Identification and clinical correlation of Non-tuberculous Mycobacteria isolates from pulmonary tuberculosis suspects with HIV co-infection at UTH, Lusaka, Zambia

By:

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A Research Dissertation Submitted to the University of Zambia in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

The University Of Zambia

School Of Medicine

Lusaka

Declaration

I, Hendrix Mundia Kangongwe, declare that this dissertation is a product of my own work. It is being submitted to the University of Zambia for the award of the Degree of Master of Science in Medical Microbiology. It has not been submitted before for any degree to this or any other University.

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Certificate of Approval

Dissertation Title**: Identification and Clinical Correlation of Non-tuberculous mycobacteria isolates from pulmonary tuberculosis suspects with HIV-co-infection at the University Teaching Hospital, Lusaka, Zambia (September, 2016).**

This dissertation by **Hendrix Mundia Kangongwe** has been approved for submission to the University of Zambia in partial fulfillment of the requirements for the degree of Master of Science in Medical Microbiology.

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Dedication

Firstly, I wish to thank the Lord God Almighty for His mercy and love, without whom I would not have accomplished this work. My special dedication goes to my dear wife Angela and indeed our wonderful children. I dedicate this work to you. Thank you for your words of encouragement, your support and indeed understanding. It really wasn't easy at times, especially when it came to apportioning resources between family and academia. Your understanding was exemplary and I am really grateful for that.

ABSTRACT

Non-tuberculosis *Mycobacteria* (NTM) are increasingly recognized as human pathogens especially with the emergence of Acquired Immunodeficiency Syndrome (AIDS). Similarity of clinical symptoms with TB, coupled with limited diagnostic capacities in tuberculosis (TB)-endemic areas has led to misdiagnosis of NTM pulmonary disease especially in Human Immunodeficiency Virus (HIV)-infected patients. The study aimed at assessing the spectrum of NTM species causing pulmonary symptoms in HIV-infected patients, with a secondary objective of assessing association of demographics, anaemia, body mass index (BMI), and CD4 count with NTM pulmonary disease. The study was a laboratory based cross-sectional study involving 56 NTM archival isolates from cultures of suspected pulmonary TB patients with HIV co-infection at the University Teaching Hospital (UTH), Lusaka, Zambia. Geno Type *Mycobacterium* CM/AS kits were used for speciation of NTM. Descriptive statistics was used to summarize data, while inferential statistics was used to evaluate associations between symptomatic NTM (dependent variable) and predictor variables. *M. immunogenum* was the most common NTM species (5.0%), followed by *M. abscessus* (3.75%), *M. smegmatis* (3.75%) *M. gordonae* (2.5%), *M. fortuitum* (1.25%), *M. intracellulare, M. mageritense, M. celatum,* and *M. chelonae,* all at 1.25% frequency of isolation. Organisms besides NTM species included members of the *M. tuberculosis complex* (MTBC) (23.75%), Gram positive bacteria with a high G+C content (43.75%) though not identified to species level. Five isolates were negative, while four could not be identified. Isolation of some pathogenic and potentially pathogenic NTM species with accompanying characteristics of clinical disease in the current study, could be suggestive of their clinical significance in HIV-infected patients in our study. The study also showed some correlations between pulmonary NTM disease and the other predictor variables (BMI, anaemia, and CD4count), though these associations were not statistically significant. The small sample size used in the study however, may not permit generalization of study findings but could form a basis for further research.

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LIST OF ABBREVIATIONS AND ACRONYMS

- AIDS Acquired Immunodeficiency Syndrome
- AFB Acid-fast bacilli
- AS Additional species
- ART Anti-retroviral therapy
- ATS American Thoracic Society
- BCG Bacillus Calmette-Guerin
- BMI Body mass index
- CM Common Mycobacteria
- CD Cluster of differentiation
- DNA Deoxyribonucleic acid
- EQA External quality assurance
- IMReT Institute for Medical Research and Training
- HAART Highly active anti-retroviral treatment
- Hb Haemoglobin
- HIV Human immunodeficiency virus
- HRCT High resolution computed tomography
- IDSA Infectious diseases society of America
- ITS Intergenic transcribed spacer
- MAC *Mycobacterium avium* complex
- MGIT *Mycobacterium* growth indicator tubes
- MOTT *Mycobacterium* other than tuberculosis
- MPB64 *Mycobacterial* protein fraction of BCG 64

- MTBC *Mycobacterium tuberculosis* complex
- NaCl Sodium chloride
- NTCP National Tuberculosis Control Programme
- NTM Non-tuberculous mycobacteria
- PCR Polymerase chain reaction
- PLWHA People living with HIV and AIDS
- PNBA *P*-nitrobenzoic acid
- rRNA Ribosomal ribonucleic acid
- TB Tuberculosis
- TCH Thiophen-2-carboxylic acid hydrazide
- UNZABREC University of Zambia Biomedical Research Ethics Committee
- UTH University Teaching Hospital
- WHO World Health Organization
- ZN Ziehl-Neelsen

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Chapter 1.0 General introduction

1.1 Background

Non-tuberculous mycobacteria (NTM) cause a variety of infections in humans (Griffith *et al*., 2007, van Ingen, 2013) with chronic, debilitating pulmonary disease among immunocompromised persons being the most common clinical manifestation (Winthrop *et al*., 2010). Infections in immunocompetent individuals, however, have been reported (Griffith *et al*., 2007). Non-tuberculous mycobacteria may be a clinically important lung disease that mimics tuberculosis (TB). However, in contrast to industrialized countries where the prevalence of pulmonary and extra pulmonary NTM disease is well documented (McCarthy *et al*., 2012), little data are available on the burden of NTM and its clinical importance in resource-poor settings (Buijtels *et al*., 2010, Chanda-Kapata *et al*., 2015, Gopinath and Singh, 2010). Similar to TB, NTM infections occur at higher frequency in HIV-infected patients (Dhungana *et al*., 2008, Nyamogoba *et al*., 2011, Mahon *et al*., 2014), especially in TB-endemic areas with a corresponding high burden of HIV and AIDS (Fair *et al*., 2007, Nyamogoba *et al*., 2012).

In most resource-limited settings, sputum smear examination for acid-fast bacilli (AFB), is still the mainstay of pulmonary tuberculosis (PTB) diagnosis (Fair *et al*., 2007, Chanda-Kapata *et al*., 2015) and like TB, NTM stain acid-fast, making the two indistinguishable by this method. Additionally, similarity of clinical symptoms with TB has led to misdiagnosis of NTM pulmonary disease, especially in Human Immunodeficiency Virus (HIV)-seropositive individuals (Buijtels *et al*., 2010, McCarthy *et al*., 2012, Yu *et al*., 2014). As a result, majority of the pulmonary diseases caused by NTM are not diagnosed to species level but are treated with conventional anti-TB treatment (ATT) which eventually fails because majority of the NTM are resistant to conventional ATT regimens (Aliyu *et al*., 2013, Yu *et al*., 2014). This can further be compounded by misdiagnosis as multi-drug resistance tuberculosis (MDR-TB) when subjected to phenotypic drug susceptibility testing. The non-responsiveness of most NTM species to conventional TB drugs pauses a serious challenge considering that these organisms have the capacity to significantly affect various systems in immunosuppressed patients (Gunaydin *et al*., 2013). Therefore, detection and identification of *Mycobacterium* species and determination of their susceptibility profiles is necessary for infection control and for the determination of epidemiology and treatment strategies (Saifi *et al*., 2013, Aliyu *et al*., 2013, Chanda-Kapata *et al*., 2015).

Despite the epidemiology of tuberculosis being well documented (Glaziou *et al*., 2013), the prevalence and epidemiology of NTM disease in many geographical regions, is largely unknown (Cassidy *et al*., 2009, Winthrop *et al*., 2010, Aliyu *et al*., 2013). However, even in the absence of documented large-scale population-based studies of prevalence or associated risk factors (Griffith *et al*., 2007, Cassidy *et al*., 2009), there is widespread belief among experts that pulmonary NTM disease is increasingly prevalent and perhaps even more prevalent than TB (Griffith *et al*., 2007, Park et al., 2010, van Ingen, 2013). Studies have shown that some NTM species are potentially pathogenic to humans particularly with increased incidence of Acquired Immunodeficiency Syndrome (AIDS) in recent years (Crump *et al*., 2009, Nyamogoba *et al*., 2011). In addition, widespread use of chemotherapeutics for interventional therapies and cancer treatment has further increased the prevalence and importance of NTM infections (Phillips and Von Reyn, 2001, Jarzembowski and Young, 2008). This is likely to be of relevance to Zambia given the high prevalence of cancer and the availability of chemotherapy besides the already high prevalence of HIV and AIDS.

It is however regrettable to note that, clinical and laboratory diagnosis of infections caused by NTM species is relatively difficult and expensive (Falkinham 3rd, 1996, De Groote and Huitt, 2006b). Diagnosis of NTM in HIV-infected patients is particularly challenging due to the high prevalence of MTBC disease which has similar microbiologic and clinical characteristics. This tends to mask disease due to NTM as both diseases can present with cavitary or non-cavitary lung disease, with a noticeable poor prognosis (McCarthy *et al*., 2012).

The low sensitivity and specificity of traditional methods such as microscopy has necessitated discovery of newer diagnostic methods with high sensitivity and specificity such as Polymerase Chain Reaction (PCR) based methods, as described in some previous studies (Shrestha *et al*., 2003, Li *et al*., 2009, Kim *et al*., 2010). However, where identification is not possible, consideration for NTM species distribution may aid clinicians in selection of empirical therapy (Phillips and Von Reyn, 2001, Wagner and Young, 2004).

1.2 Statement of the Problem

The rate of isolation of NTM and their increasing incidence in HIV-infected patients (Dhungana *et al*., 2008, Nyamogoba *et al*., 2011, van Halsema *et al*., 2015) and other immunocompromised patients (Phillips and Von Reyn, 2001, Jarzembowski and Young,

2008) has increased over the past several years. In some areas, the isolation rate of these bacteria has exceeded that of *Mycobacterium tuberculosis* complex (Dhungana *et al*., 2008, Park *et al*., 2010) resulting in a widened spectrum of clinical manifestations associated with the various NTM species. This rise in the number of NTM isolation is of concern as these organisms are both difficult to diagnose and to treat (Falkinham 3rd, 1996, De Groote and Huitt, 2006b, Gunaydin et al., 2013). In comparison to industrialized countries where the prevalence of pulmonary and disseminated disease due to NTM in people with HIV infection is well described (McCarthy *et al*., 2012), less is known about the prevalence of NTM disease in developing countries like Zambia, which still reports a high burden of TB in addition to the burden of HIV and AIDS (Fair *et al*., 2007, Buijtels *et al*., 2010, Nyamogoba *et al*., 2012). Little is also known about the significance of these organisms in Zambia and other resource-limited settings (Buijtels *et al*., 2010, Gopinath and Singh, 2010, Chanda-Kapata *et al*., 2015) and especially the extent to which they contribute to morbidity and mortality in HIV-infected patients.

1.3 Justification of the Study

The emergence of non-tuberculous mycobacteria (NTM) as opportunistic pathogens in HIV-infected patients is gaining clinical significance (Crump *et al*., 2009, Nyamogoba *et al*., 2011). An understanding of the clinical relevance of NTM isolates, particularly in settings of high HIV and tuberculosis prevalence, is important (van Halsema *et al*., 2015). Treatment of NTM disease is not directly analogous to that of TB. Similarly, *in vitro* susceptibility testing results are not well correlated with clinical response to anti-mycobacterial agents (Griffith *et al*., 2007). Furthermore, the high frequency of NTM isolation in HIV-infected individuals calls for accurate species identification prior to commencement of therapy (Lan *et al*., 2011). It's against this backdrop that this study was carried out with a view of enhancing diagnostic capacity for NTM by species identification and evaluation of clinical relevance of NTM as such information holds potential to provide useful data on epidemiology and patient management.

1.4 Research Questions

1. What is the spectrum of NTM species among HIV-infected patients presenting with TB-like disease at University Teaching Hospital, Lusaka?
2. Is there an association between demographics (gender, age), clinical features and other factors (fever, cough, chest pains, night sweats, BMI, Hb, CD4 count) and development of pulmonary NTM disease among HIV-infected patients in Zambia?

1.5 Objectives

1.5.1 Main Objective

To identify the common NTM species in HIV and AIDS patients presenting with symptomatic pulmonary disease and to correlate their clinical features and risk factors to isolated NTM species.

1.5.2 Specific Objectives

1. To determine the species diversity of NTM isolated from culture positive sputum samples of HIV-infected patients presenting with TB-like symptoms.
2. To evaluate the association of demographic characteristics (age & sex), clinical features and other factors (fever, cough, chest pains, night sweats, BMI, haemoglobin level, CD4 count) and pulmonary NTM disease in HIV-infected patients.

Chapter 2.0 Literature Review

Tuberculosis (TB) is a major global health problem, ranking above HIV and AIDS as one of the leading causes of death from an infectious disease (Dawson *et al*., 2015). It is an important health concern for developing countries (Singh *et al*., 2014) and it has also been reported as the major cause of morbidity and mortality in populations of high HIV prevalence (World Health Organization (2004). The disease is caused by species of the genus *Mycobacteria* collectively referred to *M. tuberculosis* complex (MTBC) which comprises several related species, namely *M. bovis, M. bovis*-BCG, *M. africanum, M. microti, M. cannetti*, *M. caprae*, *M. pinnipedii*, *M. suricattae*, *M. mungi*, *M*. *orygis,* and *M. tuberculosis*. However, there are other species of mycobacteria that are usually saprophytes, with potential to be opportunistic and at times even be deadly pathogens (Singh *et al*., 2014). These other mycobacteria were previously known by different names such as 'atypical', 'anonymous', '*Mycobacteria* other than tuberculosis' (MOTT) (Portaels, 1995, Katoch, 2004) or potentially pathogenic environmental mycobacteria (PPEM). The acronym non-tuberculosis mycobacteria (NTM) given by the International Working Group on Mycobacterial Taxonomy is universally accepted to refer to *Mycobacteria* other than *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium leprae* (Gopinath and Singh, 2010, Sharma, 2013, Yu *et al*., 2014). They are typically environmental organisms residing in soil and water (Falkinham, 2010). Although generally of low pathogenicity to humans, NTM can cause a wide array of clinical diseases; pulmonary disease being the most frequently seen (van Ingen, 2013).

