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Comparison of maldi-tof ms and 16s rrna sequencing for identification of a collection of clinical isolates of nonntuberculous mycobacteria in Kenya and describing their antimicrobial susceptibilities

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COMPARISON OF MALDI-TOF MS AND 16S RRNA SEQUENCING FOR IDENTIFICATION OF A COLLECTION OF CLINICAL ISOLATES OF NON-TUBERCULOUS MYCOBACTERIA IN KENYA AND DESCRIBING THEIR ANTIMICROBIAL SUSCEPTIBILITIES

By

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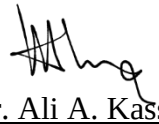

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DEDICATION

I dedicate my dissertation work to my family and friends who have supported me throughout my post-graduate training. Special thanks to Shiphrah and Esther who have been my source of joy. Not forgetting my loving mother Mrs. Racheal, without whose constant prayers and words of encouragement, I wouldn't have made it this far.

ABSTRACT

Study background: non-tuberculous mycobacteria (NTM) are members of the genus *Mycobacteria* that are ubiquitous in the environment. They are increasingly documented to cause multi-drug resistant human and animal infections in immunocompromised as well as immunocompetent hosts and thus, they are a huge burden on public health resources.

Diagnosis and treatment of these infections is particularly challenging due to their variable clinical presentation and the lack of easily accessible identification methods.

MALDI-TOF MS is a proteomic method that has gained traction in the identification of microbial organisms in clinical practice due to its fast turn-around time, high resolution, and cost-effectiveness but its utility in the identification of NTMs is still an area of ongoing research.

Broad objective: this study aimed to compare the diagnostic performance of MALDI-TOF MS against 16S rRNA sequencing as the gold standard in the identification of a collection of clinical isolates of NTMs in our laboratories in Nairobi and to describe their antimicrobial susceptibility patterns.

Study design and sites: This was a prospective laboratory-based study on clinical isolates that were collected from archived samples at Aga Khan university hospital and National TB reference laboratories, obtained between 2007 and 2021. MALDI-TOF analysis was done at the Kenyatta National hospital laboratory and 16S rRNA and whole genome sequencing at the Basel university Switzerland.

Material and Methods: sixty five archived clinical isolates were revived and analyzed using the VITEK®MS platform. Species identification was done by comparing spectra generated against spectra in the Knowledge base v3.2 database. Sequencing of the 16S rRNA genes was done for the isolates as the gold standard for identification. Resistome analysis was done from whole genome sequencing data using the CARD database.

Data management: Data were input and analyzed using Microsoft excel/ Statistical Package for Social Sciences (SPSS) software.

Results: The study found agreement between MALDI TOF MS and 16S rRNA sequencing at **81.3%** for RGM and **80.1%** for SGM. Overall, the level of agreement for NTM speciation in this study was at **81%**.

MALDI TOF, unlike sequencing, was unable to further classify the NTM isolates up to subspecies level.

Chromosomal and mutational resistance genes were found against drugs recommended for first line therapy.

Conclusion: This study showed agreement between MALDI TOF MS and 16S rRNA identification for non-tuberculous mycobacteria. The presence of several molecular markers of resistance is a finding of concern that requires further investigation.

LIST OF ABBREVIATIONS AND ACRONYMS

AFB	-	Acid-Fast Bacilli
AIDS	-	Acquired Immuno- Deficiency Syndrome
AKUHN	-	Aga Khan Hospital, Nairobi
AST	-	Antimicrobial Susceptibility Tests
ATS/IDSA	-	American Thoracic Society and The Infectious Diseases Society Of America
CARD	-	Comprehensive Antibiotic Resistance Database
HIV	-	Human Immuno-Deficiency Virus
MAC	-	Mycobacterium Avium Complex
MALDI TOF MS	-	Matrix-Assisted Laser Desorption/Ionization, Time of Flight Mass Spectrometry
MTBC	-	Mycobacterium Tuberculosis Complex
NTM	-	Non-Tuberculous Mycobacterium
NTRL	-	National Tuberculosis Reference Laboratory, Nairobi
PTB	-	Pulmonary Tuberculosis
RGM	-	Rapid Growing Mycobacteria
SGM	-	Slow Growing Mycobacteria
SOP	-	Standard Operating Procedure
STPHI	-	Swiss Tropical and Public Health Institute
TB	-	Tuberculosis
WGS	-	Whole Genome Sequencing
WHO	-	World Health Organization

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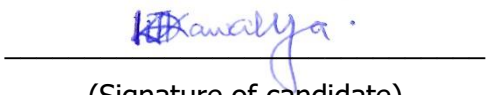
Thank you all

DECLARATION

I declare this dissertation does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university and that to the best of my knowledge it does not contain any material previously published or written by another person except where due reference has been made in the text.



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10-June-2022

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CHAPTER ONE: INTRODUCTION

Non-tuberculous mycobacteria (NTMs) like other members of the genus *Mycobacteria* are aerobic non-motile non-sporing Gram-positive acid-fast bacilli. They have a thick hydrophobic cell wall rich in mycolic acid which confers acid-fastness.

Most NTM species are non-pathogenic free-living organisms in the environment but an increasing number have recently earned recognition as important causes of human infection in immunocompromised as well as immunocompetent individuals (1).

These bacteria have the ability to form biofilm which enables them to persist in the environment as well as resist killing by antimicrobial agents and disinfectants. They also have several other mechanisms of drug tolerance/resistance which include drug efflux pumps, drug modifying enzymes as well as inducible and mutational drug resistance genes (2).

The over 190 known NTM species can be classified into two categories:

i) Rapid growing mycobacteria (RGM) which grow within 7 days on solid media. Many of these are frequently contaminants but others have been implicated in human infections. The most notably pathogenic species include *M. fortuitum* group, *M. abscessus* group and *M. chelonae* (3). These commonly infect skin, and soft tissue (4).

ii) Slow growing mycobacteria (SGM) need more than a week to grow on solid media (5). Examples include MAC, *M. kansasii, M. xenopi*, etc. These commonly infect the lungs and lymph nodes. *M. ulcerans* (causal agent for 'Buruli' ulcers) and *M. marinum* are notable for causing skin infections.

In humans, NTM disease may be grouped into 4 categories i.e., pulmonary disease, skin and soft tissue infections, visceral and disseminated infection. Visceral and disseminated infections are closely associated with immunosuppressive conditions (6).

Of these, pulmonary disease is the most common clinical presentation (7) and yet most severe due to the difficult in treating it. Looking at NTM species most frequently associated with lung disease; SGM include *M. avium* complex, *M. xenopi* and *M. kansasii*, while RGM include *M. abscessus* species (8).

CHAPTER TWO: LITERATURE REVIEW

Ecology of NTMs

Most NTMs are free-living saprophytes under various conditions in the environment. They have been isolated from both natural and man-made water sources where they persist despite disinfection. They can form biofilm in hospital water systems and equipment which makes them important causes of nosocomial infections. A case in point; *M. chimaera*, a SGM that belongs to the *M. avium* complex, was found to be the causal agent in a surge of post-op systemic infections among cardio-surgical patients after colonizing the heater-cooler systems used during the surgeries (9).

Epidemiology of NTMs

The incidence and prevalence of NTMs are on the rise globally (7). A study assessing data collected between 2008 and 2015 in the U.S. found that the incidence and prevalence of NTMs have continued to rise each year, especially in people with preexisting chronic lung disease and those above 50 years of age (10). The trend is the same worldwide and this has been suggested to be due to advancements in laboratory detection as well as the emergence of HIV/AIDS, especially in Sub-Saharan Africa (11).

Infections are most often acquired from the environment through the lungs, gastrointestinal tract, or both. Human-to-human spread is uncommon but rare cases have been documented to suggest this form of transmission for example, of *M. abscessus* in patients with cystic fibrosis (12) and *M. kansasii* between an elderly couple with pre-existing lung disease (13).

NTM infections, unlike *M. tuberculosis* complex (MTBC), are not notifiable, making epidemiological data concerning them scanty and scattered. Nonetheless, some data has shown that NTM infections vary by geographical region.

The NTM Network European Trials Group (NTM-NET) found that while *M.* avium dominated in the Americas and Europe, *M. intracellulare* dominated in South Africa and Australia (14). Also, a study in China found that higher altitudes were associated with an increase of MAC isolates whereas a decrease in altitude predicted an increase in RGM isolates (15).

In low resource countries like sub-Saharan Africa where the burden of HIV and PTB is high, many NTMs infections are misdiagnosed as tuberculosis (16).

In a systematic review that looked at 391 autopsies with histological or microbiological evidence of PTB or NTM disease in Sub-Saharan African HIV patients found that the weighted prevalence ratio of NTM versus TB was 0.16 indicating that for every seven HIV patients who died with mycobacterial infections, there was one who died with NTM infection (17).

NTMs and HIV

NTMs are facultative intracellular pathogens that can survive within macrophages (18). They are generally known to be of low pathogenicity but this changes in immunocompromised states like HIV/AIDS where they are notorious for causing disseminated infections.

MAC infections are typically seen in HIV patients with CD4 counts below 50 cells/µL, high viral loads above 1000 copies/ml, or with virological treatment failure.

WHO recommends prophylaxis using either azithromycin or clarithromycin, for disseminated MAC infection among HIV patients who are severely immunocompromised and are not on virally suppressive therapy (19).

NTMs and the One Health approach

One health is a multi-disciplinary approach integrating human, animal, and environmental health to solve complex issues like antimicrobial resistance and zoonotic diseases. Due to the ubiquitous nature of NTMs and the increasing use of pesticides and antimicrobials in the environment, it's important to examine how this may impact public health and antimicrobial resistance especially concerning NTMs.

A study in the Serengeti community in Tanzania examined NTM isolates from 472 human TB suspects, and 606 livestock, and wildlife tissue samples; and found several species with the potential to cause disease in both animals and humans (20).

NTMs have also been isolated from granulomatous lesions in farmed aquatic animals, many of which have high zoonotic potential. These included species like *M. marinum* and *M. chelonae*

(21). They therefore pose a significant public health risk, especially when factoring in their multidrug-resistant nature.

MALDI-TOF MS

Matrix-assisted laser desorption/ionization time of flight mass spectrometry is an emerging and promising proteomic technique in clinical microbiology. This technology has been around for a couple of decades but only recently has it found adaptation in microbiology as a quick and cost-effective method for identification of microbes among other functions.

Compared to conventional biochemical and molecular methods, it is simpler to use, less laborious, cheaper, very sensitive, and can provide microbe identification in just minutes (22).

MALDI-TOF MS utilizes differences in protein components of microbial cells to discriminate organisms even to species and strain level. It uses laser desorption and soft ionization technology which involves shooting a laser beam at a test sample covered by matrix on a target plate. A beam in the UV range is commonly used. The matrix for example α-cyano-4-hydroxycinnamic acid (4-HCCA) is an organic compound which assists desorption by increasing intermolecular distance between analyte particles, and preserves structural integrity of the sample during ionization by absorbing more of the energy from the laser beam.

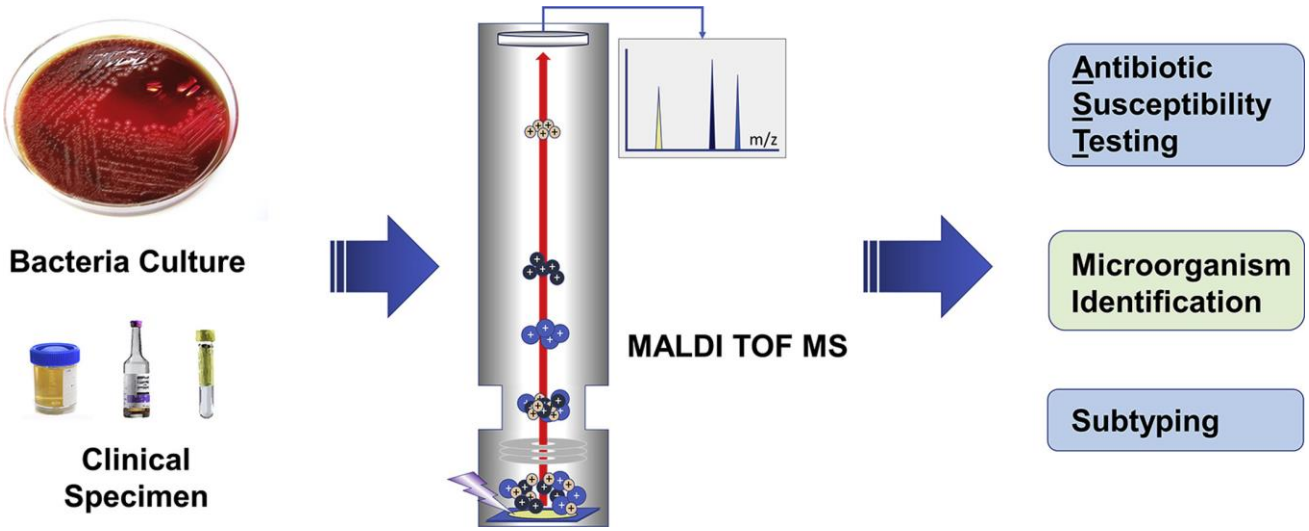


Figure 1: General Schematic for MALDI-TOF MS (23).

The sample and matrix solutions after coating onto target plate are air dried. As the matrix crystallizes, the sample co-crystallizes along with it. Upon irradiation by the laser beam, the analyte and matrix are desorbed into gaseous phase. The positively charged matrix transfer charge to the analyte particles. The protein particles that are desorbed/ionized often lie in the 2-20 kDa range, most of which are riboproteins (22).

The MALDI is coupled to a time-of-flight (TOF) mass spectrometer which uses a constant potential to accelerate the ionized particles through a vacuum tube.
The particles are separated according to their mass-to-charge ratio (m/z) and recorded as they reach the ion detector to produce a unique spectrum/peptide mass fingerprint (PMF). This is compared using analytical software to spectra of known organisms that are already in the system database to identify the unknown microorganism.

Unlike Gram negative organisms such as *Neisseria* species where whole cell samples can be coated onto target plates directly from growth media (22), mycobacteria require sample preparation prior to MALDI-TOF analysis. This is due to the complex and lipid-rich nature of the mycobacterial cell wall which limits access to the proteins in the cells, as well as for reasons related to safety (24). At the level of sample preparation, there is inactivation of the mycobacteria and disruption of their cell walls before extracting their protein components in a solvent mixture of formic acid and acetonitrile.

MALDI-TOF MS identification is limited by the robustness and quality of its spectral database and also the initial investment in purchasing the instrument is high.

The 16S rRNA is one of the most commonly used genes for bacterial phylogenetic studies and its variability allows for accurate speciation of majority of the NTMs(25). This quality makes 16SrRNA sequencing ideal as standard for comparison as a diagnostic tool.

Diagnosis of NTM pulmonary infection

Due to the ubiquitous distribution of NTMs, ATS/IDSA recommends differentiation of infection from colonization prior to initiation of therapy and/or ASTs (8). In the diagnosis of NTM chronic lung infection, it recommends demonstrating involvement by: chest imaging, AFBs on microscopy and presence of symptoms suggesting lung infection (26).

This, followed by identification of the bacilli to species level (in some cases subspecies level e.g., for *M. abscessus*) is the minimum requirement for diagnosis of NTM pulmonary disease(8).

The pathogenicity of the species identified needs to be put into consideration for example low pathogenic species like *M. gordonae* may require repeated isolation over a period of months with other supporting clinical and radiological evidence to make a diagnosis, whereas a single isolation of a more pathogenic species like *M. kansasii* may suffice in the right context (8).

Antimycobacterial susceptibility testing

Guidelines by the Clinical and laboratory standards institute (CLSI) for antimycobacterial susceptibility testing (AST) of NTMs are best adapted for the clinically relevant organisms. These include MAC, *M. Kansasii*, some RGM, *M. abscessus*, *M.* ulcerans, and *M.* marinum. The less common NTMs are still being studied.

The CLSI recommended method for ASTs of NTMs is the Mueller Hinton broth micro-dilution (27). This generates MIC (minimum inhibitory concentration) values for the various antibiotics that are interpreted as susceptible, intermediate and resistant. A standard inoculum is suspended in solution to (1-5) x 10^5 CFU/ml using a set turbidity consistent with a 0.5 McFarland standard(2).

