PREVALENCE, SPECIES CHARACTERIZATION AND FACTORS ASSOCIATED WITH NON TUBERCULOUS MYCOBACTERIA AMONG TUBERCULOSIS RETREATMENT PATIENTS SUBMITTING SAMPLES TO THE NATIONAL TUBERCULOSIS REFERENCE LABORATORY, KENYA

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Prevalence, Species Characterization and Factors associated with Non Tuberculous Mycobacteria among Tuberculosis Retreatment Patients submitting samples to the National Tuberculosis Reference Laboratory, Kenya, 2016

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DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other university.

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ABBREVIATIONS AND ACRONYMS

AFB	Acid fast bacilli
ATS	American Thoracic Society
CD4	Cluster of differentiation 4
CDC	Centres of Disease Control
CF	Cystic Fibrosis
COPD	Chronic Obstructive Pulmonary Disease
DNA	Deoxyribonucleic acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
INH	Isoniazid
IQR	Interquartile Range
MAC	Mycobacterium Avium Complex
LAM	Lipoarabinomannin
MAIS	Mycobacterium Avium Intracellulare Sacrofulaceum
MGIT	Mycobacterium Growth Indicator Tube
MOTT	Mycobacterium Other Than Tuberculosis
MTB	Mycobacterium Tuberculosis
NTM	Non Tuberculous Mycobacterium
NTRL	National Tuberculosis Reference Laboratory
PCR	Polymerase Chain Reaction

RAPD	Random Amplified Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
TB	Tuberculosis
TLC	Thin Layer Chromatography
WHO	World Health Organization

ABSTRACT

The frequency of pulmonary disease from non-tuberculous mycobacterium (NTM) is reportedly on the rise in Europe, North America, Asia and Southern Africa. In sub-Saharan Africa, information on the extent of the burden of pulmonary disease from NTM is lacking due to limitations in tools for mycobacterial species identification. Failure to characterize acid fast bacilli (AFB) positive NTM lung infections has led to their misclassification and to inappropriate treatment for pulmonary tuberculosis in developing countries. Kenya is ranked among top African countries with high Tuberculosis (TB) cases associated with high Human Immunodeficiency Virus/ Acquired Immunodeficiency Syndrome (HIV/AIDS) burden. Arguably the emergence of NTM as opportunistic infections in the HIV/AIDS patients would complicate TB management given that their treatment is not directly analogous to that of TB. This cross sectional study therefore, characterized and identified the correlates of NTM among TB retreatment patients whose samples are sent to the National Tuberculosis Reference Laboratory (NTRL). The TB retreatment patients of BACTEC™ MGIT™ (Mycobacteria Growth Indicator Tube) culture positive samples were consented and enrolled into the study. The patients' health records and case investigation forms were used to gather information associated with NTM infection.

All subjects were assigned a subject identification number (SID). All paper research records were kept in a locked filing cabinet located in a restricted-access

room at the research center in the NTRL, Nairobi. All data was entered into Microsoft Excel (USA) which was associated with SID in password protected files. Descriptive statistics (frequency and proportions) were used to summarize data and presented using tables. The overall NTM prevalence was determined for the entire patient's population. Bivariate and multivariate analyses were used to determine factors associated with NTM infection using Poisson regression at the significance level of $p \leq 0.05$. All statistical analyses were performed using STATA v 13 (StataCorp LP, Texas, USA).

The median (IQR) age of the 210 TB retreatment patients' enrolled was 35 (28 - 75) years. Slightly over fifty percent (53.8%) of the patients were females, 33.8% were aged between 25 to 34 years, 18% were smokers, while 22% were HIV positive. About 37% had respiratory symptoms while 36.2% had animal contact. Seventy-five of 210 (35.7%) of the TB retreatment patients were infected with NTM. The *Mycobacterium intracellulare* (62.7%) was the most frequent NTM observed. Others included; 16% *M. absessus,* 8% *M. fortuitum*, 5.3% *M. sacrofuloceum*, 4% *M. kansasii,* 1.3% *M. interjectum*, *1.3% M. gordonae* and *1.3% M. xenopi*. Female gender OR 1.8 (95% CI 1.1 to 3.1), residence of Eastern region OR 2.2 (95%CI 1.1 to 4.6) and Nairobi region OR 2.3 (95%CI 1.1 to 5.1), those who had respiratory symptoms OR 1.5(95% CI 1.01 to 2.5) and animal contact OR 1.7 (95%CI 1.1 to 2.7) as well as those who kept livestock OR 1.6 (95%CI 1.06 to 2.6) were likely to be infected with NTM. Infection due to NTM among TB retreatment cases is high in

Kenya and missed diagnosis jeopardizes proper management. Involvement of NTM during management of clinical pulmonary TB is important in planning for prevention and treatment of TB in Kenya especially among patients in pastoral community.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Mycobacterial infections are among the leading causes of disease in humans. In developing countries, the high incidence of tuberculosis has overshadowed the occurrence of non-tuberculous mycobacterial (NTM) infections in the past (Ferreira *et al.*, 2002). This trend is however fast changing. The incidence of NTM infections is increasing as a result of the growing number of immunodeficient patients (Ferreira *et al.*, 2002) and the use of contaminated endoscopic medical devices (Duarte *et al.*, 2009). Pulmonary disease caused by NTM has gained increased attention globally with increased incidences being reported (Hoefsloot *et al.*, 2013). By 2014 estimated prevalence of pulmonary disease caused by NTM was 33–65 per 100,000 (Morimoto *et al.*, 2014).

Globally, patients with acid-fast bacilli (AFB) positive specimens are generally presumed to be infected with *Mycobacterium tuberculosis* (MTB) and are treated with anti-tuberculosis agents and some placed in isolation rooms. Increased isolation of NTM causing mycobacterial diseases implies that more patients with AFB positive samples have received inappropriate or unnecessary empirical anti-tuberculous treatment (Hsueh *et al.*, 2006). Due to this inappropriate treatment and high treatment failure (Zheng and Fanta, 2013), the mortality of patients with NTM lung infections is on the increase (Ito *et al.*, 2012). Therefore, appropriate diagnosis and regular monitoring of NTM is crucial.

In Kenya, information on the extent of the burden of pulmonary disease from NTM is lacking due to limitations in surveillance for mycobacterial species identification (Muwonge *et al.*, 2011). The ubiquitous distribution of NTM contributes to the difficulties in interpreting positive culture results. Further, it is not mandatory to report NTM disease to the database in the Ministry of Health thus; knowledge about the epidemiology and distribution of NTM causing pulmonary disease is limited in Kenya. This study therefore seeks to evaluate the contribution of NTM among TB retreatment patients whose samples are received at the National Tuberculosis Reference Laboratory (NTRL) located in Nairobi, Kenya.

1.2 Problem statement

The burden of NTM has previously been reported to be particularly high in patients with HIV/AIDS or suspected of having pulmonary TB (Moore *et al.*, 2010). Several countries have published reports about the situation in their countries. In England, Wales and Northern Ireland an increase in NTM was reported. A report from Portugal found a high percentage of NTM strains among mycobacterial isolates (Amorim *et al.*, 2010). A multi country overview that included information from European Union (EU) countries with data up to 1996 was published by the working group of the Bacteriology and Immunology section of the International Union against TB and Lung Disease. The study concluded that there was an increase in the number of NTM in pulmonary samples collected in 2008 in 30 different countries from different continents. The study further showed that M. avium complex (MAC) bacteria predominated in most countries followed by *M. gordonae* and *M. xenopi* (Martin *et al.*, 2004). A prospective evaluation of a cohort of 721 HIV positive

patients in Abidjan, Cote d'voire, Sub-Saharan Africa found the incidence of NTM infection was 9.7 times higher among patients with baseline CD4 cell counts less than 100 cells/mm3 compared to patients with CD4 cell counts above 100 cells/mm3. Despite the high prevalence of TB infection in Kenya, data on prevalence and genotypic characteristics of NTM are nonexistent and hence the justification for this study.

The treatment of NTM disease is seldom straightforward. Diagnostic uncertainty is common and some individuals only experience minimal clinical or radiographic severity (Kobashi and Matsushima, 2003). Prolonged durations of therapy are frequently needed and eradication is unlikely. Additionally, treatment regimens are often expensive and are often poorly tolerated because of frequent side effects (Kobashi and Matsushima, 2003). Often patients describe the treatment as being worse than the disease (Griffith *et al.,* 2007). The decision to treat must be balanced between the risk of disease progression and unnecessary exposure to the cost and toxicity of medications (Griffith *et al.,* 2007). Diagnosis is therefore important and would lead to careful considerations about whether or not to treat these patients.

