**Molecular** **characterization of environmental and clinical Non tuberculous mycobacteria isolates from copper mines and miners in Zambia.**

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**ABSTRACT**

**Background**: The active and ex-miners have high risk factors for Non Tuberculous Mycobacteria (NTM) disease due to the high burden of Human Immunodeficiency Virus (HIV) infection, tuberculosis and silicosis through extensive use of aerosolised water for dust control in the mining environment. The study aimed at the isolation and characterization of nontuberculous mycobacteria from the miners and ex-miners in Zambia

**Methods:** Sputum samples were collected from 85miners and ex-miners132 water samples were collected from the mining environment. Culture of sputum and water samples was done using standard mycobacterial culture procedures. Species identification was done by sequencing the 16S – 23S rRNA Intergenic transcribed spacer (ITS) gene.

**Results:** *Mycobacterium Virginiense* was the only species isolated from humans while *Mycobacterium fortuitum* 18.94% (25/132) was the most isolated species from water. Other NTM species isolated included *Mycobacterium gordonae* 9.1 % (12/132), *Mycobacterium species* (not specified) 6.8 % (9/132), *Mycobacterium boenickei* 4.6 % (6/132). *Mycobacterium paragordonae*, *Mycobacterium fortuitum* (sub specie *fortuitum* 1.5 % (2/132), and *Mycobacterium celeriflavum* (0.8 % (1/132).

**Conclusion:** The isolation of NTM species from humans and water highlighted that active and ex-miners are at potential risks of acquiring potentially pathogenic NTM from the mining environment. The epidemiological investigation of NTM is therefore recommended. This should include sampling from environmental sources such as water source and soil.

Key words:Löwenstein-Jensen, Non-tuberculous Mycobacteria, Polymerase Chain reaction, miners, ex-miners

**1.0 INTRODUCTION**

The emerging of Non Tuberculous Mycobacteria (NTM) disease has become a major concern worldwide causing morbidity and mortality exacerbated in immunosuppressed persons mainly due to Human Immunodeficiency Virus (HIV) infection that leads to Acquired Immune Deficiency Syndrome (AIDS) (1–3).

NTM are thought to be acquired through ingestion, inoculation and inhalation during water and soil occupational activities(4–6)*.* Human-to-human transmission of NTM is generally uncommon, although there is evidence of transmission of certain NTM species such as *Mycobacterium (M) kansasii* (7).

The miners have a higher risk of having NTM disease, due to HIV infection, a high burden of tuberculosis (TB), and silicosis through extensive use of aerosolised water for dust control. It is important to rapidly and accurately identify Mycobacteria to the species level to facilitate prompt patient management. Non tuberculous mycobacteria can display clinical and radiological features similar to those exhibited by Mycobacterium Tuberculosis Complex (MTBC), hence the need for species differentiation in order to distinguish from MTBC in individuals suggestive of having tuberculosis (TB)(8). Distinguishing NTM from MBTC disease may be challenging, especially in low income countries such as Zambia where AFB smear microscopy is mainly used for MBTC diagnosis. However, even though AFB smear microscopy, allows for rapid diagnosis of mycobacteria, it does not differentiate *Mycobacterium tuberculosis* from NTM. This may lead to patients having NTM to be misdiagnosed as having TB.(9). Soil properties play an important role in promoting growth and persistence of NTM in nature thus soil of countries with high-risk of infection have higher copper and sodium levels, and lower manganese levels compared to low risk countries (10). In South African gold miners trapped dusty environments promote the aerolization of NTM that predispose miners to pulmonary NTM (PNTM), particularly caused by *M. kansasii* and Mycobacterium avium complex (*MAC)* resulting in the development of silicosis and chronic bronchitis (11).

 Worldwide, NTM have been recognized as pathogens mostly in immuno-compromised populations such as HIV/AIDS, and immuno-competent individuals(12). In the South African gold-mining there was an unusually high incidence of *M. kansasii* infection (13). In Zambia, infections due to NTM ) are slowly becoming more of a public health challenge coupled with high HIV/AIDS disease burden ((14). Zambia is second largest producers and exporters of copper in Africa behind Democratic Republic of Congo (DRC), mining activities pose a potential risk for exposure to NTM. Miners face an elevated risk of NTM exposure due to factors such as the production of dust and particulate matter, working in confined underground environments, and the of water sources (15). Despite this, no study has been conducted to understand the mining ecology related to risk of NTM infection among the copper miners and the significance of isolation of these organisms in mining environment and clinical samples. Therefore, this study aimed to isolate and characterize non tuberculous mycobacteria from the mining environment and miners/ex-miners to determine as possible sources of NTM infection for active and ex miners. Thus, identification of these Mycobacteria in clinical samples and environmental sources was important for both clinical and patient management, also help formulate policy change in understanding transmission factors that increase NTM transmission in mining environment.