For the different NTMs, varying ASTs are recommended based on correlation with clinical outcomes for example macrolides and amikacin for MAC and *M. abscessus* pulmonary disease, *erm* gene sequencing for inducible macrolide resistance in *M. abscessus*, and rifamycins for *M. kansasii* (2).

Though regarded as the gold standard, broth microdilution is laborious, complex and time consuming often requiring several weeks to get results. These reasons are prohibitive for this method especially in low resource settings like ours.

Molecular methods for detection of resistance genes are included in the guidelines to detect mutations associated with antimycobacterial resistance especially for amikacin and macrolides (27). These offer a more rapid alternative to broth microdilution.

WHO recently after a systematic analysis, endorsed a catalogue of resistance gene targets in *M. tuberculosis* complex for drug susceptibility testing (28). This was intended to encourage the use of molecular methods for resistance testing.

Treatment of NTM infection

Not all NTM lung infections require treatment. An assessment of virulence of the offending organism, risk versus benefit of treatment, patient wishes as well as availability of appropriate drugs have to be put into consideration before starting antimycobacterial treatment (8).

For treatment of NTM lung infection, ATS/IDSA recommends to use a multidrug regimen for a period of up to 12 months of culture negative sputum. For example in case of MAC lung infection, a macrolide with a rifamycin and ethambutol is recommended, with or without amikacin; and for *M. kansasii*, first line anti-TB drugs are efficacious (26). For RGM infections, therapy is tailored around in-vitro susceptibility results though often its macrolide based.

Mostly NTMs are treated with empirical regimens in the absence of AST, even though it is well known that various NTMs have unique susceptibilities and may need unique combinations of antimycobacterial therapy based on identification of the offending agent (29).

Drug dosing may be daily or thrice weekly depending on the clinical picture and ability to tolerate drugs and may last several months to years (8,26).

CHAPTER THREE: STUDY RATIONALE

Problem Statement

There is scarcity of published data on NTM clinical infections in Kenyan hospitals and practically no information on their antibiogram. HIV infection in Kenya, among other immunosuppressive conditions, increases the risk of NTM disease yet there is little to guide empirical treatment of the latter.

Diagnosis of NTM disease especially in resource limited places in Africa is a challenging task given that oftentimes investigations only go as far as chest radiography and acid-fast staining of sputum smears. Therefore, many NTM infections are misdiagnosed as TB, or worse still as multi-drug resistant TB because of their resistance to standard TB therapy.

In a clinic-based cohort study of 142 presumptive PTB in Mali, 12% (17/142) were diagnosed with NTM pulmonary disease. A portion of these (6/17), were coinfections with M. tb(16). In Botswana, a study isolated NTMs in as high as 56% of the sputum samples from 427 HIV patients with TB symptoms (30). Unfortunately, this study did not differentiate colonization from infection. Treatment of NTM colonization exposures the patients to unwarranted drug toxicities and wastage of the already limited public resources in the treatment of such patients.

Taking Kenya as an example, public laboratories that are tasked to survey multi-drug resistant tuberculosis are faced with the difficulty of speciating these infections whenever they are encountered.

MALDI-TOF MS is a very promising technique for microbial identification that has recently been made available in Nairobi, Kenya. It is a rapid alternative for the speciation of NTMs and therefore this study aimed to evaluate its diagnostic performance in comparison to 16S rRNA sequencing as the gold standard.

Despite the growing clinical importance of NTMs, infections are not notifiable and epidemiological data remains scattered and scanty with most of it coming from developed countries. Empiric treatment is therefore not guided due to lack of knowledge of local patterns of infection and drug susceptibilities.

This study and more to come, was needed to shed light on the patterns of NTM infections and their drug susceptibilities with the goal of guiding clinical practice.

Research Questions

1. How does MALDI TOF MS compare to 16S RNA sequencing in terms of diagnostic accuracy, for the speciation of NTMs?

2. What are the antimicrobial susceptibility patterns of the NTM clinical isolates?

3. What are the clinical features of patients with NTM isolates in Aga Khan university hospital, Nairobi?

Objectives

Primary objectives (Using an archived collection of NTM clinical isolates)

To compare the diagnostic performance of MALDI TOF MS to 16S RNA sequencing as the gold standard for NTM identification.

Secondary objectives (Using patient file reviews)

1. To describe antimicrobial susceptibility patterns of the NTM isolates
2. To describe clinical features of NTM infections in patients presenting at Aga Khan university hospital, Nairobi

CHAPTER FOUR: MATERIALS AND METHODS

Research design

This was a prospective laboratory-based study using an archived collection of NTMs.

Study variables

The organisms were identified to complex level and species level by MALDI TOF MS and this was compared with 16S rRNA sequencing identification.

Molecular predictions of drug susceptibilities were described from the whole genome sequencing data.

Study location

The Aga Khan University Hospital Nairobi laboratory. This site was chosen because it provided a robust collection of NTM clinical isolates including samples from sterile sites.

The National TB reference laboratory, Kenya. The site has a vast collection of NTM sputum isolates from various parts of the country.

Kenyatta National Hospital laboratory. The availability of MALDI TOF MS analysis for speciation of the isolates qualified this site as a study site.

Basel University Laboratory, Switzerland: 16S rRNA sequencing of the isolates was done here because of our established collaboration, and their vast experience and expertise in such studies.

Inclusion and exclusion criteria

NTM samples were selected consecutively based on viability of the isolates and availability of associated clinical history, from the newest sample backwards.

Study Isolates

Sixty-five NTM isolates from samples that had been archived between the period 2007 to 2021 at the Aga Khan University hospital Nairobi laboratory and respiratory samples from the National TB reference laboratory, Kenya, were revived and analyzed

Sampling procedure

The Aga Khan University, Nairobi mycobacteria laboratory is an enhanced biosafety level 2 facility, that is CAP (College of American Pathologists) accredited.

Clinical samples used, had earlier been collected from patients attending Aga Khan University hospital either as outpatients, or inpatients and from satellite labs. Handling of samples was as per SOP No: LMM:2015.01 (Appendix nine)

The samples were then processed as per SOP No: LMM 2114.01 (Appendix two) and SOP No: LMM 2128.01 (appendix eight).

Positive cultures on MGIT were ZN stained to demonstrate AFBs by microscopy and inoculated on to blood agar and MacConkey agar to exclude bacterial contamination. Positive bacterial contamination with positive AFBs, was followed by repeat decontamination.

TB GeneXpert & BD rapid tests were done to exclude MTBC as per SOP No: LMM 2111.01 (Appendix three).

Isolates with positive AFBs on microscopy, no bacterial contamination, and negative for MTBC were concluded as NTM and prepared for cryopreservation by inoculating the organisms in 2-ml cryovials containing 1.5 ml Middlebrook 7H9 medium, according to SOP No: LMM 2119 Maintenance of mycobacterial strains (Appendix five) and frozen at -85℃ where they remain viable for decades.

For this study, these clinical isolates stocked at the Aga Khan Hospital laboratory and at the National Tuberculosis reference laboratory were thawed and inoculated onto Löwenstein-Jensen (L-J) media and MGIT broth. They were then incubated at 37ºC for a maximum of 6 weeks. Positive cultures in MGIT broth were controlled for bacterial (other than mycobacteria) contamination by streaking onto blood and MacConkey agar and observing for no growth in 48hours.

Contamination was dealt with as according to SOP No: LMM 2110.01 “DEALING WITH MGIT CONTAMINATION” (Appendix four).

Presence of mycobacteria was confirmed by Ziehl Neelsen staining and microscopy for acid-fast bacilli.

Isolates from both L-J media and MGIT broth were then used for both MALDI - TOF and 16S rRNA sequencing identification.

Data collection procedures.

Sample preparation:

At the Kenyatta National Hospital laboratory, isolates grown on L-J solid media and MGIT broth were used for MALDI-TOF MS analysis. Sample preparation was done following the protocol recommended by the bioMerieux (Appendix six)

For the isolates on L-J media, a standard 1µL loop of bacterial specimen was picked and from the MGIT, 3ml of broth were centrifuged to obtain a pellet.

The organisms underwent inactivation and mechanical disruption by vortexing in a 1.5ml microtube containing 0.5mm glass beads. This step was done in a biosafety cabinet level 2.

Protein extraction step followed using 10µL formic acid 70% and acetonitrile. After centrifugation at 14000g for 2 minutes, 1 µL of supernatant was deposited on slots on the microtiter plate and allowed to dry. 1 µL of CHCA (α-Cyano-4-hydroxycinnamic acid) matrix was then added and also allowed to dry.

MALDI TOF analysis:

MALDI TOF MS analysis was performed using the VITEK® MS platform (software version 3.2 Knowledge Base – Clinical use) from bioMérieux, Marcy-l'Étoile, France. The database used for this study has unique spectra for 49 NTM taxa (Appendix seven). The VITEK MS is an automated mass spectrometry microbial identification system.

For each isolate, 2 spots were made on the plate and analyzed. Spots for quality control were also included for each run using E. coli ATCC 8739.

The protein spectra obtained were analyzed by the software comparing them with spectra in the system data base for identification of the organism

(31). Species results were recorded when both or 1 of 2 slots gave an identification while the other was unidentified.

DNA extraction.

Bacterial DNA was extracted from the 53 isolates using the Quick-DNA™ Fungal/Bacterial Miniprep kit from Zymo Research following the manufacturer's instructions(32).

Bacterial cells from LJ media picked using a 1 µL wire loop, and a pellet from centrifuging growth in MGIT tubes were used.

The bacterial cells were resuspended in 200 µL isotonic buffer and added in a Lysis tube containing bashing beads.

To the tube, 750 µL **BashingBead™ buffer** was added.

This was secured in a vortex and run at maximum speed for 10 minutes. After centrifuging the Lysis tube at 10,000g for 2 minutes, 400 µL of supernatant were added to a proprietary filter in a collection tube and centrifuged at 8000g for 1 minute.

To the filtrate in the collecting tube, 1200 µL of **Genomic Lysis buffer** was added.

Eight hundred (800) µL of this mixture was added to a DNA binding column and centrifuged at 10,000g for 1 minute. This step was repeated thus using a total of 1600 µL of mixture.

Impurities were washed from the column starting with 200 µL **DNA Pre-Wash buffer** spun at 10,000g for 1 minute, into a new collection tube.

This step was followed by a wash step with 500 µL **g-DNA Wash Buffer** added to the column and centrifuged at 10,000g for 1 minute.

With the column transferred to a clean 1.5ml microcentrifuge tube, 100 µL of **DNA Elution Buffer** was added and centrifuged at 10,000g for 30 seconds to elute the DNA.

DNA Extraction Quality control

All the DNA extracts were quality controlled using a NanoDrop spectrophotometer. The concentration ranged from 3.6 µg/ µL to 216.5 µg/µL with only 3 extracts below 11 µg/µL. The absorbances at A260/280 and A260/230 were mostly above 1.6 and below 1.9 respectively. This data showed that the DNA extracted was of good quality for sequencing.

PCR amplification of 16S rRNA gene

The 16S rRNA gene was amplified using LongAmp® Hot Start Taq DNA Polymerase (New England Biolabs) in a total volume of 50 µl. Forward primer 27F with anchor sequence (TTT CTG TTG GTG CTG ATA TTG CAG RGT TYG ATY MTG GCT CAG) and reverse primer 1492R with anchor sequence (ACT TGC CTG TCG CTC TAT CTT CRG YTA CCT TGT TAC GAC TT) were used at final concentrations of 0.2 µM each. Amplification was performed with the following PCR conditions: initial denaturation at 95 °C for 1 min, 25-30 cycles of 95 °C for 20 s, 55 °C for 30 s, and 65 °C for 2 min, followed by a final extension at 65 °C for 5 min. Amplification success was checked using agarose gel electrophoresis.

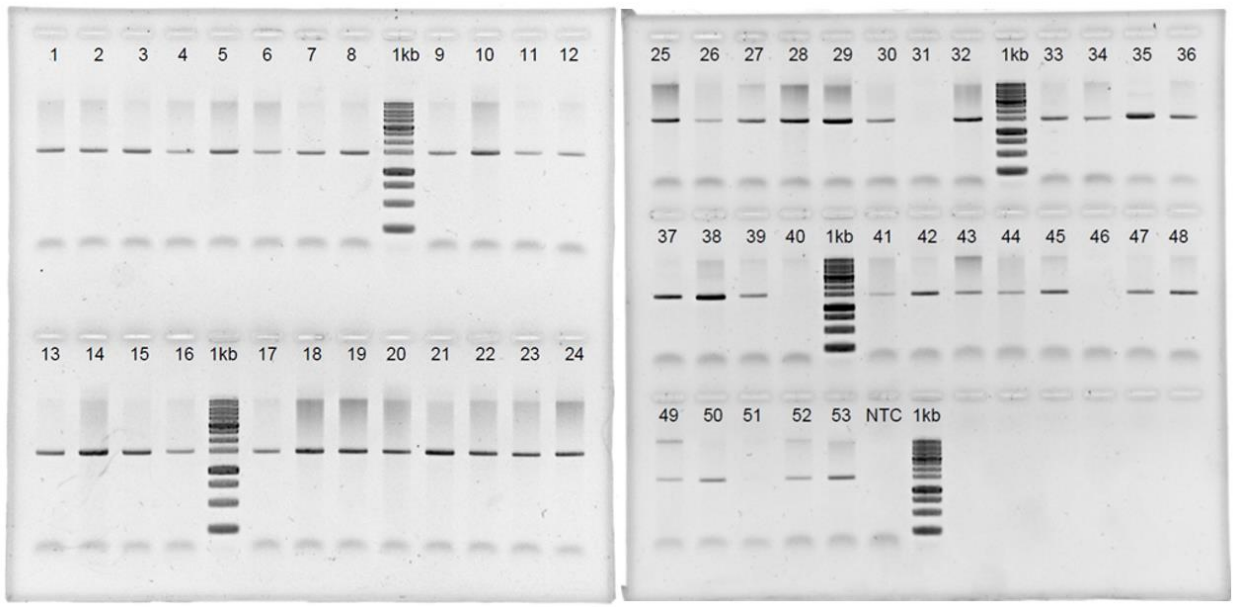


Figure 2: Gel electrophoresis of 16S rRNA amplicons

Nanopore library preparation

Amplified DNA was purified using 0.8x volume AMPure® XP beads (Beckman Coulter) and quantified with the Qubit dsDNA HS Assay Kit (Invitrogen). The sequencing library was prepared according to manufacturer's instructions using the Native Barcoding Kit 96 (SQK-NBD112.96). Briefly, 200 ng (200 fmol) amplicon DNA were end-prepped, unique barcodes were ligated and

sequencing adapters were ligated. The libraries were loaded onto the R10.4 flow cell (FLO-MIN112) and sequenced on the MinION Mk1C using high-accuracy base-calling for 2.5 hours.

16S rRNA analysis.

The 16S analysis was done using the Nanopore Epi2me 16S workflow with standard parameters. A quality score cut-off of 9 was applied. Reads between 1400 and 1800 base pairs were mapped against a 16S ribosomal RNA database. Species assignment for each sequencing read was extracted. The most common species was assigned to each sample.