1.3 Study Justification

The NTM occur in patients suffering from a range of diseases including cystic fibrosis, chronic obstructive pulmonary disease, HIV/AIDS and other immunosuppressive conditions. Studies show that NTM share clinical and radiographic similarities with MTB. The standard of care smear diagnostic test routinely used for diagnosis of NTM has low sensitivity. World Health Organization (WHO) recommends point-of-care diagnostic test Hain's Line Probe Assay (LPA) for the NTM. Therefore, a mechanism for the routine identification of NTM infection in high burden resource limited areas of the world (Kenya being one of them) is urgently needed. Since the current standard of care does not include bacterial characterization then some NTM cases with positive smears will continue to be misclassified as MTB and receive conventional TB therapy to which some of the NTM species may be resistant and majority of NTM infections will remain undetected. Limited information is available on the prevalence and genotypic characteristics of NTM among patients in Sub-Saharan Africa. Studies in 1990s in Kenya, Uganda, Tanzania and Ivory Coast concluded that disseminated NTM infections were relatively uncommon in Africa (Gilks *et al.,* 1995, Okello *et al.,* 1990). On the contrary, recent studies in Zambia and South Africa using better diagnostic tools have actually reported a high prevalence of NTM infections in HIV/AIDs cases. In Kenya, NTM infections are not well documented. The scope of this study was to intensify NTM case finding among retreatment patients in order to promote proper management and avoid unnecessary wastage of resources in terms of administration of drugs to patients.

1.4 Objectives

1.4.1 General objective

To determine the prevalence, genotypic characteristics and factors associated with NTM among TB retreatment patients submitting samples in National Tuberculosis and Reference Laboratory

1.4.2 Specific objectives

1. To determine the prevalence of NTM among samples from TB retreatment patients.
2. To determine genotypic characteristics of the NTM isolated from culture samples of retreatment patients.
3. To determine factors associated with NTM among patients submitting sputum samples to NTRL.

CHAPTER TWO

LITERATURE REVIEW

2.1 Biology of mycobacteria

Bacteria of the genus Mycobacterium are non-motile and non-sporulated rods. They are grouped in the suprageneric rank of actinomycetes that, unusually, have a high content (61-71 %) of guanine plus cytosine (G+C) in the genomic desoxyribonucleic acid (DNA) and a high lipid content in the wall, probably the highest among all bacteria (Palomino *et al.,* 2007) Mycobacterium and other closely related genera (i.e. Corynebacterium, Gordona, Tsukamurella, Nocardia, Rhodococcus and Dietzia) have similar cell wall compounds and structure and hence show some phenotypic resemblance. Several mycolic acids in the envelope structure distinguish the mycobacteria (Palomino *et al.*, 2007). Table 2.1 shows the taxonomy of Mycobacteria.

The envelope is composed of the plasma membrane, a cell wall, and an outer capsule like layer. The cytoplasmic membrane of mycobacteria does not seem to be peculiar except for the presence of some lipopolysaccharides that are anyway shared by all actinomycetales (Mahapatra *et al.,* 2005). This vital interface provides osmotic protection, regulates the traffic of specific solutes between the cytoplasm and the environment, and subsumes the cell house-keeping tasks.

Table 2.1 Taxonomy of Mycobacteria (Adapted from Palomino *et al*., 2007)

Kingdom	Bacteria
Phylum	Actinobacteria
Class	Actinobacteria
Sub-class	Actinobacteridae
Order	Actinomycetales
Sub-order	Corynebacterineae
Family	Mycobacteriaceae
Genus	Mycobacterium unique genus
Species	*M.tuberculosis, M.bovis M. africanum ,M. microti "M. canettii" ,M. caprae, M. pinnipedii, M.Xenopi etc*

The membrane contains proteins with different functions, i.e. sensors measuring the concentration of molecules in the environment, proteins trans-locating signals to genetic and metabolic machinery in the cytoplasm, enzymes involved in metabolic processes and energy generation, and carriers mediating selective passage of nutrients and ions.

The enzymes intervene in cell wall and membrane synthesis, septum formation during cell division, assembly and secretion of extracytoplasmic proteins and DNA replication (Palomino *et al.*, 2007). The outer layer of the cell wall presents an array of free lipids such as phthiocerol dimycoserosates (PDIM), phenolic glycolipids (PGL), trehalose-containing glycolipids and sulfolipids (SL). The unusual "*M.*

canettii", with its smooth colony morphology, has a unique phenolic glycolipid (Van Soolingen, 1997). Traversing the whole envelope, some glycolipids such as the phosphatidyl-myoinositol mannosides, lipomannan (LM) and lipoarabinomanan (LAM), are anchored to the plasma membrane and extend to the exterior of the cell wall. LAMs are species-specific. The mycobacterial wall also contains interspersed proteins. Some are in the process of being exported, some might be residents. Several of these proteins are responsible for cell wall construction during the life of the bacillus. There are also certain proteins called porins forming hydrophilic channels that permit the passive passage of aqueous solutes through the mycolic acid layer (Mahapatra *et al.*, 2005).

Non-tuberculous mycobacteria (NTM) also known as environmental mycobacteria, atypical mycobacteria (Griffith *et al.*, 2007) and mycobacteria other than tuberculosis (MOTT) are mycobacteria which do not cause tuberculosis or Hansen's disease (also known as leprosy).

Mycobacteria are a family of small, rod-shaped bacilli that can be classified into 3 main groups for the purpose of diagnosis and treatment (Griffith *et al.*, 2007). Mycobacterium tuberculosis complex which can cause tuberculosis are*: M. tuberculosis, M. bovis, M. africanum, M. microti, M. canetti.* Non tuberculous mycobacteria (NTM) are all the other mycobacteria which can cause pulmonary disease resembling tuberculosis, lymphadenitis, skin disease, or disseminated disease (Griffith *et al.*, 2007).

Today more than one hundred and fifty species of non-tuberculous mycobacterium (NTM) have been described (Piersimoni *et al.*, 2008). Their pathogenic potential has

been recognized since the beginning of last century. These bacteria in the past have been called atypical mycobacteria, the term first coined by Pinner (Piersimoni *et al.,* 2008).

2.2 Significance of Mycobacterium Other Than Tuberculosis (MOTT)

Mycobacteria other than tuberculosis (MOTT) have increasingly been recognized to cause pulmonary and non-pulmonary infection. Non tuberculous mycobacteria are capable of causing disease but the pathogenicity varies among the species (Van Ingen *et al.,* 2009). In general, it is a challenge to identify NTM species as a specific causal agent of disease, especially because recovery of NTM from a culture of a respiratory system may be due to contamination of the specimen or transient colonization of the patient. Therefore, the clinical significance of detected NTM in non-sterile specimens needs to be evaluated for the individual patient which is usually done in accordance with the guidelines from the American Thoracic Society (Griffith *et al.,* 2007). So far there is very limited evidence for a person to person transmission of NTM (Aitken *et al.,* 2012).

Outbreaks caused by exposure to the same reservoir have been described (Padoveze *et al.,* 2007). A wide range of animal and environmental sources such as swimming pools and aquaria as reservoirs of NTM have been documented (Padoveze *et al.,* 2007. Examples include; marine animals, tap waters and potting for *M. avium* complex and tap water for *M. gordonae*. Non tuberculous mycobacteria species have also been detected in medical devices in hospital settings (Lowry PW *et al*., 1990) and can therefore pose a risk to vulnerable hospitalized patients. For most species, no single reservoir has been identified. Although it is a challenge to link NTM to

disease in man, significant NTM disease has been described among patients suffering from cystic fibrosis (often with *M. abscessus),* chronic obstructive pulmonary disease (COPD) (Kotilainen *et al.,* 2011), HIV and other immunosuppressive conditions (Hoefsloot *et al.,* 2012) and in otherwise healthy children having cervical NTM lymphadenitis (Ding *et al.,* 2005). In addition, inhaled corticosteroids (Andrejak *et al.,* 2012) and TNF-inhibitor treatment (Kluger *et al.,* 2010) are associated with NTM disease. Host factors such as IL-12 deficiency have been described among other genetic host factors (Van de vosse *et al.,* 2004).

2.3 Non Tuberculous Mycobacterium and Pathogenesis

Out of the 95 known species of NTM, nearly one third have been observed to be associated with disease in humans. The species of NTM associated with human disease are: *M. avium, M. intracellulare, M. kansasii, M. paratuberculosis, M. scrofulaceum, M. simiae, M. habana, M. interjectum, M. xenopi, M. heckeshornens, M. szulgai, M. fortuitum, M. immunogenum, M. chelonae, M. marinum, M. genavense, M. bohemicum, M. haemophilum, M. celatum, M. conspicuum, M. malmoense, M. ulcerans, M. smegmat, M. wolinskyi, M. goodii, M. thermoresistible, M. neoaurum, M. vaccae, M. palustre, M. elephantis, M. septicum* and *M. nonchromogenicum*. Non-tuberculous mycobacteria have been reported to cause localized or disseminated disease in predisposed immune deficient individuals (Palomino *et al.,* 2007).

In non-HIV patients, different NTM may cause localized pulmonary disease, lymphadenitis, soft tissue infections, infections of joints/bones, bursae, skin ulcers and generalized disease in individuals with leukemia or in transplant patients (Pinner

et al., 2008). In HIV/AIDS patients the manifestations may range from localized to systemic disease (Wallace *et al.,* 1990). Clinical features will include local organ specific signs and symptoms to persistent high grade fever, night sweats, anaemia and weight loss in addition to nonspecific symptoms of malaise, anorexia, diarrhoea, myalgia and occasional painful adenopathy.