Non-tuberculous mycobacteria have been seen to be associated with untreated HIV infection due to decline in number and function of CD4+T-lymphocytes (Dhungana *et al*., 2008). *Mycobacteria* species are among the most common opportunistic infections in people living with HIV and AIDS (PLWHA), causing significant mortality and morbidity (Dhungana *et al*., 2008). The ubiquitous nature of these organisms significantly contributes to the ease of colonization among immunocompromised hosts such as HIV and AIDS patients to cause disease (Dhungana *et al*., 2008, Nyamogoba *et al*., 2011, Mahon *et al*., 2014). Dhungana and coworkers documented a higher prevalence of *Mycobacterium avium* complex (MAC) (41%) than of MTBC (27%) in HIV and AIDS patients in Nepal. The majority of the co-infected persons were sputum smear-negative; suggesting the value of culture in screening tuberculosis in HIV infected patients (Dhungana *et al*., 2008, Nyamogoba *et al*., 2012). The

Nepal study further highlighted the necessity of adopting new policies regarding the surveillance and treatment of MAC infection in HIV patients.

Unlike MTB, there is no evidence of human to human transmission of NTM disease (Ahmed *et al*., 2014, Mahon *et al*., 2014). Humans are thought to get infection from environmental sources with pulmonary disease probably being caused by the inhalation of aerosols containing the mycobacteria. The incidence of disease caused by NTM is somewhat independent of that of TB, but is determined by the number, distribution, and species of NTM in the environment and the susceptibility of the human population (Ioachimescu and Tomford, 2015). Considering that different NTM species have different antibiotic susceptibility patterns, their resistance to anti-tuberculosis drugs is of particular importance. Hence, accurate and early differential diagnosis of NTM is required for optimal outcomes (Singh *et al*., 2014).

Studies show that some countries report NTM prevalence rates as high as 50% among cultured mycobacteria (Park *et al*., 2010, Buijtels *et al*., 2010). In view of the high isolation rates of NTM from clinical specimens, the American Thoracic Society (ATS) published guidelines for the diagnosis of NTM disease which recommend identification of positive NTM cultures (Griffith *et al*., 2007). In line with the ATS recommendations, in case of NTM culture isolation, review of clinical records is necessary to distinguish true disease from colonization (Khatter *et al*., 2008, Winthrop et al., 2010). Accordingly, microbiologic criteria for disease have been developed by an expert ATS and Infectious diseases society of America (IDSA) committee as reported by Griffith and colleagues in 2007.These criteria, when present in the context of symptoms and characteristic radiologic abnormalities, suggest that NTM disease is present (Griffith *et al*., 2007).

2.1 Geographic diversity of NTM species

2.1.1 Global perspective

Non-tuberculous mycobacteria are widely distributed in the environment with high isolation rates worldwide (Von Reyn *et al*., 1993, Falkinham, 2002). As earlier documented, these microorganisms can be found in soil and water, including both natural and treated water sources, with Mycobacterium *kansasii*, *M. xenopi*, and *M. simiae* having been recovered almost exclusively from municipal water sources and rarely, if ever, from other environmental sources (Griffith *et al*., 2007).

A significant knowledge gap relating to the geographical distribution of NTM isolation worldwide exists (Hoefsloot *et al*., 2013). According to the study by Hoefsloot and colleagues, species identification data for 20 182 patients, from 62 laboratories in 30 countries across six continents were received, with 91 different NTM species isolated. *Mycobacterium avium* complex (MAC) predominated in most countries, followed by *M. gordonae* and *M. xenopi*. Important differences in geographical distribution of MAC species as well as *M. xenopi, M. kansasii* and rapid-growing mycobacteria were observed.

Studies carried out in India documented that the exact magnitude of NTM disease in India is not well known (Khatter *et al*., 2008, Sharma, 2013) due to failure to routinely perform culture with strict criteria in most parts of the country, thereby attributing NTM isolation to mere contamination (Sharma, 2013). This is despite the fact that these organisms have been seen to be important causes of morbidity and mortality in Western countries and India (Sharma, 2013). This highlights the need for speciation when choosing antibiotic regimens especially in immunocompromised patients, in whom the presence of any acid fast bacilli may be considered significant (Sharma, 2013).

In Bogota, Colombia, Murcia-Aranguren and others (2001) analyzed the frequency of TB and NTM in HIV infected patients. Overall 43 of 1,622 cultures (2.6%) were positive for mycobacteria. Twentytwo sputum samples were positive. Four patients were diagnosed with *M. tuberculosis* complex (1.4%). *M. avium* was isolated in thirteen patients (4.5%). They concluded that NTM infections are frequent in HIV infected patients in Bogota (Murcia-Aranguren *et al*., 2001).

In a study conducted in Southern-Central China involving 101 randomly selected NTM patients, Yu *et al.*(2014) reported that nine (8.91%) immunocompromised cases; including type two diabetes mellitus (T2DM) and HIV and AIDS were found to be infected with NTM. This study revealed the distribution and characteristics of NTM-AFB pathogen infection in Southern-Central China, and suggested that physicians should be alert of the emergence of NTM infection in AFB positive cases. Furthermore, caution must be taken when choosing chemotherapy for TB-like pulmonary infections (Yu *et al*., 2014). The identification of these pathogens in T2DM and AIDS patients suggests that non-tuberculous AFB opportunistic infections should be taken into consideration in immunocompromised patients. The researchers further echoed the similarity of clinical laboratory and image findings in patients with NTM disease with those with drug resistant TB, or non-mycobacterial AFB infections.

Majority of these patients present with AFB-positive smear results and they usually have no response to first-line and some second-line anti-TB chemotherapy (Yu *et al*., 2014).

2.1.2 African perspective

A retrospective study conducted in Oyo and Osun States in Nigeria, looked at patients who were diagnosed with pulmonary TB and had culture and drug susceptibility test results from the Institute of Tropical Medicine Antwerp, Belgium between 2007 and 2009. A total of 77 patients samples with culture results were identified, out of which 70 (90.9%) were *M. tuberculosis* isolates and 7 (9.1%) were NTM. Among the seven NTM, three were *Mycobacterium fortuitum* (*M. fortuitum*), two were *Mycobacterium intracellulare* (*M. intracellulare*) and two were *Mycobacterium chelonae* (*M. chelonae*). Of the seven, 4 (57.1%) were from previously treated patients with anti-TB drugs who were categorized as category II failure while 3 (42.9%) were diagnosed as new patients who had actually started anti-tuberculosis treatment (ATT) based on the positive smear result. It was therefore concluded that there was need for all cases of pulmonary tuberculosis to undergo sputum culture and species identification (Daniel and Osman, 2011). The researchers also suggested that there was need for a nationwide survey to quantify the burden of NTM in the country.

A study conducted in Ghana showed frequent isolation of NTM (8%) among HIV-infected patients eligible for anti-retroviral therapy (ART) (Bjerrum *et al*., 2016). Though isolation of NTM in this study was not associated with early mortality, data presented indicated that NTM could be of clinical relevance warranting increased attention and more research. Severe clinical signs and symptoms were shown to be significant predictors of NTM isolation. According to this study, significant associations were seen with regard to low CD4 count (<100 cells/µl), BMI <18.5 kg/m^2, cough ≥2 weeks or fever ≥2 weeks. *Mycobacterium avium* complex (MAC), was the most frequently isolated NTM specie in this study, followed by *M. chelonae* complex, *M. simiae*, and *M. fortuitum* complex, among others (Bjerrum *et al*., 2016).

In Kenya, Nyamogoba and colleagues sought to evaluate the prevalence of TB-HIV co-infection and demonstrate the confusion caused by NTM and HIV/AIDS co-infection in TB diagnosis and treatment (Nyamogoba *et al*., 2012). In this study, it was reported that; like elsewhere, HIV has contributed significantly to the rise in proportions of smear-negative TB cases with the study reporting ZN- smear negativity in 5.8% of the TB cases (Nyamogoba *et al*., 2012).

Studies conducted in Zambia have isolated NTM from humans and animals using a combination of phenotypic and molecular methods (Lubasi *et al*., 2009, Malama *et al*., 2014, Mwikuma *et al*., 2015). Symptomatic NTM pulmonary disease was reported to be significantly higher in HIV-seropositive individuals than their HIV-negative counterparts (Buijtels *et al*., 2010, Chanda-Kapata *et al*., 2015). These and other reported findings suggest that there is a significant correlation between HIV and NTM isolation in sputum (Griffith *et al*., 2007, Lan *et al*., 2011, Hoza *et al*., 2016). This current study provides data in support of this possible correlation.

In a cross-sectional study of the prevalence of NTM among adults aged 15 years and above, who were participants in a national TB prevalence survey in Zambia, 15.1% of presumptive TB cases had NTM, with a high rate (71%) of study participants, that were symptomatic (Chanda-Kapata *et al*., 2015). Given this high prevalence of NTM in Zambia, the load could be significantly higher in the HIV-positive group taking into account the high HIV burden in Zambia standing at 13% in the 15-49 years age bracket, with a peak of 23% in the 40-44 years age bracket as of 2013-2014 (Demographic, 2014).

In an earlier study conducted in Zambia, the authors hypothesized that there is limited information regarding the distribution of NTM in Africa, despite some studies having been conducted in this area (Buijtels *et al*., 2009). This study isolated NTM from 31 out of 180 chronically ill patients of which 19 (11%) had exclusively NTM, while 12 (7%) had NTM-MTBC co-morbidity. From the study by Buijtels and colleagues, MAC was the most isolated NTM in both patients and controls, a finding that is consistent with studies conducted in other parts of the globe (Cassidy *et al*., 2009, Simons *et al*., 2011, Gopinath and Singh, 2010, Wang *et al*., 2014). A limitation with Buijtels' study was that up to 32% of the NTM isolated from both groups (chronically ill patients and healthy controls) could not be identified to species level (Buijtels *et al*., 2009). However, the researchers indicated that the distribution of NTM in Africa may differ from that in Europe and the United States, with unidentified NTM probably colonizing persons in Africa, thereby causing disease in some instances. However, the magnitude of this problem, in addition to the problem of TB, is unknown but deserves more attention (Buijtels *et al*., 2009). Hence the current study sought to provide an insight into the distribution of NTM species in Zambia with respect to immunosuppression due to HIV and AIDS. The study also attempted to identify potential risk factors for development of NTM pulmonary disease among HIV-infected patients in Zambia.

A follow-up study examined the accuracy of the clinical diagnosis of TB based on sputum smears and x-ray in the era of increasing HIV prevalence, and also to evaluate the clinical relevance of NTM in Zambia (Buijtels *et al*., 2010). In this particular study, the researchers isolated *M. tuberculosis*, a combination of *M. tuberculosis* and NTM, and exclusively NTM from sputum at the rates of 36%, 13%, and 18% of cases, respectively. Of the presumptively diagnosed TB patients, 73% were HIV-positive of which 36% of these had positive NTM cultures. This finding was higher than in the HIV-negative group (32%), suggesting that immunosuppression played a role in development of TB-like disease in Zambia (Buijtels *et al*., 2010).

In a study conducted in Namwala district of Zambia (Malama *et al*., 2014), a range of NTM were isolated from sputum samples of suspected TB patients, gross lesions from slaughtered cattle, as well as lymph node tissue samples from Kafue Lechwe. Genotypic classification involving sequencing of the 16S ribosomal RNA gene was used for speciation of the NTM isolates from both humans and cattle. *M. intracellulare* was reported as the most isolated NTM species (Malama *et al*., 2014). This finding was in agreement with a more recent study (Mwikuma *et al*., 2015) which was primarily aimed at characterization of NTM species from clinical specimens. Isolation of NTM from human sputum and granulomatous lesions of affected animals suggested the possibility of these organisms playing a role in the observed disease pathologies (Malama *et al*., 2014).

In another study aimed at optimizing the recovery rate of *Mycobacterium* species from gastric lavages in children; out of 113 Mycobacterium species, 110 (97.3%), were MTBC, while 3 (2.7%) of the isolates, were the non-tuberculous *Mycobacterium avium* complex (MAC) and *M. kansasii*. The researchers could not however, conclude whether the NTM species were causing tuberculosis and thus suggested that these findings needed to be studied further (Lubasi *et al*., 2009). The study by Lubasi *et al*. (2009) used phenotypic methods including specific morphology in liquid media and biochemical tests such as niacin accumulation, *P*-Nitrobenzoic acid (PNBA) sensitivity, thiophen-2-carboxylic acid hydrazide (TCH) sensitivity, Sodium Chloride (NaCl) tolerance, catalase production, nitrate reduction, and growth at 25^0C and 42^0C for species differentiation within the NTM group as well as to confirm *M. tuberculosis*.

A recent study by Mwikuma and colleagues identified NTM from clinical specimen collected from four regions of Zambia. The 16S-23S rRNA gene sequence analysis of the intergenic

transcribed spacer (ITS) region of Mycobacteria was used for analysis. Out of the 91 NTM suspected isolates, fifty four (59%) isolates were identified as NTM, of which *M. intracellulare* predominated (27.8%), followed by *M. lentiflavum* (16.7%), *M. avium* (14.8%), and *M. fortuitum* (7.4%), among others. *Mycobacterium kumamotonense, M. indicuspranii, M. flavescens, M. bouchedurhonense, M. chimaera, M. europaeum* and *M. nonchromogenicum* were, according to the researchers, identified and reported for the first time in Zambia (Mwikuma *et al*., 2015). The researchers however, could not conclusively link the identified NTM species to cases as clinical data was not available and therefore, concluded that all the species identified in the study were potentially pathogenic (Mwikuma *et al*., 2015).

