zapatero *et al.,* 2018). The most prevalent NTM infection in immunocompetent children, especially those under 5 years old, is cervical lymphadenitis. Similar trend was noted in study done 4 years back from that of Miqueleiz-Zapatero et al in same country (Spain) but different region (Jiménez-Montero *et al.,* 2014). Out of 45 NTM lymphadenitis cases with microbiologic confirmation: 17 (40.5%) *M. lentiflavum* cases were noted. And most participants had a median age of 23 months. The variety and effects of non-tuberculous mycobacteria at the wildlife-livestock interface were the subject of a different investigation by Varela-Castro et al. One of the most abundant species, isolated from cattle and wild boar, was identified in the study as *M. lentiflavum*(Varela-Castro *et al.,* 2022). This raises the possibility of zoonotic transmission by indicating that *M. lentiflavum* can be found in many animal hosts. In order by Mwikuma et al to identify NTM isolated from clinical specimens carried out a study in Zambia. Along with *M. intracellulare* and *M. avium, M. lentiflavum* (16.7%) was one of the most common species with isolation (Mwikuma *et al.,* 2015). This shows that *M. lentiflavum* can be found in many populations and is not isolated to particular geographical areas. In this study, 1.8% (n=1) of the NTM isolated was *M. scrofulaceum*. In contrast with this finding, is a study done in South African gold miners where, 14% of the NTM isolates belonged to *M. scrofulaceum* (Corbett *et al.,* 1999). This finding is higher than the current study finding. There is paucity of information about *M.scrofulaceum*. Further research is needed to better understand the clinical

implications and epidemiology of *M. scrofulaceum* infections, as well as its genetic relationship with other mycobacterial species.

In conclusion, the current study's findings are in line with earlier research that revealed the presence of various NTM species in sputum samples. According to earlier studies' findings, *M. intracellulare, M. fortuitum*, and *M. avium* were the most prevalent NTM species found. These results advance knowledge of the distribution and occurrence of NTM species, which is crucial for the identification and management of NTM infections.

5.3 Antimycobacterial resistance/sensitivity patterns of the NTM isolates

The study compared the sensitivity and resistance of different drugs, including Isoniazid (INH), Rifampicin (RIF), Streptomycin (STR), Ethambutol (EMB), and Pyrazinamide (PRZ). In terms of Isoniazid, the results showed that 12.1%% of the participants who were HIV-positive coinfected with NTM were resistant compared to 8.7% HIV-negative coinfected with NTM participant. The difference in sensitivity between the two groups was statistically significant (p=0.043). This finding is in consistent with results from a study in Mumbai, India prevalence which reported 16% of NTM isolates to be resistant to INH due to −15 C→T mutation in the *inhA* gene's promoter region (Shenai *et al.,* 2009). Similarly, in Ghana a study by Addo et al reported a prevalence of 22.2% of NTM isolates to be resistant to INH by having mutations in *InhA* MUTI(C15T) (Addo *et al.,* 2017). Though resistance in Ghana was higher than the current study finding. It's important to note that the frequency and kinds of *inhA* gene mutations might change throughout time and in different regions. However, there is paucity of information especially in Africa regarding prevalence of mutations in the *inhA* gene that causes INH

resistance in NTM species. For instance, a study done by Tadesse et al in Southwest Ethiopia focused on “drug resistance-conferring mutations in MTB and excluded NTM (Tadesse *et al.,* 2016). The higher susceptibility to Isoniazid observed in the HIV-negative individuals with NTM infection in the current study may be attributed to several factors. Firstly, it is possible that the HIV-negative individuals had a less severe form of NTM infection, which could make them more responsive to treatment with Isoniazid. Secondly, the HIV-negative individuals may have had a better immune response, allowing them to effectively clear the NTM infection with the help of INH. Overall, the results of the current study suggest that HIV-positive individuals have a higher prevalence of NTM co-infection compared to HIV-negative individuals. Additionally, the study highlights the potential differences in drug susceptibility, with a higher proportion of HIV-negative individuals with NTM infection being susceptible to INH than PLWH. Further research is needed to better understand the underlying mechanisms and to develop more effective treatment strategies for individuals with NTM co-infection, particularly in the context of HIV.

This study did not register observable difference for RIF susceptibility and resistance (*P=0.181*) between the HIV-positive having NTM infection group the HIV negative but NTM infection. The current study prevalence of rifampicin resistance among HIV-positive individuals co-infected with NTM was 10%%, compared to 13%% among HIV-negative individuals. This is in consistent with a study conducted by Nwofor et al in Nigeria which reported 21.4% (3/14) of RIF resistance to be caused by NTM. And the mutations occurred in the S531L and S315T1 codons of *rpoB* genes (Nwofor *et al.,* 2015). In Iran, higher resistance to RIF by NTM than this study was noticed. 100% *M.*

simiae was observed to be resistant against RIF and INH, same full resistance was observed with MAC against RIF and INH (Akrami *et al.,* 2023). Study done in Zimbabwe reported that, the NTM species have not yet developed rifampicin drug resistance because they have not yet been exposed to the drug for a long time (Manjeese *et al.,* 2017). Perhaps this could be the reason why RIF drug resistance mutations in NTM are said to be silent because they typically occurred in the final nucleotide of codons, where amino acid sequences are extremely conserved. In another study in Peru, (TB patients), had similar finding with the current study which reported that, there is no relationship between HIV co-infection and Ant-mycobacterial drug RIF resistance (Masenga *et al.,* 2017).

Conversely, in a study conducted by Villegas et al in Peru found that rifampicin mono-resistance was associated with HIV infection (Villegas *et al.,* 2016). Though the current study didn't find an association between HIV positive co-infection with NTM, study by Villegas at el, found a weak association between HIV co-infection and rifampicin resistance. It is important to note that the studies included in this discussion were conducted in different settings. Therefore, the results may not be directly comparable due to differences in population characteristics and healthcare systems. In conclusion, the results of the current study support previous findings that HIV-positive individuals co-infected with NTM have a higher prevalence of rifampicin resistance compared to HIV-negative individuals. However, the association between HIV co-infection and rifampicin resistance is weak. Further research and interventions are needed to improve the management of rifampicin resistance and reduce the risk of poor treatment outcomes.

