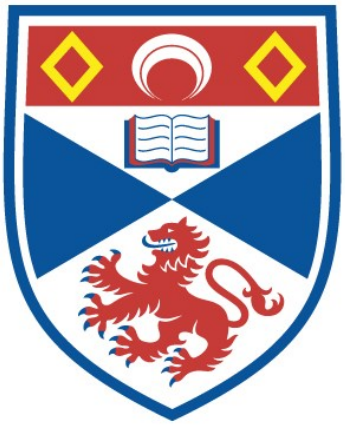
EVALUATION OF THE TUBERCULOSIS-MOLECULAR BACTERIAL LOAD ASSAY FOR TUBERCULOSIS DIAGNOSIS AND MONITORING RESPONSE TO STANDARD ANTI-TUBERCULOSIS THERAPY

Emmanual Musisi

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Evaluation of the Tuberculosis-Molecular Bacterial Load Assay for Tuberculosis Diagnosis and Monitoring response to standard Anti-Tuberculosis Therapy

Emmanuel Musisi

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This thesis is submitted in partial fulfilment for the degree of

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Acknowledgements

I would like to express a sincere gratitude to my supervisory team led on Dr Wilber Sabiiti for their guidance and mentorship throughout my doctoral research. It was a great honour for me to be integrated into St Andrews tuberculosis research group led on Prof Gillespie in the Division of Infection and Global Health, at School of Medicine. Their efforts in guiding me to writing a scientifically acceptable Thesis through series of well-balanced evaluations and positive criticisms will forever be cherished. The skills and knowledge acquired from them will guide me to further my research career.

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Dedication

This thesis is dedicated to my family: my mother Ms Namusoke Allen Joyce for her parental guidance, my wife Mrs Elizabeth Namirembe Musisi; our children: Kibalama Ddiba Elijah, Ntongo Ellaine Rachel, Nkonge Ssegawa Benjamin, and Ssentongo Mayanja Joash; aunt Prossy Namande; and my early career mentors: Professors Nicholas Walter, Harriet Kizza-Mayanja, and Dr Wamutu W Samuel**.**

Thesis abstract

Tuberculosis (TB) is a difficult disease to treat, requiring a minimum of six months on a combination of four antibiotics. This thesis reports the first systematic evaluation of the St Andrews' developed RNA-based tuberculosis-Molecular Bacterial Load Assay (TB-MBLA) for its accuracy to diagnose tuberculosis and measure treatment response in comparison to current standard-of-care tests. Presumptive TB patients were enrolled in Uganda and assessed for TB using TB-MBLA versus Xpert MTB/RIF Ultra (Xpert-Ultra) and stained smear fluorescent microscopy (SSM-FM) using sputum MGIT culture as the gold standard and reference test. Out of the 210 presumptive cases, 129 (61.4%) participants tested TB positive on the Xpert-Ultra in the sputum cohort and they were enrolled into the treatment arm and consequently monitored for six months. At baseline, 6/210 (2.9%) sputum MGIT culture results were indeterminate due to contamination, and they were excluded from the calculation of the sensitivity, specificity, and predictive values.

Sensitivity for TB-MBLA and Xpert-Ultra (95%CI) was 99%(95-100) which was higher compared to 76%(65-83) for SSM-FM. TB-MBLA specificity at 90%(83-96) was higher than the 76%(68-86) for Xpert-Ultra but less than 98%(93-100) for SSM-FM. In the treatment follow-up arm, TB positivity rates reduced for all tests. TB-MBLA positivity reduction was consistent with that of the MGIT culture but different from that of Xpert-Ultra which occurred remarkably slower. Consequently, 31 participants were still Xpert-Ultra positive at the end treatment course. Three-month post treatment follow-up of the 31 Xpert-Ultra positive cases revealed no TB both clinically and on TB-MBLA and MGIT tests. In the stool cohort, TB-MBLA detected TB in 57/100 participants including 49 who were confirmed positive for pTB on sputum MGIT culture. Fifty-seven percent (57%) of the indeterminate stool culture were positive on TB-MBLA. The findings prove that TB-MBLA's potential utility as both a diagnostic and treatment monitoring tool of TB in research and routine healthcare.

Thesis summary

Treating tuberculosis (TB) disease requires a minimum of six months on a combination therapy of antibiotic drugs. This is burdensome to TB control programmes as well as to the patients. The need for effective diagnostic and treatment monitoring tools cannot be more emphasised. These tools should ensure early detection of TB and prompt initiation of treatment, and that TB patients do not overstay on therapy inappropriately. This doctoral research evaluated a novel assay that targets 16S rRNA as a proxy for viable Mtb to inform improvements in TB diagnosis and monitoring response to treatment.

Diagnostic accuracy of TB-MBLA using sputum samples

For the first time, Tuberculosis-Molecular Bacterial Load Assay (TB-MBLA), an RNA-based technology was clinically evaluated as a diagnostic and treatment response monitoring tool for TB using sputum samples against Xpert MTB/RIF Ultra (Xpert Ultra) and stained smear fluorescent microscopy (SSM-FM) which are currently used as the standard-of-care tools in Uganda. Sputa were collected from adult persons who were presumed to have TB and analysed cross-sectionally. Findings from this investigation showed that TB-MBLA and Xpert Ultra have similar diagnostic sensitivity (95%CI) of 99% (95-100), but TB-MBLA was more specific (95%CI) at 90% (83-96) than Xpert Ultra at 76% (68-86).

Monitoring response to TB treatment

Out of the 210 participants that were enrolled into the study, 129 (61·4%) tested positive for pTB on Xpert Ultra and these were monitored for treatment response. Sputum samples were collected at weeks 2,8,17, 26 and 3 months after the end of treatment course. Positivity rates reduced for all tests, but this occurred at a remarkably slower rate for the Xpert Ultra and SSM-FM. Consequently, at the end of treatment, 31- and 6- participants were still positive on Xpert Ultra and SSM-FM, respectively.

While Xpert Ultra remained sensitive (95%CI) at 92% (62-100) at week 8 of treatment, TB-MBLA was more specific (95%CI), at 88% (80-94) and it mirrored MGIT culture at 94% agreement. At post treatment follow-up, all the 31 Xpert Ultra positive and the 6 smear positive participants were clinically well. The bacillary load measured on TB-MBLA among the smear-positive and smear- negative participants at 2 months did not statistically differ at P>0·05 despite the former having an extra month of intensive phase of treatment.

Diagnostic accuracy of TB-MBLA using stool

Stool samples were collected and processed using two methods before testing for TB. One method (n=100) utilised OMNIgene-sputum (OM-S)– a *Mycobacterium tuberculosis* preservative, and the other (n=100) utilised Phosphate buffered saline- a commonly used laboratory reagent. Ribonucleic acid (RNA) was extracted using phenol chloroform method before conducting TB-MBLA (which was done using stored stool samples). Diagnostic performance of TB-MBLA was compared with that of the Xpert Ultra and SSM-FM (which were done using fresh stool samples). Sputum MGIT culture (which was done using freshly collected sputum) was used as the gold standard and the reference test.

TB-MBLA detected Mtb in 57/100 stools of whom 49 were confirmed positive for pulmonary TB on sputum MGIT. Mean bacterial load measured on stool TB-MBLA was higher in HIV co-infected than in the HIV negative participants (p=0·04). Sensitivity of stool TB-MBLA and stool Xpert Ultra were similar but higher compared with that of the SSM-FM and stool culture. Specificity was highest for SSM-FM followed on Xpert Ultra, TB-MBLA and culture tests, respectively. Twenty six percent (26%) of the stool MGIT culture and 21% of the stool LJ culture tests were indeterminate due to contamination. Of the contaminated stool MGIT culture, 57% were positive on both TB-MBLA and Xpert Ultra.

List of Publications

1. **Emmanuel Musisi.,** Abdul Sessolo., Sylvia Kaswabuli., Josephine Zawedde., Patrick Byanyima., Shariifah Kasinga., Ingvar Sanyu., Esther Uwimaana., Stanley Walimbwa., Joseph Olore., Willy Ssengooba., Christine Sekaggya., L. Joloba., William Worodria., Laurence Huang., Stephen H. Gillespie., Derek J. Sloan., Wilber Sabiiti (2022) High Mycobacterium tuberculosis Bacillary Loads Detected by Tuberculosis Molecular Bacterial Load Assay in Patient Stool: A Potential Alternative for Non-sputum Diagnosis and Treatment Response Monitoring of Tuberculosis (*Published online 2022 Jan 12. Microbiology Spectrum; doi: 10.1128/spectrum.02100-21*)

2. **Emmanuel Musisi.,** Abdul Ssesolo., Derek J. Sloan., Stephen H. Gillespie., Wilber Sabiiti (2022) Detection and quantification of viable *Mycobacterium tuberculosis* bacilli in saline-processed stool by Tuberculosis Molecular Bacterial Load Assay: a potential alternative for processing stool (*Published online 2022 May 12. Microbiology Spectrum doi: 10.1128/spectrum.00274-22*)

Manuscripts in pre-print and under review

1. **Emmanuel Musisi.,** Adam Wyness., Sahar Eldirdiri., Evelin Dombay., Bariki Mtafya., Nyanda Elias Ntinginya., Norbert Heinrich., Gibson Sammy Kibiki., Michael Hoelscher., Martin Boeree., Stephen Henry Gillespie., Wilber Sabiti (2022) Early Depressing Effect on Abundancy and Diversity of Sputum Microbiome Followed by Recovery During Anti-Tuberculosis Treatment: A Multi-Regimen Analysis of the High-Rifampicin II- and Multi-Arm-Multi-Stage Clinical Trials. (Under review in the Lancet Microbe; pre-print copy can be accessed on http://dx.doi.org/10.2139/ssrn.4172089)

2. **Emmanuel Musisi.,** Samuel Wamutu., Willy Ssengooba., Sharifah Kasiinga., Abdul Sessolo., Ingvar Sanyu., Sylvia Kaswabuli., Josephine Zawedde., Patrick Byanyima., Praiscillia Kia., William Muwambi., Divine Tracy Toskin., Edgar Kigozi., Natasha Walbaum., Evelin Dombay., Mate Bonifac Legrady., Kizza DAVID Martin Ssemambo., Moses Jolooba., Davis Kuchaka., William Worodria., Laurence Huang., Stephen Henry Gillespie., Wilber Sabiti (2022) Accuracy of Tuberculosis Molecular Bacterial Load Assay to Diagnose and Monitor Response to Anti-Tuberculosis Therapy: A Longitudinal Comparative Study with Standard-of-Care Smear Microscopy, Xpert MTB/RIF Xpert-Ultra, And Culture (The manuscript is under review in the Lancet Microbe; pre-print copy can be accessed on; http://dx.doi.org/10.2139/ssrn.4161713)

Abstracts presented at the international conferences

1. **Emmanuel Musisi,** *et al.,* (2022) The accuracy of tuberculosis Molecular Bacterial Load Assay, Xpert MTB/RIF Ultra, smear microscopy and culture for tuberculosis treatment response monitoring: a comparative longitudinal study**.** The 53rd World Conference on Lung Health of the International Union Against Tuberculosis and Lung Disease (The Union) Virtual event October 2022

2. **Emmanuel Musisi.,** *et al.,* (2021) Tuberculosis-Molecular Bacterial Load Assay detects and quantifies viable Mycobacterium tuberculosis in stool of presumptive pulmonary tuberculosis patients EP-27-359, The 52nd World Conference on Lung Health of the International Union Against Tuberculosis and Lung Disease (The Union) Virtual event October 2021

Scientific Talks on invitation

Venue and date: Makerere University, Uganda. Date: Thursday 20th October 2022.

Audience: In attendance were Medical and Biomedical lecturers and students, as well as relevant stakeholder from the National TB reference Laboratory and the Makerere University Research and Innovation fund representatives.

Title of the presentation: Diagnostic accuracy of Tuberculosis Molecular Bacterial Load Assay among presumptive pulmonary TB patients: findings of a longitudinal study in Uganda. This talk was held at Makerere University.

Output: A policy brief which was shared with the policy makers. A copy of the policy brief is attached in appendices.

List of Figures

List of Tables

List of Abbreviations

AFB	Acid-Fast Bacilli
BCG	Bacille Calmette-Guérin
cDNA	Complementary Deoxy Ribonucleic Acid
CFP-10	Culture Filtration Protein-10
Cq	Quantification cycles
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
ESAT-6	Early Secretory Antigen Target-6
FM	Fluorescent Microscopy
HIV	Human Immunodeficiency Virus
IGRA	Interferon-Gamma Release Assay
IPT	Isoniazid Preventive Therapy
IU	International Unit
LJ	Lowenstien-Jensen culture media
LTBI	Latent Tuberculosis Infection
MGIT	Mycobacteria Growth Indictor Tube
mL	Milli litre
Mm	Millimetre
MML	Medical and Molecular Laboratory
Mtb	Mycobacterium tuberculosis
MTB/RIF	Xpert Mycobacterium Tuberculosis/Rifampicin assay
Mtbc	Mycobacterium tuberculosis complex

NAAT	Nucleic Acid Amplification test
NaLC-NaOH	N, acetyl-Cysteine-Sodium Hydroxide
OMN.S	OMNIgene•SPUTUM reagent
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PET-CT	Positron emission tomography–computed tomograph
PLWHIV	Persons living with Human Immunodeficiency Virus
PTB	Pulmonary Tuberculosis
QFT-GIT	QuantiFERON®-TB Gold blood test
rRNA	Ribosomal Riboxy Nucleic Acid
RT-qPCR	Reverse Transcriptase- quantitative Polymerase Chain Reaction
SSM	Stained Sputum Microscopy
TAT	Turnaround Time
TB	Tuberculosis
TB-MBLA	Tuberculosis-Molecular Bacterial Load Assay
TST	Tuberculin Skin Test
TTP	Time to positivity on the Mycobacteria Growth Indicator Tube culture
V3/vV4 region	Variable regions three and four of the 16S rRNA
WHO	World Health Organisation
XDR	Extensive Drug resistant tuberculosis
Xpert Ultra	Xpert Mycobacterium tuberculosis rifampicin Ultra assay
ZN	Zeil Nielsen microscopy

EDCTP	European and Developing Countries Clinical Trials Partnership
GCRF	Global Challenges Research Fund
GTC	Guanidine Thiocyanate
IDI	Infectious Diseases Institute
IDRC	Infectious Diseases Research Collaboration
IRB	Institutional Review Board
MakBRC	Makerere University Biomedical Research Centre of Excellence
MAkRIF	Makerere University Research and Innovation Fund
MDR	Multidrug Resistant Tuberculosis
mRNA	Messenger Riboxy Nucleic Acid
NIH	National Institutes for Health
NRH	Naguru Referral Hospital
OADC	Oleic acid-albumin-dextrose-catalase
PanACEA	Pan African Consortium for Evaluation of Anti-tuberculosis antibiotics
RD	Region of Difference
REC	Research and Ethics Committee
RS	Replication Synthesis Ratio
SBS REC	School of Biomedical Sciences Research and Ethics Committee
SCF-	Scottish Funding Council
SOMREC	School of Medicine Research and Ethics Committee
UK	United Kingdom

CSF	Cerebral Spinal Fluid
BD	Becton, Dickinson, and company
CRFs	Case Report Forms
EBA	Exhaled Breath Aerosols
EMB	Ethambutol
Emb B gene	Gene that encodes Ethambutol target protein
GCLP	Good Clinical and Laboratory Practice
GC-MS	Gas Chromatography–Mass Spectrometry
GLP	Good Laboratory Practice
gyrA	Alpha-subunit of Gyrase enzyme encoded by gyrA gene
ICH	International Conference on Harmonization
ICT	Immunochromatographic test
INH	Isoniazid
inhA	Gene that encodes Isoniazid target protein
katG	Catalase peroxidase enzyme encoded on KatG gene
LAMP	Loop-Mediated Isothermal Amplification
LPAs	Line Probe Assays
MTA	Transcriptional-mediated amplification
NVOCs	Non-Volatile Compounds
RIF	Rifampicin
rpoB	Beta-subunit of bacterial RNA polymerase encoded on rpoB gene
MU-JHU	Makerere University-John Hopkins University collaboration
CBC	Complete Blood Count

CAP	College of American Pathologists
CHS	College of Health Sciences
MIND	Mulago In patient Non-Invasive Diagnosis
IHOP	International HIV-Associated Opportunistic Pneumonias
IAM OLD	Inflammation, Ageing, Microbes and Obstructive Lung Disease
KCCA	Kampala Capital City Authority
MoH	Ministry of Health
BSL CAT-3	Biosafety Level Category- three
BSL II	Bio-Safety Level Category- two
LFTs	Liver Function Tests
RFTs	Renal Function Tests
ICFs	Informed Consent Forms

Table of Contents

Operational definitions

Participants population	Description
Presumptive TB case	An individual who presented with TB-like symptoms (chronic cough, sputum production, appetite loss, weight loss, fever, night sweats, haemoptysis and other).
Enrolled participants	All persons who met the eligibility criteria and voluntarily signed the Informed Consent Form.
True Positive (TP) test	Patients who presented to the clinic with symptoms of pTB and confirmed to have TB disease on the MGIT and were started on a TB treatment regimen.
True Negative (TN) test	Patients who presented to the clinic with symptoms of pTB and were not confirmed to have TB disease on Xpert Ultra and MGIT and were not started on a TB treatment regimen.
Smear-positive	Patients whose sputum tested positive with fluorescent stained smear microscopy according to guidelines specified in the Laboratory Manual.
Smear negative	Patients whose sputum tested negative with fluorescent stained smear microscopy according to the guidelines that are specified in the Laboratory Manual.
Smear indeterminate	Any indeterminate TB test results due to error or inability to produce a result from a single run.
MGIT positive	Patients whose sputum tested positive with MGIT according to the guidelines that are specified in the Laboratory Manual.
MGIT negative	Patients whose sputum tested negative with MGIT according to the guidelines that are specified in the Laboratory Manual.
MGIT indeterminate	Any indeterminate test results due to error, contamination, or inability to produce a result from a single run.
Xpert MTB/RIF Ultra positive	Patients whose sample tested positive with Xpert MTB/RIF Ultra according to the manufacturer's instructions.
Xpert MTB/RIF Ultra negative	Patients whose sample tested negative with the Xpert MTB/RIF Ultra according to manufacturer's instructions.
Xpert MTB/RIF Ultra indeterminate	Any indeterminate, error, or inability to produce a result from a single run.

Chapter One: General Introduction

1.1 Background

By late 18th Century, tuberculosis (TB) was one of the leading causes of morbidity and mortality rates in most parts of the world.[1,2] However, the advent of industrialisation saw improved household incomes, personal hygiene, welfare, and nutrition which sparked the decline of TB burden in most of the industrialised world.[1–3] This decline was accelerated by the discovery and development of the anti-TB drugs in the mid-20th century.[3,4] The new TB drugs were readily accessible in the high income, but not in the low-income countries. Thus, TB disease reduction was perceptibly significant in the industrialised nations, but the burden remained high in resource-constrained countries.[3,4] To curb TB-related infections, especially in the meagre income countries, the World Health Organisation (WHO) set out goals and milestones targeting 50% reduction of TB by the year 2025 relative to incidence levels in 2015. This decision was arrived at by the UN World Health Assembly in 2014 through the adoption of the End TB Strategy (2016–2035) as part of the Sustainable Development Goals (SDGs).[5,6]

The WHO's ambitious target of ending TB by 2035 strategy yielded some progress which witnessed a net 10% reduction in global TB incidences between 2015 and 2020, thus accounting for 50% of the first milestone of the End TB Strategy.[7–9] In the Sub-Saharan Africa, some of the high TB burden countries such as Ethiopia, Kenya, Namibia, South Africa and the United Republic of Tanzania were able to reach the End-TB milestone for the year 2020.[10] Alongside this progress, there was a remarkable increase of HIV care services, evidenced by the rise of 49% coverage of antiretroviral treatment.[10–13] Unfortunately, the outbreak of Covid-19 pandemic in the year 2019 undermined this promising progress of End-TB strategy through disruptions of the national health systems.[14] The deficit in the essential health services such as timely diagnosis, treatment and monitoring response to TB treatment caused instant reductions in TB detection rates and increased TB related deaths.[14]

1.2 Global TB related mortality

Although tuberculosis (TB) is preventable and curable, it remains one of the leading causes of ill-health and mortality worldwide.[12] Globally, the estimated number of deaths from TB increased between 2019 and 2021 which reversed years of TB mortality decline between 2005 and 2019.[18] Tuberculosis was a contributory factor to the deaths of 187,000 among the persons living with HIV (PLWHV).[18] These deaths were up from best estimates of 1.5 million in 2020 and 1.4 million in 2019 and reverted to the mortality levels in 2017.[18] In terms of global case fatality rate (GCFR), TB-related mortality rates increased from 14% in 2019 to 15% in 2020 [10,11] and this increase was believed to be due to Covid-19 outbreak, which greatly reversed the years of global progress in the reduction of TB related deaths.[10] Moreover, the estimated number of deaths officially classified as 'caused by TB (1.3 million)' in 2020 almost doubled the number of deaths caused by HIV/AIDS (0.68 million). Consequently, instead of achieving the targeted 35% reduction in the number of TB deaths between 2015 and 2020, the mortality rate in 2020 was 10% higher than what was reported in 2015. [10–12,15]

In general, the number of TB deaths increased during the Covid-19 pandemic in many of the 30 high TB burden countries.[11] For example, India alone accounted for 34% of the global TB deaths and 38% deaths among the persons living without HIV.[10] Of the three global TB watch-list countries, the Russian Federation achieved the End-TB milestone, with a cumulative reduction of 42% mortality.[10] In total, however, only 33 countries are struggling to reach the desired milestone of ending TB by the year 2035, indicating that there is urgent need to implement the key requirements of achieving the SDG.[10,11]

1.3 The Mycobacterium tuberculosis

Mycobacterium tuberculosis (Mtb) is a non-motile, non-sporulating, faintly gram-negative acid-fast bacilli. Due to the presence of the mycolic acid in its cell wall, Mtb is resistant to a wide range of antibiotics and adverse temperatures which makes it highly ubiquitous in nature. Mycolic acid rich cell wall slows down nutrient uptake and replication rates. Consequently, Mtb display a slower growth rate on artificial media which partly explains the long turnaround time (TAT) of the TB culture tests. Mtb belongs to a group of bacteria called Mtb complex (Mtbc). Members of the Mtbc group have a highly conserved genome with 99·9% similarity at the nucleotide level. The highly conserved identical genetic sequences within and between the Mtbc supports designing of the highly specific and sensitive Nucleic Acid Amplification Tests (NAAT).[16–23]

1.4.0 Pathogenesis of tuberculosis

Tuberculosis is an airborne disease that is caused by Mtb. The predilection site for TB pathogen is the lung tissue where it causes pulmonary tuberculosis (pTB). However, Mtb can spread from the lungs to affect other body organs usually causing disseminated tuberculosis. When a person with active TB coughs, it is estimated that they release aerosol droplets having between1-400 bacilli which is higher than the infectious dose. The suggested minimum infectious dose of TB is between 1-200 bacilli.[24,25] Infection with TB start when one inhales aerosol droplets which have infectious doses of Mtb. The inhaled droplets are phagocytosed by the alveolar macrophages in the lung.[26] In most cases, the innate immune defence contain Mtb infection, mainly due to the bactericidal activity of the macrophages, including pH reduction, action of lysosomal enzymes, reactive oxygen species, and nitrogen intermediates.[27,28] However, some Mtb evades the innate immune defence and continue to replicate inside the macrophages, infecting other host cells including epithelial and endothelial cells.[29]

Literature suggests that after about 2 weeks of infection, Mtb disseminate into other organs through the lymphatic system and continue attacking more and more host cells.[29–31] Once the bacteria disseminate into lymph node, dendritic cell and macrophages (professional antigen presenting cells) present the Mtb antigen to CD4+ T cells and activate the pro-inflammatory responses to initiate the adaptive immune response to infection.[30,31] This phenomenon leads to the activation of antigen-specific cells and their differentiation and expansion to effector T-cells.[32] In response, effector T-cells, including B-cells, macrophages and leukocytes migrate to the site of infection in the lung and contribute to the formation of a granuloma. Within the granuloma Mtb is contained and prevented from disseminating to other parts of the body.[29,32] Epidemiological studies have revealed that, 5% of infected patients clear the primary infection and do not develop TB disease, and that 95% have the infection in the granuloma and progress into latent TB infection (LTBI). Then, from LTBI, only 5-15% of persons eventually develop active TB disease in their lifetime.[33,34] Development of active TB disease, from LTBI starts by the replication of the viable Mtb cells inside the granuloma. When the *Mycobacteria* (Mtb) load increases the granuloma bursts and release Mtb.[35] When the TB granuloma bursts, an individual starts showing symptoms of active TB disease, may become sputum test positive for Mtb and releases infectious droplet nuclei containing Mtb when coughing or sneezing. The infectious droplets could infect another person and establish a new infection cycle.[26]

1.5.1 Diagnosis of Latent tuberculosis

Persons with latent TB infection (LTBI) are usually asymptomatic and non-infectious. Their chest Xray appears normal, and their sputum smear test is always negative which makes its diagnosis difficult. The currently available immunological tests that are used to detect LTBI include the Tuberculin skin test and the Interferon Gamma release assays. [36] A diagnosis of LTBI is important to find and treat persons who are at risk of progressing to active TB disease before they do so.

1.5.1.1 Tuberculin skin test

Tuberculin skin test (TST) consists of an intradermal injection of Mtb proteins mixture.[37] Persons with pre-existing cell-mediated immunity to tuberculin antigen develop a swelling and an induration at the site of injection within 48-72 hours.[37] The diameter of induration is interpreted as either positive or negative basing on the risk-stratified cut off points.[37,38] The test is quick and may predict progression from latent infection to active TB disease. However, it requires a return visit of 2-3 days after the injection, which calls for additional cost burden to patients in transport fares.[39] Besides, interpretation of the TST results is highly subjective and may not be reproduced.[38] Further, TST is commonly confounded by factors such as the history of gastrectomy, prior vaccination with BCG as well as being diabetic. All these confounders are prevalent in high TB burden countries.[40] Moreover, recurrent TST testing is associated with immunological recall to previous exposure to mycobacterial antigens hence exaggerating subsequent response to the test[41]. High false positivity rates are common in populations where infection with Non-Tuberculous Mycobacterium species (NTM) is higher than infection with Mtb.[38] Similarly, high rate of false negative results occurs among the persons who are living with HIV due to delayed hypersensitivity.[42,43] Nevertheless, TST is a widely applied test because it is affordable, less invasive, easy to use, and readily accessible in high burdened and resource constrained countries.[39]

1.5.1.2 Interferon gamma release assays

Interferon gamma release assays (IGRAs) measure the amount of the interferon-gamma (IFNγ) that is released in response to the following TB specific antigens: Early Secretory Antigenic Target-6 (ESAT-6), TB7.7 and Culture Filtration Protein-10 (CFP-10).[44] Unlike the Tuberculin Skin Test, IGRAs are not affected by the previous exposure to the BCG vaccine, but they are less specific since they cross-react with *Mycobacterium tuberculosis. marinum* and *Mycobacterium tuberculosis. kansasii*.[45,46]

IGRAs are less reproducible largely due to the inter-operator imprecision and immunological boosting due to the purified protein derivatives.[47,48,49] It is important to note that Interferon gamma release assays are not comparatively superior to the TST since both tests lack the accuracy that is sufficiently high to predict progression from LTBI to active TB disease.[50,51,52] However, persons with a TST sclerosis that is greater than 15 mm or a QuantiFERON®-TB Gold blood test (QFT-GIT) result that is greater than 10 IU/mL have been shown to be at higher risk of developing active TB.[53] The progression from LTBI to active TB might easily be predicted among the persons with recently acquired, than those with distantly acquired LTBI, but more investigations are needed to ascertain this observation.[54]

1.6.0 Prevention of tuberculosis

Effective TB treatment, observing cough etiquette, and effective use of vaccines can prevent or reduce transmission of TB. The Bacillus Calmette-Guerin (BCG) was developed in 1921 by knocking out the Early Secretory Antigen Target (ESAT-6) and *Mycobacterium* culture filtrate protein-10 (CFP-10) genes in the regions of difference (RD1-RD16) from the *Mycobacterium tuberculosis* to make it nonvirulent. To-date, BCG vaccine is the only approved TB vaccine, yet its efficiency is varied.[55–60]

1.6.1 Limitations of the BCG vaccine

The average efficacy of the BCG vaccine against TB meningitis and miliary disseminated TB disease in children is 86% but it is not protective against TB in adult persons.[61–63] Prior exposure to live *Mycobacterium. avium* elicits a broad immune response which is rapidly recalled following exposure to BCG vaccine. As a result, the immune response elicited by the vaccine is transient with incomplete or short lived immunity protection against TB.[64] It has been suggested that high levels of environmental mycobacteria strongly correlate with high incidences of TB although majority of the general population are vaccinated.[65] Development of a suitable vaccine takes long and requires a lot of money capital.

Different models such as non-human primate BCG infection model and human BCG challenge model to assess antimycobacterial immunity induced by the BCG and a candidate tuberculosis vaccine were described.[66–68] The subunit TB vaccines based on proteins that are expressed in the replicating stage of Mtb are not affected by exposure to the environmental mycobacteria but they are unable to prevent progression from the latent infection to active TB disease.[69] On the other hand, interventions such as Isoniazid Preventive Therapy (IPT) and Test and Treat have endured suboptimal implementation levels.[70,71]

1.7.0 Active tuberculosis

Exposure to Mtb may or may not progress to active TB disease in lifetime.[28] About 10% of persons with TB disease is due to primary infection commonly in children and the immunosuppressed persons with new TB infection.[72] About 5% of the persons with LTBI develop TB disease in the first two years of infection and the other 5% develop the disease later in life.[73] Persons with active tuberculosis commonly report recurrent fever which usually follows a daily pattern, and it increases as the day goes and it then subsides at night. However, sometimes it is associated with night sweat.[74] Although cough is often mild and non-productive in early stages of the disease, it becomes productive, and haemoptysis may occur in advanced cases of the disease. Non-pulmonary symptoms such as lymphadenopathy, unexplained fatigue, and pharyngitis may also occur.[75] Additionally, advanced TB disease may lead to anorexia, and loss of body/muscle mass.[76]

1.7.1.0 Biomarkers for tuberculosis

A biomarker is a proven measurable surrogate for a normal physiological, pathological or the effect of therapeutic or prophylaxis intervention processes.[77,78] In particular, TB biomarkers can be used for disease diagnosis, prognosis, progression from LTBI to active TB disease and monitoring the protective efficacy of TB vaccines.[79] Biomarkers for TB are categorised as bacteriological and host biomarkers.

Bacteriological biomarkers entail Mtb as a pathogen and its characteristics. Contrastingly, host-based biomarkers focus on elements and characteristics of the host's response that are specific to Mtb infection.[80] Biomarkers can be investigated at transcriptomics, metabolomics, proteomics and lipidomic levels. These can be modified to form a biosignature to develop a more suitable surrogate.[81,82] Results from the same biomarker may be inconsistent depending on geographic locations, sample collection, processing, and the analysing procedures. This phenomenon highlights the most common challenge that is associated with biomarker research and discovery. However, recent technological advancements such as high-throughput mass spectrometry and advanced bioinformatics protocols have addressed some of these challenges, leading to discovery of active pTB biomarkers with 80% accuracy in symptomatic high-risk persons. [82,83,84]

Generally, progress in the development and evaluation of TB biomarkers take a very long trajectory, which delays translation into usable tools and implementation. This process needs to be accelerated if we are to achieve the end TB strategy goal of ending TB by the year 2035 through early, affordable, faster, and correct diagnosis.

1.7.2.0 Diagnosis of active TB

Proper diagnosis of active TB is important to find and treat persons to prevent progression to severe levels which can lead to death. The common bacteriological and molecular based tests that are used to diagnose TB are discussed in this sub section.

1.7.2.1 Use of clinical symptoms to diagnose TB

TB disease is usually characterized by clinical manifestations, which distinguish it from TB infection without signs or symptoms; previously referred to as latent TB infection. Clinical symptoms are used to detect persons who are likely to have TB disease. Usually, screening for the four symptoms of cough, fever, weight loss and/or night sweats is the first key step to detect

TB in adults and adolescents, including patients living with HIV/AIDS (PLHA) regardless of antiretroviral treatment (ART) status. [85]

For infants and children living with HIV/AIDS and/or in contact with a person with infectious TB disease, screening for poor weight gain (failure to thrive) or weight loss, reduced playfulness or lethargy, fever and active cough is recommended by the WHO.[86] Sensitivity and specificity of the WHO's recommended four-symptom screening rule for tuberculosis in persons living with HIV was conducted by Hamada et al., 2018. In this analysis, 21 records involving 15427 participants of which, 1559 were confirmed to have active tuberculosis. In their final met analysis, Hamada et al., included 18 eligible studies.[87] They reported a pooled sensitivity (95%CI) of the four-symptom screening rule was lower for persons who were being treated with anti-retroviral therapy (ART) was 51.0% (28-73) which was lower than 89.4%, (83-94) for those who were ART-naïve.[87] They observed that pooled specificity (95%CI) for the participants on ART was 71% (48-86) which was higher than 28% (19-40) for those who were ART-naïve.[87] Findings of this review suggested a lower sensitivity of the WHO four-symptom screening rule among persons with HIV who are on ART than in those who are ART naive. Diagnostic accuracy of the symptom screening improved if used in addition combination with chest x-ray.[87] The review revealed that addition of any abnormal chest radiographic findings to the data in participants who were on ART improved sensitivity (95%CI) from 52.2% (38-66) to 85% (70-93) but this occurred at the expense of specificity which decreased from 56% (52-59) to 30% (26-34).[87]

1.7.2.2 Medical imaging

Over the years, chest radiography has been a useful tool for triaging and screening persons for TB with potential to increase TB case detection rates.[88] In particular, chest imaging is essential in identifying certain forms of TB, including the following pleural, pericardial and miliary TB.

Usually, bacteriological testing is limited in such cases.[89] Pleural effusion and cavitation on the chest x-ray is the commonest indicator of pTB.[90] However, cavitation is less specific since it may not differentiate between the persons with post and those with current TB disease.[91] In some cases, chest x-rays are less sensitive and they miss about 20% of the smear-negative but culture-positive patients.[92] Besides, medical imaging usually depends on skilled and experienced personnel for reliable interpretation. In some cases, these skilled personnel may not be readily available. The few who are available may be overwhelmed by the heavy work load leading to poor quality results.[93] Software for automated image reading is now available to support high throughput with minimal dependence on the radiologists. The software can be used to triage patients during case finding and to assist with prevalence surveys.[94] More sophisticated imaging methods such as chest Computerised Tomographic (CT) and Positron Emission Tomography-Computed Tomography (PET-CT) scans have a high-resolution with enhanced sensitivity and higher accuracy for miliary TB.[95,96] However, these tests are less specific and sometimes require an additional chest Xray to enhance specificity.[97,97] Nevertheless, radiological biomarkers identify more cases of child TB and LTBI cases than any of the standard-of-care tests. With specialised instrumentation, there is hope that chest imaging can improve and may be applicable in monitoring treatment response.[98,95]

1.7.2.3.0 Stained sputum smear microscopy

In resource-limited countries, stained sputum smear microscopy (SSM) is the commonest TB diagnostic test because it is simple, affordable, and quick to perform. The ease and quickness of SSM is supported by the principle based on the ability of mycolic acid in the wall of Mtb cell to prevent decolourisation of the primary dye by acid-alcohol solutions (a phenomenon referred to as acid fastness).[99] Broadly, stained smear technique for Mycobacteria is based on Carbol-fuchsin (Zeil-Neelsen) or Fluorochrome dyes.

1.7.2.3.1 Zeihl Nielsen stained smear microscopy

This technique is named after the two German doctors who developed and modified the stain: the bacteriologist Franz Ziehl (1859–1926) and the pathologist Friedrich Neelsen (1854–1898). The use of phenolic Carbol fuchsin as the primary dye in Ziehl Nielsen (ZN) for staining is based on its lipophilic nature which enables it to penetrate the waxy cell wall of the Mycobacteria.[103] Efficiency of the carbol fuchsin is enhanced by steam heating the smear. Heating melts the waxy cell wall to ease Carbol fuchsin entry into the mycobacteria cell.[103] Following decolorization with acid-alcohol, the smear is counterstained with malachite green or methylene blue which stains the background, providing a contrast colour against which the red Acid-Fast Bacilli (AFB) can be seen.[103]

Kinyoun staining is a short and simplified procedure of the conventional ZN staining method. This modification replaces the heating step with phenol-Carbol-fuchsin solution of higher concentration. However, Kinyoun procedure is not recommended for use since it is ineffective compared with the conventional ZN method.[100] To observe the AFB cells, stained smear is examined microscopically using the 100x Oil immersion objective. Acid fast bacilli appear as red, straight, or slightly curved rods, occurring singly or in small groups and may appear beaded (*Figures. 1.1.0*)

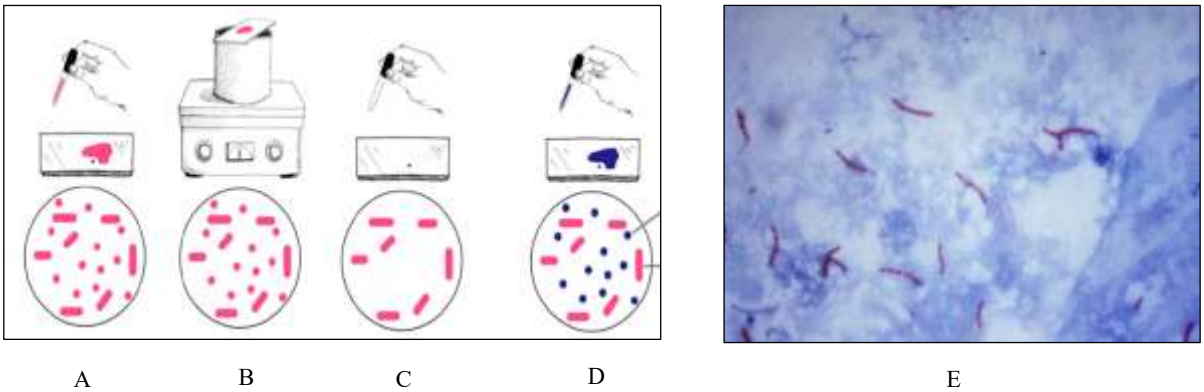


Figure 1.1.0: Procedure for Zeil-Nielsen Staining (SSM-ZN). (A) Smear is flooded (Carbolfuchsin) for 30 seconds. (B) Smear is heat fixed. (C) The smear is decolorised using acid alcohol. Methylene blue (or malachite green) is applied as a counterstain, and then rinsed with distilled water to remove the excess stain. (E) AFB appear as red rod-shaped bacteria, under X100 oil immersion. Adapted from www.labtestguide.com

1.7.2.3.2 Limitations of the Zeihl-Neelsen microscopy

Although ZN-based smear microscopy is simple and inexpensive the test is less sensitive typically requiring a minimum of 10,000 AFBs per mL as a limit of detection. Sensitivity is further reduced due to paucibacillary nature of TB in children, among persons living with HIV, and those with extra-pulmonary tuberculosis (ETB). Consequently, a negative SSM-ZN is not sufficient to exclude TB disease. Furthermore, SSM-ZN cannot differentiate Mtbc from the non-tuberculous mycobacteria infections. However, this test is widely used in resource constrained settings with literature indicating good specificity. A multi-country cross-sectional evaluation study conducted in Ethiopia, Nepal, Nigeria, and Yemen among 1,156 participants who submitted spot-spot morning sputum, and 1,199 spot-morning sputum showed that examining two SSM-ZN had a pooled sensitivity (95%CI) of 66% (62-70) which was less than 73% (69-77) for Light Emitting-Fluorescent microscopy (LED-FM).[101] Pooled specificity (95%CI) for SSM-ZN was 98% (97-98) which was higher than 91% (90-92) for LED-FM. Increasing the number of examined smears from two to three increased the SSM-ZN sensitivity from 66% (62-70) to 70·5% 66.4-74.4) [101], but specificity dropped from 98% to 96·5%.[101]

1.7.2.3.3.0 Fluorescent smear microscopy

Fluorescent stained smear microscopy **(**SSM-FM) tests is based on the ability of fluorochrome dyes such as the auramine-O to form a stable complex with the mycolic acids and the resistance of the mycobacteria to lose the primary dye because of the acid-alcohol effect. The Potassium permanganate is used as the counterstain which aids to differentiate between AFBs and artefacts. Efficiency of the SSM-FM improves if samples are properly collected, and smears are prepared and examined carefully. A sample smear which is too thick tends to flake during the staining steps and may be difficult to decolorise. Additionally, acid-fast organisms may be obscured on a thick smear. Too thin sample smears may represent ‘over dilution’ and may consequently contain less bacilli leading to false negative interpretation.[102,103]

1.7.2.3.3.1 Limitations and challenges of the fluorescent microscopy

A fluorescent stained smear microscope is usually not readily available, and it is costly which makes it inappropriate for use especially outside the National Reference and Research Laboratories. Reagents like auramine-rhodamine are potential carcinogens, the acid–alcohol and potassium permanganate are also strong irritants.[103] Besides, most strains of the rapid growers may not appear fluorescent. Excessive exposure to the counterstain may result in a loss of brilliance of the fluorescing organism. Stained smears should be examined within twenty-four hours of staining to avoid false negative interpretation that might occur when the fluorescence fades. A negative staining reaction does not indicate that the specimen will be culturally negative. Therefore, a negative SSM-FM should be confirmed with SSM-ZN and at least 100- fields should be examined before being reported as negative. An add-on culture test is necessary. Besides, Centre for Diseases Control recommends fluorochrome staining for detecting AFB in primary patient specimens because SSM-FM is on average 10% more sensitive than SSM-ZN with similar specificity at 98%. Examination for 1minute of the FM is much quicker than SSM-ZN. For example, SSM-FM examination for one minute is associated with a higher sensitivity and similar specificity to SSM-ZN examination for 4 minutes.[104]

A multi-country cross-sectional evaluation study conducted in Ethiopia, Nepal, Nigeria, and Yemen among 1,156 participants who submitted spot-spot morning sputum and 1,199 spot-morning sputum showed that examining two sputum smears yielded a pooled sensitivity (95%CI) of 72.8% (68·8%-76·5%) for LED-FM, which was higher than 65·8% (61·6%-69·8%) for SSM-ZN.[101] The same study showed that pooled specificity for 90·9% (826, 89.5%-92·2%) for LED-FM was less than 98% (826, 97·3%-98·6%) SSM-ZN.[101] Increasing the number of examined smears from two to three increased the LED-FM sensitivity (95%CI) from 72·8% (68·8%-76·5%) to 77% (3·3%-80·6%) [101] but specificity dropped from 90.0% to 88·1%.[101]

1.7.2.4.0 TB culture tests

Culture tests remain the gold standard for detecting mycobacteria in clinical specimens that are suspected to contain Mtb. Use of culture medium is mainly because it is more sensitive than smear microscopy capable of detecting less than 10 AFBs per mL in the clinical samples. Secondly, culture allows the isolation of the mycobacteria to enable speciation and identification studies. Thirdly, growth of viable bacteria on culture medium allows clinical samples to be tested for drug susceptibility. Cultivating TB involves inoculation of the clinical samples on solid medium in petri dishes, agar slants or bottles containing broths. To recover and isolate mycobacterial cells from the clinical samples, three main types of media are commonly used, and these include Egg-based, agar-based and liquid medium.[105–107]

1.7.2.4.1.0 Egg-based media

Egg-enriched media supports growth of a wider variety of mycobacteria species which is essential for biochemical tests such as niacin and catalase production tests and speciation test. Besides, egg-based media are easy to prepare, they are affordable and readily available.