2.2 Taxonomy:

The genus *Mycobacterium* is composed of more than 100 species (Gopinath and Singh, 2010, Ioachimescu and Tomford, 2015)Jang *et al*., 2014). The published mycobacterial species and subspecies are included in the List of Prokaryotic Names with Standing in Nomenclature. Mycobacterial species were classically differentiated using cultural and biochemical properties, but genetic differences are now used for this purpose (Ioachimescu and Tomford, 2015), especially 16S ribosomal RNA sequence differences. The genus *Mycobacterium* contains two obligate pathogens, the *M. tuberculosis* complex and *M. leprae*. The *M. tuberculosis* complex comprises several related mycobacteria species including *M. bovis, M. bovis*-BCG, *M. africanum*, *M. microti, M. cannetti*, M. caprae, *M. pinnipedii*, *M. suricattae, M. mungi*, *M. orygis*, and *M. tuberculosis*, which on DNA analysis are all variants of *M. tuberculosis*. Besides the already mentioned obligate pathogens of the genus *Mycobacteria*, there are other species which live freely in the environment (water and soil) and are thus often termed *environmental mycobacteria* or *non-tuberculous mycobacteria* (Ioachimescu and Tomford, 2015, Gopinath and Singh, 2010, Sharma, 2013).

2.3 Classification of NTM

Non-tuberculous *Mycobacterium* classifications have generally not been helpful to the clinician (Ioachimescu and Tomford, 2015). The most widely used classification in the past, the Runyon system, was based on microbiologic characteristics of the organisms, such as growth rate in cultures and colony pigment formation in the presence or absence of light. Familiarity with the Runyon system remains useful for presumptive laboratory identification of possible NTM pathogens. However, positive identification of NTM species is now largely

based on molecular biology techniques. Classification of NTM based on the organ system of primary involvement (e.g., lungs, lymph nodes, disseminated, skin, and soft tissue) is more useful to the clinician (Table 2.1).

Table 2.1: Major Clinical Syndromes Associated with NTM Infections.

Syndrome	**Common Causes**	**Less-Common Causes**
Pulmonary disease (especially in adults)	*M. avium-intracellulare*, *M. kansasii*, *M. abscessus*	Uncommon: *M. fortuitum*, *M. malmoense*, *M. szulgai*, *M. scrofulaceum*, *M. smegmatis*, *M. simiae*, *M. xenopi* Rare: *M.celatum*, *M. asiaticum*, *M. shimodei*
Cervical and lymphadenitis (especially children)	*M. avium*, *M. intracellulare*	*M. scrofulaceum*, *M. malmoense*, *M. abscessus*, *M. fortuitum*
Skin and soft tissue disease	*M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. marinum*	*M. haemophilum*, *M. kansasi*, *M. smegmatis*, *M. ulcerans*
Skeletal (bones, joints, tendons) disease	*M. marinum*, *M. avium* complex,	*M.haemophilum*, *M. scrofulaceum*, *M. smegmatis*,
	M. kansasii, *M. fortuitum* group, *M. abscessus*, *M. chelonae*	*M. terrae – nonchromogenicum* complex
Catheter-related infections	*M. fortuitum*, *M. abscessus*, *M. chelonae*	*M. mucogenicum*

Disseminated infection	HIV- seropositive host: *M. avium, M. kansasii*	*M. haemophilum, M. genavense, M. xenopi, M. marinum, M. simiae, M. intracellulare, M. scrofulaceum, M. fortuitum*
	HIV- seronegative host: *M. abscessus,* *M. chelonae*	*M. marinum, M. kansasii, M. haemophilum, M. fortuitum*

Taken from Ioachimescu & Tomford, 2015

Based on cultural characteristics, NTM are classically grouped into two main groups: those which form colonies on sub-culture in 7 days or less, are referred to as "rapidly growing mycobacteria" or RGM. Conversely, NTM isolates that require more than 7 days to form mature colonies on subculture are termed "slowly growing mycobacteria." (Griffith *et al*., 2007, Mahon *et al*., 2014).

Clinically, RGM have been reported to be more problematic than slow growing mycobacteria (SGM) because of their emerging importance in both sporadic infection and outbreak settings with infection ranging from asymptomatic disease with minimal clinical symptoms to severe bronchiectasis and cavitary lung disease resulting in significant morbidity and mortality (De Groote and Huitt, 2006a). Notable among the RGM is *M. abscessus* which is reportedly the most common cause of pulmonary disease due to rapidly growing mycobacteria (Griffith *et al*., 2007), with it's involvement in invasive infections in both immunocompetent and immunocompromised patients having been emphasized due to it's high *in vitro* antibiotic resistance (Huang *et al*., 2010, Lyu *et al*., 2011, Nessar *et al*., 2012). Other species among the most important RGM causing human infections besides *M. abscessus* are *M. chelonae* and *M. fortuitum* (Mahon *et al*., 2014). On the other hand, the most clinically significant NTM species among the slow growing mycobacteria besides *M. tuberculosis* are *M. avium* complex *M. kansansii,* and *M. xenopi.* The remainder of the species are mentioned below. Among the SGM, MAC has been seen to cause pulmonary disease that presents a clinical picture similar to that of TB and it is reported as the most common cause of TB-like disease in the United States (Mahon *et al*., 2014)

2.3.1 Slow-Growing Mycobacteria

The slow-growing group includes species of mycobacteria that require usually more than 7 days of incubation for mature growth; some may require nutritional supplementation of routine mycobacterial media (Ioachimescu and Tomford, 2015). The most common clinically important species found in this group include the *M. avium* complex (*M. avium* and *M. intracellulare*), *M. kansasii*, *M. xenopi*, *M. simiae*, *M. szulgai*, *M. scrofulaceum*, *M. malmoense*, *M. terrae-nonchromogenicum* complex, *M. haemophilum*, and *M. genavense*. These organisms grow best at 35° to 37° C, with the exception of *M. haemophilum*, which has a preference for lower temperatures (28° to 30° C) and the presence of iron, and *M. xenopi*, which grows optimally at 42° C. Newer isolated slow-growing species include *M. celatum*, *M. interjectum*, *M. confluentis*, *M. triplex*, *M. lentiflavum*, *M. branderi*, *M. conspicuum*, *M. cookii*, and *M. asiaticum*.

2.3.2 Rapid-Growing Mycobacteria

The rapid-growing group of Mycobacteria includes non-pigmented and pigmented species that produce mature growth on agar plates, usually within 7 days or less (Griffith *et al*., 2007). Non-pigmented pathogenic species are mostly grouped within the *M. fortuitum* complex, which includes the *M. fortuitum* group (*M. fortuitum*, *M. peregrinum*, and *M. fortuitum* third biovariant complex) and the *M. chelonae-abscessus* group (*M. chelonae*, formerly *M. chelonae* subspecies *chelonae*, *M. abscessus*, formerly *M. chelonae* subspecies *abscessus*, and *M. mucogenicum*, formerly *M. chelonae*-like organism). *M. smegmatis* may be pigmented or non-pigmented.

There has been a dramatic recent increase not only in the total number of mycobacterial species but also in the number of clinically significant species. The increase relates to improved microbiologic techniques for isolating NTM from clinical specimens and, more importantly, to advances in molecular techniques with the development and acceptance of 16S rRNA gene sequencing as a standard for defining new species (Griffith *et al*., 2007). The dramatic change in mycobacterial taxonomy came with the readily availability and reliability of DNA sequencing. Investigators recognized that the mycobacterial 16S rRNA gene was highly conserved, and that differences in the sequence of 1% or greater generally defined a new species (Tortoli, 2003, McNabb *et al*., 2004). Also critical to the dramatic change was the appearance of publicly available databases that stored the 16S rRNA gene sequences of established mycobacterial species. Recognition of a novel NTM species is now relatively

simple; that is, by performing 16S rRNA gene sequence analysis of a suspected new species and comparing the results with those in the databases (Griffith *et al*., 2007). Hence, absence of such sequences from the databases could define a new species.

2.4 Evaluation of Clinical Significance of NTM

The clinical relevance of isolated NTM is often unclear but differentiation of true infection from pseudo-infection and establishing the clinical relevance of an NTM isolate is of paramount importance since treatment of NTM disease is time consuming and often complicated (van Ingen *et al*., 2009a). To assist in this differentiation, van Ingen and colleagues recommended guidelines described by the American Thoracic Society (ATS)/Infectious Diseases Society of America (IDSA) which combines clinical and microbiological criteria for diagnosis of pulmonary NTM disease. These criteria comprise presence of clinical symptoms with appropriate exclusion of other diseases, and microbiological (positive culture from at least two sputum samples, or positive culture from one bronchial wash or lavage (Griffith *et al*., 2007).

In agreement with van Ingen *et al*., (2009), Ahmed and colleagues reported that assessment of the clinical significance of an NTM isolate is vital for establishing it's role in symptomatic patients' (Ahmed *et al*., 2014). The researchers sought to determine the distribution of NTM species and their clinical relevance at a tertiary care hospital in Pakistan. To assist in the determination of clinical relevance, data on presenting symptoms, occupation, co-morbids (e.g. malignancy, HIV infection), smoking, previous TB or TB treatment history, chronic obstructive pulmonary disease, cystic fibrosis, chemotherapy, steroid intake, and chest x-ray and/ or CT scan findings, was reviewed. The Pakistan study, thus used the ATS/IDSA criteria for diagnosis of pulmonary NTM disease (Ahmed *et a*l., 2014).

2.5 Diagnostic Criteria for NTM Lung Disease

Unlike TB, the isolation of NTM in pulmonary specimens does not equate with disease (Griffith *et al*., 2007, Johnson and Odell, 2014). Additionally, NTM infections, unlike TB, do not require public health reporting (Johnson and Odell, 2014) , resulting in failure to accurately ascertain epidemiology of NTM infections. In an effort to standardize the definition of NTM infection, the ATS/IDSA formulated and published guidelines on definition of NTM disease (Griffith *et al*., 2007). On the basis of these guidelines, the diagnosis of NTM pulmonary infection requires the presence of symptoms, radiologic

abnormalities, and microbiologic cultures in conjunction with the exclusion of other potential aetiologies (Griffith *et al*., 2007).

Despite the ATS criteria having been best described for MAC, *M. kansasii* and *M. abscessus* (Griffith *et al*., 2007), many studies and researchers have employed these criteria in the diagnosis of pulmonary NTM disease (van Ingen *et al*., 2009a, Winthrop *et al*., 2010, Ahmed *et al*., 2014).

2.5.1 Laboratory diagnostic methods for NTM

Correct species identification is of great importance because NTM species differ in their clinical relevance (Koh *et al*., 2006, van Ingen *et al*., 2009a). For example, isolation of *M. kansasii* and *M. malmoense* from pulmonary specimens (in northwestern Europe) indicates disease in >70% of all patients (Griffith *et al*., 2007, van Ingen *et al*., 2009a, Hoefsloot *et al*., 2009). To the contrary, isolation of *M. gordonae* and, to a lesser extent, *M. simiae* or *M. chelonae*, typically indicate contamination rather than true disease (Griffith *et al*., 2007, van Ingen *et al*., 2009a), whereas MAC, *M. xenopi*, and *M. abscessus* form an intermediate category between these two extremes (Koh *et al*., 2006, Griffith *et al*., 2007, van Ingen *et al*., 2009a). Singh and colleagues also echoed the importance of species identification especially in cases of patients positive for AFB and do not improve clinically (Singh *et al*., 2014).

The development of molecular biology tools for species identification in the mid-1980s was a major milestone in the diagnosis of NTM (Jagielski *et al*., 2014). Currently, the identification of clinical isolates of Mycobacteria to species level is primarily based on the characteristics of the cultured bacteria and the use of molecular techniques. Whereas conventional tests such as culture can take several weeks to perform and cannot always precisely identify the species (Bang *et al*., 2011), DNA-based techniques have revolutionized the epidemiology of TB and other mycobacterial diseases, as they allow querying the whole genome that is unique and relatively stable for each strain (Jagielski *et al*., 2014).

2.5.1.1 Phenotypic Methods

The diagnosis of TB historically relied on the identification of acid fast bacilli through microscopic examination of stained sputum smears (National Tuberculosis Guidelines, Ministry of Health, South Africa, 2014). The recommended method for staining clinical specimens for AFB, including both *M. tuberculosis* and NTM, is the fluorochrome technique, although the Ziehl-Neelsen (ZN) method or Kinyoun stain are acceptable but less sensitive alternatives

(Griffith *et al*., 2007). However, Griffith and colleagues reported that, in many cases, the NTM, especially the RGM, may be more sensitive to the AFB decolorization procedure and may not stain at all with fluorochrome stains. Therefore, if RGM are suspected, it may be prudent to use a weaker decolorizing process. Two staining methods can be used to observe acid-fast bacilli: (ZN) staining or fluorochrome-auramine staining. The staining procedure depends on the ability of mycobacteria to retain these dyes when treated with acid and alcohol solutions.

Smear microscopy, despite it's simplicity and low cost, and good specificity for TB but has very low sensitivity (Riello *et al*., 2016) in detecting TB in patients with non-cavitary pulmonary disease or low bacillary load in sputum (e.g. HIV positive patients). This low sensitivity of smear microscopy for AFB in HIV-positive patients was also documented by Nyamogoba *et al*. (2012) in a study carried out in Western Kenya. Infection due to AFB is quantified by the number of bacilli (AFB) seen on smear microscopy (National Tuberculosis Guidelines, Ministry of Health, South Africa, 2014). This diagnostic algorithm however, despite being the mainstay of TB diagnosis in several developing countries (Fair *et al*., 2007, Buijtels *et al*., 2010, Nyamogoba *et al*., 2012), cannot discriminate between NTM and MTBC.