The study also established a statistical difference between resistance of NTM to ethambutol in HIV-positive individuals (9.1%) and in HIV-negative individuals (0%). This finding indicates that there is a higher prevalence of ethambutol resistance in NTM infections among HIV-positive individuals compared to HIV-negative individuals. Consistent with the current study finding is, a systematic review and meta-analysis conducted in mainland China that investigated the prevalence and antibiotic resistance of NTM from 2000 to 2019. They reported that, the most common pre-existing conditions in NTM infected people was HIV infection with 41.33% prevalence (Zhou *et al.*, 2020). However, this study did not specifically report on the resistance of NTM to EMB, but observed that EMB was relatively the most sensitive first-line anti-TB drug for NTM (Zhou *et al.*, 2020). According to the Zhou et al. study, *M. avium* had the lowest levels of drug sensitivity among the most prevalent clinical species (26.9%, 7/26), followed by *M. abscessus* (33.3%, 9/27), *M. avium-intracellulare* (37.5%, 6/16), *M. fortuitum* (37.5%, 9/24), and *M. intracellulare* (38.5%, 10/26). But, only *M. kansasii* and *M. gordonae*, had sensitivity more than half of the tested drugs of 52% (13/25) and 66.7% (16/24), respectively. Although EMB is primarily an ant-TB drug, but it's also used for regimens for infections caused by SGM-NTM. However, it has a limited application for RGM (Brown-Elliott & Woods, 2019). A study conducted in South Korea to assess the relationships between EMB in vitro activities of MAC-PD, which is one of the primary causes of NTM-PD (Hoefsloot *et al.*, 2013), found out resistance of NTM to EMB at the rate of 87% (Moon *et al.*, 2022). This is much higher than what is reported in the current study, this could be attributed to the fact, that different studies may be conducted with different focuses and methodologies hence likely to get varying results. Another study by Heifetz, argues that some NTM species including, *M. kansasii*,

M. xenopi, M. malmoense, M. szulgai, M. marinum, M. gordonae, M. terrae, and *M. nonchromogenicum* are among the SGM that are susceptible to EMB (Heifets, 1991).

However, *M. simiae* and may be *M. asiaticum* and *M. haemophilum* are considered to be resistant to EMB. Therefore, prevalence of NTM resistant to EMB depends on the NTM species in question. In conclusion, the findings of ethambutol resistance in NTM among HIV-positive individuals align with the understanding that immunocompromised individuals, such as those with HIV infection, are at a higher risk of NTM infection, and therefore, the presence of HIV infection can weaken the immune system, making individuals more susceptible to NTM infections and potentially leading to higher rates of resistance to antimicrobial agents like EMB (Zhou *et al.,* 2020). Further research is needed to fully understand the resistance patterns of NTM to EMB and to develop effective treatment strategies for NTM infections, particularly in immunocompromised populations

Similarly, the current study noted a considerable difference of resistance of NTM to Pyrazinamide (PRZ) between HIV positive people co-infected with NTM (15.8%) compared to HIV negative people having NTM (4.3%) infection ($p=0.043$). These findings suggest a higher prevalence of Pyrazinamide resistance in NTM infections among HIV-positive individuals compared to HIV-negative individuals. A study conducted in Iran's national referral center for tuberculosis investigated pulmonary disease caused by *M. simiae* (Baghaei *et al.,* 2012). The study found out that all but one of the patients in the study were HIV-negative, suggesting that HIV infection rates were significantly lower among *M. simiae* patients. This finding indicates that the prevalence

of HIV co-infection may vary depending on the specific NTM species involved. Another study conducted in Zambia evaluated the NTM prevalence and found that clinically relevant NTM infection occurred in both HIV-positive and HIV-negative patients (Chanda-Kapata *et al.*, 2015). These findings highlight the importance of considering both HIV-positive and HIV-negative populations when studying NTM infections. Furthermore, a study conducted in Chengdu, China analyzed the clinical characteristics, drug resistance, and pathogen spectrum of patients co-infected with HIV and NTM, and the study found that Chengdu had a high incidence of HIV-infected patients (Wang *et al.*, 2019).

However, there is limited information regarding PZA susceptibility or resistance to NTM. Few studies have found out that none of the NTM species, including those that are a component of the *Mycobacterium avium* complex (MAC), have the *pncA* gene implicated in PZA activation or resistance (McGuire *et al.*, 2012). Although its exact function in NTM is still unclear, but it could be that, most NTMs lacks the third member of the operon (Rv2044c ortholog), including slow-growing species like *M. avium* (Baddam *et al.*, 2018). Additionally, Baddam *et al* further argues that, compared to *M. tuberculosis*, *M. kansasii* an NTM species which is naturally resistant to PZA, has approximately 25-fold less nicotinamidase activity and about 5-fold less Pyraminidase activity. This study emphasizes the need for comprehensive research on NTM infections in different geographical regions and populations. Although the current study findings suggest a higher prevalence of Pyrazinamide resistance in NTM infections among HIV positive individuals compared to HIV-negative individuals, but consideration should also be given to previous studies which suggests that, variations in the prevalence and

clinical characteristics of NTM infections in different populations. Further research is needed to fully understand the resistance patterns of NTM to Pyrazinamide and to develop effective treatment strategies for NTM infections, particularly in immunocompromised populations. The study's findings demonstrated that there was no discernible difference between HIV positive (84.8%) and HIV negative (95.7%) individuals who were infected with NTM in terms of streptomycin sensitivity.

Equally, the resistance of NTM to streptomycin between HIV positive cases (15.2%) and the HIV negative control group (4.3%) did not differ noticeably (P=0.181). Aminoglycoside antibiotics like streptomycin and amikacin are frequently used to treat NTM infection (Ryu *et al.,* 2016). Furthermore, they found that 82.7-88% of clinical MAC isolates were amikacin and streptomycin sensitive (Cc *et al.,* 2018). This is consistent with the finding of this investigation. The previous study by Heifets and Lindholm-Levy also established four injectable anti-tuberculosis medications, streptomycin, amikacin, kanamycin, and capreomycin to have very high bactericidal activity against *M. avium* and MTB (Heifets & Lindholm-Levy, 1989). They did not, however, estimate the sensitivity rate in their investigation. On the other hand, streptomycin-resistant NTM isolates have been discovered in earlier research (Park *et al.,* 2020). They reported that, among other isolates, 60% of those for *M. intracellulare* were found to be streptomycin resistant. According to Sreevatsan et al, mutation of the *rrs* gene at particular locations is linked to resistance to aminoglycoside antibiotics (Sreevatsan *et al.,* 1996). These results shed light on NTM resistance patterns in various groups and emphasize the need of comprehending how HIV co-infection affects NTM resistance. It is noteworthy to remember that different NTM species, patient

populations, and geographic locations can all affect the resistance patterns of NTM. Therefore, it is essential to take these aspects into account when interpreting the findings of various investigations.

HIV co-infection may not have a substantial effect on the development of streptomycin resistance in NTM, as evidenced by the lack of a significant difference in the resistance of NTM to streptomycin between HIV positive cases and the HIV negative control group in the current investigation. To fully comprehend the connection between HIV co-infection and NTM resistance, as well as the underlying mechanisms at play, more research is nonetheless required. Despite the paucity of study on this subject, studies on NTM resistance patterns in general offer some useful information. It's crucial to comprehend how HIV co-infection affects NTM resistance in order to optimize patient outcomes and treatment plans.

5.4 Molecular markers of antibiotic resistance in NTM isolates

The current study investigated specific loci in common genes shared across the genus mycobacterium associated with antibiotic resistance in MTB. There was no significant difference in the prevalence of drug resistance-associated mutations in the *rpoβ*, *katG*, and *inhA* genes between HIV positive individuals co-infected with NTM and HIV negative individuals with NTM infection. The mutations in genes have been associated with resistance to rifampicin and isoniazid (Eddabra & Neffa, 2020; Reta *et al.*, 2021). In PLWH-NTM, 9.6% of individuals had drug resistance associated mutations in the *rpoβ* gene, while in the HIV negative NTM positive, 3.2% had such mutations. Similar, 3.2% and 9.6% mutations were observed in the HIV negative and positive clinical

groups respectively for *katG*. In the case of *inhA* gene, 2.1 % of the mutations appeared in the HIV negative clinical group only.