1.7.2.4.1.1.0 Lowenstein-Jensen

Lowenstein Jensen (LJ) media is a selective medium that is used for the cultivation, isolation, and drug susceptibility testing of Mtb from clinical specimens. Lowenstein-Jensen media was first described on Lowenstein in the year 1931. The original formulation incorporated Congo red and malachite green to inhibit growth of the unwanted bacteria. In the year 1932, Jensen developed Lowenstein-Jensen media on altering the citrate and phosphate, eliminating the Congo red dye, and increasing the malachite green concentration contents of the Lowenstein formulation. [108,109] Since then, LJ culture media has undergone several modifications to improve the output and to allow different investigations to be undertaken. For example, replacement of the fat-rich glycerol with pyruvate was done to enhance growth of *M. bovis* and *M. africanum*. [110]

To determine the rate of iron uptake on the mycobacteria, LJ media can be supplemented with iron. Incorporating five percent sodium chloride can be used to characterise some species of mycobacteria while for semi quantitative analysis, LJ medium deep tubes for catalase are used. The BBL™ Mycobactosel™ Lowenstein-Jensen media Slants are supplemented with cycloheximide, lincomycin and nalidixic acid. [100,111,112]

1.7.2.4.1.1.1 Principle of the Lowenstein-Jensen medium

L-Asparagine and Potato Flour are used as sources of nitrogen and vitamins. Monopotassium phosphate and magnesium sulphate enhance organism growth and act as buffers. Malachite green inhibits growth of most contaminants while encouraging growth of mycobacteria. Egg suspension provides fatty acids and protein which are required for mycobacterial metabolism. Heating the egg suspension coagulates the albumin and a solid surface is formed. This surface supports inoculation, identification, and enumeration of the bacteria colonies. Glycerol serves as a carbon source and ingredient for the growth of the human-type tubercle bacillus while being unfavourable to the bovine type. In the Gruft method, penicillin and nalidixic acid along with malachite green prevent the growth of most of the contaminating microorganisms while promoting the earliest possible growth of mycobacteria. [113,114]

1.7.2.4.2 Ogawa Mycobacteria Growth media

Ogawa Mtb growth media is an egg-based medium with similar composition as LJ media but without asparagine. Replacing asparagine with sodium glutamate makes Ogawa medium a more affordable alternative to the LJ medium. Additionally, the concentration of the malachite green, volume of egg homogenate and pH all differ between LJ and Ogawa media.[115,116]

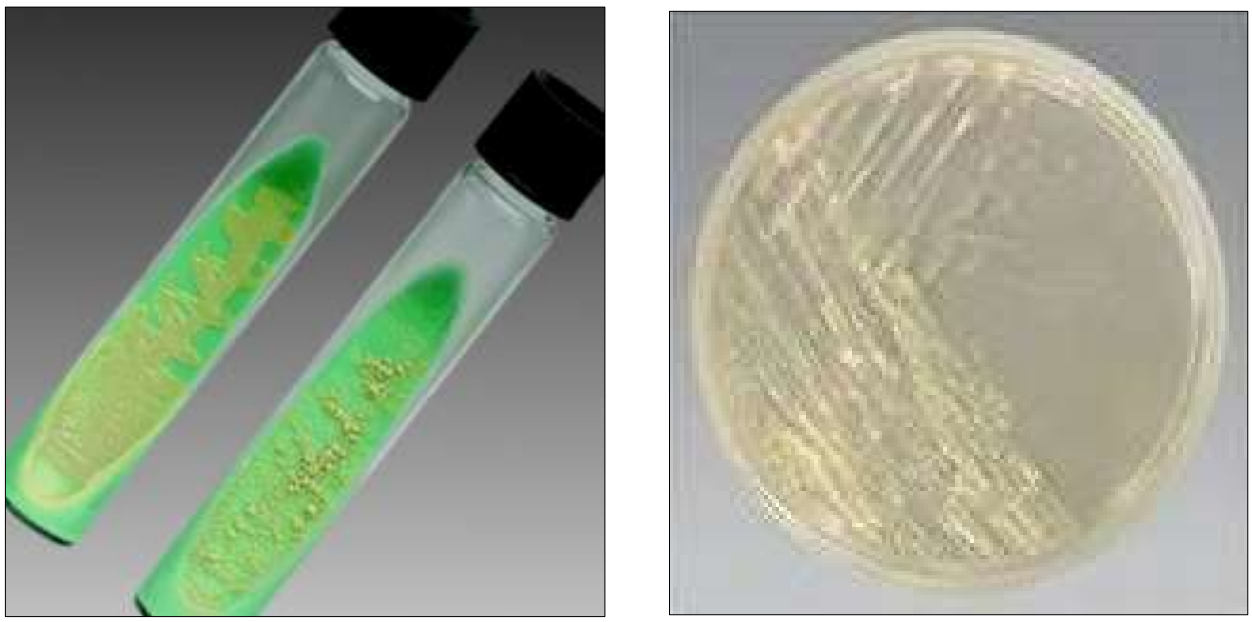
1.7.2.4.3 Challenges of the egg-based media

The main limitation of the egg-based media is the high turn-around time which may delay clinical decisions. Although mycobacteria growth can be detected in less than 21-days,

incubation proceeds for 8 complete weeks before the sample can be classified as negative. The turnaround time is even higher for samples that contain few bacilli.

1.7.2.4.5.0 Agar-based media

Agar-based medium was first described in 1958 by Middlebrook and Cohn and comes as either a 7H10 or 7H11 formulation. The composition of this medium is rich in organic salts, vitamins, cofactors, glycerol, malachite green and agar. Enriching the agar-based media with Oleic acid-albumin-dextrose-catalase (OADC) allows for qualitative procedures for isolation. Hydrolysed casein at 0.1% favours the recovery of isoniazid-resistant mycobacteria. Therefore, enriching 7H10 medium with 0.1% of the hydrolysed casein modifies it to 7H11, which enhances growth of fastidious strains of Mtb and improves susceptibility testing. Compared with 7H10 agar plates, 7H11 have been shown to be a better option for culturing multi-drug resistant strains of Mtb (MDR), hence justifying its wider applicability.[117] (*Figure 1.2.0*)



A B

Figure: 1.2.0: Growth of *Mycobacteria tuberculosis* (Mtb) on culture media: (A) Growth of Mtb in Lowenstein-Jensen media - an egg-based medium. (B) Growth of Mycobacterium tuberculosis in Middlebrook 7H11 medium - an agar-based medium. (Adapted from Mycobacteriology Laboratory, Makerere University)

1.7.2.4.5.1 Advantages of using the Agar-based media

Unlike the egg-based media, agar-based media are translucent which allows the visualisation and microscopical count of Mtb colonies as early as one week after incubation. Colony morphology of the Mtb on agar plates is distinguishable which supports the identification of mycobacteria with ease. Additionally, it is possible to perform the susceptibility testing without changing the concentration of the antibiotic which is not possible with egg-based media.[117]

1.7.2.4.5.2 Precaution measures when using agar-based media

This medium is extremely sensitive to heat and light. Therefore, to minimise loss of quality, it is better to prepare it in small quantities at a time, and it should be stored in the dark. Besides, exposure of the agar-based media to heat and day light results in the release of formaldehyde from OACD. Formaldehyde inhibits growth of the mycobacteria; therefore, it is important to always store OACD at 4^{o}C and not to heat it (but to rather thaw it to room temperature) before adding it to the agar.[118]

1.7.2.4.6.0 Liquid media

The turnaround time for the solid media culture is high and may delay clinical decision making. A shorter turnaround time is possible when the liquid culture is used. Commercial liquid-based culture systems use radiometric, colorimetric, or fluorometric detection methods in a manual, semi-automated or automated platforms.[118]

1.7.2.4.6.1.0 Manual liquid culture systems

Manual liquid culture systems lack specific instrumentation, and they are usually non-radioactive. BBL^{TM} Septi-Check^{TM} AFB from the Becton Dickinson Microbiology Systems, USA is a typical example of the manual liquid culture systems. This system is biphasic, consisting of the following media: a bottle of modified Middlebrook 7H9, a slide-plate containing chocolate agar, LJ and Middlebrook 7H11.

The blood agar test is used for the isolation- of bacteria other than the mycobacteria. Middlebrook 7H11 and the LJ media are incorporated to ensure growth of most mycobacteria species. Before inoculation of the sample, an antibiotic and enrichment supplement are added to supress the contaminating bacteria, and the bottle is upturned to inoculate the solid medium. Bacteria growth in the BBL™ Septi-Check™ AFB system is detected by observing the colonies at the surface of the broth media. BBL™ Septi-Check™ AFB system allows simultaneous detection of Mtb and NTM within three weeks of inoculation in addition to requiring a small inoculate. Most of the works, which were done in the 1990s showed that Septi-Chek AFB was more rapid with a higher recovery of Mtb than the LJ media. Besides, the system supported a wide range of biological specimens: sputum, bronchoalveolar lavage (BAL), urine, stool, and biopsy tissues; pleural, cerebral spinal fluid (CSF) and ascites fluid, but it was labour intensive. Moreover, the turn-around-time of three weeks was long enough to limit its use in clinical decision making.[119,120]

1.7.2.4.6.1.1 The BBL™ MGIT™ Mycobacteria Growth Indicator Tube

This is a simple manual fluorometric method for detection of mycobacteria growth. Each of the mycobacterial growth indicator tubes contains 4mL of the modified Middlebrook 7H9 media and a gel. The fluorescein (ruthenium) is embedded in silicone at the bottom of a 16x100 mm round-bottomed tube. When the tubes are illuminated with a long wave UV light, an intense orange light is emitted at the bottom of the tube and at the meniscus of the media for a positive test. A faint or absence of fluorescence indicates a negative test. The BBL™ MGIT™ Mycobacteria Growth Indicator Tube system does not use needles or radioactivity but rather plastic tubes to ensure safety. Evaluation studies indicated that the system had potential for routine use for both detection and drug susceptibility testing of Mtb isolates. Further, this system enabled rapid identification of Mtb and detection of resistance to antimicrobial drugs with high sensitivity and specificity.

Besides, since this system is non-radiometric method, it is considered safe to work with. However, BBL™ MGIT™ Mycobacteria Growth Indicator Tube has some limitations too. For example, it is most suitable for laboratories with small work volume which limits its applicability in the large TB reference/researchprogrammes.[121,122] (*Figure 1.3.0*)

A **B**

Figure 1.3.0: BBL™ Septi-Check™ AFB and BD-BACLTEC-MGIT. (A) BBL™ Septi-Check™ AFB; Source: http://www.bd.com/micro-biology. (B) BD-BACLTEC-MGIT Source: https://www.bd.com/en-us/offerings/capabilities/microbiology-solutions/mycobacteria-testing/bd-bactec-mgit-mycobacterial-growth-indicator-tubes

1.7.2.4.6.2 Semi-automated liquid culture systems

BACTEC 460TB is a semi-automated system. This is because the bottles which contain the culture media are incubated outside the instrument before, they are placed into the instrument. This system enhanced the recovery of mycobacteria and reduced the time that was required for mycobacteria growth in the earlier systems to less than two weeks. BACTEC 460TB system was used for the isolation of mycobacteria, differentiation, and susceptibility testing of the Mtb strains. Semi-automated liquid culture systems comprise glass vials containing 4 mL of

Midlebrook 7H12 or 7H13 (for blood samples). The medium contains palmitic acid labelled with radioactive ^{14}C. [123–125] Mycobacteria growth is detected by measuring the release of radioactive carbon dioxide with the aid of a gas flow radio counter. The released radioactivity is converted into a growth index ranging from 0-99 using standard curve plotted from released radioactivity against time. Contaminating bacteria are suppressed on adding a supplement containing several antibiotics such as Polymixin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (BACKTEC™ PANTA™ PLUS kit). To enhance Mycobacteria growth, polyoxythylene stearate (POES) is incorporated in the media. Adding 5% carbon dioxide during each reading further enhances mycobacteria growth.[123,124]

1.7.2.4.6.3.0 Automated liquid culture system

The BACTEC™ MGIT™ is a fully automated fluorometric culture system for the isolation of mycobacteria. All the data obtained with the system can be stored and processed using EpiCenter software from BD Company. A positive test is detected by a strong fluorescence from the ruthenium dye in the gel under UV light which is converted into growth units (GUs). A positive test is achieved at 75 GUs. The fluorescence intensity is directly proportional to the amount of oxygen that is consumed by the replicating bacteria. The time to detection (TTD) for a positive culture is printed as a report form provided by the system. If there is no fluorescence after 45 days, the test is reported as negative. To supress the growth of contaminating bacteria, BACKTEC™ PANTA™ MGIT supplement is added to the primary culture. The Bactec™ MGIT™ system has low contamination rates, and it can be used to perform susceptibility tests of Mtb for both first- and second- line antibiotics. However, the cost of the equipment, the required reagents and media is high, making it expensive.[118]

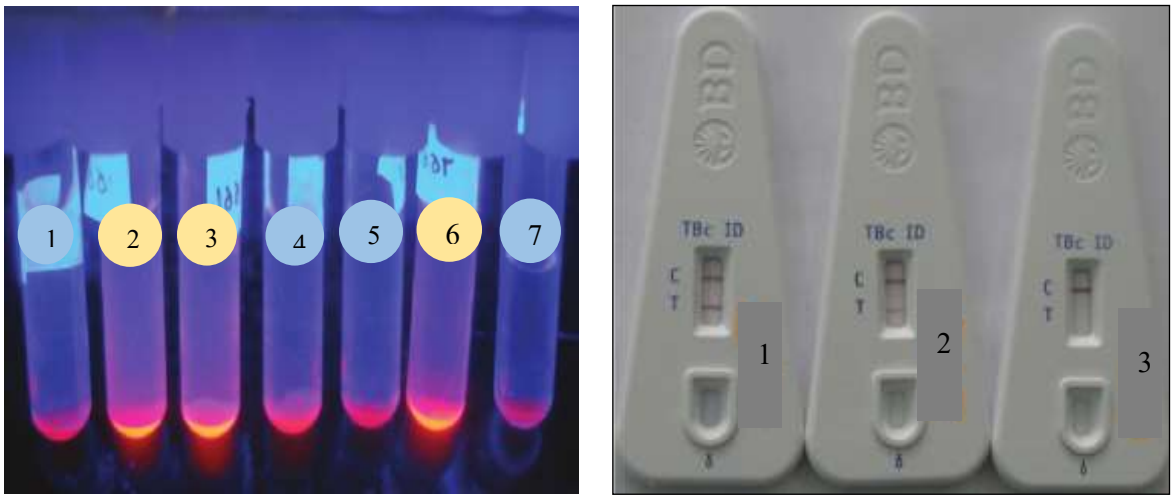
The BacT/ALERT mycobacteria detection system is a fully automated microbiology system which uses a colorimetric sensor. It provides a non-isotopic alternative for the detection of mycobacteria. This system is based on the detection of a decrease in the pH due to the accumulation of carbon dioxide from the actively proliferating bacteria. The carbon dioxide acidifies the medium and causes the colour of the sensor to turn from green to yellow and this colour change is detected by a reflectometric unit. Results are read by the system automatically every after 10 minutes using infra-red rays. Data generated are saved on the BacT/View software which minimizes error rates that are associated with manual operations.[126]

The BACTEC™ 9000 MB is an automated non-radiometric culture system for the isolation of mycobacteria. The system uses the MYCO/F medium modified from Middlebrook 7H9 media. In this system, the inoculated vials are inserted into the equipment for incubation and the results are read every after 30 minutes. The system responds to changes in oxygen concentration. Each vial contains a silicon rubber disc impregnated with ruthenium, which serves as an oxygen-specific sensor, thus enabling microorganism metabolism and growth to be detected. Oxygen consumption by microorganisms present in the medium can be observed by the increase in fluorescence and a positive reading indicates presumptive presence of viable microorganisms. This system monitors fluorescence levels and detects the growth of microorganisms using positivity algorithm. It is a rapid, sensitive, and efficient system that is suitable for the isolation of mycobacteria in clinical samples. [127–129]

1.7.2.4.6.3.1 Complementary tests to MGIT culture

The Ziel-Neelsen smear microscopy is usually done for positive MGIT culture tests to confirm the presence of the acid-fast bacilli. Chocolate blood agar is done for every positive MGIT culture test to rule out the presence of contaminating bacteria. The result of the blood agar can be interpreted as contaminated- positive or contaminated- negative.

Tuberculosis Ag MPT64 test (Dickinson and Becton Company, MD, USA) is an immunochromatographic test (ICT) that rapidly differentiates between the NTM and TB disease since MPT64 protein is only secreted by members of the Mtbc but not the NTM Mtb Ag MPT64 test is also applicable in routine Pathology laboratories as an additional diagnostic measure to strengthen the diagnosis of extra pulmonary tuberculosis in formalin-fixed biopsies, especially if culture tests are not accessible.[130,131] (*Figure 1.4.0*)



A **B**

Figure 1.4.0: MGIT culture and MPT64 test results. (A) MGIT culture tubes A2, A3 and A6 show fluorescence indicating a positive MGIT test. A1, A4, A5 and A7 show faint-to no fluorescence indicating negative MGIT result. (B) Identification of the Mycobacteria complex using TB Ag MPT64 test. B1 and B2 show red lines both in the control and test windows which indicates a positive TB Ag MPT64 test. B3 has one line in the control window indicating a negative TB Ag MPT64 test. Absence of the control band would invalidate the test. TB Ag MPT64 test with a red line in only the test window, or absence of a red line in both the test and control widows would be considered invalid (Adapted from Mycobacteriology Laboratory, Makerere University)

The fully automated BACTEC MGIT 960 system is the most used liquid culture system.[100] The World Health Organisation recommends this test as the gold standard for TB diagnosis. Most evaluation studies use BACTEC MGIT 960 system as the reference test for calculating specificity, sensitivity, and predictive values of the novel TB diagnostics. The most important advantage of using liquid culture tests is that they have higher sensitivity compared with smear microscopy and solid culture.

Ideally, liquid cultures would potentially increase the likelihood of diagnosing TB in early stages, especially the paucibacillary form of the disease; and detection of treatment failures and drug susceptibility testing (DST).[111]

However, the test has several challenges which limit its applicability for routine TB care. It is expensive requiring a category three laboratory, the turnaround time is long which would delay clinical decisions, and a lot of data is lost since some of the contaminated tests may be indeterminate.

1.7.2.5.0 Nucleic acid amplification tests

Nucleic acid amplification tests (NAATs) for TB are molecular based assays designed to target Mtbc specific gene sequences. Mature 16S and 23S ribosomal RNA, and the non-coding pre ribosomal RNA sequences have been targeted to detect and quantify viable mycobacteria. Genes that encode proteins such as mycobacterial protein fraction BCG-64kDa (MPB64), mycobacterial protein T-kDa40 (MPT40) and protein antigen have been targeted for rapid detection of Mtbc. Drug resistance determining genes gyrase subunit-beta (gyrB) and RNA polymerase-beta (rpoβ) have been incorporated as target sequences to enable testing for drug resistance. Additionally, insertion sequences IS6110 and IS986, and repetitive elements are included in the assays to improve sensitivity.[132–134] NAATs influence a variety of management decisions resulting in decreased time to diagnosis, and they could be cost saving in some subpopulations.[134,135]

1.7.2.5.1 Cobas Amplicor Mtb test

The amplicor Mtb test (Roche Diagnostic Systems, New Jersey, USA) targets Mtb specific 16S rRNA gene. Under PCR, amplicons are detected colourimtrically after hybridising to complementary oligonucleotide probes. COBAS amplicor Mtb test is the automated version of the amplicor Mtb test.

This automated version amplifies and detect Mtb automatically on the same platform using the integrated analyser. This test is intended for use on decontaminated and concentrated samples and the results are availed in seven hours, which limit its applicability as a point-of-care TB tests. To improve its robustness, COBAS TaqMan 48 analyser which runs up to 48 samples and provide results within two and a half hours was invented. Clinical evaluation investigations indicate that the modified version is highly specific and sensitive, regardless of the smear status of the participants.[136,137]

1.7.2.5.2 Gen probe assay

The Gen probe is an isothermal transcription-mediated amplification (TMA) assay which targets 16S rRNA. A specific rRNA target is amplified by the transcription of the DNA intermediate products, thus yielding multiple copies of the mycobacterial RNA. The amplicons are then detected on binding to a single stranded acridinium ester-labelled DNA probe, giving a chemiluminescence signal that is read in the illuminator. Amplification and detection are performed in a single tube and test results are availed within two and a half hours.[138,139]

1.7.2.5.3 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) test is based on auto-cycling, strand displacement DNA synthesis. This is achieved by using a DNA polymerase which has high strand displacement activity. The test applies two specially designed inner, and two outer primers supplied by the Eiken Chemical Co Ltd., Tokyo Japan. LAMP assay targets a gene that encodes DNA gyrase subunit beta. The reaction proceeds in two phases: the starting structure-producing step and the cycle-amplification step. To generate the starting structure, all the four primers are required to initiate rounds of strand displacement DNA synthesis (i.e., DNA is synthesised and denatured simultaneously). During the amplification stage, a pair of the composite primers amplifies the synthesised DNA, resulting in different sized stem-loop structure of alternatively inverted repeats of the target sequence.

This also results into cauliflower-like structures with multiple loops that are formed by annealing of the repeat sequences on the same strand. In addition, RNA transcriptase enzyme step allows the LAMP technology to use RNA as the starting material. Results can be visualised directly either by turbidometry or fluorometry method.[140,141] (*Figure 1.5.0*)

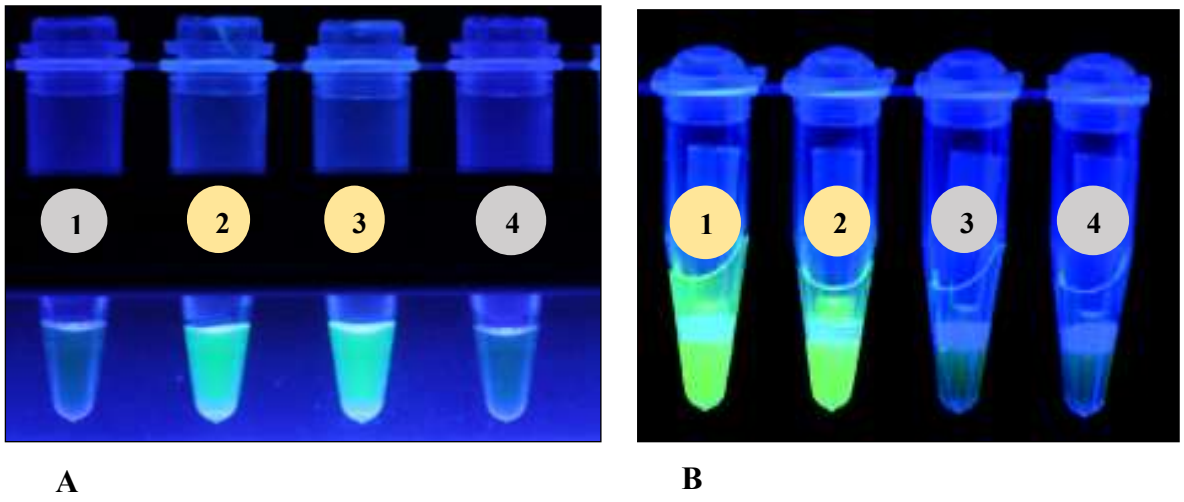


Figure 1.5.0: TB-LAMP tests. A2 and A3 indicate positive tests represented on a white precipitate of magnesium pyrophosphate. The precipitate can be observed directly on visual inspection or reading the tubes in a turbidimeter. A1 and A4 indicate negative results. B1 and B2 also indicate positive tests represented on a strong fluorescence when calcein, a chelating agent is used. In a positive test, Calcein combines with magnesium ions to yield a strong fluorescence under a UV light. Results can be inspected visually or by using spectrophotometer (Adapted from Mycobacteriology Laboratory, Makerere University)

A modified version of the LAMP assay uses a kit (Einken Chemicals, Tokyo Japan) which simplifies the DNA extraction protocol and reduces the overall turnaround time to 1.5 hours. Loop-mediated isothermal amplification assay is reproducible, affordable and requires minimal training making it applicable in resource constrained settings. However, it is associated with low sensitivity and specificity among the smear negative persons, which limits its usability in patients with paucibacillary TB which is common among the persons living with HIV, children and those who are on ant-TB treatment.[142–144]

1.7.2.5.4.0 Line Probe Assays

Line Probe Assays (LPA) are genotypic tests that rapidly identify Mtb by targeting 23S and 16SrRNA. It also detects mutations in drug resistance-determining regions such as RNA polymerase β (rpoB), Catalase G (katG), Gyrase A (gyrA), Ethambutol target protein β (embB), Region of difference 1 (RD1) and isoniazid target protein (inhA) genes.[145]

1.7.2.5.4.1 Genotype MTBDR plus assay

The genotype MTB Direct assay (Hain life sciences) is an LPA test that is used to simultaneously detect Mtb and four common Non tuberculous Mycobacteria in the clinical samples..[149] This test involves three major steps: isolation of DNA using a magnetic bead capture method, nucleic acid sequence-based amplification (NASBA), and reverse hybridisation of the amplification products to a strip-containing target-specific oligonucleotide probes.[149] The overall turnaround time is five hours which is quite high for routine TB care and management. Nonetheless, this test does not identify persons with TB drug resistance.[146]

1.7.2.5.4.2 INNO-LiPARif.TB kit

The INNO-LiPARif.TB kit (Innogenetics) detects Mtb resistance to RIF on culture isolates or sputum samples. Using this test, the Rifampicin (RIF)-resistance determining region of the rpoβ gene is amplified using a conventional PCR approach. The amplicons are hybridised to a nitrocellulose strip containing 10 specific probes: 1 probe that is specific to Mtb, 5 wild-type sensitive probes, and 4 probes for specific mutations in resistant strains.

1.7.2.5.4.3 Genotype MTBDRplus

Genotype MTBDRplus (Hain Lifescience GmbH, Nehren, Germany)is an LPA contains probes specific for *M. tuberculosis* complex, as well as probes for common rifampicin (RIF) resistance-conferring mutations and a subset of the mutations conferring resistance to isoniazid (INH).[150] The assay procedure involves DNA extraction, conventional multiplex PCR followed

by a reverse line hybridisation step. [147,148]. From systematic review works, it was shown that Genotype MTBDRplus has a pooled sensitivity and specificity of 98·4% and 98·9%, respectively, for detection of RIF resistance and 88.7% and 99·2%, respectively, for detection of INH resistance, although almost all the studies included used either cultured isolates or smear-positive respiratory specimens.[149] An initial validation study in South Africa showed that MTBDRplus was accurate for detection of resistance from smear-positive respiratory specimens and that the assay had good accuracy when applied to smear-negative respiratory specimens that contained *Mtb* in culture; 16/20 (80%) gave interpretable results for RIF, and 14/19 (74%) gave interpretable results for INH.[150]

1.7.2.5.4.4 The Genotype MTBDRsl

The Genotype MTBDRsl assay (Hain Life Sciences) is an LPA test that detects resistance to fluoroquinolones, ethambutol, aminoglycosides, cyclic peptides, and second line injectable agents. Like Genotype MTBDRplus, the test procedure for Genotype MTBDRsl involves DNA extraction, conventional multiplex PCR followed by a reverse line hybridisation step, and it can be used on culture or sputum.[151,152]

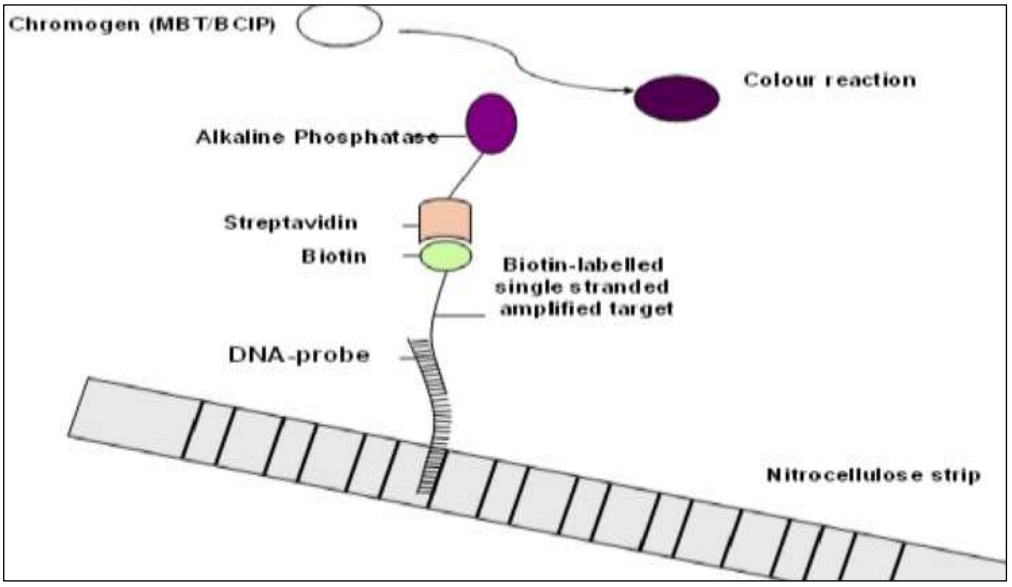


Figure 1.6.0: Line Probe Assay. For a positive test, Nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine (BCIP) in presence of alkaline phosphatase, an insoluble black-purple precipitate (Adapted from Bernad *et al.,* 2012) [123]

1.7.2.6.0 `Xpert MTB/RIF test

The first-generation commercial and laboratory-developed nucleic acid amplification tests (NAATs) were labour intensive and usually required an experienced personnel to perform them.[133] Xpert MTB/RIF assay (Xpert assay) is semi-automated with ability to detect the presence of Mtb and resistance to rifampicin by targeting the rpoβ gene which makes it suitable for routine TB management.[153,154,155] Xpert MTB/RIF assay is automated and provides results within 1 ½ hours which is key for Clinicians to make decisions early enough. Being a nucleic acid amplification test, Xpert assay is highly sensitive and specific with good positive and negative predictive values.[154–160] Among culture-confirmed child TB, Xpert assay detects up to 61% of the children who are smear-negative.[161] Among persons who live with HIV and were not being treated with the antiretroviral therapy (ART), Xpert assay increased TB case detection rates from 28% (when smear microscopy is used) to 73%. However, Xpert assay could only be used as a near point-of-care since it could not be performed at the patient's bed side.[162–166] Moreover, Xpert assay was unsuccessful in detecting persons with paucibacillary TB especially in areas of low prevalence of TB.[167] Furthermore, a drug and sensitivity test is required to validate a positive rifampicin resistance that is detected on the Xpert assay. This drawback significantly hinders the applicability of the Xpert assay as a rapid test for drug sensitivity.

1.7.2.6.1 Xpert MTB/RIF Ultra

In 2017, Cepheid developed a newer version of the Xpert system called the Xpert MTB/RIF Ultra (Xpert Ultra) to close the gaps associated with Xpert assay.[168] Xpert MTB/RIF Ultra contains IS6110 and IS1081 insertion sequence as the additional target genes with a larger reaction volume. The limit of detection of the Xpert Ultra is 15.6 bacterial colony-forming units per ml which is lower compared to the 114 colony-forming units per ml for the older version of Xpert assay.[169,170]

These qualities allow the Xpert Ultra to be a faster and more sensitive assay when compared with the older version of Xpert saasy.[171] While some studies indicate that Xpert Ultra is not statistically superior to the Xpert assay, other studies show that Xpert Ultra is more sensitive but less specific, especially in patients with paucibacillary TB.[172] Although the Xpert Ultra has some advantages over the Xpert assay, it has limitations too. Firstly, copy number of the insertion gene IS6110 widely varies in members of the Mtbc, which could reduce sensitivity.[173] Secondly, Xpert Ultra can detect DNA from dead bacilli in patients who have a history of TB.[174] Although there is no data about this, it is plausible that Xpert Ultra would falsely identify Mtb especially among patients who are being treated for TB. Thirdly, trace call positive results are not clinically conclusive since rifampicin resistance is usually indeterminate, hence requiring a repeat test. In cases where the results of the first and the second tests are discordant, clinical decision making becomes difficult.[175] Basing on its superior attributes over the first generation NAATs, it is not surprising that Xpert systems were upgraded from being used on a risk-based approach to a standard of care for TB.[176]

Sensitivity and specificity for Xpert Ultra among the children varies according to the sample used. A systematic review on Kay et al., 2022 showed that in 5 sputum studies, the Xpert Ultra summary sensitivity (95%CI) verified on sputum MGIT was 75·3% (64·3-83·8; among 127 participants and high-certainty evidence.) The same review showed that specificity (95%CI:) for Xpert ultra was 97·1% (94·7-98·5; among 1054 participants; high-certainty evidence). They noted that sensitivity and specificity were lower in Gastric aspirate compared with sputum.[177] In 7 seven studies where gastric aspirate was used, Xpert Ultra summary sensitivity (95%CI:) verified on culture was 70·4% (53·9-82·9; 120 participants; moderate-certainty evidence), and specificity (95%CI) was 94·1% (84·8 to 97·8; among 870 participants; and considering a moderate-certainty evidence).

In six stool studies, Xpert Ultra summary sensitivity verified on culture was 56·1% (39·1 to 71·7; 200 participants; moderate-certainty evidence), and specificity (95%CI:) was 98·0% (93·3-99·4); 1232 participants; high certainty-evidence).[177]

In four studies that used nasopharyngeal aspirate, a summary sensitivity (95% CI) verified on sputum MGIT culture was 43·7% (26·7-62·2; 46 participants; very low-certainty evidence), and specificity (95% CI) was 97·5% (93·6-99·0; 489 participants; high-certainty evidence).[177] In a study among adult persons living with HIV, Xpert Ultra had sensitivity and specificity (95% CI) 69% (57-80) (among 68 participants; with very low-certainty evidence) and 98% (97·to 99) (503 participants; moderate-certainty evidence), respectively.[178]

In a systematic review conducted on Zifodya et al., 2021 about the comparative performance of the Xpert Ultra and Xpert MTB/RIF for diagnosing pTB and rifampicin resistance in adults with presumptive pTB showed that Xpert Ultra's pooled sensitivity and specificity (95%CI) were 91% (86 to 95) and 96% (93-97) in 2834 participants; high-certainty evidence versus Xpert MTB/RIF pooled sensitivity 85% (79-90) and specificity 98% (97-99) among 2835 participants .[179] The difference in the accuracy of Xpert Ultra minus Xpert MTB/RIF was estimated at 6·3% (0·1 to 12·8) for sensitivity and −2·7% (−5·7 to −0·5) for specificity.[179]

In smear-negative but culture-positive participants, the review showed that pooled sensitivity was 78% (68-86) for Xpert Ultra versus 61% (48-72) for Xpert MTB/RIF, and that the pooled specificity was 96% (93-98) for Xpert Ultra versus 98·8% (98-100) for Xpert MTB/RIF.[179] In persons living with HIV, the review revealed that out that pooled sensitivity increased to was 88% (75-94) for Xpert Ultra versus 75% (59-86) for the Xpert MTB/RIF. Similarly, pooled specificity increased to 93% (82-97) for the Xpert Ultra and to 100% (99-100) for the Xpert MTB/RIF.[179]

In participants with history of TB, pooled sensitivity was 84% (73-92) for Xpert Ultra versus 82% (69-90) for Xpert MTB/RIF; pooled specificity was 88% (71- 97) for Xpert Ultra versus 97% (92-100) for Xpert MTB/RIF.[179] In their systematic review, Zifodya et al., 2021 found out that that applying the observed point estimates for the Xpert Ultra and Xpert MTB/RIF to a hypothetical cohort of 1000 patients, where 10% of the patients with symptoms are confirmed to have pTB, Xpert Ultra would miss 9 cases, which are 6/15(40%) fewer than those that would be missed on the Xpert MTB/RIF test.[179] However, Xpert Ultra would wrongly diagnose 40 persons compared with 14 persons who would be wrongly diagnosed with the Xpert MTB/RIF.[179]

1.8.0 Treatment of tuberculosis

In 1952, the TB regimen based on a combination therapy was proposed for the first time ever and its efficacy was reported over a period of years. A tolerable regimen reduces TB treatment failure, TB relapse and risk of developing drug resistant TB.[180] The use of Isoniazid and Rifampicin allowed shortening of the TB treatment from 18 months to nine months.[181–184] With addition of Pyrazinamide (PZA) treatment was further shortened to 6 months. Earlier efforts to shorten treatment to four months using a fluoroquinolone were unsuccessful. However, in April 2021, the Guideline Development Group (GDG) reviewed and considered a four-month regimen composed of rifapentine, isoniazid, pyrazinamide, and moxifloxacin. This regimen met the non-inferiority criteria set in the trial protocol and it is a possible alternative to the current standard 6-month regimen, without compromising efficacy and safety. Shorter regimen would allow faster cure and easing the burden on patients and the health care system. However, implementation and uptake of the suggested new regimen may be limited on the high cost and the limited accessibility in low-middle-income countries. [185–187]

The current standard therapy for drug sensitive TB consists of Ethambutol, Isoniazid, Pyrazinamide and Rifampicin for two months and then four months of rifampicin and Isoniazid. Although this treatment duration dropped from nine months, it is still believed that over treatment occurs, since most samples convert to culture negative on month two. Long treatment duration is associated with high levels of adverse drug reactions, poor adherence and high cost for the programmes and patients. About 15% of patients default on TB treatment due to disruptions and toxicity. Stopping TB treatment prematurely as the symptoms resolve might result in higher rates of relapse as well as risk of developing drug resistance, which prompted innovations such as directly observed therapy and provision of social support to the patients during the treatment course.[188] Unfortunately, a clinical trial to shorten treatment based on a 2-months culture negativity resulted in high levels of TB relapse.[189]

1.9.0 Biomarkers for monitoring response to TB treatment

In this section, biomarkers for monitoring TB treatment response, including measures of Mtb burden such as culture-based assays, smear microscopy are reviewed.

1.9.1 Stained sputum smear microscopy

Currently, stained sputum smear microscopy is recommended for monitoring response to TB treatment and making decision whether patients can be switched from the intensive treatment phase to the continuation phase or not. However, stained smear microscopy is observer dependent, and cannot differentiate between dead and viable Mtb, and between Mtbc and NTM. Moreover, stained smear microscopy loses its sensitivity as the bacillary load drops, due to effective TB treatment.[190,191] Therefore, a positive sputum smear at baseline and at follow-up time point are unreliable and always requires a confirmatory sputum culture test.[192–196]

1.9.2 Sputum culture

Culture outcome after two months of intensive phase treatment is the standard test for monitoring response to TB treatment.[197] Time to positivity of the liquid culture directly correlates with duration on treatment hence indicating response to treatment.[198–200] However, this marker has limited ability to predict treatment failure or relapse and a short detection phase.[201-137] On the other hand, the presence of a significant proportion of viable but non-culturable Mtb populations in sputum result in false negative culture results. As such, a negative sputum culture result may not necessarily mean absence of Mtb especially during the treatment course.[204,205] Loss of data due to contamination with other microorganisms remains a big challenge to culture test.[206,207] Besides, culture methods are laborious and technically demanding, thus limiting its applicability in low-middle income countries where TB burden is highest.[190]

1.9.3 Tuberculosis-Molecular Bacterial Load Assay

The Tuberculosis-Molecular Bacterial Load Assay (TB-MBLA) is a culture-free novel test that provides rapid quantification of viable Mtb cells in sputum samples.[208] This assay was developed as an alternative test to microscopy and culture as tools for monitoring response to TB treatment.[208–210] The uniqueness of the TB-MBLA relies in the fact that it detects and quantifies 16S ribosomal RNA (16SrRNA) and results are obtained in real time.[209] Like viral load monitoring assays in HIV patients, TB-MBLA monitors TB treatment response on measuring changes in bacteria load over the course of treatment. In a multisite study of adult TB patients, it was shown that TB-MBLA is more sensitive and reproducible than the liquid culture.[211] Quantification of the Mtb load on the TB-MBLA correlates with viable detection on the solid and liquid culture assays.[209,212] Tuberculosis Molecular Bacterial Load Assay differs from liquid culture (MGIT) in several ways.

While TB-MBLA directly measures the number of bacteria in a sample, liquid culture gives an inverse indirect measurement of the bacteria burden basing on time to detection. TB-MBLA is faster giving results in 4 hours and it is not affected on contaminants since it is highly specific to Mtbc and has potential to detect nonculturable viable bacteria.[205,213] Unlike for MGIT culture, clinical samples do not require decontamination with N-Acetyl-L-Cysteine-Sodium hydroxide (NALC-NaOH) before the test with TB-MBLA.

A study in Tanzania showed that using NALC-NaOH to decontaminate clinical samples leads to substantial loss of bacteria counts. Therefore, tests which avoid the need for decontamination are likely to offer an option to liquid culture.[214] *Mycobacterium tuberculosis* is a dangerous category B biological substance. Consequently, culturing Mtb samples must be handled in a category-3 laboratory. This requirement is expensive and may not be affordable in low- and -middle income countries.

Using a simpler and achievable conventional-heat inactivation method that may render TB samples non-infectious while preserving RNA for the TB-MBLA was described on Sabiiti et al., 2019b. They showed that heating samples could preclude the need for category 3 laboratories. If approved, this simplified approach can apply to TB-MBLA but never to MGIT culture. Basing on the data that was available on 2018, the WHO acknowledged TB-MBLA as a potential substitute for Smear microscopy and Culture assays as for monitoring TB treatment response. However, more performance data in support of the TB-MBLA assay is still needed for its endorsement.

1.10.0 Non-sputum TB samples

Although sputum is a highly variable sample, it is the standard sample for testing pulmonary TB. But young children, the terminally ill persons and those who are neurologically damaged do not easily expectorate to provide sputum in required quality and quantity. Small volume and

or salivary sputa may lead to low performance of the diagnostics tests because they may have exceptionally low or no bacteria with them. Collecting sputum for TB investigations is challenging since it requires the patient effort to expectorate the sample from deep the lungs during which aerosols are generated. The generated aerosols might expose health workers and care takers to TB. These challenges are amplified in active-case finding scenarios which require high-throughput sampling in large numbers of persons.[216,217] Consequently, obtaining an alternative/additional TB sample such as gastric or nasopharyngeal aspirates, induced sputum and broncho-alveolar lavage is critical yet the available collection methods might not be feasible or might not be readily available in resource-constrained settings where TB is endemic .[218,219,220–225] In this subsection, some of the potential alternative/additional non-sputum TB samples are discussed. These include but not limited to the following: saliva, exhaled breath concentrates, stool, and blood-based TB specific biomarkers.

1.10.1 Saliva

Saliva could be a valuable specimen for testing host biomarkers to diagnose pulmonary TB. A protein biosignature in saliva of persons with TB disease can potentially be used as a triage test.[226] However, concentration levels of the protein markers in saliva is comparatively lower than that in serum and plasma of the same patients.[226] The same applies to bacteria load which is likely to be less in saliva compared with sputum. Nevertheless, collection of saliva as a sample for TB would obviously be non-invasive and quicker compared with serum, plasma or sputum. [227, 228,229,230,231]

1.10.2 Exhaled breath concentrates

Exhaled breath aerosols (EBA) contain detectable TB-specific small metabolites, lipids, and volatile organic compounds (VOCs). Sampling these biomolecules provide a promising sample that would be easy to collect in a safe non-invasive manner.