2.4 Pulmonary infections due to NTM

Mycobacterium avium- intracellulare complex (MAC) strains have been a major cause of pulmonary and other infections in the pre-HIV/AIDS era (Wallace *et al.,* 1990). The infections were commonly seen in chronic bronchitis, bronchiectasis and in chronic obstructive airway disease in the pre-AIDS era in geriatric patients. For a long time, *M. kansasii* has been considered an important cause of pulmonary disease (Wolinsky *et al.,* 1979). *Mycobacterium scrofulaceum* has been shown to be the cause of localized pulmonary infections (Wallace *et al.,* 1990). *Mycobacterium xenopi,* an unusual bacterium with optimal growth temperature of 450 C has been encountered as a pathogen in patients with other underlying lung diseases (Ranks *et al.,* 1984). An outbreak of pulmonary disease due to *M. xenopi* from hot water supply of a hospital has been reported (Wallace *et al.,* 1990). Other pathogens reported to be associated with pulmonary infection include; *M. simiae, M. habana, M.szulgai, M. fortuitum, M. vaccae* and *M. malmoense* (Wallace *et al.,* 1990) *Mycobacterium heckeshornense* is a new slow growing species of mycobacteria which has been shown to be associated with cavitary disease in immune-competent individuals (Roth *et al.,* 2000).

2.5 Epidemiology and factors associated with NTM

Non tuberculous mycobacteria are widely distributed in the environment worldwide (Falkinham *et al.,* 2002). The organisms can be found in soil and water, including both natural and treated water sources. However, *M. kansasii, M. xenopi,* and *M. simiae* are recovered almost exclusively from municipal water sources and rarely if ever from other environmental sources. When identical methods are used, isolation rates of NTM from the environment are remarkably similar in diverse geographic areas (Von *et al.,* 1993). There is no evidence of animal-to-human or human-to-human transmission of NTM even in patients with cystic fibrosis (CF), an apparently high susceptible population and a population in which other opportunistic organisms are clearly passed between patients (Von *et al.,* 1993). There has been no documentation of human to human transmission of NTM (Olivier *et al.,* 2003). Human infection is suspected to be acquired from environmental exposures although the specific source of infection usually cannot be identified (Von *et al.,* 2002).NTM has been found to be associated with bronchiectasis, younger age, female gender and animal contact .The finding that younger age is associated with a higher risk NTM is interesting. It can be hypothesized that is partly associated with greater functional impairment among older patients (Lee *et al.*, 2015).

2.6 Incidence rates of NTM

Non Tuberculous Mycobacterium diseases have been seen in most industrialized countries, incidence rates vary from 1.0 to 1.8 cases per 100,000 persons (Horsburgh *et al.,* 1996). These overall incidence rates are estimates based on numbers of NTM isolates reported.

Mycobacterium Avium Complex is the most common NTM species causing disease but many species have been implicated (Good *et al.,* 1980). Because NTM diseases are not communicable, they are not notifiable worldwide. Although several reports have suggested that the incidence of NTM diseases have increased over the past several decades, this observation has not been conclusively established due to the lack of comprehensive surveillance efforts. The most common clinical manifestation of NTM disease is lung disease but lymphatic, skin/soft tissue and disseminated disease also occur (Good *et al.,* 1980).

In the United States between 1981 and 1983, 94% of the NTM isolates reported to Centers for Disease Control (CDC) and prevention laboratory were pulmonary, whereas 3% were lymph node and 3 % were skin/soft tissue isolation (O'Brien *et al.*, 1987). In the late 1980s and early 1990s, NTM isolates associated with disseminated disease in patients with AIDS were reported almost frequently as pulmonary isolates (Horsburgh *et al.,* 1989). More recently the CDC published the results of NTM isolates reported by state public health laboratories to the public health laboratory system (PHLS) database for the period 1993 through 1996 (CDC 1993-1996). During this period, of the NTM isolated, 75% were pulmonary, whereas 5% were from blood, 2% from skin/soft tissue and 0.4% from lymph nodes isolates. The most frequently reported potentially pathogenic species and corresponding report rates over the 4-year period (per 100,000 population) were as follows: MAC 29 to 36 isolates, *M. fortuitum* 4.6 to 6 isolates and *M. kansasii* 2 to 3.1 isolates.

Overall there is not substantially more or better information about NTM disease prevalence than that published in the 1997 ATS statement on NTM except that, in most state public health laboratories NTM isolates, especially MAC isolates are more common than *M. tuberculosis* isolates (Ostroff *et al.*, 1992).

2.7 Mechanism of Infection with NTM

The mode of transmission of NTM to humans is not clearly established. In many instances the Koch postulates do not prevail. Unlike in TB, person-to-person transmission has not been convincingly demonstrated. Although animals may serve as a reservoir for NTM, animal to human transmission is not clearly established. However, shared drinking water systems with animals may serve as a source of infection (Kankya *et al.*, 1995).

Although the exact route of NTM infection is not established with certainty, based on NTM environmental distribution, it is very likely that the organism is ingested, inhaled, or implanted. Cervical lymphadenitis due to NTM occurs more frequently in children, coincident at the time that they are exploring outdoors and secondly when there is trauma to gums due to erupting teeth (Wolinky *et al.*, 1995). Thus, it is presumed that NTM enter the tissues via the mouth. Previous infections leading to cervical lymphadenitis were most often due to *Mycobacterium scrofulaceum;* now most are due to MAC (Wolinky *et al.*, 1995).

Aerosolization of droplets small enough to enter the alveoli is the likely route of acquisition of pulmonary disease. Bathroom showers have been implicated as a primary source of exposure to aerosolized NTM (Falkiham *et al.*, 2008). Households with water heater temperatures ≤50 °C are more likely to demonstrate colonization

of their water supply with NTM than those with water heater temperature ≥55 °C (Falkiham *et al.,* 2008). Potting soils, particularly those enriched with peat, have a high concentration of NTM and dust generated from soil may produce particles small enough particles to enter the alveoli (De Groote *et al.,* 2006). However, a case controlled cohort study by Dirac looked at "aerosol-generating activities in the home and found that the only activity predictive of the development of NTM lung disease was the use of a spray bottle for watering plants (Dirac *et al.,* 2006). Contamination of hospital water supplies, medical equipment including; bronchoscopes and endoscopes and contaminated dialysis solutions, has led to both NTM colonization and nosocomial outbreaks of disease. The site of disease is dependent upon the exposure but cutaneous abscesses, pulmonary disease, meningitis, and surgical site infections have all been described (Philips *et al.,* 2001). It is hypothesized that regulations to limit hospital water system temperatures to prevent scalding hinder the control of NTM (Mandel *et al.,* 1993). Advances in DNA techniques have allowed easier and more rapid identification of the source of NTM in the setting of nosocomial outbreaks. However, recognition of nosocomial outbreaks is highly dependent upon clinical suspicion and investigation.

2.8 Identification of isolates by phenotypic characteristics

Growth rates, colony pigmentation and biochemical tests such as niacin production, nitrate reduction, tween-80 hydrolysis, arylsulphatase, urease, tellurite reduction, thiophen- 2-carboxylic acid hydrazide (TCH) sensitivity, catalase (qualitative and quantitative), growth on MaConkey agar, sodium chloride tolerance etc., are adequate to identify majority of clinically relevant mycobacteria (Vestal *et al.,*

1977). This strategy is, however, time consuming and not conclusive for many isolates with variable characters. Analysis of the lipids of mycobacteria by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) has been recommended as an alternate approach. Isolates from liquid/ solid medium can be rapidly identified (Katoch *et al.*, 1997).

Serotyping methods using serotype specific sera have been described for the members of Mycobacterium avium intracellulare scrofulacecm (MAIS) complex (Kiehn *et al.*, 1985). Some serotypes (Wallace *et al.*, 1990) of *M. avium* have been shown to be preferentially associated with disease in HIV/AIDS patients.

Isoenzyme and protein electropherogram based schemes for rapid identification and characterization of strains of *M. tuberculosis* and NTM have been developed which may be used in small laboratory settings (Sharma *et al.*, 1995).

Based on measurement of immunological divergences in the structure of certain enzyme molecules like catalase and superoxide dismutase techniques have been described for identification of mycobacteria (Shivannavar *et al.*, 1996). All these techniques need some specialized infrastructure and expertise but are not difficult to set up.

2.9 Identification and characterization of NTM by molecular methods

Based on new knowledge about the gene sequences of mycobacteria many gene probes for the identification of isolates as well as amplification of specific gene fragments from the lesions and mycobacterial culture isolates have been developed:

2.9.1 Gene probes

For the identification of important NTM several gene probes have been developed and some are also commercially available (Evans *et al.,* 1992). With the help of these probes, growth from solid slants and liquid cultures can be rapidly and reliably identified.