**2.0 MATERIALS AND METHODS**

***2.1: study Area:*** The study was conducted in Kitwe district, regarded as the third largest city in Zambia, with regard to infrastructure development and second largest in terms of geographical size and population 922,093 (16). It has a complex of Mines on its north-western and western edges. The sputum sampling was done from Occupational Health and Safety Institute (OHSI) and environmental sampling was done from copper mine named X three shafts, Shaft K, Shaft N and Shaft T.

***2.2: Study Design*:** This was a prospective cross sectional study where sputum samples from human adult (≥ 18 years old) miners and ex miners’ presented at OHSI with suspected TB (had a productive cough, loss of appetite, fever, fatigue, headache and night sweat for two weeks) or Silicosis. Water and biofilm samples were collected from common water drinking points (taps) and miner’s ablution blocks (shower heads) in copper mine named X. upon collection, samples were transported to Tropical Diseases Research Centre (TDRC) for mycobacteriological culture under cold chain. The molecular analysis of the isolates was done at the School of Veterinary Medicine, University of Zambia.

**2.3. Study Period**

The study was carried out from May, 2021 to September, 2022.

 **2.4 Laboratory Methods**

 **Sputum sample collection and processing**

 Sputum samples were collected from both active and ex miners with TB symptoms and suspected silicosis. Two spot samples were collected from each miner and ex –miner for culture (at least 15 to 30 minutes apart). A volume of about 2-10 ml each was submitted. The samples were immediately stored in a cooler box with ice packs until transported to Ndola, TDRC TB reference laboratory for processing on the same day.

At TDRC TB Reference Laboratory, samples were decontaminated using the Petroff method (17), cultured on Lowenstein-Jensen (LJ) medium, incubated at 37 °C and examined for growth weekly for at least 8 weeks and results recorded. Microscopic examination of positive cultures using Ziehl Neelsen (ZN) staining method was performed to detect the presence of AFB.

**Water samples collection and processing.**

Water and biofilm samples were collected from common communal water taps and shower rooms used by miners. Water was allowed to run through for a while and 150 ml of water was collected in sterile wilpark plastic bags from tap water while biofilms were collected by swabbing the inside part of the shower heads and water taps using sterile swabs.

Each swab was then placed in a 50 ml Falcon tubes containing 10 ml normal saline. Immediately after collection, all samples were transported to the TDRC TB laboratory under cold chain where they were processed and cultured on LJ medium.

At TDRC 100 ml of water was filtered through 0.45 µl nitrocellulose membrane filters (Millipore Corporation, Bedford, MS, USA) by vacuum filtration using a Maniford Filtration System (Sartorius AG, Goettingen, Germany). The membranes were placed in 10 ml sterile normal saline solution and the bacteria dislodged by having the surface of the membranes abraded vigorously and thoroughly with a sterile platinum inoculating loop and vortexed for 2 minutes. Swabs were also vortexed vigorously to dislodge bacteria, centrifuged and pellet resuspended in 10 ml sterile normal saline. Both sample types were decontaminated to reduce background organisms using a 1 % sodium hydroxide + 3 % Sodium dodecyl sulphate (SDS) for 15 minutes and neutralised with sterile phosphate buffer. The organisms were concentrated by centrifugation at 3800 rpm, supernatant decanted and the pellet resuspended in 2 ml sterile phosphate buffer. 500 µl of the resuspended pellet were inoculated on LJ Media. Mycobacterial growth was monitored and recorded once every 7 days until considered negative after 8 weeks. The number of colonies for positive cultures were recorded. ZN microscopy was performed on positive culture to determine Acid Fast Bacilli (AFB) and confirmed by Capilia TB – Neo kit (TAUNS Laborateries, Inc. Japan) as either NTM or MTBC following manufacturer’s instructions (capilia.co.jp/english/capilia\_tb-neo.html).

**Sequencing of 16S -23S ITS rRNA region**

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. To determine whether the PCR products were present in the samples, Electrophoresis (Mupi dexu submarine electrophoresis system- advance) was performed by running 5 μl of the PCR products on a 1.0 % agarose gel that contains ethidium bromide (0.5 - 23 - μg/ml) for 25 minutes. Thereafter, the PCR products was visualized by a UV transilluminator (IPV benchtop 3UV Transiluminator).