The gene *rpoβ* encodes the β- subunit of RNA polymerase in the mycobacterium genus (Amusengeri *et al.,* 2022). This gene has been fairly conserved across the different species in genus mycobacterium (Fedrizzi *et al.,* 2017). Single nucleotide changes in codons 516, 526 and 531 have been associated with resistance to rifampicin in MTB (Lu *et al.,* 2021).

Interestingly, the current study was able to identify D516V mutation where the codon was previously encoding aspartic acid (D) changes to valine (V) being a missense mutation in a total of 7 isolates. Moreso, in codon 526, two isolates showed the change from histidine (H) to tyrosine (Y) while one isolate in the same codon changed to aspartic acid (D) from histidine (H). All these mutations phenotypically present with resistance to rifampicin (Lu *et al.,* 2021; Reta *et al.,* 2021).

Mycobacteria contain *inhA* gene in their genomes that encodes for Enoyl-acyl carrier protein reductase enzyme (Kelley *et al.,* 1997). The inhA gene is a drug target for isoniazid. Isoniazid potency as therapy for mycobacteria may be weakened by mutations in the *inhA* gene. There are a number of SNPs that have been mapped especially the regulatory region of *inhA* gene which include T8G/A, C15T, and T16A /G (Leung *et al.,* 2006). These mutations have been associated with resistance in isoniazid (Charan *et al.,* 2020). This study found 2.7% of the NTM isolates to have the C15T missense mutation among HIV positive individuals. This implies that at codon 15 of the gene, cysteine (C) changed to threonine (T). This is consistent with findings from Mumbai, India which

observed prevalence of 16% of NTM isolates to be having −15 C→T mutation in the *inhA* gene's promoter region that caused resistance to isoniazid drug (Shenai *et al.,* 2009). However, this prevalence is lower compared to Mumbai report. Similarly, results from a study conducted in Ghana showed a higher prevalence of 22.2% of NTM isolates to be resistant to isoniazid by having mutations in *inhA* MUTI(C15T) (Addo *et al.,* 2017). The NTM species as opposed MTB can show either innate or acquired resistance to INH. While isoniazid resistance in some NTM species can be attributed to mutations in the *inhA* gene, other pathways can also result in INH resistance (Griffith *et al.,* 2007). Further, they noted that, it also depends on the NTM species and the local prevalence of resistance in a given area, the precise genetic alterations and mechanisms of resistance can change (Griffith *et al.,* 2007). Additionally, in Japan, Line Probe Assay identified the most common mutations in INH-resistant isolates as, S315T and S315N mutations in *katG* and C-15T and T-8C mutations in the promoter region of *inhA* (Mitarai *et al.,* 2012).

It's noteworthy that, the frequency and kinds of *inhA* gene mutations might change throughout time and in different regions. However, there is paucity of information especially in Africa regarding prevalence of mutations in the *inhA* gene that causes INH resistance in NTM species. Many previous studies majorly focused on MTB rather than NTM, like for the case of study done by Tadesse et al in Southwest Ethiopia focused on "drug resistance-conferring mutations in MTB and excluded NTM (Tadesse *et al.,* 2016). The lack of significant difference in drug resistance-associated mutations between HIV positive and HIV negative individuals with NTM infection suggests that HIV status may not be a major factor influencing the development of drug resistance in

NTM. These findings highlight the need for further research to better understand the factors contributing to drug resistance in NTM and to develop evidence-based treatment strategies for NTM infections.

This study reports 9.6% resistance caused by *katG* gene in the HIV positive people coinfected with NTM compared to 3.2% resistance in HIV negative people infected with NTM resulting from S315T mutation. This variant results from the change at codon 315 of the *katG* gene from serine (S) to threonine (T). The catalase-peroxidase gene (*katG* gene) is present in bacteria like mycobacteria including MTB and NTM and plays a role in isoniazid drug resistance in mycobacteria (Zhang *et al.,* 2019; Isakova *et al.,* 2018). In contrast, a study in Ghana, observed a much higher prevalence of 77.8% mutations in *katG* MUT1 (S315T1) responsible for isoniazid resistance in NTM (Addo *et al.,* 2017). Studies have shown that, NTM typically have innately reduced vulnerability to isoniazid (Reingewertz *et al.,* 2020). And the relative isoniazid sensitivity is explained by the fact that the *katG* proteins of NTM are significantly less effective at activating isoniazid than those of MTB (Reingewertz *et al.,* 2020). These findings show a divergence between the *katG* of the MTBC and the *KatG* of NTM. In another study in UK by Heym et al, reported that, the failure by NTM to activate the prodrug isoniazid, target level alterations, and variations in the C-terminal domain of *katG* are likely the causes of this enhanced resistance (Heym *et al.,* 1993). Additionally, the decrease in permeability or efflux pumps that lower intracellular concentration of isoniazid in NTM also could be the cause of resistance to isoniazid (Adams *et al.,* 2014). A number of genes in numerous biosynthetic networks and pathways are thought to be involved in the molecular processes of isoniazid resistance. Isoniazid resistance is primarily brought on

by mutations in the *katG* gene, followed by *inhA, ahpC, kasA, ndh, iniABC, fadE, furA, Rv1592c*, and *Rv1772* (Unissa *et al.*, 2016). However, according to Bakula et al, there is limited reports on how *katG* or *inhA* genes contribute to the development of the isoniazid -resistant phenotype in NTM (Bakuła *et al.*, 2018).

Globally, there is little evidence especially on the frequency of isoniazid resistance associated with *katG* gene mutations in NTM. More of the information that is currently available on *katG* gene mutations and isoniazid resistance has focused on MTB rather than NTM. However, NTM species differ from *M. tuberculosis* and typically exhibit different patterns of antibiotic susceptibility (Wu *et al.,* 2018). Given that, NTM are responsible of approximately 35% of all mycobacterial infections in humans, and more so in immunocompromised patients with a rate of about 50% (Miguez-Burbano *et al.*, 2006). Then, there is need for more research to be done to establish the prevalence of isoniazid resistance in MTB-NTM multidrug resistance caused by mutations in *katG* gene.