Despite Culture being a more sensitive diagnostic tool than smear microscopy, it's relatively expensive, not accessible to all patients, and also has a longer turn- around time. Furthermore, the ubiquitous nature of NTM can lead to contamination of respiratory specimens, thereby requiring additional identification tests such as colony morphology in liquid culture, biochemicals, and molecular tests to distinguish TB from NTM (National Tuberculosis Guidelines, Ministry of Health, South Africa, 2014). It is however, an important diagnostic tool in symptomatic patients with paucibacillary tuberculosis, such as HIV positive patients with smear negative PTB and children, due it's higher sensitivity (National Tuberculosis Guidelines, Ministry of South Africa, 2014). Mycobacterial culture is also a useful diagnostic tool in differentiation of mycobacteria species based on phenotypic and biochemical traits (Riello *et al*., 2016).

2.5.1.2 Molecular Methods

An ideal molecular typing method should accommodate the requirements with respect to both performance and analytical criteria. The desired performance parameters include technical simplicity (easiness of performance), reproducibility, robustness, time and cost effectiveness (Jagielski *et al*., 2014).

In support of the test characteristics mentioned by Jagielski and colleagues above, this study employed the molecular methods reviewed and described below for speciation of NTM isolates. In order to correlate isolated NTM species and clinical disease; the Geno Type *Mycobacterium* CM/AS (Hain Lifescience, GmbH, Nehren, Germany) which is designed to detect and identify to species level, the most clinically relevant mycobacteria species, was used in the current study. This is in line with evaluation reports by Gitti *et al*., (2006) and Richter *et al*., (2006) that document the effectiveness and reliability of the assays in identifying to species level the most clinically relevant NTM species. However, many molecular methods have been developed for identification of mycobacteria species (Lee *et al*., 2000, Mäkinen *et al*., 2002, Devulder *et al*., 2005, Jagielski *et al*., 2014).

It is hoped that the study findings will thus, add to the body of knowledge with respect to NTM species diversity and clinical relevance in HIV-infected patients in Zambia, especially that isolation of these organisms, currently does not require reporting, let alone public health notification.

2.5.1.2.1 GenoType *Mycobacterium* CM/AS

GenoType *Mycobacterium* (Hain Lifescience GmbH, Nehren, Germany) is a commercial DNA strip assay used for the detection and identification to the species level of mycobacteria obtained from positive liquid or solid cultures. It comprises two kits: the GenoType *Mycobacteria* CM (for common mycobacteria) and GenoType *Mycobacteria* AS (for additional species) assays, providing probes for 14 and 16 species, respectively (Gitti *et al*., 2006). With the CM assay, 15 patterns can be obtained from 23 species (10 individually and 13 in combination), and with the AS assay, 16 patterns can be obtained from 18 species (12 individually and 2 in combination).

The CM/AS assay is based on a multiplex PCR, targeting species-specific DNA regions combined with a reverse hybridization format (DNA strip). The specific patterns are composed of obligatory and additional facultative staining patterns that can be visually identified by clear-cut hybridization signals on the membrane strips. Whereas the CM assay allows the identification of at least the most relevant mycobacterial species, the AS assay is designed to identify further mycobacterial species.

Richter *et al*. (2006) evaluated the Geno Type *Mycobacterium* CM/AS, a commercially available DNA strip assay (Hain Lifescience, GmbH, Nehren, Germany) for the ability to differentiate mycobacterial species (Richter *et al*., 2006). The test is based on a PCR

technique targeting a 23S rRNA gene region, followed by reverse hybridization and line probe technology. Both tests were evaluated with 156 mycobacterial strains composed of 61 validly published species including different subspecies, 6 not validly published species, and 3 strains other than mycobacterial species. All strains were pre-characterized by sequencing of the 5′ region of the 16S rRNA gene and biochemical tests. In total, results for 151 strains were interpretable. Concordant results were obtained for 137 (92.6%) of 148 mycobacterial strains with the CM assay and 133 (89.9%) of 148 mycobacterial strains with the AS assay, and all three non-*Mycobacterium* species were identified. The researchers concluded that, the CM/AS assay allows rapid and specific detection of the most frequently isolated and most relevant species in a mycobacterial diagnostic laboratory (Richter et al., 2006). Identification of 37 species (22 individually and 15 as combinations of species) in a sequential analysis represents an important improvement. Moreover, with the sequential application of two different assays the balance between technical feasibility and a high rate of detection of different species is optimal.

Gitti and colleagues in 2006, compared the performance of Geno Type *Mycobacterium* CM/AS to AccuProbe test (GenProbe, San Diego, CA), a DNA probe assay targeting 16S rRNA. When the GenoType and AccuProbe (available species-kits) results were compared, full agreement was noted except for one case (Gitti *et al*., 2006). An isolate identified as *M. intracellulare* with AccuProbe was identified as *M. avium* by GenoType. In addition to the 35 isolates identified by AccuProbe, 29 isolates were identified by GenoType (1 *M. scrofulaceum*, 19 *M. fortuitum*, 6 *M. peregrinum*, and 3 *M. chelonae* isolates). Based on the results, GenoType performed well as it identified all of the different species, with the exception of a group of 12 rapid growers that probably belonged to the same species, as they gave an identical pattern when tested by GenoType *Mycobacterium* CM/AS assays. The researchers concluded that GenoType *Mycobacterium* CM/AS is a reliable, rapid, easy-to-perform, and easy-to-interpret assay.

Due to it's robustness in identifying the most common clinically relevant mycobacterial species coupled with technical simplicity and a shorter turn-around time, the Geno Type *Mycobacterium* CM/AS was used in this study.

2.6 Treatment of NTM pulmonary disease

Successful treatment of NTM infections is limited by the need for combinational therapy, administered for long durations and often associated with adverse reactions (Daley and Glassroth, 2014). For example, Griffith *et al*. (2007) recommended treatment of pulmonary

MAC infection with a three-drug treatment regimen that includes a macrolide (azithromycin or clarithromycin), rifamycin (rifampin or rifabutin), and ethambutol over a 12 month period of culture negativity. Despite the long durations of treatment, cure rates for pulmonary MAC and *M. abscessus* infections are low and coupled with high adverse reactions (Griffith *et al*., 2007, van Ingen *et al*., 2012). The limited success of current treatment regimens could be attributed to insufficient knowledge of the pharmacokinetics of the drugs used, the *in vitro* drug susceptibility of the organism, and the eventual *in vivo* treatment outcome (Griffith *et al*., 2007).

The role of *in vitro* susceptibility testing for the management of patients with NTM disease is still a subject of debate primarily due to the observation that, unlike *M. tuberculosis*, MAC response to anti-TB drugs e.g. rifampin and ethambutol may not be reliably predicted on the basis of current *in vitro* susceptibility test methods (Griffith *et al*., 2007). Furthermore, drug susceptibility profiles of NTM has considerable differences with respect to species and geographical diversities. For example, isolates of *M. fortuitum* and *M. chelonae* from a study in Central India showed 100% sensitivity to amikacin, but only two out of the three strains of *M. abscessus* tested were sensitive (Goswami *et al*., 2016). To the contrary, a study conducted in the Netherlands reported only 56% sensitivity for *M. fortuitum* while *M. chelonae* and *M. abscessus* were reported as highly resistant (Swenson *et al*., 1982).

Chapter 3.0 Methodology

3.1 Study Design

The study was laboratory based cross-sectional study.

3.2 Study Site/ Location

The study was conducted at the Institute of Medical Research and Training (IMRet) laboratory at the University Teaching Hospital, Lusaka, Zambia and the National Tuberculosis Reference Laboratory, Lusaka, Zambia.

3.3 Sampling Frame

Archived isolates from clinical trial specimens obtained from HIV and TB co-infected patients on or post-ATT were used for speciation of isolated NTM. A total of 56 archived isolates from two clinical trials: LAM- RCT (Utility and Impact of point-of-care LAM strip test for TB diagnosis in HIV-infected patients from a resource poor setting: A randomized controlled trial; NCT01770730) and TB-HAART (Effectiveness of early initiation of HAART in TB/HIV co-infected patients on TB treatment outcomes; ISRCTN77861053) studies, were used for the study.

3.3.1 Inclusion Criteria

Due to the limited number of available Isolates, all the isolates from sputum cultures from HIV and TB co-infected patients from the two studies above, were included in the study.

3.3.2 Exclusion Criteria

All isolates that did not grow on sub-culture were excluded from the study.

3.3.3 Sample Size

The required sample size was arrived at using the formula below (Daniel and Wayne, 1995):

$$n=\frac{Z^2 P(1-P)}{d^2}$$

Where; n= sample size

Z=Z value (1.96 at 95 % confidence interval)

P=Expected prevalence. In this case 7.8% or (0.078) prevalence was used. This was based on the Determine^{TM} LAM-TB randomized controlled trial (RCT) study which isolated 46 NTM out of 587 study participants from the Lusaka site, giving a prevalence of 7.8%.

d=Precision (in proportion of one; if 5%, $d = 0.05$). Hence;

$$n=1.96^2 \times 0.078 \times (1-0.078)/0.005^2$$

$$n=3.8416 \times 0.078 \times 0.922/0.0025$$

$$n=0.276/0.0025$$

$$n=\underline{110.4}$$

However, due to the retrospective nature of the study, a convenience sample of 56 archived and available isolates was used, thus n=56.

The 56 NTM isolates were obtained from two separate studies with similar characteristics of study participants. These included the LAM randomized controlled trial and TB-HAART study. However, at analysis stage, data for 15 isolates was missing from the data base and therefore, excluded in the final analysis despite the laboratory analysis having been done (Figure 3.1). The probable explanation for this is that some patients had submitted their sputum samples to the laboratory but could not make it onto the study, either they opted out or did not meet the inclusion criteria, hence their data was not captured in the data base.

A summary of the number and source of isolates used in the study is given below (Figure 3.1).

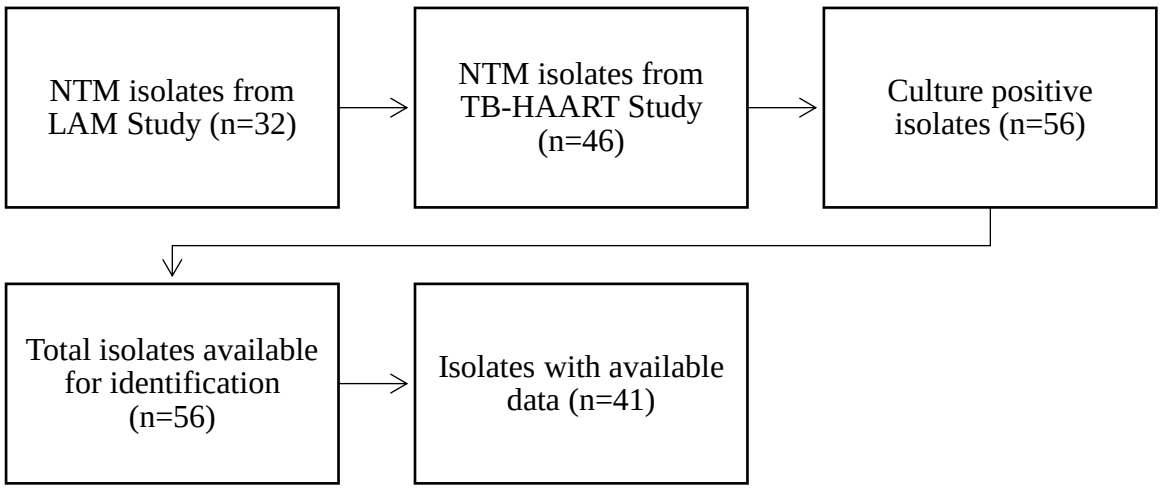

Figure 3.1: Number and source of NTM isolates used in the study

3.4. Molecular Identification

Geno Type *Mycobacterium* CM and Geno Type *Mycobacterium* AS kits (Hain Lifescience GmbH, Nehren, Germany) were used for speciation of NTM.

3.4.1 Geno Type *Mycobacterium* CM

The Geno Type *Mycobacterium* CM (for common mycobacteria) is a molecular genetic assay based on the DNA strip technology for identification of the clinically most relevant mycobacteria species from cultured material that include *M. avium ssp., M. chelonae, M. abscessus, M. fortuitum, M. gordonae, M. intracellulare, M. scrofulaceum, M. interjectum, M. kansasii, M. malmoense, M. peregrinum, M. marinum/ M. ulcerans,* the MTBC, and *M. xenopi*.

The procedure was carried out following manufacturer's instructions and involved DNA extraction from cultured material, multiplex amplification of target DNA using a thermostable DNA polymerase, and reverse hybridization of amplicons to membrane-bound probes. In order to validate the performance of the test and the reagents, each strip included 3 control zones: a conjugate control zone to check the binding of the conjugate to the strip and a correct chromogenic reaction, a universal control zone designed to detect, as known, all *Mycobacteria* and members of gram-positive bacteria with a high G+C content, and a genus control zone which detects the presence of a member of the genus *Mycobacterium* represented by specific DNA probes on the strips.

As earlier indicated, DNA extraction from cultured material was initially done. As per the protocol, genomic DNA was extracted from a one milliliter (1ml) of liquid culture, transferred into a labelled 1.5ml screw cap tube and centrifuged at 3000 xg for 20 minutes. The supernatant was then discarded leaving a pellet at the bottom of the tube.100µl of the lysis buffer (A-LYS) was added to the tube to re-suspend the pellet. The resulting mixture was incubated at 95ºC in a dry bath for 5 minutes, followed by addition of 100µl of Neutralization buffer (A-NB). Finally, the tubes were spun at full speed in a table top centrifuge for 5 minutes. The resulting solution contained free DNA of which 5µl was used for PCR.

The 5µl of DNA solution was added to the amplification mix containing the enzyme DNA polymerase making a final volume of 50µl. The amplification, which involved heat denaturation, primer annealing, and extension, was done in a GT-Q 96 Cycler (Hain Lifescience GmbH, Nehren, Germany) using the set Hot 30 programme on the thermal cycler.