Advancements in this area utilise bioaerosol sampling system and high-resolution Gas Chromatography–Mass Spectrometry (GC-MS) to allow characterisation of TB specific VOCs with a low turnaround time.[232] A systematic review of fourteen studies among 1715 participants showed that the pooled sensitivity (95%CI) and specificity (95%CI) of the electronic-nose test were 0·93% (0·82–0·97) and 0·93% (0·82–0·97), respectively, which do not meet the WHO's minimal test requirements for a triage and diagnostic.[233] Moreover, available collection methods might not be feasible or might not be readily available in resource-constrained settings where TB is endemic.[234,235]

1.10.3 Stool

Often, persons swallow expectorated sputum which ends up in the gut. Consequently, stool has been suggested as an alternative or additional sample for bacteriological confirmation of pTB disease. A systematic review of six paediatric stool studies reported that Xpert Ultra's summary sensitivity (95%CI) as verified on sputum MGIT culture was 56·1% (39-72; among 200 participants; moderate-certainty evidence), and that specificity (95%CI:) was 98%(93·3-99·4) among 1232 participants; at ahigh certainty-evidence).[177] However, stool is associated with higher error rates on Xpert MTB/RIF platforms potentially due to the clogging effect of the artefacts and presence of the PCR inhibitors in stool samples. Consequently, protocols that remove PCR inhibitors and artefacts are necessary to improve the yield of stool-based PCR tests on minimising invalid results due to error and failed tests. [236–239] Stool culture is also possible but previous studies indicate that only 50% of the persons living with HIV can benefit from this test due to the paucibacillary nature of TB disease which is common in these persons.[223,224] Similarly, stool culture has an extremely low sensitivity largely due to the contamination with other fast-growing flora which are abundant in the gut. Stool SSM also suffers from higher artefacts in stool which leads to higher false positive results.

While stool might be an easy to collect sample for TB related investigations, its wider usability is limited on the lack of faster and affordable processing methods. The currently available stool processing methods are complex, usually requiring several rounds of centrifugation. Nonetheless, stool is a potential additional sample, especially for paediatric and adult persons who find it difficult to provide sputum samples.[221,224,240]

1.10.4 Blood-based TB specific biomarkers

Persons who are infected with Mtb express unique and specific transcriptional signatures in their circulating blood and these signatures might be potential biomarkers for TB screening, diagnosis, and prognosis. Blood transcriptional markers appear early into infection with Mtb, indicating that these markers can inform development of novel tools for early TB diagnosis and monitoring treatment response.[241] Unlike for sputum, Bronchoalveolar lavage (BAL), gastric and cerebral spine fluid (CSF), and aspirates samples, blood can be collected easily which is essential for child-TB where specimen collection is a major limitation. Moreover, reproducible blood transcriptional profiles can be obtained from a small volume of blood, such a drop from a finger prick.[242–244] Longitudinal studies from South Africa described a set of transcriptional signatures of active TB which quickly diminished with successful treatment indicating potential role in monitoring treatment response.[245,246] These findings might also project the potential role of the blood-based transcriptional signatures to reveal early host response to TB infection which is key for investigations into improving treatment outcome. Although blood-based biomarkers have a great promising potential, translating these markers into practical tools has challenges too. For instance, at the present, there is no signature has been confirmed to successfully diagnose latent TB infection. [247]

1.11.0 Respiratory/Oral microbiome

Lower respiratory tract is no longer considered sterile since it harbours a diverse population of microorganisms that is collectively known as the lung microbiome. Normally, the lung microbiome would exist in an interdependent equilibrium state with the host. Related studies have indicated fluctuations in the diversity and abundance of the lung microbial communities due to TB disease.[248] A perturbed microbiome might exacerbate TB disease and accordingly impede treatment outcome. Currently, there is an urgent need to explain the effects of TB drugs on the lung microbiome since antibiotic therapies are known to impact on the human microbiome diversity and abundance.[249] This impact is reported to vary depending on a range of factors such as the antibiotic spectrum, dosage, duration of treatment, route of administration and the pharmacological properties of the drug agent.[250,251] Changes in the microbiome diversity and abundance due to antibiotic therapy may be reversible but recovery time is not predictable.[252] Besides, the processes of the host's microbial communities reversing to their original state are reported to be incomplete.[253] Generally, changes in the microbiome is critical since it might lead to losing the beneficial microorganisms that protect humans against certain opportunistic pathogens.

Changes in the diversity and abundance of the host microbiome can also lead to colonization of the drug-resistant microorganisms which are expensive to treat. For example, reduction in the abundance and diversity of the commensal flora was associated with severe inflammatory responses.[254] However, introduction of the novel sequencing technologies such as the Illumina platform provided a broader insight into the relationship between diseases, treatment and the host microbiome diversity and abundancy.[255] In these protocols, hypervariable regions of the 16SrRNA gene are usually targeted and sequenced. These genetic regions are usually conserved within but differ between different levels of the microbial classification. This unique

aspect supports easier and reliable approach to differentiate among or between the microbial organisms.[256,257]

Evaluating the adjacent regions such as regions 3/4 yields substantial differences in microbial diversity and abundacne.[258] Differences in bacterial profiles that are obtained from different hypervariable regions of the 16S rRNA gene might reflect differences in the rate of evolution of these regions. However, there is no single region that is good enough to differentiate all bacteria. Evaluating the multiple regions (V1/V2, V3, and V6/V7) improves the yield of microbial investigations but this requires standardization of the variable regions that are to be sequenced.[259,260]

Well-controlled studies evaluating the effect of TB treatment may constitute a marker for monitoring treatment outcome. These studies could allow insights into the impact of the anti-tuberculosis therapy on the diversity and abundance of lung microbiome. The association between TB drug-induced changes in the lung microbiome and clinical features of TB such as sputum clearance and body mass index could be better understood. Moreover, the association of TB-drug-induced changes in diversity and abundance of lung microbiome with conventional biomarkers such as time to TB detection on MGIT culture, contamination rates as well as treatment outcome can be investigated.

1.11.1 Limitations of host microbiome investigations

Evaluation and correct representation of the lung microbiome diversity is critical but it is limited on several factors.[261] For example, respiratory samples tend to be contaminated with the microbes in the oral cavity.[262] Direct lung tissue sampling techniques may solve this problem, but tissue sampling is invasive and may be impractical, especially in humans. Moreover, techniques such as bronchoscopies which could potentially provide protection against oral contamination are also invasive and less acceptable for research purposes.[263]

Besides, bronchoalveolar lavage and sputum which are the most accessible methods for this investigation differ greatly in terms of diversity of the microbiome they detect.[264] Moreover, the processing protocols for lung microbiome samples differ and each may have a differing impact on the quality and quantity of the extracted DNA and RNA. This challenge can be fixed on standardizing the protocols for specimen collection and preparation.[263,265]

1.12.0 Statement of the study problem

Global TB case detection, treatment success and case notification rates remain lower than the projected targets. Fast and accurate detection of TB is essential for effective treatment. Smear microscopy is the commonest TB test, but it is less sensitive especially when samples have low bacillary load. Moreover, it is incapable of differentiating between viable and dead Mtb and Mtbc form NTM acid fast bacteria. Xpert MTB/RIF is highly specific, sensitive with ability to rapidly detect rifampicin resistant. However, the current version of Xpert MTB/RIF cannot be used to monitor response to TB treatment, since it targets DNA which has a slow decay rate. While culture remains the gold standard TB test, a lot of data is lost through contaminations, and the associated high turnaround time limits its usability for routine care. Therefore, alternative, or additional tests are urgently needed to improve case detection and treatment success, as well as informing the formulation of novel TB regimens.

1.13.0 Proposed solutions to the problem

The TB-Molecular Bacterial Load Assay (TB-MBLA) was originally designed to monitor response to TB treatment. However, it has never been evaluated for diagnostic purposes and accuracy. Using the assay as a diagnostic tool may increase TB case detection, treatment success and case notification rates. Unlike the Xpert MTB/RIF and Xpert Ultra platforms which are DNA-based, TB-MBLA detects and quantifies Mtb 16SrRNA, making it suitable for monitoring treatment. Unlike culture tests, TB-MBLA is not susceptible to contamination, and it has less turnaround time, hence reducing data loss due to contaminations.

This supports quicker clinical decision making. Short turnaround time supports early treatment initiation, leading to improved treatment success rates. Moreover, TB-MBLA has the potential to amplify 16SrRNA from non-culturable yet viable Mtb. These hard-to-culture cells are largely responsible for TB relapse cases. Using TB-MBLA might support investigation of these cases and contribute to reduction of TB relapse.

1.14.0 Significance of the study

Data generated from this study have fed directly into the TB priority areas which include the following indicators: (a) increased proportion of detected relapse TB cases; (b) increased TB treatment success rates (c) adaption and scaling up of novel tools for improving TB diagnosis, treatment, and prevention, (d) increased active case finding, (e) formulation of novel regimens for shortening TB treatment.

1.15.0 Main objective

The main objective of this research was to evaluate biomarkers for *Mycobacterium tuberculosis* detection and monitoring response to anti-tuberculosis therapy.

1.16.0 Specific aims

i) In Chapter 3, the Diagnostic accuracy of RNA-based TB-MBLA was compared with the DNA-based Xpert Ultra and stained smear-fluorescent microscopy (SSM-FM) using MGIT culture as a reference. The overarching goal of this Chapter was to compare measures of the diagnostic accuracy (sensitivity, specificity, positive and negative predictive values) of TB-MBLA with those of the standard-of-care Xpert Ultra and SSM-FM.

ii) In Chapter 4, the Diagnostic accuracy of TB-MBLA were evaluated using stool as non-sputum TB sample and the accuracy measures (sensitivity, specificity, positive and negative predictive values) of stool TB-MBLA were compared with those of stool Xpert and stool SSM.

The overarching goal was to establish the usability of stool as an alternative or additional TB diagnostic sample which is urgently needed for improving TB care in children and other persons who find it hard to provide sputum.

iii) In Chapter 5, the accuracy of TB-MBLA, Xpert Ultra and SSM for monitoring response to TB treatment was assessed. The overarching goal was to establish changes in mycobacteria load, positivity rate, sensitivity, and specificity over the six-month treatment course. This investigation aimed at generating data on the possibility of diagnosing and monitoring TB treatment response using one platform. The current practice involves using a more sensitive Xpert Ultra to diagnose TB and then monitor response to TB treatment using a less sensitive SSM test.

2.0 Chapter Two: General Research Methods

2.1.0 Ethical approvals

All protocols that were used in this study were approved on the Research Regulatory bodies in Uganda and in the United Kingdom. These research approvals were granted on the University Teaching and Research Ethics Committee of the University of St Andrews UK, School of Medicine [**Approval code: MD14702**], the Makerere University School of Medicine Research and Ethics Committee [**REC Ref No. 2006-017**] and Makerere University School of Biomedical Sciences Research and Ethics Committee [**REC Ref No: SBS 529**], respectively.

2.1.1 Consultation and induction of research teams

Following the acquisition of the study approvals, the Research Assistants in Uganda (***Figure 1.0***) were inducted into the study and taken through all the research protocols. The Research Assistants and Colleagues from the TB Clinic at Naguru Referral Hospital in Uganda were given detailed information about the type of the required study participants. This information included clinical data, specimen type, specimen volume, collection, and processing methods as well as collection intervals. A consensus was reached on study participants' recruitment strategies, inclusion, and exclusion criteria. Details about data management, sample delivery, and issuance of results to the participants were discussed. We agreed to link the participants to the TB clinic for treatment initiation and follow-up. Modalities of compensation during the follow-up phase were agreed upon. Means and ways of archiving the collected specimens for the future investigations were discussed and agreed upon beforehand. Data capture forms and the study specific data and specimen databases were discussed and modified to allow easy adoption and integration into the existing workflow. The laboratory team was trained on sample processing and performing of the TB-MBLA as a way of building capacity and strengthening of the work relationship.

Figure 2.1.0: Induction of the Research Assistants and clinical team. Top left and lower left corner show training of the laboratory staff on how to extract Mtb RNA and how to prepare for the TM-MBLA PCR. The lower right corner shows the Clinical Team at Naguru Referral Hospital.

2.1.2 Informed consent for literate participants

After induction, the Clinical team was ready to identify the study participants and take them through the informed consent process. All the participants provided an informed consent before they were enrolled into the study. Informed consent forms were translated verbatim into Luganda which is the most spoken local language in Uganda. Consent forms were read to the illiterate participants/representatives in the presence of independent witnesses.

The process was interactive, during which participants were allowed to seek clarification(s) about anything in relation to the study protocols and objectives. The consent process disclosed all the information about the sample type, sample collection methods, sample volume and use of the collected samples for future TB related studies. Detailed information about the benefits and risks of study related methods were explained to the participants/representatives. The participants and/or their representatives, the clinical personnel, and witness (where applicable) signed and dated the informed consent forms accordingly. Illiterate participants/representatives marked the consent with a thumbprint and the witness attested to this process on appending a signature.

2.1.3 Confidentiality

Research records, blood, sputum, and stool specimens were identified on participant numbers and initials of their names. The original data entry forms, consent forms, initial participant questionnaire forms and all the study related material were stored and maintained under lock and key for security purposes. However, such information could only be disclosed if required on law, and to the appropriate regulatory bodies such as the Ethical Research Committees and the Uganda National Council of Science and Technology (UNCST).

2.1.4 Participant compensation and future use of stored specimens

There was no cost to the participants for examinations, laboratory tests and any indicated clinical care. As a requirement in Uganda, a separate consent form for sample storage for future TB research was filled and signed. Participants were at liberty to have their residual samples stored for future use or destroyed immediately. Even if the participant did not consent to the future use of stored samples, he or she was allowed to participate in the study.

2.1.5 Data capture and management

Clinical and laboratory data were collected and maintained in compliance with the International Conference on Harmonization (ICH) and Good Clinical Laboratory Practices (GCLP). Data from source documents (as per ICH/GCLP 1.51 definition) were captured in the laboratory test reports, clinical charts, hospital records and recorded data from automated instruments such as the Xpert Ultra. These data were consistently entered onto the Case Report Forms (CRFs). Access Data Management system was used for data capture, ensuring double data entry and data cleaning for quality control as well as an automated audit trails. Only sponsors or designated representatives and other applicable regulatory agencies were allowed to have direct access to these documents purposely for monitoring, auditing, quality assurance reviews and evaluation of safety and progress.

2.1.6 Protocol deviations and records retention

Deviations were detected on vigilant study staff, reviewing the case report forms and through the internal quality control procedures. If such deviations were serious enough to disqualify the data for analysis, that data was recorded but not included in the final statistical analysis. Case report forms and documentation related to the study were kept in a lockable cabin with a plan to retain them for a minimum of three years following the completion of the study. Any actions that would not be done according to the protocol or GCP/GCLP guidelines would be recorded as deviations from the protocol.

2.2.0 Laboratory procedures: Microbiological methods

2.2.1 Preparation of chemicals and reagents for sputum decontamination

Human sputa contain various fast growing microbial flora which contaminate culture tests. All samples were decontaminated using 2% Sodium Hydroxide/N-acetyl L-cysteine neutralised using sterile Phosphate Buffered Saline before cultivating them on culture media.

2.2.2 Sodium Hydroxide solution (4%)

To prepare 4% of NaOH (Fischer Scientific), 20 g of NaOH was dissolved in 500 mL of distilled water. The solution was autoclaved at 121°C for 20 minutes. The autoclaved solution was stored at room temperature and used within 6 months.

2.2.3 Tris-sodium citrate (2.9%)

To prepare 2.9% of Tris-sodium citrate (Fischer Scientific), 29 g of Tris-sodium citrate was dissolved in 1000 mL of distilled water. The solution was sterilised on autoclaving at 121°C for 20 minutes. The autoclaved solution was stored at room temperature and used within 6 months.

2.2.4 Phosphate buffer solution (pH 6.8)

To prepare Phosphate buffer solution (pH 6.8) (Sigma-Aldrich, UK), 4 tablets of the phosphate buffer were dissolved in 800 mL of distilled water followed on autoclaving at 121°C for 20 minutes. The solution was stored at room temperature and used within 3 months

2.2.5 Preparation of 1:1 NaOH (4 %) and Tris-Sodium Citrate (2.9%)

Fifty millilitres (50 mL) of 4% sodium hydroxide solution were added to 50 mL of 2.9% Tris-Sodium Citrate and capped securely. Depending on the workload, the volume of the solutions prepared would be scaled-up appropriately.

2.2.6 Preparation of 2% N-Acetyl L-Cysteine- Sodium hydroxide

Two grams (2 g) of N-Acetyl L-Cysteine (NALC) was dissolved in 100 mL solution of 1:1 NaOH (4 %) and Tris-Sodium Citrate (2.9%). The NALC solution was prepared and used on the same day of sample decontamination because it is known to dilapidate very quickly.

2.2.7 Mycobacteria Indicator Tubes

The ready-made Mycobacteria Growth Indicator Tubes (MGITs, Becton and Dickinson Company, MD, USA) were used for isolation of the bacteria from clinical samples.

The MGIT culture was supplemented with Oleic acid-albumin-dextrose-catalase (OADC) to enhance the growth of Mtb. To minimise sample contamination, 800 mL. of the PANTA (Becton, Dickinson and Company, MD, USA) and antibiotics (Polymyxin B, Azloxicin Nalidixic acid, Trimethoprim and Amphotericin B) were added to the Mycobacterial Growth Indicator Tubes before inoculation with clinical samples.

2.2.8 Lowenstein Jensen Medium

The ready-made Lowenstein Jensen (LJ) solid culture medium (Becton, Dickinson and Company, MD, USA) was used in parallel to MGIT culture for cultivation of Mtb from clinical samples. This is the ready-to-use medium containing selective antibiotics (PACT: Polymyxin B, Amphotericin B, Carbenicillin and Trimethoprim) to control sputum contamination.

2.2.9.0 Culture tests

2.2.9.1 Decontamination of samples for culture tests

Samples were decontaminated on mixing with equal volume of 2% NALC/NaOH and incubated at room temperature for 20 minutes before toping up with PBS to a final volume of 50 mL. The resultant mixture was centrifuged at 3000 g for 20 minutes at 4°C. The supernatant was discarded, and the pellet was re-suspended in 2 mL of PBS (PBS; pH 6.8; Becton Dickinson, Sparks, MD, USA).

2.2.9.2 Culturing decontaminated samples

Mycobacteria growth tubes were then inoculated with 500 µL of the decontaminated sample and incubated at 37°C for a maximum of 42 days. Solid culture on the LJ medium (LJ; Becton Dickinson) slants were inoculated with 1 mL of the re-suspended sediment and incubated at-37°C for a maximum of 56 days. TB-positive cultures were confirmed on the presence of acid-fast bacilli on ZN staining and presence of MPT64 antigen. Absence of acid-fast bacilli cording and growth on blood agar was recorded as contamination.

2.2.9.3 Checking for contaminants using blood agar

Blood agar culture test and Zeil Nielsen (ZN) smear microscopy were performed for all MGIT that flagged positive. About 50 µL of positive MGIT culture were inoculated on blood gar and was incubated at 37°C for 48 hours. Visible growth of colonies on blood agar after 48 hours reflected a contaminated MGIT culture and the corresponding time to positivity (TTP) was rendered invalid. For SSM-ZN, two drops of a positive MGIT culture were used to make a smear which was stained and examined. All results were reported according to the International Union against Tuberculosis and Lung Disease guidelines (*Table 2.1.0*).

Table 2.1.0: Interpretation of MGIT culture results

MGIT instrument result	ZN smear result	Blood Agar result	Final culture result	MGIT TTP
Positive	Positive	Negative	Mtb. Positive	Valid
Positive	Positive	Positive	Mtb Positive; Cont.	Valid
Positive	Negative	Positive	Mtb. Neg; Cont.	Invalid
Negative at 42 days	NA	Negative	Mtb. Negative	NA
Negative at 42 days	NA	Positive	Mtb. Negative; Cont.	NA

MGIT: Mycobacteria Growth Indicator Tube test, ZN: Zeil Nielsen, TTP: Time taken on MGIT machine to flag as positive, Cont.: contaminated test

2.2.9.4 MPT64 antigen test

Antigen MPT64 (MPT64, Becton, Dickinson and Company, MD, USA) test was performed to confirm that the final positive MGIT culture test was due to the presence of Mtbc and not NTM bacteria. A positive MGIT culture was mixed on pipetting up and down using a micropipette, and 100 µL of the MGIT suspension was applied on the lateral flow MPT64 device (TBc ID) and incubated at room temperature. Results were read after 15 minutes following the Manufacturer's instructions. A positive culture for Mtb was confirmed on

formation of two bands: one band indicating a control line (C) and the second band (T) indicating the presence of Mtb antigen in culture as described in *Chapter two*.

2.2.10 Stained smear microscopy

2.2.10.1 Ziehl-Neelsen stains

This technique uses 1% Carbol fuchsin (***prepared in Sub-section 2.2.10.4***) as the primary dye, 3% of Acid alcohol (***prepared in Sub-section 2.2.10.5***) as an acid-fast decolouriser and 0.3% of Methylene blue (***Sub-section 2.2.10.6***) as the counter stain. Basic Carbol fuchsin solution contained 10% Carbol fuchsin dye (***Sub-section 2.2.10.2***), 95% ethanol and 5% phenol (***See Sub-section 2.2.10.3***).

2.2.10.2 Preparation of 10% of Carbol fuchsin (w/v)

Ten grams (10 g) of Carbol fuchsin dye (Sigma-Aldrich, UK) was dissolved in 100 mL of 95% ethanol (Sigma-Aldrich, UK) to make 10% solution of carbol fuchsin.

2.2.10.3 Preparation of phenol (5% w/v)

To prepare phenol (5% w/v) phenol solution, 5 g of phenol crystal (Sigma-Aldrich, UK) were dissolved in 100 mL of distilled water, and this was kept out of the dark before use.

2.2.10.4 Preparation of Carbol fuchsin staining solution (1%)

Ten millilitres (10 mL) of 10% of Carbol fuchsin solution was mixed with 90 mL of 5% Phenol solution to make 1% carbol fuchsin solution. The solution was filtered using Whatman filter No.1 and stored in an amber coloured bottle which was immediately tight-capped before-storage at room temperature. The solution was used within 3 months from the date of preparation.

2.2.10.5 Preparation of Acid-alcohol (3%)

To prepare 3% of Acid-alcohol, 81 mL of 37% Hydrochloric acid (HCL) (Sigma-Aldrich, UK) was added to 919 mL of 96 % ethanol. Caution was taken to add slowly the acid to alcohol and

not vice versa, as the mixture creates heat. Using concentrated Hydrochloric acid would lead to a highly exothermic reaction, so 37% HCL was opted. The solution was stored at room temperature and used within 3 months from the date of preparation.

2.2.10.6 Preparation of methylene blue solution (0.3%)

To prepare 0.3% of Methylene blue, 3 g of Methylene blue powder (Sigma-Aldrich, UK) were dissolved in 1000 mL of distilled water. The solution was filtered and stored at room temperature and used within 2 months from the date of preparation.

2.2.10.7 Auramine O staining solution (0.1%)

Auramine-O staining solution (0.1%) was a commercial ready-to-use staining kit (Sigma-Aldrich, UK) [https://www.biognost.com/product/tb-stain-auramine-o-kit/].

2.2.10.8 Potassium permanganate (0.5%)

Potassium permanganate (0.5%) was prepared on dissolving 0.5 g of potassium permanganate powder (Sigma-Aldrich, UK) in 100 mL of distilled water. The solution was filtered and stored in the dark at room temperature.

2.2.10.9 Preparation of sputum smears

To prepare sputum smears, 0.5 mL of the 2% NALC-NaOH decontaminated sputum pellet was applied on 2 slides and a smear of about 2 cm diameter was prepared using a sterile applicator stick. The thickness of the smear was considered okay if a writing beneath the slide and the smear could be read. Before staining, smears were air dried for 30 minutes at room temperature and then heat fixed on a hotplate at 80°C for 20 minutes. One slide was stained with ZN stain and the other was stained with Auramine-O staining technique and examined on the same day.

2.2.10.10 Ziehl Nielsen staining and microscopy

Ziehl Neelsen stain was performed on flooding the smear with 1% of Carbol fuchsin with intermittent heating for 15 minutes. Heating was done slowly on passing the underneath of the slide in a blue flame (while avoiding boiling). Then the slide was allowed to stand for 5 minutes at each interval as more dye was added to avoid the smear from drying. The slides were washed with distilled water and then decolourised on flooding with 3% Acid-alcohol for 2 minutes and carefully washing with distilled water before counterstaining with 0.3% of Methylene blue for 3 minutes. The slides were rinsed with distilled water, air dried and examined using Zeiss Microscopy at 100X oil immersion objective lens. SSM-ZN smear results were graded according to the International Union for Tuberculosis and Lung Disease (IUTLD) guidelines (*Table 2.2.0*)

Table 2.2.0: Grading scale of the ZN microscopy sputum smear results

Number of fields	Smear grade
No of AFB seen per 300 fields	Negative
1-9 AFB per 100 fields	Scanty
1-9 AFB per 10 field	1+
1-9 AFB per field	2+
>9 AFB per field	3+

AFB=Acid Fast Bacilli. ZN smear grading was recorded in accordance with the International Union for Tuberculosis and Lung Disease.

2.2.10.11 Staining for Fluorescent smear microscopy

The fixed smear was stained using a fluorochrome such as rhodamine-auramine for 15 minutes before rinsing using distilled water and decolorizing with acid-alcohol for 2 minutes. Potassium permanganate was applied as a counter stain for 2-3 minutes. The counter-stained smear was rinsed with distilled water and air dried before it is examined (*Figure 2.3.0*).

2.2.10.12 Examining smears using Fluorescent Microscopy

The air-dried stained smears were examined at a magnification of X200 using a UV light from a Fluorescent Microscope. Acid-fast organisms fluoresce reddish orange against a dark background, and it was reported as positive for TB. Whereas the absence of non-fluorescing bacilli coupled with the presence of pale-yellow organisms that were quite distinct from the bright acid-fast organisms was reported as a negative test. SSM-FM were graded according to the International Union for Tuberculosis and Lung Disease (IUTLD) guidelines (*Table 2.3.0*)

Table 2.3.0: Grading scale of the FM microscopy smear results

IUATLD (1000x field=HPF) **Result:1 length = 30 fields =300 HPF)**	**Microscopy system** **Fluorescence**
Negative	Zero AFB per length
Scanty	1–29 AFB per length
1+	30–299 AFB per length
2+	10–100 AFB per field on average
3+	>100 AFB per field on average

Fluorescent Microscopy smear grading was according to the International Union for Tuberculosis and Lung Disease. AFB=Acid Fast bacilli. AFB=Acid Fast Bacilli, HPF=High Power Field

2.2.11 Quality control during reagent preparation

During preparation of the reagents, chemicals were handled in a fume hood to protect the personnel while chemicals were protected from potential contamination. Personal protective equipment including gloves, facial masks and laboratory coats were always used. Before storage, all reagent bottles were labelled with the name of the reagent such as 1% Carbol

Fuchsin, Lot number, dates of preparation and expiry (DD/MM/YYYY), storage temperature, initials of the personnel who prepared the reagent and all these details were entered in the laboratory Logbook.

2.2.12 Quality control and safety during reagent preparation

Sputum smears were prepared, and air dried in the Biosafety Cabinet Level 2 (BSL-2) to protect both the personnel and the smears from exposure and contaminants, respectively. Lead pencil was used to clearly label the frosted end of the slide with patient ID, date of preparation and staining, initials of the person who prepared and stained the smear, and type of the sample involved (i.e., sputum or stool).

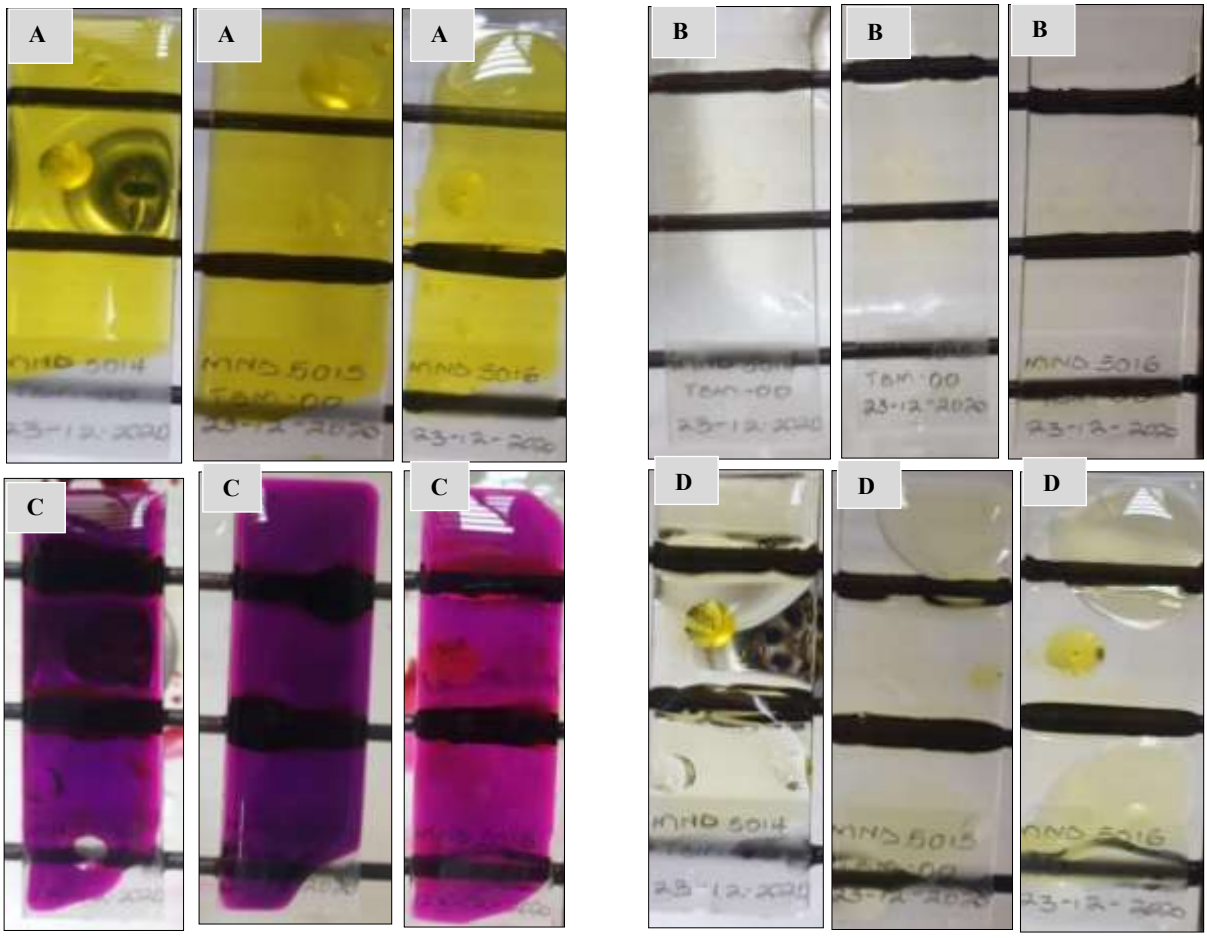


Figure 2.2.0: Staining smears for fluorescent microscopy (A) 0·1% Auramine solution for 15 minutes. (B) 0·5% Acid-Alcohol. (C) counterstaining with 0·5% potassium permanganate for 2 minutes. (D) dried in oven for 5 minutes before examining with a fluorescent microscope under X200 objective (Slides were prepared in the Mycobacteriology laboratory, Makerere University)

2.2.13.0 Molecular Assays

2.2.13.1 Xpert MTB/RIF Assay

Two millilitres (2 mL) of sputum sample were mixed with 4 mL of sample reagent (Cepheid) and incubated at room temperature for 15 minutes with intermittent shaking at 5 minutes interval. After incubation, 2 mL of the sample and buffer mixture was loaded in the Xpert MTB/RIF Ultra cartridge and scanned using the machine barcode before loading into the Xpert MTB/RIF machine for the run.

2.2.13.2 Interpretation of Xpert MTB/RIF Ultra results

Xpert MTB/RIF Ultra results were interpreted on the Xpert MTB/RIF DX System based on the measured fluorescent signals and results were displayed in the "view results window" of the machine software. Printable results were semi-quantitively displayed as indicated in *Table 2.4.0*

Table 2.4.0: Xpert MTB/RIF Ultra results and interpretation

Results displayed	**Semi-quantitation**	**Interpretation**
MTB DETECTED	High	Positive
MTB DETECTED	Medium	Positive
MTB DETECTED	Low	Positive
MTB DETECTED	Very Low	Positive
MTB DETECTED	Trace	Positive
NO MTB DETECTED	..	Negative

Rifampicin susceptibility result **Displayed result**	**Interpretation of the result**
Rif Resistance DETECTED	Mutation was detected in the rpoβ gene
Rif resistance INDETERMINATE	Mtb concentration was lower than the limit of detection needed for mutations in rpoβ gene to be detected.
Rif Resistance NOT DETECTED	No mutation was detected in the rpoβ gene

2.2.13.3 Preparation for Tuberculosis-Molecular Bacterial Load Assay

The Tuberculosis-Molecular Bacterial Load Assay (TB-MBLA) is a molecular test for rapid quantification of viable Mtb direct from clinical patient samples. The test detects and quantifies Mtb on targeting free Mtb16S-ribosomal RNA (16S-rRNA) via the reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR).

2.2.13.3.1 Reagents for RNA extraction: Guanidine thiocyanate

Guanidine thiocyanate (GTC) was used to preserve RNA from degrading due to RNases activity. In a fume hood, 200 g of GTC powder (Sigma-Aldrich, UK) was dissolved in 120 mL of molecular grade water. The solution was mixed thoroughly and incubated at 37°C overnight to further dissolve the GTC. The next day, the tube was stirred thoroughly to dissolve any undissolved GTC. Forty millilitres (40 mL) of 1 M Tris-HCl; pH 7.5 (Sigma Aldrich, UK) was added, and the volume was adjusted to 396 mL with molecular grade water. GTC was activated on adding 4 mL of β-mercaptoethanol (Sigma Aldrich, UK) and stored in aliquots of 4 mL at -80°C to make a final volume of 400 mL of GTC solution. During the incubation sessions, GTC was protected from light on wrapping the bottle in aluminium foil.

2.2.13.3.2 Ice cold ethanol (70% v/v)

Aliquots of 50 mL absolute ethanol were prepared in a 50 mL falcon tube and frozen at -80°C until use. To prepare 70% ethanol, 35 mL of absolute ethanol was added to 15 mL of molecular grade water (Qiagen, UK) and mixed up and down ten times before freezing at -80°C until use.

2.2.13.3.3 Sputum samples for RNA extraction

Three spot sputa were pooled and homogenised using sterile beads and vortex mixer. One millilitre (1 mL) of the homogenised sample was mixed with 4 mL of the thawed activated GTC solution within 1hr of sample of collection. Samples which were homogenised in GTC solution were batched at -80^{o}C until the time of RNA extraction, on average, every fortnight.

2.2.13.3.4 Preparing standard curves

Standard curves were prepared using 10-fold serial dilutions of the RNA extracts of known concentration (Concentration 10^6 eCFU per mL). Briefly, 10 µL of the neat RNA extract was added to 90 µL of the molecular grade water (Sigma-Aldrich, UK) and serially diluted up to 10^{-6} dilutions. Standard curves were prepared using the same Rotor Gene5plex Platform (Corbett Research LTD, UK) that was used for testing the clinical samples.

2.2.13.4 RNA extraction

Prior to RNA extraction, 100 µL of the extraction control (concentration; 10^6 eCFU per mL) was spiked into the clinical sample. The extraction control was used to assess the efficiency of the extraction process and control for the presence of inhibitors during the RT-qPCR step. RNA extraction was performed following the TB-MBLA protocol. Briefly, Mtb cells were lysed using RNAPro Blue Kit protocol before extraction of the nucleic acid using the chloroform-phenol technique. DNA was removed on using the Ambion Turbo DNase kit (Life Technologies, UK).

2.2.13.4.1 Removal of DNA from the nucleic acid extract

Briefly, 11 µL of the DNAse mix (1 µL of Turbo DNase I enzyme: 10µL of 10x buffer) was mixed with 100 µL of RNA extract and incubated at 37°C for 30 minutes. An additional 1 µL of DNase enzyme was added to RNA extract and incubated for another 30 minutes at 37°C. The activity of the DNase was stopped on adding 10 µL of DNA inactivation reagent and incubated at ambient temperature with intermittent mixing at an interval of 5 min for a total of 15 min. The extract was centrifuged at 13,000xg for 2 minutes and 110 µL of the supernatant containing pure RNA was harvested and tested immediately or stored at -80ºC until the time of TB-MBLA testing.

2.2.13.5 Reagents for RT-qPCR *(Details in appendix II)*

2.2.13.5.1 Stock concentration of the primers (100 µM)

Primers and probes for Mtb and extraction control were supplied in a lyophilized form. The stock concentration of 100 µM was prepared according to the manufacturer's instructions. Primers were dissolved using RNase free molecular grade water (Qiagen, UK). Probes were reconstituted using the probe dilution buffer supplied on the manufacturer.

2.2.13.5.2 Working concentration of the primers (10 µM)

Probe solutions for Mtb 16sRNA and extraction control were needed. To prepare 10 µM of each of the forward and reverse primer mix, 80 µL of RNase-free water was mixed with 10 µL of each of the stock solution of the forward primer (100 µM) and 10 µL of the stock solution of each of the reverse primer (100µM). The primer mix was stored at -20°C until use.

2.2.13.5.3 Working concentration of the probe (20 µM)

Probe solutions for Mtb 16sRNA and extraction control were needed. To prepare the 20 µM of each of the probe solution, 20 µL of each of the stock solution (100µM) was mixed with 80 µL of the RNase free molecular grade water (Qiagen, UK). The probe solution was stored in the -20°C freezer until use. An aliquot of 0.2 µL of the probe was used for each sample reaction to make a final concentration of 0.2 µM. During preparation and storage, the probes were protected from light since they are light sensitive.

2.2.13.5.4 RT-PCR conditions and analysis of results

The RT-qPCR cycling conditions were as follows: 50°C for 30 min for reverse transcription, 95°C for 15 min to activate Taq polymerase, 94°C for 40 sec for 40 cycles to allow the primers and probes to anneal; and 60°C for 60 sec to allow acquisition in Green (FAM-dye) and Yellow (HEX-dye) channels. The RNA from Mtb were detected in the green channel and the extraction control in the Yellow Channel.

Analysis of results was done on setting a threshold at 0.01, selecting slope correctly and removing the outlier at 10%. Only the sigmoid-shaped amplification curves above the set threshold line were true amplification curves.

2.2.13.5.5 Master mix for RT-PCR reaction set up

Two sets of master mix for the quantitative RT-PCR containing reverse transcriptase enzyme (RT+) and without the enzyme (RT-) were prepared using the Quantitec multiplex PCR mix (Qiagen, Germany).

Table 2.5.0: TB-MBLA PCR master mix

Reagent	Vol.(µL) per RT+ reaction*	Vol.(µL) per RT- reaction**	Total vol. (µL) for RT+ reactions$^{\beta}$	Total vol.(µL) for RT- reactions$^{\beta\beta}$
Quantitec mix	10	10	--	--
Mtb 16S primer mix (F+R)	0.4	0.4	--	--
EC primer mix (F+R)	0.4	0.4	--	--
Mtb 16S-FAM probe	0.2	0.2	--	--
EC probe	0.2	0.2	--	--
RT enzyme	0.2	0	--	--
RNAs free water	4.6	4.8	--	--
Total master mix volume	**16**	**16**	**--**	**--**

*Each sample was run in duplicate for the RT+ and in singlet reaction for the RT- mix. **The RT- reactions were included in each run and for each sample to ensure that the amplification during RT-PCR was due to RNA template and not DNA. $^{\beta}$ Total volume for RT+ reactions were calculated on multiplying the total number of the samples on 2. $^{\beta\beta}$ Total volume for RT- reactions was multiplied on the total number of samples.

Molecular biology grade water was used as a no template control for each run to check if there was contamination during extraction and preparation of the master mix. Sequence specific primers and Taqman dual labelled probes for Mtb-16S rRNA and for EC target were procured from MWG Eurofins, Germany.

2.2.14.0 Collection of stool samples

Stool samples were collected on the day of patient enrolment using a flushable Hystool® stool collection device (https:/www.hystool.co.uk)

2.2.14.1 Attaching the stool collection bag on the toilet

The backing paper was peeled off from the adhesive pads on the corners of the stool collection bag. The bag was opened as wide as possible and stuck to the toilet seat using the adhesive pads.

2.2.14.2 Utilising the toilet

A piece of a toilet paper was folded and placed at the bottom of the stool bag to prevent direct contact between the bag and stool because the loose stool could burst the bag. The patient sat on the toilet seat and used it normally. Care was taken for stool not to pass through the bag. Participants were advised to try as much as possible not to pee in the stool because this could contaminate the sample or dissolve it. A portion of the stool (about two scoops) was picked into the sample container using a scoop which is attached to the cap of the stool container.

2.2.14.3 Flushing the toilet after stool collection

Sticker pads were unpeeled from the toilet seat allowing the gab, sticker, and contents to fall into the toilet and flushed normally. The stool bag used could dissolve in water and the sticker paper was biodegradable.

2.2.14.4 Processing stool samples

Spot stool (~6.0g) were self-collected on the participants into a sterile pre-labelled stool container and split into 2 equal portions on the laboratory technologist. One portion was homogenised in 10 mL phosphate buffered saline (PBS) and the other was mixed in equal volume of OMNIgene-sputum reagent (OM-S; DNA Genotek, Inc., Ottawa, Canada) and incubated at room temperature for 15 minutes.

OMNIgene-sputum reagent decontaminates TB samples while preserving *Mtb* viability. The resulting suspension was pelleted twice in OM-S at 3,000 × *g*, for 20 and 10 min, respectively. The resulting pellet was re-suspended in 6 mL of phosphate-buffered saline before being aliquoted into four different portions of 1 mL each to be tested on auramine O smear microscopy (smear), Xpert ultra, MGIT and LJ culture tests. The remaining portions were banked at -20°C and tested on TB-MBLA in a batch on all specimens concurrently at once. In addition to stool samples, sputum samples were collected and tested on the same day using Sputum MGIT culture and Xpert Ultra.

2.2.15.0 Sample size calculation

Sample size calculation was based on data from the Uganda's National TB data [266] and a method described on Buderer.[267] Using Buderer's method, a minimum required samples size was 125 participants at baseline to achieve statistical power.

$$N=\frac{Z^2\,\alpha_{/2}\,Sp(1-Sp)}{d^2\,X(1-P)}$$

Z_1- $\alpha_{/2}$: Standard normal deviation = 1.96
Sp: Anticipated assay specificity = 0.84
d: Absolute precision on either side of Sn and Sp = 0.1
P: Prevalence of TB in Uganda = 0.6

For treatment follow up arm, to get a minimum of 100 cases of bacteriologically confirmed pTB, we proposed to screen 1608 cases with TB-like clinical signs. This estimation was based on the Uganda National TB prevalence survey which showed that Greater Kampala has twice as high, 0.5% the national TB prevalence. At the national level, 3·1% (160/5144) cases were bacteriologically confirmed to have TB. We therefore estimated that with double TB prevalence rate in Greater Kampala, we would need fewer cases to achieve 100 bacteriologically confirmed TB cases. Indeed, we screened 210 participants to get 129 bacteriologically confirmed pTB positive cases who were then recruited into treatment follow up arm.

