ISOLATION AND MOLECULAR CHARACTERIZATION OF NON-TUBERCULOUS MYCOBACTERIA OF PUBLIC HEALTH SIGNIFICANCE FROM HUMANS AND WATER IN NAMWALA DISTRICTS OF ZAMBIA

BY

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The University of Zambia

DECLARATION

The contents of the dissertation are the author's own works. The dissertation has not previously been submitted for the award of degree to any University.

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Ngula Monde

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Date

DEDICATION

To my loving and supportive husband Elijah Kabelenga, my lovely daughters Grace and Atowe and my mother Nosiku Monde. I love you all!!!.

CERTIFICATE OF APPROVAL

This dissertation submitted by NGULA MONDE is approved as fulfilling part of the requirements for the award of the degree of MASTER OF SCIENCE IN PUBLIC HEALTH at the University of Zambia.

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ABSTRACT

Globally, opportunistic infections due to environmental Non-tuberculous Mycobacteria are increasingly becoming a public health threat due to the Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome pandemic. In Zambia, Non tuberculous Mycobacteria are gaining recognition as pathogens of public health significance. However, there is scanty information on the isolation and speciation of these organisms for better patient management, which could consequentially reduce the burden of these infections. Given the above information, the thrust of this study was to isolate and characterize NTM of public health significance from humans and water in Namwala district of Zambia.

The study was a cross-sectional study were 306 sputum samples from adult human patients with suspected Tuberculosis were collected from four health centres in Namwala district. Additionally, 149 water samples were collected from different water drinking sources such as Tap water, Borehole water, rivers, wells and streams. Standard TB culture methods were employed to isolate Nontuberculous Mycobacteria. The isolates were then characterized using the *16S-23S* internal transcribed spacer region Sequencing.

One hundred and fifty three (153) individuals with suspected TB were sampled and 7(4.6%) were found to have NTM with *M. arupense* (3, 2%) being the most common organism. Out of the 149 water samples collected, (23, 15%) NTM were isolated with the common species being *Mycobacterium gordonae (*5, 3%), *Mycobacterium senegalense* (3, 2%), *Mycobacterium peregrinum* (3, 2%) *and Mycobacterium fortuitum* (3, 2%). *Mycobacterium avium* and *Mycobacterium fortuitum* were both isolated from humans and water.

The NTM species isolated in this study are potentially pathogens that have been found to be associated with pulmonary infections. The isolation of Non tuberculous Mycobacteria in water could possibly indicate that the source of infections of these mycobacteria in infected individuals of Namwala district could be linked to drinking water.

The isolation of NTM from both humans and water in Namwala district has highlighted the public health significance of these mycobacteria. We therefore, recommend further studies to be done to fingerprint the Non tuberculous Mycobacteria isolates so as to establish the epidemiological link using other molecular methods like Mycobacterium Interspersed Repetitive Units Variable Number Tandem Repeats.

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LIST OF ABBREVIATIONS

AFB	Acid-Fast Bacilli
AIDS	Acquired Immunodeficiency Syndrome
BD	Becton Dickinson
BSC	Biological Safety Cabinet
CDC	Center for Disease Control
COPD	Chronic Obstructive Pulmonary Disease
CPC	Cetylpyridinium Chloride
CSO	Central Statistics Office
DHMT	Ndola Health Management Team
DNA	Deoxyribonucleic Acid
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
IUATLD	International Union against Tuberculosis and Lung disease
KH2PO4	Mono-potassium phosphate
LJ	Löwenstein-Jensen
MAC	Mycobacterium avium Complex
MDR	Multidrug Resistance
MGIT	Mycobacteria Growth Indicator Tube
MIRU	Mycobacterium Interspersed Repetitive Units
MTC	Mycobacterium tuberculosis complex
NaHPO4	Disodium phosphate
NALC	N-Acetyl L-Cysteine
NaOH	Sodium hydroxide
NTM	Non-tuberculous Mycobacteria
PCR	Polymerase Chain reaction
PRA	PCR Restriction enzyme analysis
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium dodecyl sulphate
SE	Standard Error

TB	Tuberculosis
VNTR	Variable Number Tandem Repeats
WHO	World Health Organization
ZDHS	Zambia Demographic Health Survey
ZN	Ziehl Neelsen

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

The non-tuberculous Mycobacteria (NTM) include those *Mycobacterium* species that are not members of the Mycobacterium tuberculosis complex (MTBC), the causative agent of pulmonary tuberculosis (TB) and *Mycobacterium leprae* (Asiimwe et al., 2013; Malama et al., 2014). They are normal inhabitants of natural waters, drinking waters, animals, birds and soils(Muwonge et al., 2012; Ricketts et al., 2014; Velayati et al., 2014). Thus, the environment is the primary source of infection of these mycobacteria to humans. Human-to-human transmission of NTM is generally uncommon, although there is evidence of transmission of certain NTM species such as *Mycobacterium kansasii* (Ricketts et al., 2014). NTM have also been recovered from meat, fish, dairy products, fruits, vegetables and especially unpasteurized milk, suggesting a zoonotic potential for these bacteria (Konuk et al., 2007).

Although members of the MTC are largely known to cause the majority of the mycobacterial infections worldwide, NTM are also increasingly becoming more of public health significance (Kankya et al., 2011).

There are 172 different species of NTM but the most important and potentially pathogenic of these are *M. avium, M. intracellulare, M. kansasii, M. xenopi, and M. abscessus (*Kankya et al., 2011). These organisms are capable of causing pulmonary disease, disseminated disease and localized lesions, mostly in immuno-compromised individuals. Pulmonary

disease is the most frequently encountered and has been reported to account for up to 94% of cases of NTM disease (Panagiotou et al., 2014). People living with the Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (HIV/AIDS), represent some of the most vulnerable populations to NTM infection, as well as a higher risk for complications and poor disease outcomes. Those receiving immunosuppressive therapy secondary to organ transplantation, cancer, autoimmune disease and those with diabetes mellitus may also be more susceptible to NTM as compared with the general population (Akbar et al., 2015).

Non-tuberculous Mycobacteria can display clinical and radiological features similar to those exhibited by MTBC, hence the need for species differentiation in order to distinguish from MTBC in individuals suggestive of having TB. Distinguishing between MTC and NTM disease may be challenging, especially in low income countries such as Zambia where AFB smear microscopy is mainly used for MTC diagnosis. However, even though AFB smear microscopy, allows for rapid diagnosis of mycobacteria, it does not differentiate *M. tuberculosis* from NTM. This may lead to misdiagnosis of TB (Chanda-Kapata et al., 2015).

In Zambia, NTM have also been recognized as pathogens of major public health significance with the advent of Human HIV/AIDS (Buijtels et al., 2005; Buijtels et al., 2009; Malama et al., 2014). However, only scanty studies have demonstrated the isolation and characterization of these mycobacteria in humans and water.

1.2 STATEMENT OF THE PROBLEM

Globally NTM have been recognized as pathogens especially in immuno-compromised populations including HIV/AIDS, as well as immuno-competent individuals (Ahmed et al., 2013a). The importance of NTM infections in humans in sub-Saharan Africa has recently received increased attention (Kankya et al., 2011) as these NTM infections are both difficult to diagnose and treat. Treatment also varies according to the NTM species involved (Ahmed et al., 2013a). NTMs are generally resistant to standard first line anti-TB therapy (Muwonge et al., 2011) and presumably many of these cases would be considered treatment failures, and subsequently treated for multi-drug resistant (MDR) disease (Maiga et al., 2012).

In Zambia, infections due to non-tuberculous mycobacteria (NTM) are slowly becoming more of a public health challenge with the HIV/AIDS disease burden (Malama et al., 2014a). However, there is limited information regarding the isolation and speciation of NTM from humans and environment such as water. Failure to identify and characterize these NTM may lead to their misclassification and mistakenly treated for pulmonary tuberculosis. Thus, identification of these Mycobacteria in clinical samples and environmental sources is important for both clinical and patient management.

1.3 RATIONALE

The recently notable increase in the number of NTM infections globally, has made it clinically important to rapidly and accurately identify Mycobacteria to the species level to facilitate prompt patient management. Therefore this study will provide information on the circulating species of NTM in humans and water in Namwala District of Zambia. This will

help the clinicians to properly manage patients with NTM infections with regard to targeted treatment. Furthermore, the study will help policy makers to better understand the factors increasing transmission of NTM in humans, hence, help in the design and implementation of preventive measures.

1.4 OBJECTIVES

1.4.1 General Objective

To isolate and characterize Non-tuberculous Mycobacteria of public health significance from humans and water in Namwala District of Zambia.

1.4.2 Specific Objectives

1.4.1.1 To isolate and Characterize Non tuberculous Mycobacteria from humans and water using DNA sequencing.

1.4.1.2 To establish the public health significance of the isolated Non-tuberculous Mycobacteria

1.5 RESEARCH QUESTIONS

- What are the Non-tuberculous Mycobacteria species circulating in humans and drinking water in Namwala District?
- What is the public health significance of Non-tuberculous Mycobacteria in Namwala District of Zambia?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Background

Members of the Mycobacterium tuberculosis complex (MTC) are widely known to be responsible for the majority of mycobacterial infections. However, environmental opportunistic infections due to Non-tuberculous Mycobacteria (NTM) are also becoming a public health problem (Kankya et al., 2011). These organisms constitute the majority of species in the genus mycobacteria (Tortoli, 2003). The past decade has seen not only a notable increase of new NTM species but also an increase in the isolation of NTM (Daley & Griffith, 2010). This increase could be attributed to a number of factors, including increase in the number of immuno-compromised population including those with HIV/AIDS, availability of new and more sensitive molecular methods for NTM identification from specimens, increase in knowledge and interest in the isolation of all *Mycobacterium* species and impact of human activities on the ecology of NTM (Ahmed et al., 2013; Mwikuma et al., 2015).

Non Tuberculous Mycobacteria have thus far been associated with disease in humans and have been reported to cause pulmonary, lymph node, skin, soft tissue, skeletal, and disseminated infections as well as nosocomial outbreaks related to inadequate disinfection/sterilization of medical devices (Katoch 2004; Kankya et al., 2011).

The most important potentially pathogenic NTM in humans are *M. avium, M. intracellulare, M. kansasii, M. xenopi, and M. abscessus*. However, an increasing number of NTM species previously considered non-pathogenic, have also been shown to cause infections in humans (Kankya et al,. 2011). A study based on data from a number of

geographically distant regions in Asia showed that the *M. avium* complex was responsible for 56% of clinically relevant pulmonary disease (range 40–81%), *M. abscessus* (35%), *M. chelonae* (31%), *M. kansasii* and scrofulaceum (17%), *M. celatum* (9%), *M. szulgai* (6%), *M. fortuitum (*5%), *M. gordonae* (2%) and *M. terrae* (1%) (Simons et al., 2011).

2.2 Distribution of NTM in the environment

Non-tuberculous Mycobacteria are widely distributed in nature and has been isolated from natural open bodies of waters, biofilms, soil, tap water, waters used in showers and surgical solutions. They may also be found in drinking water distribution system (Malama et al., 2014c; Muwonge et al., 2012; Ricketts et al., 2014). A majority of these NTM habitants are shared with humans and this is a major determinant in disease acquisition (Falkinham, 2009). A study done in Queensland Australia showed the isolation of pathogenic NTM from water, biofilms and aerosols in the households of patients with NTM disease with the most common NTM species being *M. avium, M. kansasii, M. lentiflavum and M. abscessus* (Thomson et al., 2013). Another study done by Falkinham using DNA fingerprint analysis revealed that household water contains mycobacterial species which are a source of infection in patients with NTM disease (Falkinham, 2009). Despite this availability of information on the isolation of NTM in humans and water, the distribution of NTM in most parts of the world, Zambia included, is not yet fully understood.

2.3 Prevalence of NTM infection

The prevalence of NTM infections and NTM lung disease have increased worldwide since the 1950s (Kim et al., 2014). Most of the epidemiological data on NTM lung disease has

been generated from studies in North America, where there is increasing awareness of the clinical significance of NTM disease (Ingen et al., 2011). A study done by Cassidy et al among Oregon residents from 2005 -2006, Oregon USA, reported an annual prevalence of 7.2 cases per 100,000 persons. The prevalence was significantly higher in females than males and was highest in persons above 50 years (Cassidy et al., 2009). Another report from Oregon, indicated the overall prevalence of NTM pulmonary disease in a two year period to be 8.6 cases per 100,000 and increased to 20.4 cases per 100,000 among those at least 50 years old (Winthrop et al., 2006). Studies from Canada (Marras et al., 2007), Australia (Thomson et al., 2013), Taiwan (Lai et al., 2010), Netherlands (Ingen et al., 2009) have also reported an increase in the prevalence of NTM. A study done by Marras et al (2007) reported an increase in the isolation prevalence of all NTM species in Ontario, Canada, from 9.1 cases per 100,000 persons in 1997 to 14.1 cases per 100,000 populations in 2003.

In sub-Saharan Africa, Zambia inclusive information on the extent of the burden of NTM infection is lacking mainly due to limitations in tools for mycobacterial species identification, these diseases are often underdiagnosed or misdiagnosed as tuberculosis and no system for notification exists as in the case for *M. tuberculosis*. There are only a few studies documenting the prevalence of NTM. Botha et al (2013) have reported the following NTMs to have been commonly isolated in human specimens in Southern Africa. Namely; *M. abscessus, M. avium, M. chelonae, M. elephantis, M. fortuitum, M. gordonae, M. intracellulare, M. interjectum, M. paraffinicum, M. senegalense* and *M. simiae.* A recent study done in Uganda showed the prevalence of NTM in infant TB suspects to be 3.7%

while that in adolescent suspects to be 4.6% with the most common NTM species being *M. fortuitum* (Asiimwe et al., 2013).