The current investigation established 9.6% of PLWH coinfected with NTM to have drug resistance-associated mutations in the *rpoβ* gene, while those who were HIV negative, but infected with NTM were 3.2% had such mutations. The beta subunit component of RNA polymerase in bacteria is encoded by the highly conserved gene *rpoB.* And more than 95% of RIF-resistant mutations in mycobacteria are linked to *rpoB* gene alterations because RIF acts on the RNA polymerase subunit encoded by the *rpoB* gene. (Park *et al.,* 2020; Zaw *et al.,* 2018). Antimycobacterial resistance in NTM species has been documented in Iran, 60% of NTM species, including MAC, *M. simiae,* and *M. kansasii*

(apart from 17 RIF-resistant *M. kansasii*), were resistant to RIF because of mutations at positions 1249, 1356, 1407, 1479, 1533, and 1536 of the 350-bp fragment of the *rpoB* gene (Akrami *et al.,* 2023). This study reported lower frequency of NTM-RIF resistance due to mutation of *rpoB* gene in relation to a study conducted by Nwofor et al in Nigeria where a frequency of 21.4% (3/14) of NTM isolates to be RIF resistant with mutations in occurring in the S-531L and S315T1 codons of *rpoB* genes was reported (Nwofor *et al.,* 2015). In Africa, study done in Zimbabwe reported NTM species that have *rpoB* gene mutations but their clinical importance may differ (Manjeese *et al.,* 2017). For instance, they argue that, *rpoB* mutations have been linked to rifampicin resistance in some NTM species, including MAC just like in MTB.

But the report further suggests that, because the NTM species have not yet been exposed to rifampicin for a lengthy period of time, they have not yet evolved rifampicin drug resistance. Perhaps this could be the reason why RIF drug resistance mutations in NTM are said to be silent because they typically occurred in the final nucleotide of codons, where amino acid sequences are extremely conserved. (Manjeese *et al.,* 2017).

Conclusively, lack of Clinical and Laboratory Standards Institute (CLSI) recommendations of defined cutoff values for *rpoB* gene (Cowman *et al.,* 2016). And the fact that many regions of *rpoB* gene have been proposed to be involved in RIF resistance in various research, are the two limiting factors with the *rpoB* gene sequencing (Jo *et al.,* 2017). More so, lack of reference strain sequencing databases might potentially contribute to improper alignment and misidentification of RIF resistance caused by *rpoB g*ene. Similar view is shared by Moon et al, who argued that, although recent studies described an increased treatment failure rate for patients with high MICs for RIF, the subject remains controversial (Kwon, Daley, *et al.,* 2019). This is because, cutoff points for resistance to rifampin is only laboratory derived (van Ingen *et al., 20*12), but most studies did not show the relationship between in vitro activities of rifampin and clinical outcomes (Research Committee of the British Thoracic Society, 2001). It is also noteworthy to say, that apart from the frequently studied mutation in *rpoB* gene that causes most of the RIF resistance in mycobacteria, more *rpoA* and *rpoC* mutations were discovered (Comas *et al.,* 2012), and despite not being linked to rifampin resistance, *rpoA* and *rpoC* mutations are known to provide compensatory mechanisms that offset the otherwise lower fitness cost of significant *rpoB* mutations. Consequently, as much as *rpoB* gene mutation is correlated to RIF resistance in Mycobacterium especially in MTB. The function of *rpoB* mutations is less known in NTM hence more research needs to be done.

5.5 Association between molecular markers of antibiotic resistance in NTMs and the clinical outcomes in HIV

The current study revealed that HIV-positive individuals with NTM coinfection who also had the *inhA* drug resistance gene were twice as likely to be underweight compared to HIV-negative individuals with NTM, but were less likely to be immunosuppressed or have high HIV viremia, suggesting that the inhA gene may play a role in the emergence of underweight status in HIV-positive individuals with NTM coinfection. The particular genetic modifications and methods of resistance can vary depending on the NTM species and the local incidence of resistance in a given area (Griffith *et al.,* 2007). However, there is paucity information about the frequency of *inhA* gene changes that lead to isoniazid resistance in NTM species, particularly in PLWH. The complexity of isoniazid's mode of action emphasizes the need for additional study to clarify the molecular causes of drug resistance and the ways in which isoniazid influences various enzymes in the mycobacterial cell. Study by Abate et al reported that, malnutrition can contribute to underweight status in HIV-positive people. Severe acute malnutrition kills more HIV-positive people than HIV-negative people (Abate *et al.,* 2020). In addition, People who are malnourished and also infected with HIV are at an increased risk of morbidity and mortality. Moreover, immune dysfunction brought on by HIV raises the danger of opportunistic infections and may have an impact on children's nutritional health. HIV infection typically leads to nutritional deficiencies through decreased food intake, malabsorption, and increased consumption and excretion of nutrients. Losing weight may hasten death. The same study indicated that a combined prevalence of underweight and wasting among children with HIV was reported to be 41.63% in East Africa (Abate *et al.,* 2020). Clinical implications of this study's findings include the

possibility that people with HIV and NTM co-infection who have the *inhA* drug resistance gene to INH may be more likely to be underweight. This emphasizes how crucial it is to manage and treat co-infected people while considering the genetic profile of NTM strains otherwise the morbidity and mortality rate is bound to be high.

Furthermore, the lack of a correlation between the cases and immunosuppression raises the possibility that variables other than immunological status may affect how clinical outcomes in NTM infections develop. As a result of this research, it was found out that HIV-positive NTM-infected people with the *inhA* drug resistance gene had a higher likelihood of being underweight than HIV-negative NTM-infected people. To fully comprehend the molecular underpinnings of medication resistance in NTM infections and how it affects clinical outcomes, more study is required.

On the other hand, people with HIV who both had NTM and the *katG* drug resistance gene also had a higher likelihood of being underweight. This suggests that the *katG* gene may contribute to the emergence of underweight individuals in this population. The catalase-peroxidase gene is present in bacteria like in genus *Mycobacteria* including NTM is referred to as the *"katG* gene"(Zhang *et al.,* 2019). And, it is involved in the activation of the prodrug isoniazid. There are limited studies correlating the underweight PLWH co-infected with NTM that have resistant *katG* gene. However, abate et al alludes to the fact that, malnutrition can contribute to underweight status in HIV-positive people co-infected with NTM which eventually kills more HIV-positive people than HIV-negative people (Abate *et al.,* 2020). According to study by Reingewertz et al. NTM often have naturally lower vulnerability to isoniazid hence the reason for presence of drug resistant *katG* gene in NTM (Reingewertz *et al.,* 2020). Moreover, immune

dysfunction brought on by HIV raises the danger of opportunistic infections and may have an impact to PLWH coinfected with NTM like drug resistant gene which typically leads to nutritional deficiencies through decreased food intake, malabsorption, and increased consumption and excretion of nutrients. More research is necessary to completely understand the molecular basis of *katG* gene drug resistance in NTM infections especially in PLWH and how it impacts clinical outcomes.