2.2.16 Statistical Analyses

Means and medians were calculated using standard formulae. Differences in baseline clinical characteristics were compared using Mann-Whitney U-test for continuous variables. TB-MBLA quantification cycles were converted to bacterial loads using a standard curve and reported as estimated colony forming units per mL (eCFUs per mL) and all were log transformed using a standard formula in excel before statistical analyses. Mean cycle thresholds of the Xpert ultra were obtained using TB-specific probes, including the insertion sequences. Quantification cycles for Xpert ultra and TB-MBLA, and the TB-MBLA bacterial loads between HIV-negative and HIV-positive participants were compared using Mann Whitney U-test. Correlations of the time-to-positivity for MGIT culture and quantification cycles were performed using Spearman's correlation test. Statistical significance was considered at probability value less than 0.05. Sensitivity was calculated as a proportion of the results that were positive on both the index test (TB-MBLA) and the reference test (True positive tests) to all results that were positive on the reference test (True positive + False negative). Specificity was calculated as a proportion of the results that were negative on both the index test (TB-MBLA) and the reference test (True negative tests) to all results that were negative on the reference test (True negative + False positive tests). Positive predictive value was calculated as a proportion of true positive results to the summation of true positive and false positive results. Negative predictive value was calculated as a proportion of true negative results to summation of true negative and false negative results. STATA version 15.1 tool (StataCorp, College Station, Texas, USA) was used to achieve the diagnostic accuracy estimates at the confidence interval of 95%.

3.0 Chapter Three

Evaluation of the diagnostic accuracy of the Tuberculosis-Molecular Bacterial Load Assay among presumptive pulmonary tuberculosis adult patients

3.1.0 Background

Globally, 10 million persons were estimated to have developed TB in 2020, and 30% of them were believed to have missed diagnosis or not notified to the National Authorities.[268] In the year 2018, the United Nations high-level meeting on tuberculosis and the political Heads at local and international levels committed to support endeavours towards upscaling TB diagnosis and treatment.[268] Subsequently, a reduction of 9% in TB incidence and a 14% drop in TB-related deaths were noted between 2015 and 2019, but this progress was too slow to achieve the set goals.[268] Timely diagnosis and treatment reduce TB-related morbidity and mortality which justifies the demand for more suitable tools.

Stained smear microscopy (SSM) is the commonest tool for TB diagnosis because it is fast, affordable, but requires significant training to perform it well. However, smear microscopy is observer dependent and cannot differentiate between dead and viable Mtb, and between Mtb complex and non-Mtb. It is associated with low sensitivity especially among the persons living with HIV and those with low bacterial loads, hence limiting its applicability.[192–196] Culture is considered to be the optimal confirmatory test for TB diagnosis and a reference for other TB tests, yet it has several limitations: it is slow and requires an expensive high containment laboratory.[269,270] Molecular-based assays have potential to solve the challenges that are experienced on the conventional TB diagnostic methods since they are usually faster, reproducible, not compromised on contamination and have been proven to be highly specific and sensitive for Mtb.[271,272] Accordingly, Xpert Ultra- a modified version of the Xpert MTB/RIF assay is now recommended as an initial TB diagnostic and rifampicin resistance test in all adults and children with signs and symptoms of pulmonary TB.

However, trace call positive cases are not clinically conclusive since rifampicin resistance is usually indeterminate hence requiring a second run. In cases where the results of the first and the second run are discordant, making clinical decision becomes difficult which potentially delay clinical interventions.[175] Tuberculosis-Molecular bacterial load assay (TB-MBLA) is a novel RNA based assay that detects and quantifies viable Mycobacterium tuberculosis (Mtb) bacilli in sputum.[273,274] In 2018, the TB-MBLA was noted on the WHO to be a biomarker with potential to replace smear and culture for monitoring TB treatment response.[275] TB-MBLA targets the abundant Mtb16S ribosomal RNA specifically and the test is highly sensitive with good positive predictive values.[276]

3.2.0 Demographics of Uganda

Uganda is located astride the Equator positioned in the Eastern region of Africa. On average, Uganda is about 1,100 metres (3,609 Ft.) above sea level and much of its border is lakeshore. Such environment supports the vector-, pathogen-host interactions, partly explaining why Uganda is burdened on lots of infectious diseases. There are four main administrative divisions called regions which include the Central, Western, Eastern, and Northern. The four regions are further sub-divided into 15 sub-regions which are constituted on 121 districts. The current study was based in Kampala, which is the most populated district with about 1,659,600 million persons. (*Figure 3.1.0*) The current growth rate of Uganda is estimated to be at 3.32%. On average, every adult female has approximately 5.7 children. At this growth rate, over one million persons are added to the population annually. It is estimated that each household has an average of 4.5 members which may partly explain the high prevalence of tuberculosis due to congestion, inadequate aeration and hygiene, and limited access to nutritious food. All these lifestyle factors are risk factors for TB transmission in case one of the household member has active TB disease.

Figure 3.1.0: Map of Uganda. The current study was conducted at a Referral Hospital which is in Kampala. The Hospital serves persons around the central region of the country. Because central region is cosmopolitan, there are chances that results obtained from this region are more representative than results from any other region.

Based on these estimates, Uganda's population is projected to exceed 100 million persons on the year 2050. This enormous increment in population creates a huge challenge to the already dilapidated public health systems, in the sector of the infectious disease control. (www.DHSprogram.com; Uganda Population 2022 (Demographics, Maps, Graphs) (worldpopulationreview.com; World Bank, 2018, Uganda; Population Reference Bureau 2018, Uganda)

3.3.0 Epidemiology of tuberculosis in Uganda

Uganda is one of the 30 World Health Organisation designated countries with a high burden of TB and HIV infections and diseases. In 2019, Uganda notified 65,897 TB cases to the World Health Organisation. In the same year, 88,000 persons fell ill of TB, and an estimated 15,600 persons died. Men constitute more than half of the TB cases (56 %) in the country and the children account for 13 % of the total cases. In 2019, only 65,897 TB cases were diagnosed and reported to the National TB and Leprosy Programme (NTLP). This indicates that nearly 22,103 (~25%) TB cases were either not diagnosed or they were diagnosed but they were not notified to the NTLP.[12]

3.4.0 TB screening and Management in Uganda

A patient presenting with any one of the following unexplained ailments/conditions is screened for TB: persistent cough for 2 or more weeks, persistent fever for 2 or more weeks, noticeable weight loss, excessive night sweats, and if it is a child, unexplained poor weight gain and/or history of contact with a person with confirmed TB. This patient is considered a presumptive TB case and he/she qualifies to undergo investigations for active TB disease.

3.4.1 TB diagnosis and management using Xpert MTB/RIF assay

For health facilities with functional Gene Xpert platforms, Xpert MTB/RIF assay is done in parallel with rapid screening of HIV. If Mtb is detected and it is rifampicin sensitive, the patient

is initiated on the standard first line TB treatment. If Mtb is detected and it is rifampicin resistant, the patient is treated as MDR-TB at a designated MDR-TB initiation Hospital. Additionally, a sample is referred for a baseline smear and TB culture to perform the 1st and 2nd line drug sensitivity testing (DST). In case of an indeterminate rifampicin result, the patient is initiated on the first line standard TB treatment regimen, but Xpert MTB/RIF assay test is repeated using another sample to aid in the review for the rifampicin resistance. If the test is negative, further clinical evaluation and X-ray test are performed. If the patient is likely to have clinical TB, he/she is started on the first line TB treatment. Otherwise, the patient is treated for bacterial infections using the broad-spectrum antibiotics and subsequently monitored for two weeks. When the patient fails to respond to the broad-spectrum antibiotics after 2 weeks, he/she is re-assessed for TB.

3.4.2 TB diagnosis and management using smear microscopy

If the facility lacks a Xpert platform which is the case in most of the peri urban and rural Health facilities, a smear microscopy is performed in parallel with HIV test. If the result is positive, the patient is started on the first line TB treatment, and a sample is referred for Xpert MTB/RIF assay to rule out rifampicin resistance. Negative smear samples are referred for Xpert MTB/RIF assay. If all the results are negative, the patient is treated for bacterial infections with broad spectrum antibiotics. If the patient does not respond to broad spectrum antibiotics in 2 weeks, he/she is re-assessed for TB.

3.5.0 Recording and reporting

All diagnosed TB patients i.e., those who are drug resistant, sensitive, or indeterminate are recorded in the unit TB register and subsequently included in the facility quarterly (HMIS 106a, Health Management Information System, section 106a). Notification reports and all rifampicin resistant TB patients should be notified in the weekly (HMIS: Health Management Information

System, section 033b). In addition, rifampicin resistant TB patients are recorded in the district line list and the Drug resistant TB register at the treatment initiation facility.

3.6.0 Justification for undertaking this work in Uganda

Tuberculosis-Molecular Bacterial Load Assay has the potential to achieve the following: 1) reduce turnaround time leading to early treatment initiation, 2) reduce loss of data through contamination, and 3) increase TB detection rates and subsequent case notification rates. All these attributes feed directly into the Uganda's National priority area which is "to reduce the TB incidence from 201/100,000 to 117/100,000 on 2025/26" through: (a) increased proportion of new/relapse TB cases detected from 75% to 90%, (b) increased treatment success from 72% to 90%, (c) adaption and scaling up of new tools for improving diagnosis, treatment, and prevention of TB, and (d) increased active case finding at all Health facilities in the country.

3.6.1 Main Objective

The main objective of this Chapter was to evaluate the diagnostic accuracy of the tuberculosis-Molecular Bacterial Load Assay in comparison with the standard-of-care tests using sputum sample: smear microscopy and DNA-based Xpert MTB/RIF Ultra using sputum MGIT culture as the reference test.

3.6.2 Specific Objectives

i. To establish the sensitivity, specificity, and predictive values of the tuberculosis-Molecular Bacterial Load Assay among the adult TB patients.
ii. To ascertain the sensitivity, specificity, and predictive values of the standard of care tests: smear microscopy and Xpert Ultra using the same samples from the same participants.
iii. To subjectively compare the sensitivity, specificity, and predictive values of the tuberculosis-Molecular Bacterial Load Assay with the standard of care tests: smear microscopy and Xpert Ultra.

3.7.0 Study sites and Design

This study was conducted at Naguru General Hospital and Makerere University, Kampala Uganda. Naguru General Hospital is also known as China-Uganda Friendship Hospital, Naguru. The Hospital is located along the Naguru Road, Nakawa Division, Kampala District. This location is about 4 Km East of the central business district of Kampala. Naguru General Hospital serves the residents of Kampala metropolitan area and other Ugandans from different parts of the country. To-date, Naguru General Hospital consists of four operating rooms, a maternity ward, a paediatric unit, a teenage centre, a blood bank, a radiology department equipped with a CT scan, functional TB, and HIV clinics. The laboratory department is furnished with a functional Xpert MTB/RIF Ultra machine, fluorescent microscope, centrifuges, biosafety cabinet level II (BSL-II) among other standard laboratory requirements to handle clinical samples for microbiological investigations. This study was nested in a longitudinal cohort of persons with pneumonia at Naguru Hospital. This cohort is known as the "Mulago In-patient Non-Invasive Diagnosis-International HIV-Associated Opportunistic Pneumonias-Inflammation, Aging, Microbes and Obstructive Lung Disease" (MIND-IHOP-IAM OLD) study.

The parent study is sponsored on Makerere University and the University of California, San Francisco, funded on the National Institute of Health (NIH). The primary objective of the MIND-IHOP-IAM OLD study is to investigate the frequency, quantity, and diversity of bacterial, mycobacterial, fungal, and viral bugs in the respiratory and non-respiratory human specimens on using microbiologic, serologic, and nucleic-acid amplification techniques, seeking to establish the relationship between the presence of these organisms and clinical outcomes.

3.7.1 Sites for participant enrolment

Study participants recruitment was done at the TB Clinic and the triage station of the Naguru General Hospital. Blood and sputum samples collection, HIV testing, complete blood count (CBC), liver and renal function tests (RFT/LFTs), smear microscopy, and Xpert Ultra assays were done at the MIND-IHOP-IAM OLD study laboratory. During the enrolment sessions, study nurse and the student screened potential participants from the outpatient department, at the triage station and or TB-clinic. Patients who qualified and consented to participate in the study provided clinical and laboratory data.

3.7.2 Sites for laboratory services

Circulating levels of the $CD4^+/CD8^+$ counts were done at the Makerere University-John Hopkins University (MU-JHU) Core Laboratory. Solid and liquid culture tests were done at the Mycobacteria Laboratory which is Biosafety category 3 facility (BSL CAT3) found in the Department of Medical Microbiology at the College of Health Sciences (CHS), Makerere University, Kampala Uganda. Both facilities; (MU-JHU) Core Laboratory and Mycobacteria Laboratory which is Biosafety category 3 facility are certified on the College of American Pathologists (CAP) society.

3.7.3 Study design

Participants were screened basing on the guidelines of the Uganda National Tuberculosis and Leprosy programme. Specimens were collected cross-sectionally and processed accordingly. Participants were identified and consented on the study nurse, except in a few cases where a clinician assessment was deemed necessary.

3.7.4 Inclusion criteria

The WHO-recommended tuberculosis screening guidelines were followed. Consequently, participants were enrolled into the study project after meeting the following criteria:

i. Being able to provide a written informed consent or oral witness for the illiterate patient
ii. Being male or female adult within the age limit of 18-65 years
iii. Meet criteria for a presumptive TB case
iv. Being able to produce an adequate volume of sputum of 2 mL or higher
v. Being a newly identified with active TB with/without a history of TB treatment
vi. Willingness to be enrolled into the treatment response follow-up arm
vii. Being able to return to the Hospital for follow-up visits if included in the follow-up arm
viii. Having accessible Physical address and contact reference for easy follow-up

3.7.5 Exclusion criteria

Patients were excluded based on any of the following criteria:

i. Being terminally ill with a high likelihood of missing the treatment follow-up
ii. If patient was unable to produce required sputum volume
iii. Having a history of drug resistance

3.7.6 Study related risks and challenges

3.7.6.1 Loss of confidentiality due to participating in the study

Loss of confidentiality would cause stigma especially to participants that had been found to be positive for TB, HIV, or both. In this case, all personnel involved in data collection were trained on how to oversee patient data with care. Consequently, no identifiable information was used. We ensured that participant information on the data capture forms were put in a unique folder that was labelled with a unique identifier number before keeping them in a lockable cabin with a restricted access.

3.7.6.2 Exposure of the study personnel to TB

To mitigate the risk of catching infection, personal protective equipment. Were availed and all personnel involved in data collection were sensitised on using these tools. For example, sputum

expectoration was done in a designated well aerated open space. Processing of sputum and blood samples was strictly done in the biosafety cabinet. Protective gears such as the World Health Organisation approved N95 nasal masks. gloves and face googles were always worn during data collection. A specimen rejection criteria put in place, and this was utilised to reject all specimens that were delivered in leaky and soiled containers.

3.7.6.3 Mitigating contracting Covid 19 on study personnel

Continuous training in infection control in the context of Covid 19 was conducted. The training was aimed to equip the personnel with knowledge to assess the risk of Covid 19 among the patients and to emphasize the infection control measures. All staff were provided with alcohol-based hand rubs in addition to the existing hand washing facilities to enable them wash regularly. To maintain social distancing and decrease of congestion, staff members were encouraged to work on alternate days. Mandatory cleaning of the surfaces and outdoor meetings were emphasised and implemented. Free access to Personal Protective Equipment (PPE) including face shields, gaggles' scrubs, aprons, N95, face masks and gloves was ensured.

3.7.6.4 Mitigating study participants from contracting Covid 19

All patients were provided with protective face masks during their interaction with the study staff. Patients were provided with hand washing facilities, and they were encouraged to effectively wash their hands before interaction with study staff. In addition to routine work, patients were screened for possible Covid 19 infection and in case they were graded as high risk, they were referred to the Hospital-managed isolation unit for further management. Moreover, a maximum of 2 participants were scheduled for follow up visit points to avoid congestion at the data collection station.

3.7.6.4 Effect of Covid 19 on execution of the current study activities

At the start of the Covid 19 related restrictions on movement, we experienced a slow enrolment. To mitigate this, as a team we worked with the TB Clinic/Programme colleagues to increase patients' screening points. Research participants were engaged to lead us to their contacts who had TB-like symptoms. In n this case, motorcycle riders were deployed to get to these persons and shuttle them to the clinic for potential enrolment into the study. As Covid 19 lock downs dragged on, supplies from overseas delayed due to restrictions in shipment from abroad. To mitigate this, we leveraged on our collaborators, and we made sure to refund/replace the borrowed supplies.

3.7.7 Withdrawal from the study

Participants' withdrawal from the study was voluntary and due to death, transfer out of the study area or withdrawal of consent to continue participating in the subsequent follow up visits. Participants who withdrew from the study were helped to start and or continue with their TB treatment in case they were positive for TB. The patient ID of the participants who withdrew from the study were maintained for inclusion in statistical analysis and all the related documents were updated. Date and reason of withdrawal was indicated on the patient file for future reference.

3.7.8 Data collection network

The study nurse collected the clinical data, including patient demographics, TB history and other relevant information. Patient clinical information was captured onto the respective primary source documents, signed, and dated before handing them over to data clerk for transferring the same information into the electronic database. Before the patient were referred for specimen collection, they were clearly informed of the specimen type and volume of the sample that is required. Patients' decision was always respected.

3.8.1 Chest radiographs

Standard chest radiographs were done in the Radiology Department of Naguru General Hospital. Antero-Posterior views were evaluated for TB, using a standard reporting form on the Study Pulmonologist to classify the outcomes of Chest-Xray readings. Results from the chest x-ray were primarily for clinical management of the patients but not for research purposes. However, reference could be made where the circumstances required so. If the patient had TB symptoms but the sputum was negative, and the chest x-ray was highly suggestive of pTB, that participant would be asked to supply another sputum sample to be re-examined to confirm absence of bacteriologically confirmable TB disease.

3.8.2 Laboratory and clinical data collection and management

Before the specimens were collected, the detail of specimen collection procedures was revealed to the patients, and their decision was considered. Specimens were collected from patients who provided full consent and it was done on the student and a designated Phlebotomist under the supervision of a Clinician when it was required. All specimen containers were clearly labelled and capped to make sure that there is no leakage and that the containers are not soiled.

Specimen in their containers were added to the Ziplock and handed over to the laboratory runner to deliver them to the respective laboratories. All specimens were accompanied with well filled test requisition forms. The clinical data and specimen collection information were captured onto the respective data capture forms and log forms. Data capture forms were then handed over to the data entrant for entry into electronic database. Specimen information (type, collection date, volume, identification number, and the position in a cryovial for archived samples) was also captured into the separate specimen database. Hard copies of the test results from the respective laboratories were delivered on the laboratory runner. These results were captured into the results capture forms and handed to the data entrant for capture into the electronic database.

After data was captured into the electronic database, all hard copies were archived in patient unique file and stored into a lockable cabin. All dates were uploaded onto the Central server which is only accessed on selected persons. Before data analysis, electronic copies were retrieved and cleaned-up to rid typos before statistical data analysis.*(Figure 3.2.0)*

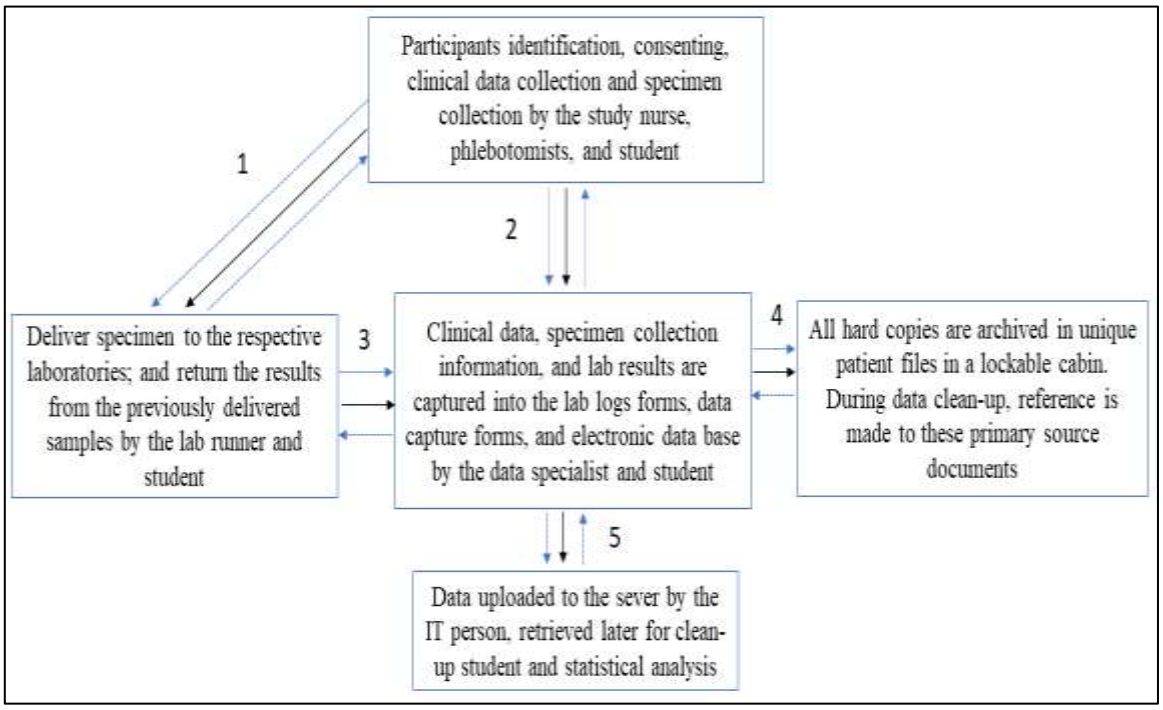


Figure 3.2.0: Patient identification, data collection and information flow. The blue dotted lines indicate that when information was unclear, the concerned party was contacted either on emails or a phone call and a response was delivered on the same means. **1:** Specimen in their containers were added to the Ziplock and handed over to the laboratory runner to deliver them to the respective laboratories. All specimens were accompanied with well filled test requisition forms. **2:** The clinical data and specimen collection information were captured onto the respective data capture forms and log forms. Data capture forms were then handed over to the data entrant for entry into electronic database. Specimen information (type, collection date, volume, identification number, and the position in a cryovial for archived samples) was also captured into the separate specimen database. **3:** Hard copies of the test results from the respective laboratories were delivered on the laboratory runner. These results were captured into the results capture forms and handed to the data entrant for capture into the electronic database. **4:** After data was captured into the electronic database, all hard copies were archived in patient unique file and stored into a lockable cabin. **5:** All dates were uploaded onto the Central server which is only accessed on selected persons. Before data analysis, electronic copies were retrieved and cleaned-up to rid typos before statistical data analysis.

3.8.3 Processing and examination of blood specimen

About 4-5ml of the venous blood was collected and used to measure the complete blood count, renal, and liver function tests. Blood was also used to assess for the presence or absence of

HIV, and to measure the circulating levels of the $CD4^{+}/CD8^{+}$. During counselling, testing and issuing results, the Uganda National HIV Counselling and Testing guidelines were followed.[277]

3.8.4 Processing and examination of sputum specimens

Each participant's sputum sample was examined using four different tests including the following: TB-MBLA, Xpert Ultra, MGIT culture, and SSM-FM. Participants were requested to provide three serial spot sputa for us to be able to get enough sample volume. Because sputum is variable, the three sputa were pooled and homogenised immediately before equal volume aliquots were assigned to different tests as shown in the *Figure 3.2.0* below.

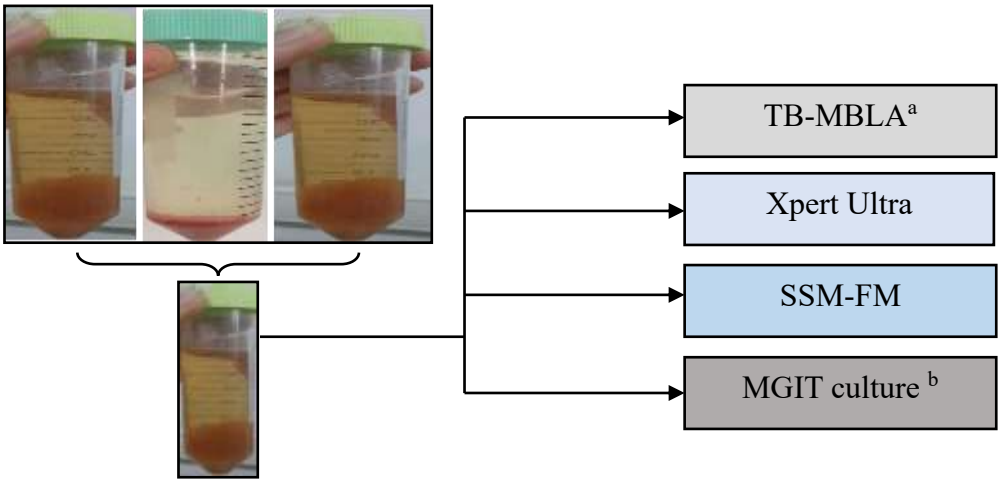


Figure 3.3.0: Sample collection and processing before examination. TB-MBLA: Tuberculosis Molecular Bacterial Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM: Stained smear microscopy, MGIT: Mycobacteria Growth Indicator Tube. [a]TB-MBLA was the study index test. B MGIT was the gold standard and reference for TB-MBLA, Xpert Ultra and SSM. Participants provided 3 spot sputa which were pooled and homogenised using sterile beads and vortex mixer to improve specificity, sensitivity and reduce on the cost per test. Each pooled and aliquoted into 4 portions. One portion was tested on either TB-MBLA, Xpert Ultra, culture or smear (**Source: The picture was taken during specimen collection at Naguru referral hospital, Uganda**).

Each mL of sputum for TB-MBLA were preserved in 4 mL of GTC containing 1% mercaptoethanol as detailed *Chapter Two*. Pooling the three sputa was preferred to ensure that a single homogeneous sample was assessed on all test platforms: TB-MBLA, Xpert Ultra, SSM-FM and MGIT culture. Literature review indicated that sample pooling improves sensitivity, specificity, and that it saves on the investigational costs.[278]

3.8.5 Laboratory investigations

Xpert MTB/RIF Ultra was performed for each baseline sample to assess for the presence or absence of Mtb in patients' sputa. One millilitre (mL) of the homogenised sputum sample was used as detailed in *Chapter Two*.

3.8.5.1 Stained sputum smear microscopy

Sputum smears were prepared on applying 100 µL of the pelleted sputa on a labelled glass slide. Sputum was diluted on distributing it evenly to make a uniform smear as described in Chapter Two. Smears were air dried for 30 minutes and then heat-fixed at 50°C for 20 minutes in an oven before staining and examining them as detailed in *Chapter Two*.

3.8.5.2 MGIT culture and confirmation of Mtb

Before the culture tests were done, sputa were decontaminated as described in Chapter Two. The MGIT culture tubes that flagged positive in the BACTEC MGIT 960 instrument were further examined on SSM-ZN to confirm the presence of AFB. Cultures that were AFB positive on the SSM-ZN were tested with the MPT64 antigen test to further confirm the presence of Mtb and exclude non-tuberculosis mycobacteria. Blood agar was performed to rule out contaminants and exclude false positive signals of the MGIT instrument due to contamination, changes in oxygen level and pH of the medium as detailed in *Chapter Two*.

3.8.6.0 TB-MBLA

Samples for TB-MBLA were batched and processed on weekly basis. Before batching, 1 mL the pooled sputum was added to 4 mL of GTC that was already activated with beta mercaptol ethanol and stored at -80ºC for a fortnight. This storage process preserve the bioavailability of the RNA. Within two weeks, RNA extraction was performed as described in *Chapter two*. The TB-MBLA and standard curves were done as detailed in *Chapter Two*.

3.9.0 Results

3.9.1 Participants' enrolment and their baseline characteristics

A total of 236 persons were screened for TB between the period of September 2019 to February 2022. Out of the 236 screened patients, 210 (89%) were enrolled into the study this was because of the following reasons: i) Fifteen (6.4%) of the 236 persons that were screened objected to participate in the study because they were staying far from the TB Clinic, and it would have been hard for them to return for the scheduled visit points. ii) Ten (4.2%) persons withheld their consent to participate in the study. ii) One of them (0.4%) failed to provide sputum sample. (*Figure 3.1.0*).

The enrolled participants were young adults with median (IQR) age of 35 years (27-44). Majority of the participants 135 (64%) were of male gender. Overall, 72 (66%) of the 210 participants were living with HIV 24 (23.3%) of whom were confirmed to have pTB coinfection with a median CD4 cells count 227 cells per µl (IQR: 54-345). Most of the 210 participants 206 (98%) reported unexplained cough for two or more weeks and 54 (27%) of these had blood in their sputum. Overall, 159 (75.7%) participants reported unexplained weight loss. Of these, 86 (53%) were confirmed to have pTB, and 67(42%) were pTB negative according to MGIT culture. Gene Xpert MTB/RIF Ultra detected TB in 129 participants. The gold standard test (sputum MGIT culture) confirmed pTB in 103 (49%) participants whereas Xpert Ultra identified 129 (61·4%) participants. Out of the 210 enrolled participants, 6(2·9 %) were excluded from the final analysis because their MGIT culture test was indeterminate due to contamination. Median body mass indices: 19 kg/m^2 for all participants, 20 kg/m^2 for participants that were confirmed to have pTB, and 18·8 kg/m^2 for those who were pTB negative according to MGIT culture, were all within the 'healthy range' value 18·5-24·9. Heart rate, respiratory rate, oxygen saturation as well as body temperature did not significantly differ among participants who were confirmed to have pTB and those that were not(*Table 3.1.0*).

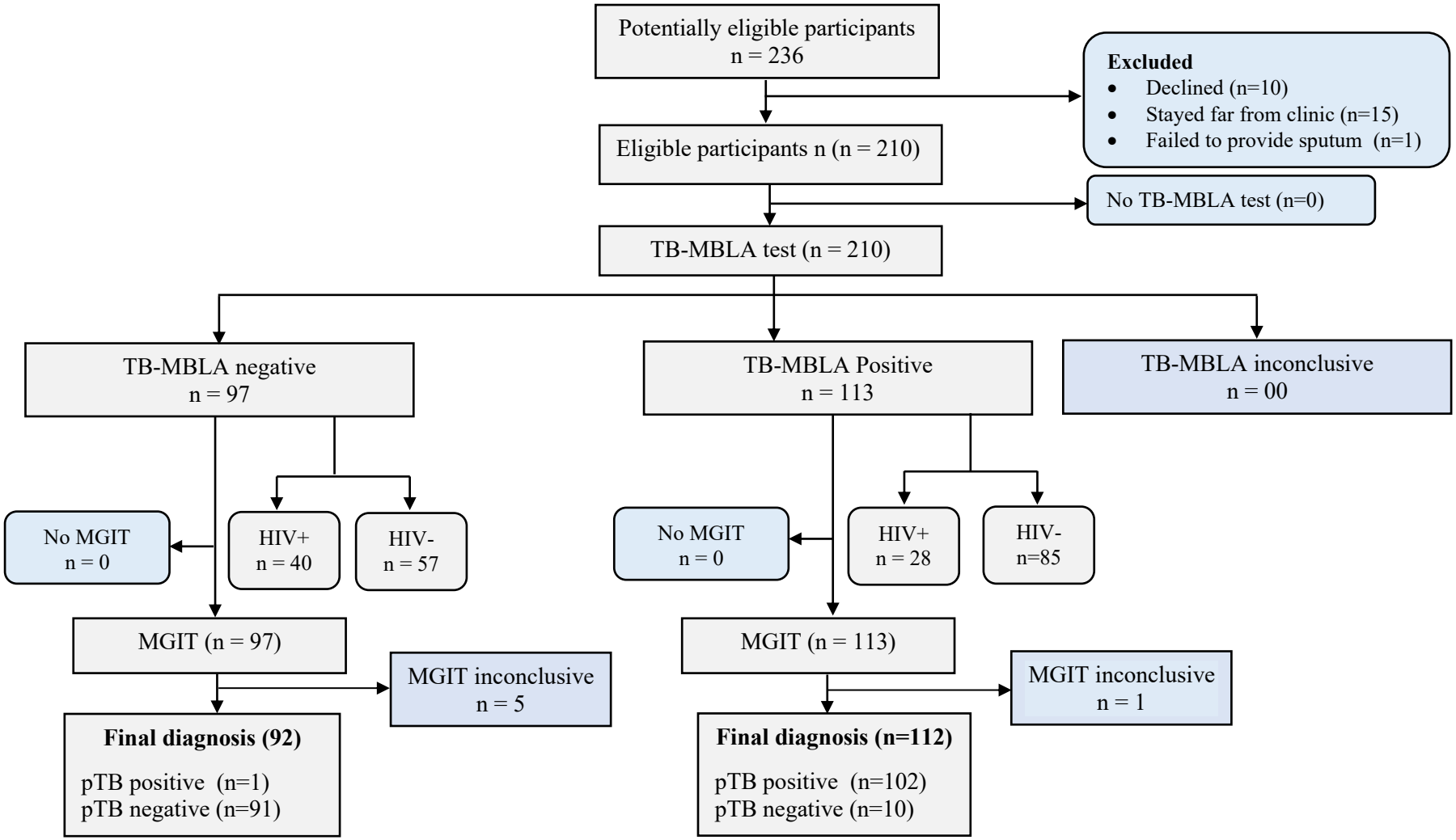


Figure 3.4.0: Participant enrolment. Potentially eligible persons were n=236. Only n=210 participants were enrolled into the study because n=10 withheld consent, n=25 stayed far from clinic and would not come back on the scheduled follow up visit, n=I failed to provide sputum sample. Six out of the n=210 participants had their MGIT culture result indeterminate due to contamination on bacteria other than Mtb. MGIT culture as a gold standard TB test identified n=103 participants as positive and n=101 as negative. TB-MBLA, which is the index test identified n=114 participants as positive and n=97 as negative. Twenty-eight of the n=113 TB-MBLA positive cases were living with HIV and n=85 were living without HIV. After excluding the indeterminate MGIT culture results, positive TB-MBLA results were n=112, and negative TB-MBLA results were n=92. n=101 results were positive on both the gold standard test (MGIT culture) and the index test (TB-MBLA).

Table 3.1.0: **Baseline participants' demographic characteristics**

Demographic characteristics	Persons with indicated pulmonary tuberculosis (pTB)				
	Overall N[1] = 210	**Positive [a] n=103 (49%)**	**Negative n=101(48%)**	**Contaminated n= 6 (3%)**	**p-value [c]**
Age (Years)	35(27-44)	32(26-43)	35(30-47)	37(30-48)	0.6
Male	135(64)	78(75)	54(53.5)	3(50)	..
Evening fevers	174(82.9)	92(89.3)	77(76.2)	5(83.3)	..
Weight loss >5%	159(75.7)	86(83.5)	67(66.3)	6(100)	..
Cough >2weeks	206(98)	102(99)	98(97)	6(100)	..
Haemoptysis	54(27)	28(27.2)	25(24.8)	1(16.7)	..
Heart rate (%)	98(85-112)	104(88-114)	93(79-104)	104(66-123)	0.27
Respiratory rate (%)	22(20-28)	24(20-28)	21(20-26)	20(18-23)	0.27
Oxygen saturation (%)	96(94-98)	96(94-97)	97(94-98)	97.5(97-98)	0.82
Living with HIV/AIDS	72(66)	24(23.3)	44(43.6)	4(66.7)	..
Antiretroviral therapy use	35(63)	7(6.8)	25(24.8)	3(50)	..
CD4 (cells/μL)[b]	222(54-381)	227(57-345)	183(52-601)	514(113-922)	0.44
CD8 (cells/μL) [b]	585(413-874)	589(459-872)	585(410-897)	631(466-883)	0.68
CD4/CD8 [b]	0.28(0.1-0.5)	0.24(0.1-0.5)	0.3(0.1-0.9)	0.6(0.25-1.1)	0.51
Body mass index	19(17-22)	20.1(17-24)	18.8(17-21)	24(18-27)	0.2
Body temperature	37(36-37)	36.6(36.4-37)	36.5(36-37)	37(37-37)	0.81

N[1]: 210 refers to all participants who were enrolled into the study. MGIT culture test confirmed pTB in 103 participants, and 101 participants were confirmed negative for pTB. Six (3%) MGIT culture results were indeterminate due to contamination. Categorical data are n/N (%); Quantitative data are median [IQR]; [a] Bacteriologically confirmed positive or negative TB cases using sputum MGIT; [b] CD4 and CD8 and (CD4/CD8) were measured and (calculated) only for HIV positive participants (n=72). [c] P-value compares persons who were bacteriologically confirmed to have pTB and those without pTB

3.9.2 Mtb bacillary load and quantification cycles

Mycobacteria load measured using TB-MBLA, Cq values, and time-to-positivity were obtained for only Mtb positive results. Of the 113 TB-MBLA positive results, 102 (90·3%) were also positive on MGIT , and 1(0·9%) was contaminated with MGIT culture and was excluded from the analyses. The overall mean ± standard deviation bacteria load of these 112 samples was 4·8 ± 1·5 $\log_{10}$ eCFU per mL. Twenty-eight (25%) of the TB-MBLA-positive-HIV positive participants had mean bacteria load of 3·8±1·6 $\log_{10}$ eCFU per mL. For participants who were TB-MBLA-positive but HIV negative (n=84), mean bacteria load was 5·2±1·3 $\log_{10}$ eCFU per mL representing a 1·4$\log_{10}$ eCFU per mL logs higher (t-test , p>0.0002) compared to those who were positive on TB-MBLA and HIV testing. Median (IQR) Cq value for TB-MBLA was 20 (17-25). Median (IQR) Cq was 25 (20-28) in TB-MBLA-positive-HIV positive participants (n=28), and 20 (16-23) in TB-MBLA-positive-HIV-negative participants (n=84). For Xpert Ultra (n=126), median Cq value was 20 (18-23). In Xpert Ultra-positive-HIV-positive participants (n=34), median Cq was 23 (20-28), and 19 (18-22) in the Xpert Ultra-positive-HIV negative (n=92). (*Table 3.2.0*)

Table 3.2.0: **Bacterial load, Quantification cycle values, and MGIT TTP**

Test	Bacterial Load (Mean±SD log10 eCFU/mL)	Quantification cycle (Cq)(Median[IQR]	MGIT TTP (Median days[IQR])
TB-MBLA[a]			
Overall[b] (n=112)	4·8 ± 1·5	20 (17-25)	--
HIV positive (n=28)	3·8±1·6	25(20-28)	--
HIV negative (n=84)	5·2±1·3	20(16-23)	--
MGIT			
Overall[b] (n=103)	--	--	7(5-10)
HIV positive (n=24)	--	--	9·5(6·5-13)
HIV negative (n= 79)	--	--	6(5-9)
Xpert Ultra			
Overall[b] (n=126)	--	20(18-23)	--
HIV positive (n=24)	--	23(20-28)	--
HIV negative (n=92)	--	19(18-22)	--

TB-MBLA: Tuberculosis Bacterial load assay, MGIT: Mycobacteria Growth Indictor Tube, Xpert Ultra: Xpert MTB/RIF Ultra; [a] Index test. [b] Sample size excludes those which were indeterminate on MGIT culture

Spearman correlation test showed a positive correlation of the overall Cq values of TB-MBLA and Xpert Ultra possibly indicating that at baseline, both tests detect viable Mtb. Overall median values for the quantification cycles values 20 (IQR:17-25; n=112) for the TB-MBLA and 20 (IQR:18-23; n=126) for the Xpert Ultra were comparable indicating that both tests measured similar bacteria burden in the sputum sample (*Figure 3.2.0*).

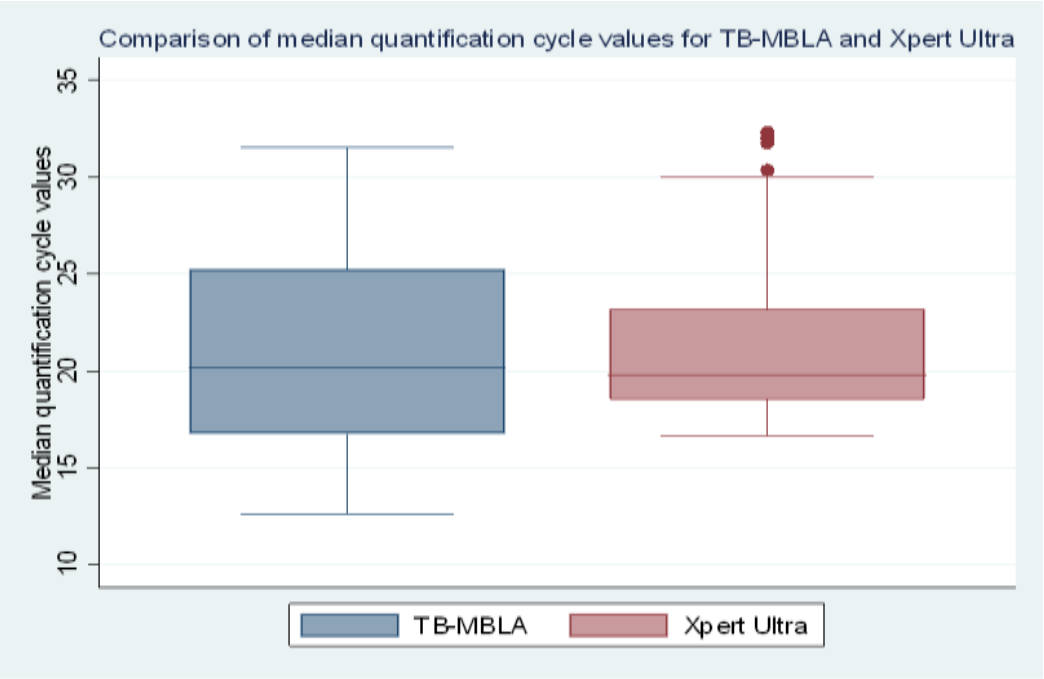


Figure 3·5·0: Comparison of median quantification cycles values of the index test (TB-MBLA) and standard- of-care test (Xpert Ultra). TB-MBLA: Tuberculosis Molecular Bacterial Load Assay, Xpert Ultra: Xpert MTB/RIF assay.

3.9.3 Discordant results

Although TB-MBLA concurred with MGIT culture test at 94% (kappa=0·89), Xpert Ultra at 90·2% (Kappa statistics (k)=0·8), and SSM at 62·5% (Kappa statistics (k)=0·22), there were some discordances. Test results which were negative on Xpert Ultra, MGIT and SSM-FM but positive on TB-MBLA were pooled and excluded those which were self-repeating. We noted that 33(29·4%) of the 112 TB-MBLA positive samples were negative on the pooled test results. Considering MGIT test alone, ten results were TB-MBLA positive but were negative on MGIT test.