In Zambia, NTM are also slowly being acknowledged as pathogens of human and animal infections (Malama et al., 2014b). However, little is known on the distribution of these organisms in humans as well as environment such as water. Only a limited number of studies have highlighted the isolation of NTM species in humans and there have been no published studies on the isolation of NTM in water. A study conducted by Buijtels et al (2005), in Katete, Sesheke and Chilonga districts of Zambia on the clinical relevance of NTM, reported the isolation of NTM from various specimens of patients including ones that are usually sterile (Buijtels et al., 2005). In Katete district, NTM were isolated from both patients and controls and the most frequently isolated NTM species was *M. avium Complex (MAC).* Other NTM species isolated were *M. porcinum* and *M. peregrinum* (Buijtel et al., 2009). In the recently conducted national TB prevalence survey, the prevalence of NTM in TB symptomatic participants aged 15 years and above was reported to be 1,477/100,000 (95 % CI 1010–1943). The NTM prevalence was higher among rural than urban participants and it increased with participant's age (Chanda-Kapata et al., 2015).

2.4 Transmission and Risk factors associated with NTM infection

Transmission of NTM infection occurs by inhalation or ingestion of aerosolized organisms from soil, dust or water (Falkinham et al., 2009). Damaged skin tissues favour infections by particular NTM species including *M. abscessus* (Buijtels et al., 2005). Lack of documented evidence of direct human to human and animal to human transmission of NTM

infection in the past years has led to the belief that environment is the only source of infection. However, Ricketts et al (2014) has documented human to human transmission of *Mycobacterium kasansii* in a husband and wife living in East London, UK, an area with a high incidence of tuberculosis.

There is evidence that food of animal origin could also be a source of NTM infections to human (Falkinham, 2009; Muwonge et al., 2010).

Several Risk factors for NTM infection have been documented in literature (Falkinham, 2009), and these may be divided into underlying medical conditions, living and work environment, and patient demographic features. Underlying medical conditions likely provide the most powerful risk factor for NTM infection. These risk factors may be divided into impairment of local pulmonary defences and generalized immune defects, for example Cystic Fibrosis (CF) and HIV, respectively. Preexisting lung disease, such as silicosis and other pneumoconiosis, chronic obstructive pulmonary disease (COPD), bronchiectasis, prior tuberculosis, pulmonary alveolar proteinases, and esophageal motility disorders are also associated with NTM infection (Katoch et al., 2007; Faria et al., 2015). Alcohol abuse, diabetes mellitus, malignancy and smoking have also been identified as important risk factors (Katoch et al., 2007).

Living in urban versus rural settings has also been associated with NTM colonization with the most commonly documented environmental risk factor being the work environment, more especially mining and other heavy industries such as smelting. Residence in areas where these industries dominate may also be a risk factor (Buijtels et al., 2005).

Patient demographic features such as age, sex or a combination of the two have also been identified as risk factors for NTM infection (Buijtels et al., 2009).

2.5 Clinical Manifestation of NTM Infections

Non-tuberculous Mycobacteria disease most commonly presents as pulmonary manifestations. However, superficial lymphadenitis, skin and soft tissue infection and disseminated disease, are of clinical importance as well (Botha et al., 2013). Pulmonary disease accounts for up to 90% of all cases. Clinical presentation of pulmonary disease due to NTM may be similar to tuberculosis with the following clinical signs: low fever, night sweats, anaemia, weight loss, malaise, anorexia, diarrhoea and painful adenopath (Katoch 2004). Progressive pulmonary disease is primarily caused by *M. avium* complex (MAC) and *M. kansasii*. Other species which cause lung disease include *M. abscessus*, *M. fortuitum*, *M. xenopi*, *M. malmoense*, *M. szulgai*, *M. simiae*, and *M. asiaticum* (Buijtels et al., 2005).

Clinical features for NTM infections affecting other sites of the body are usually local organ specific signs and symptoms such as cellulites and lymphadenitis (Tortoli, 2009). Skin and soft tissue infections usually occur from percutaneous inoculation (e.g.; trauma surgery) and are caused primarily by *M. marinum* and *M. ulcerans*, *M. chelonae* and *M. fortuitum*. They are characterized by granulomatous lesions developing few weeks after innoculation (Tortoli, 2009).

Cervical lymphadenitis, especially in children younger than five years of age, is caused mostly by MAC and *M. scrofulaceum.* Infection is limited to cervical and mandibular

lymph nodes. Swelling of the lymph nodes is usually the first evidence of infection, although a draining sinus can result if the infection is untreated (Primm et al., 2004)

Disseminated infections of NTM are rare and most commonly caused by *M. chelonae*, *M. abscessus* and *M. fortuitum*. Disseminated NTM infections develop almost always in severely immunocompromised such as HIV-infected patients. The respiratory apparatus and the gastrointestinal tract are the two major routes of infection. The main symptoms and signs include high fever, diarrhoea, weight loss, abdominal pain, sweating, hiccups, aneamia, hepatomegalia and splenomegalia (E. Tortoli 2009).

2.6. Laboratory Diagnosis of NTM

2.6.1 Smear Microscopy

Direct Smear microscopy is the most commonly used method throughout the world for the detection of Acid Fast bacilli (AFB) in clinical specimens (Muwonge et al, 2014). The recommended microscopy method for staining clinical specimens for AFB is the fluorochrome technique, although the Ziehl-Neelsen (ZN) method is also acceptable but has a low sensitivity, with detection limit of between 5,000–10,000 bacilli/ml of sample (Steingart et al., 2006).

Most health centers in developing countries, Zambia inclusive, depend on smear microscopy for diagnosis of tuberculosis. However, this method does not distinguish MTC from NTM (Katale et al., 2014). Pulmonary TB shares clinical signs with NTM disease, hence causing clinical and diagnostic challenges (Asiimwe et al., 2013).

2.6.2 Culture Techniques

The isolation of Mycobacteria on culture provides the definitive diagnosis of disease due to NTM (Siddiqi, 2006). At present, mycobacterial culture can be performed on conventional egg based solid medium such as Lowenstein- Jensen (LJ) (BD Diagnostic) and Stonebrink media (BD Diagnostic) or agar based media such as Middlebrook 7H10 and 7H11 (BD Diagnostic) and liquid media such as Middlebrook 7H9 (BD Diagnostic) (WHO, 2012).

2.6.2.1 Solid Media

Solid media allows for detection of mixed cultures and contaminants and colonial morphology which helps in the identification of mycobacteria (Isenberg, 1998).Unfortunately, the growth of mycobacteria requires a protracted period of 4–8 weeks, hence, delaying appropriate treatment (Muwonge et al., 2014a).

2.6.2.2 Broth Media

Liquid culture systems such as BACTEC and Mycobacteria Growth Indicator Tube (MGIT) (BD Diagnostics), on the other hand offer a more sensitive and rapid alternative to conventional solid culture and yield better recovery and faster growth of mycobacteria (Nyendak et al., 2009) but have a draw-back of being expensive (Muwonge et al., 2014a). One of the most widely used broth systems is the non-radiometric MGIT, which contains a modified Middlebrook 7H9 broth in conjunction with a fluorescence quenching–based oxygen sensor to detect mycobacterial growth. As the mycobacteria grow and deplete the oxygen present, the indicator fluoresces when subjected to ultraviolet light (Griffith et al., 2007).

2.6.2.3 Incubation of NTM Cultures

The optimal temperature for most cultures for NTM is between 28°C and 37°C. Most clinically significant slowly growing mycobacteria grow well on primary isolation at 35°C to 37°C Cultures for Rapidly Growing Mycobacteria (RGM) and *M. marinum* should be incubated at 28°C to 30°C, but, optimal recovery of all species may require duplicate sets of media at two incubation temperatures. Most NTM grow within 2 to 3 weeks on subculture, however, in order to detect *M. Ulcerans* or *M. genavense,* cultures should be incubated for at least 8 to 12 weeks. Rapidly growing mycobacteria usually grow within 7 days of subculture (Griffith et al., 2007).

2.6.3 Identification of NTM

Identification of mycobacteria at species level is very important for patient management and this is usually done by phenotypic tests which are based on a panel of biochemical tests, pigmentation, and growth characteristics (Katoch et al., 2007). However, these methods are laborious and time consuming requiring 2-6 weeks for completion. Moreover the rapid increase of newly described species, the repetition of tests in cases of ambiguous results and some species being biochemically inert or extremely slow growing have made the use of these traditional methods more complex and outdated (Therese et al., 2009). Based on these inherent drawbacks of phenotypic methods, most mycobacteriology laboratories have opted for alternative methods for species identification of clinical strains. The development and application of various molecular biology techniques in the last two decades has led to rapid identification of mycobacteria, universality of results which allows comparisons of identification among different laboratories and improved accuracy in

identification (Patel et al., 2000; Therese et al., 2009). The rapid methods used for identification of NTM include, DNA probes (AccuProbe, Gen-Probe, San Diego, CA), High-Performance Liquid Chromatography (HPLC), Polymerase Chain Reaction (PCR)-restriction enzyme analysis (PRA) or sequencing of the 16S ribosomal DNA (rDNA) (Pauls et al., 2003).

2.6.3.1 DNA Probes

The development of commercial DNA probes (AccuProbe; GenProbe, San Diego, CA) for identification of mycobacteria has provided a rapid and specific approach. They are developed for the identification of the *M. tuberculosis* complex, the *M. avium* complex, *M. avium*, *M. intracellulare*, *M. gordonae*, and *M. kansasii* (Richter et al., 1999). The tests are based on species-specific DNA probes that hybridize with rRNA released from bacteria. The probes are labeled with acridinium ester, and results are measured with a luminometer (Soini & Musser 2001). However, DNA probe technology suffers from limitation of cost and requires several probes and cover only a limited range of mycobacterial species (Katoch 2004; Therese et al., 2009).

2.6.3.2 High Performance Liquid Chromatography (HPLC)

The analysis of lipid fractions has contributed significantly to the knowledge of *Mycobacterium* species. Complex high-molecular-weight β-hydroxyl fatty acids with a 22 or 24-carbon alkyl chain at the α-position are structural characteristics of mycolic acids (MAs), a type of fatty acid found in the *Mycobacterium* spp. cell wall. Several methods of fatty-acid analysis have indicated that MAs are species or group specific (Adam et al.,

1896). High Performance Liquid Chromatography (HPLC) analysis of MAs is a reliable method for the detection of mycobacteria species, because of the rapid, species specific and reproducible nature of the method. Also this method is relatively inexpensive and has been found to be a more rapid alternative laboratory technique than the use of commercial nucleic acid probes (Glickman et al 1994). Identification of mycobacteria by HPLC is therefore, performed by comparing fingerprint patterns obtained from each clinical sample with those from the reference strains (Adam et al., 1896). Though HPLC is used for identification in some research laboratories, it also has limitations in the identification of mycobacteria with similar profiles particularly the rapid growers, which, now include many new species (Therese et al., 2009).

2.6.3.3 PCR Restriction Enzyme Analysis (PRA)

PCR Restriction enzyme Analysis (PRA) is a rapid DNA method which is based on the amplification of a 441bp DNA fragment of the *hsp*65 gene encoding the 65-kDa heat shock protein, followed by digestion with two restriction enzymes, *BstE*II and *Hae*III (Telenti et al., 1993). The products of digestion are analyzed electrophoretically, e.g., by agarose or polyacrylamide slab gel(Hernandez et al., 1999). PCR Restriction enzyme Analysis is the most widely used method but has not been applied for many of the new taxa. Interpretation of results is also challenging for species with unique patterns or multiple patterns of sequences (Therese et al., 2009).

2.6.3.4 DNA Sequencing

Sequencing is a PCR-based technique which has become the gold standard for identification of *Mycobacterium* species. The method consists of PCR amplification of

Mycobacteria DNA with genus-specific primers and sequencing of the amplicons on an automated sequencer. Once the sequences have been determined, they are compared with a library of known sequences in databases available on the internet. The target most commonly used is the gene coding for the 16S rRNA. Two other targets that have also proven useful in identification are the 16S ribosomal, the internal transcribed spacer (ITS) region and the *hsp*65 gene (Telenti et al., 1993; Harmsen et al., 2003).

The 16S rRNA is an approximately 1500 nucleotides sequence encoded by the 16S ribosomal DNA (rDNA), which is a highly conserved gene with regions common to all organisms (conserved regions) and also areas where nucleotide variations occur (variable regions). For purposes of mycobacterial identification, sequence analysis focuses on two hypervariable sequences known as regions A and B. The sequence of region A is usually adequate to identify most NTM species, although sequencing of region B may be necessary, especially in the identification of undescribed species or those species which cannot be differentiated by sequence of the region A alone (Griffith et al., 2007). The 16S rRNA gene has been sequenced from a large number of *Mycobacterium* species, and the identification method based on this gene has been evaluated extensively in diagnostic laboratories (Soini and Musser, 2001). The use of molecular techniques that target the 16S rRNA gene has greatly contributed in the accurate identification and classification of mycobacteria (Katale et al., 2014). However, some members of clinically significant mycobacterial complex groups such as *M. kansasii* complex (*M. kansasii* and *M. gastri*), *M. fortuitum* complex (*M. fortuitum, M. chelonae*and *M. abscessus*), *M. avium* complex (*M. avium* and *M. interacellurae*), *M. farcinogenes* complex (*M. farcinogene s*and *M.*

senegalense) and *M. terrae* complex *(M terrae, M. nonchromogenicum,* and *M triviale)* are difficult to be differentiated from each other due to a very high degree of similarity in their 16S rDNA sequence (Shojaei et al., 2012). Therefore, the 16S -23S internal transcribed spacer region DNA sequencing was selected for use in this study.