Following the PCR, reverse hybridization of PCR products to membrane-bound probes was done in a GT Blot 48 automated system (Hain Lifescience GmbH, Nehren, Germany) following a GT Fast V2 programme. Initially, 20µl of denaturation solution (DEN, blue) was added to the corner of each of the wells used, followed by addition of 20µl of amplified sample to the solution while mixing well by pipetting up and down. Appropriately labelled strips were then placed in each well and the subsequent steps which included addition of hybridization buffer, stringent wash solution, conjugate, substrate, and finally the rinse solution, were carried out using the set GT Fast V2 programme on the GT Blot automated system. At the end of the run, the strips were removed from the wells and placed on appropriately labelled interpretation charts for species identification.

The results were interpreted according to the interpretation charts provided with the kit following manufacturer's instructions. Identification of *Mycobacterium* species was done with the aid of species- specific patterns that could be visually read by clear-cut hybridization signals on the membrane strips.

3.4.2 Geno Type *Mycobacterium* AS

The Geno Type *Mycobacterium* AS (for additional species), like the CM kit, is based on the DNA-strip technology. It permits the identification of the following *Mycobacterium species*: *M. simiae, M. mucogenicum, M. goodii, M. celatum, M. smegmatis, M. genavense, M.*

lentiflavum, M. herckeshornense, M. szulgai/ M. intermedium, M. phlei, M. haemophilum, M. kansasii, M. ulcerans, M. gastri, M. asiaticum, and *M. shimoidei.*

The assay procedure, from extraction of genomic DNA to interpretation of results, is as described above for the Geno Type *Mycobacterium* CM assay.

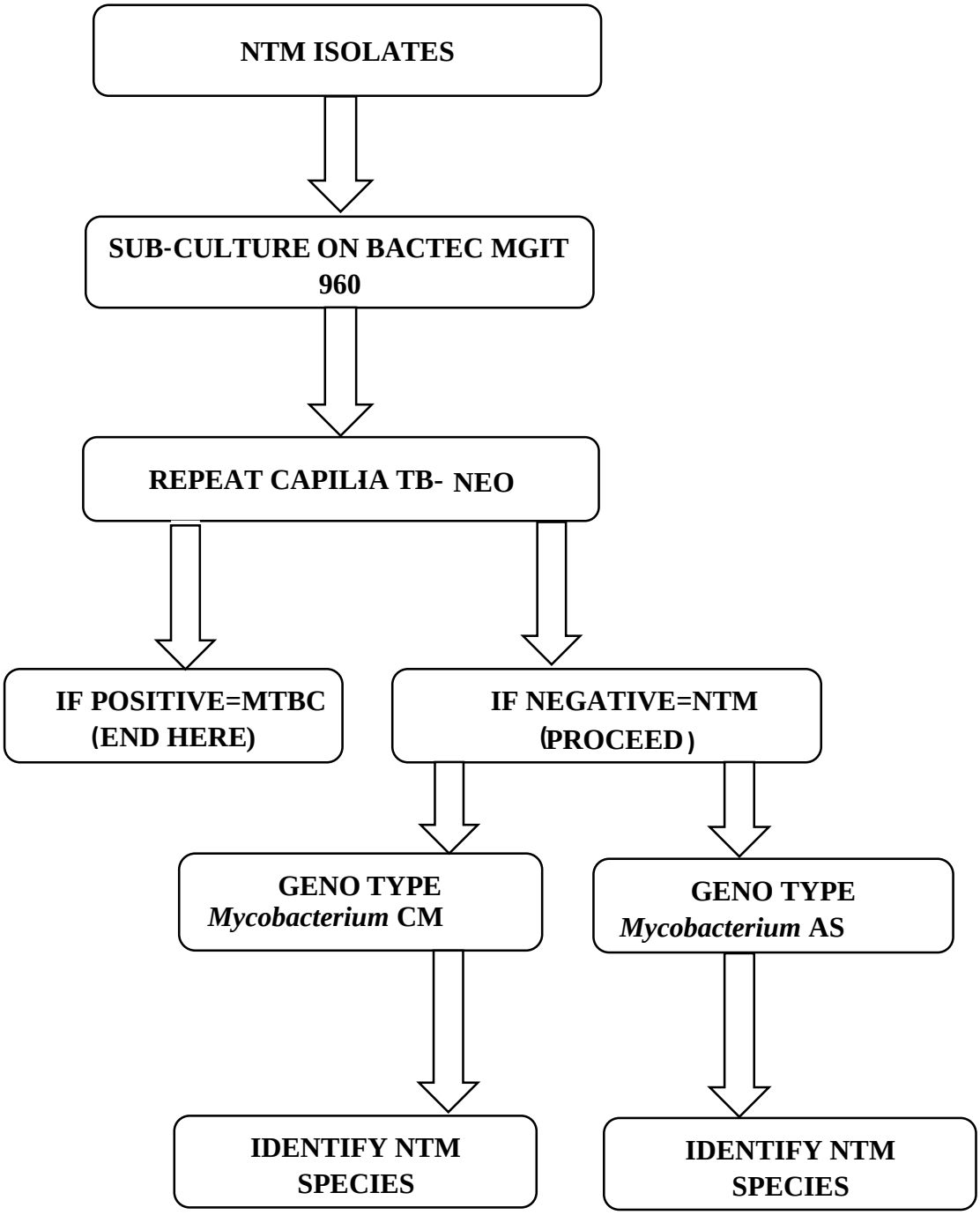

Figure 3.2: Sample process flow

Archived isolates were initially sub-cultured on *Mycobacterium* growth indicator tubes (MGIT). Capilia TB (TAUNS Laboaratories Inc. Japan) was repeated to rule out MTBC and only isolates negative for Capilia TB test were further identified using the Geno Type *Mycobacterium* CM/AS molecular assays. The process is described in the flow chart above (Figure 3.2).

Table 3.1: Table of variables

Characteristic	Variable type	Data type	Measurement scale
Symptomatic NTM pulmonary disease	Outcome (dependent)	Dichotomous (Yes/No)	Nominal
Age	Independent	Categorical (20-29, 30-39, 40-49, ≥50)	Ordinal
Sex	Independent	Categorical (Male/Female)	
BMI	independent	Categorical (<18.5kg/m^2, 18.5-24.9kg/m^2, ≥25kg/m^2)	Ordinal
Haemoglobin level	Independent	Categorical (<8g/dl, 8-11.9	Ordinal
CD4 count	Independent	Categorical (<200 cells/µl, 200-349 cells/µl, ≥350 cells/µl)	Ordinal
Clinical features:	Independent	Categorical (no, mild, moderate)	Ordinal
Night sweats	Independent		
Chest pains	Independent		
Cough	Independent		
Fever	Independent		

The variable types and their measurement scales are summarized above (Table 3.1). In order to measure associations between the predictor and outcome variables, all the variables were categorized.

3.5 Data Analysis

Data was entered on Microsoft Excel spread sheets and analyzed using STATA version 12. Descriptive statistics were used to summarize data while Chi-square test or Fisher's exact test was used for proportions or categorical variables. Logistic regression analysis was used to determine the association between independent and dependent variables. Odds ratios (OR) with 95% confidence intervals (CI) were calculated to assess the effect of demographics (age and sex), clinical features (fever, cough, chest pains, night sweats) and other factors (BMI, Hb, CD4 count) on symptomatic NTM pulmonary disease. *p*-value < 0.05 was considered statistically significant. The clinical features as recorded in the data base, were categorized as normal, mild, and moderate.

Chapter 4.0 Results

Initially, 56 archived isolates from two studies were sub-cultured on *Mycobacterium* growth indicator tubes (MGIT) and all isolates were culture positive. The isolates were further confirmed to be NTM by Capilia TB-Neo (TAUNS Laboratories Inc. Japan) prior to identification by the Geno Type *Mycobacterium* CM (for common mycobacteria) and Geno Type *Mycobacterium* AS (for additional species). Of the 56 isolates, data on 41 isolates, was available for analysis (Table 4.1). The remaining 15 did not have clinical and demographic data but the laboratory analysis was done.

Table 4.1: Background characteristics of participants from whom the isolates were obtained.

Characteristic	Frequency	%
Sex		
1. Male	23	(56.1)
2. Female	18	(43.9)
Total	**41**	**(100)**
Age group		
1.20-29 years	6	(14.6)
2.30-39 years	20	(48.8)
3.40-49 years	10	(24.4)
4.50 years and above	5	(12.2)
Total	**41**	**(100)**
Body mass index		
1. <18.5kg/m^2	19	(52.8)
2. 18.5-24.9kg/m^2	15	(41.7)
3. ≥25kg/m^2	2	(5.5)
Total	**36**	**(100)**
Cd4 count		
1. <200 cells/µl	6	(15.0)
2. 200 349 cells/µl	14	(35.0)
3. ≥350 cells/µl	20	(50.0)
Total	**40**	**(100)**
Haemoglobin Level		
1. <8g/dl	6	(15.4)
2. 8-11.9g/dl	27	(69.2)
3. ≥12g/dl	6	(15.4)
Total	**39**	**(100)**

Table 4.1 above gives a summary of the background characteristics of the study participants from whom the isolates used in the study were obtained. The data was further used for

evaluation of NTM clinical significance by assessing each of the variables as an independent risk factor for NTM pulmonary disease.

4.1 Demographic characteristics of participants from whom isolates were obtained

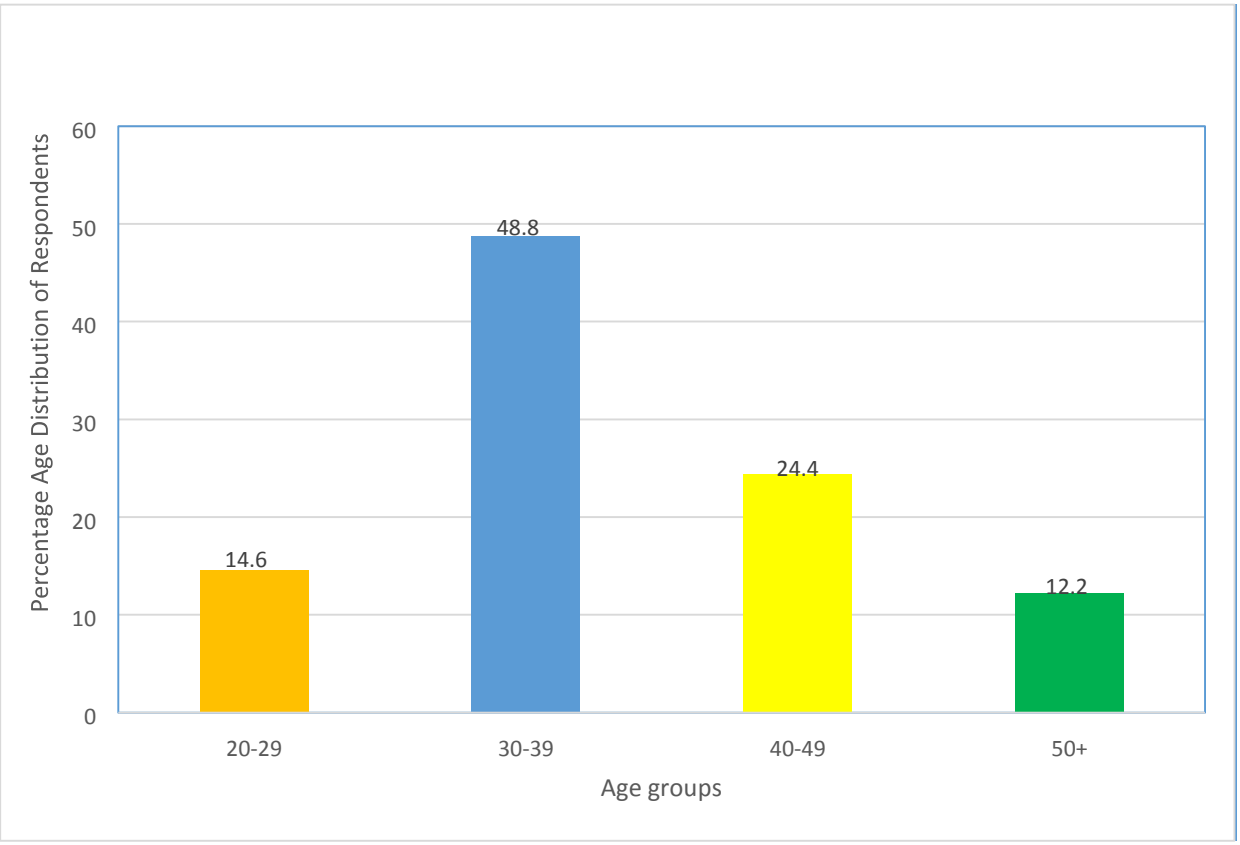

Figure 4.1: Age distribution of participants in the study

The data presented showed age categories ranging from 20-29 to ≥50 years, with respective percentage distributions (Figure 4.1). It is evident that majority of the participants fell in the 30-39 years age bracket representing 48.8%, while those in the ≥50 years were the least represented age group (12.2%).