Whole genome sequencing

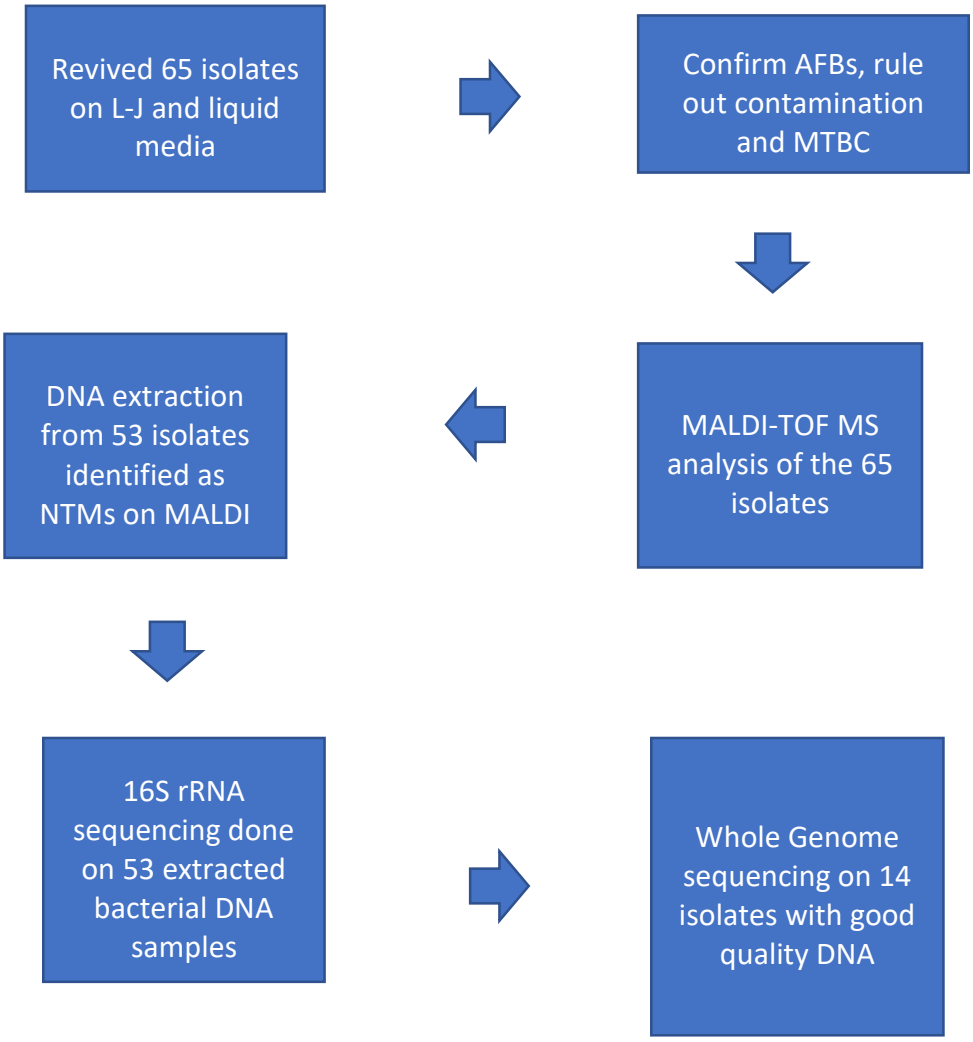
Nanopore library preparation

Extracted genomic DNA was quantified with the Qubit dsDNA HS Assay Kit (Invitrogen). Samples with DNA concentrations >33 ng/ µl were selected for whole genome sequencing. The sequencing library was prepared according to manufacturer's instructions using the Native Barcoding Kit 96 (SQK-NBD112.96). Briefly, 400 ng genomic DNA was nick-repaired, end-prepped, unique barcodes were ligated, and sequencing adapters were ligated. The libraries were loaded onto the R10.4 flow cell (FLO-MIN112) and sequenced on the MinION Mk1C using high-accuracy basecalling for 72 hours.

Data analysis

Sequences shorter than 1000 base pairs were removed. A quality score cut-off of 9 was applied. Sequences were aligned to the NCBI reference sequence of the species assigned by the 16S rRNA sequencing. Samples with sequencing coverage above 15 for >90% of the reference genome were subjected to de novo assembly. De novo assembly was conducted using canu 2.2 with parameters "canu -genomeSize = 5.5m maxInput Coverage = 100 minInput Coverage = 10 stopOnLowCoverage=10 -nanopore" at sciCORE (http://scicore.unibas.ch/) scientific computing core facility at University of Basel. The assemblies were annotated using RAST (Rapid Annotations using Subsystems Technology). Resistome analysis was performed using the Resistance Gene Identifier pipeline from the The Comprehensive Antibiotic Resistance Database (CARD).

Work flow.


Revived 65 isolates on L-J and liquid media
Confirm AFBs, rule out contamination and MTBC
MALDI-TOF MS analysis of the 65 isolates
DNA extraction from 53 isolates identified as NTMs on MALDI
16S rRNA sequencing done on 53 extracted bacterial DNA samples
Whole Genome sequencing on 14 isolates with good quality DNA

CHAPTER FIVE: RESULTS

Data management.

Data analysis was done using Microsoft Excel and Statistical Package for Social Sciences. Level of agreement was calculated to evaluate the performance of MALDI-TOF and output in terms of percentages of correct identifications.

Sample description

A total of 65 NTM isolates were revived on both Lowenstein Jensen media and MGIT broth, and analyzed by MALDI TOF MS.

Figure 3: Pigmented (L) and non-pigmented NTM isolate (R)

Of these, 12 were non-NTM environmental contaminants as shown in the table below.

Table 1: non-NTM identifications on MALDI TOF MS

serial no.	Media	MALDI ID
84	Solid	Chryseobacterium indologenes
85	Solid	Strep infantarius ssp coli
86	Solid	Actinomyces naeslundii
87	Solid	Ochrobactrum anthropi
88	Solid	Finegoldia magna
89	Solid	Prevotella ruminicola
90	Solid	Rhizorhapis suberifaciens
91	Liquid	Ewingella americana
92	Liquid	Bacillus cereus group
93	Liquid	Bacillus cereus group
94	Liquid	Achromobacter denitrificans
95	Liquid	Cutibacterium acnes

Fifty-three (53) NTM isolates with the relevant clinical history were identified on MALDI TOF MS analysis. Of these, 40 (75.5%) were clinical isolates archived at Aga Khan university hospital, Nairobi (AKUHN). The other 13 (24.5%) NTMs were from clinical isolates kept at National Tuberculosis Reference Laboratory, Kenya (NTRL). This is shown in Table 2 below.

Table 2: source of the NTM isolates

Sample source

		Frequency	Percent
Valid	AKUHN	40	75.5
	NTRL	13	24.5
	Total	53	100.0

These isolates were obtained from patients with varied characteristics. Their age ranged from 3 years to 77 years with a mean and median age of 44.5 years. This is summarized in figure 4

Statistics

Age

N	Valid	50
	Missing	3
Mean		44.52
Median		44.50
Skewness		-.133
Std. Error of Skewness		.337
Minimum		3
Maximum		77
Percentiles	25	32.75
	50	44.50
	75	56.25

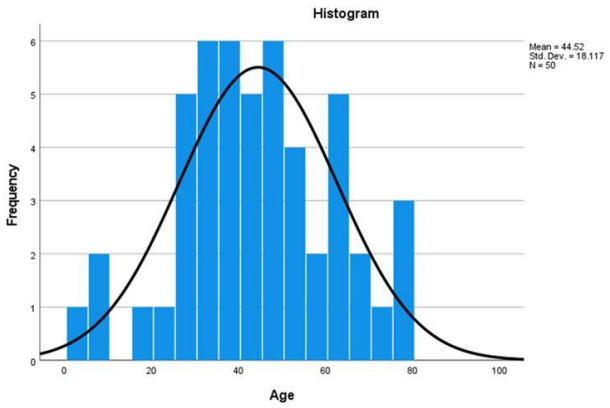


Figure 4: Age distribution of patients who provided samples for the NTM isolates

Prior history of treatment for pulmonary tuberculosis was not uniform between the sample source sites.

In our study, all the 13 isolates collected at NTRL were from patients previously treated for pulmonary tuberculosis. These were referred either as disease relapses or drug resistant infections. This was not the same for isolates from AKUHN where only 4 isolates were from patients previously treated from TB. Overall, 17/47 isolates were from patients with a history of prior treatment for tuberculosis.

Of the isolates obtained from AKUHN from patients with known HIV serostatus, the majority (21/33) were from HIV seropositive pateints. From NTRL, only 1/11 patients were HIV seropositive. Overall, 50% of the isolates of those with available HIV serostatus, were from HIV infected patients.

All the study patients were symptomatic with the majority (45/50, 90%) having respiratory symptoms. Most of the patients from AKUHN also had constitutional symptoms (31/35, 88.6%) that included fever, night sweats and significant weight loss. From NTRL, only 6/13 (46%) of the patients had documented constitutional symptoms.

The patients from whom the study material was obtained also had had diagnostic imaging done on them that included chest x-rays, and CT scans. Overall, 36/47 (76.6%) of the patients had imaging evidence of disease. Details are summarized in Table 3 below.

Table 3: Summary of clinical features of patients with NTM disease

		Sample source	
		AKUHN	NTRL
		Count	Count
TB treatment	absent	30	0
	present	4	13
HIV status	negative	12	10
	positive	21	1
Resp Sx	absent	5	0
	present	32	13
Constitutional Sx	absent	4	7
	present	31	6
Imaging	absent	6	5
	present	28	8

The isolated NTMs were grown from various clinical specimen including respiratory samples (15.1% Bronchial wash, 73.6% sputum), extrapulmonary samples (9.4% tissue that consisted of bone marrow, lymph node, pericardial, peritoneal, wound and corneal scraping) and pleural fluid (1.9%). This is as shown in Table 4 below.

Table 4: Distribution of clinical samples of the NTM isolates

Sample

		Frequency	Percent
Valid	Bronchial wash	8	15.1
	Pleural fluid	1	1.9
	Sputum	39	73.6
	Tissue	5	9.4
	Total	53	100.0

The distribution of isolates as grown on both LJ solid media and MGIT broth is shown in Table 5 below.

Table 5: Media used for growing the NTM isolates

Media

		Frequency	Percent
Valid	Liquid	34	64.2
	Solid	19	35.8
	Total	53	100.0

MALDI TOF Analysis

Of the 53 isolates identified on the VITEK® MS platform as non-tuberculous mycobacterial species, identifications fell into 11 taxa which included 4 taxa of rapidly growing mycobacteria (*M. abscessus, M. fortuitum, M. mucogenicum and M. neoaurum*) and 7 taxa of slow growing mycobacteria (*M. avium, M. gordonae, M. haemophilum, M. intracellulare, M. kansasii, M. simiae* and *M. szulgai*).

The identification was up to species and complex level as shown in the table below.

Table 6: NTM taxa identified on MALDI TOF MS

				Count
Runyon class	RGM	MALDI ID	M.abscessus	3
			M.fortuitum group	8
			M.mucogenicum	3
			M.neoaurum	2
	SGM	MALDI ID	M.avium	6
			M.gordonae	5
			M.haemophilum	1
			M.intracellulare	10
			M.kansasii	11
			M.simiae	3
			M.szulgai	1

Two replicate smears had been made on the MALDI micro-titer plate and identifications for these isolates were made on either both slots or 1 of 2 slots with no identification on the other.

Of the identifications of rapidly growing mycobacteria (RGM), 11/16 (68.8%) were identified on both slots on the MALDI micro-titer plate. Comparing with isolates that were identified as slow growing mycobacteria, 16/37 (43.2%) were identified on both slots. All were high confidence level identifications.

Table 7: Consistence of MALDI TOF identifications

Runyon class * ID SLOTS Crosstabulation

Count

		ID SLOTS		
		1	2	Total
Runyon class	RGM	5	11	16
	SGM	21	16	37
Total		26	27	53

16S rRNA sequencing analysis.

Of the 53 NTM isolates, 47 (88.6%) were identified by 16S rRNA sequencing as Mycobacterium species (5 amplified on repeat PCR and 6 (11.3%) were identified as environmental bacteria likely contaminants).

The sequencing reads showed highly consistent identifications with 44/47 (93.6%) identifications giving a single species identification with > 54% of the reads used. Three isolates had reads for 2 organism identifications (*M. haemophilum* and *Pseudomonas fluorescens* at 66.7% and 26.8% of the reads respectively, *M. kansasii* and *Microbacterium lacticum* at 40.3% and 36.7% of the reads respectively, *M. saskatchewanense* and *M. angelicum* at 47.4% and 32.2% of the reads respectively). The table below shows the detailed results from the 16S rRNA sequencing based identification.

Table 8: Detailed results from 16S rRNA sequencing

no.reads	Species 16S	Reads for species	Percent for species	Species 16S (second)	Reads for species (2nd)	Percent for species (2nd)
6509	M. simiae	6256	96.1%			
2705	M. kansasii	2596	96.0%			
7935	M. simiae	7611	95.9%			
1967	M. persicum	1878	95.5%			
4624	M. parascrofulaceum	4412	95.4%			
5609	M. persicum	5351	95.4%			
3263	M. gordonae	3112	95.4%			
5100	M. avium subsp. avium	4862	95.3%			
5049	M. neoaurum	4802	95.1%			
5214	M. paraintracellulare	4958	95.1%			
2825	M. gordonae	2685	95.0%			
5427	M. kansasii	5152	94.9%			
3124	M. persicum	2965	94.9%			
3738	M. paraintracellulare	3547	94.9%			
4476	M. gordonae	4240	94.7%			
4172	M. neoaurum	3952	94.7%			
5307	M. abscessus subsp. bolletii	4977	93.8%			
4535	M. aubagnense	4233	93.3%			
5658	M. fortuitum subsp. fortuitum	5248	92.8%			
1796	M. kansasii	1649	91.8%			
5284	M. neworleansense	4837	91.5%			
6819	M. phocaicum	6180	90.6%			
3221	M. avium subsp. avium	2912	90.4%			
2568	M. conceptionense	2310	90.0%			
2173	M. intracellulare subsp. chimaera	1953	89.9%			
1535	M. simiae	1334	86.9%			
7489	M. avium subsp. avium .	6345	84.7%			
6487	M. gordonae	5349	82.5%			
7302	M. fortuitum subsp. fortuitum	6012	82.3%			
8279	M. paraintracellulare	6689	80.8%			
5375	M. avium subsp. avium	4309	80.2%			
4913	M. persicum	3881	79.0%			
1333	M. tuberculosis	1034	77.6%			
4169	M. avium subsp. avium	3122	74.9%			
11890	M. paraintracellulare	8748	73.6%			
9758	M. conceptionense	7161	73.4%			
5895	M. neworleansense	4241	71.9%			
2627	M. kansasii	1815	69.1%			
7227	M. abscessus subsp. bolletii	4978	68.9%			
7476	M. haemophilum	4990	66.7%	Pseudomonas fluorescens	2004	26.8%
5439	M. abscessus subsp. bolletii	3621	66.6%			
9890	M. conceptionense	6371	64.4%			
2635	M. avium subsp. avium	1689	64.1%			
5438	M. peregrinum	3267	60.1%			
7979	M. simiae	4344	54.4%			
7202	M. saskatchewanense	3413	47.4%	Mycobacterium angelicum	2316	32.2%
6598	M. kansasii	2661	40.3%	Microbacterium lacticum	2424	36.7%

The mixed reads were likely due to contaminating genetic material in the samples. Of note, one sample was identified as *M. tuberculosis*.

Phylogenetic analysis

Using the MEGA genetics analysis software, the near complete 16S rRNA DNA reference sequences for the 18 mycobacterial taxa identified among the study isolates were input from the NCBI GenBank.

These sequences were aligned; unaligned sequences on both ends were trimmed. Phylogenetic analysis was then done using the maximum likelihood phylogenetic method, bootstrapped at 1000 replications and using the **general time reversible substitution model** to generate a cladogram.