Similar to the *inhA* gene, the *katG* gene did not significantly correlate with viral load or immunosuppression. Contrary to these findings, where PLWH co-infected with NTM had no correlation with Immunosuppression, a study by Bonard et al reported that, patients with baseline CD4 cell counts < 100 cells/mm^3 had a 10 times greater risk of NTM infection, according to a prospective cohort study in Cote d'Ivoire of HIV-positive patients (Bonard *et al.*, 2004). Bonet et al. support the findings of Bonard and colleagues. According to their study in the provincial reference hospital in Cambodia, they discovered that disseminated NTM disease occurs more frequently after the CD4 cell count falls below 50 cells/L (Bonnet *et al.*, 2017). Similar trend was observed in South East Asia, where it was noted that, NTM infections particularly MAC occur worldwide in adults and children with severe immunodeficiency and a CD4 count that is often less than 50 cells/L (Borand *et al.*, 2019). Lack of information on the epidemiology and clinical impact of NTM isolation in HIV-infected people from countries with a high TB incidence and inadequate resources is potentially increasing the morbidity and mortality of immunocompromised individuals. this study did not find the correlation between PLWH co-infected with NTM and plasma viral load. There are limited authorities over the same. However, study conducted by Gautama et al in India,

observed that, there is a correlation of CD4 counts and plasma viral load with opportunistic infections in PLWH (Gautam *et al.,* 2009). The results support earlier research that found a link between NTM infections and HIV co-infection as well as the existence of drug-resistant genes in NTM and MTB. These results underline the need for additional study as well as thorough diagnosis and therapeutic approaches for NTM infections, particularly in HIV-positive people.

The current study did not find a significant link between underweight, immunosuppression, viral load, and HIV-positive individuals co-infected with NTM that have the *rpoB* drug resistance gene. Given that there is no correlation between, immunosuppression, viral load, or underweight in HIV-positive people co-infected with NTM that had *rpoB* drug resistance gene, it is possible that other factors are at play. The complex relationships between viral co-infections, medication resistance, and clinical outcomes in HIV-positive people who are also co-infected with NTM require more study. The beta subunit component of RNA polymerase in bacteria is encoded by the highly conserved gene *rpoB* and, more than 95% of RIF-resistant mutations in mycobacteria are linked to *rpoB* gene (Park *et al.*, 2020; Zaw *et al.*, 2018).

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. This study established from genotypic characterization of drug resistant NTMs that *M. intracellularae* and *M. fortutium* were the most common NTMs among the study participants and are likely being misdiagnosed for MTB at in HIV patients attending the comprehensive care clinic at BCRH. This study was able to genotype nine (9) different types of NTMs among the study participants.

2. Susceptibility patterns generated from MIC assays across the panels of antimycobacterial drugs on the NTM isolates revealed Ethambutol to be the most effective drug with most isolates being sensitive to it. On the contrary, rifampicin was the less sensitive drug with isoniazid being slightly better. There was no difference insensitivity/resistance between HIV negative and positive participants for all the antibiotics used except for Ethambutol which was significantly less sensitive in the HIV positive clinical group.

3. The current study found mutations in *rpoβ*, *katG* and *inhA* genes often associated with antibiotic resistance in MTB among the NTM isolates. Analysis of the *rpoβ* gene associated with resistance to rifampicin revealed D516Y, H526Y and H526Y as the SNPs. Variation of the *katG* was affecting codon 315 only with the mutation being S315T while in *inhA* was C15T. The mutations in the both *katG*

and *inhA* being linked with isoniazid resistance. The identified markers have previously been reported in MTB suggesting horizontal gene transfer between members of the mycobacterium genus or selection pressure by the NTM genomes on the different targeted genes during antimycobacterial therapy.

4. This study found mutations in the *inhA* loci to be associated with wasting/underweight among HIV infected individuals. Participants who presented the C15T mutation were twice likely to be underweight compared to those who did not have this mutation in the *inhA* gene. The other clinical outcomes in HIV including CD4 T cell count (immunosuppression) and RNA copies (viral load)/viral suppression were not associated with any of the molecular markers.

6.2 Recommendations

6.2.1 Recommendations for action

1. Since there's considerable incidence of *M. intracellularae* and *M. fortuitium* among HIV patients seeking care at the comprehensive care clinic, it is critical to improve diagnostic protocols that enable precise differentiation between NTMs and MTB. By integrating genotypic characterization techniques in conjunction with conventional diagnostic methods, it is possible to prevent misdiagnoses and guarantee the implementation of suitable treatment strategies.

2. Ethambutol demonstrates the highest efficacy as a drug against NTM isolates, as determined by susceptibility patterns. Therefore, its utilization should be prioritized in treatment guidelines, specifically for patients living with HIV.

Nevertheless, the diminished susceptibility to Ethambutol that was noted among individuals living with HIV highlights the criticality of customizing treatment plans in accordance with HIV status, possibly incorporating dosage modifications or combination therapies to guarantee effectiveness.

3. Antibiotic resistance-associated mutations in the *rpoβ*, *katG*, and *inhA* genes underscore the criticality of maintaining ongoing surveillance of NTM isolates. This surveillance should encompass not only conventional antimycobacterial drugs but also other antibiotics that are commonly used in clinical practice, in order to effectively guide treatment decisions and monitor emergent resistance patterns.

4. The correlation observed between *inhA* gene mutations and underweight/wasting in individuals infected with HIV highlights the criticality of incorporating nutritional screening and support into the overall provision of HIV care. It is recommended that clinicians integrate genetic markers that are linked to clinical outcomes into their regular evaluation protocols. This would enable the timely detection of patients who are susceptible to malnutrition, facilitating prompt intervention via nutritional counselling and support services.

6.2.2 Recommendations for future studies

1. Conduct additional genotypic characterization of NTMs in order to gain a deeper understanding of their genetic diversity, prevalence, and distribution. This may facilitate the development of more precise diagnostic and therapeutic approaches.

2. Antibiotic susceptibility testing for NTMs should be expanded to include a wider variety of antimycobacterial medications. This could aid in the fight against antimicrobial resistance and yield significant insights regarding efficacious treatment alternatives.

3. Undertake longitudinal investigations to examine the efficacy of treatments for NTM infections, with a specific focus on individuals who are HIV-positive. This can aid in the identification of variables that impact the success or failure of treatments and the optimization of therapeutic strategies.

4. Specifically, investigate the molecular mechanisms that contribute to antibiotic resistance in NTMs, with an emphasis on gene mutations including *rpoβ, katG*, and *inhA*. Gaining insight into the mechanisms underlying resistance can aid in the advancement of innovative treatment approaches and targeted therapeutics.

5. Investigate the effects of horizontal gene transfer among mycobacterium species on the development of antibiotic resistance as well as the impact of selection pressure exerted by NTM genomes on antimycobacterial therapy-targeted genes.

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APPENDICES

Appendix I: Informed Consent Form for The Participants

Title of Research: Molecular markers in antibiotic resistant Non-tuberculous Mycobacteria isolates among HIV patients presenting with presumptive tuberculosis in Bungoma County

Researcher: Ronald Wamalwa, PhD student, Masinde Muliro University of Science and

Technology

Purpose of the Study

You are being asked to participate in this research entitled above. For you to be able to decide whether to participate in this project, you need to understand what the project is about, as well as the possible risks and benefits in order to make an informed decision. This describes the purpose, procedures, possible benefits, and risks. It also explains how your personal information will be used and protected. Once you have read this form and your questions about the study are answered, you will be asked to sign it. This will allow you to participate in this study.