2.9.2 Gene amplification methods

Polymerase chain reaction (PCR) techniques for detection and rapid identification of various clinically relevant mycobacteria have been developed which include methods for concentration and detection of *M. avium, M. intracellulare* from the clinical specimens (Chen *et al.*,1996), *M. paratuberculosis* from clinical specimens and milk (Vary *et al.*,1990). PCR assays using genus/ group specific amplification followed by restriction analysis have been described for the analysis of gene regions like 65 kD (Rodrigo *et al.,* 1992) and rRNA gene region which have been found to be useful for identification of different mycobacteria (Vannechoutte *et al.,* 1993).

Most important and specific approach mainly applicable at reference level involves the amplification of 16S rRNA followed by sequencing and several species have been identified for the first time using this approach (Rogall *et al.,* 1990). These PCR methods can be used for direct detection of mycobacterial pathogens (Chen *et al.,* 1996) as well as for rapid identification of clinical isolates of NTM (Rodrigo *et al.,* 1992).

2.9.3 DNA fingerprinting techniques

DNA fingerprinting techniques are of interest for identifying the subtypes/strains which would be more commonly associated with disease and also to investigate

hospital acquired infections. Several DNA fingerprinting techniques have been investigated on the diversity in NTM (Falkinham *et al.,* 1999). Techniques like pulsed field gel electrophoresis (Vanitha *et al.,* 2003) random amplified polymorphic DNA (RAPD) - arbitrary PCR (Kauppihen *et al.,* 1994), rRNA gene polymorphism (Katoch *et al.,* 1987), typing using different insertion/repeat element (Tortoli *et al.,* 2000), plasmid typing and single gene polymorphism (Soini *et al.,* 1996) have been successfully used for molecular typing of NTM. Insertion sequence based RFLP methods have been described to be useful for characterization of *M. hemophilum, M. avium* as well as *M. kansasii.* Using these methods certain RFLP types of *M. avium* have been shown to be closely linked with disease in Europe and African (Portaels *et al.,* 1991). In India, such information about molecular types of NTM is very scanty.

2.9.4 Determination of sensitivity profiles

The drug susceptibility profile of NTM is usually quite different from *M. tuberculosis*. Firstly, these organisms are usually sensitive at high concentrations of antitubercular drugs (Wallace *et al.,* 1990), thus higher cut off values for deciding sensitivity/resistance are recommended. Secondly, rapid growing mycobacteria are usually resistant to rifampicin and isoniazid (INH) whereas these are sensitive to drugs like new generation macrolides, cephalosporins and sulphones. The media usually recommended for the sensitivity screening of M. tuberculosis are used for NTM also. Other media like chocolate agar/supplemented with ferric ammonium salts/mycobactins, will be needed for the sensitivity screening of fastidious species.

Newer techniques like BACTEC™ MGIT™ and E test have been also found to be quite useful for sensitivity determination of rapid as well as slow growing NTM (Steadham *et al.*, 1995).

2.10 Treatment of NTM Infections

There are numerous clinical challenges regarding the treatment of NTM pulmonary infections. The duration and toxicity of anti- mycobacterial therapy must be balanced against the often indolent clinical course and the patients' other co-morbidities (Thorax *et al.*, 2009). It is often appropriate to observe a patient's clinical course before embarking on therapy. There is currently a paucity of data on the natural history of untreated NTM infections adding to the difficulties of clinical decision-making. Kikuchi *et al*, have demonstrated that specific genotypic patterns of MAC may predict clinical behavior (Kikuchi *et al.*, 2011). If confirmed by subsequent investigations, these data may augment clinical decision making on directed drug therapy.

Patients with advanced lung disease in need of transplantation present particular challenges when they develop NTM pulmonary infections. It is debated if listing for transplant should be deferred until completion of therapy. Because of the long duration of therapy for NTM, it is the authors' opinion that listing these patients for bilateral lung transplants should not be delayed, as the involved lungs will be resected at the time of transplantation. Directed NTM therapy should be initiated and continued in the post-transplant period, although the duration of post-transplant therapy is not well defined (Kikuchi *et al.*, 2011).

Recommended antibiotic therapy for the most common causes of pulmonary infections is outlined below. For details regarding therapy of other NTM infections, the reader is referred to the ATS consensus statement. Monitoring patients for signs of drug toxicity is required during therapy. Attention to adequate nutritional intake and weight stability is important as gastrointestinal side-effects, such as nausea and vomiting, are commonly seen in conjunction with the macrolide agents, rifampin, and rifabutin. It is especially important that weight is monitored in patients with nodular bronchiectasis given the propensity of these patients to be quite thin (Thorax *et al.*, 2009).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Study area

This study was carried out at the National Tuberculosis Reference Laboratory (NTRL) in Nairobi, Kenya. This is a government (public) institution under the National Public Health Laboratories (NPHLs) of the Department of Preventive and Promotive Health. It is mandated to act as a reference point for diagnosis of tuberculosis through the provision of microscopy, mycobacterium culture and drug sensitivity testing (DST) services for patient care and management. It is also mandated to develop national policies in relation to TB Laboratory protocols/procedures, operational research, coordination of quality assurance, planning and coordination for TB diagnostic performing sites. This laboratory was selected because it offers specialized TB testing with modern sensitive and specific techniques such as the BACTEC™ MGIT™ (Mycobacteria Growth Indicator Tube) and Hain molecular test. Sputum samples are received in this laboratory from all over the country. These samples are generally drawn from both new and retreatment patients but majority from the retreatment cases.

3.2 Study Population

Culture samples with positive growth on the machine received from TB retreatment cases for routine surveillance of TB were used for this study. The BACTEC™ MGIT™ 960 System monitors the tubes for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument-positive; i.e., the test sample contains viable organisms. Culture tubes which remain negative for a

minimum of 42 days (up to 56 days) and which show no visible signs of positivity are removed from the instrument as negatives.

3.2.1 Inclusion Criteria

Culture samples from TB retreatment patients that had initial positive growth on the BACTEC™ MGIT™ 960 machine.

3.2.2 Exclusion Criteria

Culture samples from presumptive TB patients. (These are patients who present with symptoms or signs suggestive of TB previously known as a TB suspect).

3.3 Study Design

This was a descriptive cross-sectional study.

3.4 Sample Size Determination

Sample size was determined using Cochran's formula of 1977,

$$n = \left(\frac{z}{m}\right)^2 p(1-p)$$

Where;

- z is the critical value based on the desired confidence level (e.g., z = 1.96 for 95% confidence level);
- m is the margin of error or precision of the estimate in this case m=0.05.
- p is the estimated value of the proportion. In this study, p is the proportion of NTM in new TB suspects in a Nigerian study (16%) (Aliyu *et al.*, 2013).

Thus = 1.962 X 0.16 X 0.84 / 0.052 = 206. For this study a round value of 210 culture samples with positive growth on the BACTEC™ MGIT™ 960 were selected and processed.

3.5 Sampling design

Purposive sampling design was used where culture samples from 210 patients with TB retreatment were collected from December 2014 to March 2015. All samples that had been incubated for detection of mycobacterial growth and had positive growth on the BACTEC™ MGIT™ 960 were tested to detect Non Tuberculous Mycobacteria (NTM). Cultures suggestive of Non-tuberculous mycobacterial infections were sub-cultured and characterized. All culture samples from retreatment cases with positive growth from the BACTEC™ MGIT™ 960 were picked consecutively until the desired number of 210 was attained.

3.6 Quality Assurance

Standard operating procedures were adopted, especially those pertaining to identification of samples, analysis and posting of results. Each culture sample had a negative and a positive control for purposes of quality control. The researcher maintained the code of ethics governing research including maintaining recruitment and inclusion criteria. Eventually data were cleaned and counterchecked for errors before the actual analysis and results presentation.

3.7 Data collection

3.7.1 Patients' Socio- demographic, clinical and Environmental Exposure Factors

A case investigation form was used to collect data on socio-demographic, clinical and exposure factors to NTM including sex, age, type of patient, marital status, education level, occupation, HIV status, history of diabetes mellitus, smoking, alcohol use, presence of respiratory symptom, environmental exposures (farming and animal contact) and clinical variables (smear results) (Appendix 1). Further, patients' health records cards were used to collect data on place of birth, treatment regimen, patient classification, previous culture results and CD4 count (Appendix 2).

3.7.2 Mycobacterial Culture

The processed samples (0.2 ml) was used to inoculate 3% Lowenstein Jensen (LJ) medium and cultured for 8 weeks in an incubator at 35-37℃ under 5-10% CO2. Samples (0.5 ml each) was also used to inoculate Mycobacterium Growth Indicator Tube (MGIT) medium after mixing with Polymyxin B, Amphotericin, Nalidixic acid, Trimethoprim and Azlocillin containing antimicrobial agent mixture (PANTA) supplement according to the manufacturer's protocol. The tubes were then incubated for 6 weeks in the BACTEC™ MGIT™ 960 system (Becton Dickinson). If fluorescence was detected in the tube, the test was considered positive. The mycobacterial isolates were identified as M. tuberculosis complex or species of NTM using Hain's GenoType® Mycobacterium CM and GenoType®

Mycobacterium AS Molecular Genetic Assays, following manufacturer's instructions.