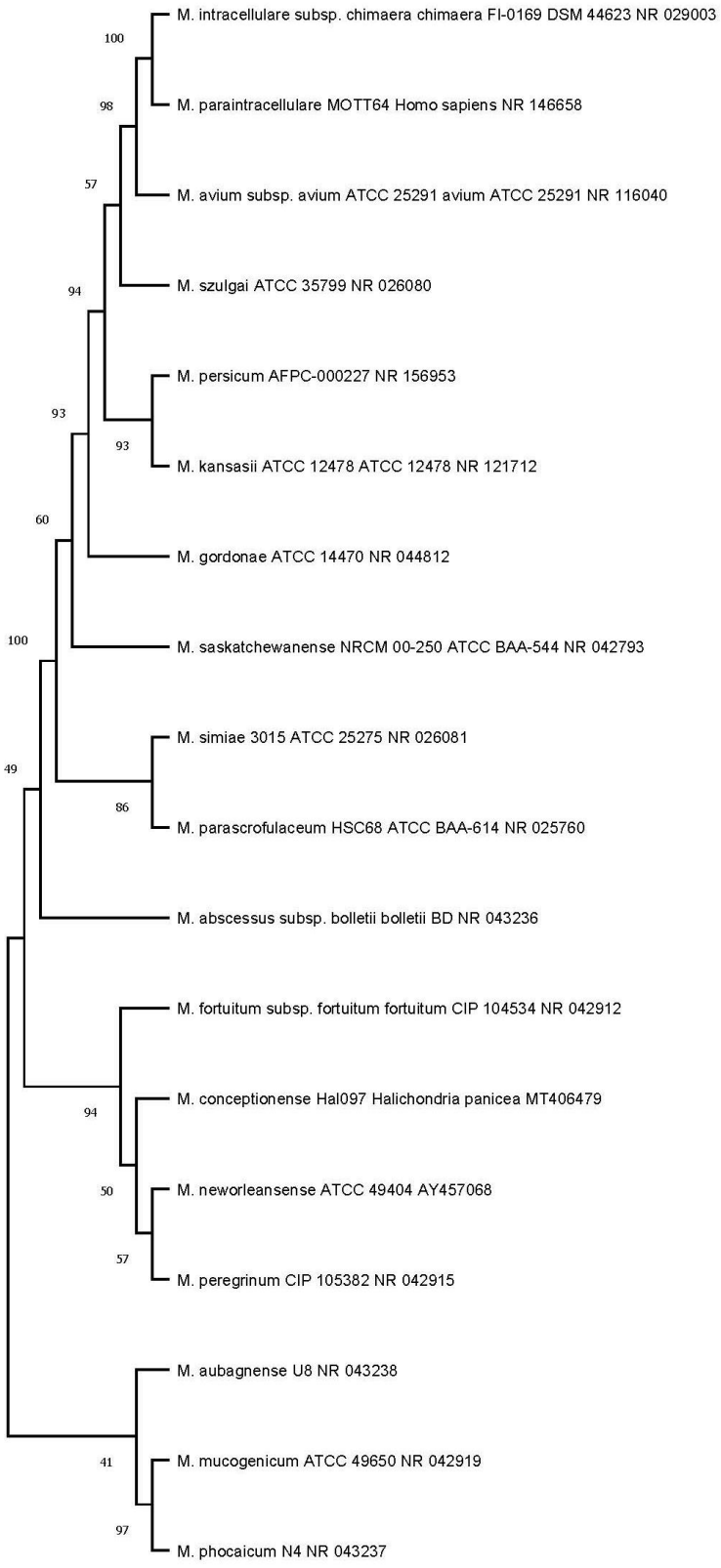


Figure 5: Cladogram for the NTM isolates

Comparing MALDI TOF against 16S rRNA sequencing identification

Comparing the species identifications by MALDI TOF MS against 16S rRNA sequencing revealed good agreement for most of the isolates.

For the isolates that MALDI TOF identified as *M. abscessus* (2), *M. avium* (5), *M. neoaurum* (2), *M. simiae* (3); there was complete agreement with 16S rRNA sequencing with sequencing further characterizing the isolates to subspecies level. These included *M. abscessus* MALDI taxon identified as *M. abscessus subsp. bolletii*, and *M. avium* for *M. avium subsp. avium*.

For the M. fortuitum group, MALDI identifications matched those of sequencing for 7/8 isolates. Of these, sequencing further speciated the isolates for example the taxa *M. conceptionense, M. neworleansense, M. peregrinum* and *M. fortuitum subsp. fortuitum*, all belong under the fortuitum group. A single isolate of *M. persicum* was mis-identified as *M. fortuitum* group by MALDI TOF.

For M. intracellulare identifications, 5/8 were in agreement with sequencing results with more detailed speciation and cross identification. These included *M. intracellulare subsp. chimaera* and *M. paraintracellulare* (cross-ID). The 3/8 mis-identifications *as M intracellulare* were *M. abscessus subsp. bolletii, M. kansasii*, and *M. simiae*.

For *M. gordonae* as identified on MALDI TOF, there was agreement with sequencing identification for 4/5 of the isolates. A single isolate of *M. parascrofulaceum* was mis-identified as *M. gordonae* on MALDI.

MALDI identification *M. kansasii* had 7/9 matched results with sequencing; 3 being cross identification with *M. persicum*. The mis-identified isolates were *M. conceptionense* and *M. tuberculosis*.

M. mucogenicum had 2/3 match-identifications with *M. aubagnense* and *M. phocaicum* on sequencing which belong in the *M. mucogenicum* group. A single isolate of *M. avium subsp. avium* was mis-identified as *M. mucogenicum* of MALDI.

There was one isolate of M. saskatchewanense that was mis- identified on MALDI TOF as M. szulgai. This data is summarized in the table below.

Table 9: Diagnostic agreement between MALDI TOF and 16S rRNA sequencing

						Count
MALDI ID	M.abscessus	Species 16S rRNA	M. abscessus subsp. bolletii	Agreement	yes	2
	M.avium	Species 16S rRNA	M. avium subsp. avium	Agreement	yes	5
	M.fortuitum group	Species 16S rRNA	M. conceptionense	Agreement	yes	2
			M. fortuitum subsp. fortuitum	Agreement	yes	2
			M. neworleansense	Agreement	yes	2
			M. peregrinum	Agreement	yes	1
			M. persicum	Agreement	no	1
	M.gordonae	Species 16S rRNA	M. gordonae	Agreement	yes	4
			M. parascrofulaceum	Agreement	no	1
	M.haemophilum	Species 16S rRNA	M. haemophilum	Agreement	yes	1
	M.intracellulare	Species 16S rRNA	M. abscessus subsp. bolletii	Agreement	no	1
			M. intracellulare subsp. chimaera	Agreement	yes	1
			M. kansasii	Agreement	no	1
			M. paraintracellulare	Agreement	yes	4
			M. simiae	Agreement	no	1
	M.kansasii	Species 16S rRNA	M. conceptionense	Agreement	no	1
			M. kansasii	Agreement	yes	4
			M. persicum	Agreement	yes	3
			M. tuberculosis	Agreement	no	1
	M.mucogenicum	Species 16S rRNA	M. aubagnense	Agreement	yes	1
			M. avium subsp. avium	Agreement	no	1
			M. phocaicum	Agreement	yes	1
	M.neoaurum	Species 16S rRNA	M. neoaurum	Agreement	yes	2
	M.simiae	Species 16S rRNA	M. simiae	Agreement	yes	3
	M.szulgai	Species 16S rRNA	M. saskatchewanense	Agreement	no	1

Of the SGM, misidentifications as RGM on MALDI included *M. persicum (M. fortuitum), M. avium subsp. avium (M. mucogenicum)*, and RGM misclassified as SGM included *M. conceptionense (M. kansasii), M. abscessus subs. bolletii (M. intracellulare)*.

SGM misidentified as SGM included *M. saskatchewanense (M. szulgai), M. kansasii (M. intracellulare), M. simiae (M. intracellulare), M. tuberculosis (M. kansasii)* and *M. parascrofulaceum (M. gordonae)*

An agreement was found between MALDI TOF MS and 16S rRNA sequencing at **81.3% (13/16)** for RGM and **80.1 (25/31)** for SGM. Overall, the level of agreement for NTM speciation with MALDI TOF in this study was at **81% (38/47)**. Unlike sequencing, MALDI TOF was

unable to further classify the isolates up to subspecies level. This was summarized in the table below

Table 10: Summary of agreement by taxa

MALDI ID * Agreement Crosstabulation

Count

		Agreement		
		no	yes	Total
MALDI ID	M.abscessus	0	2	2
	M.avium	0	5	5
	M.fortuitum group	1	7	8
	M.gordonae	1	4	5
	M.haemophilum	0	1	1
	M.intracellulare	3	5	8
	M.kansasii	2	7	9
	M.mucogenicum	1	2	3
	M.neoaurum	0	2	2
	M.simiae	0	3	3
	M.szulgai	1	0	1
Total		9	38	47

Of the taxa that were misidentified on MALDI TOF MS, 6/9 were SGM. This difference was not found to be statistically significant at p= 0.96 (significant at < 0.05, Pearson Chi-square)

Table 11: Summary of the diagnostic disagreements

MALDI ID	**Species 16S rRNA**	**Agreement**	**Runyon**
M.szulgai	M. saskatchewanense	no	RGM
M.intracellulare	M. abscessus subsp. bolletii	no	RGM
M.kansasii	M. conceptionense	no	RGM
M.mucogenicum	M. avium subsp. avium	no	SGM
M.fortuitum group	M. persicum	no	SGM
M.intracellulare	M. kansasii	no	SGM
M.gordonae	M. parascrofulaceum	no	SGM
M.intracellulare	M. simiae	no	SGM
M.kansasii	M. tuberculosis	no	SGM

Table 12: Crosstabulation of Runyon class vs agreement

		Agreement		
		no	yes	Total
		Count	Count	Count
Runyon	RGM	3	13	16
	SGM	6	25	31

Chi-Square Tests

	Value	df	Asymptotic Significance (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.002[a]	1	.960		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.003	1	.960		
Fisher's Exact Test				1.000	.642
N of Valid Cases	47				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.06.

b. Computed only for a 2x2 table

Analysis of resistance genome

Whole genome FASTA sequences for 14 samples were analyzed for resistance genes using the Resistance Gene Identifier (RGI 5.2.1) web portal on the Comprehensive Antibiotic Resistance database (CARD 3.2.2) online platform.

Chromosomally encoded resistance genes were found against several drug classes including aminoglycosides, macrolides, and tetracyclines.

The results were as follows.

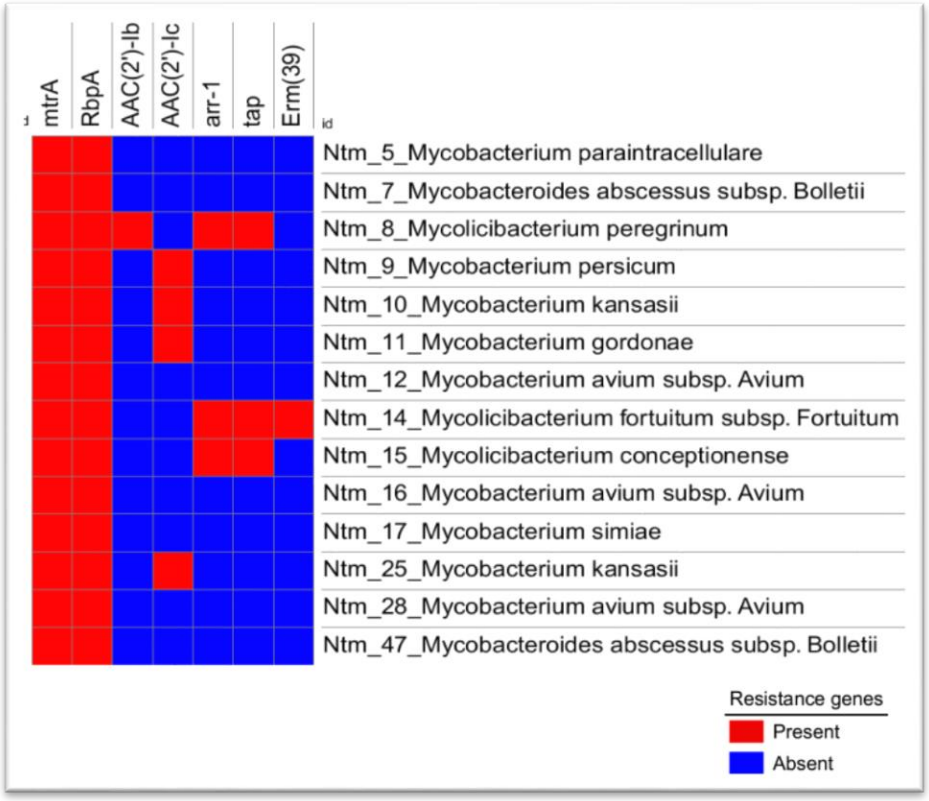


Figure 6: Summary of chromosomally encoded resistance genes

Erm; ribosomal RNA methyltransferase gene (inducible macrolide resistance)

Tap; major facilitator superfamily (MFS) antibiotic efflux pump gene (confers resistance to tetracyclines)

arr-1; rifampin ADP-ribosyl-transferase (Arr) gene (confers resistance to rifamycins)

AAC(2′)-Ib/c; chromosomally-encoded aminoglycoside acetyltransferase gene (confers resistance to aminoglycosides)

RbpA; RNA-polymerase binding protein gene (confers resistance to rifamycins)

mtrA; resistance-nodulation-cell division (RND) antibiotic efflux pump (confers resistance to penam, a macrolide antibiotic)

Table 13: Summary of drug resistance mutations in study isolates

Isolate	Species	*rrl* A2145G, A2045G	*rrl* A2274G	rrl A2059G	murA C117D	ribD G8R	Drug
5	M. paraintracellulare						capreomycin, clarithromycin, fosfomycin
7	M. abscessus subsp. bolletii						macrolide
8	M. peregrinum						fosfomycin
9	M. persicum						capreomycin, clarithromycin, fosfomycin
10	M. kansasii						capreomycin, clarithromycin, fosfomycin
11	M. gordonae						clarithromycin, fosfomycin
12	M. avium subsp. avium						capreomycin, clarithromycin, fosfomycin
14	M. fortuitum subsp. fortuitum						fosfomycin
15	M. conceptionense						fosfomycin
16	M. avium subsp. avium						capreomycin, clarithromycin, fosfomycin
17	M. simiae						clarithromycin, fosfomycin, Para-aminosalicylic acid
25	M. kansasii						capreomycin, clarithromycin, fosfomycin
28	M. avium subsp. avium						capreomycin, clarithromycin, fosfomycin
47	M. abscessus subsp. bolletii						macrolide antibiotic

CHAPTER SIX: DISCUSSION.

This study aimed at comparing the utility of using MALDI TOF MS versus using 16S rRNA in the identification for non-tuberculous mycobacteria. MALDI TOF MS identified the 53 NTM isolates to complex/species level by comparing the protein spectral fingerprints generated from the organisms against the Knowledge base v3.2 database.

16S rRNA gene sequence is approximately 1500bp and highly conserved across bacterial species but yet contains variable regions that are species-specific. These variable regions are useful for species identification.

Sequencing classified 42 isolates into 18 non-tuberculous mycobacterial taxa. These, unlike the 11 MALDI taxa were identifications up to subspecies level. This is attributed to the high discriminatory power of the 16S rRNA sequence. Of note is that while the current VITEK MS has spectra for 49 NTM taxa, the 16S rRNA sequence NCBI (National Center for Biotechnology Information) database has sequence entries of over 190 NTM taxa.

The commonest NTM species isolated in this study were *M. kansasii*, *M. avium* and *M. fortuitum*. These are all pathogenic NTMs. *M. kansasii* and *M. avium* are both SGM that have long been known to cause disease. *M. kansasii*, unique for its photochromogenicity, causes pulmonary disease that is indistinguishable from tuberculosis while *M. avium* is known to cause disseminated infection especially in HIV/AIDS patients. Among the RGM that are known to cause infections, *M. fortuitum* stands out as a frequent cause of non-pulmonary disease(33).

In this study, 4 isolates were identified as *M. persicum*, a member of the *M. kansasii* complex that was originally isolated from respiratory samples of patients with pulmonary disease in Iran (Persia). *M. persicum* is almost phenotypically identical to *M. kansasii* (34) . Its pathogenic potential can only be speculated as of today, from its isolation from clinically relevant samples (35). Considering that the VITEK MS database v3.2 used in this study did not include unique spectra for *M. persicum*, 3/4 of these isolates were identified by MALDI TOF as *M. kansasii*. (One isolate was mis-identified as *M. fortuitum*).

It is important to further speciate members of the *M. fortuitum* complex due to the varied presence of inducible drug resistance genes like the *erm* gene. For example, studies suggest that *M. conceptionense* may not have a functional erm gene (36). This information may be necessary when initiating empiric treatment where such isolates may be macrolide susceptible.

This study also found that; in contrast to MALDI TOF, sequencing further speciated members of the fortuitum group to include *M. conceptionense*, *M. peregrinum*, *M. fortuitum* and *M. neworleansense*. Although the VITEK MS data (Appendix seven) contained unique spectra for *M peregrinum*, and *M. fortuitum subsp. fortuitum*, no subspecies identifications were made for these isolates.

Our phylogenetic analysis, consistent with previous studies shows that *M. paraintracellulare* and *M. intracellulare* are monophyletic(37). MALDI TOF cross-identified these isolates because of their phenotypic similar and also because the MALDI database did not have unique spectra for *M. paraintracellulare*.