The 16S- 23S rRNA internal transcribed spacer region has been considered a suitable target for differentiating species and has been used to distinguish clinically relevant *Mycobacterial* species (Katoch et al., 2007). The ITS between the 16S and 23S rRNA gene is approximately 270 to 360 bp but can vary in size from species to species (Heekyung park et al, 2000). The application of the 16S-23S internal transcribed spacer gene sequence has had a great impact on identification and classification of Mycobacteria. The ITS region has a greater discriminatory power than the 16S rDNA (Shojaei et al., 2012).The 16S-23S rRNA internal transcribed spacers contain enough sequence diversity to distinguish all clinically important Mycobacteria except for the members of the *M. tuberculosis* complex. These target genes also allow for differentiation of *M. kansasii* and *M. gastri*. Further, because of the intraspecies variation observed in the 65-kDa protein gene, this target can also be used for distinguishing clones of certain mycobacterial species (Soini & Musser 2001).

2.7 Treatment of NTM

Treatment of NTM varies according to the NTM species involved, its susceptibility profile, the disease site and distinction between slow versus rapid growing NTM. Treatment of these infections usually involves a combination of drugs with in-vitro activity against a particular NTM isolate (Ahmed et al., 2013). For most slow growing strains, the regimen

comprises of rifampicin and ethambutol and a macrolide administered for 18-24 months. In cases of severe disease, amikacin or streptomycin should be added in the initial 3-6 months. For rapid growing species, treatment regimens are based on in-vitro Drug susceptibility testing (DST) results (Brown-Elliott et al., 2012; Mirsaeidi et al., 2014)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Design

This was a prospective cross-sectional study where sputum samples from human adult (≥ 15 years old) presumptive TB patients and water samples were collected and analyzed. Sputum samples were collected from adult patients seeking medical attention at four local health facilities in Namwala. Water samples were collected from common water drinking points such as communal taps, open wells, streams and rivers. Focal persons in these areas helped in accessing these water collection points.

3.2 Study Site

The study was conducted in Zambia in the rural district of Namwala. The district is situated in the Southern Province of Zambia and is one of the districts with a large number of

Pastoral farmers. Four sampling areas were selected and these are; Namwala central, Maala, Kabulamwanda and Chitongo as shown in figure 3.1. Namwala is one of the districts in Zambia with the highest TB prevalence in both animals and humans (Malama et al., 2014c; Munyeme et al., 2009). It covers an estimated total area of about 10,000 square kilometers and lies between latitudes 15 and 17°S of the equator and longitude 25 and 27°E. The greater area of its traditional land is covered by the Kafue Flood plains of the Kafue River. This offer nutritive varieties of rich lush green grass for both cattle and wildlife for a greater part of the year than the surrounding Savannah woodlands (Munyeme et al., 2010). Namwala district supports approximately 30 000 herds of cattle and approximately 44,000 Kafue lechwe (Malama et al., 2014c) and agriculture is the main economic activity. Humans and animals commonly share the same microenvironments such as water drinking points, especially in dry seasons (Phiri, 2006), increasing the possibility of interspecies disease transmission or contamination of water sources, which could potentially lead to a zoonotic transmission to humans (Katale et al, 2014).

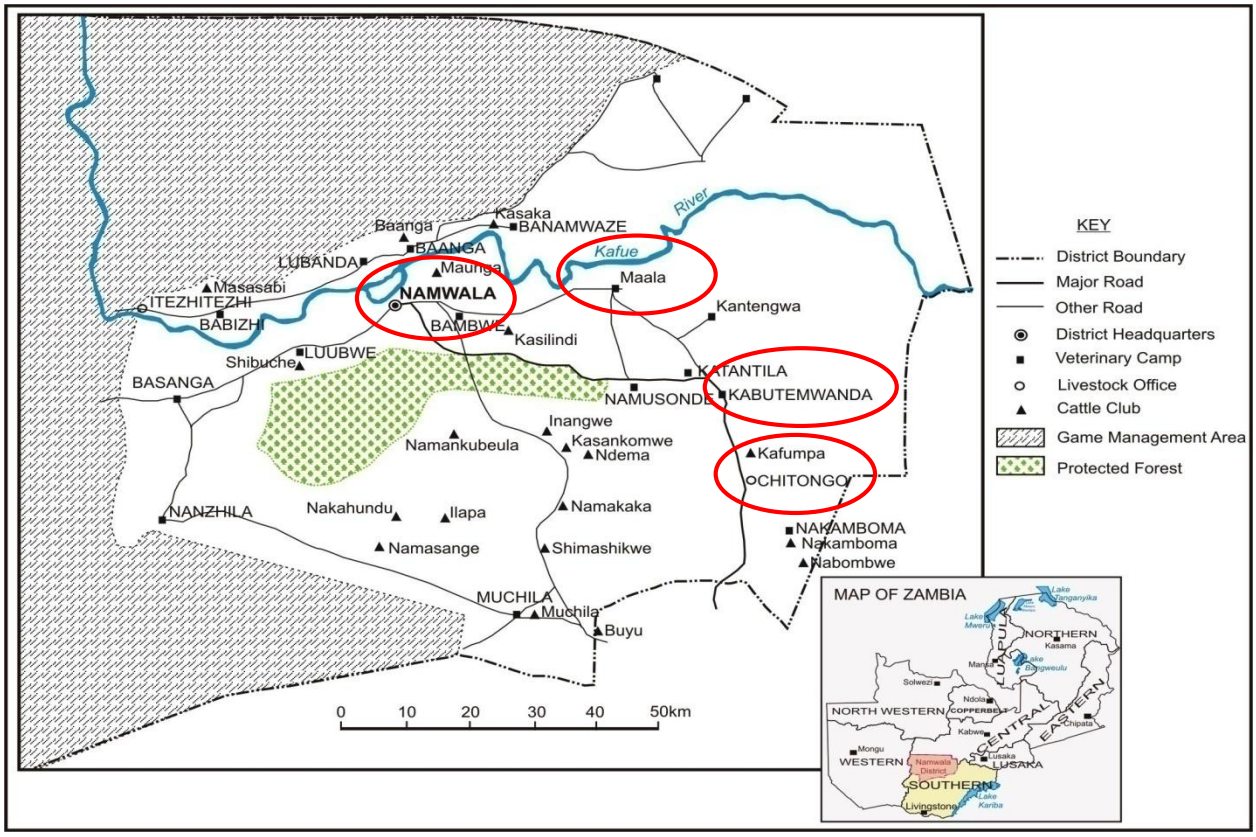

Figure 3.1: Map of Namwala district showing the four study sites (Map by Musso Munyeme)

3.3 Study Period

The study was carried out between October 2015 and March 2016.

3.4 Study Population

The study targeted adult human presumptive TB patients seeking medical care at health facilities in Namwala district. Four (4) health facilities were selected for sampling of human sputum samples. These comprised of Namwala District Hospital, Maala Rural Health Centre, Kabulamwanda Rural Health Centre and Chitongo Rural Health Centre. The patients presented with TB symptoms such as chronic (Defined as ≥ two weeks) productive cough, loss of appetite, fever, fatigue, headache and night sweats. Since the selected health centers in Namwala are able to deliver TB treatment, all the patients

diagnosed with TB in the study were treated at these health facilities according to the national TB guidelines in line with the WHO treatment guidelines.

3.4.1 Inclusion Criteria

Male and female adult (>15 years old) TB suspected patients seeking medical attention at the selected health facilities were included in this study.

3.4.2 Exclusion Criteria

Patients with other illnesses were not included in the study.

3.5 Sampling Frame

Patients who presented with TB symptoms to the health facilities in the four study sites were sampled. The health facilities comprised of Namwala District Hospital, Maala Rural Health Centre, Kabulamwanda Rural Health Centre and Chitongo Rural Health Centre. Additionally, water was sampled from various common water drinking points such as borehole, Taps, wells, streams and rivers in the same four study areas where the patients resided.

3.6 Sample Size Determination

The sample size required for this study was calculated using the sample size formula given below.

$$n = \frac{Z_{\alpha}^{2} pq}{L^{2}}$$

where:

n= sample size required

Z_{α}= 1.96 is the value of the standard normal distribution corresponding to a significant level of α (alpha) for a 2-sided test at the 0.05 level.

p= a priori estimate of the proportion of samples containing NTM

q= 1-p

L= The precision of the estimate (allowable error), equal half of the confidence interval.

3.6.1 Human Samples (Sputum samples)

Fifty percent (50%) was used as an estimate of the proportion of sputum samples containing NTM since the prevalence of NTM is not known for Namwala District and Zambia. The target sample size was 384. However 306 sputum samples were collected. The target sample size was not met because of the sampling period was short.

$$n = \frac{1.96^2 \times (0.5 \times (1 - 0.5))}{(0.05)^2}$$

n = 384 sputum samples

3.6.2 Water Samples

A prevalence of NTM of 4.5% for water samples was used based on a study done by Kankya et al (2011). The calculated sample size was 66 water samples from all the four

study sites. However, 149 water samples were collected from the four study sites. More water samples were collected in order to increase the power and validity of the study.

$$n = \frac{1.96^2 \times (0.045 \times (1 - 0.045))}{(0.05)^2}$$

n = 66 water samples

3.7 Sampling Procedure

Patients who presented to the health facilities with TB symptoms were purposively selected. A semi structured questionnaire was administered to study participants who consented by signing a participant consent form (Appendix 2). This was followed by submission of two sputum samples. The questionnaire was administered in the language of their choice (Appendix 3). Water samples were also conveniently sampled from various common water drinking points such as borehole, Taps, wells, streams and rivers. A biodata sheet was used to capture information such as water source, quality of water and sampling area (Appendix 4).

3.8 Isolation of NTM from humans and water

3.8.1 Sample Collection, Transportation and Storage

3.8.1.1 Sputum samples

Sputum samples were collected from patients with respiratory symptoms. Sputum samples were collected in sterile containers with a tight-fitted lid or cap. Two samples were collected from each patient. A volume of about 2-10 ml each was submitted. After routine microscopy with the Ziehl–Neelsen (ZN) staining technique at the study health facilities, the samples were stored in cetylpyridinium chloride (CPC) medium (Sigma-Aldrich, Steinheim, Germany) and kept at ambient temperature until they were taken to Ndola at Tropical Diseases Research Centre (TDRC), TB reference laboratory for further processing.

3.8.1.2 Water Samples

Water samples were collected at various common drinking points such as wells, boreholes, tap and streams in the community of the study sites. One hundred and fifty milliliters (150ml) of water was collected in sterile whirlpak plastic bags. For borehole and tap water sources, the water was allowed to run through for a while and then collected. Immediately after collection, all samples were placed in a cool box containing ice packs for transportation to the laboratory where they were stored in CPC media until processing at TDRC, Ndola.

Figure 3.2: One of the water collection points in Namwala (Photo by Ngula Monde)

3.8.2 Isolation of NTM

3.8.2.1 Sputum Sample Processing

Samples were placed in the Biological Safety Cabinet Class 2 were processing was done (Figure 3.3). The samples were decontaminated using the Petroff method (Petroff 1915). The purpose of sample decontamination is to liquefy the sample, remove contaminants and concentrate the AFB if present in the sample. In this procedure 4% Sodium Hydroxide (NaOH) was used. The NaOH solution was mixed with an equal quantity of sodium citrate solution (2.9%) to make a working solution, in which N-Acetyl L-Cysteine (NALC) was added. An equal quantity of NaOH-NALC-citrate and sputum was mixed into a 50ml centrifuge tube under a Biological Safety Cabinet (BSC) Class 2. The mixture was then

vortexed lightly for about 15-30seconds and then allowed to stand for15 minutes at room temperature. Thereafter, phosphate buffer (pH 6.8) (a mixture of 9.47g Na_2HPO_4 in 1litre distilled water and 9.07g KH_2PO_4 in 1litre distilled water) was added up to the 45ml mark of the centrifuge tube and centrifuged at a speed of 3000xg for 15minutes. After centrifugation, the tubes were allowed to stand for 5minutes to allow aerosols to settle. The supernatant was then carefully decanted into a suitable container containing a mycobactericidal disinfectant. Thereafter, 2ml of phosphate buffer (pH 6.8) was added to the pellet and then resuspended with the help of a pipette or vortex mixer. Each sample was cultured on both glycerated and pyruvated Löwenstein-Jensen (LJ) media and incubated at 37°C. The cultures were examined for growth weekly for 8 weeks, after which results were obtained and recorded. Microscopic examination of positive cultures by ZN Method to done to detect AFB.