The mean bacteria load for these 10 samples was 3·8 ± 1·33 log_{10}eCFU per mL, which was 1·0log_{10} eCFU/mL less than the average bacteria load (4·8logs) in all study participants. Three 3 (2·6%) samples were positive on TB-MBLA but negative on Xpert Ultra. The mean bacteria load in these three samples was 2·1log_{10}eCFU per mL, which was almost half the average bacteria load (4·8 logs) for the entire study population. Focusing on SSM-FM, 34 (30·4%) samples were positive on TB-MBLA but negative on SSM-FM. The mean bacteria load in these 34 samples was 3·4 log_{10} eCFU per mL which was 1.4log10eCFU per mL less than the (4·8logs) bacteria load for the overall study population. On average, samples that were positive on TB-MBLA but negative on MGIT, Xpert Ultra and SSM-FM had an average bacterial load of 1·5logs below the average bacterial load of the study population.

3.9.4 Xpert Ultra 'Trace call' results

'Trace call result' is a new category of results on the Xpert Ultra platform that can be used to make clinical decision. Overall, out of the enrolled 210 participants, Xpert Ultra detected Mtb in 129 participants of whom 3(2·3%) had indeterminate MGIT culture results. Ten (7·8%) of the 129 Xpert Ultra positive results were 'trace call' category. Out of the 10 trace call results, 2(20%) results were also positive on both TB-MBLA and MGIT culture. The mean bacteria load of these two samples was 4·6 log_{10} eCFU per mL which was like 4·8 log_{10} eCFU per mL bacteria load for the overall TB positive study population.

Median quantification cycle value of these two samples was 25·3 which was also similar to average Cq value 25 for the overall population. Median MGIT time to positivity for the MGIT culture test of these two samples was 10-days. Although 'trace call' results guided TB treatment initiation, majority of these results were negative 8(80%) on TB-MBLA and MGIT culture, and 9(90%) on SSM-FM. Importantly, all the 10 trace call results were associated with 'indeterminate rifampicin resistance' result (*Table 3.3.0*).

Table 3.3.0: **Relationship between 'Trace call' results with other tests results [n=10]***

Test	Test results n/N (%)	
	Positive	**Negative**
TB-MBLA[a]	2(20)	8(80)
MGIT culture	2(20)	8(80)
SSM-FM	1(10)	9(90)

[a] index test. *None of the 'samples with 'trace call' had a corresponding MGIT culture as indeterminate.

3.9.5 Measures of diagnostic accuracy in all study population participants [n=204]

Test diagnostic performance was measured against MGIT culture test. Out of the 210 enrolled participants, 6(2.9%) were excluded from diagnostic accuracy statistical analysis because they were indeterminate on MGIT culture. Therefore, diagnostic accuracy analysis was done for 204 (97%) participants. We noted that TB-MBLA had a diagnostic sensitivity (95%CI) of 99% (95-100), and specificity (95%CI) of 91% (83-96). Although sensitivity at 99% (95-100) was similar for TB-MBLA and Xpert Ultra assays, specificity at 78% (68-86) was lower for Xpert Ultra when compared to 90% (83-96) for the TB-MBLA test. SSM-FM was the least sensitive test at 75% (65-83) but it was the most specific test at 98% (93-100). (*Table 3.4.0*)

3.9.6 Measures of diagnostic accuracy in persons living with HIV [n=68]

Being HIV positive may lower the sensitivity of TB diagnostic tests due to low bacillary load in sputum. We found out that that in persons living with HIV, TB-MBLA and Xpert Ultra had similar sensitivity of 100% which was almost two-folds higher than 54%(33-74) for SSM-FM test. The NPV (95%CI) 100% (91-100) for TB-MBLA and 97% (85-100) for Xpert Ultra were similar. The positive predictive value (95%CI) of TB-MBLA was 89% (71-98) which was 17% higher than 72% (53-86) for the Xpert Ultra test. SMM-FM was the most specific test at 98% (87-100) with PPV (95%) of 93% (66-100) (*Table 3.4.0*).

Table 3.4.0: **Measures of diagnostic accuracy in the total study population [n=204]**

Tests	Mean value (95% CI) for[b]							
	TP	**TN**	**FN**	**FP**	**Sensitivity(%) (95%CI)**	**Specificity(%) (95%CI)**	**PPV(%) (95%CI)**	**NPV(%) (95%CI)**
TB-MBLA[a]	102	91	1	10	99(95-100)	90(83-96)	91(85-96)	99(94-100)
Xpert Ultra	102	77	1	24	99(95-100)	76(68-86)	82(74-89)	99(93-100)
SSM-FM	77	99	26	2	75(65-83)	98(93-100)	97(91-100)	79(71-86)

TB-MBLA: Tuberculosis Molecular Bacterial Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM-FM: Stained Sputum Smear-Fluorescence Microscopy, MGIT: Mycobacteria Growth Indicator Tube. PPV: Positive Predictive Value, NPV: Negative Predictive Value [b] Sputum MGIT culture was used as the reference., TP: True positive results, TN: True negative results, FN: False negative results, FP: False positive results, [a] TB-MBLA was the index test.

Table 3.4.1: **Measures of Diagnostic accuracy in persons living with HIV [n=68]**

Test	Mean value (95% CI) for[b]							
	TP	**TN**	**FP**	**FN**	**Sensitivity(%) (95%CI)**	**Specificity(%) (95%CI)**	**PPV(%) (95%CI)**	**NPV(%) (95%CI)**
TB-MBLA[a]	24	40	4	0	100(86-100)	93(81-99)	89(71-98)	100(91-100)
Xpert Ultra	23	33	11	1	100(79-100)	81(63-90)	72(53-86)	97(85-100)
SSM-FM	13	43	1	11	54(33-74)	98(87-100)	93(66-100)	79(65-89)

TB-MBLA: Tuberculosis Molecular Bacterial Load Assay, Xpert Ultra; Xpert MTB/RIF Ultra, SSM; Stained Sputum Smear-Fluorescence Microscopy, MGIT: Mycobacterial Growth Indicator Tube, PPV: Positive predictive value, NPV: Negative predictive value, TP: True positive results, TN: True negative results, FP: False positive results, FN: False negative results. [S]putum MGIT culture was used as the reference test, [a] TB-MBLA was the index test

3.9.7 Smear negative participants [n = 124]

Out of the 124-sputum smear-negative results, 26 (20·96%) were confirmed to have Mtb on the sputum MGIT culture, 34 (27·42%) were positive on the TB-MBLA with mean bacterial load ± SD; 3·38 ± 1·33 eCFU per mL; 48/124 (38·7%) were positive Xpert Ultra test. Twenty-four (19·4%) of the 124 samples were positive on all the three tests i.e., TB-MBLA, Xpert Ultra and MGIT. Nine samples were positive on TB-MBLA but negative on MGIT culture. Seven (77·8%) of the 9 samples were also positive on Xpert Ultra. The average bacteria load of the 9 samples that TB-MBLA positive but MIT negative was 3·47 $\log_{10}$ eCFU per mL. Three samples were positive on TB-MBLA but negative on Xpert Ultra, 1 (33%) of which was also positive on MGIT culture. Mean bacteria load of these 3 samples was 3·51 log eCFU per mL. Two samples were positive on TB-MBLA but negative on both TB-MBLA and Xpert Ultra with average bacteria load 2·26 $\log_{10}$ eCFU per mL. Sixteen samples were positive on Xpert Ultra but negative on both TB-MBLA and sputum MGIT. (*Figure 3.4.0)*.

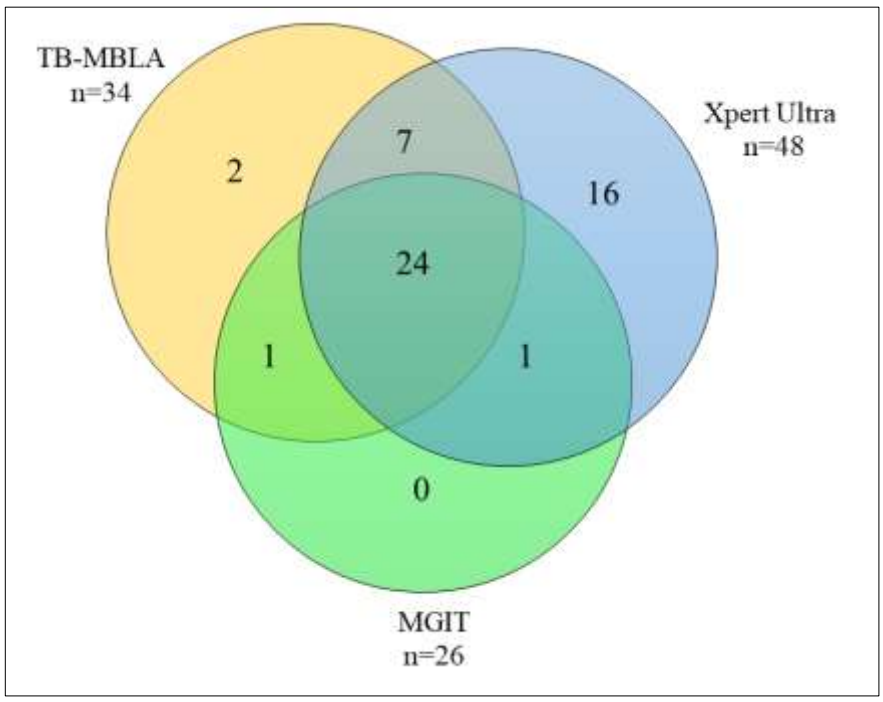


Figure 3.6.0: Positive test results among the smear negative participants. Out of the 26 samples confirmed pTB positive, 25 (96·2%) were also positive on both TB-MBLA and Xpert Ultra. Twenty-four (19·4%) samples were positive on all the three assays. Thirty-one (25%) tested positive on both TB-MBLA and Xpert

The 48-smear negative but Xpert Ultra positive samples were further categorised according to positivity grades. Eight of the 48 (16·7 %) were graded as 'Trace', 21(43·8%) were graded as 'low', 8(16·7%) were graded as 'very low', 5(10.4%) were graded as 'medium' and 5 (10·4%) were graded as 'high'. Out of the 48 Xpert Ultra positive but SSM-FM negative samples, 31(64·5%) were also positive on TB-MBLA with mean bacterial load ± SD, 3·50 ± 1·33 eCFU per mL. Sixteen (33·3%) of the 48 samples were negative on TB-MBLA but positive on Xpert Ultra. These sixteen samples had lower mycobacteria load ranging between 'low' to 'trace' according to Xpert Ultra grading.

3.9.7.1 Measures of diagnostic accuracy in smear negative participants [n=124]

The sensitivity (95%CI) of TB-MBLA among the participants who had a negative SSM-FM test result was 96% (80-100), and specificity (95%CI) was 92% (84-96). Sensitivity of the Xpert Ultra 96% (84-100) was similar to that of the TB-MBLA but specificity 78% (69-86) less compared to 92% (84-96) of the TB-MBLA. The PPV (95%CI) of the TB-MBLA was 76% (58-89) and was higher than 53% (39-69) of the Xpert Ultra. On contrast, the NPV was high at 99% for both TB-MBLA and Xpert Ultra tests. The trend of the sensitivity, specificity and predictive values was similar in participants who were negative on SSM-FM and living with HIV (*Table 3.5.0*).

Table 3.5.0: Diagnostic accuracy among SSM-FM negative participants [n=124]

Test	Performance in all SSM-FM negative study participants							
	TP	**TN**	**FP**	**FN**	**Sn% (95%CI)**	**Sp(%) (95%CI)**	**PPV(%) (95%CI)**	**NPV(%) (95%CI)**
TB-MBLA[a]	25	90	8	1	96(80-100)	92(84-96)	76(58-89)	99(94-100)
Xpert Ultra	25	76	22	1	96(84-100)	78(69-86)	53(39-69)	99(93-100)
Diagnostic accuracy of TB-MBLA and Xpert Ultra in PLWHIV with negative SSM-FM[52]								
TB-MBLA	11	39	2	0	100(72-100)	95(84-99)	85(55-98)	100(91-100)
Xpert Ultra	10	33	8	1	91(59-100)	80(65-91)	56(31-79)	97(84-100)

TP: True positive cases, TN: True negative cases, FP: False positive cases, FN: False negative cases, Sn: Sensitivity, Sp: Specificity, Sputum mycobacteria Growth Indicator Tube culture was used as the reference standard test. PLWHIV: Persons Living With HIV

3.10.0 Discussion

Using Molecular assays can potentially increase TB detection rates and the number of TB patients who could start on the treatment early enough. These assays also improve treatment outcome and reduce TB transmission rates. In this Chapter, a novel-RNA based TB-MBLA was evaluated for its diagnostic accuracy among the presumptive TB patients. The diagnostic accuracy of the TB-MBLA was compared with smear microscopy and Xpert Ultra.

TB-MBLA was sensitive and specific with higher positive predictive value, indicating that it has potential role in TB diagnosis. Although sensitivity was similar for TB-MBLA and Xpert Ultra, specificity was higher for TB-MBLA. MGIT culture which was used as the reference test could have underestimated the specificity of the Xpert Ultra since it is likely to be less sensitive than the Xpert Ultra. This observation indicates the limitation of using a bacteriological test as a standard reference for the more sensitive and specific molecular-based assays. It also justifies the need for a more accurate and reliable molecular TB surrogate method to function as the standard reference for the nucleic acid amplification-based tests.

In the year 2018, the WHO noted that TB-MBLA has a potential to replace culture and smear for monitoring TB treatment response. The findings from the current study show that TB-MBLA has a higher degree of concordance with MGIT culture than SSM-FM and Xpert Ultra, indicating that TB-MBLA is a likely to be a more suitable molecular test that can replace MGIT culture as a standard reference for TB molecular assays.

Being HIV positive is an important risk factor for getting TB disease and getting ill from the TB. It is therefore important to have a test and sampling strategies that improve TB detection accuracy in persons living with HIV. From literature review, Xpert Ultra is noted to have a pooled sensitivity of 69% (57-80) in persons living with HIV.[279] In the current study, the sensitivity of TB-MBLA and Xpert Ultra among the persons living with HIV was 100%.

The higher sensitivity of these assays may be attributed to the fact that we used a pooled sample which reportedly improve estimates of diagnostic accuracy.[278]

The observation in this study that 8/10 (80%) -9/10 (90%) of the trace positive Xpert Ultra cases were negative on TB-MBLA, MGIT culture and SSM-FM tests may suggest a possibility of over-diagnosis. However, since the current study was conducted in a high TB and HIV prevalence country[10,11,11,15] and enrolled participants who were screened positive for TB-like symptoms with no history of prior TB treatment, it is plausible that treating patients who were trace call was beneficial. However, future studies should investigate such 'trace call' results in countries with low TB and HIV prevalence, focusing on asymptomatic persons with a history of treated TB. These investigation will be needed to unravel the time taken for Xpert Ultra positive but culture or TB-MBLA negative patients to also become Xpert Ultra negative.

Prior studies indicate that Xpert Ultra has a higher pooled sensitivity and specificity among the smear-negative TB samples.[280,281] Our findings concur with this fact. We observed that TB-MBLA and Xpert Ultra tests identified more positive cases in the smear-negative participants further suggesting the potential advantage of these assays over the bacteriological tests. Of Importantly, TB-MBLA could have a comparative advantage in routine TB care over the Xpert Ultra because we found out that it has a higher positive predictive values: 92% versus 82 in entire study population, 89% versus 72% in those living with HIV, and 76 versus 54 in those with smear negative TB. The observation that TB-MBLA detected TB that was otherwise missed on SSM-FM (n=34), MGIT culture (n=10), and Xpert Ultra (n=3) indicates an added value for this novel tool. Since the measured bacteria load was below the average for the entire study population, it is plausible that TB-MBLA has potential to detect persons who present with low bacteria load. This is important to guide clinicians to initiate treatment early.

To date, smear microscopy is a rapid and cheap method to detect AFB, but this requires at least 5,000–10,000 bacilli cells per mL of sputum to yield a positive result.

It is, therefore, possible that samples with lower bacillary load would yield a negative result for TB. Nevertheless, smear microscopy is the most used TB test in the resource constrained settings, implying that a substantial number of persons often miss accurate diagnosis and treatment. This presents a huge challenge for the TB Control Programmes in poor countries, since persons with smear-negative pulmonary TB are about 10 to 12.5% as infectious as smear-positive cases and they considerably contribute to TB transmissions.[282,283] Liquid sputum culture has a high sensitivity and can detect as low as 10 viable bacilli per mL of sputum sample and may identify TB patients who are missed out on the smear microscopy. However, culture requires a longer time, extending up to 4 weeks before a positive result can be issued, and requires category three containment. This causes delays in making clinical decisions.[284]

3.11.0 Conclusion

Using sputa samples from the same presumptive TB patients, molecular-based assays were found to exhibit high sensitivity, specificity exceeding the minimum WHO Target Profile Product [285] in the general study population, in study population participants who were living with HIV, and those who were smear negative. In this study, the two molecular assays (TB-MBLA and Xpert Ultra) identified pTB positive cases that were missed on the SSM-FM and MGIT culture. However, TB-MBLA and MGIT culture concurred on the negativity of some cases that were positive on the Xpert Ultra, demonstrating similar specificity between these two assays. Taken together, the findings of this study show that TB-MBLA is sensitive and specific with higher positive predictive value and can potentially be used as a diagnostic and treatment monitoring tool.

3.12.0 Limitations of the current study

The main limitation of the study is the dependence on MGIT culture as the reference test for the molecular based assays. Culture is an imperfect surrogate and may have distorted the results of the diagnostic performance of the molecular tests. Secondly, our findings are further limited on the small sample size and being undertaken at only one site and fewer sampling points. While findings show that TB-MBLA has potential as both a diagnostic tool, in its current state, the lengthy processing protocol requires it to undergo technical modification to automate its operation, which will make it more rapid for wider applicability in areas with limited workforce.

4.0 Chapter Four

Accuracy of the Tuberculosis Molecular Bacterial Load Assay to monitor response to anti-tuberculosis therapy in comparison to the conventional standard of care sputum smear microscopy, Xpert MTB/RIF Ultra, and culture

4.1.0 Introduction

Despite the seemingly steady improvements over time, Tuberculosis (TB) treatment success rate remains lower especially among the persons living with HIV at 77% compared to 86% among the general TB population.[11,12] All TB patients should be monitored closely to detect interrupted treatment, slow treatment response or even failure, on the same day of sample collection. Effective monitoring of the patients' response to TB treatment is key for guiding clinicians in making timely treatment decisions to minimize TB transmissions, risks of developing drug resistance, dilapidated health, and deaths.[187] However, efforts to detect TB treatment failure or disruptions early into therapy are hindered on the lack of optimal and effective tools for monitoring TB treatment response.[286]

Accurate tests reflecting the changes of viable Mtb during TB therapy are crucial for making correct clinical decisions on the progress of the patients' treatment.[287,288] Patient clinical symptoms such as cough, sputum production, loss of weight, loss of appetite, night sweats and chest pain are associated with active TB disease and are used to clinically monitor response to TB drugs[289,290] However, some of the TB symptoms clear too fast while others persist even after treatment. For example, Hales et al., showed that 20% of the patients continued to present with productive cough after treatment completion, and that this cough was not associated with TB disease but other ailments.[291] Similarly, Bark et al., reported that 13% of the patients in present with cough at month-6 of treatment.[292] To-date, bacteriological investigations including liquid culture (MGIT) and stained smear microscopy (SSM) appear to be most suitable treatment monitoring tools based on some inherent positive attributes.

For example, liquid culture is sensitive and it can detect as low as 10 bacilli per millilitre of a clinical sample.[128] Additionally, liquid culture can be used to identify drug resistant Mtb and to isolate viable Mtb, which allows investigations that require viable organisms.[128] However, liquid culture is associated with several limitations including the following: i) this test requires expensive high containment laboratories and highly trained personnel to perform, ii) It is associated with a long turnaround time, and iii) loss of data due to contamination on other fast-growing bacteria that are available in the clinical samples.[269,270]

Consequently, stained sputum smear microscopy (SSM) has been used as the standard of care test for monitoring response to TB treatment until recently when the WHO recommended Xpert MTB/RIF Ultra as an add-on test.[293] Stained smear microscopy test is fast and affordable which explaining its wider applicability. [195] However, SSM is observer dependent, and it requires significant training and experience to competently interpret results.[196] Moreover, SSM test cannot differentiate between dead and viable Mtb, and between Mtbc and NTM.[199] Further, SSM is associated with low sensitivity, especially among the persons living with HIV and those with low mycobacterial loads such as those on TB treatment.[192–196]

Molecular-based assays may have potential to solve the limitations of the conventional diagnostic methods in monitoring treatment response. This is because Molecular-based assays are generally fast, reproducible; they are not compromised on contamination, yet they have been proven to be highly specific and sensitive.[275] For example, the Xpert Ultra is being adopted on the TB Control programmes as a standard of care for TB diagnosis because it is highly sensitive, and specific even in persons living with HIV and those with smear negative TB. Xpert Ultra cartridge derives its improved sensitivity from a bigger sample volume per reaction which doubles the amount of sample DNA that can be assessed. The additional multi copy gene probes and primers which allow identification of paucibacillary samples which are IS*6110*/IS*1081* positive but *rpoB* negative. [271,272]

Nevertheless, the Xpert Ultra cartridge targets Mtb-DNA as a proxy for Mtb in a clinical sample. It is commonly understood that DNA is a stable macro biomolecule that is not cleared rapidly after Mtb is killed, implying that there is a high chance of giving false positive results among patients who are being treated for treatment.[294] Because of its attributes, Xpert Ultra is now recommended as an initial TB diagnostic test and rifampicin resistance test in all adults and children with signs and symptoms of pulmonary TB.[178] However, as in much as the Xpert Ultra is highly sensitive in both smear negative and general population of TB patients, the new results category known as the 'trace call' positive is not clinically conclusive, since the associated rifampicin resistance test is usually indeterminate, hence requiring a second run.[178] In cases where the results of the first and second test runs are discordant, making a clinical decision becomes difficult, which potentially delays subsequent clinical assistance to patients.[175]

Much as molecular based assays are highly promising tools for improving TB diagnosis, there is no molecular test that has been validated for detection of viable Mtb in the clinical samples to reflect the progress of anti-TB treatment in routine a clinic setting. Monitoring TB treatment response is essential for understanding whether a patient is or not responding to anti-TB medications, and to identify poor responders with a higher risk of developing drug resistance or treatment failure in the early stages of the treatment.[295] Overall, effective monitoring of patients' response to TB treatment could lead to a better usage of anti-TB drugs, reduce treatment costs for patients, health facilities and the costs associated with the evaluation of new anti-TB antibiotics.[295] Tuberculosi*s*-Molecular bacterial load assay is a novel RNA based assay that detects and quantifies viable *Mycobacterium tuberculosis* (Mtb) bacilli in clinical samples.[273,274] In 2018, WHO noted that TB-MBLA has potential to replace SSM and culture as a tool for monitoring response to TB treatment.[275]

However, lack of evidence on the performance, suitability and comparative advantage over the SSM and culture limited immediate role out of TB-MBLA in TB clinical settings.[296] TB-MBLA targets the abundant Mtb16S-ribosomal RNA specifically and the test is highly sensitive with good positive predictive values.[276] Previously, TB-MBLA was evaluated as a treatment monitoring tool on samples that were already confirmed TB positive on other tests such as SSM which made it impossible to calculate specificity and predictive values.[296] TB-MBLA has the potential for routine monitoring of response to TB treatment but so far, there is not enough supporting data which limit its roll out into TB care settings. In this chapter, accuracy of the TB-MBLA to monitor response to anti-tuberculosis therapy in comparison to the conventional standard of care sputum smear microscopy, Xpert MTB/RIF Ultra, and culture was investigated among the ambulatory and hospitalised adult presumptive TB patients in Uganda, East Africa.

4.1.1 Management of TB in Uganda

After confirmation of the presence of Mtb, the patient is registered for anti-TB treatment and receive the 6-month standard anti-TB treatment. Treatment is administered on out-patient basis at the nearest health facility. This lessens the transport burden and increase retention in care. The nurses responsible for Directly Observed Therapy ensure that every dose that is given to the patient is swallowed and documented appropriately on the patient treatment card.

4.1.2 TB treatment response assessment in Uganda

Monitoring response to TB treatment is performed on examining sputum using SSM at months 2, 5 and 6 after the treatment initiation for the new active TB cases. For the re-treatment cases, monitoring treatment response is done at months 3, 5 and 7 after the treatment initiation. When at the end of intensive phase treatment, sputum SSM is positive, the intensive treatment phase is extended for 1 extra month. At the end of the extended intensive treatment phase, another sputum sample is collected and used for culture and drug susceptibility testing (DST).

If the DST result is suggestive of resistance to the first line TB medication, that patient is switched to the second line TB treatment regimen. If the DST is negative, that patient is switched to the continuation treatment phase. A sputum specimen for culture and DST is also collected when a patient has a smear positive at month -5 and -6 of anti-TB treatment to detect the possibility of MDR-TB and/or treatment failures.

4.1.3 Rationale for exploring the utility of TB-MBLA in clinical settings

Liquid culture is sensitive but using it for monitoring treatment response does not provide real time results for clinical management of TB treatment. Typically, culture test requires at least two months or more to obtain a phenotypic DST result after sample collection. Consequently, there is an urgent need for a rapid, sensitive, and specific, yet affordable test to monitor response to TB treatment. This would provide valuable information for early treatment clinical decision making. Presently, there is no proven molecular test for the detection of the decreasing load of viable Mtb signifying effective treatment. Monitoring response to TB treatment is key for understanding whether the patient is responding to TB medications or not. Monitoring response to TB treatment identifies patients who are poor responders with a higher risk of developing drug resistance and/or treatment failure.

4.1.4 Main objective

The overall objective of this Chapter was to investigate the utility of TB-MBLA for monitoring response to TB treatment in comparison with the standard of care methods in Health facility centres. Effective monitoring of response to TB treatment can lead to a better use of anti-TB drugs, lessen treatment costs burden for patients, Health facilities and Governments.

4.1.5 Specific aims

The specific objectives of this Chapter were to:

1) Evaluate the accuracy of the TB-MBLA for monitoring TB treatment response compared to bacteriological tests: sputum SSM and culture. Accuracy was measured as sensitivity, specificity, and predictive values.

2) Compare the performance of TB-MBLA for treatment response monitoring to Xpert Ultra test. Accuracy was measured as sensitivity, specificity, and predictive values.

3) Describe the changes in bacterial loads measured on the TB-MBLA and the cycle threshold cycles that are generated on the Xpert Ultra during the TB treatment course.

4.2.0 Methods

4.2.1 Ethics approvals

The project was approved on the Makerere University School of Medicine Research and Ethics committee [*REC REF No. 2006-017*] and Makerere University School of Biomedical Sciences Research and Ethics Committee [*REC REF No: SBS 529*] respectively. Before enrolment, participants consented to utilizing their biological samples and clinical data for this study. All the study related activities were conducted according to the guidelines outlined in the Good Clinical and Laboratory Practice manual.[297]

4.2.2 Participants and Study design

We conducted a longitudinal prospective study using spot sputa that were obtained from persons who were being investigated for pTB, and later confirmed positive and treated. This sub study was nested within a cohort of persons with pneumonia at Naguru Referral Hospital, Kampala (Uganda) for the period between 2019 and 2022.[298] Adult participants (≥18 years of age) who were coughing for at least 2 weeks with/without night sweats, with apparent weight loss; with/without symptoms of extrapulmonary tuberculosis were enrolled.

4.2.3 Study specimens during treatment monitoring phase

All participants were invited to the study site and requested to provide 3 spot sputa which were pooled and homogenized at week 0, and then at weeks 2, 8, 17 and 26 after the initiation of TB treatment. The homogenized sputa were aliquoted into 4 portions, each 1mL and assessed using Xpert Ultra, smear microscopy, Lowenstein-Jensen, and Mycobacteria Growth Indicator Tube culture tests. Aliquots for TB-MBLA were preserved in Guanine thiocyanate and stored at -20^{o}C until use. Xpert Ultra was used as the standard of care for tuberculosis and a basis for treatment initiation as described in Chapter Two.

4.2.4 Placement of participants on the Standard TB Treatment regimen

Participants who were positive on Xpert Ultra were initiated on the Standard TB treatment consisting of Rifampicin (R), Isoniazid (H), Pyrazinamide (Z) and Ethambutol (E) for the first two months and then continuation phase with R and H for additional four months (2HZRH +4HR) following the National Leprosy and Tuberculosis Programme guidelines. Treatment was administered on the respective Health facility Nurses.

4.2.5 Follow up visits during treatment monitoring phase

The National Leprosy and Tuberculosis Programme (NTLP) schedules for routine monitoring of response to TB treatment involves sampling the patients at month 2 to decide whether they can be switched to the continuation phase or extend their treatment for an extra month, and then at months 5 to check for treatment failure or success. Follow-up visits for the current study were selected to match the NTLP schedules, to reduce the burden of transport cost and to save the patients' time. An additional week 2 visit, which is not part of the routine NTLP monitoring visit was included to capture early information on patient response to treatment during the first 2 weeks of TB therapy.

4.2.5.1 Tracing of study participants during treatment monitoring phase

Contact details for each participant such as physical address and phone number(s) were captured into the respective patient file at enrolment. The study Nurse explained to the patient all the details of the subsequent study visit dates which were scheduled for the whole duration of 6 months of treatment. Participants were reminded through a phone call at one week, and then two days to their next visit. A scheduled home visit was only arranged for participants who missed a visit date and those who were not reachable through phone calls.

4.2.6 Clinical assessment of treatment response

Each patient was screened for clinical symptoms of TB at baseline visit and resolution of these clinical symptoms in subsequent follow up visits. Participants were given clear instructions on how to take medications on the study Nurse. At a fortnight interval, participants were contacted using a phone call and asked about their general health. During this phone call, participants were reminded to adhere to the pill ingestion instructions. They were also asked to mention any unusual condition that they might have experienced after starting TB treatment. In case an adverse reaction to treatment was reported, participants would be called back to the Hospital to get the necessary intervention and care outside the scheduled visit points.

4.2.6.1 Assessment of participants' adherence to treatment

During treatment follow up visits, adherence to treatment instructions was based on self-reporting approach. Participants were probed on the study Nurse to establish if they were adhering to the pill ingestion instructions. In addition, drug cards and pills counting were reviewed to reconcile with what the participants self-reported. Any discordance was further interrogated to ensure that participants successfully adhered to treatment.

4.2.6.2 Transport of participants during treatment monitoring

Participants used the local public transport means to get t o and from the health facilities. In cases where participants had no transport to attend the scheduled follow up visit, the study courier/driver in coordination with the designated study staff arranged for a home visits to transport those participants to the health facility. Participants were provided with N95 masks to mask up while in the public transport vehicles. This was important to minimize transmissions of TB.

4.2.6.3 Compensation of patients during monitoring response to treatment

A non-coercive compensation was approved on Makerere University School of Medicine Research and Ethics committee [REC REF No. 2006-017] and Makerere University School of Biomedical Sciences Research and Ethics Committee [REC REF No: SBS 529]. Subsequently, each participant was re-imbursed a sum of 25,000 Uganda shillings (equivalent of to £5) to compensate for their transport cost and lunch per visit point. When the participant required additional clinical investigation (s), they were helped to draw and deliver the required samples to the Hospital laboratory. Results from these tests were interpreted on the study doctor who would prescribe the necessary treatment or refer the patient accordingly. Blood transfusion and rehydration (with Dextrose 5 or normal saline) were freely offered for those participants who required these remedies. After specimen collection, all participants were supplied with 500 mL of Ribena juice at no cost.

4.2.6.4 Xpert MTB/RIF Ultra for monitoring treatment response

One mL of the homogenized sputa was mixed with 2 mL of the sample reagent buffer (Cepheid, Sunnyvale, CA, USA) and then assessed according to the Manufacturer's protocol. Results were automatically generated semi quantitatively on the Xpert Ultra platform. All samples were examined using the same Xpert Ultra machine as detailed in Chapters Two and Three. [299]

4.2.6.5 Stained smear microscopy for monitoring treatment response

One mL of each homogenized sputum sample was sedimented at 3000 g for 10 minutes and a smear (1-2cm diameter) was prepared from the sediment and stained using auramine-O-staining technique guidelines (Merck, Darmstadt, Germany). Stained sputum smears were examined under ultraviolet-based fluorescent microscope at X400 magnification as detailed in Chapters Two and Three.

4.2.6.6 Culture test for monitoring response to treatment

MGIT tubes were then inoculated with 500 µL of the decontaminated sample and incubated at 37°C for a maximum of 42 days. Solid culture on the LJ medium (LJ; Becton Dickinson) slants were inoculated with 1 mL of the re-suspended sediment and incubated at 37°C for a maximum of 56 days. TB-positive cultures were confirmed on the presence of acid-fast bacilli on Ziehl–Neelsen staining and presence of MPT64 antigen. Absence of acid-fast bacilli cording, and growth on blood agar was recorded as contamination. All results were reported according to the International Union Against Tuberculosis and Lung Disease guidelines as described in Chapters Two and Three.[300]

4.2.6.7 TB-MBLA for monitoring response to TB treatment

Total Mtb rRNA was extracted using a method described elsewhere [208,301] and then tested at 0.1 dilution. TB-MBLA was performed basing on the duplex reverse transcriptase-real time qPCR principle targeting both *Mycobacteria tuberculosis complex* and the extraction control on a RotorGene 5plex platform (Qiagen, Manchester, UK). Primers and TaqMan dual-labelled probes were manufactured on MWG Eurofins, Germany. The qPCR cycling conditions were as reported on Honeyborne *et al*., [301] Quantification cycles (Cq) readouts were converted to bacterial load using a standard curve that was customized for the site's qPCR platform and recorded as estimated colony forming units per mL (eCFU per mL).[208]

4.3.0 Results

4.3.1 Participant demographic characteristics

The 126 participants that were enrolled into the treatment response monitoring follow up arm of the current study were young adults with median age of 33 years (IQR: 26-43), of whom 80(63.5%) were male gender. Out of the 126 participants, 112(89%) reported having experienced evening fevers, 125 (99%) reported unexplained cough for at least two weeks before coming to the hospital, and 106 (84.1%) reported unexplained weight loss. However, body mass index of 18.8 kg/m^2 was within the normal range. Thirty-four (26.%) of the 126 participants were living with HIV with median (IQR3) CD4 cells count 222 cells/µl (63-349) (*Table 4.1.0*).

Table 4.1.0: Demographic data at baseline of the study participants

	Quantitative data; Median (IQR) **Qualitative data; n/N (%)**
Characteristics	**Participants with pTB (N= 126)[a]**
Age (Years)	33(26-43)
Male gender	80(63.5)
Evening fevers	112 (88.9)
Weight loss>5%	106 (84.1)
Cough >2weeks	125 (99.2)
Haemoptysis	35 (27.8)
Heart rate (%)	104 (91-113)
Respiratory rate	24 (20-28)
Oxygen saturation	96 (94-97)
Living with HIV/AIDS	34 (26.4)
Antiretroviral therapy use	10 (7.9)
CD4(cells/µL) [b]	222 (62.5-349)
Body mass index (kg/m^2)	18.8 (17.7-21.0)
Body temperature (^oC)	36.6 (36.4-37)

[a] N=126 excludes (n=3) participants whose sputum MGIT was contaminated. CD4 cell counts were measured for only those participants who were HIV positive. Weight loss, evening fevers and recurrent coughs were self-reported on the participants

4.3.2 Participants follow-up

At baseline, Xpert MTB/RIF Ultra (Xpert Ultra) test detected Mtb in samples from129 participants and these participants were included into the treatment response monitoring arm. Three (2.3%) of these 129 participants had contaminated sputum MGIT culture test results and were excluded from the final statistical analyses. Along the treatment course, some participants declined to provide the sputum sample, some defaulted on treatment, some died, and some were transferred out of the study clinic to other TB care facilities. Therefore, 121/126 (96%) participants provided samples at week 2, 112/126 (88.9%) at month 2, 105/126 (84.9%) at month- 4, and 95/126 (75.4%) at the end of treatment month -6. During the treatment response monitoring course, 7/126 (5.6%) declined to give a sample at some point, 4/126 (3.2%) defaulted on treatment, 7 (5.6%) died, 8/126 (6.3%) opted to be transferred to other TB clinics, and 5/126 (4%) were lost to follow-up. (*Figure 4.1.0*).

Overall, there was a noticeable reduction in the positivity rate for all the TB tests during the treatment follow-up relative to the baseline number of pTB positive cases, but this occurred remarkably slower for the Xpert Ultra test. For instance, at week- 8 into treatment, the number of positive cases reduced from 70 to 19 for TB-MBLA test, from 81 to 12 for MGIT, from 105 to 83 for Xpert Ultra, and from 64 to 13 for SSM-FM test, respectively. At the end of TB treatment, there was no positive result for TB-MBLA, and sputum MGIT. On the contrary, 31/126 (24.6%) and 6 (4.8%) were still positive for the Xpert Ultra and SSM-FM respectively (*Figure 4.1.0*).

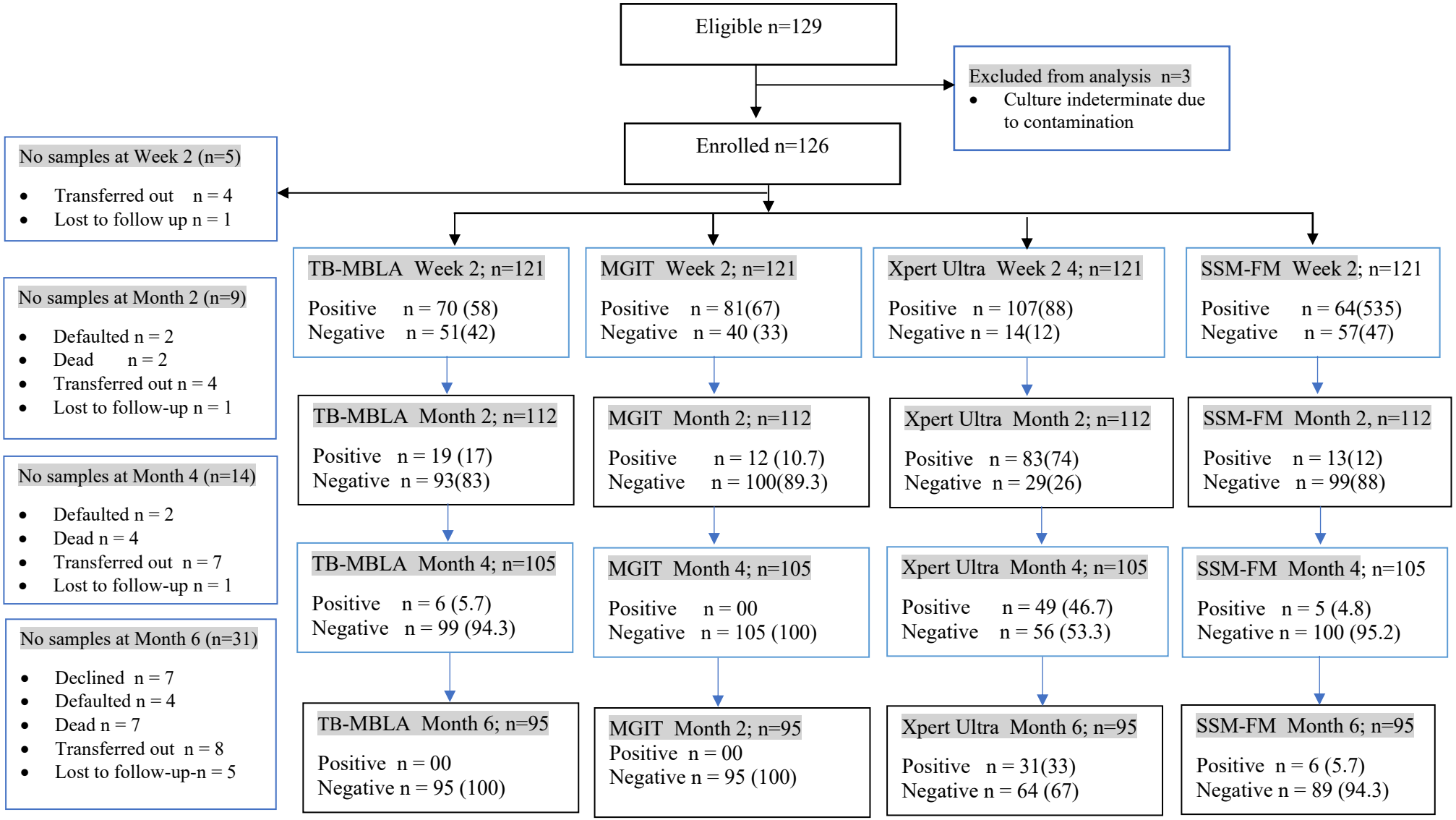


Figure 4.1.0: Participant enrolment and sputum collection during treatment follow-up. TB-MBLA; Tuberculosis-Molecular Bacterial Load Assay, Xpert Ultra was used as the standard of care, and it was used as the basis for treatment initialization. Participants were treated with the first line standard TB regimen and subsequently followed up at week 2, and months 2, 4 and 6. Quantitative data is n/N(5).

4.3.2 Changes in bacteria load over the treatment course

Decrease in the positivity rates was corroborated on the reduction in the mycobacteria load. For example, relative to the bacteria load at baseline, the mean bacteria load measured on TB-MBLA reduced on 1.4log_{10} eCFU per mL, 2.3log_{10} eCFU per mL, 2.6log_{10} eCFU per mL to no detection at weeks 2, months- 2,4, and 6, respectively. Similar trend was observed for the time-to- positivity of MGIT culture test grade. For the Xpert Ultra Cq values remained constant at months -2, 4 and 6, this trend was like that of the SSM-FM grades (*Table 4.2.0*)

Table 4.2.0: Changes in bacteria load over the treatment course

Tests	Categorical data is n/N(%) Quantitative data are median (IQR)			
	Week 2	**Month 2**	**Month 4**	**Month 6**
TB-MBLA[a] positivity rate	70/121 (57·9)	19/112 (16·9)	6/105 (5·7)	0/95 (0)
TB-MBLA quantification cycle values	26 (23-29)	28 (26-30)	30 (29·6-30·4)	36.2 (35·1-36·1)
TB-MBLA, bacteria load (Log_{10} eCFU/mL)	3·5 (2·6-4·4)	2·6 (2·2-3·3)	2·3 (2-2·3)	-------
Xpert Ultra positivity results	110/121 (91·0)	84/112 (75·0)	49/105 (46·7)	31/95 (32·6)
Xpert Ultra Cq values	20·2 (18·6-23)	25 (21-28)	26·2 (23-30)	26 (21-30)
SSM-FM positivity rate	65/121 (53·7)	13/112 (11·6)	6/105 (5·7)	6/95 (6·3)
SSM-FM, median grade	2 (1·5-3)	2 (2-2)	1 (1-2)	1 (1-2)
MGIT[b] culture positivity rate	81/121 (66·9)	12/112 (10·7)	0/105 (0)	0/95 (0)
MGIT culture TTP (days)	13 (11-16)	22.5 (12-27)	-------	------

TB-MBLA: Tuberculosis Molecular Bacteria Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM-FM: Stained Sputum Smear- Fluorescent Microscopy, MGIT: Mycobacteria Growth Indicator Tube, TTP: Time-to-positivity, Cq=Quantification cycle value. [a] TB-MBLA was the index test. [b] Sputum MGIT culture was standard reference test for other tests. Quantitative data is n/N (%)

4.3.3 Concordant test results during treatment follow-up

At week 2 of treatment samples were collected from 121 participants of whom, 70 (57·8%) were positive and 51 (42·2%) were negative on TB-MBLA. We also noted that 36/51 (70·6%) samples were negative on TB-MBLA and MGIT culture, and 66/70 (94.3%) were positive on both tests. The overall agreement between TB-MBLA and MGIT was 84·3% (Kappa=0·67). We noted that both TB-MBLA and SSM-FM were negative in 44/51(86·3%) samples, and positive in 13/70 (18·6%). Like for MGIT culture, the overall concordance between TB-MBLA and SSM-FM tests was substantial at 83·7%; kappa=0·67. We observed that 14/51 (27·5%) samples were negative on both TB-MBLA and Xpert Ultra., and 70/70 (100%) were positive on both TB-MBLA and Xpert Ultra tests. (*Table 4.3.0*).