To obtain genomic DNA for sequencing, the QIAamp method for DNA extraction was used.(18), then polymerase chain reactions (PCRs)was performed, after that sequencing was performed for the 16S – 23SrRNA Internal Transcribed Spacer region with primers Sp1 (5′-ACC TCC TTT CTA AGG AGC ACC-3′) and Sp2 (5′-GAT GCT CGC AAC CAC TAT CCA-3′) (19). The sequencing was done using an Applied Bios system, (AB3130) sequencing machine at the University of Zambia, School of Veterinary Medicine. 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 that 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 31 Information, http://blast.ncbi.nlm. nih.gov/). The isolate identification 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 ≥ 85 were accepted (20).

**Statistical analysis**

The Data from laboratory analysis was entered in Excel Spread sheet and imported into STATA version 14 (STATA Corp, College Station, Texas) for descriptive and statistical analyses.

**Ethics approval and consent to participate**

Ethical clearance to conduct the study was granted from “Excellence in Research Ethics and Science” (Ref. No. 2022-June-2022). Permission to conduct the study at OHSI and X mine was obtained from the National Health Research Authority, Ministry of Health and X named mine management as well as OHSI Management. The study did not pose any risk to the participants and confidentiality was maintained. Written consent was sought from the participants and the objective of the study was properly explained to the participants.

**3.0: RESULTS**

**Demographic characteristics of study participants**

 **Gender**

 The 85 participants participated in the study were males only and consented to take part in the study. Each submitted two sputum samples resulting in a total of 170 sputum samples.

 **Age**

The median age and interquartile range (IQR) was 50.2 (45 to 57) years. The age range between 41-60 had the highest number of participants 70.6% (120/170) followed by the age range between 21-40 20% (34/170) (Table 1).

**Table 1**: Proportion of age by categories

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Age Group** | **N** | **Proportion** | **SE** | **95% CI** |
| 21-40  | 34 |  20 % | 0.0307692 | 14.6 - 26.8 % |
| 41-60 |  120  | 70.6 % | 0.0350496 | 63.3 - 77.0 % |
| >60 | 16 | 9.4 % | 0.0224609 | 05.8 -14.9 % |

**Isolation of NTM in sputum and water samples**

Out of 85 study participants, NTM was isolated from 1 (1.2%) miner.

Seventy-two 72/132 (54.5%) NTM were obtained on culture from water and biofilms samples using ZN method and confirmed by Capilia Neo test. Water samples yielded 31 (43.1 %) NTM recovery while biofilm yielded 41 (56.9 %). Fifty-seven 57/72(79.2%) were characterized to specie level and 15 (20.8%) were uncharacterized. The majority of NTM were isolated from shaft K (42.1%) followed by shaft N (31.0%) and least was shaft T (26.3%).

Identification of NTM using DNA sequencing from Humans, water biofilms.

The Two 2/170 (1.2%) NTMs isolated from one individual were characterized to species level and the species identified was *Mycobacterium virginiense****.***

 57 NTM isolates were characterized to species level the most prevalent NTM species isolated from water and swabs samples were *Mycobacterium fortuitum* 18.9 % (25/132), *Mycobacterium gordonae* 9.1 % (12/132), *Mycobacterium species* 6.8 % (9/132), *Mycobacterium boenickei* 4.6 % (6/132). *Mycobacterium paragordonae* 1.5 % (2/132) and *Mycobacterium fortuitum* (*sub specie fortuitum*), and *Mycobacterium celeriflavum* (Table 2)

**Table 2** Distribution of NTM species isolated from Humans and water

|  |  |  |  |
| --- | --- | --- | --- |
| **NTM species** | **N** | **Frequency %** | **95 CI%** |
| **Humans(N=170,n=2)** |  |  |  |
| *M. virginease* | 2 | 1.2 | 0.14 – 4.19 |
| **Water & Biofilms** |  |  |  |
| *M. fortuitum* | 25 | 18.9 | 12.65 – 26.68 |
| *M. gordonae* | 12 | 9.1 | 4.79 -15.34 |
| *Mycobacterium species* | 9 | 6.8 | 2.65 – 11.59 |
| *M. boenickei*  | 6 | 4.6 | 1.69 -9.63 |
| *M. fortuitum(sub specie fortuitum)* | 2 | 1.5 | 0.18 – 5.32 |
| *M. paragordonae* | 2 | 1.5 | 0.18 -5.32 |
| *M. celeriflavum* | 1 | 0.8 | 0.02 - 4.15 |