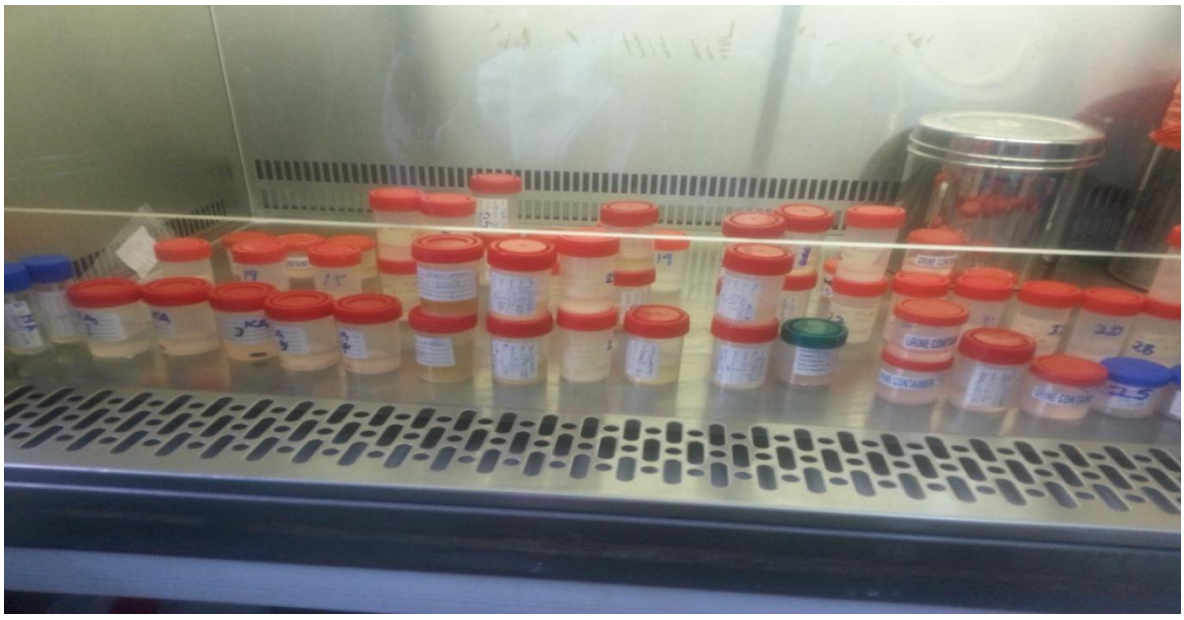

Figure 3.3: Sputum samples for processing under a Biological Safety Cabinet (Photo by Ngula Monde)

3.8.2.2 Water Sample Processing

Hundred and Fifty (150ml) of water was filtered through 0.45µl nitrocellulose membrane filters (Millipore Corporation, Bedford, MS, USA) by vacuum filtration using a Manifold Filtration System (Sartorius AG, Goettingen, Germany). The membranes were transferred into sterile screw-capped specimen containers with 10ml of 0.005% cetylpyridiniumchloride (CPC) (Sigma-Aldrich, Steinheim, Germany) to reduce background organism levels. The surface of the membranes was abraded vigorously and thoroughly with a sterile plastic inoculating loop. The samples were then exposed to the decontaminant at room temperature for 30min to remove unwanted bacteria. The mixture was then centrifuged at 3000xg for 20minutes. The pellet was washed twice with 1ml sterile normal saline and finally re-suspended in 1ml sterile distilled water. The suspension (0.5ml) was inoculated on LJ slants and incubated at 37°C.

The cultures were examined for growth for up to 8 weeks, after which definitive results were obtained and recorded. Microscopic examination of positive cultures was done using Ziehl Neelsen staining method to detect the presence of AFB.

3.8.2.3 Preliminary Identification of NTM

Mycobacterial growth from sputum samples was preliminary identified as either MTBC or NTM using Capilia TB-Neo (TAUNS Laboratories, Inc. Japan), an immunochromatographic method which can detect MPT64, a protein specifically secreted by *M. tuberculosis* complex and not produced by NTM, was performed according to the manufacturer's instructions.

3.8.3 Molecular Characterization of NTM

3.8.3.1 DNA Extraction and PCR

To obtain genomic DNA for sequencing, the Trisol method for DNA extraction was used. Firstly the tubes were arranged and labelled with the sample identification number. Beads were then added to the tubes and 500µl of Trisol reagent added. One Hundred microlitres (100µl) of the mycobacteria isolates were inoculated into the trizol reagent. The mixture was then vortexed using a mini Roto Vortex machine (Fisher Scientific). The tubes were then placed in a micro smushi100R machine for 1minute at 3500rpm. This was followed by centrifugation in a High speed refrigerated micro centrifuge Mx 2007 (Eco-Tomy) at 10,000rpm for 10 minutes to remove the debris. The supernatant was removed from the tubes to remain with the pellet at the base of the tubes. Absolute ethanol (500µl) was added to the tubes and centrifuged at 10,000rpm for 15 minutes. The supernatant was again removed and 250µl of 70% ethanol added to the tubes to wash the DNA. The tubes were again centrifuged at 10,000rpm for 15 minutes, supernatant removed and thereafter the tubes were inverted to dry. After the tubes had dried, 20µl of TE buffer was added to re-suspend the DNA pellet and 1% (2µl) of the DNA was used for the PCR amplification.

In the PCR reaction, the 16S – 23S rRNA Internal Transcribed Spacer region was amplified with primers Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) and Sp2 (5′-GAT GCT CGC AAC CAC TAT CCA-3′) in a 20µl reaction volume (10x buffer 2µl, dNTP 1.6µl, forward or reverse Primers 0.8µl, distilled water 13.7µl, Taq polymerase enzyme 0.1µl and DNA template 2µl). The thermocycles included initial activation of the enzyme (95°C for 1 minute), 35 cycles of denaturation (95°C for 30 seconds), annealing (55°C for 30

seconds), extension (72°C for 1 minute), and the last extension at 72°C for 5 minutes. To determine whether the PCR products were present in the samples, Electrophoresis (Mupid-exu submarine electrophoresis system- advance) was performed by running 5µL of the PCR products on a 1.0% agarose gel that contained ethidium bromide (0.5µg/ml) for 25 minutes. Thereafter, the PCR products were visualized by a UV transilluminator (IPV benchtop 3UV Transiluminator).

3.8.3.2 DNA Sequencing of the 16S-23S ITS Region

3.8.3.2.1 DNA Purification for DNA target

Prior to sequencing, PCR products were purified using the Wizard SV Gel and PCR Clean Up System Kit (Promega, USA) according to manufacturer's instructions.

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3.8.3.2.2 Sequencing Process

The gene target used in this sequencing reaction was the 16S- 23S rRNA ITS region. The sequencing reaction was performed using the following primers: primers Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) and Sp2 (5′-GAT GCT CGC AAC CAC TAT CCA-3′). The 20µl sequencing master mix comprised of; Bigdye 1.0µl, 5x buffer 3.5µl, primers 1.0µl, distilled water 13.0µl and DNA 1.5µl. This reaction was performed for both the forward and reverse reactions. The reaction was then placed in the thermal cycler with the following conditions: initial denaturation at 96ºC for 1 minutes, followed by 25 cycles of denaturation at 96 ºC for 10 seconds, annealing at 50 ºC for 5seconds and extension at 60 ºC for 4 minutes. The reaction was run for 2 hours.

After sequencing reaction, excess dye terminators and primers were removed from the cycle sequencing reaction using the EDTA/NaOac/ Ethanol precipitation method. To each well 2µl of 125mM of EDTA and 2µl of 3M Sodium Acetate were added. The mixture was then mixed by tapping. Thereafter, 90µl of 100% ethanol was added and incubated at room temperature for 10 minutes. The tubes were then centrifuged at 1500rpm for 20 minutes at 4°C and supernatant removed. Two thousand microlitres (2000µl) of 70% Ethanol then added to the tubes and the mixture was centrifuged at 15000rpm for 5 minutes and supernatant removed. This step was repeated and afterwards supernatant removed.

The PCR tubes were then wrapped in alluminium foil and dried in a Vacuum drier (Micro Vac$_{TM}$, TOMY) for 10 minutes. After drying, the tubes were removed from the vacuum drier and placed in the Thermocycler (Applied Biosystem) for 2 minutes to denature the DNA. The tubes were then removed from the thermocycler and placed on ice. Two hundred microlitres (200µl) of the sample was then loaded into the sequencer Machine (Applied Biosystem, AB 3130) and allowed to run for 8 hours.

3.8.3.2.3 Sequencing Data Analysis

The sequence data was assembled and edited using the ATGC software. The software was used to assemble each forward and reverse sequence into a consensus sequence, which was then edited to resolve base pair ambiguities between the two strands by evaluation of the electropherograms. Each consensus sequence was compared to available sequences in GenBank by the NCBI Blast sequence alignment tool (National Centre for Biotechnology

Information, http://blast.ncbi.nlm. nih.gov/). The isolate was determined to species level based on the maximum score and maximum identity values on NCBI Blast alignment, a maximum score and maximum identity of ≥ 98% were accepted.

3.9 Data Analysis

The Data from the questionnaire, biodata sheet and laboratory analysis were entered in Excel Spread sheet and subsequently imported to STATA version 12(STATA Corp, College Station, Texas) for final descriptive and statistical analyses. Proportions of NTM from humans and water were computed. Associations between categorical variables and the outcome variable were assessed using Pearson chi-square test. The multiple logistic regression model was used to examine association of various predictors with the outcome. A p value less or equal to 0.05 at 95% CI was considered statistically significant.

3.10 Ethical consideration

Patient's confidentiality was maintained by using study numbers instead of patient's name. However, when the sample was positive for mycobacteria, the number was traced back to the patient's name so as to refer the patient to the clinician for treatment.The study questionnaire and sputum samples were assigned and identified by study numbers for confidentiality. The information sheet was read to the study participants by a community health worker who explained the details of the study in English and or in Tonga before obtaining informed consent.The protocol was therefore implemented in such a way as to have minimum interference with routine work at the health facilities. Ethical clearance to conduct the study was sought and granted from ERES (Excellence in Research Ethics and Science) converge IRB (Ref.No. 2015-Aug-002) (Appendix 6). Approval to conduct the study was obtained from the Directorate of Research and Graduate studies (DRGS) through

the Assistant Dean, Postgraduate (Appendix 5) and permission to conduct the study in Namwala District was granted by the National Health Research Authority, Ministry of Health (Appendix 7).

CHAPTER FOUR

4.0 RESULTS

4.1 Descriptive Results for human sampling

4.1.1 Summary of Study Areas

A questionnaire survey was conducted and 153 questionnaires were administered in the four study areas in Namwala district and these were broken down as follows; Namwala Central (*n*=62), representing 40.5% of the respondents at 95% CI (32.7% - 48.4%), Kabulamwanda (*n*=41) representing 26.8% of the respondents at 95% CI (19.7% - 33.9%), Chitongo (*n*=12), representing 7.8% of the respondents at 95% CI (3.5%-12.2%), Maala (*n*=38), representing 24.8% of the respondents at 95% CI (17.9% - 31.7%) (Table 4.1)

Table 4.1: Descriptive summary of study areas and proportional sampling

Study areas	*N*	Proportion	SE	95% Confidence Interval (CI)		
Namwala Central	62	40.5%	0.039	32.70%	:	48.40%
Kabulamwanda	41	26.8%	0.036	19.70%	:	33.90%
Chitongo	12	7.8%	0.022	3.50%	:	12.20%
Maala	38	24.8%	0.035	17.90%	:	31.70%

N= Number of study participants, SE= Standard Error

4.1.2 Sex

Out of the 153 participants included in this study, 40.7% (95% CI: 39.10-55.10) were males and 52.9% (95% CI: 44.9-60.9) were females, with 100% data for gender being available. All study participant submitted two sputum samples and a total of 306 samples were collected (Table 4.2).

Table 4.2: Proportion of sex of study participants

Sex	*N*	Proportion	SE	95% Confidence Interval		
Male	72	40.7%	0.04	39.10%	:	55.10%
Female	81	52.9%	0.04	44.90%	:	60.90%

4.1.3 Age

The median age and interquartile range (IQR) was 36 (28 to 41) years. Age was categorized into four groups with the following populations in each group: 0-20 years: 6 participants 3.9% (95%CI: 0.81-7.03), 21-40 years: 92 participants 60.1% (95% CI: 52.28 - 67.98), 41-60 years: 50 participants 32.7% (95%CI: 25.6 – 40.20) and 61-100 years: 5 participants 3.3% (95% CI: 20.42 – 6.12) (Table 4.3).

Table 4.3: proportion of age by categories

Age Group	*N*	proportion	SE	95% Confidence Interval		
0-20	6	3.9%	0.016	0.81%	:	7.03%
21-40	92	60.1%	0.04	52.28%	:	67.98%
41-60	50	32.7%	0.038	25.16%	:	40.20%
61-100	5	3.3%	0.014	0.42%	:	6.12%

4.2 Isolation of NTM from Humans

Based on morphological characteristics and acid fastness by the ZN method, 13.1% (20) mycobacteria were detected among 153 study participants. Of these 4.6% were identified

as NTM, while 8.5% were identified as MTC. The overall prevalence of NTM in humans was 4.6% and it ranged from 1.2% to 7.9%. The area prevalence of NTM in humans according to study site was as follows: Maala 7.9% (95% CI 0% - 16.7%), Kabulamwanda 4.9% (95% CI 0% - 11.6%), Namwala central 3.3% (95% CI 0% - 7.7%) (Table 4.4).

Table 4.4: Prevalence of NTM in humans by Study Site

Site	**N**	**No. of NTM isolated**	**Prevalence**	**95% CI**
Namwala Central	62	2	3.3	0 - 7.7
Kabulamwanda	41	2	4.9	0 - 11.6
Chitongo	12	0	0	-
Maala	38	3	7.9	0 - 16.7
Total	**153**	**7**		

The proportion of NTM positives was also higher in females 7.4% (95% CI 1.6 – 13.2) than in males 1.4% (95% CI 0 – 4.1). The proportion of NTM positives were also seen to be high in the age group of 21-40 years 5.4% (95% CI 0.7 – 10.1) (Table 4.5).

Table 4.5: Proportion of NTM positives by sex and Age

Variable	**Level**	***n***	**% proportion**	**95% CI**	**P-value**
Sex	Male	1	1.39	0 - 4.1	0.079
	Female	6	7.41	1.6 - 13.2	

Age Category	0 - 20 years	_	_	_	
	21 -40 years	5	5.4	0.7 - 10.1	1.000
	41 - 60 years	2	4.0	0 - 9.5	
	61 - 100 years	_	_	_	

4.3 Isolation of NTM in Water

Thirty two (32) NTM isolates were obtained on culture from 149 water sample. The majority being isolated from Maala, followed by Chitongo with the lowest being in Namwala central (Figure 4.1). NTM were also more isolated from borehole water sources followed by river, dam and stream water sources and lowest in tap water source (figure 4.2). Both scenarios were confirmed using a Mean Smoother scatter plot incorporating a trend line (Figures 4.1 and 4.2).