MALDI TOF mis-identified 9 isolates. Of these, 4 taxa are not included in the VITEK MS knowledge base v3.2 database. These include *M. conceptionense, M. persicum, M. saskatchewanense,* and *M. parascrofulaceum.* A study that compared spectra databases showed that the VITEK MS was more likely to mis-identify a microorganism if it were absent from its database (38).

One isolate of M. tuberculosis was mis-identified as M. szulgai on MALDI TOF. Since both organisms have unique spectra in the VITEK clinical-use database, it is unclear as to how this was possible but NTM-M.tb mixed infection could be argued.

The identification of *M. persicum* and *M. conceptionense,* pathogens of unclear risk as *M. kansasii* and *M. fortuitum* respectively, which are organisms with established pathogenicity and drug resistance profiles highlights the short-comings of the VITEK MALDI TOF MS platform in failing to further classify NTMs beyond species level.

Molecular markers of resistance

Macrolides and amikacin are the basis of therapy for MAC and *M. abscessus* pulmonary disease (8). This current study found no inducible *erm* resistance genes in the *M. abscessus* isolates but found mutations in the 23S rRNA gene *rrl* gene conferring resistance to macrolides (A2059G) and capreomycin (A2145G, A2045G). This study is in agreement with other studies that have documented *M. abscessus* as one of the most difficult to treat infection due to its several mechanisms of resistance to antimycobacterial drugs including macrolides, aminoglycosides and rifamycins (39)(40)(41).

The isolates belonging to the *M. avium-intracellulare* complex had mutations in the *rrl* gene conferring resistance to capreomycin (A2145G, A2045G), clarithromycin (A2274G) and Fosfomycin (murA C117D). Though these mutations may suggest macrolide resistance, this phenotype has been documented to occur at low rates even in patients on chronic macrolide treatment (42,43). Nonetheless, presence of the resistance mutations raises possibility of poor clinical response to treatment.

Rifamycins are the backbone for the treatment of *M. kansasii* infections. Isolates belonging to the *M. kansasii* group including *M. persicum* all had the RbpA gene that increases tolerance to rifamycin. Rifamycin resistance in mycobacteria is commonly associated with presence of the rpoB gene (44,45). This was not found in isolates in this study. One isolate of *M. persicum* was fond to have the arr-1 gene which has been associated with resistance to rifamycins. The arr-1 gene was first isolated from *M. smegmatis* but has since been documented to be widely distributed among bacterial phyla (46).

M. fortuitum infections can be treated with a two-drug regimen based on in vitro ASTs comprising fluoroquinolones, doxycycline, amikacin, or sulfonamides (47). Isolates belonging to the *M. fortuitum* group had chromosomally encoded genes conferring resistance to tetracyclines (tap). The *M. peregrinum* isolate (isolate 8) also had resistance genes to aminoglycosides - AAC(2')-Ib. Unlike the M. fortuitum subsp. fortuitum isolate, both *M. conceptionense and M. peregrinum* isolates lacked the inducible erm gene suggesting possible susceptibility to macrolides.

Of note, all isolates had the RbpA gene suggesting tolerance to rifamycins. The RbpA gene is known to encode for RNA polymerase (RNAP) binding protein A (RbpA) which is a transcription factor required for viability and tolerance to rifampicin in mycobacteria(48).

It is important to keep in mind that genotypic resistance markers may not necessarily translate to phenotypic resistance due to several mechanisms that may affect gene expression including environmental and epigenetic factors(49).

CHAPTER SEVEN: CONCLUSION

This study found the level of agreement between MALDI TOF MS and 16S rRNA identification for non-tuberculous mycobacteria at 81.3% for RGM and 80.1% for SGM. Overall agreement was at 81%. MALDI TOF identification was limited to species and complex level. None of the isolates was identified to subspecies level, a limitation that is crucial especially for some species like M. abscessus and M. fortuitum where subspecies identifications may help guide therapy. This limitation is most likely attributed to the diversity of the VITEK MS (clinical use) spectral database.

Nonetheless, this finding is in agreement with other studies that have proposed MALDI TOF MS as a rapid and cost-effective alternative to 16S rRNa sequencing, for identification of non-tuberculous mycobacteria from clinical samples.

The presence of resistance genes in the isolates against antibiotics recommended for therapy may suggest resistance. This, in concert with recommendations for treatment of NTM pulmonary disease(8) based on susceptibility testing results, emphasizes the need for susceptibility driven therapy over empirical treatment.

Isolates belonging to the M. fortuitum group other than M. fortuitum subsp. fortuitum may be macrolide susceptible.

Limitations

Due to the COVID pandemic, travel restrictions as well as restrictions on shipping of the clinical isolates both delayed the study considerably and limited the extent of analysis originally intended for these isolates.

This study was also unable to correlate the findings of molecular markers of resistance with resistance phenotypes. Nonetheless, these findings still raise awareness to the possibility of multi- drug resistance among NTM clinical isolates and the need for phenotypic susceptibility testing.

CHAPTER EIGHT: ETHICAL CONSIDERATIONS

This study was given ethical approval from the institution's ethics review committee.

All isolates were de-identified / anonymized and assigned study codes for identification.

Patient files were reviewed for predetermined salient associated clinical features. Confidentiality was upheld. All physical study documents were kept in a desk cabinet under lock and key. All soft files were kept in a secure folder on a computer that was password protected and accessed only by the investigator.

CHAPTER NINE: RECOMMENDATIONS

Aga Khan hospital, Nairobi has been outsourcing all NTM isolates for speciation, a process that is not only protracted but also expensive. As we move to acquire MALDI TOF MS, the data generated from this study provides information about the performance of this method for the speciation of local NTM isolates against established methods like 16S rRNA sequencing.

From the findings of this study, MALDI TOF MS performs satisfactorily well in the identification of clinically relevant NTM species and therefore recommended for this purpose in routine clinical isolates.

From the whole genome data, several molecular drug resistance markers against recommended antimycobacterial agents were found. This data can be used in clinical practice to encourage susceptibility testing prior to initiating treatment, a practice that already is encouraged over empirical therapy approach.

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APPENDICES

APPENDIX ONE: DATA COLLECTION TOOL

NON-TUBERCULOUS MICOBACTERIA STUDY DATA COLLECTION TOOL

SAMPLE DESCRIPTION

SPECIMEN NO: **SAMPLE TYPE:**

SERIAL NO: **DATE OF COLLECTION:**

CLINICAL DATA

AGE: **SEX:**

SYMPTOMS:

- ☐ **RESPIRATORY**
- ☐ **CONSTITUTIONAL**

HIV STATUS: **PRIOR TB TREATMENT**

STATUS: (Y / N)

OTHER COMMOBIDITIES:

SAMPLE PROCESSING

DATE OF 1ST SUBCULTURE:

- ☐ **AFBs PRESENT**
- ☐ **NO BACTERIAL GROWTH**

DATE OF 2ND SUBCULTURE:

GROWTH ON L J MEDIA:

MALDI TOF MS ID:

16S rRNA SEQUENCING ID:

DRUG SUSCEPTIBILITY PROFILE: RIFAMPICIN ☐ ISONIAZID ☐

ETHAMBUTOL ☐

AMIKACIN ☐ MACROLIDE ☐ QUINOLONE ☐

SIGNATURE:

APPENDIX TWO: SOP No: LMM 2114.01 SPECIMENS PROCESSING FOR ISOLATION OF MYCOBACTERIA USING BD MGIT™ SYSTEM

1. PURPOSE/INTRODUCTION:

1.1. PURPOSE: This SOP gives direction on how to process specimens for isolation of Mycobacteria using BD MGIT™ system, at The Aga Khan University Hospital- Nairobi, Department of Pathology, Microbiology Section.

1.2. INTRODUCTION: Pre analytical specimen processing in the diagnosis of pathogenic mycobacteria is very important. Inadequate or improper preparation of a patient specimen submitted for mycobacterial testing can lead to contamination of cultures and subsequent delays in reporting clinically significant results.

2. SCOPE / RESPONSIBILITY

2.1. SCOPE: This document applies to all Laboratory Technologists in Microbiology Section of the Pathology Department.

2.2. RESPONSIBILITY: It is the responsibility of all the microbiology staff to adhere to this SOP

3. DEFINITIONS AND ABBREVIATIONS:

3.1. SOP -Standard Operating Procedures
3.2. AKUHN- Aga Khan University Hospital Nairobi
3.3. N/A- Not Applicable
3.4. LMM- Laboratory Microbiology and Molecular Biology
3.5. MGIT- Mycobacterium Growth Indicator Tube

4. SPECIMEN:

Recommended Specimens	-Collection Notes	-Pre-Analytical Processing
-Sputum -Specimens other than sputum (extra-pulmonary): -Pus and other mucopurulent specimens -Gastric aspirates -Bronchial washings -Laryngeal swabs -Tissue -Urine	-Specimens should be collected aseptically in sterile containers with a tight-fitted lid or cap.	-Process clinical specimens as soon as possible or refrigerate them at 2-8^{o}C

-Other body fluids		

5. EQUIPMENT / SUPPLIES/ REAGENTS:

-Equipment	Supplies	Reagents
-Centrifuge(preferable refrigerated) with a minimum 3000 x g force and safety shield/caps -Vortex mixer -Timer	-Disposable 50 ml plastic tubes (Falcon tubes) -Sterile transfer pipettes	-Sterile NaOH-NALC-sodium citrate solution -Phosphate buffer pH 6.8 (0.067M).

6. SAFETY PRECAUTIONS

6.1.1. Personal protective equipment including proper protective gown, gloves and respirator masks (N95) must be worn at all times in the Bio-safety Level 3 areas.

6.1.2. Exercise the normal precautions required for handling infectious material.

6.1.3. Dispose of all waste materials in accordance with local guideline.

6.1.4. Perform all mycobacteria specimen processing in a Bio-safety Level 3 suite with a biological safety cabinet Class ll

6.1.5. Perform all specimen manipulation, and smear preparation in a Class ll BSC over disinfectant soaked towels or absorbent surface liners.

6.1.6. BSC must be certified at least annually to ensure that HEPA filters are functioning properly and that airflow rates meet specifications.

6.1.7. Immerse used transfer pipettes and sticks in discard container with an appropriate disinfectant. Tightly seal discard container prior to removal from the BSC.

7. METHODOLOGY:

7.1. PRINCIPLE OF PROCESSING

7.1.1. Sputum specimens are viscous materials contaminated with normal flora. Other specimens may also contain contaminants and/or require freeing of the TB bacilli from the specimen.

7.1.2. Processing involves pre-treatment of the specimen:

7.2. Digestion: to free the TB bacilli from the mucus, cells or tissue in which they may be embedded. The digestion agent is N-Acetyl-L-Cysteine (NALC). Na Citrate is applied to bind heavy metal ions that could inactivate the NACL. NACL, a mucolytic agent, liquefies sputum releasing trapped contaminants in addition to freeing TB bacilli.

7.3. Decontamination: to eradicate contaminants that grow more rapidly than TB and would interfere with the ability to recover TB. The decontaminating agent

is NaOH

7.4. Homogenization of the digested materials

7.5. Concentration of the TB bacilli by centrifugation before smear preparation and media inoculation

8. PROCEDURE

8.1. Sputum

8.1.1. If specimen is not collected in a 50 ml centrifuge tube, transfer it to a 50 ml centrifuge tube with a screw cap.

8.1.2. Add NaOH-NALC-sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap.

8.1.3. Vortex lightly or hand mix for about 15-30 seconds. Invert the tube so the whole tube is exposed to the NaOH-NALC solution.

8.1.4. Wait 15-20 minutes (up to 25 minutes maximum) after adding the NaOH-NALC solution. Vortex lightly or hand mix/invert every 5-10 minutes or put the tubes on a shaker and shake lightly during the whole time.

8.1.5. Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder (30-35 grams) directly to the specimen tube. Mix well.

8.1.6. At the end of 15-20 minutes, add phosphate buffer (pH 6.8) up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark). Mix well (lightly vortex or invert several times). Addition of sterile water is not a suitable alternative for the phosphate buffer.

8.1.7. In the BSC, load the diluted specimens into aerosol free safety centrifuge cups.

8.1.8. Centrifuge the specimen at a speed of 3000 g or more for 15-20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.

8.1.9. After centrifugation, allow tubes to sit for 5 minutes to allow aerosols to settle. Then carefully decant the supernatant into a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid. Add a small quantity (1-2 ml) phosphate buffer (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.

8.1.10. Use the resuspended pellet for making smears and for inoculation of MGIT tubes and other media.

8.2. Specimens other than sputum (extra-pulmonary)

8.2.1. Pus and other mucopurulent specimens

8.2.1.1. If the specimen is thick or mucoid and less than 10 ml in volume, digest and decontaminate with NaOH-NALC method similar to the procedure used for sputum specimens.

8.2.1.2. If the specimen is not thick, it may be treated with 2-4% NaOH. The concentration of NaOH depends upon the contaminating bacteria expected to be present in the specimen.

8.2.1.3. If the volume is over 10-12 ml, process only 10 ml or first concentrate by centrifugation at 3000x g for 15-20 minutes. In such a situation, if the specimen is thick, liquefy the specimen by adding a small quantity of NALC only (50-100 mg powder) and mix well.

8.2.1.4. After the concentration step, resuspend the sediment in 5 ml sterile water, decontaminate with NaOH and concentrate again by centrifugation

8.2.1.5. Always resuspend the sediment (pellet) in buffer to reduce the pH.

8.3. Gastric aspirates

8.3.1. Concentrate by centrifugation before decontaminating. Resuspend the sediment in about 5ml of sterile water and decontaminate with NaOH-NALC or 2-4% NaOH as recommended for sputum.

8.3.2. After decontamination, concentrate again prior to inoculation of the sediment In to culture media.

8.3.3. Due to the low pH, gastric aspirates should be processed as soon as possible (within 4 hours of collection).

8.3.4. If the specimen cannot be processed quickly, it should be neutralized with NaOH before transportation or storage.

8.4. Bronchial washings

8.4.1. All other pulmonary specimens, such as bronchial washings (BAL) may be treated as sputum.

8.4.2. If the specimen is up to 10 ml in volume, process the whole specimen. For larger volumes, concentrate the specimen by centrifugation (3000x g, 15-20 minutes).

8.4.3. If the specimen is thick or mucoid, liquefy by adding a small quantity of NALC powder (50-100mg). After centrifugation, resuspend the sediment in 5 ml sterile water and decontaminate like sputum.

8.5. Laryngeal swabs

8.5.1. Transfer the swab into a sterile centrifuge tube and add 2 ml sterile water. If necessary, break off the swab stick so the cap of the centrifuge tube can be placed on it and tightened.

8.5.2. Add 2 ml of NaOH-NALC solution replace the cap and mix well in a vortex mixer. Let stand for 15 minutes.

8.5.3. Remove the swab by with forceps, squeezing the liquid out of the swab and discarding it.

8.5.4. Fill the tube with phosphate buffer. Mix and centrifuge at about 3000x to3500 g for 15-20 minutes.

8.5.5. Discard the supernatant fluid and resuspend the sediment in 1-2ml sterile buffer. Use this suspension for smear and culture.

8.6. Tissue

8.6.1. Homogenize the tissue in a tissue grinder with a small quantity of sterile saline or water (2-4 ml).

8.6.2. All steps must be done in a biological safety cabinet (BSC) and all equipment must be sterile.

8.6.3. Decontaminate the homogenized specimen following the same NaOH-NALC procedure as in sputum

8.6.4. After resuspension of the sediment with phosphate buffer, inoculate 0.5 ml MGIT tube. If the tissue grinder is not available, use a mortar and pestle.

8.6.5. Tissue may also be placed in a Petri dish with sterile water (2-4 ml) and be torn apart with the help of two sterile needles or a sterile scalpel blade. Work under the hood and use sterilized materials.

8.7. Urine

8.7.1. As a routine isolation method, a totally voided, early morning urine specimen is used for mycobacterial culture. Pooled or mid-stream urine specimens are not recommended.

8.7.2. The specimen is concentrated by centrifugation using several 50 ml centrifuge tubes (with screw caps) for at least 20-25 minutes.