**4.0 DISCUSSION**

This study explores the isolation and characteristics of NTM in the mining environment and among miners and ex-miners in Zambia. Nine NTM species were isolated and characterized, with three, namely *M. Virginiense, M. fortuitum*, and *M. gordonae*, identified as potential pathogens associated with various infections, especially among immunocompromised individuals, including those with HIV, TB, and silicosis. The isolation of *M. virginiense* from an ex-miner raises intriguing questions about the zoonotic transmission of this NTM, possibly linked to occupational or environmental interactions. Despite low NTM prevalence among miners due to strict infection control measures, environmental water systems showed a higher rate, suggesting a possible source of human infections. The findings stress the necessity for advanced molecular studies to establish epidemiological links and evaluate NTM transmission risks in mining settings.

In this study, one NTM species and eight NTM species were isolated and characterised from humans and mining environments respectively. This study reports for the first time in Zambia the isolation of *M. Virginiense* from clinical samples of an ex-miner which has the potential to cause tenosynovitis and osteomyelitis in immune-compromised individuals. Other studies done elsewhere also identified *M. virginiense* from pulmonary samples (21)though it could not be proven to be the cause of the pulmonary symptoms as the patient's lung had suffered complete destruction due to *M. massiliense* infection previously, and subsequent CT scans showed neither improvement nor deterioration following the identification of *M. virginiense.* (21)

*Mycobacterium virginiense* is a newly described member species of the Mycobacterium terrae complex which is recognised as of clinical importance (22) and causes tenosynovitis and osteomyelitis. It’s a slow-growing NTM first identified in 2016. It was first isolated from humans in 2018 by Vasireddy and colleagues (21) in South Korea, Since the first report in 2016, two more *M. virginiense* have been isolated, one from a mud specimen of a swine farm in Japan (23) and another from swine faecal specimen. The *M. virginiense* isolated in Zambia could be an imported case by an expatriate who had harboured the infection during working time or period when he was engaged by X named copper mine, or the ex-miner could have interacted with an infected swine as this NTM is also found in swine and soil (23).

Only a few cases of *M. virginiense* have been reported worldwide, and it has been acknowledged to be a major contributor to tenosynovitis and osteomyelitis though its status as a genuine respiratory pathogen has yet to be conclusively determined (21)Inconsistent to our study findingsin South African gold miners the prevalence of NTM among the miners was found to be high among the symptomatic HIV-positive miners with the most prevalent species identified being *M. Kasnsasii* (26%) *M. fortuitum* (18%) and *MAC* 18% (24) .

A range of potentially pathogenic NTM species were isolated in water and biofilms in the present study and the most common organism was *M. fortuitum,* followed by *M. gordonae, Mycobacterium species, M. boenickei, M. paragordonae, M. fortuitum (sub specie fortuitum),* and *M. celeriflavum*. This is in agreement with another study done in Zambia which isolated *M. fortuitum* as the most common NTM species isolated in water and different to a study by Monde et al. 2018 in which *M. gordonae* was the most isolated NTM species in water (25). These results indicate that the prevalence of certain NTM species may be a global phenomenon, suggesting similar environmental conditions or factors that may contribute to the consistent isolation of specific NTM species across diverse geographic locations(26)

*M. fortuitum* and *M. gordonae* are potentially pathogenic and have been associated with causing diseases in both immunocompetent and immunocompromised individuals(25)*M. fortuitum and M. fortuitum* *sub species fortuitum* belong to *Mycobacterium fortuitum complex*, a rapid growing mycobacteria which is the most common cause of pulmonary infection in immunocompromised patients, in addition it has the potential to cause diseases affecting the skin, soft tissues and post-surgical wounds(25) The *M. fortuitum* group comprises *M. fortuitum*, *M. peregrinum*, *M. senegalense*, *M. alvei*, *M. houstonense*, *M. neworleansense*, *M. boenickei*, *M. septicum*, and *M. porcinum* (27). It’s the most common species that has been isolated in water and this is in agreement with the study done by Velayati and colleagues (28).

On the other hand,*M. gordonae* is recognized as a common contaminant in laboratory and tap water and most prevalence species isolated from municipality and natural water sources. It occurs mostly as a contaminant or colonizer in immune competent people. However, it has the potential to be pathogenic in certain individuals, leading to systemic symptoms and, in advanced HIV patients, it may result in disseminated disease (29).