At month 2, sputum samples were collected from 112 participants of whom, TB-MBLA tested positive in 19 (17%), and 93(83%) were negative. TB-MBLA and MGIT culture were negative in 89/93 (95·7%) samples, and positive in 8/19 (42·1%) samples. On overall, concordance between TB-MBLA and MGIT culture was 86·6%; Kappa=0·44). We noted that both TB-MBLA and SSM-FM were negative in 86/93 (92·5%), and positive in 6/19 (31·6%). On the overall, TB-MBLA and SSM-FM concurred at 82·14%; kappa=0·28. We also observed that 14/93(15.1%) were negative on both TB-MBLA and on Xpert Ultra, and that 15/19 (78·9%) samples were positive on both TB-MBLA and Xpert Ultras. (*Table 4.3.0*).

At month 4 into TB treatment, 105 participants provided sputum samples of which, 99 samples of the 105 were negative on TB-MBLA, MGIT culture and SSM-FM tests, respectively. The molecular assays, (TB-MBLA and Xpert Ultra) were negative for 55 out of the 105 sputum samples.*(Table 4.3.0)*. At the end of treatment, samples were collected from 95 participants and all the 95 samples were negative on both TB-MBLA and MGT culture; 89/95 (93·7%) samples were negative on both TB-MBLA and SSM-FM, and 64/95 (67·4%) samples were negative on both TB-MBLA and Xpert Ultra. *(Table 4.3.0)*

Table 4.3.0: Discordant/concordant results on the same sample during treatment monitoring

		Standard of care tests					
		MGIT		Xpert Ultra		SSM-FM	
Visit point	**Index test (TB-MBLA)**	Positive n/N(%)	Negative n/N(%)	Positive n/N(%)	Negative n/N(%)	Positive n/N(%)	Negative n/N(%)
Week2 (n=121)	Positive (n=70)	66(94.3)	4(5·7)	70(100)	-------	57(81·4)	13(18·6)
	Negative (n=51)	36(70·6)	15(29.4)	37(72·6)	14(21·4)	7(13·7)	44(86·3)
Month2 (n=112)	Positive (19)	8(42·1)	11(57·9)	15(78·9)	4(21·1)	6(31·6)	13(68·4)
	Negative (93)	4(4·3)	89(95·7)	69(75)	24(25)	7(7·5)	86(92·5)
Month 4 (n=105)	Positive (6)	-------	6(100)	5(83·3)	1(16·3)	1(16·3)	5(83·3)
	Negative (99)	-------	99(100)	44(44·)	55(55·6)	------	99(100)
Month 6 (n=95)	Positive (00)	-------	------	-------	-------	-------	-------
	Negative (n=95)	-------	95(100)	31(32·6)	64(67·4)	6(6·6)	93(93·4)

TB-MBLA: Tuberculosis Molecular Bacteria Load assay, MGIT: Mycobacteria Growth Indicator Tube, Xpert Ultra: Xpert MTB/RIF ultra, SSM-FM: Stained Sputum Fluorescent Microscopy

4.3.3 Positive Xpert MTB/RIF Ultra cases at the end of treatment [n=31]

At the end of treatment, 31/95 (32·6%) participants remained positive on Xpert Ultra. Semi-quantitative grading varied among the 31 results that were positive on Xpert Ultra varied. Fifteen out of the 31 (48·4%) were 'Low', 10 out of the 31(32%) were 'Very low', 5 out of the 31 (16·1%) were 'Trace calls' and 1 out of the 31(3·2%) was graded 'median. Three months after the end of treatment, 3 (9·7%) of the 31 Xpert Ultra positive participants remained positive but with no TB-like clinical symptoms, 7(22·6%) had converted to negative, and 21(67·7%) did not return to the clinic visit since they insisted that they were clinically well. (*Table 4.3.0*)

TABLE 4.3.1: Comparison of 'Trace call' results

	Xpert Ultra		TB-MBLA		SSM-FM		MGIT	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Week0	10	..	2/10	8/10	1/10	9/10	2/10	8/10
Week2	5/9	4/9	..	9/9	1/9	8/9	..	9/9
Week8	3/9	6/9	1/9	8/9	..	9/9	..	9/9
Week17	2/9	7/9	..	9/9	1/9	8/9	..	9/9
Week26	2/6	4/6	..	6/6	..	6/6	..	6/6

Seven (70%) of these ten participants were co-infected with HIV and all were treated for tuberculosis without repeating the test and all had indeterminate rifampicin results. At week2, one (10%) failed to expectorate. At weeks 8 one participant had died while at week 26, one more participant died, one failed to expectorate; one was transferred out on request. Importantly, only one participant was positive on Xpert Ultra throughout the treatment. All other tests agreed on the negativity of the results that were otherwise trace positive with Xpert Ultra.

4.3.4 Relationship between treatment outcomes at 8 and 26 weeks

At 8 weeks into treatment course, samples were collected from 112 participants, of whom 20 (16.9%) were positive on TB-MBLA with median Cq value 28 and mean bacteria load 2.7 ± 0.6$\log_{10}$ eCFU per mL. Six (30%) out of the 20 participants who were positive on TB-MBLA were also positive on SSM-FM. Mean bacterial load 2.64 ± 0.8$\log_{10}$ eCFU for participants who were positive on TB-MBLA, and SSM-FM was 0.16$\log_{10}$ eCFU per mL which was less than 2.8±0.6 $\log_{10}$ eCFU per mL for those who were TB-MBLA positive but smear negative. Eighty-four (74.3%) of the 112 participants were positive on Xpert Ultra (with median Ct value 22.7).

Thirteen (15.5%) of the 84 participants who were positive with Xpert Ultra were also positive on SMM-FM. The 13 participants who were positive on SSM-FM at week 8 received one extra month of the intensive phase treatment before switching them to the continuation treatment phase. Despite this discordance, treatment success rate was comparable. A phone call follow-up at three months after the end of treatment revealed that none of the participants with successful treatment outcome had TB-like symptoms.

TABLE 4.4.0: Treatment outcomes at 6 months among positive cases at 8 weeks

	TB-MBLA			**Xpert Ultra**		
	Overall (N =20)	**Treated for extra month (n=6 [30%])**	**Not treated for extra month (n=14 [70%])**	**Overall (N = 84)**	**Treated for extra month (n=13 [15.5%])**	**Not treated for extra month (n=71 [84.5%])**
Successful	18(90)	6(100)	12(85·7)	70(83·3)	9(69·2)	61(86)
Failure	None	None	None	1(1·2)	1(7·7)	None
Mortality	1(5)	None	1(7·1)	2(2·4)	1(7·7)	1(1·4)
Lost to follow-up	None	None	None	4(4·8)	1(1·7)	3(4·2)
Others	1(5)	None	1(7·1)	7(8·3)	1(1·7)	6(8·4)

Data are n/N (%); Success refers to a negative sputum MGIT culture result with no clinical symptoms, Failure refers to positive sputum MGIT culture with/out clinical TB symptoms. Others include those who declined/failed to give a sputum sample, and those who opted out of the study. When the participants who belonged to 'others' and those who were lost in follow-up were considered, success rate was 100% for TB-MBLA-with treatment extension, 92% for TB-MBLA-without treatment extension; and 92% for Xpert Ultra-with treatment extension, and 98% for Xpert Ultra-without treatment extension.

4.3.5 Measures of diagnostic accuracy during treatment monitoring

Tests' diagnostic performance was measured against sputum MGIT culture test as a standard reference test. At week 2, samples were obtained from 121 participants. TB-MBLA had a sensitivity (95%CI) of 87% (77-93). Although sensitivity at 99% (93-100) was higher for Xpert Ultra, its specificity at 33% (19-50) was lower when compared to 92% (79-98) for TB-MBLA and SSM-FM tests. Positive predictive value 96% (87-99) for TB-MBLA was comparable to 95% (87-99) for SSM-FM and were both higher than 76% (67-84) for Xpert Ultra. (*Table 4.5.0*) Compared with diagnostic accuracy estimates at week 2, sensitivity dropped from 87% (77-93) to 67% (35-90) at month 2 for TB-MBLA, from 74% (64-83) to 50%(21-79) for SSM-FM, and from 98.8% (93-100) to 92% (62-100) for Xpert Ultra test. Similarly, positive predictive value dropped from 96% (87-99) to 40% (19-64) for TB-MBLA, from 95% (87-99) to 42% (19-75) for SSM-FM, and from 76% (67-84) to 13% (7-22) for Xpert Ultra test.(*Table 4.5.0*)

At months 4, it was impractical to calculate the sensitivity since the standard reference test (sputum MGIT culture) tested negative for all the samples much as TB-MBLA, Xpert Ultra and SSM-FM were positive in 6/105 (5·7%), 48/105 (45·7%) and 7/105 (6·7%) samples, respectively. However, specificity (95%CI) could be calculated, and it was 94·4% (90-99) for TB-MBLA, 55% (20-64) for Xpert Ultra, and 97·5% (89-99) for SSM-FM.(*Table 4.5.0*)

At month 6, the standard reference test (MGIT culture) was negative for all the 95 samples which made it impossible to calculate the sensitivity estimate of the index test and the standard of care tests. Specificity (95%CI) could only be calculated for Xpert Ultra and SSM-FM and these were 67·4% and 95%, respectively. (*Table 4.5.0*)

TABLE 4.5.0: Measures of diagnostic accuracy during treatment

	TB-MBLA[a]				Xpert Ultra				SSM-FM			
	TP=66	**TN=36**	**FP=4**	**FN=15**	**TP=80**	**TN=13**	**FP=27**	**FN=1**	**TP=61**	**TN=37**	**FP=3**	**FN=20**
Week2 (n=121)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV(%) (95%CI)	NPV(%) (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV(%) (95%CI)	NPV(%) (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV(%) (95% CI)	NPV(%) (95%CI)
	87 (77-93)	92 (79-98)	96 (87-99)	77 (62-88)	98·8 (93-100)	33 (19-50)	76 (67-84)	93 (66-100)	74 (64-83)	92 (79-98)	95 (87-99)	63 (49-76)
	TP=8	**TN=89**	**FP=11**	**FN=4**	**TP=11**	**TN=28**	**FP=72**	**FN=1**	**TP=6**	**TN=93**	**FP=7**	**FN=6**
Month 2 (n=112)	Sn(% (95%CI)	Sp(%) (95%CI)	PPV(%) (95%CI)	NPV(%) (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV% (95%CI)	NPV% (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV(%) (95%CI)	NPV(%) (95%CI)
	67 (35-90)	88 (80-94)	40 (19-64)	96 (89-99)	92 (62-100)	26 (18-36)	13 (7-22)	96 (81-100)	50 (21-79)	93 (86-97)	46 (19-75)	94 (87-98)
	TP=0	**TN=101**	**FP=4**	**FN=0**	**TP=0**	**TN=57**	**FP=48**	**FN=0**	**TP=0**	**TN=100**	**FP=5**	**FN=0**
Month 4 (n=105)	Sn(%) (95%CI)	Sp(%) (95%CI)	PPV(%) (95%CI)	NPV(%) (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV% (95%CI)	NPV% (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV%) (95%CI)	NPV%) (95%CI)
	--------	96.2	-------	-------	------	54.3	--------	--------	-------	95.2	--------	-------
	TP=0	**TN=95**	**FP=0**	**FN=0**	**TP=0**	**TN=64**	**FP=31**	**FN=0**	**TP=0**	**TN=89**	**FP=6**	**FN=0**
Month 6 (n=95)	Sn(% (95%CI)	Sp(%) (95%CI)	PPV(%) (95%CI)	NPV(%) (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI)	PPV% (95%CI)	NPV% (95%CI)	Sn(%) (95%CI)	Sp(%) (95%CI	PPV%) (95%CI)	NPV%) (95%CI)
	--------	100	--------	-------	-------	67.4	--------	--------	-------	95	-------	------

TB-MBLA: Tuberculosis Molecular Bacteria Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM-FM: Stained sputum Smear-Fluorescent microscopy, TP: True positive cases, TN: True negative cases, FP: False positive cases, FN: False negative results. Sn: sensitivity, Sp: Specificity, PPV: Positive Predictive values, NPV: Negative predictive value. [a] TB-MBLA was the Index test which was tested against sputum MGIT culture

4.4.0 Model-Based description of Xpert Ultra and TB-MBLA results during TB treatment monitoring

It was observed in Chapter 4.1.0 that the positivity rate for the Xpert Ultra dropped slowly at 74% and 31/95 (33%) on 2 and 6 months of TB treatment, respectively. For that reason, a Model-Based approach to explain the delayed conversion to negative on the Xpert Ultra assay was sought.

4.4.1 Description of the model

To obtain a quantitative measure of DNA and 16S rRNA elimination rate during treatment with standard TB regimen, time variation of the quantification cycles (Cq) measured on Xpert Ultra test and TB-MBLA was modelled on an exponential saturation function.[302] When the concentration of the target nucleic acid molecules was immeasurable, a Cq value of 40 was used in the analysis. Consequently, the Cq values traced an increasing trajectory over the time between the pre-treatment bacterial load and the vanishing concentration of the target nucleic acid molecule. This time dependence phenomenon was modelled on the function below:

$$\mathrm{Cq}(t) = \left(40 - \mathrm{Cq}_0\right)(1 - \exp(-\mathrm{Rt})) + \mathrm{Cq}_0 \text{.....equation (1.1.1)}$$

Cq_0; is the quantification cycle value that corresponds to a pre-treatment bacterial load. Cq_{40}; is the quantification cycle value that corresponds to the immeasurable bacterial load during TB treatment.

The rate of increase of the quantification cycle (Cq) is described on a single exponential rate constant, R, which has a dimension of 1/time. Other functions exhibiting similar saturation behaviour may have been chosen, but their precise forms, however, are of little importance for the purpose of this analysis. To find a model that describes the time dependence of the Cq values more accurately, significantly more time-points would be required with more frequent sampling at the initial period of the treatment. Furthermore, it is expected to be determined on multiple parameters, because not only the rate of bacterial killing but also the degradation of

the free bacterial DNA or 16S rRNA would need to be considered. The former may depend upon the combination and dosage of drugs, and it is likely to change during treatment with the metabolic state of the bacteria.

The function in equation 1.1.1 was chosen primarily because it is simple, and that only one parameter, R, needs to be fitted in the experimental data. As the Cq value is directly proportional to the negative logarithm of the bacterial load, log *c*, the exponential increase of Cq corresponds to an exponential decrease of log *c*, *i.e.*, the order of magnitude of the number of bacteria. This implies that log *c* decreases at a constant specific rate, that is, on a constant percentage of the instantaneous value of log *c.* over a unit time. Instead of the exponential rate constant, *R*, the Cq(t) function in equation 1.1.1 may be expressed in terms of a more intuitive time parameter, $T_{99\%}$, corresponding to the time required to reach 99% of the total increase of Cq, as below:

$$\mathrm{Cq}(t) = \left(40 - \mathrm{Cq}_0\right)\left(1 - exp\left(\frac{ln(0.01)}{T_{99\%}}t\right)\right) + \mathrm{Cq}_0 \quad \text{.....equation(1.1.2)}$$

It is easy to show that after the $T_{99\%}$ elimination time, $\log(c(T_{99\%})) = \log(c_0)/100$ or equivalently, $c(T_{99\%}) = (c_0)^{1/100}$. In other words, $T_{99\%}$ corresponds to the timepoint when the order of magnitude of the initial mycobacterial load is decreased on a factor of 100. For instance, if the pre-treatment mycobacterial load were 10^8 eCFU per mL, it would decrease to $10^{0.08}$eCFU per mL $\simeq$ 1.2eCFU per mL on the $T_{99\%}$ timepoint.

4.4.2 Findings of the model-based analysis

On fitting the exponential saturation curve given in equation 1.1.2 to each set of the measurements for each patient, separately, the $T_{99\%}$ elimination time constants can be determined. The obtained $T_{99\%}$ parameters are shown in *Figure 4.2.1* for 94 patients corresponding to measurements of CT values *via* TB-MBLA and 5 different genes used in Xpert Ultra.

Sorting $T_{99\%}$ values in increasing order within each set of measurements illustrated patient-to-patient variability more clearly and the differences between the two methods and between genes in Xpert Ultra. Figure 4.2.1 shows that lower $T_{99\%}$ elimination time constants were obtained for most patients from TB-MBLA measurements when compared to Xpert Ultra. As the ratio of live to dead bacteria in the sample is independent of the measurement technique, these results indicate that free bacterial 16S rRNA degrades more rapidly than free bacterial DNA. Moreover, when the 5 different genes used in Xpert Ultra are compared we noted that the $T_{99\%}$ elimination time constants for the IS1081gene were higher than those corresponding to rpoB1-4. This might be explained on preferential cleavage of the free bacterial DNA within the rpoB1-4 genes. (Figure 4.5.0). The reason behind these unexpected findings warrants further investigation in the future.

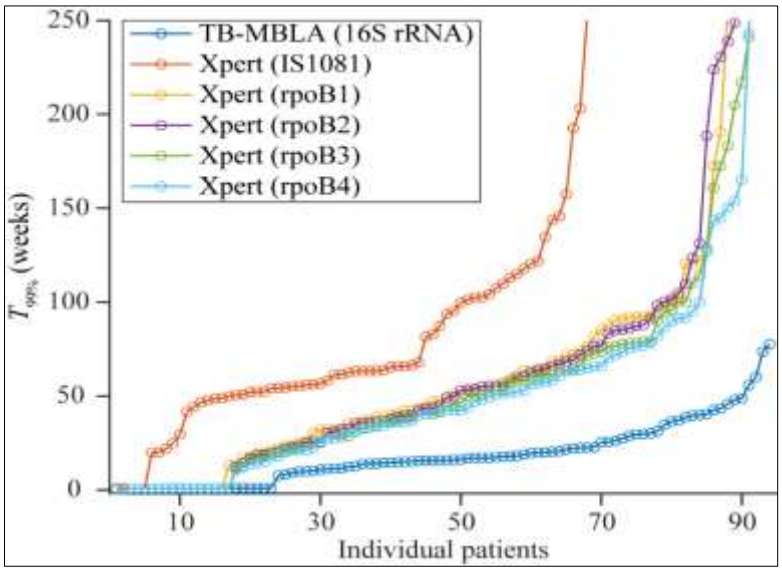


Figure 4.2.1: Elimination time constants of the nucleic acid molecules (DNA and 16S rRNA) in each individual sputum sample over the period of treatment monitoring.

4.4.3 Nucleic acid elimination rates from TB-MBLA and the 5 genes in Xpert Ultra

To compare the elimination rates obtained from TB-MBLA and those of the 5 genes in Xpert Ultra, the median $T_{99\%}$ values of the patients were calculated in each case. When the Cq values did not follow the expected increasing trend over time, the curve described on equation (1.1.2) did not fit the experimental data well, and low correlation coefficients were obtained.

When the R^2 value of a fit was lower than 0.5, the corresponding $T_{99\%}$ values were disregarded for the calculation of the medians, owing to their large uncertainty. The median $T_{99\%}$ elimination time constants are summarised in *Table 4.2.1*. To calculate the overall central value for Xpert Ultra, the median was calculated from all the retained $T_{99\%}$ parameters including all five genes.

Table 4.5.1: Elimination rates for 16S rRNA and DNA

Method	***T*99% (weeks)**
TB-MBLA	15.7
Xpert Ultra – gene IS1081	63.2
Xpert Ultra – gene rpoB1	48.4
Xpert Ultra – gene rpoB2	49.3
Xpert Ultra – gene rpoB3	46.0
Xpert Ultra – gene rpoB4	42.1
Xpert Ultra (overall)	51.6

4.4.4 Expected time dependence of the CT values

To illustrate the expected time dependence of the CT values, the $T_{99\%}$ elimination time constants given in *Table 1* may be substituted into equation (1.1.2) to plot CT as a function of time. This is shown in figure 4.2.2, where CT_0 was taken to be 19, which is close to the median of the measured CT_0 values in each case. In *Figure 4.2.2(B),* vertical dashed lines indicate the respective $T_{99\%}$ elimination times.

It is clear from the values in *Table 4.2.1* and *Figure 4.2.2* that the Ct values of the 16S rRNA-based TB-MBLA increased 3.3 times more rapidly than those of the DNA based Xpert Ultra. That is, a DNA-based PCR measurement yields a positive result for 3.3 times longer i.e., in the case of the Xpert Ultra- than TB-MBLA for the same sample, regardless of the cut-off value considered as limit of detection.

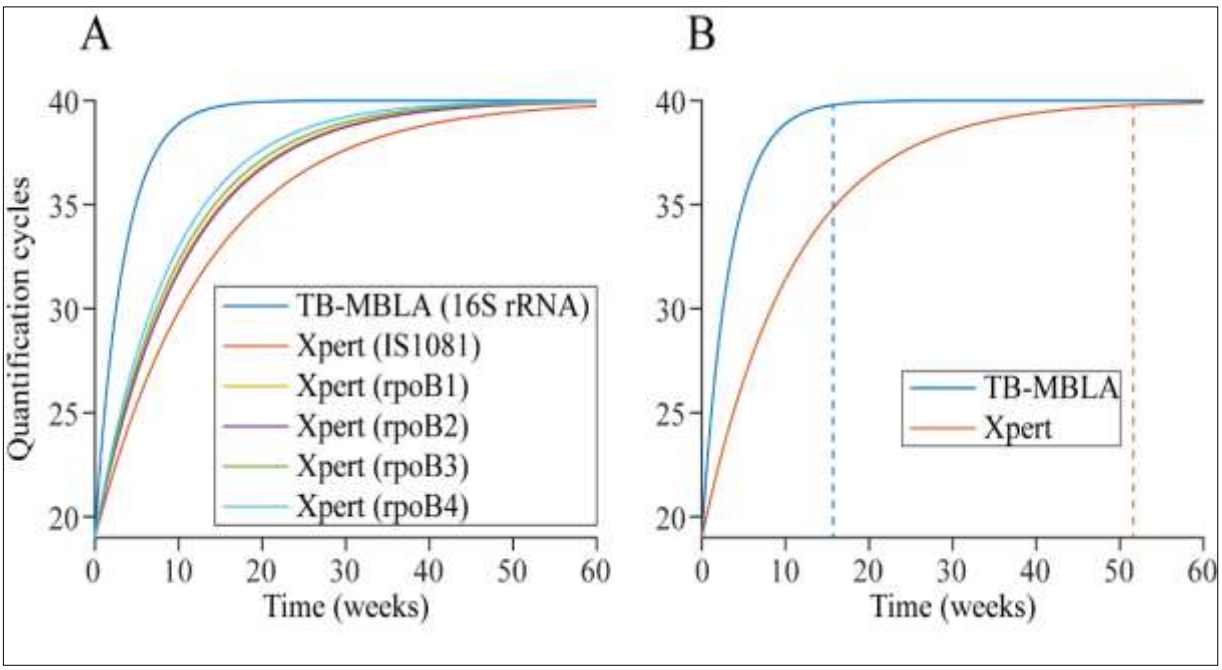


Figure 4.1.2: Changes of Cq vlues over time. In figure A, curvves show changes in the Cq values as a results of changes in 16SrRNA, and the individual five genes that are amplified on the Xpert Ultra. In figure B, a blue curve shows changes in Cq values as levels of 16srRNA changes during treatment. Red line indicates chsnges in median Cq value of the five genes that were amplified The Cq values of the 16S rRNA (TB-MBLA) increase approximately 3.3 times more rapidly than those of the mycobacterial DNA (Xpert Ultra). In figure 4.4.4 (B), vertical dashed lines show the respective $T_{99\%}$ elimination times.

4.5.0 Discussion

The present findings show that TB-MBLA mirrors sputum MGIT culture during treatment response monitoring. These results indicate the suitability of TB-MBLA as an additional or alternative tool for monitoring response to TB treatment. Our findings further reveal that 2 months sputum smear microscopy (SSM-FM) test is insufficient to inform decision to extend the intensive treatment phase as already indicated elsewehere.[303]

Our results confirm the unsuitability of the Xpert Ultra test as a tool for monitoring response to TB drugs and support the use of TB-MBLA for monitoring TB treatment response. From this study, it can be concluded that Xpert Ultra is a highly sensitive assay for the first line diagnosis of tuberculosis but less sensitive test for monitoring treatment response.[303] The high specificity of TB-MBLA during treatment indicates that the test would rarely yield a positive result for persons who have successfully responded to TB treatment. This attribute gives TB-MBLA a comparative advantage over the Xpert Ultra test as a tool for monitoring response to TB treatment. When Xpert Ultra was used for monitoring response to treatment, specificity significantly reduced indicating the possibility of yielding a positive results for persons who could have successfully responded to TB drugs.

Technically, specificity for Xpert Ultra in our study could have been underestimated on sputum MGIT culture which was used as the reference test. However, using sputum MGIT culture has an advantage because, it is the most stringent definition of a true positive TB disease especially in a setting where TB is highly prevalent.[114] Using a less stringent reference test/algorithm could have yielded a higher specificity for Xpert Ultra. [304] but the definition of a composite reference test has never been standardised for adult patients that are presumed to have pTB. In this study, we used MGIT culture because of the following reasons: i) our study population was selected from patients that were already confirmed to have TB-like symptoms, ii) the study was conducted in a country where TB is highly prevalent, iii) we used sputum which is the

accepted superior TB sample, yet we had observed that our study participants had a high average mycobacteria load (4.8log estimated colony forming units per mL of sputum sample). Sputum smear microscopy is widely used for pre-treatment diagnosis of TB and as the standard for monitoring response to treatment. The wide use of smear is perhaps due to its affordability and ease of use, but its utility as a treatment response marker is compromised on low sensitivity and inability to distinguish the viable from dead bacilli making it unsuitable for monitoring response to TB drugs and to inform clinical decisions during treatment monitoing.[305]

Our study further shows that the Xpert Ultra quantification cycles change slowly, reflecting slow degradation of DNA from bacilli that may have been killed on therapy. This renders the Xpert Ultra less specific as patients progress on treatment. Before treatment is initiated, Xpert Ultra appears to reflect the DNA from viable bacilli. This is demonstrated in baseline Xpert Ultra and TB-MBLA quantification cycles which were similar and strongly correlated with time-to positivity of the sputum MGIT culture.

While all the 95 participants at 6 months of treatment were negative with TB-MBLA and sputum MGIT culture, 31/95 (33%) of them were still positive with Xpert Ultra. We noted that the rate of change of the quantification cycles per week for the Xpert Ultra test during TB treatment increased 3.3 times less than that of the TB-MBLA. This finding indicated a delayed clearance of the Mtb-DNA compared to Mtb-16SrRNA. This observation may explain why 33% of the 95 patients at month 6 of treatment remained positive for Xpert Ultra but negative for TB-MBLA and MGIT culture. These findings corroborate the findings of Friedrich et al. (2013), which showed that 27% of the study patients were still Xpert MTB/RIF positive at 6 months, and concluded that Xpert MTB/RIF was unsuitable tool for monitoring response to TB treatment.[306]

Friedrich et al. (2013) study further demonstrated that positivity rate of Xpert MTB/RIF assay declined in a linear fashion as opposed to the non-linear (biphasic) form of resolution which was shown on smear, and culturea.[307] But compared to the 2013 Friedrich et al. study, a slightly higher positivity rate of the Xpert Ultra test was noted in the current study at the end of TB treatment. This difference may be attributed to the enhanced sensitivity of the Xpert Ultra cartridge compared to the older version of Xpert MTB/RIF assay. Indeed, 5 of the 31 Xpert Ultra positive results at the end of treatment were 'trace calls' positive results.

Accurate early markers of poor TB prognosis are still needed to minimize potential over treatment. Overtime, a 2-month sputum smear result has had a positive impact on informing TB treatment outcome but with several challenges, which limit its impact on treatment success rate.[308] The present finding that at two months, SSM-FM missed 14 participants who were otherwise positive with T B-MBLA further points to its low sensitivity and inadequacy to inform extension of the intensive phase treatment.

In the year 2018, the World Health Organisation cited TB-MBLA as a potential replacement of the stained smear test as a tool for monitoring TB treatment response.[275] The current study findings confirm this early promise. TB-MBLA is a fully quantitative test that measures viable Mtb in clinical samples in a shorter time. Whether the use of 2-month TB-MBLA to monitor treatment response would reveal the actual mycobacterial load present in the patient's sample needs to be investigated. Results from TB-MBLA could provide further useful information for prognosis and inform treatment clinical decisions at the end of the intensive phase treatment.

The strength of the current study is that it was nested in a bigger parent longitudinal study with experienced research team who guided on collection of high-quality data coupled with high retention rate of the participants into TB care. Moreover, there was no data lost due to invalid/indeterminate Xpert Ultra test.

Moreover, sputum MGIT culture contamination rate of 2.9% was lower than the average laboratory contamination rate, which is 8%. The low contamination rate was attributed to good sputum collection and processing practices including training patients to expectorate properly, and careful decontamination protocol.

4.6.0 Study limitations

The main limitation of this study is the dependence on MGIT culture as the standard reference test for the molecular based assays. MGIT culture is an imperfect surrogate that might have underestimated the specificity and sensitivity of the molecular tests. Using sensitivity and specificity correction method such as the described on Staquet et al.,[309] or Brenner et al.,[310] could have improved the measures of diagnostic accuracy. However, getting a logical corrected sensitivity and specificity depends on selecting a good known sensitivity and specificity of the reference standard. At the present, the sensitivity and specificity of the sputum MGIT culture vary according to study population, and sample used. Corrected sensitivity and specificity is also affected on 'conditional dependency effect' of the tests. [311]

The Second limitation of the current study is that we used a small sample size (n=206) from only one site in Uganda. This could have limited our statistical powers to have strong and conclusive inference message. Nevertheless, our findings reveal that TB-MBLA has the potential to be used as a treatment monitoring tool. On the contrary, these findings show that Xpert Ultra, in its current state, is unsuitable for monitoring treatment response and requires further technical improvements to prevent the assay from detecting DNA from dead bacteria. However, in its current state, the lengthy processing protocol requires further technical modification(s) such as automation, which will make it more rapid which will support its wider applicability in areas of limited trained workforce. Larger studies with longer and multiple follow-ups are needed to clarify the prognostic relevance of TB-MBLA assay results for the prediction of treatment failure or relapse.

5.0 Chapter Five

Detection and quantification of *Mycobacterium tuberculosis* on Tuberculosis-Molecular Bacterial Load Assay in patient stool

Note: Findings of this Chapter can also be accessed on line: doi.10.1128/spectrum.02100-21 for OM-S processed stool, and doi.org/10.1128/spectrum.00274-22 for saline processed stool

5.1.0 Introduction

Sputum is the standard sample for TB diagnosis and treatment monitoring, but production of a good sputum sample is impeded on patient age, health status and other conditions, as well as time of collection.[312,313] Sputum quality and volume mostly depend on patient effort and attitude during expectoration, severity of lung disease, and time of collection. For example, weak patients such as those who are terminally ill or neurologically damaged usually provide sputum which is of low quality and small volume.[314] Low quality samples limit the accuracy of TB tests. Small sample volumes cannot support parallel investigations which are necessary to understand comparative advantages of different tests. Besides, sputum is highly heterogenous in that samples from TB cavitation are more likely to carry with them a higher mycobacterial load than the sample from outside the cavitation hence necessitating alternative or additional samples.[315,316] Alternative samples include bronchial alveolar lavage, gastric aspirates, nasal pharyngeal aspirate, and others[317–320] as described in previous Chapters**.** However, wider applicability of the alternative samples is usually limited on sophisticated collection and processing methods, leaving sputum the most recommended sample. [317,318,320] Often, persons swallow sputum, which ends up in the gut, and hence, stool has been suggested as an alternative or additional sample type for bacteriological confirmation of pulmonary TB.[321–323] However, sensitivity and specificity of the Xpert MTB/RIF evaluation of stool varied depending on the population and laboratory processing methods. [324]

A study in Pakistan showed that stool Xpert TB had a sensitivity of 88.9% (95% CI 50.7-99.4) and a specificity of 95% (95% CI 81.8-99.1), with positive predictive value and negative predictive values at100% and 82.1%, respectively.[325] A study among adult persons in the South east Asia showed that stool culture detected TB in 50% of the persons living with HIV.[326] In Burkina Faso, which is an endemic TB country, sensitivity of the stained stool smear microscopy and liquid culture were 60% and 33%, respectively.[327] In a study that was conducted at Nolungile Clinic, Khayelitsha, South Africa and at a tertiary paediatric hospital, Red Cross Children's Hospital, in South Africa showed that sensitivity of stool Xpert Ultra was 47.1% (95%CI: 26.2–69.0) , and that specificity was 99.0% (95%CI: 94.4–99.8). [328]

In a study that was conducted on Banada et el., to investigate a novel sample processing method for rapid detection of TB in the stool of paediatric patients using the Xpert MTB/RIF it was found out that the assay sensitivity was 85% (95% CI 0.6–0.9), and 84% (95% CI 0.6–0.96) for 0.6g and 1.2g stool samples, respectively, and a specificity of 100% (95% CI 0.77–1) and 94% (95% CI 0.7–0.99), respectively.[329]

In 2019, the Guideline Development group of the WHO recommended Xpert MTB/RIF Ultra to improve TB diagnosis and rifampicin resistance using stool samples but the results, so far, show low diagnostic yield [313,330–334] The low performance may be attributed to the low mycobacteria load in stool [311], possible inhibition of the PCR assays, as well as clogging of the Xpert pipettes on the artefacts in stool. With these observations, it is evident that novel tools for stool TB diagnosis are needed. [313,335–338] The TB-MBLA has potential to replace smear and culture tests. [339–342]

Tuberculosis Molecular Bacterial Load Assay targets 16S rRNA which is highly abundant in Mtb cell compared with DNA and this may increase the probability of detecting Mtb 16S rRNA in a sample with low bacterial load.

This novel assay has potential to detect and quantify Mtb in non-sputum samples, but its utility in stool is not yet known.[343,344] The overall objective of this chapter was to investigate the value of stool as a sample type for diagnosis of tuberculosis was highlighted. While other studies have used DNA-based assays like the Xpert MTB/ RIF and culture to detect Mycobacterium tuberculosis in stool, this was the first study that has applied TB-MBLA, an RNA-based assay, to quantify TB bacteria in stool.

5.1.0 Specific Aims

The specific aims of the Chapter included:

1) To explore the ability of TB-MBLA to detect and quantify viable Mtb in stool samples and correlate the bacterial loads with MGIT culture time-time to positivity to confirm that TB-MBLA detects viable Mtb.

2) To evaluate the diagnostic accuracy of TB-BLA on stool and compare its performance with the routine standard sputum smear microscopy, Xpert Ultra, and culture tests.

3) To compare performance TB tests on stool processed using OM-S and PBS to assess the wider applicability of stool for TB diagnosis.

5.1.0 Schematic presentation of stool-based study

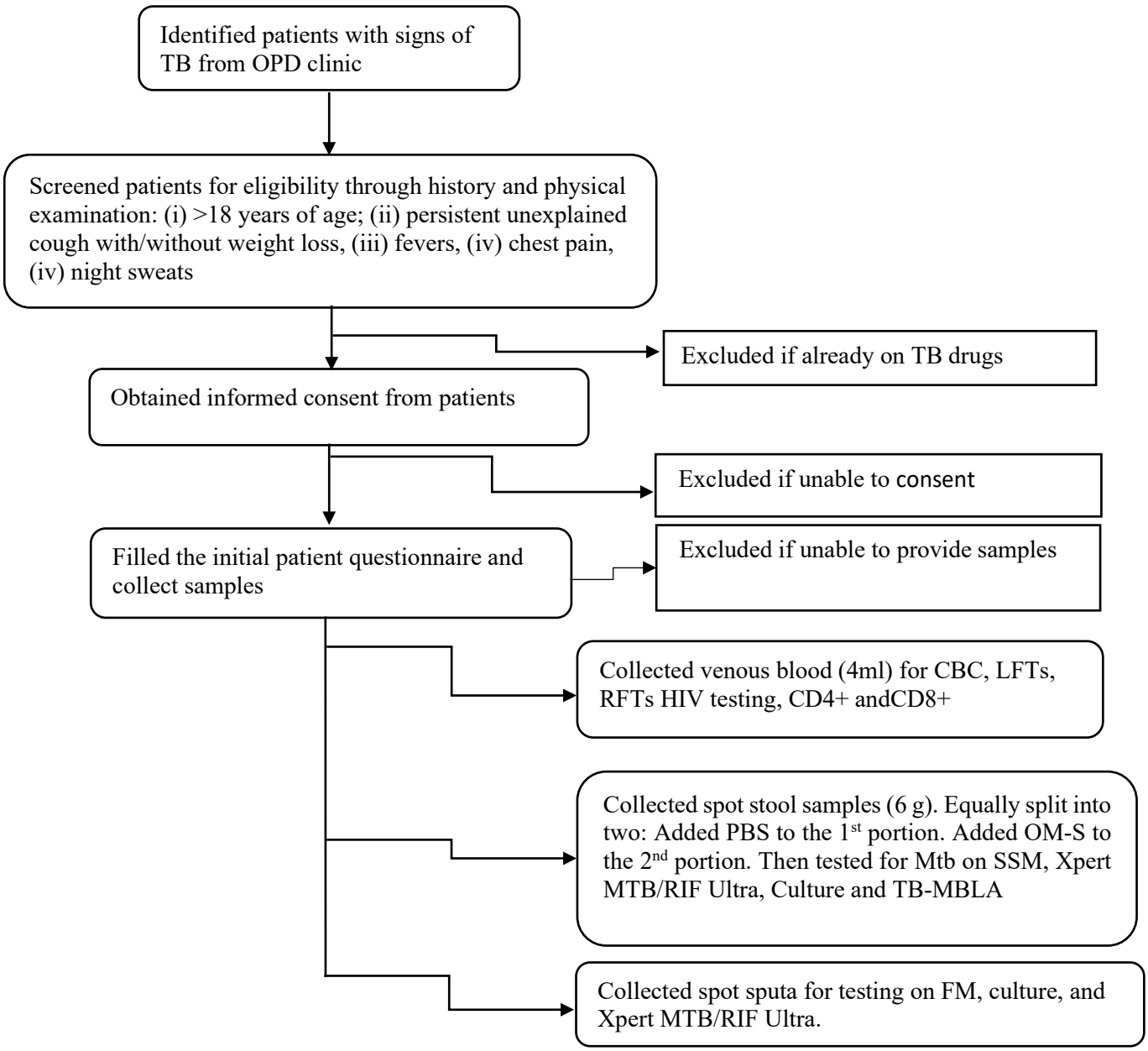


Figure 5.1.0: Schematic of study design OPD: Outpatient Department, TB: Tuberculosis, CBC: Complete Blood Count, OM-S: OMNIgene. SPUTUM, SSM: stained smear microscopy, PBS: Phosphate Buffered Saline. Results of CBC, HIV and TB (sputum DFM and Xpert MTB/RIF Ultra) were issued on the same day for clinical patient management. To note, TB-MBLA test was done on frozen stool but not on sputum samples.

5.2.0 Methods

5.2.1 Research ethics

Both the parent and the current studies were approved on the Makerere University School of Medicine Research and Ethics committee [*REC REF No. 2006-017*] and Makerere University School of Biomedical Sciences Research and Ethics Committee [*REC REF No: SBS 529*] respectively. All participants provided an informed consent as described in Chapters Three and Four. All other Good Clinical and Laboratory Practice guidelines were observed. [345]

5.2.2 Study site

The research work was a retrospective laboratory-based study that utilized stool specimens. The study was nested on a longitudinal cohort of persons with pneumonia at Naguru Referral Hospital, Kampala (Uganda). The index test (TB-MBLA) investigation was done in the Medical and Molecular Laboratories, Limited (MML) which are in the Department of Medical Microbiology, Makerere University College of Health Sciences, Kampala Uganda.

5.2.3 The Parent study

Mulago Inpatient non-invasive diagnostic study (MIND-IHOP-IAM GOLD) is a longitudinal medical research group that is sponsored on Makerere University and the University of California, San Francisco, and is funded on the National Institute of Health (NIH). The main objective of the MIND-IHOP study is to investigate the frequency, quantity and diversity of bacterial, mycobacterial, fungal and viral bugs in the respiratory and non-respiratory specimens on using microbiologic, serologic and nucleic-acid amplification techniques to determine the relationship between the presence of these organisms and the clinical outcomes. [346,347]

5.2.4 Recruitment for stool study cohort on the parent study

The parent study identified, through the Triage and Chest clinic of Naguru Referral Hospital, Kampala, the study participants. Participants were male and female adults (≥18 years) in their sound mind state to be able to give an informed consent. Before enrolment, participants had to have at least one or a combination of TB-like-signs symptoms including, persistent unexplained cough, unexplained loss of weight, unexplained night fevers with profuse sweating, history of contact with patients who had been confirmed to have TB disease. Persons who were found to have TB disease were started on treatment.

5.2.5 Data and specimen collection

The parent study collected clinical data (HIV status, HIV treatment status, CD4 counts, respiratory rate, heart rate, body mass index, prior TB treatment, prior exposure to TB persons, and chest X-ray. Laboratory data included stool and sputum smear, sputum and stool Xpert MTB/RIF Ultra, sputum, and stool culture; HIV status and CD4 cell counts. Both clinical and laboratory data were collected and maintained in compliance with the International Conference on Harmonization and Good clinical laboratory practice. At enrolment, participants were identified and consented as described in previous Chapters. Sputum, blood, and stool samples were collected and processed as described in Chapter Two.

5.2.6 The current study

The current study utilised stored stool to assess the performance of standard of care tests and the novel RNA-based tool (TB-MBLA) to diagnose TB. Utilised samples were collected from patients who had been bacteriologically confirmed TB positive and TB negative against sputum MGIT culture as the reference and gold standard test.

5.2.7 The following inclusion criteria was considered for the current study

i. Availability of sufficient stool sample volume

ii. Unmistakably labelled samples that could easily be linked to the clinical and laboratory data

iii. Availability of matching patient history and clinical data

iv. Availability of matching baseline TB results from smear, Xpert Ultra and culture tests

v. Sample(s) being stored at the right temperature (-20^{o}C)

5.2.7 Data required for the current study

i. Demographic data: Age and gender

ii. Clinical data: HIV status, HIV treatment status, CD4 counts, respiratory rate, heart rate, body mass index, Chest X-ray, body temperature

iii. Laboratory data: standard of care and reference TB investigations which included, smear microscopy, Xpert Ultra, and TB culture

5.2.8 Stool samples in the biorepository

In this exploratory study to investigate the potential use of stool to diagnose TB, a convenient sample size of 100- paired archived stool was randomly selected and used. A sample size of 100 -paired archived stool samples were used because that what my Ph.D. budget could support. On the time of this study, there were 600 stored stool samples. Systematic random sampling selection method was conducted to obtain 100 samples which were processed using OMNIgene.SPUTUM (OM-S). OMNIgene.SPUTUM reagent decontaminates TB samples while preserving Mtb viability (DNA Genotek, Ottawa, ON, Canada) reagent. Corresponding stool sample aliquots which were processed using phosphate buffered saline were also selected and matched with OM-S processed stool. Therefore, a total of 200 samples were selected and examined.