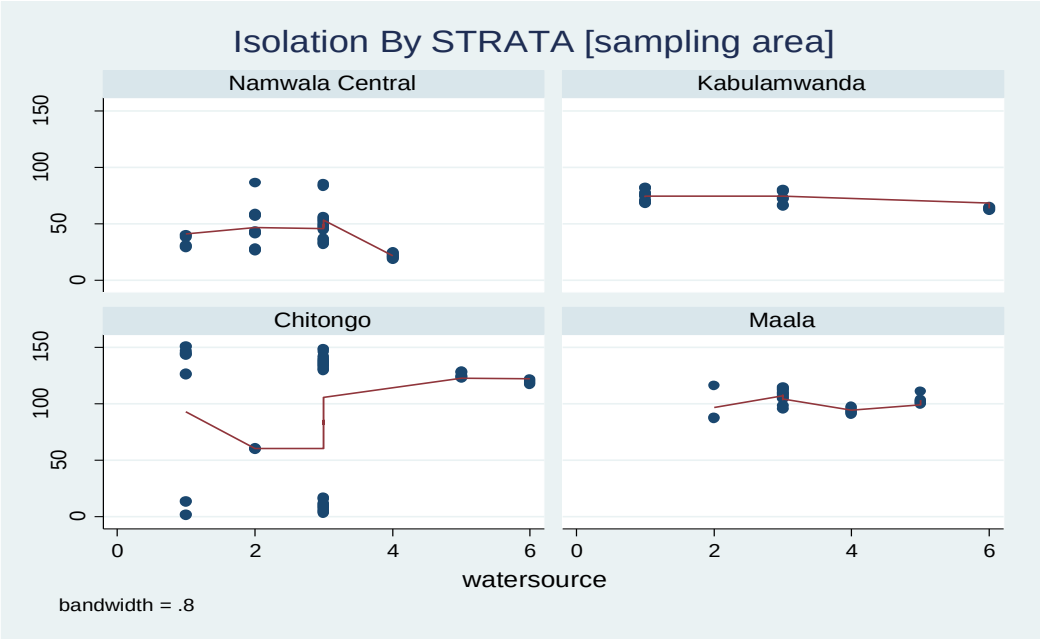

Figure 4.1**:** Isolation of NTM according to study site using a mean Smoother scatter Plot

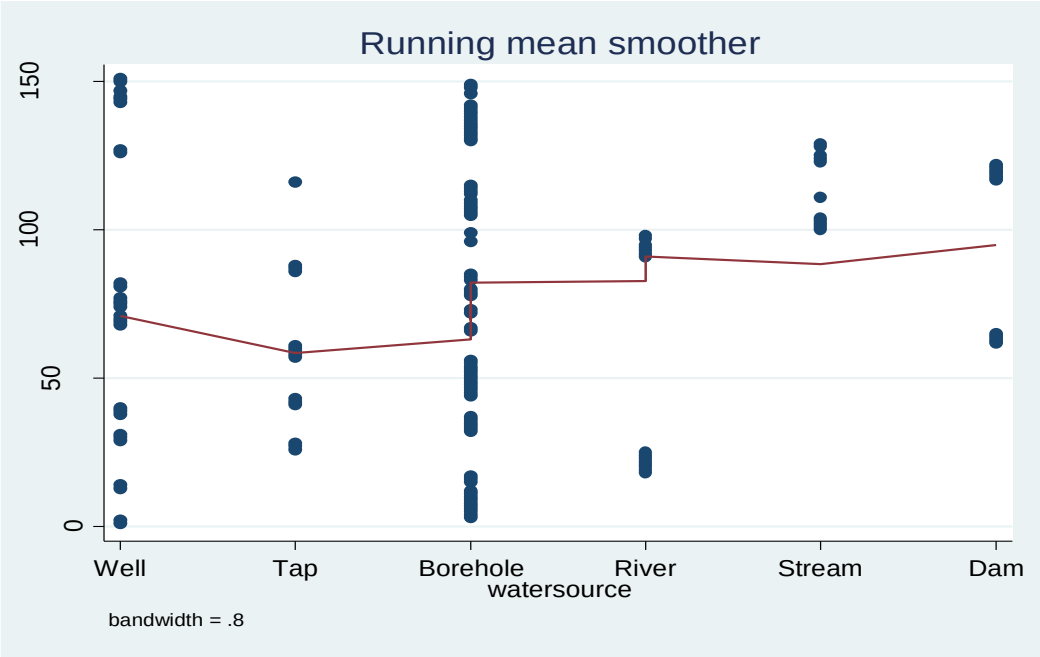

Figure 4.2: Isolation of NTM according to water sources using a mean Smoother scatter Plot

4.4 Molecular Characterization of NTM from Humans and Water

From human samples only 4.6% (7/153) were positive for NTM with the most prevalent species being *Mycobacterium arupense* 2% (3/153). Of the 32 NTM isolates obtained on culture from water samples, 23 were characterized to species level, two were identified as *Novosphingobirium pentamativorans* and *Cynoglossus semilaevis piwi*. The remaining 7 were uncharacterized. The most prevalent NTM species isolated from water samples were *Mycobacterium gordonae* 3.4% (5/149), *Mycobacterium senegalense* 2.0% (3/149), *Mycobacterium peregrinum* 2% (3/149) and *Mycobacterium fortuitum* 2% (3/149). *Mycobacterium avium* and *Mycobacterium fortuitum* were both isolated from humans and

water. Maala had the highest number of NTM species identified while Chitongo had the least number of NTM species (Figure 4.3).

Table 4.6: NTM species isolated from humans and water

NTM species	*n*	Frequency (%)	95% CI
Humans (N=153, *n*=7)			
M. arupense	3	2.0	0 – 4.2
M. abscesus	1	0.7	0- 1.9
M. avium	1	0.7	0 – 1.9
M. fortuitum	1	0.7	0 - 1.9
M. nebraskense	1	0.7	0 – 1.9
Water (N=149, *n*=23)			
M. gordonae	5	3.4	0.4 – 6.2
M.senegalense	3	2.1	0 – 4.3
M, fortuitum	3	2.1	0 – 4.3
M. peregrinum	3	2.1	0 – 4.3
M. kumamotonense	1	0.7	0 – 2.0
M. simiae	1	0.7	0 – 2.0
M. avium	1	0.7	0 – 2.0
M. gilvum	2	1.3	0 – 3.2
M. flavescens	1	0.7	0 – 2.0
M. housetonense	1	0.7	0 – 2.0
M. smegmatis	1	0.7	0 – 2.0
M. parafinicum	1	0.7	0 – 2.0

Namwala central

Mk, Mpa, Mfl, Mh, Mg, Mku, Ms

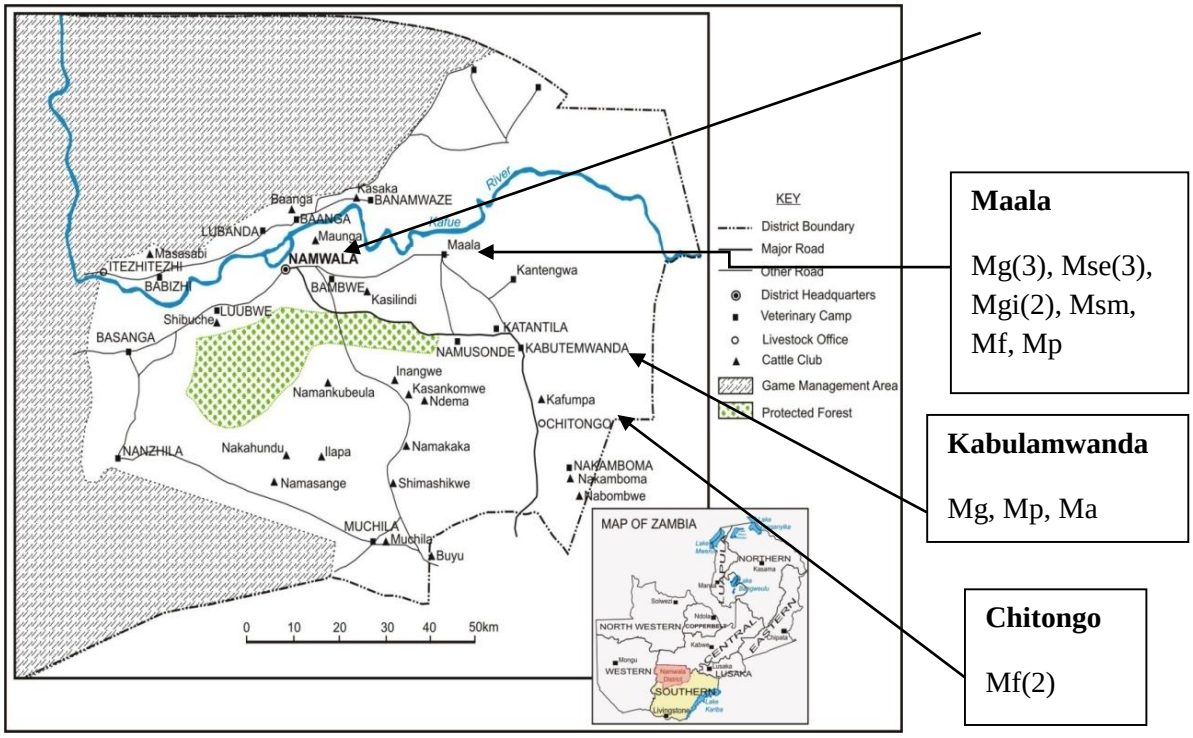

Figure 4.3: Map of Namwala district showing the characterized NTM species in water in the four study sites. Namely, Mk *(M. kumamotonense*), Mpa *(M. parafinicum)*, Mfl *(M. flavescens)*, Mh *(M. housetonense)*, Mg *(M. gordonae)*, Mgi *(M. gilvum*), Ms (*M. simiae*), Mp (*M. peregrinum)*, Mse *(M. senegalense)*, Msm *(M. smegmatis*), Mf *(M. fortuitum*)

4.5 Factors associated with NTM infections

4.5.1 Univariables associated with NTM infections

A number of factors were investigated for association with NTM infections in this study. The following factors were found to be associated with NTM infections; Marital status with the divorced category showing an association (p- 0.002), practices for safe drinking

water with use of chlorine (p- 0.046) and letting water to settle (p- 0.038) being associated with NTM infections and lastly culture results (p- 0.0001) Table 4.7.

Table 4.7: Univariate analysis of Risk factors

Variable	Level	Coeficient	T test	p-value	95% CI
Marital status	Married	_	_	_	_
	Single	-0.014	-0.37	0.713	0 - 6.3
	Widow	_	_	_	_
	Separated	_	_	_	_
	Divorced	0.43	3.14	**0.002**	0.56 - 8.1
Practice to make water safe to drink	Use of chlorine	-0.22	-2.02	**0.046**	0
	Use of water filters	-0.25	-1.07	0.287	0 - 21.1
	Let water to settle	-0.25	-2.09	**0.038**	0
	Not applicable	-0.2	-1.87	0.063	0 -1.2
Culture results	Positives	0.39	9.21	**0.0001**	30.5 - 47.2

4.5.2 Multivariate analysis

Variables considered to be significant on univariate analysis were included in a multiple regression model Table 4.8. The following variables were identified by the model to be associated with NTM infections. Marital status, with the divorced category showing an

increase of 0.63 in NTM infections (coef 1.68, 95% CI 34.3-100.7; p- 0.00.01) than the other categories within marital status, practices for safe drinking water with NTM infections being reduced by 0.18 (Coef -0.18, p-0.033) and by 0.16 (Coef -0.16, p-0.0001) with use of chlorine/bleach in drinking water and use of water filters for drinking water respectively and finally culture results, showing an increase of 0.34 in NTM infections in individuals with positive culture results (Coef 0.34, 95% CI 26.0 – 42.7; p-0.0001).

Table 4.8: Multiple regression Model

Variable	Level	Coeficient	T test	p-value	95% CI
Marital status	Single	-0.006	-0.2	0.839	0 - 5.6
	Divorced	0.68	4.02	**0.0001**	34 – 100
Practice to make water safe to drink	use of chlorine	-0.18	-2.16	**0.033**	0
	use of water filters	-0.84	-3.39	**0.0001**	0
	Let water to settle	-0.16	-1.77	0.079	0 - 1.8
Culture results	Positives	0.34	8.15	**0.0001**	26.0 - 42.7

CHAPTER FIVE

5.0 DISCUSSION

Non-tuberculous Mycobacteria have gained clinical and public health significance in the last few decades in both immuno-compromised and immuno-competent individuals (Ahmed et al., 2013b). Water is a documented source of NTM infection in humans, but it is not the only source (Thomson et al., 2013). Currently, very little data exists on the NTM species distribution in humans and water in Africa, Zambia inclusive. It was against this background that this present study was formulated. To the best of our knowledge, this is the first study in Zambia to isolate and characterize NTM from humans and water.

In the present study, a range of NTMs from humans and water in Namwala district, an agro-pastoral district located in Southern Province of Zambia were isolated. The overall prevalence of NTM in humans in Namwala district was found to be 4.6%. This is comparable to a study done in Shanghai China, were the overall prevalence of NTM in humans was 5.9% (Wu et al., 2014). Another study done in Nigeria showed a prevalence of 15% (Aliyu et al., 2013). Although, the prevalence of NTM may seem to be low in our study, the presence of NTM species is associated with a high likelihood of disease and thereafter mortality (Katoch, 2004; Chimara et al., 2008; Kankya et al., 2011). The prevalence of NTM was about three times higher in females than in males and was high in the younger age group of 21 to 40 years. However, these results were not statistically significant ($p>0.05$). The high prevalence of NTM in females could partly be attributed to female's high medical care seeking behavior which leads to high chances of them being diagnosed with disease (WHO, 2007). These findings were in agreement with a study done

by Cassidy et al (2007), Oregon, USA, in which a significantly higher prevalence of NTM was reported in females than males. The reasons for the observed high prevalence of NTM in the age group of 20 to 40 years in the present study, is not clearly known but it could be due to the fact that this is an active age group both physically and sexually, hence prone to diseases such as HIV which compromises their immune system rendering them susceptible to infections such as NTM infections. Recent studies conducted in Zambia have actually reported a high prevalence of NTM infections in HIV/AIDS patients (Buijtels et al., 2009; Chanda-kapata et al., 2015).