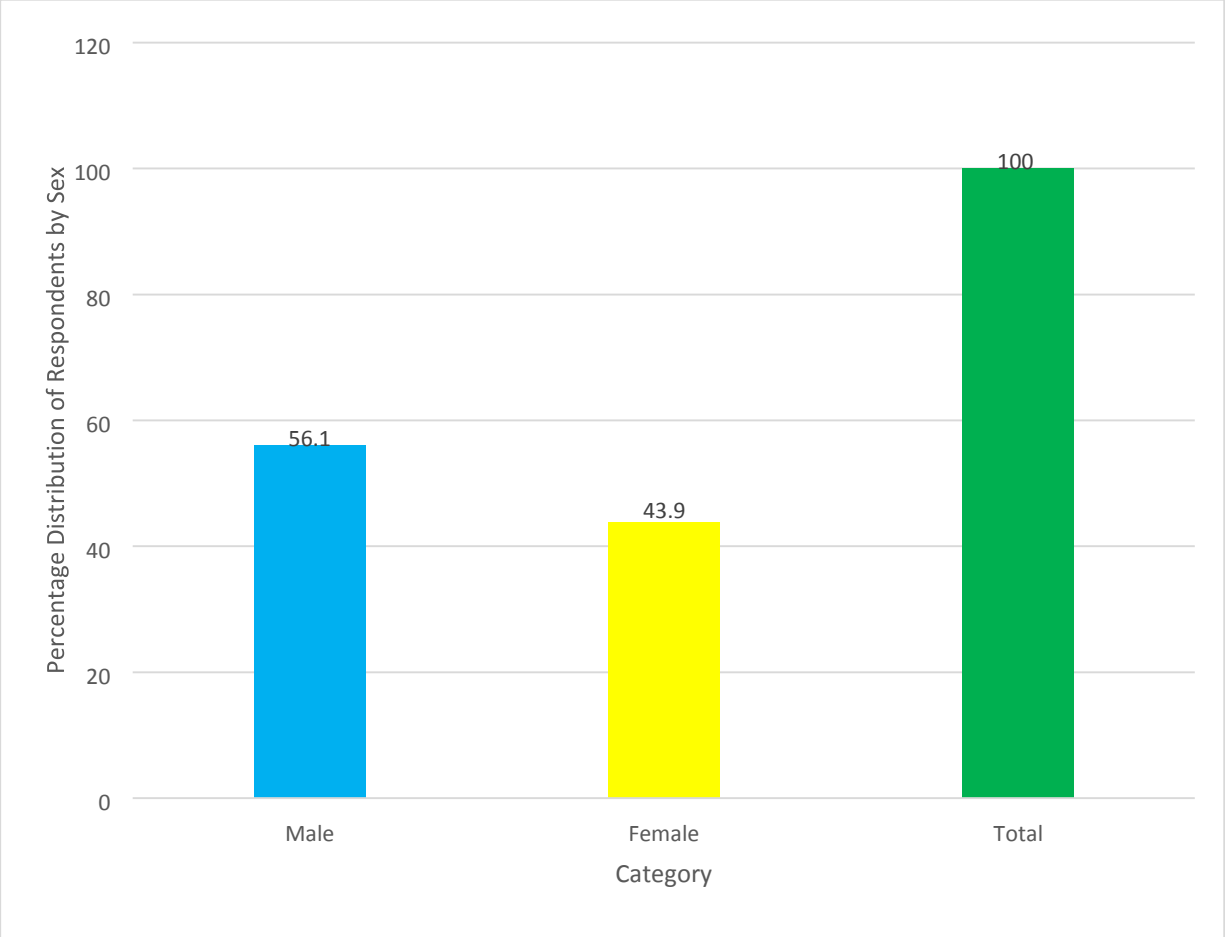

Figure 4.2: Distribution of participants in the study by sex

The above figure shows the distribution of study participants with respect to gender. The data obtained showed that the proportion of males was slightly higher than that of females (Figure 4.2).

4.2 Laboratory identification of isolates

4.2.1 Auramine smear microscopy

From the positive cultures, smears were made and stained using the Auramine method and examined under fluorescence microscopy for identification of acid-fast bacilli. Of the 56 NTM isolates, 2 were smear negative.

4.2.2 Geno Type *Mycobacterium* CM

Using this method, 7 different species of NTM were identified. *M. immunogenum* (n=4) was the most isolated species, followed by *M. abscessus* (n=3), *M. gordonae* (n=2), *M. intracellulare* (n=1), *M. fortuitum* (n=1), *M. mageritense* (n=1), and *M. chelonae* (n=1). Three

isolates could not be identified, while five isolates did not yield positive amplicons upon amplification. Besides NTM species, other organisms detected included some members of *M. tuberculosis* complex (12) and Gram positive bacteria with a high G+C content (27) which, however, could not be identified to species level. A summary of the results obtained by the Geno Type *Mycobacterium* CM is given below (Figure 4.1).

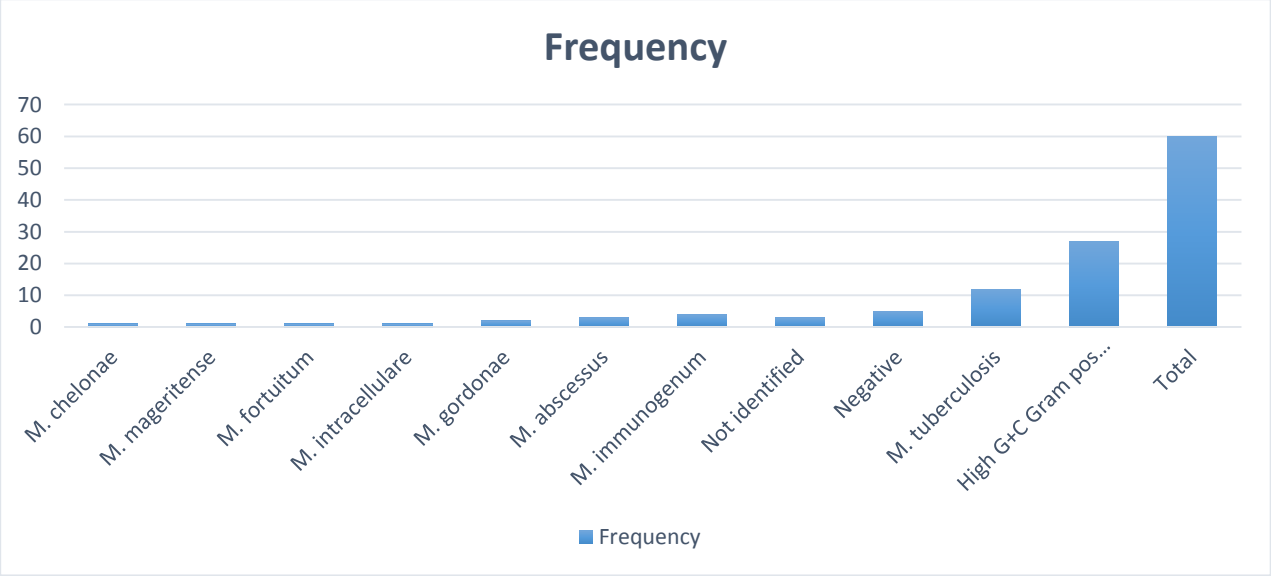

Figure 4.3 Identity and frequency of isolates by Geno Type *Mycobacterium* CM

4.2.3 Geno Type *Mycobacterium* AS

The Geno Type *Mycobacterium* AS, in comparison to the CM kit, could only identify two different species of NTM; namely *M. smegmatis* (n=3) and *M. celatum* (n=1). One isolate could not be identified, while three isolates did not yield positive amplicons upon amplification. Non-NTM species detected by this method included *M. tuberculosis* complex (n=16) and Gram positive bacteria with a high G+C content (n=32). A summary of the results obtained by the Geno Type *Mycobacterium* AS is given below (Figure 4.2).

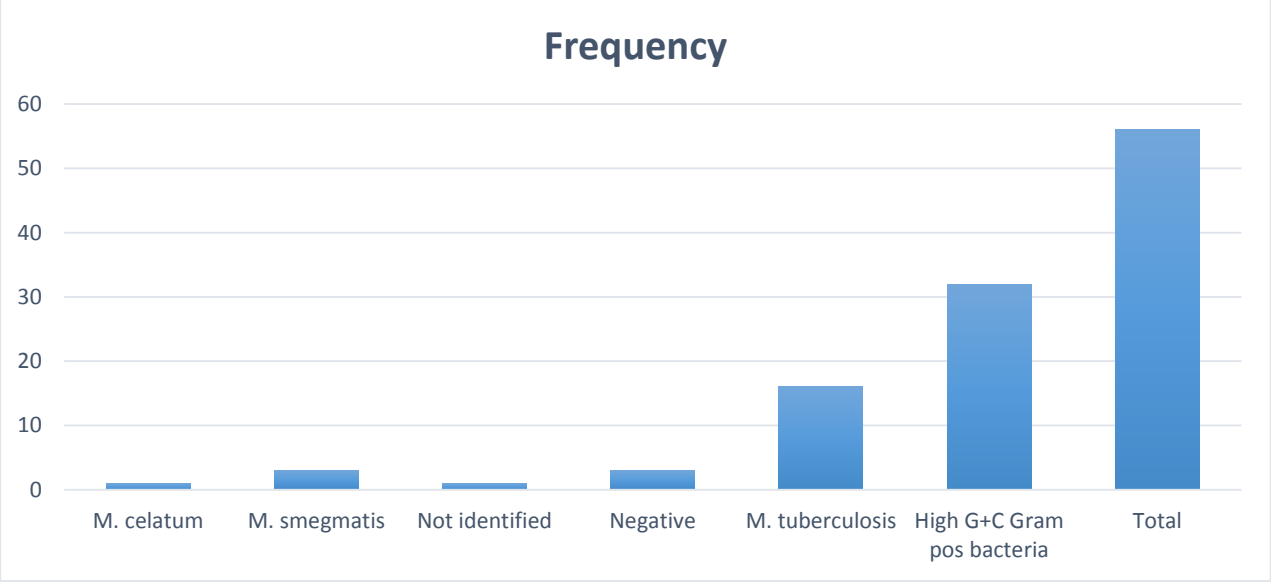

Figure 4.4 Identity and frequency of isolates by Geno Type *Mycobacterium* AS

Table 4.2: Identification of isolates by the Geno Type *Mycobacterium* CM/AS assays

Serial No.	Identity	Frequency	%
1	*M. immunogenum*	4	5
2	*M. abscessus*	3	3.75
3	*M. smegmatis*	3	3.75
4	*M. gordonae*	2	2.5
5	*M. fortuitum*	1	1.25
6	*M. mageritense*	1	1.25
7	*M. celatum*	1	1.25
8	*M. chelonae*	1	1.25
9	*M. intracellulare*	1	1.25
10	*M. tuberculosis* complex	19	23.75
11	Gram positive bacteria with a high G+C content	35	43.75

12	*Mycobacterium* species not identified	4	5
13	Negative	5	6.25
14	**Total**	**80**	**100**

Table 4.1 above shows a summary of the results obtained by the molecular methods. From the table, nine NTM species were identified by a combination of both the Geno Type *Mycobacterium* CM and Geno Type *Mycobacterium* AS kits. It is also evident that organisms other than NTM were also identified, though not to species level.

4.2.4 Comparison of Geno Type *Mycobacterium* CM and AS results

Table 4.3: Comparison of Geno Type *Mycobacterium* CM and AS results.

STUDY ID	Geno Type *Mycobacterium* CM	Geno Type *Mycobacterium* AS	Concordance
3101	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3019	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3129	*M. tuberculosis* complex	High G+C G+ve bacteria	No
3153	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3166	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3169	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3182	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3206	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3260	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
3455	High G+C G+ve bacteria	*Mycobacterium spp*. not identified	No
41379	Negative	Negative	Yes
41401	*Mycobacterium spp*. not identified	*M. smegmatis*	No
41411	*M. abscessus*/ *M. immunogenum*	*M. celatum*	No
41413	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41417	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41433	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41513	Negative	High G+C G+ve bacteria[1]	No
41515	*Mycobacterium spp*. not identified	High G+C G+ve bacteria	No
41523	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41525	Negative	High G+C G+ve bacteria	No
41531	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41537	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41558	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
41560	High G+C G+ve bacteria	*M. smegmatis*	No
41582	High G+C G+ve bacteria	High G+C G+ve bacteria[2]	Yes
41586	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes

41604	*M. tuberculosis* complex	High G+C G+ve bacteria	No
41611	Negative	Negative[2]	Yes
41650	Negative	Negative	Yes
41668	*M. fortuitum*/ *M. mageritense*	*M. smegmatis*	No
41673	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
41212	*M. gordonae*	*M. tuberculosis* complex	No
41424	Negative	High G+C G+ve bacteria[1]	No
41514	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
41545	*M. intracellulare*	*M. tuberculosis* complex	No
41546	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41571	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41581	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41667	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
41674	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
41686	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
	[1]*Positive results were only obtained after a repeat PCR run with more amplification cycles*		
	[2]*Two isolates with different laboratory identification numbers were found for these study participants*		

LAB.ID	**Geno Type *Mycobacterium* CM**	**Geno Type *Mycobacterium* AS**	**Concordance**
T4346	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
T4352	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
T3924	*M. abscessus*/ *M. immunogenum*	*M. tuberculosis* complex	No
T3927	*M. chelonae*/ *M. immunogenum*	High G+C G+ve bacteria	No
T3928*			
T3929	*M. gordonae*	*M. tuberculosis* complex	No
T4028	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
T4029	*M. tuberculosis* complex	High G+C G+ve bacteria	No
T4102	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
T4103	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
T4124	High G+C G+ve bacteria	High G+C G+ve bacteria	Yes
T4126	*M. tuberculosis* complex	*M. tuberculosis* complex	Yes
	*Isolate not found during laboratory analysis		

Results obtained by the above two methods were compared and a summary of the results obtained is shown above (Table 4.2). Concordant results were seen in 35/53 (66.04%) isolates analyzed. The table also shows that two of the isolates were assigned two different laboratory accession numbers, while one isolate could not be traced and therefore, could not be analyzed.