5.2.8.1 Systematic random sampling for stool samples

Although a convenient sample size of 100-paired (100 processed using OMS and 100 processed using PBS) from presumptive TB patients was targeted to be used for the current study, a systematic random sampling technique was used to randomly obtain the required samples from the biobank. Consequently, a fixed sampling interval was calculated as a ratio of total banked stool samples to the target sample size (K=N/n). That is, (600/100 = 6). A start sampling point was selected between 1 and the fixed sampling interval, i.e., start sampling point on subtracting one form the fixed sampling interval i.e., K-1: 6-1=5. Therefore, in each cryovial box containing 81 stool samples, the start sampling point was the 5th sample and, thereafter, every 6th sample was selected until we obtained 100 random samples. Samples that were processed using PBS were stored in a separate cryovial box. Therefore, a sample in PBS with similar identification number as the selected OM-S processed stool were selected and paired.[348]

5.2.9.1 Laboratory investigations for OM-S and saline processed stool

Xpert MTB/RIF Ultra (Xpert Ultra), stained smear microscopy-Fluorescent Microscopy (SSM-FM), MGIT ,and LJ culture tests were performed on the same day of specimen collection. Contrastingly, TB-MBLA test was performed using archived stool samples which had been stored at -20oC for 18 months. Specimen collection and processing procedures were performed as detailed in Chapter Two.

5.2.9.2 Xpert MTB/RIF Ultra for stool processed in OM-S

Briefly, 1 mL of the homogenized sample was mixed with 2 mL of the reagent buffer and tested per the Cepheid protocol (Cepheid, Sunnyvale, CA, USA). The same Xpert Ultra platform was used for all the samples. Results were automatically generated and categorized as ‘negative’, ‘trace’, ‘scanty’, ‘very low’, ‘low’, ‘medium’, or ‘high’ Participants with positive results were initiated TB treatment. Patients with negative results were referred for further investigations.

5.2.9.3 Smear microscopy for stool processed with OM-S and PBS

Sputum and all stool smears were processed and examined. Briefly, 1 mL of the homogenized stool was sedimented and a smear (1-2 cm) was prepared, air dried as described in Chapter Two. Briefly, the dried smear was stained for 15 min using a 0.5% solution of auramine-O (Merck, Darmstadt, Germany), decolorized for 2 min in 3% acid alcohol, and counter stained for 1 min using 0.5% potassium permanganate solution as described in Chapter Two. Smears were examined within 1 h under a fluorescent microscope at x400 magnification and results were interpreted as described in Chapter Two. Results were shared with the team in TB Clinic before recording them onto the designated data capture forms and laboratory results log sheets (*Appendices xxxx* and *xxxx*) and into the electronic database. Samples were processed and decontaminated as described in Chapter Two.

5.2.9.4 Fresh stool culture

Stool MGIT culture was done using freshly collected stool samples. MGIT culture tubes were inoculated with 1 mL of the decontaminated sample and incubated at 37°C for a maximum of 42 days. TB-positive cultures were confirmed on the presence of acid-fast bacilli on Ziehl–Nielsen staining and presence of antigen MPT64 as prepared as descried in Chapter Two. Presence of contaminants was assessed using blood agar culture as described in the Methodology. Results were shared with the team in the TB Clinic on a phone call before recording them onto the designated data capture forms and laboratory results log sheets (*Appendix II* page *231*) and into the electronic database. Lowenstein-Jensen slants were inoculated with 1 mL of the decontaminated sample and incubated at 37°C for a maximum of 56 days. Results were reported according to the International Union against Tuberculosis and Lung Disease guidelines.

5.2.9.5 TB-MBLA for stool processed in OM-S and PBS

The same protocol described in Chapter Two for sputum TB-MBLA was adopted. Thawed stool samples were spiked with 100 µL of the extraction control and homogenised on pipetting up and down. RNA was isolated using the Fast Prep RNA pro blue kit (MP Biomedicals, Santa Ana, CA, USA). Duplex reverse transcriptase qPCR targeting both Mtb and the extraction control was performed on a RotorGene 5plex platform (Qiagen, Manchester, UK). Primers and TaqMan dual-labelled probes used were manufactured on MWG Eurofins, Germany. The qPCR cycling conditions were as described in Chapter Two. Stool TB-MBLA quantification cycles (Cq) values were converted into bacterial load using a standard curve and reported as estimated colony forming units per mL (eCFU per mL).

5.3.0 Statistical analyses of data generated from stool

Differences in baseline clinical characteristics were compared using Fisher's exact test and Mann-Whitney U-test for categorical and continuous variables, respectively. Bacterial load results (eCFU per mL) were log transformed before statistical analyses. Negative, positive, and overall (Kappa -scores) percent concordance between stool tests and Sputum MGIT culture were calculated using STATA version.15.1 (StataCorp, College Station, Texas, United States). Sensitivity analysis method was used in calculation of specificity and sensitivity to minimize the interpretation bias due to contamination[349]. STATA version.15.1 against Sputum MGIT culture as the reference test was used to calculate the sensitivity and specificity at 95% confidence interval. Statistical significance was considered at probability value less than 0.05. Contaminated stool culture results could not be interpreted as either positive or negative. Status of these results was assessed for positivity or negativity using stool TB-MBLA, stool Xpert Ultra and stool smear, and thereafter referred to them as "resolved". To ascertain whether the status of the "resolved" result was valid, their agreement with the corresponding Sputum MGIT culture using Kappa-statistics was established.

5.4.0 Results

5.4.1 Characteristics of participants who provided stool samples

Majority of the participants were young adults aged 34 years (IQR: 25-342). Most of the participants, 53/100 (53%) were females. Among the participants who were bacteriologically confirmed positive for pTB, 20 (33%) were living with HIV-infection with median CD4 cells count 71 cells per µl, including 5 (8%) who were on HIV treatment. All participants reported at least one of the following symptoms: unexplained persistent fever, weight loss, and cough prior to enrolment (*Table 5.1.0*).

Table 5.1.0: Participant demographic and clinical characteristics

		Participants with indicated pulmonary TB status [b]	
Characteristics [a]	**Overall (n=100)**	**Positive (*n* = 61 [61%])**	**Negative (*n* = 39 [39%])**
Age (years.)	34(25-42)	33(25-41)	36(26-45)
Female Gender	53(53)	32(52.5)	21(53.9)
Living with HIV	36(35)	20(33)	16(41)
ART use	20(38)	10(16.4)	10(26)
CD4 (cells/µL)[c]	110(44-228)	71(26-171)	170(66-254)
BMI (Kg/m^2)	20(18-22)	19.7(18-23)	19(17-21)
Alcohol use	66(66)	42(69)	24(61)
Smoking	21(21)	12(20)	9(23)
Fever	79(79)	48(78.7)	31(79)
Weight loss >5%	87(87)	54(88.5)	33(84)
Cough >2weeks	100(100)	61(100)	39(100)
HR (%)	100(84-111)	100(84-111)	101(81-111)
RR (%)	22(20-26)	22(20-26)	24(20-27)

[a] HIV: Human immunodeficiency virus, ART: Antiretroviral Therapy, BMI: Body Mass Index, HR: Heart Rate, RR: Respiratory Rate. [b] Bacteriologically confirmed positive or negative TB cases., [c] CD4 cell counts were measured for HIV-infected adults only (n = 36).

5.4.2 Mtb bacillary load measured on TB-MBLA in stool processed using OM-S

Bacteria load was measured using TB-MBLA test. TB-MBLA PCR output is quantification cycles (Cq) values. These quantification cycle values are then converted to bacterial load on extrapolating on a standard curve and expressed as the estimated Colony Forming Units per millilitre of a sample. In this objective, the overall mean (± SD) bacterial load in stool (n=100) was 5.1±1.59 $\log_{10}$ eCFU per mL. We noted that stool samples from participants living with HIV had mean (± SD) bacterial load of 5.67±1.7 $\log_{10}$ eCFU per mL which was higher than 4.83±1.59 $\log_{10}$ eCFU per mL in stool samples of the participants who were living without HIV negative, (p=0.04). As expected, Cq values and the bacterial load measured on stool TB-MBLA showed a strong negative correlation (r = -0.99). (*Figure 5.1.1)*.

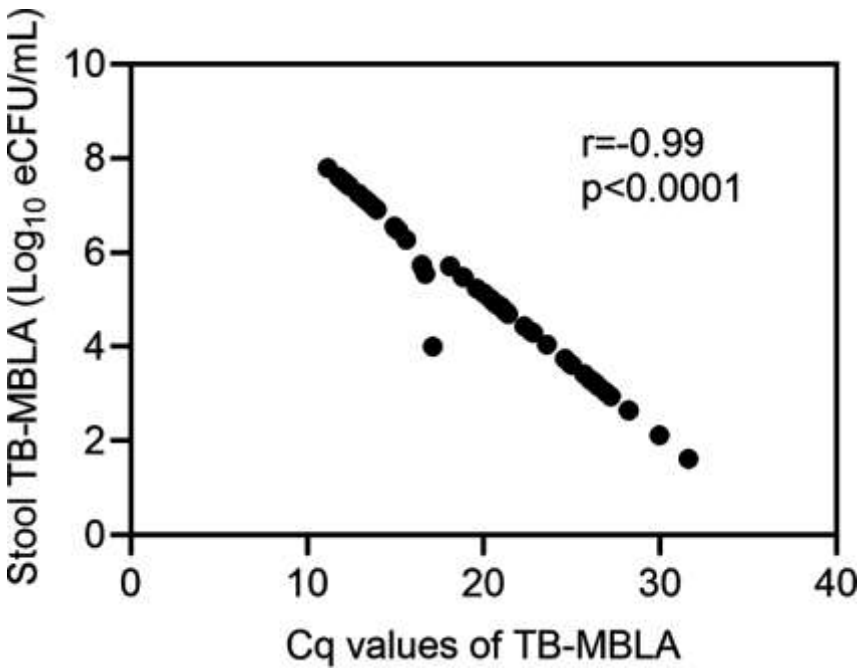


Figure 5.1.1: Correlation between the Cq values and bacteria load in OM-S processed stool. Correlation of the bacterial load and the quantification cycles was determined using the spearman rank sum test. The graph was constructed using GraphPad Prism software. The molecular bacterial load (log10 eCFU per mL) and the Cq values showed a strong negative correlation (r = -0·99). This result confirms that the bacterial loads are direct inverse derivative of the quantification cycles.

5.4.3 Concordance between sputum MGIT culture and stool processed in OM-S

Stool TB-MBLA detected and quantified Mtb in 49/61(80.3%) sputum MGIT culture positive participants and in 8/39 (20.5%) sputum MGIT culture negative participants. Stool Xpert Ultra identified Mtb in 51/61(83.6%) sputum MGIT culture positive participants and in 4/38(10.5%) sputum MGIT culture negative participants. Stool-stained smear (SSM-FM) detected Mtb in 48/61(78.7%) sputum MGIT culture and in 8/39 20.5%)(sputum MGIT culture negative participants. Stool MGIT culture detected Mtb in 39/61(63.9%) sputum MGIT culture positive participants. Stool LJ culture detected the lowest, 27/61(44.3%) sputum MGIT positive cultures (*Figure 5.1.2*).

The overall percent stool- positivity and negativity concordance to Sputum MGIT culture was higher in molecular assays compared with non-molecular assays. Using Kappa analysis, the two molecular assays strongly agreed with sputum MGIT culture at 81% (κ=0.6) for TB-MBLA, and 87% (κ= 0.72) for Xpert Ultra. Among the non-molecular assays, stool smear had the highest concordance with the Sputum MGIT culture at 63% (κ=0.34) followed on MGIT stool at 46% (κ=0.2) and stool LJ at 42% (κ=0.2) respectively (*Table 5.1.2*).

Table 5.1.2: **Concordance of stool assays and sputum MGIT culture**

Test[a]	Percent agreement			Kappa(κ)	Strength
	Positive	Negative	Overall		
TB-MBLA[b]	82	89	84	0.67	Substantial
Xpert MTB/RIF Ultra	85	90	87	0.72	Substantial
MGIT culture	62	60	61	0.2	Poor
LJ culture	43	80	56	0.19	Poor
SSM-FM	42	97	61	0.31	Fair

[a]TB-MBLA: Mycobacteria Tuberculosis Molecular Bacterial Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, MGIT: Mycobacterial Growth Indicator Tube, LJ: Löwenstein-Jensen, SSM-FM: Stained Stool Smear-Fluorescence Microscopy. [b] TB-MBLA test was done using stool frozen at −20°C for 18 months.

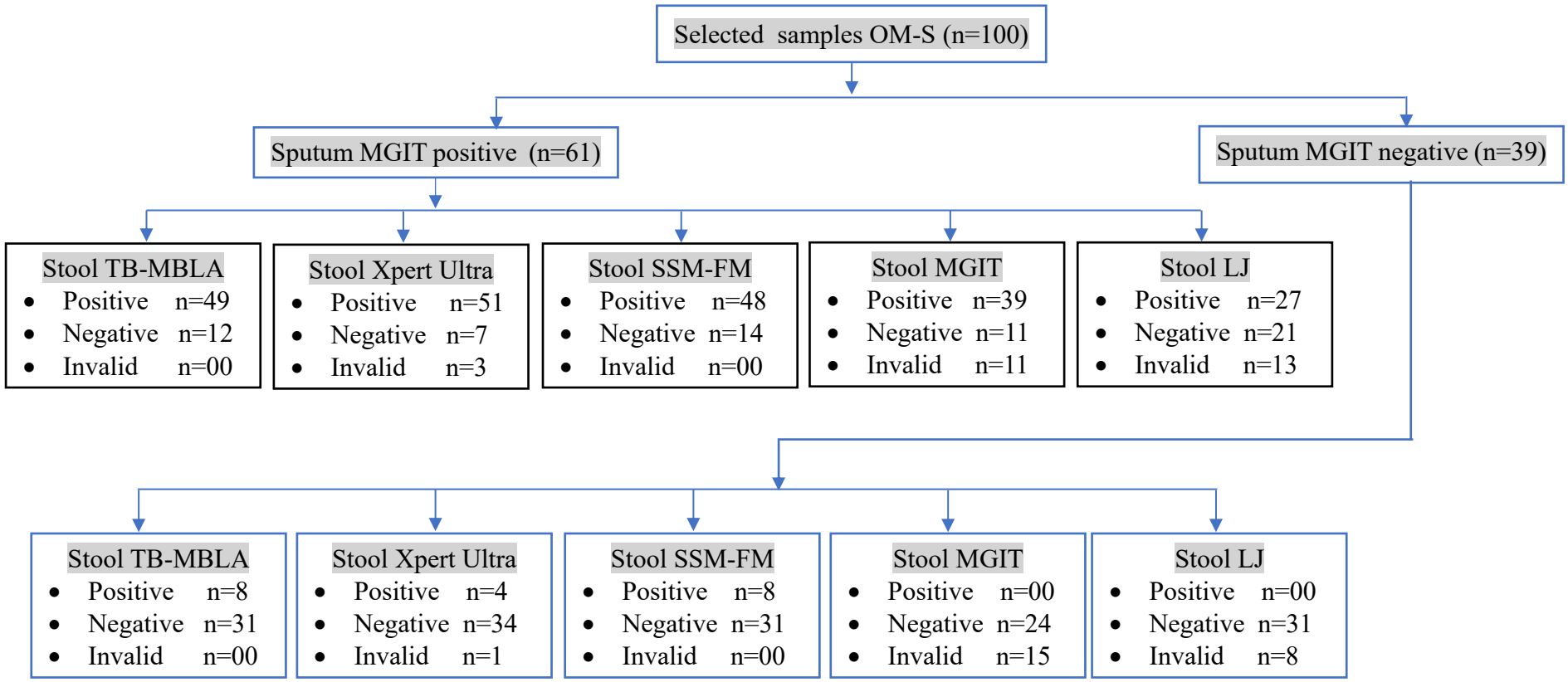


Figure 5.1.2: Flow chart showing the numbers of patients and samples and test results. Stool TB-MBLA: Tuberculosis Mycobacteria Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM-FM: Stained Stool Smear- Fluorescent Microscopy, stool LJ: Lowenstein–Jensen culture, stool MGIT: Mycobacteria Growth Indicator Tube.

5.4.4 Concordance within TB positive stool assays for stool processed in OM-S

Out of the 100 stool samples, 14 (14%) were consistently positive with all the 5 tests i.e., stool TB-MBLA, tool Xpert Ultra, stool SSM-FM, stool MGIT and tool LJ. Concordance was highest between the two molecular tests, stool-TB-MBLA and stool-Xpert Ultra, 45/100 (45%). Combined between stool TB-MBLA and combined stool MGIT and stool-LJ cultures showed a concordance of 21/100 (21%). We noted that concordance was least at 14/100 (14%) in comparison to other tests.

5.4.5 Indeterminate culture results for stool processed in OM-S

Twenty six percent 26/100 (26%) and 21/100 (21%) of the stool MGIT and stool LJ culture were respectively indeterminate due to contamination. Twelve samples 12/26(46%) of the indeterminate stool MGIT culture were positive on stool TB-MBLA and stool Xpert Ultra. Considering LJ culture, 14/21 (67%) of the indeterminate samples were positive on stool TB-MBLA whilst 12/21 (57%) were positive on stool Xpert Ultra. Overall, indeterminate results that were resolved on stool TB-MBLA and stool Xpert Ultra were concordant with sputum MGIT culture at 81% (κ=0·54) and 85% (κ=0·73) respectively, suggesting that they were true positives. In contrast, a weak positivity and negativity concordance of the results was observed and, these were resolved on stool SSM-FM and sputum MGIT culture at 43% (κ=0·2) and 62% (κ=0·2) respectively. (*Table 5.1.3*)

Table 5.1.3: Indeterminate results that were resolved on TB-MBLA and Xpert Ultra

		No. (%) Indicated result in:					
		Stool TB-MBLA		Stool Xpert Ultra		Stool SSM-FM	
	No. (%) of indeterminate culture	Positive	Negative	Positive	Negative	Positive	Negative
MGIT	26(26)	12(46)	14(54)	12(46)	14(54)	2(8)	24(92)
LJ	21(21)	14(67)	7(33)	12(57)	9(43)	5(24)	16(76)

[a]MGIT: Mycobacterial growth indicator tube; LJ: Lowenstein-Jensen. [b] Contaminated culture results that were neither positive nor negative. [c]TB-MBLA: Tuberculosis Molecular Bacterial Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM-FM: Stained Sputum Smear-Fluorescent Microscopy

5.4.6 TB-MBLA and Xpert Ultra quantification cycles for stool processed in OM-S

To compare the quantification between stool TB-MBLA and stool Xpert Ultra, samples that were positive on both assays were considered. Mean Cq values of all the TB specific probes of the Xpert Ultra were calculated and compared with the Cq values of the stool TB-MBLA. A Mann Whitney test showed the median (IQR) Cq value of the stool TB-MBLA, 20·3 (15·4-24·8) which was significantly lower than that of stool Xpert Ultra at 25.1 (22-28) ($p<0·00001$; n = 45). To minimize bias, the same volume that was used for Xpert Ultra was used for TB-MBLA. A Spearman's correlation analysis showed non-significant positive correlation between the stool TB-MBLA- and stool Xpert Ultra- Cq values ($r = 0·17$, $p = 0·25$).

5.4.7 Sensitivity and specificity for stool processed in OM-S

Using Sputum MGIT culture as the reference test, sensitivity (95% CI) of stool assays was 80% (68-89) for TB-MBLA, 90% (79-98) for Xpert Ultra and both were higher compared with 44% (32-58) for smear; 64% (51-76) for MGIT and 62 (45-77) for LJ stool cultures. Specificity at 95% confidence interval was highest for smear at 97% (87-100) followed on 91% (76-98) for Xpert Ultra; 79% (63-90) for TB-MBLA; 80% (64-91) for LJ and 62% (45-77) for MGIT (*Table 5.1.1*).

Table 5.1.4: Results for analysis of diagnostic accuracy of stool assays

					Mean value (95% CI) for[a]:			
Test	TP	FP	TN	FN	Sn(%) (95%CI)	Sp(%) (95%CI)	PPV(%) (95%CI)	NPV(%) (95%CI)
TB-MBLA[b]	49	8	31	12	80(68-89)	79(63-90)	86(74-93)	72(56-85)
Xpert Ultra	51	4	34	7	90(79-98)	91(76-98)	86(70-95)	86(70-95)
SSM-FM	48	8	31	13	44(32-58)	97(87-100)	96(82-100)	53(41-65)
MGIT culture	39	-	24	11	64(51-76)	62(45-77)	52(37-67)	52(37-67)
LJ culture	27	-	31	21	44(32-58)	80(64-91)	48(35-61)	48(35-61)

TB-MBLA: Tuberculosis Molecular Bacteria Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM-FM: Stained Sputum Smear-Fluorescent Microscopy, MGIT: Mycobacteria Growth Indicator Tube, LJ: Lowenstein Jensen media, Sn: Sensitivity, Sp: Specificity, PPV: Positive Predictive Value, NPV: Negative Predictive Value. TP: True Positive result, FP: False Positive result, TN: true negative result, FN: False Negative result

5.4.8 TB-MBLA positive but sputum MGIT negative for stool processed in OM-S

Eight stool samples were positive on TB-MBLA, but their corresponding Sputum MGIT cultures were negative. Further analysis revealed that 6 of these 8 stool samples had at least one positive corresponding sputum test: 4 of them were positive on both sputum smear and sputum Xpert Ultra and 2 were positive on only sputum smear, but negative on sputum Xpert Ultra. The remaining 2 stool samples were negative on all the test methods.

5.4.9 TB-MBLA negative but sputum MGIT positive for stool processed in OM-S

Twelve stool samples tested negative on TB-MBLA, but their corresponding Sputum MGIT cultures were positive. Compared with the corresponding stool Xpert Ultra, 6 samples were negative, 5 were positive and detected as 'low' (n = 4), and 'trace' (n = 1)); and 1 was invalid. The mean MGIT time-to-positivity (TTP) of the sputa that correspond to negative stool samples on both stool TB-MBLA, and stool Xpert Ultra was 11 days, indicating a moderately high bacillary load in sputa. Means (Mean ± SD) of Cq values of the extraction control in stool samples that were positive on stool TB-MBLA at 24·1 ± 1·9, and for the negative stool samples at 24·1±2·3 did not differ significantly (p=0·48), thus indicating absence of inhibition. Additionally, the average Cq value of 7/12 (58·3) stool samples was 0, indicating the absence of any amplification on TB-MBLA, but 5 out of the samples showed late amplification with average Cq value 32·76 and mean bacteria load 0·9 log10 eCFU per mL.

5.4.10 Time to positivity and quantification cycle values for stool processed in OM-S

Overall, mean ± SD MGIT TTP at 12·3 ± 6·4 days was higher for stool MGIT culture than 7·1 ± 3·1 days for sputum MGIT culture. The relationship between the quantification cycle **(**Cq) values for both stool TB-MBLA and stool Xpert Ultra did not significantly correlate with either stool MGIT TTP or sputum MGIT TTP (*Figure 5.4.0*).

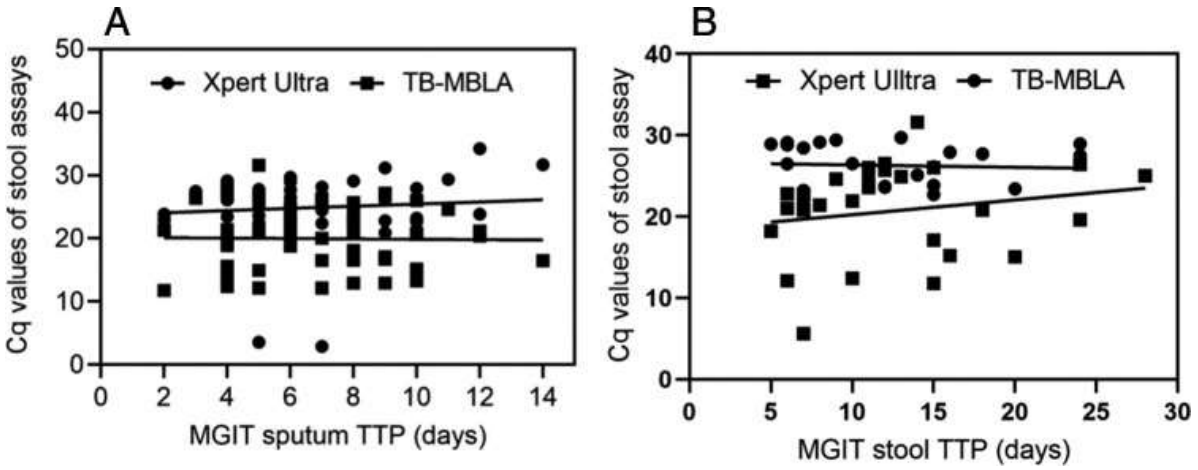


Figure 5.1.3: Correlation of TTP for stool culture with quantification cycles. (A) Relationship between the C_q values of stool TB-MBLA or the C_q values of stool Xpert ultra and the Sputum MGIT culture time to positivity (TTP) (days). The Spearman regression R^2 values were 0·000 and 0·13 for stool TB-MBLA and stool Xpert ultra, respectively. (B) Relationship between the C_q values of stool TB-MBLA or the C_q values of stool Xpert ultra and the MGIT stool culture TTP (days). The Spearman regression R^2 values were 0·02 and 0·04 for stool TB-MBLA and stool Xpert Ultra, respectively. Overall, we did not find a significant correlation between C_q values and MGIT TTP.

5.5.0 Results for stool samples processed using phosphate buffered saline

5.5.1 Bacillary load in stool processed with saline

The overall mean bacterial load (Mean ± SD) measured on stool TB-MBLA was 4.28 ± 0.95 log_{10} eCFU per mL. Stool of participants living with HIV had mean bacterial load of 4.25 ± 1.09 log_{10} eCFU per mL and, this did not significantly differ from that of the participants who were not co-infected with HIV (4.30 ± 0.86 log_{10} eCFU per mL; p>0.05).

5.5.2 Sensitivity and specificity of stool processed with saline

Using Sputum MGIT culture as the reference test, sensitivity (95%CI) for stool TB-MBLA was 77% (65-87) and was higher than 48% (37-63) stool-SSM-FM, 35% (23-47) stool MGIT culture, and 18% (10-30) for stool LJ culture. Specificity (95%CI) for stool TB-MBLA at 90% (73-96) was consistent with that of stool SSM-FM at 98% (86-100) but were both higher than 5% (1-43) for stool MGIT culture at and 49% (29-63) for stool LJ culture at (*Table 5.2.0*)

Table 5.15: Diagnostic accuracy of assays for PBS-processed stool

Test[a]					Mean value (95%CI) for[b]			
	TP	FP	TN	FN	Sn(%) (95%CI)	Sp(%) (95%CI)	PPV(%) (95%CI)	NPV(%) (95%CI)
TB-MBLA[c]	47	4	35	14	77(65-87)	90(73-96)	92(79-97)	71(79-97)
SSM-FM	29	29	37	01	48(37-63)	98(86-100)	50(83-100)	95(43-68)
MGIT culture	18	9	2	6	35(23-47)	5(13-43)	67(31-61)	25(8-30)
LJ culture	11	2	19	13	18(10-30)	49(29-63)	85(22-58)	61(14-35)

TB-MBLA: Tuberculosis Molecular Bacteria Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra, SSM-FM: Stained Sputum Smear-Fluorescent Microscopy, MGIT: Mycobacteria Growth Indicator Tube, LJ: Lowenstein Jensen media, Sn: Sensitivity, Sp: Specificity, PPV: Positive Predictive Value, NPV: Negative Predictive Value. TP: True Positive result, FP: False Positive result, TN: true negative result, FN: False Negative result

5.5.3 Indeterminate samples in saline processed stool

Sixty-five out of the 100 samples were contaminated (69%) with MGIT and 55/100 (55%) were contaminated with stool LJ culture. We noted that 36(55%) of the 65 contaminated stool MGIT results were negative on stool TB-MBLA, and that 29/65(44.6%) were negative with stool TB-MBLA. Considering stool LJ culture, 29/55 (53%) of the contaminated samples were negative with stool TB-MBLA whilst 26/55 (47%) were positive with stool TB-MBLA. For We also observed that 49/65 (75%) of the contaminated stool MGIT results were negative with SSM-FM, and that 16/65(25%) were positive with stool SSM-FM. For stool LJ, 35/55(64%) were negative with stool SSM-FM, and that 20/55(36%) were positive with stool SSM-FM. Overall, indeterminate results that were resolved on stool TB-MBLA and stool SSM-FM were concordant with sputum MGIT culture at 76% (κ=0·54) and 61% (κ=0·31) respectively, suggesting that they were true positives.(*Table 5.6.0*)

Table 5.1.6: Indeterminate results that were resolved on TB-MBLA and Xpert Ultra

		No. (%) of resolved results [c]:			
		Stool TB-MBLA		Stool SSM-FM	
	No. (%) of indeterminate culture result	Positive	Negative	Positive	Negative
MGIT culture	65(65)	29(45)	36(55)	16(25)	49(75)
LJ culture	55(55)	29(53)	26(47)	20(36)	35(64)

[a] MGIT: Mycobacterial Growth Indicator Tube, LJ: Lowenstein-Jensen.· [b] Contaminated: culture results that were neither positive nor negative. [c] TB-MBLA: Tuberculosis Molecular Bacterial Load Assay, Xpert Ultra: Xpert MTB/RIF Ultra. SSM-FM: Stained Stool Smear-Fluorescent Microscopy

5.5.4 Stool TB-MBLA positive but sputum MGIT negative samples

Four (4%) of the 100 stool samples were positive on stool TB-MBLA, but the corresponding sputum MGIT cultures were negative. The average Cq of these samples was 28·8 and mycobacteria load was 4·3 ± 0·9$\log_{10}$ eCFU per mL which was ~0·02logs higher than the average (4·28 ± 0·95 $\log_{10}$ eCFU per mL) mycobacterial for the study population.

Three (75%) of the 4 stool samples were also positive on the corresponding sputum Xpert Ultra, of which, 2 were categorized as 'high' and 1 as 'very low' which suggests that these three samples were likely true Mtb positive. The remaining sample was also negative on the corresponding sputum Xpert Ultra.

5.5.5 Stool TB-MBLA negative but sputum MGIT positive samples

Fourteen (14%) of the stool TB-MBLA results were negative but the corresponding sputum MGIT cultures were positive. The MGIT TTP of the sputa that correspond to the stool samples that were negative on stool TB-MBLA was 8·3 days. The overall mean (± SD) Cq value of the extraction control in samples that were positive with stool TB-MBLA was 25·2 ± 2·2, and 24·9± 1·9 for the samples that were negative. A Mann-Whitney U test showed that Cq values did not differ significantly (p=0·48) potentially indicating absence of inhibition of the PCR amplification in both stool B-MBLA and stool Xpert Ultra tests. Importantly, all the 14 samples that were negative on stool TB-MBLA but negative on the corresponding sputum MGIT culture had a Cq value of zero, indicating an absolute absence of amplification.

5.5.6 Time to positivity and cycle quantification values for PBS-stool

Samples that were processed with PBS were not tested on Xpert Ultra due to lack of cartridges. Overall, the mean ± SD MGIT TTP at 10·4 ± 7·4 days was higher in stool samples than in sputum samples at 5.7 ± 2.7 days. The relationship between Cq values and the MGIT TTP values in PBS-processed stool was investigated. The mean Cq values for stool TB-MBLA correlated weakly with stool MGIT TTP (r = 0·26; p = 0·3), perhaps indicating the effect of long storage of stool samples at -20°C before testing with TB-MBLA.

5.5.7 Comparison of results for PBS- and OM-S processed stool

The TB-MBLA positivity rate in PBS processed stool was 53/100 (53%), 4% less than OM-S processed stool in presumptive cases. Positivity rate was 47/61 (77%) if cases that were confirmed for TB on MGIT culture were considered. Mean bacillary load was $4{\cdot}28 \pm 0{\cdot}95 \log_{10}$ eCFU per mL in PBS processed stools, on average $0{\cdot}8$ $\log_{10}$ eCFU/mL less than the load that was detected in OM-S processed stool, Mann Whitney p=0·003. TB-MBLA sensitivity and specificity (95% CI) were 77% (65-87) and 87% (73-96), respectively and were consistent with OM-S processed stool. TB-MBLA positive predictive value in saline processed stool was 92%, 6% higher than OM-S processed stool. MGIT culture contamination rate at 35% in PBS processed stool was 23% higher than in OM-S proceed stool. (*Table 5.7.0*)

Table 5.1.7: Comparison of PBS versus OM-S processed stools

	Data for:		
	OM-S processed stool (n=100)	**PBS processed stool (n=100)**	**P-Value**
Confirmed pTB on Sputum MGIT (no. [%]	61(61)	61(61)	-
Positive on stool TB-MBLA only (no. [%])	8 (8)	4 (4)[a]	-
Positive on both Sputum MGIT culture and stool TB-MBLA* (no. [%])[b]	49(49)	47(47)[a]	-
Bacterial load ($\log_{10}$ eCFU per mL)[c]	5·1±1·59	4·28±0·95	0·003
Median quantification cycle (Q1-Q3)	20(15-25)	22 (2 -25)	0·002
Stool MGIT contaminated (no. [%])	26(26)	69(69)	-
MGIT contaminated but TB-MBLA positive (no. [%])	12(46)	35(51)	-
Sensitivity (%[95%CI])	80(68–89)	77(65-87)	-
Specificity (%[95%CI])	79(63–90)	90(76-97)	-
PPV (%[95%CI])	86(74–93)	92(81-98)	-
NPV (%[95%CI])	72(56–85)	71(57-83)	-

[a] Forty-seven samples were sputum MGIT culture-stool TB-MBLA positive, while 4 samples were stool TB-MBLA positive only. Overall stool TB-MBLA positivity was 51% (51/100 samples) or 77% (47/61 samples) considering sputum MGIT culture as the gold standard . [b] Sputum MGIT was used as the gold standard and reference test for TB-MBLA. [c] Bacterial load values were log transformed before the mean was calculated

5.6.0 Discussion

Stool is an easy to obtain sample and could enhance TB diagnosis in persons who cannot provide adequate sputum, yet MGIT culture which is the current confirmatory test for TB has low sensitivity for stool. In this study, the ability of molecular-based fully quantitative assay, TB-MBLA to detect and quantify viable Mtb in frozen stool samples was assessed. For stool that was processed with OM-S, TB-MBLA did not achieve the minimum optimal sensitivity (79% versus 90%) WHO TTP for a triage test. For stool that was processed with PBS, TB-MBLA surpassed the minimum optimal specificity (90% versus 80%) WHO TTP for a triage test. It is likely that using fresh stool sample can improve the sensitive and specificity to the WHO recommended estimates levels. The results provided evidence of high bacillary load in the stool samples in persons living with HIV and those without, indicating that the assay has broad applicability in multiple settings. It is not clear whether the higher bacillary load observed in the stool of HIV positive patients was due to dissemination of bacilli from respiratory tract to the gut or not. A larger scale study is needed to establish the cause of this difference and its underlying mechanisms.

Although TB-MBLA had significantly lower Cq (higher bacterial load), Xpert Ultra had higher sensitivity and specificity than TB-MBLA. The lower TB-MBLA Cq could be explained on the fact that TB-MBLA detects the more abundant rRNA compared to DNA which is detected on Xpert MTB/RIF. *Mycobacterium tuberculosis* has been shown to contain 700 ribosomes per cubic micrometre of cytoplasm, implying that higher amount of rRNA is available for detection[350]. Ideally, this would mean higher sensitivity on TB-MBLA compared to Xpert Ultra, but in the present study, the reverse was true. One reason to explain this inconsistency could be that Xpert Ultra targets multi-copy Mtb gene targets, enabling it to detect fewer bacilli in a sample with a lower bacillary load.[351]

Furthermore, unlike Xpert Ultra, the specimen processing for TB-MBLA (particularly the initial centrifugation step to harvest Mtb cells from sputum) has been shown to cause substantial loss of the viable Mtb bacilli that do not easily sediment. Consequently, this could reduce assay sensitivity[352]. Thirdly, the loss of Mtb cell viability due to prolonged sample storage at -20ºC may have compromised the sensitivity of TB-MBLA. Using sputum MGIT culture as the reference test could have underestimated the sensitivity and specificity of the stool assays. Whether using correction methods as that described elsewhere [353–355] can improve the sensitivity and specificity of stool TB-MBLA need to be investigated in future.

In principle, there should be a correlation between rRNA, and DNA detected in the same cell. However, data analysis showed non-significant correlation between stool- TB-MBLA and Xpert Ultra Cq values. This may require further analysis in a large-scale study, to ensure that all analyses are done on freshly collected stool samples to eliminate the confounding loss of viability caused on storage conditions. Overall, the current results concur with the findings from other studies where molecular assays for stool were shown to have higher diagnostic accuracy than the stool culture assays, when sputum MGIT culture was used as the reference test.[356,357] The low diagnostic accuracy of stool culture may partly be attributed to the higher contamination rates and possible Mtb growth inhibition and/or reduction of viable bacillary load due to the killing effect of the decontamination methods. These factors further highlight the importance of sample processing methods.[214]

In this study, stools were processed using OM-S reagent. OM-S suppresses contaminants but delays Sputum MGIT time to culture positivity (TTP).[358,359] It is possible that the same effect occurred in our stool samples, hence the absence of correlation between MGIT TTP and the Cq values of the molecular tests. Other studies have shown inverse correlation of MGIT TTP and Cq values.[207,301,360]

We observed that MGIT TTP for saline processed stool was less on 2 days compared with the OM-S processed samples. However, there was a ~1log eCFU/mL drop in quantifiable bacterial load in saline processed stool compared to OM-S processed stool. This could be explained on the fact that TB-MBLA was performed on stool samples that had been stored at -20ºC for over a year, the conditions under which Mtb RNA preserving ability may have been lower than OM-S. This means that PBS processed stools might achieve similar sensitivity as OM-S if TB-MBLA is performed on the freshly prepared stools.

In this study, 12 samples from the pTB confirmed cases on sputum MGIT culture were not amplified on stool TB-MBLA. Whether this was due to inhibition could not be ascertained because the spiked extraction control was efficiently recovered as demonstrated on qPCR amplification. It can, however, be hypothesized that the loss of Mtb viability during stool storage at -20ºC compounded on some loss of RNA during extraction may have led to non-detection on TB-MBLA.[352] Also noted was that in 5 of the 12 TB-MBLA-negative samples, Xpert Ultra positivity was low/trace, implying fewer bacilli in the samples prior to storage at -20ºC. Some stool samples showed late amplification, and these were reported as negative basing on the cut off value of 30. Whether the Cq cut off value of 30 wrongly placed these samples under "TB negative" category could not be explained. Future studies need to investigate the Cq cut off value for stool.

Using Molecular based assays for stool has potential to increase TB case detection in both adults and children because they use Mtb specific primers and probes which are not affected on non-Mtb flora present in stool. In this study, it is shown that 26% of the tested stool samples were contaminated (grew non-TB flora) on MGIT stool culture and 21% on LJ stool culture. But all contaminated culture samples had a definitive (positive or negative) result on TB-MBLA.

To confirm validity of these results, percent agreement to the reference standard test was calculated and found to range between 81- 85%, implying that over 80% of the resolved results were valid and could be used to inform clinical decision making. Unlike Xpert Ultra which detects non-viable Mtb bacilli due to persistent DNA after cell death, TB-MBLA has been shown to be sensitive to agents that reduce cell viability. [361,362]

In its present form, however, TB-MBLA assay is labour intensive, requiring a lot of "hands-on" and samples must be run in batches which prevents its use in "real time". Nonetheless, even in its current form, TB-MBLA is more rapid than culture. If the protocol is shortened, TB-MBLA could provide a result quick enough for the Clinicians to make suitable decisions early. Diagnosis in children and immune compromised persons is difficult, usually depending on the unreliable clinical symptoms. Using stool TB-MBLA could improve confidence of Clinicians to initiate or withhold treatment. Placing patients on appropriate treatment early enough minimizes the risk of TB transmission and associated mortality.

Smear microscopy is the fastest and the most accessible test making it the commonest TB diagnostic in resource-constrained settings.[363] However, like in sputum [364], its sensitivity in stool is lower than that of molecular tests and may not give reliable yields in samples that have less than 10000 bacilli/mL as reported elsewhere. [365,366] Detection of substantial amount of bacillary load in both HIV negative and positive stool is a testament of sensitivity of stool as a sample type worth considering among samples of primary diagnosis of TB. Further studies should investigate the mechanism underlying the higher bacillary load in HIV positive compared to HIV negative patients. Using stool might provide an easier way to enhance diagnosis of the gastrointestinal TB. Gastrointestinal TB is a life-threatening form of TB that Control programs have not accorded due attention, perhaps because it is less transmissible yet challenging to diagnose.[367] TB-MBLA detected and quantified Mtb in 8 stool samples among the Sputum MGIT negative participants, 2 of which did not have any corresponding positive

sputum test. Whether these cases represented gastrointestinal TB or not could not be ascertained in this study because we lacked data about the relevant descriptors such as colitis symptoms and Bristol Stool Chart scales. Future evaluations to unveil the use of stool specimens to diagnose gastrointestinal TB are urgently necessary.

5.7.0 Limitations of stool-based investigations

The sample size and use of stored samples for TB-MBLA could have limited the statistical power and performance of the assay. A sample size that is too small increases the likelihood of a Type II error which skews the results, decrease the statistical power of the study, and underestimate the specificity and sensitivity of the assays. Storing samples at -20^{o}C for 18 months could have degraded some rRNA hence lowering the sensitivity of TB-MBLA. Nevertheless, in this Chapter, usability of the TB-MBLA to detect and quantify Mtb in stool with a high level of accuracy is demonstrated. These findings indicate the potential role of stool TB-MBLA in facilitating clinical decision making. Besides, TB-MBLA is a rapid test and might enable rapid detection and quantification of Mtb in clinical specimens. Molecular testing of stool may facilitate TB diagnosis in patients who fail to produce sputum and in screening of high-risk groups.

5.8.0 Recommendations for the follow up on stool-based investigations

Future studies will explore the diagnostic accuracy and treatment response monitoring utility of TB-MBLA in a larger sample size utilizing fresh stool among patients who are unable to provide sputum samples. Design of these investigations will modify the current stool processing methods and TB-MBLA protocol to make it shorter and to minimize the loss of Mtb bacilli during the centrifugation steps. Additionally, future studies will explore the role of stool TB-MBLA to study the mycobactericidal effect of the novel TB regimens in patient populations where sputum production is often problematic, including children.

6.0 Chapter six

6.1 General discussion

The primary objective of TB treatment is to eliminate all the Mtb bacilli to achieve a permanent TB cure.[368] Therefore, rapid and accurate tests that can be used to diagnose and monitor treatment response quickly are urgently needed. Current standard of care-stained sputum smear microscopy and assessment of culture negativity at the end of two months of treatment are inefficient for monitoring response to treatment.[369] Stained smear microscopy (SSM-FM) has low sensitivity and may not distinguish live bacilli from those that are dead following initiation of treatment. Sputum culture takes long to yield results making it less useful for prompt treatment decision making. Besides, the current practice of the TB programmes involves diagnosing TB using a more sensitive Xpert Ultra and subsequently monitoring response to treatment using a less sensitive SSM-FM.