8.7.3. Resuspend the sediment in each tube with 1-2 ml sterile water and then pool together (total volume 5-10 ml).

8.7.4. Decontaminate the concentrated specimens with 4% NaOH for 15-20 minutes.

8.7.5. After decontamination, proceed in a manner similar to sputum

8.8. Other body fluids

8.8.1. Body fluids, such as CSF, synovial fluid and pleural fluid are collected aseptically and thus can be inoculated into MGIT medium without decontamination (with the addition of PANTA).

8.8.2. However, since sterility is not guaranteed, it is recommended these specimens should be lightly decontaminated.

8.8.3. If the specimen volume is more than 10 ml, concentrate by centrifugation at about 3000-3500x g for 15-20 minutes.

8.8.4. Liquefy thick or mucoid specimens prior to centrifugation by adding NALC powder (50-100 mg).

8.8.5. After centrifugation, resuspend the sediment in about 5 ml of saline and then decontaminate following the procedure similar to that for sputum.

8.8.6. Isolation of mycobacteria from blood specimens has not been evaluated thoroughly.

8.8.7. A few studies have been published or presented where blood was used with MGIT System after lysis centrifugation.65 BACTEC Myco/F Lytic

medium is recommended for isolation of mycobacteria and fungi from blood samples.

9. PROCEDURE NOTES

9.1. Temperature increase during centrifugation increases the killing effect on mycobacteria which will decrease the positivity rate and increase time-to-detection.2

9.2. A refrigerated centrifuge with at least 3000x g force is ideal. If a refrigerated centrifuge is not available, avoid temperature build-up, especially if the room temperature is high. Add refrigerated (chilled) phosphate buffer before centrifugation which should help in keeping the temperature low.

9.3. Other reagents during the digestion/decontamination step should not be refrigerated but kept at room temperature. Lower temperatures reduce the digestion decontamination process of NaOH-NALC.

10. INTERFERING SUBSTANCES

10.1. N/A

11. RESULTS

11.1. N/A

12. INTERPRETATION OF RESULTS

12.1. N/A

13. QUALITY CONTROL

13.1. N/A

14. QUALITY CONTROL MATERIALS

14.1. N/A

15. CALIBRATOR

15.1. N/A

16. CALIBRATION

16.1. N/A

17. QUALITY CONTROL RESULT

17.1. N/A

18. REFERENCES

18.1. MGIT™ Procedure Manual by Salman H. Siddiqi, Ph. D

APPENDIX THREE: SOP No: LMM 2111.01 THE BD MGIT™ TBc Identification Test (TBc ID)

1. **PURPOSE/INTRODUCTION:**
   1.1. This SOP gives direction on the procedure for the identification of *M. tuberculosis* complex using the BD MGIT TBc kit, at The Aga Khan University Hospital-Nairobi, Department of Pathology, Microbiology Section.

2. **SCOPE / RESPONSIBILITY:**
   2.1. This document applies to all Laboratory Technologists in Microbiology Section of the Pathology Department.

3. DEFINITIONS AND ABBREVIATIONS:
   3.1. SOP-Standard Operating Procedures
   3.2. AKUHN- Aga Khan University Hospital Nairobi
   3.3. N/A-Not Applicable
   3.4. LMM-Laboratory Microbiology and Molecular Biology
   3.5. MGIT-Mycobacterium Growth Indicator Tube

4. SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
This test is designed to identify MTbc from AFB smear-positive **MGIT** tubes (4 mL and 7 mL). The presence of AFB in a positive **MGIT** tube should be confirmed using an AFB smear prior to conducting the test	N/A	Positive **MGIT** tubes can be stored at 2–37°C for up to 10 days after **MGIT** tube positivity and prior to testing with the TBc ID device. If necessary, positive **MGIT** tubes may be stored and maintained at -20 to 8°C for up to two months.

5. EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment	Supplies	Reagents
-Pipettor (capable of delivering 100 µL), timer.	sterile pipette tips	**BD MGIT™** TBc ID test devices

6. SAFETY PRECAUTIONS

6.1.1. Personal protective equipment including proper protective gown, gloves and respirator masks(N95) must be worn at all times in the Bio-safety Level 3 areas.

6.1.2. Exercise the normal precautions required for handling infectious material.

6.1.3. Dispose of all waste materials in accordance with local guideline.

6.1.4. Perform all mycobacteria specimen processing in a Bio-safety Level 3 suite with a biological safety cabinet Class ll

6.1.5. Perform all specimen manipulation, and smear preparation in a Class ll BSC over disinfectant soaked towels or absorbent surface liners.

6.1.6. BSC must be certified at least annually to ensure that HEPA filters are functioning properly and that airflow rates meet specifications.

6.1.7. Immerse used transfer pipettes and sticks in discard container with an appropriate disinfectant. Tightly seal discard container prior to removal from the BSC.

7. METHODOLOGY:

7.1.1. Explanation and priniciple of The **BD MGIT™**

7.1.2. TBc Identification Test Tentative differentiation may be made by the following observations:Rate of growth. Generally, *M. tuberculosis, M. bovis* and, to some extent, *M. kansasii* are slow growers and take a longer time to turn positive in a MGIT tube as compared to other mycobacteria (NTM).In liquid medium growth, *M. tuberculosis* appears granular, while growth of most NTM does not show flakes or granules; rather it forms uniform slight turbidity (except *M.kansasii*).Smear made from a positive MGIT broth also helps in tentative differentiation of *M.tuberculosis* complex from other mycobacteria. Cultures belonging to the TB complex form typical clumps and serpentine cords while other mycobacteria appear as loose, smaller clumps and cording or single cells *M. kansasii* may be difficult to differentiate because its morphology is closer to *M. tuberculosis*. The identification of isolates grown in MGIT tube by molecular methods such as AccuProbe® (GenProbe Corp.) has been reported with good results.

7.1.3. The **BD MGIT™** TBc Identification Test (TBc ID) is a rapid chromatographic immunoassay for the qualitative detection of *Mycobacterium tuberculosis* complex (MTbc) antigen from AFB smear-positive **BD MGIT** tubes. The device will detect the following species of the MTbc: *M. tuberculosis, M. bovis, M. africanum,* and *M. microti*. Modern procedures used to identify *M. tuberculosis* from liquid culture include nucleic acid probes and gas-liquid / high performance chromatography. It is important to distinguish non-tuberculous mycobacteria (NTM) from *Mycobacterium tuberculosis* complex (MTbc). The **BD MGIT** TBc ID procedure identifies MTbc from culture grown in liquid media and requires no sample preparation. The total assay time is 15 minutes with reactivity determined by visual color development. It detects MPT64, a mycobacterial protein fraction that is secreted from MTbc cells during culture. When samples are added to the test device, MPT64 antigen binds to anti-MPT64 antibodies conjugated to visualizing particles on the test

strip. The antigen-conjugate complex migrates across the test strip to the reaction area and is captured by a second specific MPT64 antibody applied to the membrane. If the MPT64 antigen is present in the sample, a color reaction is produced by the labeled colloidal gold particles and is visualized as a pink to red line.

7.1.4. Procedure

7.1.4.1. Remove the TBc ID device from its foil pouch immediately before testing. Place the device on a flat surface. Test devices may be stored at 2–35°C. DO NOT FREEZE. Devices must be at ambient room temperature at time of testing.

7.1.4.2. Label one device for each sample to be tested.

7.1.4.3. Thoroughly mix the sample (AFB smear-positive **MGIT** tube) by inverting or vortexing. Do not centrifuge.

7.1.4.4. Remove cap from **MGIT** tube and using a sterile pipette tip, pipette 100 µL of the sample into the sample well (as indicated by the teardrop S) of the appropriately labeled device. Tightly replace cap on **MGIT** tube. Start timer for 15 min.

7.1.4.5. Read result at 15 min and record test result. Do not interpret test after 60 min.

7.2. PROCEDURE NOTES AND LIMITATIONS OF THE TEST

7.2.1. AFB smear-positive **MGIT** tubes can be tested in the TBc ID device within 10 days after **MGIT** tube positivity.

7.2.2. If devices are refrigerated, they must be brought to ambient room temperature in the foil pouch prior to testing.

7.2.3. For *in vitro* Diagnostic Use

7.2.4. •Do not use devices beyond the expiration date.

7.2.5. Do not reuse the device.

7.2.6. •Use a clean, sterile pipette tip for each sample.

7.2.7. Do not test clinical specimens directly in this device (e.g., human body fluid, tissue, sputa and bronchial lavage fluid).

7.2.8. Used test devices may contain viable MTbc which could be infectious. Used devices should be discarded according to your institutional guidelines or Standard Precautions requirements.

7.2.9. •Processed sputum and other non-sterile specimens are typically cultured in the **MGIT** system for the growth and detection of mycobacteria. As a result, primary cultures can contain non-AFB microorganisms. If non-AFB organisms predominate in a positive **MGIT** culture their growth and metabolism may interfere with the **BD MGIT** TBc ID performance. Care must be taken when using this test with polymicrobic cultures containing both AFB and non-AFB organisms. As a guideline, only use the TBc ID test on a positive **MGIT** tube if AFB-positive organisms predominate on the smear.

Performing the test on a very turbid positive culture (>1.0 McFarland) containing an overgrowth of non-AFB organisms may cause a false positive result.

8. Limitations

8.1. This test does not rule out the presence of other mycobacterial or mixed bacterial infections.

8.2. This test is unable to differentiate between MTb complex (MTbc) organisms.

8.3. This test should not be used solely for the determination of MTbc infection. The test results are to be used in conjunction with information available from the patient's clinical evaluation and other diagnostic procedures.

8.4. A negative result does not always rule out the possibility of infection with MTbc. The device is unable to detect MTbc when a mutation arises in the MPT64 gene. The test results are to be used in conjunction with information available from the patient's clinical evaluation and other diagnostic procedures.

8.5. Some substrains of *M. bovis* BCG among *M. tuberculosis* complex produce no MPT64 antigen and will therefore result in a negative test result with the device.

8.6. There are reports of rare strains of MTbc that produce MPT64 antigen below detectable limits.

8.7. False positive results may be observed in the presence of protein A-producing strains of bacteria (e.g. *Staphylococcus aureus*).

9. INTERPRETATION OF RESULTS

9.1. **Positive Test for TBc (MPT64 antigen present) -** A pink to red line appears at the Test "T" position and the Control "C" position in the read window. This indicates MPT64 antigen was detected in the sample. The intensity of the C and T lines may vary. The background area should be white to light pink.

9.2. **Negative Test for TBc (no MPT64 antigen detected) -** No pink to red line is visible at the Test "T" position of the read window. This indicates that MPT64 antigen was not detected in the sample. A line at the Control "C" position read window indicates proper performance of the test procedure. The background area should be white to light pink.

9.3. **Invalid Test -** The test is invalid if no pink to red line is visible at the Control "C" position in the read window or if the background area color inhibits test interpretation. If invalid, the sample must be retested with a new device.

10. REPORTING OF RESULTS

10.1. **Report positive Test Should** as MTb complex (MTbc).

10.2. **Negative Test** Should be reported as Acid-Fast Bacilli, non-MTbc.

10.3. **Invalid Test** Do not report results.

11. QUALITY CONTROL

11.1. Each device contains both positive and negative internal/procedural controls. The appearance of a control line in the read window at the Control "C" position provides an internal positive control that validates the proper reagent function

and assures that the correct test procedure was followed. The membrane area surrounding the test and control lines is the internal negative control for the device. A background area that is white to light pink indicates that the test is performing correctly. Positive and negative external controls should be tested in the same manner as test samples to provide a means of external quality control. Positive Control:

11.2. A positive **MGIT** tube prepared by growing a known isolate of MTbc. This should yield a positive result.

11.3. Negative Control: An uninoculated **MGIT** tube. This should yield a negative result.

11.4. External controls should be run, at a minimum, for each new lot or each new shipment received. If the controls do not perform as expected, do not report sample results.

12. QUALITY CONTROL MATERIALS

12.1. ATCC H 37 RV

13. CALIBRATOR

13.1. N/A

14. CALIBRATION

14.1. N/A

15. QUALITY CONTROL RESULT

15.1. A positive test: A pink to red line appears at the The Test “T” position and the Control “C” position in the read window.

15.2. Negative Test : No pink to red line is visible at the Test “T” position of the read window

16. REFERENCES

17. **MGIT** TBc ID kit insert

APPENDIX FOUR: SOP NO: LMM 2110.01 DEALING WITH MGIT CONTAMINATION

1. **PURPOSE/INTRODUCTION:**
   1.1. This SOP gives direction on the procedure for dealing with MGIT contamination, at The Aga Khan University Hospital-Nairobi, Department of Pathology, Microbiology Section.

2. **SCOPE / RESPONSIBILITY:**
   2.1. This document applies to all Laboratory Technologists in Microbiology Section of the Pathology Department.

3. **DEFINITIONS AND ABBREVIATIONS:**
   3.1. SOP-Standard Operating Procedures
   3.2. AKUHN- Aga Khan University Hospital Nairobi
   3.3. N/A-Not Applicable
   3.4. LMM-Laboratory Microbiology and Molecular Biology
   3.5. MGIT-Mycobacterium Growth Indicator Tube
   3.6. NTM-Non-tuberculous mycobacteria

4. **SPECIMEN:**

Recommended Specimens	Collection Notes	Pre-Analytical Processing
N/A	N/A	N/A

5. **EQUIPMENT / SUPPLIES/ REAGENTS:**

Equipment	Supplies	Reagents
-Microscope Warmer at 65ºC - 70ºC -Bunsen burner -Incubator at 37ºC±1ºC -Inoculating loops -Centrifuge with a minimum 3000x g force and safety shield/caps (refrigerated centrifuge is preferred) Vortex mixer	-Immersion oil -Clean glass slides -Disposable 50 ml plastic tubes (Falcon tubes) -Cotton plugged pipette tips -Transfer pipettes	-Clean glass slides -Blood Agar Plates -MaConkey Agar Plates Ziel-Neelsen staining reagents -Gram stain reagents Middlebrook 7H10 or 7H11 agar plate -4% sterile NaOH solution Phosphate buffer pH 6.8

-Timer -Pipettes -BACTEC™ MGIT™ 960 System		MGIT medium -MGIT growth supplement -MGIT PANTA™

6. SAFETY PRECAUTIONS

6.1.1. Personal protective equipment including proper protective gown, gloves and respirator masks(N95) must be worn at all times in the Bio-safety Level 3 areas.

6.1.2. Exercise the normal precautions required for handling infectious material.

6.1.3. Dispose of all waste materials in accordance with local guideline.

6.1.4. Perform all mycobacteria specimen processing in a Bio-safety Level 3 suite with a biological safety cabinet Class ll

6.1.5. Perform all specimen manipulation, and smear preparation in a Class ll BSC over disinfectant soaked towels or absorbent surface liners.

6.1.6. BSC must be certified at least annually to ensure that HEPA filters are functioning properly and that airflow rates meet specifications.

6.1.7. Immerse used transfer pipettes and sticks in discard container with an appropriate disinfectant. Tightly seal discard container prior to removal from the BSC.

7. METHODOLOGY:

7.1. Liquid media are more prone to contamination than solid media. It is extremely important to process specimens with extreme care, adhering very closely to procedures and recommendations. Following are guidelines for controlling excessive media contamination (for further details, please refer to Appendix C-3 Troubleshooting of MGIT Procedure Manual).

7.2. Bacterial contamination

7.3. The incidence of contamination with bacteria (other than mycobacteria) varies from

7.4. laboratory to laboratory depending upon several factors. According to the CDC guidelines, up to 5% contamination rate is acceptable in cultures of clinical specimens on solid media. A general recommendation is that 5% ± 2% is acceptable for all media types. However, for liquid media, slightly higher contamination may be accepted (up to 7-8%). Very low contamination rate (less than 3%) may indicate too harsh a decontamination process, which would also affect growth of mycobacteria and may reduce the positivity rate and increase time-to-detection of positive mycobacterial culture. On the other hand, a higher contamination rate (above 8%) may be due to the following reasons:

7.5. Improper or under decontamination of specimen.

7.5.1.1. Very mucoid specimens that are hard to liquefy may result in high contamination.

7.5.1.2. Long storage and transportation time of the specimen after collection. In such situations, especially in hot weather, bacteria tend to overgrow and then are hard to kill by routine decontamination procedure.

7.5.1.3. Use of non-sterile materials such as pipettes, tubes, etc.

7.5.1.4. Sometimes if reagents are prepared, stored in bulk and used for long periods of time, they may become contaminated.