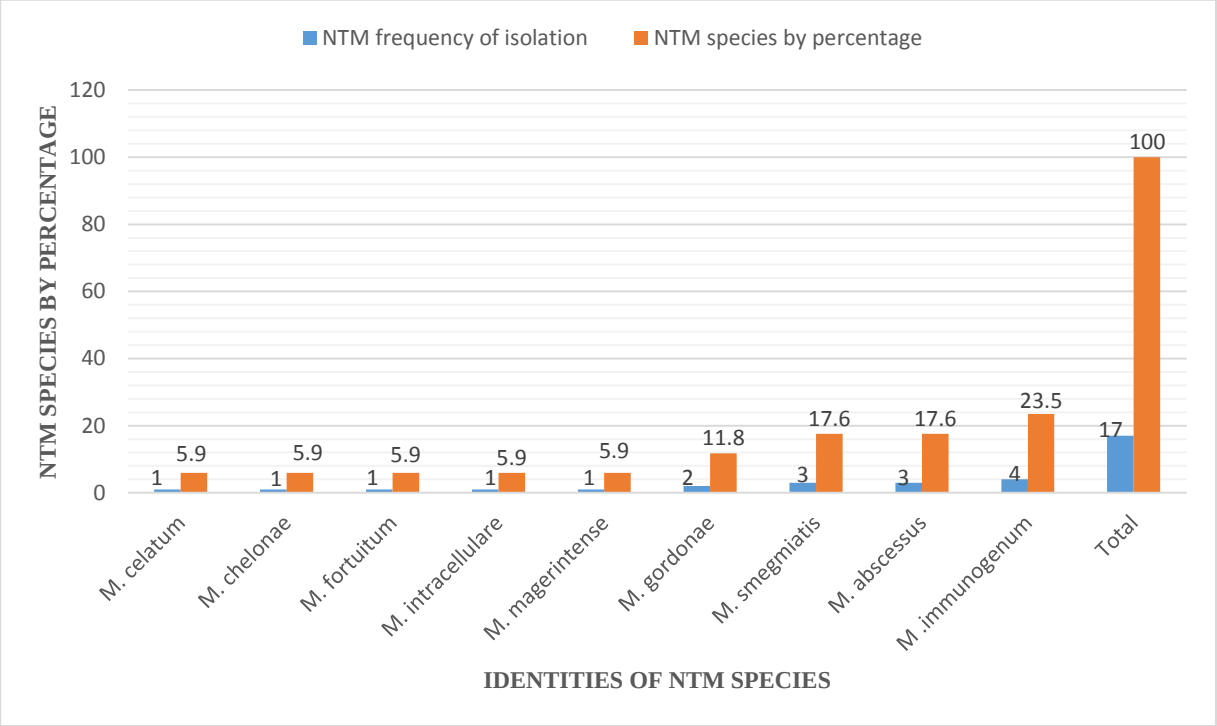

Figure 4.5: Frequency and identities of isolated NTM species

The 56 available isolates were subjected to molecular analysis using Geno Type *Mycobacterium* CM/AS kits (Hain Lifescience GmbH, Nehren, Germany). Nine (09) different species of NTM with varying frequencies of isolation, were identified. Figure 4.1 above provides a summary of the identified NTM species.

Table 4.4: Spectrum and frequency of isolated NTM species

Identity	NTM frequency of isolation	NTM species by %
M. celatum	1	5.9
M. chelonae	1	5.9
M. fortuitum	1	5.9
M. intracellulare	1	5.9
M. magerintense	1	5.9
M. gordonae	2	11.8
M. smegmiatis	3	17.6
M. abscessus	3	17.6
M .immunogenum	4	23.5
Total	**17**	**100**

Isolated NTM species in the current study as well as their frequencies of isolation are shown above (Table 4.3). *M. immunogenum* was the most frequently isolated species (23.5%), while *M.*

celatum, *M. chelonae*, *M. fortuitum*, *M. intracellulare*, and *M. mageritense* were the least isolated species (5.9%).

4.2.6 Sample of results obtained by the two molecular assays

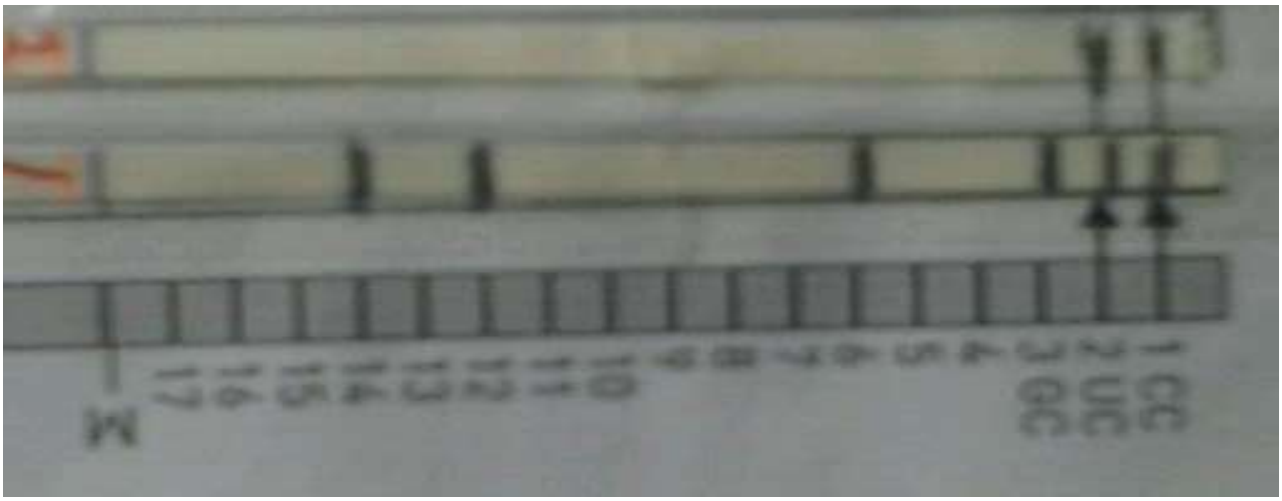

Figure 4.6: Geno Type *Mycobacterium* AS showing *M. celatum* (band pattern: 1, 2, 3, 6, 12, 14) and high G+C Gram positive bacteria (band pattern: 1, 2).

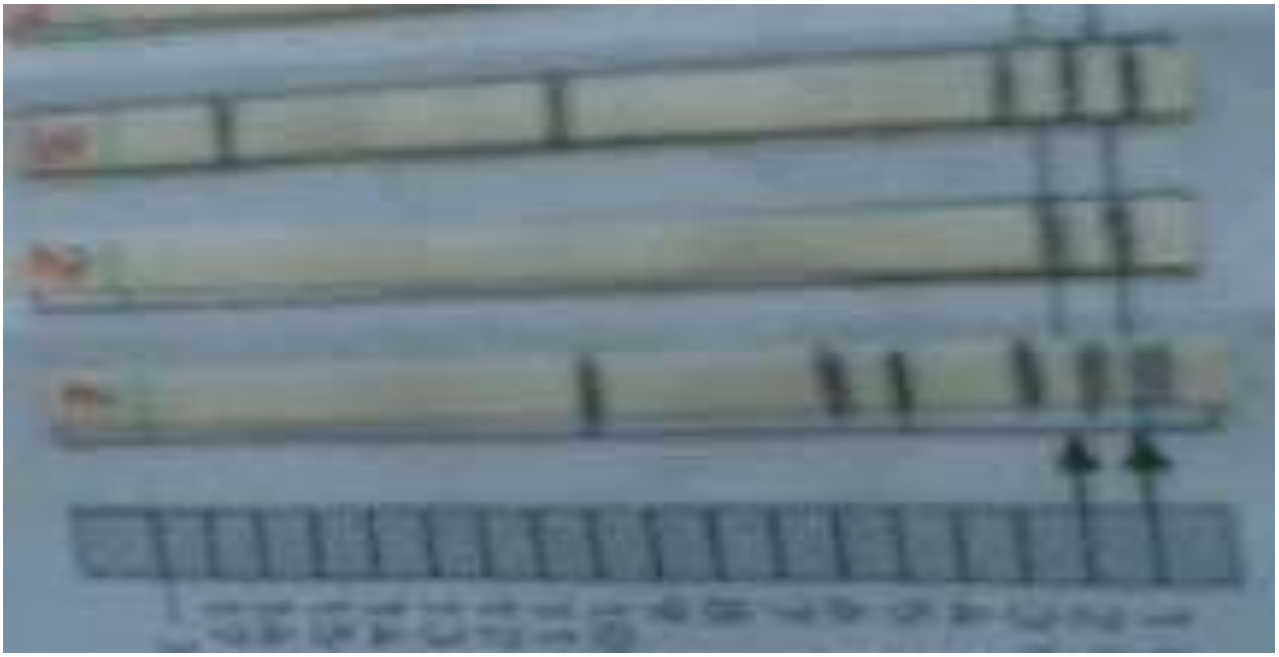

Figure 4.8: Geno Type *Mycobacterium* CM showing *M. abscessus*/ *M. immunogenum* (band pattern: 1, 2, 3, 5, 6, 10), high G+C Gram positive bacteria (band pattern: 1, 2), and *MTB*C (band pattern: 1, 2, 3, 10, 16).

Sample pictures of the band patterns obtained by the two assays are shown above (Figures 4.2 and 4.3). Figure 4.2 shows the band patterns obtained by the Geno Type *Mycobacterium* AS representing *M. celatum* and a high G+C Gram positive bacteria, while figure 4.3 shows band patterns obtained by the Geno Type *Mycobacterium* CM representing *M. abscessus*/ *M.*

immunogenum, a high G+C Gram positive bacteria, and a member of the *M. tuberculosis* complex.

Table 4.5: Association of demographic (age & sex) and other factors (BMI, Hb, CD4 count) with symptomatic NTM disease

	Symptomatic NTM		
Characteristic	**No**	**Yes**	**P-value**
Age group			0.645
1. 20-29 years	3 (10.7)	3 (25.0)	
2. 30-39 years	15 (53.5)	5 (41.6)	
3. 40-49 years	6 (21.4)	3 (25.0)	
4. 50 years and above	4 (14.3)	1 (8.3)	
Total	**28 (100)**	**12 (100)**	
Sex			0.738
1. Female	12 (42.8)	6 (50.0)	
2. Male	16 (57.2)	6 (50.0)	
Total	**28 (100)**	**12 (100)**	
Body mass index			0.585
1.<18.5kg/m^2	14 (53.9)	4 (44.4)	
2. 18.5-24.9kg/m^2	11 (42.3)	4 (44.4)	
3. ≥25kg/m^2	1 (3.8)	1 (11.2)	
Total	**26 (100)**	**9 (100)**	
Cd4 count			0.442
1. <200 cells/µl	3 (10.7)	3 (27.3)	
2. 200-349 cells/µl	10 (35.7)	3 (27.3)	
3. ≥350 cells/µl	15 (53.6)	5 (45.4)	
Total	**28(100)**	**11 (100)**	
Haemoglobin Level			0.542
1. <8g/dl	3 (10.7)	3 (27.3)	
2. 8-11.9g/dl	20 (71.4)	7 (63.6)	
3. ≥12g/dl	5 (17.9)	1 (9.1)	
Total	**28(100)**	**11 (100)**	

†Pearson Chi-square test (or Fisher's exact test where expected frequency in a cell was less than 5) was used to establish statistical significance set at 95% C.I. with p-<0.05, considered statistically significance.

Table 4.5 above shows the association of demographic and the other predictor variables with symptomatic NTM pulmonary disease. Though some associations maybe clinically relevant, none of the predictor variables showed statistically significant differences.

Table 4.6: Association of clinical symptoms with symptomatic NTM disease

	Symptomatic NTM		
Characteristic	**No**	**Yes**	**P-value[†]**
Cough			**<0.001**
1. No	24 (100)	1 (16.7)	
2. Mild	0 (0.0)	5 (83.3)	
Total	**24 (100)**	**6(100)**	
Fever			
1. No	25 (100)	3 (50.0)	**0.001**
2. Mild	0 (0.0)	2 (33.3)	
3. Moderate	0 (0.0)	1 (16.7)	
Total	**25 (100)**	**6 (100)**	
Night Sweating			**<0.001**
1. No	25 (100)	3(50.0)	
2. Mild	0 (0.0)	3 (50.0)	
Total	**24 (100)**	**6(100)**	
Chest pains			**<0.001**
1. No	25 (100)	3(50.0)	
2. Mild	0 (0.0)	3 (50.0)	
Total	**24 (100)**	**6(100)**	

[†]Pearson Chi-square test (or Fisher's exact test where expected frequency in a cell was less than 5) was used to establish statistical significance set at 95% C.I. with p-<0.05, considered statistically significance.

Table 4.6 above shows the relationship between TB-associated clinical signs and symptoms with symptomatic NTM disease. As can be seen from the table, clinical symptoms associated with TB were also significantly associated with symptomatic NTM disease, p-≤0.001.

Table 4.7: Assessment of demographics (age & sex), BMI, CD4 count, and haemoglobin level as risk factors for symptomatic NTM disease.

Characteristic	OR	95% CI	P-value[†]
Age group			
1. 20-29 years	Ref.		
2. 30-39 years	0.3	.05-2.2	0.255
3. 40-49 years	0.5	.06-4.2	0.521
4. 50 years and above	0.2	.02-3.8	0.317
Sex			
1. Female	Ref.		
2. Male	0.8	.2-2.9	0.678
Body mass index			
1. <18.5kg/m^2	Ref.		

2. 18.5-24.9kg/m^2	1.3	.258-6.3	0.767
3. ≥25kg/m^2	3.5	.17-69.3	0.411
Cd4 count			
1. <200cells/µl	Ref.		
2. 200-349cells/µl	0.3	.04-2.3	0.251
3. ≥350cells/µl	0.3	.05-2.2	0.255
Haemoglobin Level			
1. <8g/dl	Ref.		
2. 8-11.9g/dl	0.4	.06-2.2	0.257
3. ≥12g/dl	0.2	.01-2.9	0.239

† Pearson Chi-square test (or Fisher's exact test where expected frequency in a cell was less than 5) was used to establish statistical significance set at 95% C.I. with p-<0.05, considered statistically significance.