Explanation of the study

Non-tuberculous mycobacteria (NTMs) are ubiquitous, free living, environmental saprophytic microorganisms. They are mostly found in water, soil, biofilms, aerosols, vegetation, animals and human. NTMs belong to the genus *Mycobacterium* which also comprise of *Mycobacterium tuberculosis* (MTB). NTMs and MTB appear similar based on microscopy, radiology and clinical symptoms, consequently, this may lead to misdiagnosis. Bungoma is

among the Counties in Kenya with a high tuberculosis (TB) incidence and prevalence among HIV/AIDS patients. Therefore, this study seek to establish the antimicrobial resistance (AMR) pattern in NTMs isolated from HIV-1 patients, establish molecular markers of AMR in NTMs and the association of the resistance pattern with clinical outcomes that is underweight, immunosuppression and viral suppression. The results of this study will help to inform Bungoma County and the country at large the status of antimicrobial resistance among HIV-1 patients presenting with NTMs and provide a platform for policy makers to develop ways of effectively treating NTMs in HIV/AIDS individuals.

Risks and Benefits

There is minimal discomfort you will feel when exactly 3ml venous blood sample will be drawn by a trained phlebotomist. However, it is a bearable process. Participants will be counselled by a trained counsellor prior to collection of blood. Those patients will be referred for anti-tuberculous therapy if they are confirmed to have TB. Participants will benefit from peer counseling services at the comprehensive care centers.

Confidentiality

The participant's anonymity and confidentiality shall be maintained at all times. All the information will be stored in password protected computers that are accessible only by the principal investigator. Serial numbers will used to code the samples. Names will not be used at any point and is of no purpose for this study

Contact Information

If you have any questions regarding this study, please contact Ronald Wamalwa, on phone number 0720469159 or the Secretary MMUST-IREC, P.O BOX 190-50100 Kenya. Telephone number: 056-31375

Declaration

Having read and understood the purpose of the study, I willingly accept to participate in the study.

Participant's Name……Signature…… Date…….

Principal investigator……. Signature……. Date.....

Note: Below are some of the key contacts

Principal investigator: Ronald Wamalwa- 0720469159

Co-investigator: 1. Dr. Bernard Guyah - 0739730843

2. Dr Nathan Shaviya -072300008

Appendix II RESEARCH AUTHORIZATION FOR DATA COLLECTION -

COUNTY GOVERNMENT OF BUNGOMA

Email: bungomadhospital@yahoo.com
Telephone: +254727592119
When replying please quote
REF: BCH/BGM/ERC/VOL.I/106

BUNGOMA COUNTY HOSPITAL
P.O. Box 14-G.P. O-50200
BUNGOMA

DATE: 15TH FEBRUARY 2023

DEPARTMENT OF HEALTH AND SANITATION

RONALD WAMALWA

REF: NACOSTI/P/23/22686

RE: RESEARCH AUTHORIZATION FOR DATA COLLECTION -NO. ERC/106-02/2023

This is to inform you that, Bungoma County Referrals Hospital Ethics Review Committee (BCRH ERC) acting on behalf of the Bungoma County Department of Health, has received and authorized your data collection for the protocol titled: ***"Molecular Markers of Antibiotic Resistant Nontuberculous Mycobacteria Isolates among HIV Patients Presenting with Presumptive Tuberculosis in Bungoma County".*** The approval period shall expire 15th February, 2024.

The approval is subject to compliance with the following requirements:

1. Only approved documents including informed consent and study instruments will used.

2. All changes including amendments, deviations and violations are submitted for review and approval by the BCRH ERC.

3. Death and life-threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to BCRH ERC within 24 hours of notification.

4. Any change, anticipated or otherwise that may increase the risks or affected safety of welfare of the study participants and others or affect the integrity of the research must be reported to BCRH ERC within 24 hours.

5. Clearance for export of biological specimens must be obtained from relevant institutions.

6. Submission of a request for renewal of approval should be done at least 60 days prior to expiry of the approval. Attach a comprehensive progress report to support the renewal.
7. Submission of an executive summary report within 90 days upon completion of the study to BCRH ERC.
8. Submission of quarterly progress report to the BCRH ERC and dissemination of preliminary findings at the end of the study is expected from the researcher.

This authorization should be attached to your research license from National Commission for Science, Technology and Innovation (NACOSTI) and also other necessary clearances. Preliminary dissemination of your findings to BCRH is mandatory prior to publications.



Dr
CHAIRMAN, ETHICS AND RESEARCH COMMITTEE
BCRH- BUNGOMA
Copyy to Director, Health services

Appendix III TPT FORM

REPUBLIC OF KENYA

MINISTRY OF HEALTH

Intensified Case Finding (ICF) / Tuberculosis Preventive Therapy (TPT) Record Card

Name:_____ Sub County Registration No._____ CCC No._____

Age: (Years)_____ Months: _____ Sex: Male ☐ Female ☐ Weight (Kg):_____ Height :_____

Physical Address: _____ National ID/NEMIS:_____ Landmark:_____

Contact telephone: _____ Supporter's Name: _____ Supporter's Cell phone Number:_____

Date	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-
1. Cough of any duration (Y/N)														
2. Fever (Y/N)														
3. Noticeable weight loss/Failure to Thrive /Poor weight gain (Y/N)														
4. Night sweats (Y/N)														
5. Reduced playfulness/lethargy/ irritability														
6. Contact with a TB case														

(Key: Y-Yes; N – No)

- If "Yes" to any question; take a detailed history, examine the patient and do sputum examination (sputum smear or GeneXpert).

Record your action in the table below

- If "No" to question 1-5 above, consider TPT eligibility and initiate workup for TB Preventive Therapy and repeat screening on subsequent visits.

Action taken/Date	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-	-/ /-
Gene Xpert (MTB Detected/MTB Not Detected)														
TB-LAM (should be done alongside Xpert) (Pos/Neg)														
Chest x-ray (Normal (N) /Suggestive of TB (S)														
Start anti-TB (Y/N)														
Invitation of contacts (Y/N)														
Evaluated for TPT (Y/N)														

TB Preventive Therapy client work up

Ask for the following		
Yellow coloured urine (Y/N)		
Numbness/ burning sensation in the hands or feet (Y/N)		
Examine for the following		
Yellowness of eyes (Y/N)		
Tenderness in the upper right quad-rant of the abdomen (Y/N)		
If the client has any of the above history or examination findings, defer TPT; manage the underlying condition and re-evaluate on next visit.		
If no to all the above initiate TPT and repeat evaluation on subsequent visit		
Indicate results (if available)		
1. Liver function test	ALT	Date:
	AST	Date:
2. TST		Date:
3. IGRA		Date:

Date started on TPT____/____/____	HIV Status_____
On ART? (Y/N)	ART Regimen_____
If on ART, date started ART____/____/____	

Treatment Options (Regimen)	Tick (√)	Dosage
3RH (75/50mg)		
3HP		
6H		

TPT Outcome (Tick √)	
Event	Date
Treatment Completed	
Lost To Follow Up	
Discontinued*	
Died	
Transferred Out	

*Reason for discontinuation	
Poor Adherence	(Tick √)
Adverse drug reaction	
Active TB disease	
Others	
Indication for TPT	
Household contact	
PLHIV	
HCW & Other Facility staff	
Prison Setting	
Other Clinical risk group	