*M. cereriflavum,* it’s a rapid growing scotochromogenic *Mycobacterium,* first characterized and isolated in clinical specimen in 2015 in Iran from pulmonary samples (30). *M. boenickei* is a rapid growing and mostly commonly found in soil, dust and water. It belongs to a family of *Mycobacterium fortuitum* biovariant complex and it’s known to cause a wide variety of significant diseases in humans and are resistant to first line anti tuberculosis drugs(31).

*Mycobacterium species (*sub species unknown*)*, also known as atypical mycobacterium can be rapid or slow glowers NTM that cause a number of diseases in both children and adults who are both immune competent and immune suppressed (32,33). They are found in naturally and human- made environments, they also inhibit plumbing and human water treatments sources mainly in urban areas. They are highly resistant to water treatment chemicals used in treating water sources and colonizes drinking water, showers as well as hot tubs (34).

The current study found a high isolation of NTMs from shower heads biofilms compared to tap water which had the lowest number of NTM isolated. The findings from our study are similar to studies of other researchers done elsewhere(29,35,36, highlighting showerhead biofilms as a significant source of NTM, yielding higher NTM quantities compared to water samples. This phenomenon may be attributed to the enrichment effect facilitated by the waxy properties of bacteria, providing resistance to shear forces during shower operations (35). This high isolation rate of NTMs in shower heads could partly be due to high levels of organic matter and soil in waters (29) contributing to the survivability of the mycobacterial flora, this trend was observed in shafts T and N, where water, piped from the mine's underground systems, stored in tanks, and used for various purposes, facilitated biofilm formation (37). The piping systems in these shafts may contribute to biofilm growth, possibly influenced by certain materials like iron pipes (37). In contrast, the lower mycobacterial load in tap water from shaft T could be linked to the lethal effect of chlorine (25)**,** as this shaft receives chlorinated water from the local supply company, unlike the other two shafts. These findings underscore the prevalence of mycobacteria in biofilms within water distribution systems, emphasizing the need for further research on their implications for human health, particularly in mining environments.

The limited occurrence of NTM in active and ex-miners in this study is attributed to strict infection control measures imposed on miners diagnosed with TB in Zambia. According to the 1999 Workers' Compensation Act, individuals diagnosed with TB are prohibited from returning to work in the mines. Additionally, ex-miners undergo monthly screenings for TB and silicosis, with those diagnosed with TB promptly receiving treatment. In contrast, the study reveals a high prevalence of NTMs in water samples collected from three mining shafts, with 54.5% of the samples testing positive for NTM on culture. These NTMs, mainly saprophytic isolates, pose a potential risk to miners, especially those with compromised immune systems. The relatively high proportion of NTM species obtained through culture from mining environmental water distribution systems indicates a potential source for human infection. There is therefore need to conduct further studies to fingerprint the NTM isolates so as to establish the epidemiological link and assess the risk of NTM transmission in mining settings with more advanced molecular tests like whole genome sequencing.

**5.0 CONCLUSION**

The isolation of NTM species from humans, *Mycobacterium virginiense* and water, *Mycobacterium fortuitum* highlighted that active and ex miners are at potential risks of acquiring pathogenic NTM from mining environment. The epidemiological investigation of NTM in the study area is therefore recommended. This should include sampling from environmental sources such as water source before water distribution to ascertain source of contamination and soil.

**Abbreviations**

AFB: Acid-Fast Bacilli; AIDS: Acquired Immunodeficiency Syndrome; CI: Confidence Interval; DNA: Deoxyribonucleic Acid; HIV: Human Immunodeficiency Virus; LJ: Löwenstein-Jensen; MTC: Mycobacterium tuberculosis complex; NCBI: National Centre for Biotechnology Information; NTM: Non-tuberculous Mycobacteria; PCR: Polymerase Chain reaction; TB: Tuberculosis; WHO: World Health Organization; ZN: Ziehl Neelsen

**Conflicts of Interest:** The authors declare that there is no conflict of interest regarding the publication of this article.

**Ethics Consideration**

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**Availability of data and materials**

The data sets and materials used in the analysis of this current study are readily available from the corresponding author and can be accessed upon reasonable request.

**Authors’ contributions**

**OBS**, collected samples, analyzed the data and drafted the manuscript, **ICB** edited the manuscript, **JC**, supervised data collection at TDRC, **GC** supervised data collection, **BMH**, analyzed the data and supervised data collection, **VD**, helped in data analysis, **SZ**, analyzed and revised the manuscript, **MZ** revised the manuscript and helped me in lab work, **MM**, conceptualized the study, drafted the manuscript and supervised laboratory work. All authors read and approved the final version of the manuscript.

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