An assessment of demographics, BMI, haemoglobin level and CD4 count as independent risk factors for NTM pulmonary disease, was done (Table 4.7). From the table, patients who were 50 years and above were 80% less likely to have symptomatic NTM disease, while males were 20% less likely to have the disease. With regards to BMI, those that were overweight were 3.5 times more likely to have NTM disease. Patients with CD4 count greater or equal to 350 cells/µl and those with haemoglobin level greater than 12g/dl, were 70% and 80% respectively, less likely to be associated with the outcome. The above associations were however, not statistically significant.

Chapter 5.0 Discussion

Non-tuberculous mycobacteria (NTM) have been shown to be significant contributors to TB-like disease in HIV-seropositive individuals (Khatter *et al*., 2008, Griffith *et al*., 2007, Buijtels *et al*., 2010, Hoza *et al*., 2016). In symptomatic patients, evaluation of NTM clinical significance is vital for establishing it's role in the observed symptomatology (Van Ingen *et al*., 2009b, Ahmed *et al*., 2014, Koh *et al*., 2006), especially in immunocompromised hosts in which the presence of any acid-fast bacilli, may be considered significant (Sharma, 2013).

This study identified *M. immunogenum* as the most frequently isolated NTM species from the 56 archived isolates. The organism is a rapid growing mycobacterium (RGM), closely related to *M. abscessus* and *M. chelonae* (Wilson *et al*., 2001, Griffith *et al*., 2007). Clinically, it has been isolated from skin lesions, corneal ulcers, joint fluid, central venous catheter sites, and blood (Wilson *et al*., 2001). Pulmonary disease with this organism has also been reported (Moore *et al*., 2000). In a more recent study, *M. immunogenum* has also been associated with central nervous system infections, though the clinical significance of this organism and other rapidly growing mycobacteria from brain abscesses, is unclear (Greninger *et al*., 2015). Consistent with Moore *et al*., (2000), *M. immunogenum* was isolated from pulmonary specimen, in the current study and it's isolation could possibly point to it's role in causing clinical symptoms in patients from whom it was isolated.

With regard to clinical significance, majority of the isolated NTM species in this study have been reportedly isolated from clinical specimen of pulmonary and extra-pulmonary origin (Griffith *et al*., 2007, Khatter *et al*., 2008, van Halsema *et al*., 2015, Hoza *et al*., 2016). Among the species isolated in the current study, *Mycobacterium intracellulare*, *M. fortuitum*, *M. chelonae*, *M. gordonae*, and *M. celatum*, have been isolated from sputum samples of HIV seropositive patients (Khatter *et al*., 2008, van Halsema *et al*., 2015, Hoza *et al*., 2016, Bjerrum *et al*., 2016). Other species among the identified NTM species which have been isolated from pulmonary disease patients include *M. abscessus* and *M. chelonae* (Griffith *et al*., 2007, Wallace *et al*., 2014). The latter is reportedly the most common cause of pulmonary disease due to rapidly growing mycobacteria (Griffith *et al*., 2007), with it's involvement in invasive infections in both immunocompetent and immunocompromised patients having been emphasized due to it's high *in vitro* antibiotic resistance (Huang *et al*., 2010, Lyu *et al*., 2011, Nessar *et al*., 2012). *Mycobacterium abscessus* was identified as the second most isolated

NTM species in this study, after *M. immunogenum*. This finding suggests the organism's involvement as aetiological agent of TB-like disease among study participants.

The third ranked NTM species among the identified isolates was *M. smegmatis*. The organism is a rapid growing mycobacterium producing colonies on egg medium in 2 to 4 days. Though commonly considered saprophytic, *M. smegmatis* has been implicated in rare cases of pulmonary, skin, soft tissue, and bone infections (Mahon *et al*., 2014). In the current study, it is likely that the organism could have played a role in causing symptoms consistent with TB in some of the study participants.

Mycobacterium gordonae was found to be the fourth ranked in frequency of isolation in the current study. It is a pigmented, slowly growing mycobacteria usually isolated as yellow/orange colonies after 3 weeks or more at 37ºC. Epidemiologically, *M. gordonae* is frequently encountered in the environment and in clinical laboratories but is almost always considered non-pathogenic (Griffith *et al*., 2007). Studies conducted in Croatia and Tanzania reported *M. gordonae* as the most frequently isolated among pulmonary NTM isolates (Jankovic *et al*., 2013, Hoza *et al*., 2016). On the basis of clinical significance, the Croatian study reported that only a small percentage of all patients with *M. gordonae* met the criteria for probable disease (Jankovic *et al*., 2013). It is therefore, not clear whether isolation of this NTM species in the current study was suggestive of clinical disease or perhaps mere colonization as strict criteria for case definition could not be met due to insufficient data.

The remainder of the identified NTM isolates included *M. fortuitum*, *M. intracelluare*, *M. celatum*,
M. chelonae, and *M. mageritense*, all at 1.25% frequency of isolation. Of these, *M. chelonae* and *M. fortuitum* are among the three most important rapidly growing mycobacteria causing human infections besides *M. abscessus* (Mahon *et al*., 2014). *M. chelonae* is found in the environment and associated with many of the same opportunistic infections as *M. fortuitum* and it is also the species of rapidly growing mycobacteria most likely isolated from disseminated cutaneous infections in immunocompromised patients (Mahon *et al*., 2014). Both *M. fortuitum* and *M. chelonae* have been associated with a variety of infections of the skin, lungs, bone, central nervous system, and prosthetic heart valves (Mahon *et al*., 2014). As earlier indicated, *M. intracellulare*, *M. fortuitum*, and *M. celatum* have been isolated from pulmonary specimen of immunocompromised patients due to HIV and AIDS (Khatter *et al*., 2008, van Halsema *et al*., 2015, Hoza *et al*., 2016). It could therefore, be possible that isolation of these organisms in the current study, was correlated with the observed

characteristics of pulmonary disease similar to PTB. *M. mageritense*, another rapidly growing mycobacterium, has been reported in mycobacterial outbreaks of furunculosis associated with footbaths at nail salons in the United States of America (Winthrop *et al*., 2002, Sniezek *et al*., 2003, Gira *et al*., 2004). It is not clear whether the organism was pathogenic in the current study.

Another striking observation of this study is the high numbers of non-NTM organisms detected, including *M. tuberculosis* complex and Gram positive bacteria with a high guanine plus cytosine content. This is despite the fact that all isolates were initially categorized as NTM based on Capilia TB rapid test (Capilia TB-Neo ver 1.1. 2013). According to the performance characteristics of the Capillia TB test, it is designed to detect the *Mycobacterial* protein fraction from BCG 64 (*MPB64*) antigens specifically produced by *M. tuberculosis* complex. However, a mutation in the base sequence of this gene will result in a mutant in which the expression of the *MPB64* protein is incomplete, thereby giving a false negative result. This finding is particularly of concern as patients could have been misdiagnosed as not having TB when in fact not. The non-responsiveness of such patients to conventional ATT regimens, could perhaps be attributed to multi-drug resistant (MDR) or even extensively resistant TB (XDR). The immunocompromised nature of the patients put them at high risk of infection to both mycobacterial and non-mycobacterial acid-fast bacilli with similar microbiological and clinical characteristics as TB (Sharma, 2013, Yu *et al*., 2014).

From the study, it was noted that among patients with Gram positive bacteria with a high guanine plus cytosine content, some patients presented with signs and symptoms of TB, while others did not. Of the 35 reported cases, 13 (37.1%) had symptoms characteristic of pulmonary TB. These were categorized as one case with fever only, one with cough only, one with cough and chest pains only, one with cough, fever, and night sweats only, one with cough, chest pains and night sweats only, and one case with all the four symptoms (fever, cough, chest pains, night sweats). The remainder of the cases did not have data on clinical signs and symptoms, but had radiological evidence of pulmonary TB. Six of these showed potentially active TB, while one had uncertain TB, with one case of no TB on chest radiography. Based on the above noted observations, it is clear that organisms other than mycobacteria, can mimic pulmonary tuberculosis in presentation. Therefore, accurate and timely differential diagnosis is paramount prior to making a TB case definition and commencement of therapy.

In this study, five isolates did not yield positive results upon PCR amplification as shown in table 4.1 and 4.2 in the results section. The likely explanation for this observation is that the target DNA in the samples was below detectable levels. This could be justified by the fact that some of the initially negative samples, did yield positive results upon repeat PCR runs with increased amplification cycles. Other than the negative samples, four of the isolates could not be identified to species level by either of the molecular kits used (Geno Type *Mycobacterium* CM/AS), a limitation also echoed by other researchers (Nyamogoba *et al*., 2011, Nyamogoba *et al*., 2012, Aliyu *et al*., 2013).

5.1 Evaluation of clinical relevance and potential risk factors for NTM disease

Unlike some studies conducted previously in Zambia (Lubasi *et al*., 2009, Malama *et al*., 2014, Mwikuma *et al*., 2015, Chanda-Kapata *et al*., 2015), clinical relevance of NTM in the current study, was evaluated using a combination of microbiological (culture and microscopy), haematological and immunological parameters (haemoglobin level and CD4 count), body mass index (BMI) as well as TB-associated symptoms (fever, cough, chest pains, night sweats), categorized as normal, mild, and moderate (Table 4.5). In addition, potential risk factors of early mortality (Gupta *et al*., 2015) namely; low baseline CD4 count, male gender, low BMI, anaemia, age greater than forty years, among others, were assessed as independent risk factors for NTM pulmonary disease.

Despite some studies having reported increasing age as being well correlated with symptomatic NTM disease (Koh *et al*., 2006, Aliyu *et al*., 2013, Chanda-Kapata *et al*., 2015), this particular study reported no significant association between age and symptomatic NTM pulmonary disease. This finding is consistent with Lan *et al*. (2011). However, majority of the participants in the current study (both the symptomatic and non-symptomatic NTM groups), fell in the age bracket 30-39yrs. With regard to gender, studies conducted elsewhere have documented a higher NTM prevalence in males than females (Lan *et al*., 2011, Nyamogoba *et al*., 2012, Saifi *et al*., 2013). However, a recent study in Zambia, showed no significant difference in the prevalence of symptomatic NTM by sex (Chanda-Kapata *et al*., 2015). In agreement with Chanda-Kapata and colleagues, this study did not find a statistically significant difference with respect to gender and symptomatic NTM pulmonary disease. Being underweight has also been reported to be a significant predictor of NTM disease (Pettipher *et al*., 2001, Aliyu *et al*., 2013), with weight loss having been reported in 100% of patients with disseminated MAC infection in a South African cohort. This study, however,

does not report a significant difference between patients who were underweight and those who were normal among the symptomatic NTM group (Table 4.4). A correlation between low CD4 count and NTM isolation from clinical specimens of HIV infected individuals (Khatter *et al*., 2008, Crump *et al*., 2009, Lan *et al*., 2011), has also been reported. However, the current study does not report any difference in terms of percentage of patients with a CD4 count lower than 200cells/µl and those with CD4 counts between 200-349 cells/µl within the symptomatic NTM group. To the contrary, a higher proportion of symptomatic NTM patients, was seen in participants with a CD4 count higher than 350 cells/µl (Table 4.4).

In this study, TB-associated symptoms (fever, cough, chest pains, night sweats), demographics (age, sex), haematological (haemohlobin level), immunonlogical (CD4 count), and BMI, were all evaluated against the outcome (symptomatic NTM pulmonary disease). Statistically significant results were seen with respect to TB-associated symptoms, p-≤ 0.001 (Table 4.5), whereas there were no significant correlations between the other predictor variables and the outcome as shown in Table 4.4 above. However, patients with symptomatic NTM pulmonary disease were more likely to be in the age group 30-39yrs, with a CD4 count ≥350cells/µl, BMI of <18.5-24.9kg/m^2, and a haemoglobin level between 8-11.9g/dl. The above associations, as already mentioned, were not statistically significant despite some associations being clinically significant.

6.0 Limitations of the study

The study had proposed to complement the Geno Type *Mycobacterium* CM/AS kits with 16S rRNA sequencing, but due to limited resources, this could not be done. The study also proposed to employ the ATS/IDSA criteria for evaluation of NTM clinical significance in pulmonary disease patients which require clinical, radiological, and microbiological evidence and appropriate exclusion of other diagnoses. However, due to limited data, these criteria could not be strictly followed. Following modifications of the above criteria by some authors (Andréjak *et al*., 2010, McCarthy *et al*., 2012), it was possible to assign the participants to the NTM suspect group as determined by positive culture of at least one sputum sample and presence of clinical signs and symptoms consistent with pulmonary disease as well as abnormal chest radiograph. The retrospective nature of the study could not allow for a larger sample size with more data that could otherwise have been useful in generalizing study findings.

7.0 Conclusion

This study identified nine different species of NTM, namely: *M. immunogenum*, *M. abscesus*, *M. smegmatis*, *M. gordonae*, *M. chelonae*, *M. fortuitum*, *M. intracellulare*, *M. celatum*, and *M. mageritense*. Isolation of some pathogenic and potentially pathogenic NTM species with accompanying characteristics of clinical disease in the current study could be suggestive of their clinical significance in HIV-infected patients in our study. The study also showed some correlations between pulmonary NTM disease and the other predictor variables (BMI, anaemia, and CD4 count), though these associations were not statistically significant. Failure to strictly follow the ATS/IDSA criteria for pulmonary NTM case definition due to insufficient data coupled with the small sample size used in the study however, may not permit generalization of study findings but could form a basis for further research.

8.0 Recommendations

1. A prospective study with a larger sample size, taking the form of a randomized controlled trial or case control study, is recommended for accurate determination of the true prevalence and clinical relevance of NTM and other non-mycobacterial AFB pathogens in people living with HIV and AIDS in Zambia.

2. Molecular diagnostic technologies should be scaled up for ease of access to all patients to facilitate timely and accurate differentiation of mycobacterial and other acid-fast bacterial pathogens for better treatment outcomes of HIV-infected patients.

9.0 References

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10.0 Appendices

10.1 Appendix 1: Ethics approval letter

10.2 Appendix 2: Clearance to submit proposal for ethics review

10.3 Appendix 3: Permission to use stored isolates and patients' data