3.7.2.1 Mycobacterium CM and GenoType AS Molecular Genetic Assay

From the liquid media, 1 ml. Pellet bacteria was directly applied by spinning for 15 min in a standard table top centrifuge with an aerosol- tight rotor in a class II safety cabinet at approximately 10000 x g. The supernatant was discarded and bacteria re-suspended in 100 µl of water by vortexing. Bacteria were then incubated for 20 min at 95°C in a waterbath. A further incubation was done for 15 min in an ultrasonic bath followed by spinning for 5min at full speed. 5 µl of the supernatant was drawn for PCR. Amplification was done by preparing the amplification mix (45 µl) in a DNA- free room. The DNA sample was then added in a separate room. Amplification products were stored at +8 to –20°C. For checking the amplification reaction, 5 µl of each sample was directly applied to a 2% agarose gel without the addition of loading buffer.

During hybridization, 20µl of denaturation solution was dispensed in a corner of each of the wells used. 20µl of amplified sample was added to the solution followed by pipetting and mixing then incubated at a room temperature for 5 minutes. While incubation was taking place, strips were taken out of the tube using tweezers and marked with a pencil underneath the colored marker. 1 ml of the pre-warmed hybridization buffer was added to each well and then the tray was shaken gently until a homogenous color had formed. Strips were then placed in each well completely covered by the solution. The tray was then placed in shaking water bath for 30 minutes at 45°C. The hybridization buffer was then completely aspirated

using a Pasteur pipette that was connected to a vacuum pump. I ml of stringent wash solution was then added to each strip and incubated for 15 minutes at 45°C in a twincubator. This was followed by removing the stringent wash solution completely then pouring out wash solution in a waste container. All remaining fluid was removed by turning the tray upside down and gently striking it on absorbent paper. Each strip was washed once in 1 ml rinse solution for 1 minute on twincubator. The rinse solution was then poured out after incubation.

1 ml of diluted conjugate was added to each strip incubated for 30 minutes on shaking platform. After removing solution, each strip was washed twice for 1 minute with 1 ml of rinse solution and once for 1 minute with 1ml of distilled water on twincubator. I ml of diluted substrate was added to each strip and incubated without shaking for 20 minutes. Reaction was then stopped by briefly rinsing twice with distilled water. Using tweezers, strips were removed from the tray and dried between two layers of absorbent paper.

3.7.2.2 NTM identification

Cultures with positive growth on BACTEC™ MGIT™ 960 machine and presence of AFB but were negative for MTB complex using the SD-Bioline assay were sub-cultured in Lowenstein Jensen (LJ) media. SD Bioline TB Ag MPT64 Rapid Assay is a rapid immunochromatographic identification test for the *M. tuberculosis complex*. Biochemical, immunological and molecular biological characterization of *Mycobacterium tuberculosis* has led to the identification of several antigens which may be useful in the development of improved diagnostic methods in order to discriminate between the *M. tuberculosis* complex and Mycobacteria other than M.

tuberculosis (MOTT bacilli). *M. tuberculosis* has been known to secrete more than 33 different proteins. One of the predominant proteins, MPT64 was found in the culture fluid of only strains of the *M. tuberculosis* complex.

3.7.2.3 SD- Bioline Assay

A 100 µl supernatant of sample from liquid culture was added to a 100 µl of suspended liquid cultures in buffer into the sample well. After 15 minutes, interpretation of the test was done. A positive test for SD Bioline assay showed 2 or 3 bands including the ''c'' line while a negative test showed one ''c'' line in result window.

Those that subsequently grew on LJ medium were considered to be NTMs and were characterized with the PCR based Genotype CM/AS assays. Thus, following the testings, the individuals in this study who visited the TB testing centers were identified into four groups: MTB cases, NTM cases, NMY (not mycobacterium though positive for AFB) cases and those negative for AFB due to lack of bacterial growth or growth of contaminants.

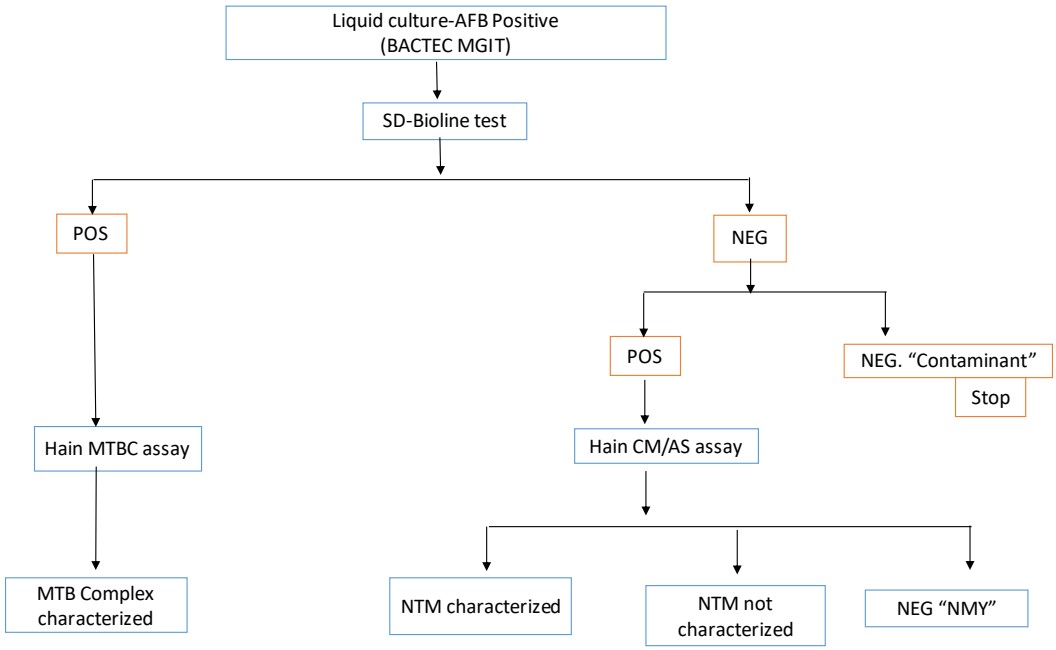

Figure 3.7.2.3 Flow chart showing Mycobacterial Detection

3.8 Data Management

All subjects were assigned a subject identification number (SID). All paper research records were kept in a locked filing cabinet located in a restricted-access room at the research center in the NTRL in Nairobi. All data entered into Microsoft Excel 2007 (Microsoft,USA) which was associated with SID in password protected files.

3.9 Data analysis

Descriptive statistics (frequency and proportions) were used to summarize data and presented using tables. The overall NTM prevalence was determined for the entire patient's population. Bivariate and multivariate analyses were used to determine factors associated with NTM infection using Poisson regression at the significance level of $p \leq 0.05$. All statistical analyses were performed using STATA v 13 (StataCorp LP, Texas, USA).

3.10 Ethical Considerations

The study was conducted according to the Declaration of Helsinki and International Conference on Harmonization Guideline on Good Clinical Practice (ICH-GCP). The protocol were reviewed and approved by Jaramogi Oginga Odinga Teaching and Referral Hospital's Ethics and Review Committee (JOOTRH-ERC) prior to any protocol-related procedures being conducted (Appendix 1). The investigator informed the ERC as to the progress of the study on a regular basis per the ERC requirements, but at minimum once a year. To maintain confidentiality, initials and coded numbers were used to identify the participants' source documents and study reports. All study records were maintained in a secured location. Patient information were not obtained or released without written permission from the participant/participant's legally authorized representative except as necessary for monitoring of the study.

CHAPTER FOUR

RESULTS

4.1 Descriptive characteristics of study population

Table 4.1 summarizes the descriptive characteristics of the study population.

Out of the 210 TB retreatment patients enrolled in the study, their mean age was 38.09 (SD 14.2) years and a median (IQR) range of 35 (28 to 75) years. There were two age group peaks; those aged 25 to 34 years (33.8%) and those aged 35 to 44 years (24.8%). Those aged less than 15 years were the least common (χ^2 = 116.8; df = 6; P = 0.001).

There was near equal proportion of female patients (113; 53.8%) to the male patients 97 (46.2%) and the distribution by gender was not significantly different (χ^2 = 1.219; df = 1; P = 0.27). Similarly, there was no statistically significant difference in gender across all age groups (χ^2 = 9.125; df = 6; P = 0.167). Among those aged 25 to 34 years, were 62% female while 38% were male while those aged 35 to 44 years, 51.9% were female while 48.1% were male as shown on table 4.1.

Slightly above a quarter of the patients 27.1% were from central part (Embu,Kiambu and Murang'a) of Kenya followed by 24.3% from Eastern (Kitui, Machakos, Marsabit, Meru and Wajir) and Western (Kisumu and Kakamega) part each (Table 4.1). About 14.8% were from Nairobi and only 9.5% from Rift Valley (Kajiado and Narok)(χ^2 = 23.61; df = 4; P = 0.001).

4.1.1 Marital status

Slightly over three quarters (77.6%) of the patients were married while 22.4% who were not currently married (single, separated, divorced or widowed) ($\chi^2 = 64.07$; df = 1; P = 0.001).