7.6. (For details of troubleshooting, refer to Appendix C-3 of MGIT Procedure Manual).

7.7. **Detection of contamination:**

7.7.1. Growth of contaminated bacteria will result in positive

fluorescence. It is important to observe all fluorescent positive MGIT tubes visually for turbidity and to make an AFB smear. If a MGIT tube broth is heavily turbid, contamination is suspected even if the AFB smear is positive. Usually contaminating bacteria cause heavy turbidity, although *M. tuberculosis* growth appears as particles without any significant turbidity, while some of the NTM may produce light turbidity. Contamination may be confirmed by the following method:

7.7.1.1. Make a smear and stain with Ziehl-Neelsen stain. Presence of non-acid-fast contaminated bacteria on smear confirms contamination. Sub-culture a loopful of the liquid culture suspension on each of a Blood Agar Plate and a MacConkey Plate.

7.7.1.2. Several specimens (4) may be carefully inoculated on a plate (small streak for each specimen, properly labeled).

7.7.1.3. Divide the plate and identify specimen number by a marker.Incubate these subcultures at 36ºC±1ºC and observe after 24-48 hours. If contaminating growth appears, confirm again by Gram and ZN stain.

7.7.1.4. If contamination is confirmed repeat the isolation procedure (see below).

7.8. **How to control high contamination rate:**

7.8.1. The following are steps to help reduce a high contamination rate during isolation of mycobacteria from clinical specimens. Review all the procedures and make sure all recommended steps are followed closely. If high contamination persists, take the following measures:

7.8.2. Increase the NaOH concentration (not more than 1.5% final concentration in the specimen). The increase in NaOH is known to decrease contamination rate. Do not increase the exposure time more than 25 minutes to NaOH-NALC solution.

7.8.3. Increase the concentration of PANTA.

7.8.3.1. PANTA concentration may be increased by reconstituting with a smaller volume of Growth Supplement. However, the increase of PANTA concentration should be carefully evaluated since higher concentration of some antimicrobials present in PANTA may adversely

affect growth of some species of mycobacteria other than *M.tuberculosis*. Instead of 15.0 ml use 10.0 ml to reconstitute PANTA. Add the regular ml volume in the MGIT tube.

7.8.4. Do not change the NaOH concentration and PANTA at the same time. Try one procedure at a time and document improvement of results.

7.8.5. If there seems to be a common source of contaminating bacteria (same kind of bacteria contaminating repeatedly), check sterility of all reagents. It is a good practice to dispense small quantities of reagents and use only one at a time.

7.8.6. Leftovers should be discarded or re-sterilized.Try to reduce time between collection of specimens and processing. If a specimen

7.8.7. needs to be stored, use refrigeration.

7.8.8. Transport specimen with ice and in an insulated chest, especially in hot weather.

7.8.9. Inverting the tube during the decontamination process helps decontaminate the inside surface of the top of the tube.

7.8.10. If there is a persistent *Pseudomonas* contamination problem, oxalic acid procedure is known to be more efficient for killing these bacteria. However, it has not been validated for MGIT. Azlocillin in PANTA is very effective in the inhibition of *pseudomonas* growth; increasing the PANTA concentration may help.

7.9. Isolation of mycobacteria from contaminated or mixed cultures

7.9.1. **Decontamination of contaminated culture**: Usually more than one specimen is collected from a patient and it is not necessary to salvage a contaminated specimen if other specimens from the same patient are positive and not contaminated. However, if it is critical to have results on a particular specimen that was contaminated, the contaminated broth may be reprocessed to recover mycobacteria.

7.9.1.1. Transfer the entire MGIT broth into a 50 ml centrifuge tube.

7.9.1.2. Add an equal quantity of 4% sterile NaOH solution.

7.9.1.3. Mix well and let stand for 15-20 minutes, mixing and inverting the tube periodically.

7.9.1.4. Add phosphate buffer pH 6.8 after 15-20 minutes up to 40 ml mark. Mix well.

7.9.1.5. Centrifuge at least at 3000x g for 15-20 minutes.

7.9.1.6. Pour off the supernatant fluid.

7.9.1.7. Re-suspend the sediment in 0.5 ml of buffer and mix well.

7.9.1.8. Inoculate 0.5 ml into a fresh MGIT tube supplemented with MGIT growth supplement/PANTA.

7.9.1.9. Leave inoculated tubes at room temperature for 30 minutes, and then place in the instrument and follow for observation of growth.

7.10. Isolation of mixed mycobacterial culture on Middlebrook Agar Plate

7.10.1. AFB-positive cultures with more than one mycobacterial species may be separated by

7.10.2. streaking a small loopful of positive broth on a Middlebrook 7H10 or 7H11 agar plate.

7.10.3. Spread the loopful on the full surface of the plate to achieve isolated colonies. Incubate the inoculated plate at 37ºC±1C in a plastic bag. Periodically observe for growth (up to 6 weeks).

7.11. Cross-contamination

7.11.1. Cross-contamination of mycobacteria from specimen to specimen is also known in mycobacteriology laboratories. Usually it happens during the processing of specimens, especially at the time when a NaOH-NALC solution is added to the specimen or when a buffer is added to the tubes. Aerosol generation or splashing during the addition causes cross-contamination by contaminating the next tube or by contaminating the reagent stock solution.

7.11.2. Touching the lip of the specimen tube with the reagent container during pouring or adding of the reagent may also lead to high contamination. Sometimes stock solution of a reagent gets contaminated with mycobacteria commonly found in water (*M. gordonae, M. xenopi*). Aliquoting small quantities reduce the chances of cross-contamination. In the event of a cross-contamination episode, all reagents, equipment and biosafety cabinets must be thoroughly checked.

7.12. PROCEDURE NOTES

7.12.1. N/A

8. INTERPRETATION OF RESULTS

8.1. N/A

9. QUALITY CONTROL

9.1. N/A

10. QUALITY CONTROL MATERIALS

10.1. N/A

11. CALIBRATOR

11.1. N/A

12. CALIBRATION

12.1. N/A

13. QUALITY CONTROL RESULT

13.1. N/A

14. REFERENCES

14.1. MGIT™ Procedure Manual by Salman H. Siddiqi, Ph. D

APPENDIX FIVE: SOP NO LMM 2119.01: MAINTENANCE OF MYCOBACTERIAL STRAINS

1. PURPOSE

1.1. This SOP is to describes optimal storage conditions for mycobacterial strains (clinical and reference strains) to ensure their viability and retention of biological characteristics over time**.**

1.2. INTRODUCTION:

1.2.1. The storage and maintenance of mycobacterial reference strains and clinical isolates is an important part of good laboratory practice in a mycobacteria laboratory.

1.2.2. The storage of reference- and control strains facilitates a reliable control on intra- and inter-test reproducibility. It also helps to study various aspects of the epidemiology of tuberculosis it is important to be able to study isolates of individual patients or of patients associated with particular place- or time factors. To make this possible it is crucial to store these isolates in the best possible manner which reduces all possible alterations to minimum for the longest possible time.

1.3. PREAMBLE:

1.3.1. Date of revision**.**

1.3.2. Changed front page format; removed date and signature requirements for the approval matrix, Added title of the reviewers**.**

2. SCOPE /RESPONSIBILITY

2.1. SCOPE

2.2. This document applies to all Laboratory Technologists working in Tb Lab in Microbiology Section of the Pathology Department**.**

2.3. RESPONSIBILITY: It is the responsibility of all the microbiology staff to adhere to this SOP.

3. DEFINITIONS AND ABBREVIATIONS:

3.1. SOP-Standard Operating Procedures

3.2. **AKUHN-** Aga Khan University Hospital Nairobi

3.3. **N/A-**Not Applicable

3.4. **LMM-**Laboratory Microbiology and Molecular Biology

4. SPECIMEN:

Recommended Specimens	Collection Notes	Pre-Analytical Processing
Mycobacterial strains	N/A	N/A

5. EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment	Supplies	Reagents
BSC, class II, (annually certified) Freezer at –18 °C and/or –70 °C	Solid cultures in tubes/vials Liquid cultures in tubes/vials Cryovials with screw-caps Cryoboxes	Middlebrook 7H9 broth

6. SAFETY PRECAUTIONS

6.1.1. Personal protective equipment including proper protective gown, gloves and respirator masks(N95) must be worn when handling infectious materials**.**

6.1.2. Exercise the normal precautions required for handling infectious material.

6.1.3. Dispose of all waste materials in accordance with local guideline.

6.1.4. Perform all mycobacteria specimen processing in a Bio-safety Level 3 suite with a biological safety cabinet Class ll

6.1.5. Perform all specimen manipulation, and smear preparation in a Class ll BSC over disinfectant-soaked towels or absorbent surface liners.

6.1.6. BSC must be certified at least annually to ensure that HEPA filters are functioning properly and that airflow rates meet specifications.

6.1.7. Immerse used transfer pipettes and sticks in discard container with an appropriate disinfectant. Tightly seal discard container prior to removal from the BSC

7. METHODOLOGY:

7.1. PRINCIPLE AND EXPLANATION

7.1.1. Mycobacterial strains must be stored in conditions that preserve their viability and protect specific strain characteristics**.**

7.2. PROCEDURE

7.2.1. Short term storage

7.2.1.1. Cultures on egg-based medium should be preferably stored at 2–8 °C and may be maintained viable for up to 1 year. These cultures may also be kept at room temperature (maximum 20 °C, in air-conditioned rooms if necessary), in the dark, but media quality may deteriorate and viability may be affected.

7.2.1.1.1. Storage of liquid cultures for more than 1 month is not recommended since media quality deteriorates rapidly and affects viability while clumping in liquids makes the determination of bacterial concentration highly unpredictable. Moreover, liquid media are more prone to contamination.

7.3. Long-term storage

7.3.1. Scrape as many colonies as possible from an egg-based medium slant. Suspend colonies in a 2-ml cryovial containing 1.5 ml Middlebrook 7H9 medium. Store at –18 °C for several years or at –70 °C for decades.

7.3.2. The viability of tubercle bacilli declines much more rapidly at –18 °C than at – 70 °C: only 1% are still viable at –18 °C after 2 years compared with 100% at – 70 °C. It is thus crucial to prepare the heaviest bacterial load possible in order to compensate for the loss of viability. In deciding whether they actually need a –70°C freezer, laboratories must consider the objectives of long-term storage of mycobacterial cultures. Storage of cultures for up to 5 years after the initial isolation can be achieved at –18 °C provided that cultures (or subcultures) showed luxuriant growth and were abundant. A freezer at –70 °C may be required for national reference laboratories involved in research programmes or long-term epidemiological studies.

7.4. Long-term storage of reference cultures

7.4.1. Reference strains for quality control of drug susceptibility testing and medium preparation (M. tuberculosis H37Rv) must be permanently maintained in the laboratory. To avoid serial subculturing, which could lead to genetic drift and alter the phenotypic biological characteristics of strains, it is advisable to adopt the following procedure:

7.4.2. Scrap as many colonies as possible from egg-medium slants of the reference strain. Suspend colonies in a tube containing 15 ml Middlebrook 7H9 medium.

7.4.3. Dispense the suspension in 10 cryotubes, 1.5 ml per tube. Label each tube with the strain reference and date. Store at –18 °C or –70 °C.

7.4.4. Thaw one tube and inoculate 10 tubes of egg-based medium, which will be used as the source of bacilli for quality control whenever necessary. Thaw another tube when the 10th tube source is exhausted

7.5. Waste management

7.5.1. Dispose of cultures by autoclaving – both liquid and solid

7.6. PROCEDURE NOTES

7.6.1. N/A

8. INTERPRETATION OF RESULTS

8.1. N/A

9. QUALITY CONTROL

9.1. N/A

10. QUALITY CONTROL MATERIAL

10.1. N/A

11. CALIBRATOR

11.1. N/A

12. CALIBRATION

12.1. N/A

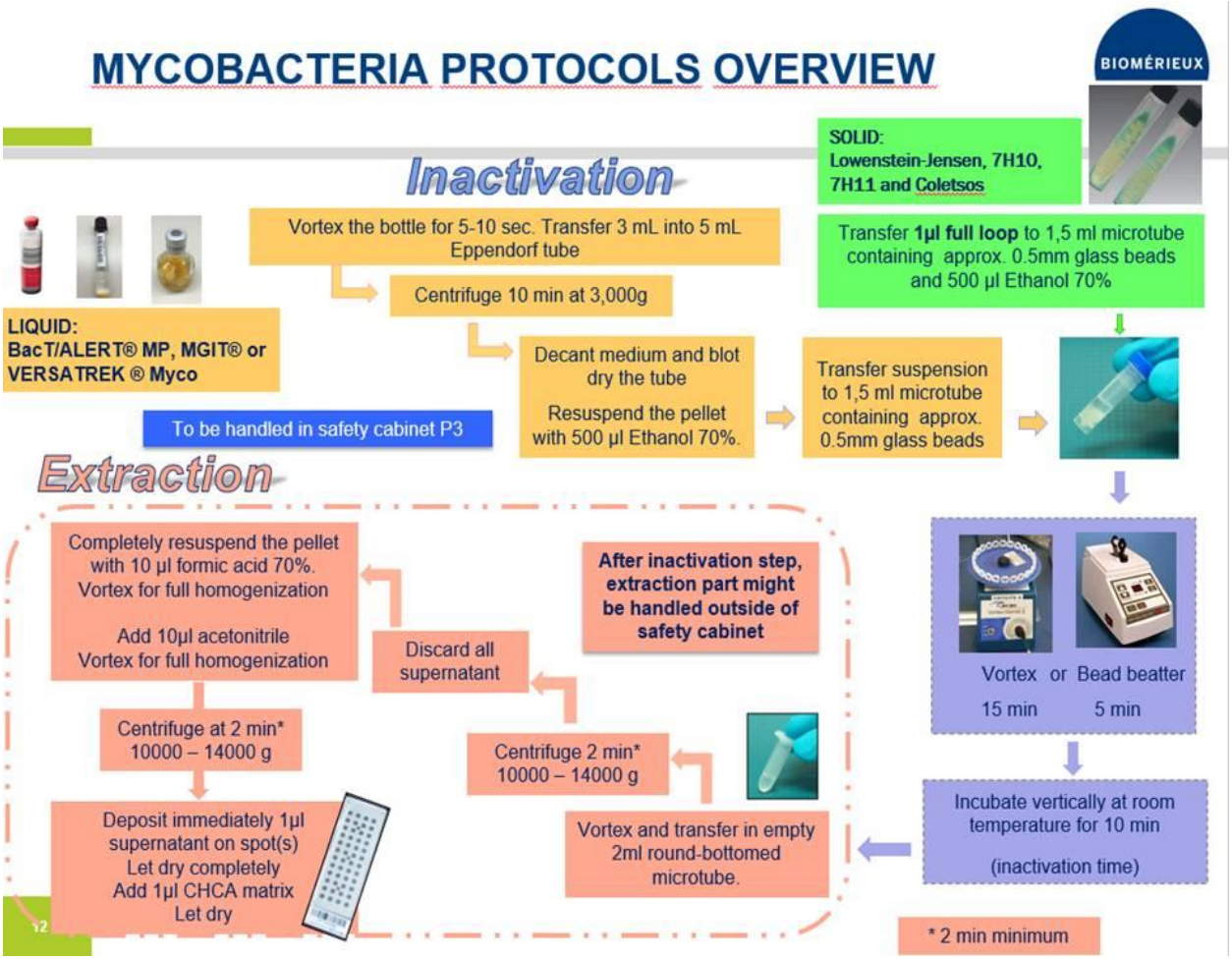
13. RESULT.