In the current study, out of 149 water samples collected from various study sites in Namwala district, 32 (21.5%) samples were positive for NTM on culture, indicating that NTM thus far are relatively common in water sources of Namwala district. Although a recent study carried out in Sri Lanka showed a high frequency of isolation of mycobacteria from aquarium water and surface water (rivers, streams) with the most common NTM to be *Mycobacterium fortuitum* (Edirisinghe 2014), the present study, found a high isolation rate of NTM in borehole water sources while Tap water had the lowest. This high isolation rate of NTM in borehole could be due to high levels of organic matter and soil in borehole waters contributing to the mycobacterial flora. The piping systems used in borehole may also support the formation of biofilms which favour the growth and multiplication of NTM (Thomson et al., 2013). Some pipe materials have been shown to contribute to biofilm formation particularly iron pipes (Thomson et al., 2013) and the capacity of mycobacteria to form biofilms has been demonstrated elsewhere (Falkinham 2009; Covert et al., 1999). A study done by Schulze-Robbecke et al (1992), German, found that 90% of biofilm

samples from pipes of various water distribution systems contained mycobacteria, signifying that mycobacteria biofilms are present in water piping systems .The low mycobacterial load in tap water sources observed in this study could probably be related to the lethal effect of chlorine to mycobacteria, since tap water is usually chlorinated in Namwala district by the local water supply company.

The isolation rate of NTM in both water and human samples was also highest in Maala as compared to the other study sites. This can be attributed to the fact that Maala is surrounded with open grasslands with a lot of Dambos, rivers and pypyrus flora, with reeds that enhance the formation of microfilms and microfilms are known to contain (Falkinham 2009).

Upon characterization of NTM isolated from humans and water, a wide range of pathogenic and potentially pathogenic NTM species were obtained.

A total of seven NTM species from human sputum samples were identified, with the most prevalent organism being *Mycobacterium arupense*. This is the first study in Zambia to report the isolation of *Mycobacterium arupense* in human sputum samples. Cloud et al, (2002), USA, identified *Mycobacterium arupense* for the first time in human clinical samples. Other Studies done by Masaki et al, (2006), Japan, Tsai et al, (2008), Taiwan and Neonakis et al, (2010), Greece, all reported the isolation of *Mycobacterium arupense* in human sputum samples and in all these four studies *Mycobacterium arupense* was reported to be of clinical significance. *Mycobacterium arupense* is a novel *Mycobacterium* species potentially pathogenic and has been associated with causing human pulmonary infections

(Slany et al., 2010). Therefore, *Mycobacterium arupense* isolated from humans in the current study is of clinical significance as the patients from whom it was isolated from presented with symptoms of pulmonary infection.

Other NTM species that were isolated from humans were *Mycobacterium abscesus*, *Mycobacterium avium*, *Mycobacterium fortuitum* and *Mycobacterium nebraskense*. All these species have been found to cause disease in immunocompromised and immunocompetent individuals. *Mycobacterium abscessus* has been reported to be the leading cause of lower respiratory tract infections among the rapidly growing NTM species (Jarzembowski, 2008; Wang et al., 2010). *Mycobacterium avium* has also been known to cause opportunistic infections in humans and animals (Falkinham 1996) while *Mycobacterium nebraskense* is an NTM pathogen that has been associated with cause of nodular pulmonary disease (Rahbar et al., 2010).

Drinking water has been suggested as a reservoir for many pathogenic and potentially pathogenic NTM such as *M. avium complex, M. gordonae, M. kansasii, M. fortuitum, M. simiae, M. senegalense, M. chelonae* and *M. xenopi* (Falkinham et al., 2001; Primm et al., 2004; Thomson et al., 2013). In this study, a number of pathogenic and potentially pathogenic NTM species were isolated from water samples of Namwala district. The most prevalent NTM species was *Mycobacterium gordonae*, followed by *Mycobacterium fortuitum*, *Mycobacterium senegalense*, and *Mycobacterium peregrinum*. This is in partial agreement with the findings of the study conducted by Thomson et al, (2013), Brisbane, Australia, were *Mycobacterium gordonae* was also the most commonly isolated species in natural and municipal water. *Mycobacterium gordonae* is one of the least pathogenic of the

mycobacterium species isolated. It is usually a contaminant or colonizer in immunocompetent individuals, however, it has been found to be pathogenic in HIV infected patients who are severely immunocompromised (Barber et al., 1991). Apart from AIDS, underlying immunue suppression, advanced structural lung disease such as emphysema, pneumoconiosis, alcoholism, chronic lung disease, diabetes mellitus and malignancy have all been reported as risk factors for infections due to *Mycobacterium gordonae* (Barber et al., 1991; Weinberger et al., 1992). Therefore, *Mycobacterium gordonae* isolated from clinical samples should not be automatically considered as a contaminant or colonizer especially in immunocompromised patients or patients with the above mentioned risk factors.

Mycobacterium fortuitum, Mycobacterium senegalense and *Mycobacterium peregrinum* all belong to the *Mycobacterium fortuitum complex*. A study done by Velayati et a, (2015), Iran, also found *Mycobacterium fortuitum* to be the most common species in water (Velayati et al., 2015). Immunocompromised individuals are more likely to be infected with *Mycobacterium fortuitum* from water sources and infections caused by this organism include skin and soft tissue infections, lymphadenitis, pulmonary infections and catheter related infections (Falkinham 1996).

Mycobacterium senegalense and *Mycobacterium peregrinum* have also been isolated in water elsewhere (Dantec et al., 2002; R. Thomson et al., 2013). They are potentially pathogenic NTM species and have been implicated to cause disease both in immunocompetent and immunocompromised persons (Todorova et al., 2015). The isolation of *Mycobacterium senegalense* in water sources of Namwala district is another key finding in our study. *Mycobacterium senegalense* is a rapidly growing mycobacterium

species that has been reported to cause disease among cattle in east Africa (Hamid et al., 2002). Therefore, isolation of this NTM species in water in the present study poses a risk to cattle of Namwala District because this is a pastoral area (Malama et al., 2014). Further, all *Mycobacterium senegalense* species isolated in this study were from Maala which and this area has the largest number of cattle in Namwala.

Further, *Mycobacterium fortuitum* and *Mycobacterium avium* were both isolated in humans and water and both organisms are pathogenic and have been associated with pulmonary infections. The isolation of these organisms both from humans and water indicates that environmental contact through water could probably be the source of infections in affected individuals in this study. Therefore, this has highlighted the public health significance of these Mycobacteria in Namwala Distric of Zaambia.

This study has also identified factors that are significantly associated with NTM infections. These factors include; being divorced in the marital status group, use of chlorine and water filters as practices to having safe drinking water and finally having positive culture results from atleast two samples.

Although no literature was found on the association between marital status and NTM infection, this study has shown that divorced patients had more NTM infections. This could be attributed to stress which divorced patients encounter, eventually lowering their immune system, making them susceptible to infections. Stress has been documented to lower the immune system of the affected individuals (Segerstrom and Miller., 2004).

Additionally, the present study has shown that use of chlorine and water filters as practices to having safe drinking water reduces the likelihood of having NTM infections. Mycobacteria are highly resistant to chlorine but effective chlorine disinfection can be achieved for certain NTM species at exposure greater than 1 mg l^{-1} for longer than 2 hours (Taylor et al., 2000).

Water filtration has also been shown to reduce NTM numbers, but without changing the filter regularly (less than 3 weeks), the filter can become a source of infection (Rodgers et al., 1999). Since filters require to be replaced regularly, they tend to be expensive and in a rural setup like Namwala district, not everyone would afford to use these filters. Hence, this may require the intervention of health authorities.

Furthermore, the findings from this study have shown that positive culture results from atleast two separate sputum samples submitted by one patient increases the likelihood of having NTM infection. Unlike tuberculosis, a single positive sputum culture for NTM is not necessarily considered diagnostic of NTM lung disease. However, when two or more sputum cultures are positive, the diagnosis of disease is more likely (Daley & Griffith 2010). In a study from Japan, 98% of patients with two or more positive sputum cultures for MAC had evidence of progressive disease (Tsukamura 1991).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

This study has shown the isolation of NTM species from both humans and water with *Mycobacterium arupense and Mycobacterium gordonae* being the most common NTM species respectively. This indicates that water is an important environmental source harboring pathogenic NTM. Further, the wide presence of NTM in the water sources of Namwala is a potential public health hazard especially for the immunocompromised individuals. Therefore, the isolation of NTM from humans and water in Namwala district highlighted the public health significance of these mycobacteria

6.2 Recommendations

- There is need for accurate and sensitive molecular techniques for speciation of NTM to be rolled out in all the culture facilities country wide.
- There is need for local authority to promote disinfection of water at the point of use. This would involve use of chlorine, boiling of water and use of water filters.
- Further studies should be conducted to fingerprint the NTM isolates so as to establish the epidemiological link. This can be archived by using other molecular methods like MIRU- VNTR (Muwonge et al 2014).
- Another study is required to assess the Drug susceptibility patterns of the prevalent NTM species in Zambia, so as to guide clinicians in terms of treatment regimen.

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8.0 APPENDICES

Appendix 1: The Informed Consent Form

Informed Consent Form

Title: Isolation and molecular characterization of Nontuberculous Mycobacteria of public health significance in humans and water.

Hello, my name is Ngula Monde. I am conducting a research in fulfilment of my project requirements for the Master of Science in Public Health at the University of Zambia, School of Veterinary Medicine.

My study is looking at the circulating Non tuberculous Mycobacteria species of public health significance in humans and drinking water in Namwala district. There has been an increase in the isolation rate of Non-tuberculous Mycobacteria infections in humans in sub-Saharan Africa with the advent of HIV/AIDS. Transmission to humans may occur through . In Zambia the Non-tuberculous Mycobacteria species have also been recognized as pathogens of major public health significance but have not been extensively investigated. If you have symptoms such as cough persisting for at least two weeks, loss of appetite, fever, fatigue, headache, night sweats or symptoms in any part of the body persisting for more than two weeks, you will be requested to take part in this study.Your participation in this study will help provide the much needed information on the circulating Non-tuberculous Mycobacteria species in Namwala districts, which will further help in patient management with regards to treatment.

Therefore, you are kindly being requested to participate in the study.If you choose to participate in this study, you will be asked to sign a consert form and provide two sputum samples, skin scraping, urine or pus sample depending on signs and symptoms you have. Also, you will be requiered to answer a 5-10 minutes questionare. You are free to ask any question before and after the sample has been taken.

No other examinations that are not for the purposes of this study will be carried out on the sample you will provide.

As part of the sample collection, less or no pain or discomfort is expected from the client and as part of the questionaire, you will be asked questions some of which may be personal. The whole process will be short and professionally done.

There will not be any immediate benefit to you if you choose to participate. However, many people may benefit in future in terms of treatment.

Participation in this study is voluntary and be assured that the information you will provide and also the results of the test will be strictly confidential. This information will be restricted to the researchers and the principal supervisor for the duration of the study. The sample will be retained for a maximum of six months before they are discarded, just in case there is need for reprocessing the sample. The results of the test will help to determine whether you have mycobacterial infections and arrange for medical attention.

For further information you may contact the following;

Ngula Monde:	The Chairperson
The researcher	ERES Converge IRB
TEL: +260977857835	33 Joseph Mwilwa Road, Rhodes Park
	Rhodes Park, Lusaka
Email:nkabelenga@yahoo.co.uk	Email: eresconverge@yahoo.co.uk

Appendix 2: The Participant Consent Form

Participant Consent Form

I ……have agreed to take part in this research which is studying *the Isolation and Molecular characterization ofNon-tuberculous Mycobacteria of public health significance in Humans and water in Namwala district of Zambia*. This is in order to know the Non-tuberculous Mycobacteria species circulating in this district and also to know the sources of infections of these mycobacteria so as to help with patient management with regards to treatment. I confirm that the study has been adequately explained to me and I understand the risks involved, if any.

I am participating voluntarily and I understand that I can withdraw at any time without any repercussions.

I am further aware that the information I give will be treated in confidence and I will not be personally identified.

I agree to provide necessary information and submit sputum, skin scraping, urine or pus sample.

Participant signature……Date……..

Participant thumbprint in the box above

Name of researcher: Ngula Monde…… Signature…….

For further clarification you may contact the following:

Ngula Monde:
The researcher
TEL: +260977857835

The Chairperson
ERES Converge IRB
33 Joseph Mwilwa Road, Rhodes Park

Rhodes Park, Lusaka

Email:nkabelenga@yahoo.co.uk Email: eresconverge@yahoo.co.uk

Appendix 3: The Questionnaire

THE UNIVERSITY OF ZAMBIA

SCHOOL OF VETERINARY MEDICINE

QUESTIONNAIRE

Title: *Isolation and Molecular characterization of Non-tuberculous Mycobacteria of public health significance from humans and water in Namwala District of Zambia*

Date…….. Area/Village.....

PATIENT ID……INITIALS…… SERIAL #.....

SEX…….. AGE…….....

DEMOGRAPHIC DATA

MARITAL STATUS…… OCCUPATION…… RESIDENCE……...

Animal Ownership

Q1. Do you keep cattle?

0: Yes ……..... ☐

1: No ……..... ☐

Animal Contact

Q2. Have you been in contact with animals recently?

0: Yes… ……..... ☐

1: No… ……..…….....…….....…….....……..... ☐

Use of animal products

Q3.Do you handle beef products?

0: Yes …….....☐

1: No …….....☐

If so specify the type of products…….....

Q4.Do you drink raw milk (unprocessed)?