4.1.2 Occupation

The distribution of patient's occupation was as follows; close to a quarter 39.5% practised farming, 30.5% were unemployed, 21.9 % were in various forms of employment while only about 8.1% were housewives ($\chi^2 = 45.04$; df = 3; P = 0.001).

4.1.3 Education Level

Half of the patients (50.5%) had secondary level education followed by primary education (43.3%) and the least 1.9% had tertiary level education ($\chi^2 = 163.6$; df = 3; P = 0.001).

Table 4.1: Descriptive characteristics of the study participants

Characteristics	Sample size		χ2	df	P value
	N	%			
Age in Years					
Mean (± SD 14.3) = 38.09					
Median = 35					
Range = 75(12-87)					
10-14	1	.5			
15-24	28	13.3			
25-34	71	33.8			
35-44	52	24.8	116.8	6	0.001
45-54	29	13.8			
55-64	17	8.1			
>64	12	5.7			
Gender					
Female	113	53.8	1.219	1	0.27
Male	97	46.2			
Body Mass Index					
Mean (± SD 2.56) = 19.24					
Median = 18.6					
Range = 16.2(13.2-29.3)					
<18.5 (Underweight)	104	49.5			
18.5-24.9 (Normal)	105	50	102.03	2	0.001
25-29.9 (Overweight)	1	.5			
Region					
Central	57	27.1			
Eastern	51	24.3			
Nairobi	31	14.8	23.61	4	0.001
Rift valley	20	9.5			
Western	51	24.3			
Marital status					
Married	163	77.6	64.07	1	0.001
Not Married*	47	22.4			
Occupation					
Employment	46	21.9			
Farming	83	39.5	45.04	3	0.001
Housewife	17	8.1			
Student	64	30.5			
Education Level					
Primary	91	43.3			
Secondary	106	50.5	163.6	3	0.001
Tertiary	4	1.9			
Non-Formal Education	9	4.3			

No - Number; % - Percentage; χ2 - Chi square; df - Degree of freedom; SD- standard deviation; * Not Married (Single, Separated, Divorced, Widowed); P - Level of significance; P ≤ 0.05 indicates the relationship is significant

4.2 Baseline characteristics of study patients

Table 4.2 highlights the baseline characteristics of the study participants as described below.

4.2.1 Respiratory symptoms

About 36.7% of the patients had respiratory symptoms (presence of fever $>38^0$c and at least sore throat or cough) ($\chi^2 = 14.93$; df = 1; P = 0.001).

4.2.2 HIV infection

About 22% of the patients were HIV infected while 78.1% were HIV negative ($\chi^2 = 66.31$; df = 1; P = 0.001).

4.2.3 Immunological status

For those patients who were HIV infected, the mean CD4 was 238.6 (SD 138.3) ranging 529 (41 to 570) cell/mm3. At the time of study, majority of the patients 67.4% had CD4 count below <350 cell/mm3 verses 32.6 patients who had CD4 > 350 cell/mm3 ($\chi^2 = 187.63$; df = 1; P = 0.001).

4.2.4 Animal and livestock contact

Close to a quarter of the patients (36.2%) had contact with animals (these were patients who kept livestock and had previous contact with animals) compared to the majority (63.8%) who had no animal contact ($\chi^2 = 16.02$; df = 1; P = 0.001). Similarly, about a quarter (33.8%) of the 36.2% were livestock farmer while majority (66.2%) who were not practicing animal farming ($\chi^2 = 22.02$; df = 1; P = 0.001)

4.2.5 Smoking and alcohol

About 18% were smokers while majority 81% were not smokers (χ^2 = 80.48; df = 1; P = 0.001). Similarly, only 14.8% reported consuming alcohol while the majority 85.2% did not consume alcohol (χ^2 = 104.31; df = 1; P = 0.001).

Table 4.2: Baseline characteristics of the study participants

Characteristics	Sample size		χ2	df	P value
	N	%			
Respiratory Symptoms (Presence of fever and at least, sore throat, or cough)					
Yes	77	36.7	14.93	1	0.001
No	133	63.3			
HIV status					
Positive	46	21.9	66.31	1	0.001
Negative	164	78.1			
CD4 for HIV positive Mean (± SD 138.3) = 238.6 Median = 243 Range = 529(41-570)					
<350	31	14.8	187.63	2	0.001
>350	15	7.1			
History of Diabetes Mellitus					
Yes	54	25.7	49.54	1	0.001
No	156	74.3			
Smoking					
Yes	40	19	80.48	1	0.001
No	170	81			
Alcohol consumpsion					
Yes	31	14.8	104.31	1	0.001
No	179	85.2			
Animal Contact					
Yes	76	36.2	16.02	1	0.001
No	134	63.8			
Keep Livestock					
Yes	71	33.8	22.016	1	0.001
No	139	66.2			

No - Number; % - Percentage; χ2 - Chi square; df - Degree of freedom; SD- standard deviation; P - Level of significance; P ≤ 0.05 indicates the relationship is significant

4.3 Prevalence of NTM

Figure 4.3 highlights the prevalence of NTM among the study participants.

Out of the 210 patients' samples analyzed, 75 (35.7%) were positive for NTM species based on the Hain's Genotypic CM/AS assay test, while the rest 135 (64.3%) had *M. tuberculosis*. A total of eight different mycobacterium species were found causing NTM among these patients. These included 47/75 (62.7%) *M. intracellulare*, 12/75 (16%) *M. absessus*, 6/75 (8%) *M. fortuitum*, 4/75 (5.3%), *M. sacrofuloceum*, 3/75 (4%) *M. kansasii*, 1/75 (1.3%) *M. interjectum*, 1/75 (1.3%) *M. gordonae* and 1/75 (1.3%) *M. xenopi*.

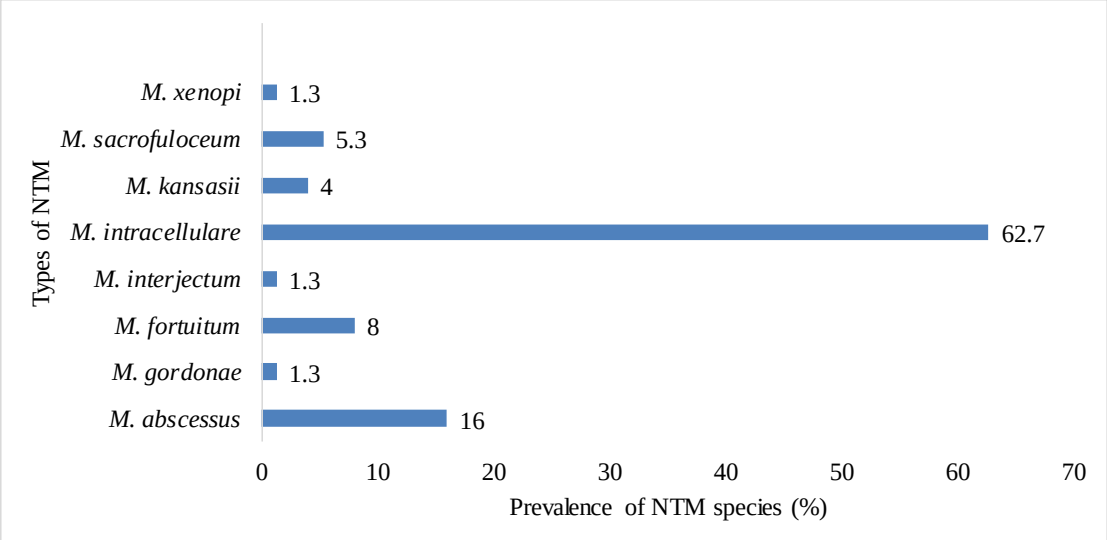

Figure 4.3: Prevalence of NTM among the study participants

4.4 Socio-Demographic factors associated with NTM infection

Table 4.4 summarizes the socio-demographic factors associated with NTM infection among TB retreatment patients in Kenya. In the bivariate analyses, female gender was more likely to be infected with NTM than males (OR 1.8, 95% CI 1.1 to 3.1). Patients residing in the Eastern (OR 2.2, 95% CI 1.1 to 4.6) and Nairobi (OR 2.3, 95% CI 1.1 to 5.1) areas of Kenya were more likely to be infected with NTM compared to those residing in the Western regions of Kenya.

Patients who were farmers (OR 1.6, 95% CI 0.9 to 2.7), who had tertiary level education (OR 2.2, 95% CI 0.9 to 5.5) and those who were from Central region (OR 2.1, 95% CI 0.97 to 4.3) were near likely to be infected with NTM. Other factors we did not find associated with NTM infection both in bivariate and in multivariate included; age, marital status, and occupation and education level.