13.1. N/A

14. QUALITY CONTROL RESULT

14.1. N/A

APPENDIX SIX: MALDI TOF MS PROTOCOL



APPENDIX SEVEN: VITEK MS KNOWLEDGE BASE V3.2 DATABASE

NTM species available on the VITEK MS Knowledge base v3.2 databas
Mycobacterium abscessus
Mycobacterium agri
Mycobacterium arupense
Mycobacterium asiaticum
Mycobacterium aurum
Mycobacterium avium
Mycobacterium brisbanense
Mycobacterium celatum
Mycobacterium chelonae
Mycobacterium cosmeticum
Mycobacterium □avescens
Mycobacterium alvei
Mycobacterium farcinogenes
Mycobacterium fortuitum
Mycobacterium fortuitum ssp fortuitum
Mycobacterium houstonense
Mycobacterium peregrinum
Mycobacterium porcinum
Mycobacterium senegalense
Mycobacterium gastri
Mycobacterium genavense
Mycobacterium goodii
Mycobacterium gordonae
Mycobacterium haemophilum
Mycobacterium immunogenum
Mycobacterium intracellulare
Mycobacterium kansasii
Mycobacterium kubicae
Mycobacterium lenti□avum
Mycobacterium mageritense
Mycobacterium malmoense
Mycobacterium marinum
Mycobacterium mucogenicum
Mycobacterium nebraskense
Mycobacterium neoaurum
Mycobacterium para□hicum
Mycobacterium phlei
Mycobacterium scrofulaceum
Mycobacterium shimoidei
Mycobacterium simiae
Mycobacterium smegmatis
Mycobacterium szulgai
Mycobacterium triplex
Mycobacterium africanum
Mycobacterium bovis
Mycobacterium canettii
Mycobacterium tuberculosis
Mycobacterium vaccae
Mycobacterium xenopi

APPENDIX EIGHT: SOP No: LMM 2128.01 PRIMARY ISOLATION OF MYCOBACTERIA USING BD MGIT™SYSTEM

1. PURPOSE/INTRODUCTION

1.1 PURPOSE: This SOP describes the procedure for primary isolation of Mycobacteria using the BACTEC MGIT 960 system, at The Aga Khan University Hospital-Nairobi, Department of Pathology, Microbiology Section.

1.2 INTRODUCTION: The mycobacteria growth indicator tube is designed for the rapid detection of mycobacteria in all types clinical specimens. The BACTEC MGIT 960 instrument is a fully automated system that exploits the fluorescence of an oxygen sensor to detect growth of mycobacteria in culture.

1.3 PREAMBLE:

1.3.1 Date of revision.

1.3.2 Changed front page format; removed date and signature requirements for the approval matrix,Added title of the reviewers.

2. SCOPE/RESPONSIBILITY

2.1 SCOPE: This document applies to technologists in Microbiology Section of the Pathology Department.

2.2 RESPOSIBILITY: It is the responsibility of all the microbiology staff to adhere to this SOP

3. DEFINITIONS AND ABBREVIATIONS:

3.1 ABBREVIATIONS:

3.1.1 SOP Standard Operating Procedures

3.1.2 AKUHN Aga Khan University Hospital Nairobi

3.1.3 N/A Not Applicable

3.1.4 LMM Laboratory Microbiology and Molecular Biology

3.1.5 QC Quality Control

3.1.6 MGIT Mycobacterium Growth Indicator Tube

3.1.7 NTM Non Tuberculous Mycobacteria

3.2 DEFINITIONS:

3.2.1 N/A

4. SPECIMEN:

Recommended Specimens Collection Notes Pre-Analytical Processing

See PECIMEN PROCESSING FOR ISOLATION OF MYCOBACTERIA USING BD MGITTM SYSTEM SOP N/A N/A

5. EQUIPMENT / SUPPLIES/ REAGENTS:

EquipmentSupplies Reagents

-Centrifuge with a minimum 3000x g force and safety shield/caps (refrigerated centrifuge is preferred)

- Vortex mixer

-Timer

-Pipettes

-Disposable 50 ml plastic tubes (Falcon tubes)

-Cotton plugged pipette tips

-Transfer pipettes

MGIT medium

MGIT growth supplement MGIT PANTA™

-BACTEC™ MGIT™ 960 System

6. SAFETY PRECAUTIONS

6.1 Personal protective equipment including proper protective gown, gloves and respirator masks (N95) must be worn at all times in the Bio-safety Level 3 areas.

6.2 Exercise the normal precautions required for handling infectious material.

6.3 Dispose of all waste materials in accordance with local guideline.

6.4 Perform all mycobacteria specimen processing in a Bio-safety Level 3 suite with a biological safety cabinet Class ll

6.5 Perform all specimen manipulation, and smear preparation in a Class ll BSC over disinfectant soaked towels or absorbent surface liners.

6.6 BSC must be certified at least annually to ensure that HEPA filters are functioning properly and that airflow rates meet specifications.

6.7 Immerse used transfer pipettes and sticks in discard container with an appropriate disinfectant. Tightly seal discard container prior to removal from the BSC.

7. METHODOLOGY:

7.1 Principle of the BACTEC™ MGIT™ 960 System:

7.1.1 The MGIT (Mycobacteria Growth Indicator Tube) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0 ml of modified Middlebrooks 7H9 broth base.

7.1.2 This medium is terminally sterilized by autoclaving. An enrichment, MGIT 960 Growth Supplement, is added to make the medium complete. This Growth Supplement is essential for growth of many mycobacteria, especially those belonging to M. tuberculosis complex.

7.1.3 The enrichment must be added to the MGIT medium prior to inoculation of a specimen. MGIT growth supplement contains 15 ml of the following approximate formula:

7.1.3.1 Bovine Albumin --50.0 gm

7.1.3.2 Dextrose --20.0 gm

7.1.3.3 Catalase --0.03 gm

7.1.3.4 Oleic Acid -- 0.1 gm

7.1.3.5 Polyoxyethyline state (POES) ---- 1.1 gm

7.1.4 MGIT growth supplement is a sterile product. Handle aseptically. Do not

use if turbid or if it appears to be contaminated. It is important to add the growth supplement in the biological safety cabinet to avoid contaminating the medium.

7.1.5 Addition of the MGIT PANTA, a mixture of antimicrobial, is necessary to suppress contamination. Each vial of MGIT PANTA (for MGIT 960) contains a lyophilized mixture of the antimicrobials with the concentrations, at the time of production, as follows:

7.1.5.1 Polymyxin B 6,000 units

7.1.5.2 Amphotericin B 600 µg

7.1.5.3 Nalidixic Acid 2,400 µg

7.1.5.4 Trimethoprim 600 µg

7.1.5.5 Azlocillin 600 µg

7.1.6 In addition to Middlebrook 7H9 liquid media, the MGIT tube contains an oxygen-quenched fluorochrome, tris 4, 7-diphenyl-1, 10-phenonthroline ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube.

7.1.7 During bacterial growth within the tube, the free oxygen is utilized and is replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in fluorescence within the MGIT tube when visualized under UV light.

7.1.8 The intensity of fluorescence is directly proportional to the extent of oxygen depletion. MGIT tubes may be incubated at 37ºC and read manually under a UV light or entered into a MGIT 960 instrument where they are incubated and monitored for increasing fluorescence every 60 minutes. Growth of bacteria as well as mycobacteria increases the fluorescence. In case of M. tuberculosis, at the time of positivity, there is approximately 105 – 106 colony forming units (CFU) per ml of medium.

7.1.9 The instrument declares a tube negative if it remains negative for six weeks (42 days). The detection of growth can also be visually observed by the presence of a non-homogeneous light turbidity or small granular/flaky appearance in the medium. Growth of some NTM (most commonly rapid growers) results in light turbidity, while contaminating bacteria generally produce heavy turbidity.

7.2 PROCEDURE

7.2.1 RECONSTITUTING PANTA

7.2.1.1 Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completely dissolved. Reconstituted PANTA can be used for up to 5days if stored at 2-8°C. Add 0.8 ml of this enrichment to each MGIT tube.

7.2.1.2 The enrichment with reconstituted PANTA should be added to the MGIT medium prior to inoculation of specimen in MGIT tube.

7.2.1.3 Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/ PANTA.

7.2.2 INOCULATION OF MGIT MEDIUM

7.2.2.1 Label MGIT tubes with specimen number.

7.2.2.2 Unscrew the cap and aseptically add 0.8 ml of MGIT growth supplement/PANTA to each MGIT tube. Use of an adjustable pipette is recommended.

7.2.2.3 Using a sterile pipette or a transfer pipette, add up to 0.5 ml of a well-mixed processed/concentrated specimen to the appropriately labeled MGIT tube. Use separate pipette or pipette tip for each specimen.

7.2.2.4 Immediately recap the tube tightly and mix by inverting the tube several times. Wipe tubes and caps with a mycobactericidal disinfectant and leave inoculated tubes at room

temperature for 30 minutes. Work under the biological safety cabinet for the specimen inoculation.

7.2.3 INOCULATION OF ADDITIONAL MEDIA

7.2.3.1 Inoculate onto solid medium 0.1 to 0.25 ml (2-3 drops) of processed/concentrated specimen.

7.2.4 INCUBATION

7.2.4.1 All inoculated MGIT (7mL) tubes should be entered in the BACTEC MGIT 960 instrument after scanning each tube (refer to the BACTEC MGIT 960 Instrument Manual for details).

7.2.4.2 Keep the cap tightly closed and not to shake the tube during the incubation. This helps in maintaining the oxygen gradient in the medium. The instrument maintains 37ºC ± 1ºC temperature. Since the optimum temperature for growth of M. tuberculosis is 37ºC, make sure the temperature is close to 37ºC.

7.2.4.3 Note: If a specimen is suspected of containing mycobacteria which require an optimum temperature other than 37°C (for example, M. haemophilum, M. marinum, M. chelonae and M. ulcerans require 30°C), then two sets of media should be inoculated, one in the instrument at 37ºC and the other in an outside incubator at 30ºC. These tubes can be monitored by using a UV light source (Wood's lamp) and can also be checked visually (refer to the BACTEC MGIT 960 Manual).

7.2.5 Length of incubation: MGIT tubes should be incubated until the instrument flags them positive. After a maximum of six (6) weeks, the instrument flags the tubes negative if there is no growth.

7.3 PROCEDURE NOTES

7.3.1 It is customary to use two different types of media for maximum recovery of mycobacteria. With the MGIT system, maximum recovery of mycobacteria may be achieved by using an additional solid medium, most commonly an egg-based medium such as LJ is used.

7.3.2 Volumes greater than 0.5 ml of decontaminated specimen may disturb the pH of the medium and may cause false fluorescence. This may also increase contamination or otherwise adversely affect the performance of the MGIT

medium

7.3.3 One of the major sources of contamination in MGIT medium is environmental contaminants introduced during addition of growth supplement.

8. INTERFERING SUBSTANCES

8.1 N/A

9. RESULTS.

9.1 Positive MGIT tube flags positive, indicating the presence of bacteria and the instrument will show it's the growth units.

9.2 A Negative tube will only flag as negative after 6 weeks of incubation.

10. QUALITY CONTROL

10.1 Every time inoculation is done a negative control must be processed and incubated with the samples for contamination monitoring.

11. QUALITY CONTROL MATERIALS

11.1 Use 5 ml of phosphate buffer

12. CALIBRATOR

12.1 N/A

13. CALIBRATION

13.1 N/A

14. QUALITY CONTROL RESULT

14.1 The negative control should show no growth within the incubation protocol period. If the negative control shows positive fluorescence, check for the presence of bacteria/mycobacteria. If positive for growth, investigate procedures and all the reagents for possible source of contamination.

15. APPENDICES:

15.1 Document change history.

15.2 SOP distribution listing.

15.3 SOP training log.

APPENDIX NINE: SOP No: LMM:2015.01 COLLECTION AND TRANSPORT OF SPUTUM SPECIMENS.

1. **PURPOSE:**

1.1. This SOP gives instructions on how to collect and transport sputum specimens for microbiology analysis at The Aga Khan University Hospital, Nairobi-Department of Pathology, Microbiology Section.

1.2. **INTRODUCTION.**

1.2.1. Good quality sputum samples are essential for accurate microbiological diagnosis of pneumonia, but also acute tracheitis and bronchitis. Sputum cultures are routinely used for patients with chronic and often progressive suppurative lung diseases such as Cystic Fibrosis and Primary Ciliary Dyskinesia. However, samples contaminated with oropharyngeal secretions and saliva are difficult to interpret and can be misleading.

1.3. **Preamble:**

1.3.1. SOP review date.

1.3.2. Changed front page format; removed date and signature requirements for the approval matrix, Added title of the reviewers.

2. **SCOPE / RESPONSIBILITY:**

2.1. **SCOPE:**

2.1.1. This document applies to all Laboratory Staff in Microbiology Section of the Pathology Department.

2.2. **RESPONSIBILITY:**

2.2.1. Technical heads and appropriate laboratory staff are responsible for ensuring this SOP is implemented as written.

3. **DEFINITIONS AND ABBREVIATIONS:**

3.1. **ABBREVIATIONS:**

3.1.1. SOP Standard Operating Procedures

3.1.2. AKUHN Aga Khan University Hospital Nairobi

3.1.3. N/A Not Applicable

3.1.4. LMM Laboratory Microbiology and Molecular Biology.

4. SPECIMEN:

Recommended Specimens Collection Notes Pre-Analytical Processing

-N/A - N/A - N/A

5. EQUIPMENT / SUPPLIES/ REAGENTS:

Equipment Supplies Reagents

-N/A -Clean,dry,wide-necked,leak-proof container N/A

6. **SAFETY PRECAUTIONS**

6.1. Wear appropriate protective equipment such as gloves, Lab coats or aprons, eyewear or masks when handling infectious material

6.2. Dispose of all waste materials in accordance with local guideline.

6.3. Wash hands particularly after collection and handling of clinical specimen. Generally, follow universal safety precautions when in the Laboratory.

6.4. Use leak-proof containers in a sealed plastic bag for transport and storage.

6.5. When a sputum specimen is being collected, adequate safety precautions should be taken to prevent the spread of infectious organisms.

7. **METHODOLOGY/ PROCEDURES**:

7.1. **TEST PRINCIPLE**

7.1.1. N/A

7.2. **PROCEDURE:**

7.2.1. Give the patient a container and request him / her to cough deeply to produce a sputum specimen.

7.2.2. NOTE: -The specimen must be sputum, not saliva. Sputum is best collected in the morning soon after the patient wakes up and before mouth wash.

7.2.3. The laboratory recommends collecting three sputum specimens for acid-fast smears (spot, morning, spot) and culture in patients with clinical and chest x-ray findings compatible with tuberculosis. These three samples should be collected at 8–24-hour intervals and should include at least one first morning specimen.

7.2.4. Specimens must be delivered to the laboratory promptly; specimens that cannot be processed within one hour of the time of collection should be refrigerated during transport to and storage in the laboratory prior to processing. This will decrease overgrowth with contaminating organisms likely to be present.

7.2.5. Label the container with patient's name, date and collection time.

7.2.6. Deliver to the Laboratory with as little delay as possible.

7.3. PROCEDURE NOTES:

7.3.1. If the patient is a young child and it is not possible to obtain sputum, gastric washing can be used for the isolation of M.tuberculosis but not for other respiratory pathogens.

7.3.2. Specimens for isolation of S. pnemoniae and H.influenzae must never

be refrigerated.

7.3.3. Specimens for isolation of M.tuberculosis should be delivered to the laboratory within 2 hours, or kept at 4ºC (to slow down the multiplication of commensals) until delivery is possible.

8. INTERFERING SUBSTANCES

8.1. N/A

9. QUALITY CONTROL

9.1. AKUHN's Laboratory specimen rejection and acceptance criteria will be used to ensure that high quality specimens are accepted for testing in the microbiology laboratory. Trends of specimen rejection rate will be used to initiate corrective and preventive action to improve the quality of specimens at the source. Records for all quality control results will be reviewed by the section head or designee and records maintained in accordance with AKUHN document and record retention policy.

9.2. The Quality Manager or designee will do random checks to ensure that this SOP is implemented as written. If Quality Control yields unexpected results, investigations will be done and corrective and preventive action initiated, implemented, reviewed and closed. All corrective and preventive action should be documented in the corrective action form.

10. QUALITY CONTROL MATERIALS

10.1.1. N/A

11. CALIBRATOR

11.1. N/A

12. CALIBRATION

12.1. N/A

13. RESULTS

13.1.1. N/A

14. QUALITY CONTROL RESULTS

14.1. N/A.

15. APPENDICES:

15.1. Document change history.

15.2. SOP distribution listing.

15.3. SOP training log.

16. REFERENCES

16.1. Medical Laboratory Manual for Tropical Countries Volume II Microbiology by Monica Chees brough

16.2. WHO's Basic Laboratory Procedures in Clinical Bacterial