0: Yes …….....☐

1: No …….....☐

Q5.Which is your main source of milk?

0: Own cows…….....☐

1: Other farmers…….....☐

2: Market…….....☐

3: Others specify …….....

Q6. Do you eat raw meat or undercooked meat?

0: Yes…….....☐

1: No…….....☐

If so specify the type of products…….....

Q7. Do you slaughter animals for meat?

0: Yes …….....☐

1: No …….....☐

Q8. If yes how often do your slaughter (or participate in slaughtering) animals?

0: At least once a year…….....☐

1: At least twice a year….....☐

2. At least every month…..☐

4. Special events (weddings and funerals).....☐

5. Others…….....

Drinking water sources

Q9. What is your main source of drinking water?

0: Own well…….....☐

1: Tap…….....☐

2: Bore hole……...☐

3: River…….....☐

4: Others specify …….....

Q10. Are the sources of water used for both humans and animals

0: Yes …….....☐

1: No …….....☐

Q11. Do you do anything to the water to make it safe?

0: Yes …….....☐

1: No …….....☐

Q12. What do you usually do to make the water safe to drink?

0: Boil…….....☐

1: Add bleach/Chlorine…….....☐

2: Use water filter…….....☐

3: Let it stand and settle…….....☐

4: Others specify…….....☐

Humans clinical signs

Q13. Clinical symptoms lasting for atleast two weeks.

0: Fever…….....☐

1: Chills…….....☐

2: Two weeks cough…….....☐

3: Body weakness (Fatigue)..…….....☐

4: Headaches…….....☐

5. Night sweats……...☐

6: Others (specify)…….....

Laboratory Finding

Q14. AFB Smear results

0: Negative…….....☐

1: Positive …. ……..…….....☐

Q15. Culture results

NTM: Positive …… Negative……

THE END

Appendix 4: Bio Data Sheet for Water Samples

THE UNIVERSITY OF ZAMBIA

SCHOOL OF VETERINARY MEDICINE

Bio Data Sheet- Water Samples

Title: *Isolation and Molecular characterization of Non-tuberculous Mycobacteria of public health significance from humans and water in Namwala District of Zambia*

Sample No.	Site of water collection e.g. Tap, well, river	Village Name	Volume of sample collected	Quality of water	GPS

Appendix 5: letter of approval from PG

THE UNIVERSITY OF ZAMBIA
SCHOOL OF VETERINARY MEDICINE
OFFICE OF THE ASSISTANT DEAN (POSTGRADUATE)

Telephone: 293727
Telegrams: UNZA LUSAKA
Telex: UNZALU ZA 44370
Fax: 293727/253952
School Fax: 293727
Vet. Clinic Telephone: 291515

P.O. Box 32379
Lusaka, Zambia

Your Ref:

Our Ref:

17th July, 2015

Mr. Ngula Monde
C/O Disease Control
School of Veterinary Medicine
University of Zambia
P.O. Box 32379
LUSAKA

Dear Mr. Monde,

RE: APPROVAL OF RESEARCH PROPOSAL

At the meeting of the School Board of Graduate Studies held on 2nd July, 2015, your research proposal entitled ***'Isolation and Molecular Characterisation of Non Tuberculous Mycobacteria of Public Health Significance from Humans, Animals and Water in Namwala District of Zambia'*** was tabled and discussed. I am therefore pleased to inform you that the research proposal was subsequently approved by the Board.

On behalf of the Board, I wish you success as you apply for ethical approval and carry on with you research activities.

Yours sincerely

Prof. B. M. Hang'ombe
ASSISTANT DEAN (PG), SCHOOL OF VETERINARY MEDICINE

Cc *Director, DRGS*
Dean, School of Veterinary Medicine
Head, Disease Control Department
Dr. Musso Munyeme, Disease Control Department
Dr. Adrian Muwonge, (University of Edinburgh
Dr. Sydney Malama, University of Zambia
Prof. Mwanza, Clinical Studies

Appendix 6: Letter of approval from Eres converge

33 Joseph Mwilwa Road
Rhodes Park, Lusaka
Tel: +260 955 155 633
+260 955 155 634
Cell: +260 966 765 503
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No. 00011697

25th September, 2015

Ref. No. 2015-Aug-002

The Principal Investigator
Ms. Ngula Monde,
Tropical Diseases Research Centre
P.O. Box 71769,
NDOLA.

Dear Ms. Monde,

RE: ISOLATION AND MOLECULAR CHARACTERIZATION OF NON TUBERCULOUS MYCOBACTERIA OF PUBLIC HEALTH SIGNIFICANCE FROM HUMANS AND WATER IN DISTRICT OF ZAMBIA.

Reference is made to your corrections. The IRB resolved to approve this study and your participation as principal investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2015-Aug-002**
Approval and Expiry Date	Approval Date: 25th September, 2015	Expiry Date: 24th September, 2016
Protocol Version and Date	Version-Nil	24th September, 2016
Information Sheet, Consent Forms and Dates	• English.	24th September, 2016
Consent form ID and Date	Version-Nil	24th September, 2016
Recruitment Materials	Nil	24th September, 2016
Other Study Documents	Questionnaire.	24th September, 2016
Number of participants approved for study	192	24th September, 2016

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- ERES Converge IRB does not "stamp" approval letters, consent forms or study documents unless requested for in writing. This is because the approval letter clearly indicates the documents approved by the IRB as well as other elements and conditions of approval.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Dr. E. Munalula-Nkandu
BSc (Hons), MSc, MA Bioethics, PgD R/Ethics, PhD
CHAIRPERSON

Appendix 7: Letter of authorization to conduct research in Namwala District

THE NATIONAL HEALTH RESEARCH AUTHORITY
C/O Ministry of Health
Ndeke House
P.O. Box 30205
LUSAKA

In reply please quote:
MH/101/23/10/1
No.....

29 October, 2015

Ms. Ngula Monde
University of Zambia, School of Veterinary Medicine
P. O. Box 32379
Lusaka

Dear Ms. Monde

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Isolation and Molecular Characterization of Non Tuberculosis Mycobacteria of Public Health Significance from Humans and Water in Namwala District of Zambia."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been approved to carry out the above mentioned exercise on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Dr. I. Muteba
For/Director
National Health Research Authority

Appendix 8: Paper I (Published in Journal of Tuberculosis Research)

A Review of Tuberculosis in Ndola District of Zambia

Ngula Monde[1, 3], Musso Munyeme[3], Sydney Malama[2*]

[1]Department of Biomedical Sciences, Tropical Diseases Research Center, P.O. Box 71769, Ndola

[2]Health Promotions Unit, Institute of Economic and Social Research, University of Zambia, P.O. Box 30900, Lusaka.

[3]Department of Disease control, School of Veterinary Medicine, University of Zambia, P. O. Box 32379, Lusaka

*Corresponding author: sydneymalama1971@gmail.com

Abstract

Background

Tuberculosis (TB) remains the most frequent and important infectious disease causing morbidity and mortality globally. The World Health Organization estimates the incidence of all forms of TB in Zambia at 444/100,000. Tuberculosis case notification rates have increased eightfold over the past two decades and this is largely due to the concurrent Human Immunodeficiency Virus epidemic. The cornerstone of Tuberculosis control is early case detection and treatment which is promoted by Direct Observed Treatment-Short course strategy. This paper reviews the available information in English on TB situation in Ndola district of Zambia with the purpose of assessing successes recorded over the reviewed period

Results

This review has noted a reduction in the number of new cases of TB recorded, improved diagnosis and treatment success of TB in Ndola district over the reviewed period.

Conclusion

This review has observed a reduction in the number of new cases of TB recorded in Ndola district over a period of ten years due to the strategies put up by the National TB Program as well as the effective implementation of the strategies by the District Medical team. These strategies included the Directly Observed Therapy Short course (DOTS).

Key words: Ndola, Tuberculosis, Zambia

Introduction

Worldwide, Tuberculosis (TB) remains the most frequent and important infectious disease causing morbidity and mortality. Tuberculosis is caused by members of the Mycobacterium tuberculosis complex (MTC), which includes *Mycobacterium tuberculosis, Mycobacterium bovis, Mycobacterium africanum, Mycobacterium caprae,*

Mycobacterium microti, *Mycobacterium pinnipedii* and *Mycobacterium canettii* [1,2]. Approximately one third of the world's population is infected with bacteria belonging to the MTC complex, with Sub-Saharan Africa having the highest annual incidence since the advent of HIV and AIDS [3,4].

Zambia with a population of 13 million people [5], is an example of a country highly affected by TB (Ayles et al., 2009). Despite a long-running National Tuberculosis and Leprosy Program (NTLP), Zambia has seen a rapid increase in TB cases, especially after 1983, synchronous with the beginning of the HIV era in Zambia [6]. The World Health Organization estimates the prevalence of all forms of TB in Zambia at 444/100,000 and ranks Zambia as ninth in the world [7]. High risk of infection is particularly seen in specific sub-groups such as HIV infected persons, pregnant women, children and prisoners [8]. The HIV prevalence of 14.3% country wide and 22% in the capital Lusaka, continues to fuel the TB epidemic with WHO estimating that 70% of Zambian TB patients are infected with HIV [9], however, this varies from the different provinces ranging from about 50% in North-Western Province to more than 80% in Lusaka province [10]. Other factors which are exacerbating the TB burden in Zambia include high poverty levels, poor housing [5], limited TB control strategies in congregate settings and challenges of TB diagnosis in pediatric patients [11]. The disease burden varies among provinces, with highest notification rates being reported from provinces along the line of rail: Lusaka (28% in 2004) and Copperbelt (28% in 2004) followed by Southern Province (10%) and low rates (5 to 6%) from other provinces [12]. The rapid increase of tuberculosis in Zambia from 1985 to 2004 (Figure 1) was mainly attributed to the HIV epidemic, but other factors like

population growth, urban overcrowding and improved case detection could have also contributed [13].

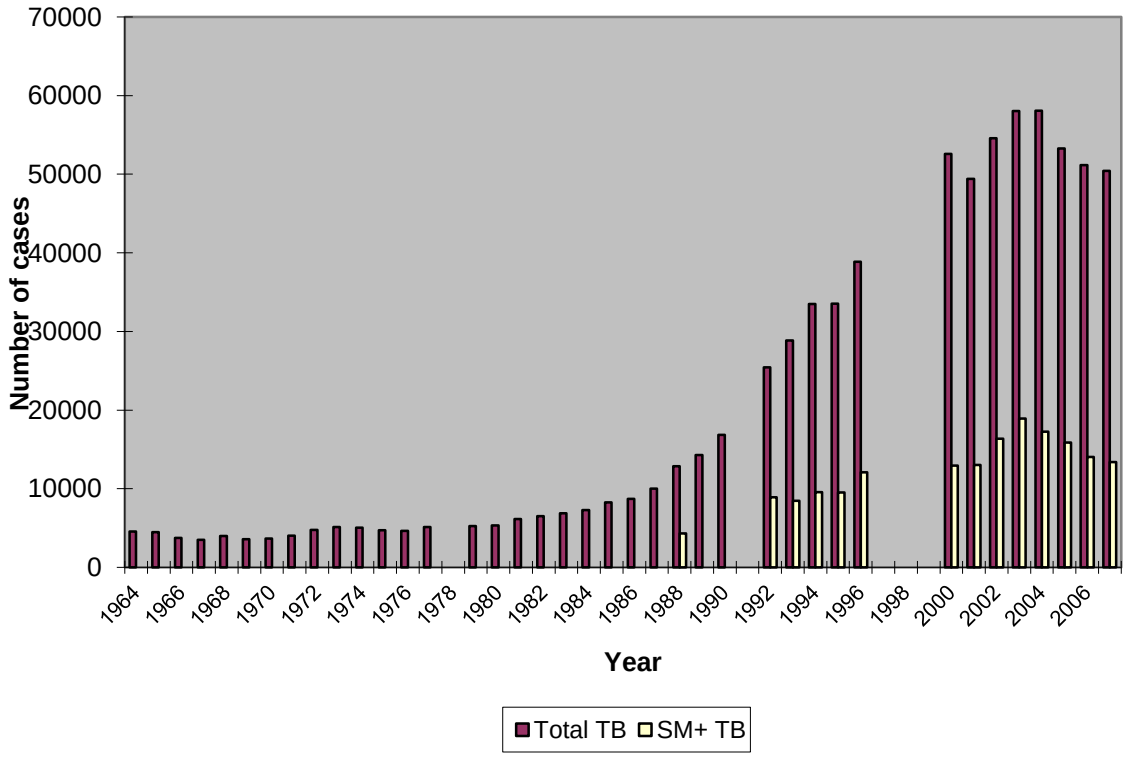

Figure 1: Notifications of tuberculosis all forms and new smear positive cases in Zambia, 1964-2007

Source: NTLP manual

Zambia has made good progress in TB control since 2007 with the estimated prevalence rates showing a downward trend and having very good treatment outcomes in new cases and retreatment cases regardless of the HIV status of the patients [10].

Mycobacterium tuberculosis is usually transmitted to humans by inhalation of aerosol droplet nuclei containing tubercle bacilli which are expelled by an individual with active pulmonary TB[7]. Although *M. tuberculosis* infection is the most common cause of human tuberculosis, unknown proportion of cases are due to *M. tuberculosis*, data on the

prevalence of human disease due *to M. bovis* in Zambia is limited, due to technical problems posed by identification of this species, such as trained personnel and laboratory facilities and only smear microscopy is performed on sputum samples to identify AFB [14].