Table 4.4: Socio-Demographic variables associated with NTM infection

Socio-Demographic variables	Sample size	NTM infection N	NTM infection %	Bivariate OR (95% CI)	Multivariate OR (95% CI)
Age Group					
10-14	1	0	0	0.2 (0.6 - 1.6)	
15-24	28	13	17.3	0.1 (0.2 - 1.6)	
25-34	71	22	29.3	0.4 (0.4 - 1.3)	
35-44	52	17	22.7	0.5 (0.5 - 1.6)	NS
45-54	29	12	16	0.6 (0.2 - 2.9)	
55-64	17	6	8	0.9 (0.7 - 1.6)	
>64	12	5	6.5	Referent	
Gender					
Female	113	42	37.2	**1.8 (1.1 - 3.1)**	NS
Male	97	33	34	Referent	
Marital status					
Married	163	56	34.4	0.8 (0.4 - 1.3)	NS
Not Married*	47	19	40.4	Referent	
Occupation					
Employment	46	10	21.7	0.6 (0.3 - 1.4)	
Farming	83	41	49.4	1.6 (0.9 - 2.7)	NS
Housewife	17	4	23.5	0.7 (0.2-2.2)	
Student	64	20	31.3	Referent	
Education Level					
Primary	91	32	35.2	1.1 (0.6-1.8)	
Secondary	106	37	34.9	1 (0.5-1.8)	NS
Tertiarry	4	3	75	2.2(0.9-5.5)	
Non-Formal Education	9	3	33.3	Referent	
Region					
Central	57	23	40.4	2.1(0.97-4.3)	
Eastern	51	21	41.2	**2.2(1.1-4.6)**	NS
Nairobi	31	14	45.2	**2.3(1.1-5.1)**	
Rift valley	20	7	35	1.7(0.67-4.68)	
Western	51	10	19.6	Referent	

N - Number; % - Percentage; OR - Odds ratio; CI - confidence interval; ND - Not Done; NS - Not significant

* Not Married (Single, Separated, Divorced, Widowed); Central - Embu, Kiambu and Muranga

Eastern - Kitui, Machakos, Marsabit, Meru, Wajir; Rift Valley - Nakuru, Kajiado and Narok

4.5 Clinical and exposure factors associated with NTM infection

Table 4.5 summarizes the clinical and exposure factors associated with NTM infection among the participants in this study. Patients who had respiratory symptoms (fever $>38^0$ c and at least sore throat or cough) (OR 1.5, 95% CI 1.01 to 2.5) were more likely to be infected with NTM than those who did not have these symptoms. Patients who had animal contact were more likely to be infected with NTM compared to those without (OR 1.7, 95% CI 1.1 to 2.7). Further, those patients who kept livestock were more likely to be infected with NTM than those who did not (OR 1.6, 95% CI 1.06 to 2.6).

Further, patients who were HIV positive (OR 1.8, 95% CI 1.1 to 3.1) were near likely to be infected with NTM than those who were negative for HIV.

The patient's body mass index, those that were diabetic or those who smoked or consumed alcohol were not associated with NTM infection both in bivariate and in multivariate analyses.

Table 4.5: Clinical and exposure factors associated with NTM infection

Clinical and Exposure variables	Sample size	NTM infection N	NTM infection %	Bivariate OR (95% CI)	Multivariate OR (95% CI)
Respiratory Symptoms (Presence of fever and at least, sore throat, or cough)					
Yes	77	36	46.8	**1.5(1.01-2.5)**	1.95(0.99-2.5)
No	133	39	29.3	Referent	
HIV status					
Positive	46	23	50	**1.5(0.96-2.5)**	NS
Negative	164	52	31.7	Referent	
Body Mass Index (kg/m^2)					
<18.5 (Underweight)	104	37	35.6	ND	ND
18.5-24.9 (Normal)	105	37	35.2	Referent	
25-29.9 (Overweight)	1	1	100	ND	
History of Diabetes Mellitus					
Yes	54	20	37	1.3(0.61-1.71)	NS
No	156	55	35.3	Referent	
Smoking					
Yes	40	17	42.5	1.2(0.71-2.1)	NS
No	170	58	34.1	Referent	
Alcohol consumpsion					
Yes	31	11	35.5	0.9(0.5-1.8)	NS
No	179	64	35.8	Referent	
Animal Contact					
Yes	76	38	50	**1.7(1.1-2.7)**	NS
No	134	37	27.6	Referent	
Keep Livestock					
Yes	71	35	49.3	**1.6(1.06-2.6)**	NS
No	139	40	28.8	Referent	

N - Number; % - Percentage; OR - Odds ratio; CI - confidence interval; ND - Not Done; NS - Not significant

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Prevalence of NTM

Although success in TB elimination efforts has reduced incidence of *M. tuberculosis* related diseases in Kenya, apparent increase in non-tuberculous mycobacterial disease has occurred. In Kenya and many countries, there are no nationally representative data about the prevalence of NTM related diseases (Billinger *et al.*, 2009). The current study therefore provides vital information that will contribute significantly in the management of pulmonary infections in Kenya. The overall prevalence of NTM in this study was found to be 35.7%. This significantly high prevalence of NTM among patients with pulmonary disease is worrying. Low NTM prevalence rates have been reported elsewhere; in Iran, Tabarsi *et al.*, (2009) reported a prevalence of 11.4% NTM among patients with multiple drug resistance histories. In Thailand, Srisuwanvilai *et al.* 2008 reported NTM prevalence of 11%. In Nigeria, 15% of patients who sought clinical treatment for TB were caused by NTM (Aliyu *et al.*, 2013). Other studies have reported high NTM infection. In Taiwan, Sun *et al.* (2009) showed 43.3% NTM prevalence while Makarova *et al.*, 2009 showed a higher NTM prevalence of 65.4%.

5.1.1 Genotypic characteristics NTM

The NTM complex were caused majorly by *M. intracellulare* (62.7%), followed by *M. absessus,M.fortuitum, M. sacrofuloceum, M. kansasii, M. interjectum, M. xenopi and M.gordonae.* These findings are similar to those found in Japan (Ito *et al.*,

2012), in East Asia (Simons *et al.,* 2011), in South American (Hoefsloot *et al.,* 2013) in Nigeria (Aliyu *et al.,* 2013), in Canada and European countries (Marras *et al.,* 2007; Cassidy *et al.,* 2009).

5.1.2 Factors associated with NTM infection

Female gender was a strong predictor for NTM infection in the current study. This finding is similar to that of Cassidy *et al.,* (2009) in Oregon, US which showed a strong association of NTM frequent infection in women than men. Our findings are in line with published reports from institutional cases series and experts who believe the epidemiology of this disease has changed during the last several decades to affect women more frequently than men (Griffith et al., 2007; Kim *et al.,* 2009).

In a study of 35 patients, Danley measured the levels of dehydroepiandrosterone-sulfate (DHEA-S), estrone, and ultrasensitive estradiol blood samples. They found that women with MAC infection had lower DHEA-S levels, but not lower estrogen levels, compared with control subjects. There was no relationship between BMI and hormone levels in their study population. Although the study makes an interesting observation, the small sample size makes it difficult to make definitive conclusions Danley *et al.,* 2014). This could be a possible explanation as to why female gender was a strong predictor of NTM infection in this study.

The above studies suggest a role for estrogen in immune response to NTM. However, the mechanisms by which estrogen enhances host defense to NTM remains to be defined. In an excellent manuscript published by Chan and Iseman, the contribution of sex hormones and the mechanism by which they might modulate the NTM response was reviewed. They hypothesize that hormones such as

Luteinizing hormone (LH) and estrogen may contribute to altered leptin and adiponectin levels that in turn may modulate the host response in terms of cytokine and chemokine expression (IL-10, TGF-β).

Region of origin contributed to the risk of NTM infection. Eastern and Nairobi parts of Kenya are generally dusty (Eastern) and crowded (Nairobi). These regions are characterized with a pocket of poor nutrition, poor hygienic measures in the surrounding environment which have been shown to increase the risk for NTM disease (Marras *et al.,* 2002; Gupta *et al.,* 2009). Higher risks for environmentally acquired pulmonary mycobacterial infections have been previously reported for individuals with occupational exposures to dust (Tiwari *et al.,* 2007). Most regions of Eastern Kenya and pockets of Nairobi are characterized by crowding and hot dusty environment which could explain high prevalence rates in these regions.

Animals contact and livestock keeping was a significant risk factor for NTM infection in this study. NTM are ubiquitous opportunistic pathogens. Humans and their agronomic animals are literally surrounded by NTM thus in developing countries, the dietary habit of people, close physical contact between humans and animals, and inadequate disease control measures in animals and humans facilitate the transmission of the disease between animals and humans (Ameni *et al.,* 2013).

Respiratory symptoms (fever >38.0°C), sore throat, cough) were associated with NTM infection in the current study. This observation mirrors what has been observed in other studies which have shown prior tuberculosis or people with pre-existing lung conditions, chronic obstructive pulmonary disease, lung damage due to occupational exposures to dusts (e.g., mining), cystic fibrosis, cystic fibrosis

mutation, a-1-antitrypsin deficiency (Griffith *et al.,* 2007; Hamilton *et al.,* 2008; Ameni *et al.,* 2013).