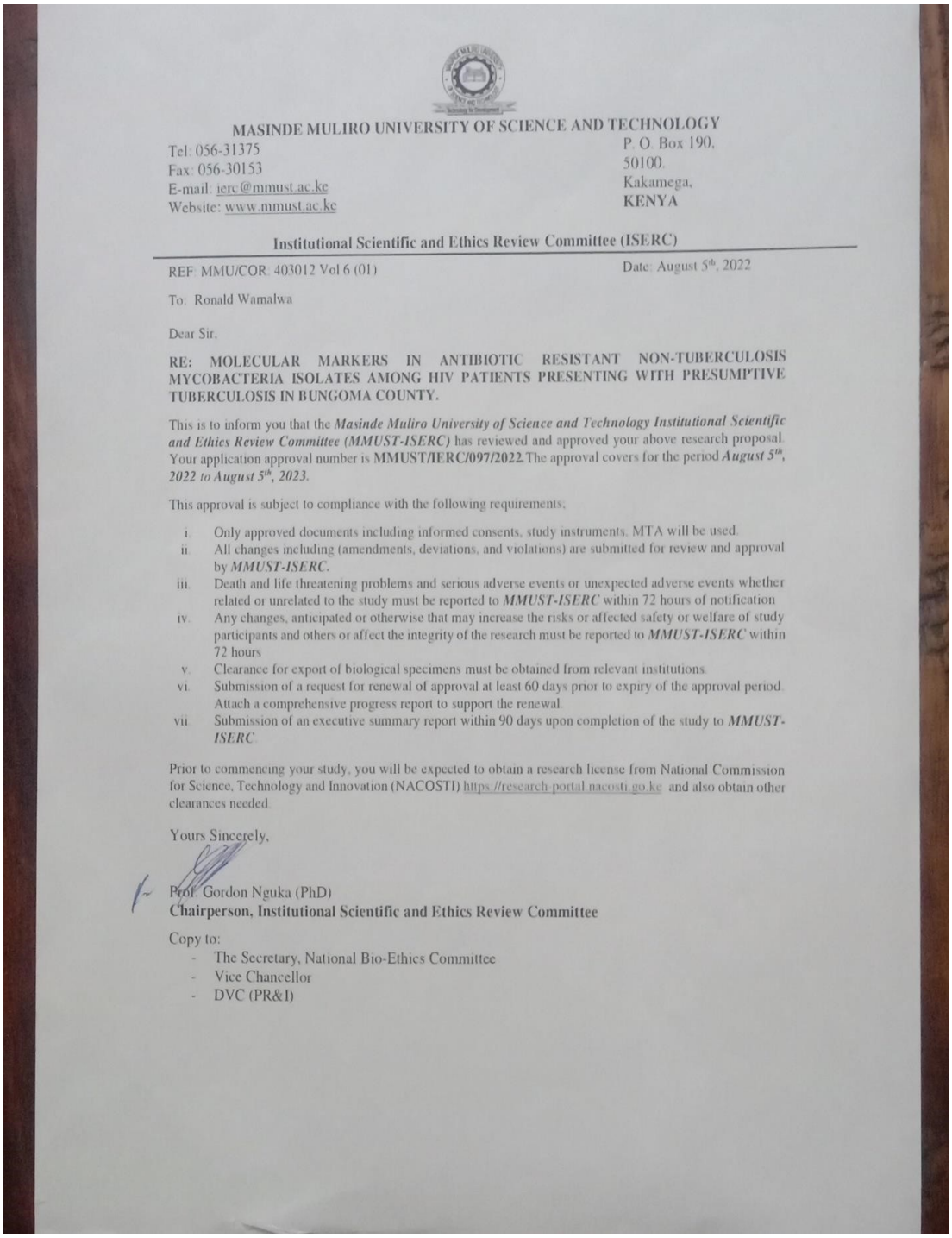
TPT due date	Date collected TPT	Weight (kg)	Hepatotoxicity (vomiting, right upper quadrant abdominal pain, yellow urine or eyes)		Peripheral Neuropathy Does client have any of the following in the limbs? Numbness, tingling or burning sensation		Hypersensitivity/rash		Others e.g gastrointestinal disturbances		Adherence Monitoring
			Yes (state action taken)	No	Yes (state action taken)	No	Yes (state action taken)	No	Yes, state effect and action taken	No	If non adherent, state the action taken e.g. decision made to stop TPT, adherence counseling, etc)

Comments (Remarks)



September 2020
MOH/DNTLD/ICFIPT/01

Appendix IV: MMUST-ISERC Approval



MASINDE MULIRO UNIVERSITY OF SCIENCE AND TECHNOLOGY

Tel: 056-31375
Fax: 056-30153
E-mail: ierc@mmust.ac.ke
Website: www.mmust.ac.ke

P. O. Box 190,
50100,
Kakamega,
KENYA

Institutional Scientific and Ethics Review Committee (ISERC)

REF: MMU/COR: 403012 Vol 6 (01) Date: August 5th, 2022

To: Ronald Wamalwa

Dear Sir,

RE: MOLECULAR MARKERS IN ANTIBIOTIC RESISTANT NON-TUBERCULOSIS MYCOBACTERIA ISOLATES AMONG HIV PATIENTS PRESENTING WITH PRESUMPTIVE TUBERCULOSIS IN BUNGOMA COUNTY.

This is to inform you that the ***Masinde Muliro University of Science and Technology Institutional Scientific and Ethics Review Committee (MMUST-ISERC)*** has reviewed and approved your above research proposal. Your application approval number is **MMUST/IERC/097/2022**. The approval covers for the period ***August 5th, 2022 to August 5th, 2023.***

This approval is subject to compliance with the following requirements;

i. Only approved documents including informed consents, study instruments, MTA will be used.
ii. All changes including (amendments, deviations, and violations) are submitted for review and approval by ***MMUST-ISERC.***
iii. Death and life threatening problems and serious adverse events or unexpected adverse events whether related or unrelated to the study must be reported to ***MMUST-ISERC*** within 72 hours of notification
iv. Any changes, anticipated or otherwise that may increase the risks or affected safety or welfare of study participants and others or affect the integrity of the research must be reported to ***MMUST-ISERC*** within 72 hours
v. Clearance for export of biological specimens must be obtained from relevant institutions.
vi. Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. Attach a comprehensive progress report to support the renewal.
vii. Submission of an executive summary report within 90 days upon completion of the study to ***MMUST-ISERC.***

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

Yours Sincerely,

Prof. Gordon Nguka (PhD)
Chairperson, Institutional Scientific and Ethics Review Committee

Copy to:
- The Secretary, National Bio-Ethics Committee
- Vice Chancellor
- DVC (PR&I)

Appendix V: Clearance from NACOSTI

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No:52737

Date of Issue: 12/January/202

RESEARCH LICENSE



This is to Certify that Mr.. Ronald wamalwa of Masinde Muliro University of Science and Technology has conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in B MOLECULAR MARKERS IN ANTIBIOTIC RESISTANT NON-TUBERCULOUS MYCOBACTERIA ISOLATES AMONG HIV PATIENTS PRESENTING WITH PRESUMPTIVE TUBERCULOSIS IN BUNGOMA COUNTY for the period end 1 /January/2024