Diagnosis of Tuberculosis in Zambia

The hallmark of TB control is early case detection and treatment which is promoted by Direct Observed Treatment-Short course (DOTS) strategy [6,15]. While WHO target detection rate is 75% of new Smear positive cases and cure rates of 85%, Zambia's performance is only at 49% and 75% respectively [7]. The WHO estimates that Zambia has a case detection rate of approximately 80% [7], meaning that approximately 12 000 TB cases go undiagnosed each year. The public and semi-public sectors notify 90% of cases; the remainder is from a limited number of private sector facilities that collaborate with the NTLP. Case notification data from Zambia indicate that rates of TB infection and disease have increased eightfold over the past two decades, largely due to the concurrent HIV epidemic [16,17].

In Zambia, TB is mainly diagnosed by microscopy, using Ziehl–Neelsen (ZN) stains [18]; culture and DST have also been performed since the late 1990s [19]. The ZN method is rapid and inexpensive and highly specific. However, the main limitation of the method is its low sensitivity, with a detection limit of between 5,000–10,000 bacilli/ml of sample, particularly in HIV co-infected patients [20]. With these limitations of ZN technique Zambia has rolled out the use of LED fluorescent microscopy in most of the TB diagnostic centers in the country.

The laboratory gold standard for diagnosing TB is mycobacterial culture [21]. Mycobacterial culture is much more sensitive than microscopy and allows the recovery of the bacteria for other studies, such as drug susceptibility testing and genotyping [21]. In Zambia, due to limited infrastructure in most health facilities, culture is only performed in the three TB reference laboratories situated in Lusaka and Ndola districts and a few selected private institutions.

Drug susceptibility testing to 1st line drugs is mainly performed using convertional methods such as the proportional method using Lowestein- Jensen (L-J) and liquid medium based methods such as Mycobacterial Growth Indicator Tube (MGIT) system 960 (BD diagnostics, USA). Culture and DST is recommended to be performed only on samples from all patients enrolled on treatment as retreatment cases; those who fail to respond to treatment and all those who had interrupted treatment. The drugs routinely tested for are rifampicin, isoniazid, ethambutol and streptomycin (Kapata et al, 2013).

In recent years, a number of alternative diagnostic tests that use immunological and molecular methods have been developed. Many molecular methods have been developed for direct detection, species identification and drug susceptibility testing of mycobacteria [22]. One of the new Rapid molecular test that has the potential to substantially improve and accelerate the diagnosis of TB and drug resistance TB is the GeneXpert MTB/RIF diagnostic test. The Xpert MTB/RIF system is a recently developed TB-specific application, designed to detect *M. tuberculosis* as well as rifampicin resistance-conferring mutations directly from sputum [23]. The recent WHO endorsement of Xpert MTB/RIF is a good development; however, the technology in Zambia is being implemented in phases

and only in selected health facilities. This is because financing the implementation of such a molecular test is a major undertaking [19,24]. Another molecular method being used in Zambia is the Line Probe Assay (Hain Lifescience, Germany). The Line Probe Assay (1st line) is a DNA strip test that allows simultaneous molecular identification of tuberculosis and the most common genetic mutations causing reistance to rifampicin and isoniazid (FIND- line probe assay, 2015). However, this method is being used in the Reference laboratories where culture, biosafety and PCR facilities exist. Although molecular methods are rapid and results are obtainable within a limited period of time, these techniques need more advanced and sophisticated infrastructure and their introduction in the country like Zambia on routine bases requires careful planning and additional human and financial resources [13].

Treatment of TB in Zambia

The standard treatment regimen for new case has an initial phase lasting 2 months using for 4 drugs, isoniazid, rifampicin, pyrazinamide, and ethambutol. In the continuation phase lasting 4 months , 2 drugs, isoniazid and rifampicin are used [19,25]. WHO recommends that national TB control programmes should ensure that supervision and support are provided for all TB patients in order to achieve completion of the full course of therapy [26]. Treatment success rate for new TB smear positive cases has greatly improved from 77% in 2002 cohort to 88% for the 2012 cohort in Zambia. A study done by Kapata et al, where data on the trends of TB burden over the past two decades was reviewed, indicated that the cure rates almost doubled in the last decade from 47% in 2000 to 82% in 2009. The treatment success rate also increased from 66% in 2000 to 86% in 2009 (Kapata et al., 2011)

TB situation in Ndola District

Ndola district is located on the Copperbelt of Zambia and is the third largest city in Zambia with a population of 455, 194 [5]. Ndola lies between latitudes 120° and 160° South of the Equator and between 250° and 30° east (Figure2).

Figure 2: Map depicting Ndola district of Zambia. Source: City of Ndola- District situation analysis, 2005

Ndola district has 31 health facilities: 28 primary health centers or clinics, two referral hospitals and one infectious disease reference laboratory. Most of these health centres provide TB diagnosis and treatment.

Sputum smear microscopy still remains the cornerstone for diagnosis of TB in Ndola District. A study done by Mulenga et al., 2010 showed that the majority of MTBC isolates

in Ndola belongs to the SAF1 family with a high clustering rate indicating high recent transmission of TB [6]. This emphasizes on the importance of early diagnosis and timely treatment. Differentiation within the Mycobacterium tuberculosis complex is necessary for epidemiological purposes and some individual patient treatment [27]. Since TB in Ndola is mostly diagnosed by sputum smear microscopy (SSM) which cannot differentiate members of the complex. The epidemiology of TB remains largely unknown, hence, posing challenges in TB control strategies. Routine acid-fast bacilli (AFB) culture and identification of isolates are not performed at each TB diagnostic centre due to lack of well-equipped laboratory facilities [18]. However, the district has one regional TB laboratory which performs culture only follow up cases such as treatment failures, defaulters etc.

TB intervention strategies in Ndola District

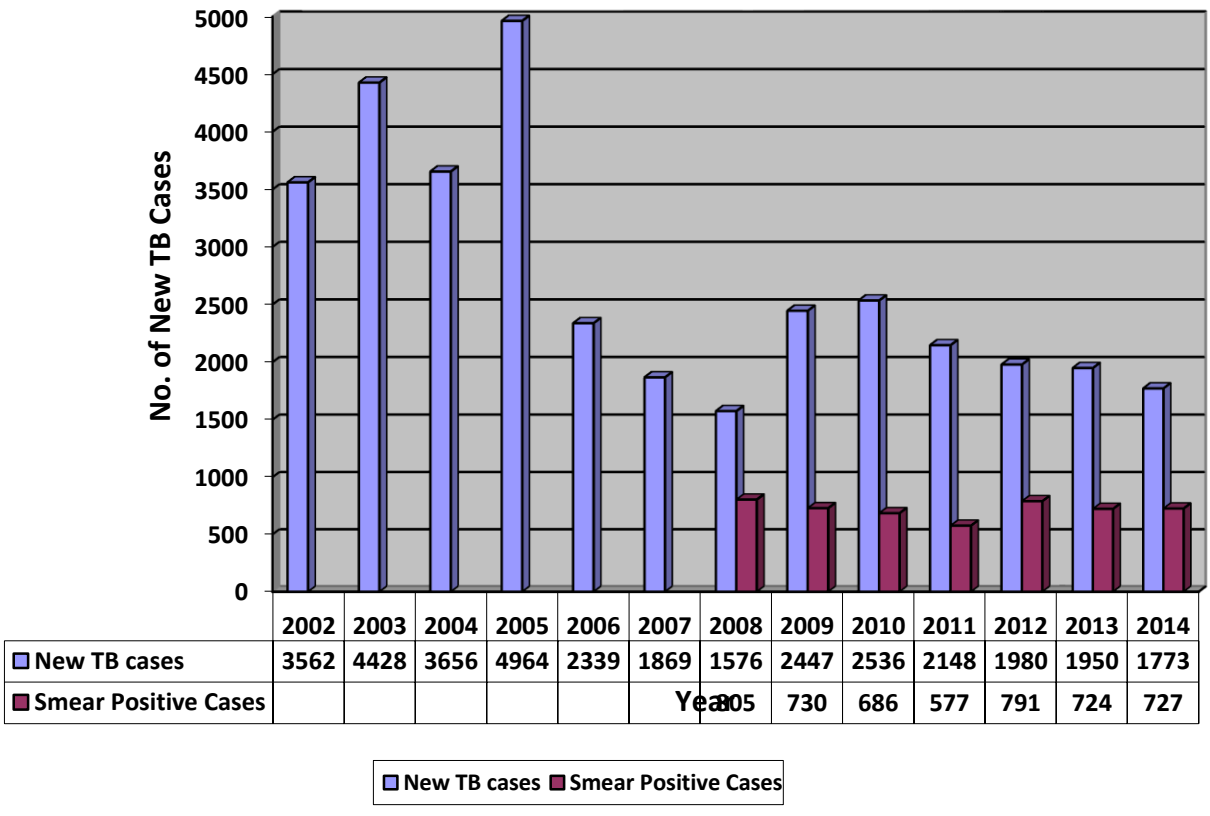

Figure 3: Number of New TB cases and Smear positive cases from 2002 to 2014 Ndola District. Source: Ndola DHMT data, 2002-2014

Data from Ndola DHMT show that there has been a downward trend in the number of new cases of TB from 2002 to 2014 in Ndola urban district (Figure 3). These could be due to improvement in TB intervention strategies such as Directly Observed Therapy Short course (DOTS) implementation. DOTS remain at the heart of the strategy to stop TB. DOTS consists of 5 main technical elements; political commitment, case detection through quality assured bacteriology, standardized treatment with supervision and patient support, an effective drug supply and management system and a monitoring and evaluation system that incorporates impact measurement [15]. Zambia adopted the WHO-recommended DOTS strategy as its primary approach for tuberculosis (TB) control in 1993 and has since achieved 100% coverage. Ndola district is one of the earliest to have implemented DOTS in Zambia [15]. In an effort to improve case management, Ndola District has benefited from the use of community volunteers and treatment supporters in their TB programme to assist in ensuring patients complete treatment. DHMT data for Ndola district for 2014 indicate a relatively high treatment success rate of 91% [28]. High treatment success rates may contribute to deceased notification rates and may indicate decreased TB transmission in the general public (Kapata te al, 2013).There has also been an improvement in diagnosis of TB, meaning that all the TB suspects are being confirmed as cases. Some years back, most cases of pulmonary tuberculosis were diagnosed through clinical diagnoses and light microscopy which has proved to be less sensitive. This means that in some cases, patients

were diagnosed with TB disease on the basis of their signs and symptoms or using chest X-ray, even if their specimen does not contain *M. tuberculosis* resulting in over diagnosis of TB. Most of the diagnostic centres in Ndola district are performing smear microscopy using the more sensitive and WHO recommended LED fluorescence Microscopy. The NTLP has also rolled out the use of GeneXpert technology in selected health facilities with the help of partners such as TB Care II. There has also been an effective screening of TB contacts. It is recommended that household contacts be screened for symptoms of disease and Isoniazid preventive therapy (i.e. daily Isoniazid for at least 6 months) offered to children aged less than 5 years along with all HIV-infected children [29]. This is to reduce the risk of infection.

There has also been an effective supply of anti TB drugs in the district. The district has not experienced any shortages of these drugs for the past three to four years, this could also contribute to the experienced downward trend in new TB cases [30].

The other reasons for the downward trend could be weaknesses in the routine TB surveillance. The routine surveillance data may have gaps due to recording bias or weaknesses. This observed downward trend also entail that the National TB control programme is performing very well in reducing the burden of TB in the district. This is in terms of provision of quality diagnostic and treatment services for TB cases at every level of health delivery. In order to provide quality TB services, the NTLP has developed guidelines and standards to explain the different steps involved in TB management, including the National TB Guidelines and the TB Manual. Separate guidelines also exist for adult TB, MDR TB, TB/HIV, TB infection control, and procurement and supply management. Standards on diagnosis and treatment and laboratory manuals have also been

developed; as well as forms, formats, and a standardized system for record keeping; and systems for supervision, monitoring and evaluation [28]. Ndola district has also implemented the TB infection control demonstration project at most of its diagnostic centres through the Ministry of Health (MOH) and the Ministry of Community Development Mother and Child Health (MCDMCH) with the support of TB CARE I and TB CARE II partners. The goal of the project was to provide safe work practices in order to reduce TB transmission among people living with HIV (PLHIV) and Health Care Workers (HCWs). The implementation process was aimed at strengthening the district healthcare system in implementing TB Infection Control, acknowledging occupational risk for the health care workers acquiring TB infection and developing TB disease [30].

Conclusion

This review has observed a reduction in the number of new cases of TB recorded in Ndola district over the reviewed period due to the strategies such as DOTS put up by the National TB Program as well as the effective implementation of these strategies by the District Medical team. However, TB is a disease that has always been, and still is associated with poverty, HIV/AIDS and deprivation. Therefore, the eventual eradication of this ancient scourge will surely go hand in hand with the creation of a caring national society and improved living standards of the people.

Recommendations

- Newer diagnostics technologies for the rapid diagnosis of active TB cases and drug resistance testing such as the GeneXpert MTB/RIF technology needs to be fully

funded by the NTLP in order to sustain its implementation in the selected health facilities in Ndola.

- There is also need to improve case detection and treatment of TB in children.
- There is need to ensure that all the necessary materials are provided to all TB diagnostic centers to ensure accurate and prompt diagnosis of TB.
- There is need for studies to be done on Non tuberculous Mycobacteria and ascertain its prevalence in humans in Ndola district.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NM contributed to reviewing literature and drafting of the manuscript, MM contributed to the writing of the manuscript, SM contributed to the conception, reviewing literature and editing of the manuscript. All authors read and approved the final manuscript.

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Abbreviations

CSO	Central Statistics Office
DHMT	Ndola Health Management Team
DOTS	Direct Observed Treatment Short course
LED FM	Light Emitting Diode Fluorescence Microscopy
MCDMCH	Ministry of Community Development, Mother and Child Health

MDR	Multidrug Resistance
MOH	Ministry of Health
NTLP	National Tuberculosis and Leprosy control Programme
PLHIV	People Living with HIV
WHO	World Health Organization
ZDHS	Zambia Demographic Health Survey

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