Although the current study observed a near association of HIV/AIDS infection with NTM infection, studies have shown that generally, NTMs are increasingly being isolated among HIV/AIDS positive and negative cases of TB in Sub-Saharan Africa (Buijtels *et al.,* 2005; Crump *et al.,* 2009; Buijtels *et al.,* 2010). A prospective evaluation of a cohort of 721 HIV/AIDS positive patients' in Abidjan, Cote d'Ivoire, Sub-Saharan Africa found the incidence of NTM infection was 9.7 times higher among patients with baseline CD4 cell counts less than 100 cells/mm3 compared to patients with CD4 cell counts above 100 cells/mm3 (Bonard *et al.,* 2004). In addition to HIV/AIDS disease, NTMs have been reported to be more common among persons with occupational exposure to dust (Amorim *et al.,* 2010). Occupational dust exposure in HIV/AIDS positive subjects accelerates the risk of pulmonary infection with both TB and NTM (Park *et al.,* 2009). In addition, the risk of reactivation of latent mycobacterial infections including NTM is found to be higher in patients receiving treatment with tumor necrosis factor inhibitors like infliximab and this is becoming more prevalent in Europe and United States (Agliari *et al.,* 2013).

Other risk factors that we either did not measure or did not found associated with NTM infection includes; age which Aliyu (2013) showed was more common among older age groups. Fish exposure risk for infections with *M. marinum* infection while children aged below 5 years are at risk for cervical lymphadenitis caused more typically by *M. avium* (WHO, 2011). Immunodeficiency, due to HIV-infection is a

risk factor for *M. avium* (WHO, 2009). The disease was also reported to be higher among farmers (Gopinath and Singh, 2010).

5.2 Conclusions

Based on the findings of this study the following conclusions can be drawn.

1. The study found a high prevalence (35.7%) of NTM in patients seeking for TB retreatment.
2. This study re-affirms the predominance of *Mycobacterium intracellulare* (62.7%) as the highest contributor to NTM infection and to a less extent *M. kansasii, M. interjectum, M. gordonae* and *M. xenopi.*
3. The factors that were found to be associated with NTM were female's gender, residency, presence of respiratory symptoms, animal contact and livestock keeping.

5.3 Recommendations

In view of the conclusions highlighted above, this survey highlights the growing importance of NTM as a pathogen, the importance of routine, standardized surveillance, and the need to find out how best to manage NTM since their regimen vary from those of *M. Tuberculosis*.

Persons in regions with large livestock and are exposed to animals, testing for NTM especially when they present with any TB like symptoms . Further such persons in this environment should be sensitized about the consequences of animal contact in NTM epidemiology.

The involvement of environmental hazard in transmission of NTM should be made a public health priority. Particularly patients with respiratory diseases, pre-existing

lung conditions, chronic obstructive pulmonary disease, lung damage due to occupational exposures to dusts e.g. farmers should be coarsely monitored and evaluated for the presence of NTM prior to TB management. NTM are environmental pathogens that deserve further examination as important causes of public health morbidity.

Despite these recommendations, several limitations are notable from this study which needs to be taken into consideration. Firstly, is that the study was a cross-sectional thus unable to adequately evaluate causal and temporal associations between HIV, smoking and mycobacterial infections. Secondly the findings may only represent facility-based TB patients whereas findings from other patients who did not visit these healthcare facilities may differ. Bias in this study may have been introduced by giving only TB retreatment patients visiting the facility a chance to participate while new cases had been left out.

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APPENDICES

APPENDIX 1: CASE INVESTIGATION FORM

Section A: Patient demographics (Indicate an X for a positive answer)

1. Patient Study ID……
2. Date Registered
3. Sex: Male…….. Female…….. (Indicate X if male or female)
4. Patient's Age……. (Years)
5. Type of Patient: New Patient……Previously treated case…… (Indicate an X)
6. Marital Status: Single…….Married……separated……. Divorced……Widowed…… (Indicate an X for a positive answer)
7. Highest Education: No formal education……...Primary…… Secondary……College……..University……..other (specify)
8. Occupation: Formal Employment…….Informal employment……..… self-employment….. Unemployed……..Student…… Farmer…… Other (Specify)……

Section B: HIV STATUS

9. HIV serological status Positive…… Negative… Not done….. Declined…...

10. Have you ever been diagnosed as having the following?

i) Diabetes……. ii) Tuberculosis……iii) Respiratory symptom (For respiratory symptoms, the patient should have a record of at least two of the following signs or symptoms: fever (>38.0°C), sore throat or cough, this should be assessed from the records of the patient).

(Specify)……

11. Have you ever smoked cigarette/Tobacco? Yes……. No…….

12. Have you ever drunk alcohol? Yes……. No……

13. If yes, are you a current drinker? Yes……. No……..

14. If yes, how many days per week do you drink alcohol?

15. Do you practice farming? Yes…….. No…….

16. If yes, for how long have you been farming? (Months)……..

17. In the last 6 months, have you come in contact with animals? Yes….. No….

18. Do you keep livestock? Yes…… No……..

19. If yes, for how long have you been keeping livestock? (Months)…..

Section C: FIELD MICROSCOPY RESULTS

20. Date of sputum collection……/……/……

21. FM Sputum results

AFB	Actual No	1+	2+	3+	Negative
SPOT					
Morning					

Section D: SPECIMEN FOR REFERRAL TO NTRL

22. Date shipment of spot specimen to NTRL…… /…… /……..

Day Month Year

Responsible Officer……. Date…….

APPENDIX 2: PATIENT HEALTH RECORDS DATA

Registration Number ……

Name of the referral hospital ……

Gender (Male or female ……..

Age (in years): ……

Place of birth: ……...

Date when treatment started: ……

Disease classification (Pulmonary or non-pulmonary): ……

Patient classification

A. New B. Smear positive relapse C. Smear negative/extra pulmonary relapse D.

Failure E. Return after default F. Transfer In: ……

The regimen: ……...

Sputum smear examination: ……

Culture Result: ……

Drug Sensitivity Testing Results (R for resistant or S for sensitive: ……

HIV status: ……

CD4 Count: ----------

APPENDIX 3: INFORMED CONSENT

Title of study: Prevalence and Characterization of Non Tuberculous Mycobacteria among retreatment cases attending National Tuberculosis Reference Laboratory. For questions about the study, contact: Jacqueline J. Limo (0722-662929) of NTRL.

Description: You are requested to provide us with your sputum sample to help us identify non tuberculosis mycobacteria. These are mycobacteria other than tuberculosis that complicate Tuberculosis treatment. We would also like to analyze your patient profile. Therefore, some of your demographic information will be recorded for epidemiological reasons.

Risks and benefits: We will do our best to make sure that the personal information gathered during this study is kept private and we do not see any other potential risk of this study to you. There is no monetary benefit for your participation in this study. The benefit which may reasonably be expected to result from this study includes your contributions to efforts to provide measures to promote early accurate TB diagnosis and result dissemination for eventual TB management in Kenya. Your decision whether or not to participate in this study will not affect your current benefits (if any) you get from your work at the TB site.

Time involvement: This study will take about 30 minutes of your time.

Subject's rights: If you have read this form and have decided to participate in this project, please understand your participation is voluntary and you have the right to withdraw your consent or discontinue participating at any time without penalty.

You have the right to refuse to answer particular questions. Your individual privacy will be maintained in all published and written data resulting from the study.

I have read this form or had it read to me in a language that I understand. I have discussed the information with study staff. My questions have been answered. My decision whether or not to take part in the study is voluntary. If I decide to join the study, I may withdraw at any time. By signing this form, I do not give up any rights that I have as a research participant.

Participant Name	Participant Signature/ Thumb print	Date
_____	_____	_____
Study Staff Conducting	Study Staff Signature	Date
_____	_____	_____

APPENDIX 4: ETHICAL APPROVAL FROM JARAMOGI OGINGA ODINGA TEACHING AND REFERRAL HOSPITAL

MINISTRY OF HEALTH

Telegrams: "MEDICAL", Kisumu
Telephone: 057-2020801/2020803/2020321
Fax: 057-2024337
E-mail: ercjootrh@gmail.com
When replying please quote
ERC.1B/VOL.I/130
Ref: .....

JARAMOGI OGINGA ODINGA TEACHING & REFERRAL HOSPITAL
P.O. BOX 849
KISUMU
24th September, 2014
Date .....

Dear Jacqueline,

RE: FORMAL APPROVAL TO CONDUCT RESEARCH TITLED: *"PREVALENCE AND CHARACTERIZATION OF NON-TUBERCULOUS MYCOBACTERIUM AMONG RETREATMENT CASES IN NATIONAL TUBERCULOSIS REFERENCE LABORATORY"*

The JOOTRH ERC (ACCREDITATION NO. 01713) has reviewed your protocol and found it ethically satisfactory. You are, therefore, permitted to commence your study immediately. Note that this approval is granted for a period of one year (24th September, 2014 to 24th September, 2015). If it is necessary to proceed with this research beyond the approved period, you will be required to apply for further extension.

Also note that you will be required to notify the committee of any protocol amendment(s), serious or unexpected outcomes related to the conduct of the study or termination for any reason.

Finally, note that you will also be required to share the findings of the study in both hard and soft copies upon completion.

The JOOTRH ERC takes this opportunity to thank you for choosing this institution and wishes you the best in your endeavours.

Yours sincerely,

WILBRODA MAKUNDA,
For: **SECRETARY – ERC,**
JOOTRH – KISUMU.

APPEDIX 5: PUBLICATION