License No:NACOSTI/P/23/22686

52737

Applicant Identification

Director

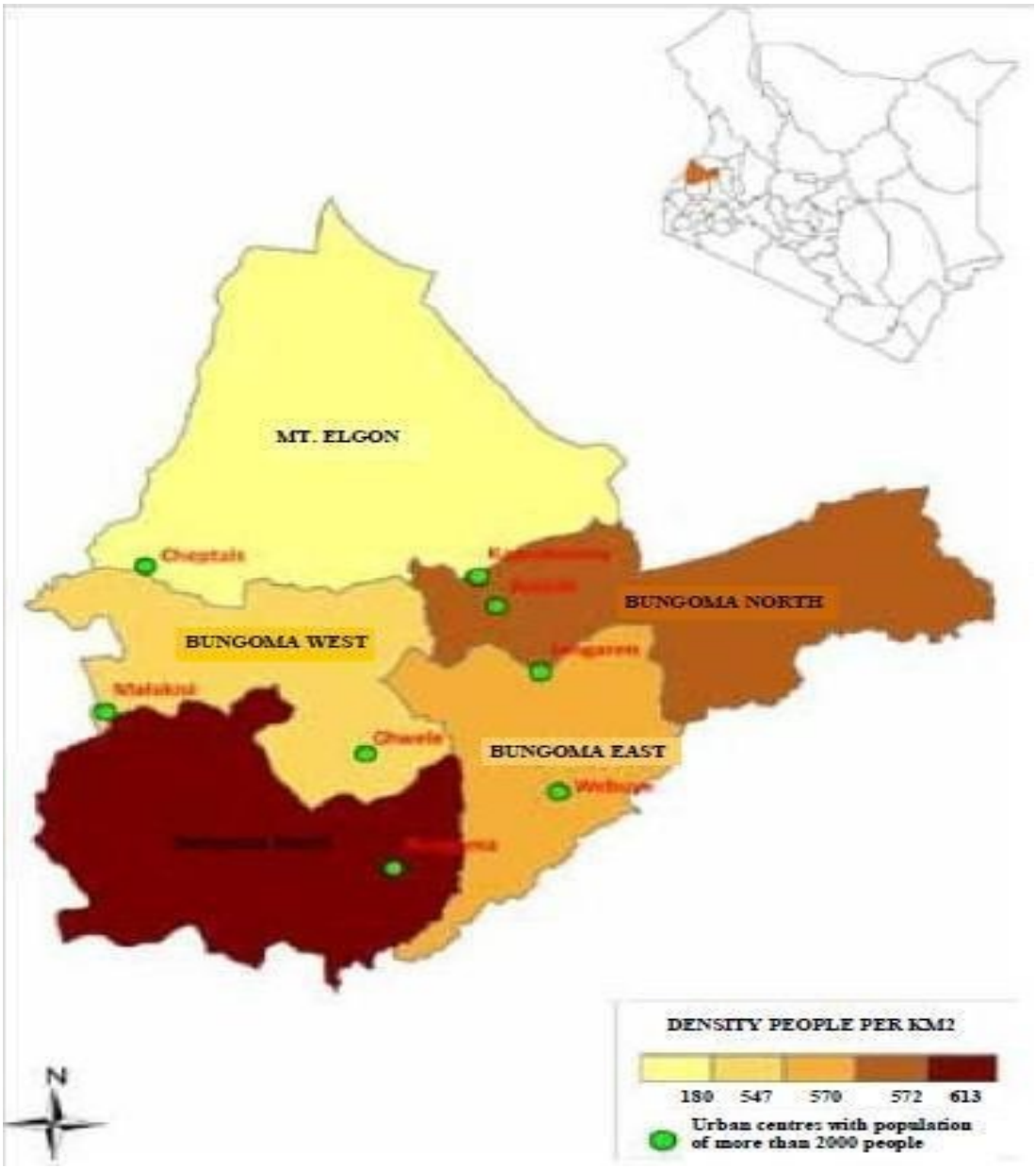
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity c
Scan the QR Code using QR scanner

See overleaf for

Appendix VI: Map of Bungoma county



Appendix VII: Antimicrobial breakpoints

Antimicrobial Agent		MIC breakpoints (μg/ml)		
		Susceptibility	Intermediate	Resistance
Macrolides	CLR[a]	≤2	4	≥8
	AZM[c]	≤2	4	≥8
Rifamycins	RIF[a]	-	-	>1
	RFB[a]	-	-	>2
Aminoglycosides	STR[a]	-	-	≥5
	GEN[b]	<=4	8	>=16
	KAN[a]	-	-	≥4
	TOB[a]	≤2	4	≥8
	NEO[b]	-	-	>=10
	AMK[a]	≤16	32	≥64
Fluoroquinolones	MXF[a]	≤1	2	≥4
	CIP[a]	≤1	2	≥4
	LVX[a]	≤2	4	≥8
Cephalosporin	FOX[a]	≤16	32-64	≥128
	CMZ[d]	≤16	32	≥64
Tetracyclines	TCY[b]	<=4	8	>=16
	DOX[a]	≤1	2-4	≥8
	MNO[a]	≤1	2-4	≥8
Glycylcycline	TGC[a]	≤1	2-4	≥8
Sulfonamides	SOX[a]	≤38	-	≥76
Carbapenems	IMP[a]	≤4	8-16	≥32
Oxazolidinones	LNZ[a]	≤8	16	≥32
Lincosamides	CLI[b]	<=0.5	1-2	>=4
Penicillins	OXA[b]	<=2	-	>=4
Polypeptides	TEC[b]	<=8	16	>=32
	VAN[b]	<=2	4-8	>=16
Others	CFZ[e]	-	-	>1
	INH[a]	-	-	≥1
	EMB[a]	-	-	≥4
	ETH[a]	-	-	>5

a denotes the breakpoints coming from **Susceptibility Testing of Mycobacteria, Nocardia, and Other Aerobic Actinomycetes; Approved Standard–Second Edition**. *CLSI document M24-A2*.
b denotes the breakpoints coming from Performance Standards Antimicrobial Susceptibility Testing-27th Edition. *CLSI document M100*.
c, d, e denote the breakpoints coming from [18–20], respectively.
d, S, susceptible, I, intermediate susceptible, and R, resistant.

CLR-Clarithromycin, AZM-Azithromycin. RIF-Rifampicin, TFB-Rifabutin, STR-Streptomycin, GEN-Gentamycin, KAN-Kanamycin, TOB-Tobramycin, NEO-Neomycin, AMK-Amikacin, MXF-Moxifloxacin, CIP-Ciprofloxacin, LVX-Levofloxacin, CMZ-Clofazimine, TCY-Teicoplanin, DOC-Doxycycline, MINO-Minocycline, TGC-Tigecycline, COX-Cefoxitin, IMP-Imipenem, LXZ-Linezolid, CLI-Clindamycin, OXAOxacillin, TEC-Tetracycline, VAN-Vancomycin, CFZ-Cefmetazole, INH-isoniazid. EMB- Ethambutol, ETH-Ethionamide