The TB-MBLA has the potential to improve monitoring of TB treatment response on allowing diagnosis and monitoring to use the same test. However, the performance of TB-MBLA in clinical settings and the benefits of the standard of care tests throughout the course of TB regimen remain less documented. Therefore, this Ph.D. thesis evaluated TB-MBLA as a potential and accurate alternative for the diagnosis and TB treatment response monitoring, with ability to give results in the shortest time possible.

In Chapter Three, the diagnostic accuracy of TB-MBLA was evaluated among the presumptive TB patients in Uganda - a high TB burden setting according to the WHO report 2021. Sputum TB-MBLA had a sensitivity of 99% which is higher than 90%- the recommended WHO optimal minimum sensitivity for a triage test. Sputum TB-MBLA specificity of 90% was higher than 80%- the WHO recommended optimal minimum specificity for a triage test. These findings indicate that sputum TB-MBLA is suitable for diagnosing TB.

In a high TB prevalence setting. Overall, TB-MBLA and Xpert Ultra demonstrated similar diagnostic performance which was higher than that of SSM-FM and reaffirmed the advantage of molecular based assays. According to the WHO, TB screening should be offered to all persons living with HIV at enrolment stage, diagnosis and during all follow-up visits. The lower bacillary load among the HIV positive participants might indicate that this group was identified before TB disease got severe. Such observation may be a direct benefit of the strategy to intensify active TB case finding among the persons living with HIV. Similarly, the fact that majority of bacteriologically pTB confirmed participants were not living with HIV might point to the benefits of TB preventive treatment in reducing the risk of developing active TB.[370] Similar strategies might benefit the persons who are not living with HIV, yet at risk of acquiring TB disease, but cost-benefit analysis must be considered.

Diagnosis of TB in HIV co-infected persons is difficult due to a high frequency of smear-negative disease. However, Xpert MTB/RIF technology narrowed this gap due to its sensitivity.[371] The findings indicate that TB-MBLA is useful in this subpopulation of patients, since it has same sensitivity like that of the Xpert Ultra, besides higher positive predictive value and specificity. The same trend of results was reflected in the participants who were smear negative, regardless of their HIV status. The low specificity of Xpert Ultra could be due to 'trace call results', which were coded negative on the less sensitive liquid culture that was used as the reference test.

Although a study on Rimal *et al*. shows that the diagnostic performance of Xpert assay is almost at par with the liquid culture test in smear-negative sputum samples[372], a molecular based reference may be more suitable in assessing the diagnostic performance of the nucleic acid amplification tests. However, in the WHO-recommended algorithm, Xpert assay was found to offer only a slight sensitivity gain and was projected to have major resource implications.[373]

In Chapter Four, the accuracy of TB-MBLA in monitoring treatment response to anti-tuberculosis therapy was determined and compared with the standard of care tests SSM-FM, Xpert Ultra and culture. This study has revealed that Xpert Ultra is more persistently positive with 33% of the cases still positive at completion of treatment compared to 27% reported for the older version of Xpert assay. It can be hypothesized that this difference in the positivity rate at the end of treatment might be caused on the trace call results, which are usually detected on the insertion gene probes.[374] This proposition is supported, at least in part, on the fact that majority of the positive Xpert Ultra results at the end of treatment were graded as 'trace call'.

On the other hand, SSM-FM sensitivity was low and turned negative faster than the positivity of the other bacteriological measures following the initiation of treatment. Analysis of the month two smear microscopy positive- versus SSM-FM negative- cases but positive with TB-MBLA revealed that SSM-FM was insufficient to inform an extra month of intensive treatment. In contrast, TB-MBLA positivity resolved this in a manner that is consistent to MGIT culture. This performance makes TB-MBLA with laboratory turn-around time of 4h, a more suitable biomarker for monitoring response to anti-tuberculosis therapy than the currently used stained smear microscopy and liquid culture. However, more performance data is needed from multi-site study designs with bigger sample size and improved the statistical power. Nevertheless, this study has an added value in that it is the first study that has compared ribosomal RNA-based assay (TB-MBLA) against DNA-based assay (Xpert Ultra) for pre-treatment diagnosis of TB and monitoring treatment with a six-month standard TB regimen. As a result, the utility of TB-MBLA for accurate diagnosis of TB has been demonstrated and the limitation of Xpert Ultra and SSM for monitoring TB treatment response was further confirmed.

In Chapter Five, the use of stool as an additional/alternative sample for the diagnosis of active tuberculosis was considered. While previous studies employed DNA-based assays like the Xpert MTB/RIF, and bacteriological tests such as culture to detect Mtb in stool,[375,376] TB-MBLA, an RNA-based assay, was, for the first time, applied to detect and quantify TB bacteria in stool. TB-MBLA detected and quantified viable Mtb bacilli in the stool, thus raising its utility as an alternative/additional to sputum sample type for TB diagnosis and monitoring response to treatment. Furthermore, evidence is provided to the fact that TB-MBLA for stool is sensitive and specific, suggesting its probable usefulness in clinical decision making. DNA based assays are capable detecting DNA from already dead Mtb in stool hence having an RNA based assay is an added advantage. The present results provide additional evidence that high bacillary loads are present in the stool of persons living with HIV and those without, indicating a possible broader application of TB-MBLA in multiple settings. Stool is an easy-to-obtain sample that could enhance TB diagnosis in persons who cannot provide adequate sputum, and yet, MGIT culture, which is the current confirmatory test for TB has low yields for stool.[376]

However, high microbial density and diversity in stool compromises the specificity and sensitivity of culture-based tests due to overgrowth of non-*Mtb* flora. Because of this, TB-MBLA becomes not only the most sensitive and specific test, but also suitable tool for the detection and quantification of viable TB bacteria in stool. Most importantly, this Chapter raises the possibility of using a non-sputum alternative/additional sample type for diagnosis of TB among persons who have difficulty in producing sputum. Furthermore, this Chapter highlights the effective use of PBS as a cheap working solution in the preparation of stool for TB-MBLA and other molecular applications in both resource- rich and limited settings.

6.3.0 Implications of the Findings

Overall, the results of this thesis show the striking potential of TB-MBLA for diagnosis of pulmonary TB and monitoring response to TB treatment. Since this study has shown that TB-MBLA is more sensitive than SSM-FM and MGIT culture, it can provide rapid, accurate and informative results needed for the clinical management of patients at TB Clinics. SSM-FM and MGIT culture tests take too long to yield results and are associated with a substantial data loss through contaminations. The present results show that TB-MBLA quantifies TB bacillary load directly from both sputum and stool samples. Being able to quantify TB in stool could provide easier means of diagnosing and monitoring response to TB treatment in the young children, terminally ill and neurologically damaged patients, who are unable to provide sputum samples. Because TB-MBLA provides accurate and reliable results on the viable Mtb remaining during anti-TB treatment, Clinicians can make correct and timely decisions that are not based on the less sensitive stained smear microscopy and culture tests. However, further studies are urgently needed to investigate the applicability of stool TB-MBLA for diagnosis and monitoring response to TB treatment and its potential role in childhood TB clinical trials for new drug regimens.

7.0 Future studies building on doctoral research

1. Host Gene signature-based tool for early diagnosis of TB infection

The current diagnostic tests for active TB diagnosis focus on the detection of Mtb bacilli within sputum samples and are, thus not useful for diagnosing latent TB infection. Similarly, immunological tests, although approved on the USA Food and Drug Administration as diagnostic tests for latent TB infection, they are not suitable for active TB, since they fail to differentiate latent TB from active TB disease. The symptomatic detection of TB disease is too late to stop its transmission. On the time of detection, the patient will have transmitted TB infection to many others. Therefore, early detection before persons develop symptoms is crucial to prevent severe TB disease and transmission to others.

Host blood RNA-based PCR tests were developed to achieve the following: i) Detection of the latently infected persons before they progress to active disease, and ii) Detection of the active TB in sub-populations who cannot produce sputum. TB infection elicits specific gene expression in the human host to suppress or eliminate the infection. These genes can be detected in blood with relatively good specificity and sensitivity. However, none of the gene signatures has been adopted as a simple, accurate, and affordable near point-of-care diagnostic test. Prospectively, a customised quantitative PCR assay will be evaluated based on TB-specific human genes as a near point-of-care diagnostic and treatment monitoring tool for TB. Undertaking an in-depth, rapid, and accurate screening of latent TB infection is critical to prevent and control TB transmission. Therefore, innovations that rapidly and accurately identify TB patients who would benefit from Isoniazid Preventive Therapy intervention must be prioritised.

A blood-based TB-specific qPCR test should be able to assist the Clinician to easily identify persons who would benefit from the Isoniazid Preventive Therapy intervention. Generally, the current tests for TB are validated for testing TB disease using expectorate sputum, yet some persons like the young children and immune-compromised patients usually find it difficult to produce adequate sputa samples, leading to low case detection, risk of infection and high mortality rates. Consequently, confirmation of tuberculosis in such groups of persons may be based on proposed blood-based assay. Lastly, a blood-based assay may be more accurate for child TB because of its paucity bacillary nature in this subgroup.

As part of this study, each participant provided a blood sample which was used to evaluate selected host TB-specific biomarkers. A biobank was created to store all the blood specimen. A database of the associated clinical data was also created in a format which allows easy retrieval and linkage of the two datasets. A Reverse Transcriptase-Real Time Polymerase Chain Reaction (RT-qPCR) was developed targeting three panels of host genes that are differentially expressed in the presence of TB. The established biobank will provide the required samples to evaluate the performance of the developed host RNA-based quantitative real time PCR as a diagnostic and treatment monitoring tool. We believe that blood-based markers have the potential to increase asymptomatic and symptomatic TB case detection on using a more accessible sample in persons living with HIV and paediatric patients who struggle to produce sputum sample.

This potential was visible during the preliminary work in which 8 genes were investigated using human lung cell line. It was observed that 4 of these genes could distinguish TB infection from no TB infection [Mann-Whitney U test, $p=0.0012$, 0.0042, 0.0009 and 0.022 for genes 1, 2, 7, & 8, respectively after 72h of incubation at $37^{o}C$.

The genes were evaluated on a training set of blood RNA samples from 35 Ugandan adults, 21 (60%) of whom had been confirmed to have pulmonary TB using Xpert Ultra and MGIT culture. Different genes singly and in combination with either of the other genes distinguished TB positive from TB negative cases with specificity of 90-100%, Mann-Whitney U test, p = 0.004.

From these preliminary findings, future studies may be conducted to explore the following:

1) Description of the quantitative read-out of a customised quantitative real time PCR of a host gene signature assay amongst presumptive TB patients with Xpert Ultra-confirmed pTB, presumptive TB patients without Xpert Ultra-confirmed pTB, patients with other respiratory infections, latent TB infection patients (also defined as asymptomatic study participants with positive QuantiFERON test), healthy persons defined as asymptomatic study participants with negative QuantiFERON test.
2) Systematic evaluation of the accuracy (sensitivity, specificity, positive- and negative-predictive values) of the gene panels and select the most suitable one
3) Identification of most accurate gene signature in Objective 2 and development of a kit to be evaluated in a large sample size of participants from different demographic and geographic backgrounds.

2. Utility of TB-MBLA for the diagnosis of childhood tuberculosis and monitoring response to anti-tuberculosis drugs

Childhood tuberculosis is a leading cause of morbidity and mortality worldwide. It is known that timely diagnosis and initiation of treatment reduce childhood mortality from TB to near zero. The under-diagnosis of tuberculosis in children in Uganda and globally poses a challenge for the End TB strategy and requires thorough evaluation of new methods and samples. The use of an alternate samples such as stool in TB diagnosis would ease sample collection and

ensure timely accurate diagnosis, prompt treatment and, therefore, improved treatment outcomes. Collection of alternative samples such as secretions from the stomach and nose might be invasive and harmful. Unfortunately, the current stool processing methods are complex and are associated with high Xpert Ultra errors and higher culture contamination rates. While culture remains the gold standard test for diagnosis of TB, obtaining results takes longer, which delays initiation of treatment.

Based on the findings in Chapter Five, a cross-sectional and subsequently longitudinal study will be to evaluate the usability of TB-MBLA for childhood TB in comparison to SSM, Xpert Ultra and culture. The use of stool samples to diagnose pulmonary tuberculosis in children has the potential to significantly increase the proportion of child patients. This will serve to inform policy and improve the control strategies for tuberculosis in Uganda. Furthermore, it will decrease the time to diagnosis on decreasing the difficulty in sample collection from child patients, therefore improve outcomes of children with pulmonary tuberculosis. The Molecular Bacterial Load Assay has revolutionised TB diagnosis. Moreover, the TB-MBLA has multiple benefits compared to conventional diagnostic tests. The TB-MBLA is unaffected on other organisms usually present in samples. It has a turnaround time of less than 24 hours and, can be used as a biomarker of monitoring treatment response as it responds rapidly to changes in *Mycobacterium tuberculosis*.

Potential impact on policy and/or programs

The current policy on childhood TB treatment outcome and diagnosis is performance of Xpert MTB/RIF and microscopy. However, existing data shows that Xpert MTB/RIF misses a substantial number of children that have TB while smear microscopy is highly subjective and cannot differentiate between dead and viable Mtb. Besides, Mtb culture assay as the current gold standard is associated with high turnaround time and high contamination rates. TB-MBLA has a short turnaround time, it is not reader dependent, and it is free from contaminations,

meaning that it can be a better replacement or alternative test to the conventional tests. However, there is not enough evidence to support its clinical application. Providing data on the diagnostic accuracy of the TB-MBLA in children will contribute to the performance evidence of this tool. Such evidence is needed on the World Health Organization before it endorses this assay.

4. Effect of TB drug regimens on the abundancy and diversity of sputum microbiome

Background: Respiratory tract microbiota acts as the gate keeper for respiratory health. Using new drug combinations, attempts have been made to shorten treatment of tuberculosis (TB), but the impact of such drug combinations on the respiratory microbiome have not been studied. With funding from the European and Developing Countries Clinical Trials Partnership and German Ministry of Education and Research, we used total RNA and V3-V4 16S rRNA gene sequencing, sputum microbiome of participants under the HIGHRIF2 and Multi-Arm-Multi-Stage clinical trials were analysed across a 3-month treatment period. Participants were treated with standard TB regimen Isoniazid(H)-Rifampicin-(R600mgor10mg/kg)-Pyrazinamide(Z)-Ethambutol(E) in comparison to investigational regimens containing fixed dose, R900mg and R1200mg under HIGHRIF2 and/or differing doses of rifampicin (10mg/kg, 20mg/kg, 35mg/kg including novel combinations replacing ethambutol with SQ109(Q) or moxifloxacin(M) in the Multi-Arm-Multi-Stage study.

Findings: We found out that different anti-TB regimens and dosages have different effects on the sputum microbiome. The standard first-line regimen, HRZE appeared soft on microbiome, causing increase in taxa evenness and no significant reduction of diversity. Within the backbone of the standard regimen, increasing the dose of rifampicin alone required up to 35mg/kg to achieve significant reduction of microbiome, which did not recover to pre-treatment level on month-3 of treatment follow-up. A less rifampicin dose of 20mg/kg supplemented with 400mg moxifloxacin achieved significant reduction of microbiome

diversity but recovered to pre-treatment level on month-3 of treatment. The drug SQ109 appeared to have added no significant value added to the performance of anti-TB regimen as well as effect on microbiome. Most importantly, Mtb did not show recovery across regimens, indicating potential selective elimination of Mtb, and the promise that novel optimal anti-TB regimens to shorten treatment course are achievable without universally damaging the beneficial respiratory microbiome. Further large-scale longitudinal studies will be needed to ascertain whether it is only Mtb that is eliminated and what implications this has on the recovering microbiome and treatment outcome A manuscript detailing these fundings can be accessed online (Under review; http://dx.doi.org/10.2139/ssrn.4172089)

From these preliminary findings, future studies may be conducted to explore the following:

1. Does the recovery of microbiome while still under antibiotic pressure mean replenishment from dietary sources or acquisition of antibiotic resistant genes?
2. What are the implications of anti-TB therapy induced microbiome dysbiosis on patient long-term physiology, post-TB lung disease and other health outcomes?
3. Can the understanding of the antibiotic-microbiome-physiology/immunity interaction shift the paradigm of drug safety analysis from host-focused to an integrated approach including the host and their microbiome?

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APPENDICES:

RESEARCH AND ETHICS

University of St Andrews | FOUNDED 1413

School of Medicine Ethics Committee

23 January 2020

Emmanuel Musisi
School of Medicine

Dear Emmanuel

Thank you for submitting your ethical application which was considered at the School Ethics Committee meeting on 21 January 2020.

The School of Medicine Ethics Committee, acting on behalf of the University Teaching and Research Ethics Committee (UTREC), has approved this application. It is the responsibility of the researcher to ensure all other necessary approvals are in place before commencing the research, such as risk assessment and permission to recruit staff and students in the School. The particulars relating to the approved project are as follows:

Approval Code:	MD14702	**Approved on:**	21 January 2020	**Approval Expiry:**	21 January 2025
Project Title:	Evaluating the accuracy of tuberculosis Molecular Bacterial Load Assay (TB-MBLA) for detection of Mycobacterium tuberculosis and monitoring response to anti-tuberculosis medicine				
Researcher(s):	Emmanuel Musisi				
Supervisor(s):	Dr Wilber Sabiiti				

The following supporting documents are also acknowledged and approved:

1. Response to SEC feedback
2. Patient Questionnaire
3. Consent forms
4. Project extension confirmation
5. Correspondence with Makerere University

Approval is awarded for 5 years, see the approval expiry data above.

If your project has not commenced within 2 years of approval, you must submit a new and updated ethical application to your School Ethics Committee.

If you are unable to complete your research by the approval expiry date you must request an extension to the approval period. You can write to your School Ethics Committee who may grant a discretionary extension of up to 6 months. For longer extensions, or for any other changes, you must submit an ethical amendment application.

You must report any serious adverse events, or significant changes not covered by this approval, related to this study immediately to the School Ethics Committee.

School of Medicine Ethics Committee
Dr Morven Shearer, SEC Convenor/Gill Rhodes, SEC Administrator
School of Medicine, University of St Andrews, North Haugh, St Andrews, Fife. KY16 9TF
T: 01334 461733 E: medethic@st-andrews.ac.uk

Approval is given on the following conditions:

- that you conduct your research in line with:
  - the details provided in your ethical application
  - the University's Principles of Good Research Conduct
  - the conditions of any funding associated with your work
- that you obtain all applicable additional documents (see the relevant webpage for guidance) before research commences.

You should retain this approval letter with your study paperwork.

Yours sincerely

Dr Morven Shearer
Convenor of the School of Medicine Ethics Committee

cc. Dr Wilber Sabiiti

INFECTIOUS DISEASES RESEARCH COLLABORATION

INFECTIOUS DISEASES RESEARCH COLLABORATION (IDRC)

6th January 2020

University Teaching and Research Ethics Committee,
University of St Andrews,
School of Medicine
St Andrews KY16 9TF

Dear Sir/Madam

RE: **Emmanuel Musisi's PhD research at IDRC**

I hereby certify that Mr Musisi's proposed doctoral research "Evaluating the accuracy of tuberculosis Molecular Bacterial Load Assay (TB-MBLA) for detection of *Mycobacterium tuberculosis* and monitoring response to anti-tuberculosis medicine", will be nested within the IDRC,s **Mulago Inpatient Noninvasive Diagnosis (MIND)** study. MIND study was approved by the Makerere University School of Medicine Research and Ethics Committee(SOMEREC) and has over 10 years cross-sectional and cohort study experience with established infrastructure including support personnel , data management and access to laboratory facilities required for consistent participant enrolment, follow-up and generation of quality data. After review of Mr Musisi's protocol i confirm the following:

1. Emmanuel's research will not involve collection of new additional samples from participants. The same sample for MIND will also be analysed by Emmanuel's research.
2. No new recruitment method or protocol method – participants will follow the approved MIND protocol and consent process.
3. Surplus Samples related to Mr Musisi's study will be shipped to St Andrews for further analysis subject to material transfer agreement approval by Uganda National Council of Science and Technology and the University of St Andrews.

We welcome the proposed research at IDRC and highly commend it for your ethical approval. For further clarification, please feel free to contact me on the contacts given below.

Sincerely

Dr William Worodria MBChB, MMed, PhD
Senior Consultant Physician
MIND study site Principal Investigator
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COLLEGE OF HEALTH SCIENCES
SCHOOL OF MEDICINE

RESEARCH ETHICS COMMITTEE

July 2, 2020

Dr. William Worodria
Department of Internal Medicine

Category of review
[ ] Initial review
[ ] Continuing review
[X] Amendment
[ ] Termination of study
[ ] SAEs

Dear Dr. Worodria,

Re: REC REF No. 2006-017

Title: "Mulago inpatient Noninvasive diagnosis-International HIV-associated opportunistic pneumonia"

Your proposal entitled **"Mulago inpatient Noninvasive diagnosis-International HIV-associated opportunistic pneumonia"** initially reviewed and approved by the School of Medicine Research and Ethics Committee on January 31st, 2006.

On May 12th, 2020, you requested for permission to make some changes in the study and informed consent form: to remove some of the co-investigators names from the protocol Charles Chiu, MD University of California San Francisco, USA, Greg Dolganov, PhD Stanford University, USA, Yong Huang, MD University of California San Francisco, USA, Joseph Kovacs, MD NIH, Bethesda, Maryland, USA, Henry Masur, MD NIH, Bethesda, Maryland, USA, Michael McCune, MD University of California San Francisco, USA and include Dr. Crystal North from Harvard University as a new co-investigator, to update background section with the latest findings from previous studies from the current IAM OLD cohort, add new sub aims 1) to evaluates mechanisms of COPD development, 2) To study the influence of air pollution exposure on the relationship between HIV infection and lung function among never smokers, to amend the study procedure by adding a detailed section of measurements of Air pollutants (PM2.5 exposure) using Ultrasonic Personal Air Samplers, to include the PM2.5 exposure measurement as one of the follow up procedures for patients already enrolled in the study.

To eliminate one of the TB diagnostic sub Aim 3 under which evaluation of a stool processing method for detection of Mycobacterium tuberculosis using Direct Fluorescent Microscopy, GeneXpert MTB/RIF and Culture Assays was done. All specific objectives, sponsors and procedures related to this sub aim have been removed. To include one new sub aim under AIM 3 which evaluates TB diagnostics, propose to study a new MTB nucleic

acid amplification-based test called the Molecular Bacterial Load Assay (MBLA). To include new follow up visits of 2 weeks, 2 months and 4 months for newly enrolled TB study patients. Separate consent forms to re-consent the study participants already enrolled in the cohort have been developed so as to participate in studying the new sub objectives and to consent for some of the new procedures including measurement of Diffusion Lung capacity, Personal air pollution levels of PM25 and collection of saliva and semen for Cytomegalo virus testing. To add a statement indicating it's optional for male HIV positive participants to offer the study Semen. A new consent form 'IAM OLDA has been developed and the other the current consent forms have been updated to include new procedures and follow up periods for newly enrolled patients who will eventually participate in studying the new sub study objectives. Further stated that all study staff who are to participate in the spirometry and bronchoscopy procedures will strictly use enhanced complete full body Personal Protective equipment.

The committee considered these changes on 01st July 2020. On behalf of the committee, I am glad to inform you that these changes have been approved. You may now proceed with the study. Please forward regular reports on your study to the committee.

MAKERERE UNIVERSITY
SCHOOL OF MEDICINE
APPROVED
★ 02 JUL 2020 ★
RESEARCH & ETHICS COMMITTEE
P.O BOX 7072, KAMPALA

Yours sincerely,

Assoc. Prof. Ponsiano Ocama
Chairperson School of Medicine Research & Ethics Committee

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COLLEGE OF HEALTH SCIENCES
School of Biomedical Sciences
Department of Immunology and Molecular Biology

February 2, 2021

Dr. Erisa Mwaka
Chair, SBS-REC
MakCHS

Dear Chair,

RE: COMPLETION OF CORRECTIONS TO MUSISI EMMANUEL'S PROTOCOL TITLED "IDENTIFICATION AND EVALUATION OF BIOMARKERS FOR *MYCOBACTERIUM TUBERCULOSIS* DETECTION AND MONITORING RESPONSE TO ANTI-TUBERCULOSIS THERAPY"

By copy of this letter I confirm that the Mr. Musisi Emmanuel has completed addressing comments, concerns, corrections and suggestions that arose during the review of the above protocol. As the assigned reviewer I send him back to you for your clearance.

Yours Sincerely.

Dr. Bernard S. Bagaya, PhD
SBS REC Member and reviewer

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COLLEGE OF HEALTH SCIENCES
SCHOOL OF BIOMEDICAL SCIENCES
RESEARCH AND ETHICS COMMITTEE

4th Feb 2021

SBS-849

To Mr. Musisi Emmanuel
Principal Investigator
Uni. Of St. Andrew's

Category of review
[X] Initial review
[ ] Continuing review
[ ] Amendment
[ ] Termination of study
[ ] SAEs

Decision of the School of Biomedical Sciences Research and Ethics Committee (SBS-REC) at its 103rd REC meeting held on 26th Nov 2020.

In the matter concerning the review of a proposal entitled, **"Identification and evaluation of biomarkers for Mycobacterium TB detection and monitoring response to Anti TB." SBS-REC - 849**

The investigators have met all the requirements as stated by **SBS-REC** and therefore, the proposal version 1 of January 2021 is **APPROVED.**

The approval granted includes all materials submitted by the investigators for **SBS-REC** review and is valid until **25th Nov 2021**.

Please note that the annual report and the request for renewal where applicable, should be submitted six weeks before expiry date of approval.

Any problems of a serious nature related to the execution of the research protocol should be promptly reported to the **SBS-REC**, and any changes to the research protocol should not be implemented without approval from **SBS-REC**, except when necessary to eliminate apparent immediate hazards to the research participant(s)

You are required to register the research protocol with the Uganda National Council for Science and Technology (**UNCST**) for final clearance to undertake the study in Uganda.

MAKERERE UNIVERSITY
SCHOOL OF BIOMEDICAL SCIENCES
VALID UNTIL
25 NOV 2021
RESEARCH & ETHICS COMMITTEE
P. O. BOX 7072, KAMPALA

Dr. Emsa Mwaka
Chairperson, School of Biomedical Sciences Research and Ethics Committee.

UNIVERSITY YA CALIFONIA, SAN FRANCISCO, MULAGO HOSPITAL, MAKERERE UNIVERSITY, OKUKIRIZA OKWETABA MU KUNOONYEREZA KWA MIND

ABANOONYEREZI:

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OKWANJULA: Kuno kunonyereza kwa kisawo nga kusasulibwa ettendekero ekulu erya Makerere ne ttendekero ekulu ye Califoniya, San Francisco. Okunonyereza kuno kujja kusasulirwa ebitongole ebya National Institute of Health(NIH) ne. Ndi mukwanaganya wa byakunonyereza era nja kunnyonnyola ku kukunyereza kuno. E'siimu zaffe ziri, 0757247007, 0752900230 ne 0773834149

EKIGENDERERWA; Okunonyereza kuno kukolebwa okusobola okuyiga ku buzibu bwa lubyamira, okusobola okuzula lubyamira amangu ate nokusobola okutebereza ebiva mu ku jjanjaba. Abantu nga 1100 bebajja okwetaba mukunonyereza kuno. Tukusaba okwetabemu kubanga kizuuliddwa nti oyina akawuka ka mukenenya(HIV) oba oyina obubonero bwbulwadde bwa mawwuggwe (Pnuemonia). Twala obudde bwo okusalawo oba wetabamu. Bwoba olina by'obuuza, oyinza okubuza omuntu yenna akola ku musomo guno oba okubuza nze.

Okunonyereza kuno kujja kumala enyaka ebnairi. Kulunaku lwono kkiriza Okwetaba mukunonyereza kujja twala esaawa nga bbiri n'edakiika atano (2hrs 50mins) nga tonaba kusibulwa. Oluvanyuma lw'okusibulwa tujja kukubira esimu era kino kijja kutwala edakika nga kumi (10). Bwonooba okkirizza okujja mudwaliro olukyaala lujja kutwaala eddakika nga nk

ENKOLA EGOBERERWA

- **Okubuzibwa, Okukeberebwa engeri gy'ossamu nokwekkanya embeera y'obulamubwo:** Tujja kukubuza ebikwatako nebikwata kubulwaddebwo. Oyinza obutadamu ebibuzo ebimu singa owulira nga tibukuwadde mirembe naye era newetaba mukunonyereza kuno.
  Nja kebera engeri gyosaamu, ndabe ebyava mukwekebejja n'ebyava mu bye watuwa okebera ndyoke netegereze embeera yobulamu bwo.

- **Ekifananyi Kyamawugwe:** Tujja kukuba ekifanyi kya mawugwe oluvanyuma filimu yeekifananyi tujikube ekifananyi ngatukozesa Kamera eyomulembe kitusobozese okutereka ekifanyi ekyo.

- **Okufuna ekikolondolwa:** Tujja kukujjako ekikolondolwa leero ne nkya. Naye nga tetunakujjako kikolondolwa, tuyinza okusaba onyumunguze mu kamwa namazzi amayonjo. Tujja kukebera oba olina obulwadde bwakafuba era tuyinza okukusaba okunusa omukka gw'amazzi agalimu omunnyo kikusobozese okufuna ekikolondolwa. Bwekinazulwa nti olina obulwadde bwa Kafuba, tujja kusaba otwongere ebikolondolwa ebilala eby'okwekebejja bwoba okiliziganya nakyo.

- **Okubudabudibwa n'okukebelebwa obulwadde bwa Mukenenya:** Abababudabuda okuva mu Routine Counseling and Testing Service bajja kukyalira era batubulire ebinaba bivudde mu kebelebwa.

- **Okujjibwako Omusayi:** Tujja kukujjako obujiko 4.5 obw'omusaayi bw'onobera owerebwa ekitanda, tujja kukujjako obujiko 3.5 ku lukyala olusoka ate era tukujeko akajiko kamu singa okeberebwa amawugwe. Gujja kukozesebwa okumanya kiki ekileta obulwadde bwa mawugwe, nokupima obutafali bwolina obulwanyisa obulwadde ekinayamba abasawobo okukulabirira obulungi. Ekisera kyetunamala nga tukulondola tujja kukunganya obujiko bw'omusayi obuli wakati wa 3.5-4. Bwekinazulwa nti olina obulwadde bwa Kafuba, tujakusaba otwongere omusayi omulala, singa obera oyagadde.

- **Okwoza Amawugwe:** Abasawo bwe banalaba nga kyetagisa, bajja kozesa ebyuma byabwe okwoza mumawugwe okuzula lubyamira. Okwoza kuno kutekwa kukolebwa ku makya nga tonalya kintu kyonna.Bwoba wetaze okebelebwa kuno, abasawo bajjakuwa akayiso okukiriza okusa obulungi nga'bakwoza mumawugwe n'okukakanya nga bakukolako ate no'ku kakanya kubulumi bwoyinza okuwulira mu kamwa oba mumumiro nga bakukolako. Nga tebanakwoz'amawugwe ojjya'kusabibwa obutalya n'obutanywa kintu kyona okuva kusawa nya ezekiro ekinakesa olunnaku abasawo lwebategese oku'kwoza amawugwe. Bwo'ba ne'dagala lyotera okumira kumakya, oyinza okulimira nga abasaawo tebanaba kwoza'mawugwe naye nga okozesa amazzi matono ddala okulimira. Bwo'ba olina obulwadde obuziyiza okusa, nga asiima, abasawo bajja kusoka okuwa edagala elyo'kuyambako okusa obulungi nga lino'edagala baliyisa mu musiwa oba ojja kusabibwa okussa ennyo oliyingize mumawuggwe n'omuka gw'ossa. Abasaawo bajja kupiima pulesa, bapiime engeri gyo'ssa mu ate bakutekeko akuma akapiima engeri omutiima gwo gwegukuba mu. Nga bino byonna babikola okulaba nti olibulungi nga bakwoza mumawugwe, era bajja kubipima nga tebanaba kukolako, nga bakukolako n'oluvanyuma nga bamaze okukolako. Muk'woza amawugwe, abasaawo bayisa akaseke mukamwa ko,okutusa mumawugwe bakakozese okuyisa mu amazzi agatukula nga gegano gebasiika mu nga gamazze okwoza amawugwe, nebagakebera okuzula lubyamira bwoba omulina. Kino bajja kiddamu emirundi etaano oba mukagga okufuna amazzi agamala

okukeberebwa. Tujja na kuyisa ka bulaasi akatono enyo emirundi mukaaga mu mawugwe go kituyambe okukebera ebiri mu mawugwe go.

- Tujja kwongera tukujjeko ebyokwekebejja ebirala. Ate tukusabe onyumunguze obugiko bubiri obwa mazzi agalimu omunyo okumala edakika emu oluvanyuma olindemu obutikitiki butano olw'owande mukakopo kaffe. Oluvanyuma tujja kuyisa kululimu lwo akakolokota olulimi.
- **Okutuwa obubi bwo**:Tujja kusaba bwo'genda emanjju okufuna ku'mpitambi. Tujja kusaba oyambale gilavu ezalabba okunganye sampolo y'obubi bwo mu kakopo ng'okozesa akagiko. Ng'omaliriza okuteka obubi mu kakopo gezaako nyo okulaba nti okasanikidde bulungi. Bw'oba okiriza, byetukujeeko oku kebelebwa tujja bitereka tubikozese mukunoonyereza mubiseera ebijja mumaso.
- **Okutuwa Enkwaanso:** Okunonyerezza kwaffe okwaasooka kwalaga nti waliwo akawuka akayitibwa Cytomegalo virus (CMV) mubasajja abayina akawuka ka mukenenya (siliimu) nga kano kalowoozebwa okuleeta endwadde ezamawugwe. Akawuka kano aka CMV kasinga kubeera mu nkwaaso zabasajja. Naffe kyetuva tukusaba okutuwa enkwaso zino bwoba oli musajja ate nga oyina akawuka ka mukenenya tu kakebere oba mwekali okusoboła okutegeera engeri je kaleetamu endwadde ezomu mawuggwe. Kino kijja kuba kya kyeyagalile mu basajja abayina akawuka aka silimu. Bwonooba toyagadde tuwa nkwaaso tekijja kulemesa kwetaba mu bikolwa ebilala ebiri mu kunonyereza kuno. Bwonooba okkiriza okutuwa enkwaaso tujja kukusaba ozifunire ewaka mungeri yo kwemazisa wekka. Kino tujja kukusaba okikole nga wayisewo esaawa asatu mu mukaaga nga tewegasse na mwagalwa oba mukyala wo. Ojja kusabibwa okuleeta nekwaaso ezo mu dwalilo mu saawa nya okuva lwe wemazisizza. Naye bwekiba nga enkwaaso ozifunye nga mu saawa ezo enya tojja kusoboła kuba ku dwaliro tujja kukusaba otereke enkwaaso zo mu filiigi. Nansi waffe ajja kukuwa endagililo engeli joonoteeka enkwaso zino mu kacupa kaanaba akuwadde olwo bonomala ojje nako mu dwaliro ku lunaku lwemunalagaana.
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- **Olukalala lwebibuzo ebikwatagana ku ndyaayo n'embera y'ebyenda byo**: Singa osalawo okuleta sampolo y'obubi, tugya kukubuza ebibuzo ebikwatagana n'embera yebyobulamu bwo mu byenda, endyayo, ebyokulya n'okunywa. Bino bijja kutuyamba okwetegereza ebiri musampolo y'obubi bwo
- **Okkulondoola Ebikwatako:** Singa osangibwa nga oyina akafuba wabula nootenyigira mu kunoonyereza kwokutunula mumawugwe tujja kukukubira essimu ate singa tetusobodde kukufuna ku simu tujjaa kukozesa ennamba y'essimu endala gyonooba otuwadde kuntandikwa ekyokwetaba mu kunoonyereza kuno. Tujja kukutukirira ku mukutu gw'essimu oluvanyuma lw'emyezzi ebiri okuva kulunnaku lwewatandika okwenyigira mu kunonyereza kuno. Ojja kusabibwa okudayo mu ddwaliro oluvanyuma lwa weeki bbiri , emyezi ebiri ,emyezi ena emyezi mukaaga okuva lwootandika obujjanjabi bwa TB.), Oluvanyuma tujja kukusaba okukomawo mu ddwaliro oluvanyuma lwe emyezi esatu nga omaze obujjanjabi(emyezi ngamwenda(9) okuva lwootandika obujjanjabi), n'oluvanyuma kumyezi kuminetaano nga omaze obujjanjabi (lemyezzi nga Abiri mu gumu (21) okuva lwootandika obujjanjabi). . Oluvanyuma ojja kusabibwa okudayo mu ddwaliro omulundi gumu buli mwaaka okumala emyaaka etaano. Bwetunakusanga nga toyina kafuba, ojja kusabibwa okudayo mu ddwaliro oluvanyuma, ngawayiseewo emyeezi ena, ngawayiseewo emyeezi kumi, ngawayiseewo emyeezi kuminamukaaga ne kumyeezi abiri mumunaana okuva na leero. Oluvanyuma naawe ojja kusabibwa okudayo mu ddwaliro omulundi gumu buli mwaaka okumala emyaaka etaano Tujja kukubuza ebibuzo buli lukyaala lo muddwaliro era ojja kusabibwa okukola bino wamanga:

- **Okugibwako Omusayi:** Bwonosangibwa nga oyina akafuba tujja kukunganya obugiko bw'omusayi obuwerako ku nkyala zo ezinadirira. . Tujja kukujako wakati wa miilu ataano ne nkaaga muttaano ezomusaayi leelo, mumweezi gwoonomalilamu obujjanjabi, emyezi esatu nga omaze obujjanjabi ku myezi kuminetaano nga omaze obujjanjabi. Oluvanyuma lweezo enkyaala, ojja kujibwaako omusaayi nga ozzeeyo mu ddwaliro omulundi gumu buli mwaaka okumala emyaaka etaano. Bwetunakusanga nga toyina kafuba, tujja kukujako wakati wa miilu ataano ne nkaaga muttaano ezomusaayi leelo, ngawayiseewo emyeezi ena, ngawayiseewo emyeezi kumi, ngawayiseewo emyeezi kuminamukaaga ne kumyeezi abiri mumunaana okuva na leero. Oluvanyuma lweezo enkyaala, ojja kujibwaako omusaayi nga ozzeeyo mu ddwaliro omulundi gumu buli mwaaka okumala emyaaka etaano Dokita wo watusaba okukwooza mumawugwe tujja kukujako miilu zomusayi endala kuminataano kulunaku lwetunayoza mumawugwe.
- **Enkalala z'ebibuzo:** Ojja kubuzibwa ebibuzo ebikukwatako, obubonero bwolina n'embera yo ey'ebyobulamu. Nga bino bifanagana nebibuzibwa mu nkyalo zo ez'eddwaliro ezabulijjo. Okwongereza kubino, ojja kubuzibwa ebibuzo ebikwatagana ku nkozesa yo eyasigala, omwenge, nebiragalalagala ebirala.
- **Okugezesa enkola ya amawugwe goo (Spirometry):** Tugenda kukebera engeri empewo gyetambula mu mumawugwe go. Ogenda kussiza omukka mu ka tuubu nga kali ku kamaciini. Okukeberebwa kuno kugenda kudibwamu ng'owerebwa eddagala eriyamba mu kussa eriyitibwa Albuterol, oba Salbutamol) nga liyamba okugulawo empewo gyeyita tumanye oba eddagala lino likuyambako mu kussa obulungi. Ojja kusabibwa okuddamu omutendera guno emirundi egiwerako tusobole okufuna ebipimo byenzisaayo ebitufu. Tujja kugatako okupima engeri amawugwe go je gatuusa omukka gwoyingiza nga ossa mu musayi Empima eno eyitibwa Diffusion Lung capacity mu lufutifuti.
- ***Okupima obutwa mu mukka gwossa:*** Ojja kuyitibwa ojje ku ddwaliro saawa ana-munaana nga olukyala lwetwakuwa telunatuuka. Ojja kuweebwa akuuma akatono akapima embeera yoomukka gwossa eeka nejotambulila. Tujja kusasulila ebisale ebyentambula yo bwe tunaaba tukuyise okukima akuuma kano. Akuuma kano kayitibwa Personal sampler. Ojja kusomesebwa engeri yokwambalamu akuuma kano okumala enaku bili. Ojjakudda nakuuma kano mu dwalilo nga olukyaala lwetwakuwa lutuuse. Okupima embeera yoomukka gwossa kijja kukolebwa okutandankira ku nkyaala zino. Bwoba wasangibwa nakawuka aka TB enkyalo zijja kuba luvanyuma lwe emyezi esatu nga omaze obujjanjabi (emyezi ngamwenda (9) okuva lwootandika obujjanjabi), n'oluvanyuma kumyezi kuminetaano nga omaze obujjanjabi (lemyezzi nga Abiri mu gumu (21) okuva lwootandika obujjanjabi). Bwoba tewasangibwa nakawuka ka TB enkyalo zijja kuba ngawayiseewo emyeezi ena, ngawayiseewo emyeezi kumi, ngawayiseewo emyeezi kuminamukaaga ne kumyeezi abiri mumunaana okuva na leero. Oluvanyuma ojja kusabibwa okudayo mu ddwaliro omulundi gumu buli mwaaka okumala emyaaka etaano.

OBUZIBU N'EBIYINZA OKUKUTUKAKO

- **Okwoza Amawugwe:** Oyinza okukolola, Okulakira, oba obutasa bulungi. Oyinza okufunamu olusujja sujja oluvanyuma lwa kabanga akatono nga omazze okunazibwa mumawugwe. Oyinza okufuna empewo mumawugwe, amawugwe okuvamu omusayi, oba elimu ku mawugwe okulekera okukola, naye bino tebitela kubawo. Era sikyabulijjo nti oyinza okulemererwa okusa kululwo oba okufa oluvanyuma lwa kino.

- **Empitambi:** Mukufuuna empitambi tujja kusaaba okwewala okujikwatamu era tujja kuwa ebyokukozesa. Abantu abamu bayinza okuwulira obuzibu m'ngeri empitambi gy'ewunya era bayinza oku sindukirira ememe ob'okusesema

Okutuwa Enkwaanso: Tewali kizibu ku mubiri kijja kukutuukako nga otuwadde enkwaaso naye oyiza okufunamu ensonyi, okuswaalamu, okwelalikililamu, nokufunamu okutuyana okutonotono nomutima okweyongramu okukuba entunnunsi entonotono.

- **Enkola y'okugyamu ekikolondolwa, Okumunyunguza mukamwa, okalakata kululimi, n'okunaza mukamwa:** Oyinza okuwulira obuzibu mu kussa nga okolebwako oba okulakirwa oba okuwulira ng'ayagala okusesema.
  Tukusaba otubulire singa oba olina obuzibu bwofunye olw'o kunonyereza kuno.

- **Okujiibwako Omusayi:** Oyinza okuwulira obulumi bwe mpiso nga eyita mu lusussulwo . A wafumitibbwa empiso wayinza okudugalirira oba okola ekizigira. Naye bwofuna obulumi okutwala akabanga, okw'okyerera oba nga wagonderera, tegeza abasawo.
  Omusaayi gwo bweguba mutono, oyinza okuwulira nga onafuye oluvanyuma lw'okujjibwako omusaayi. Tujja kebera omusaayi gwo bwekinasangibwa nti mutono tujja kwewala okukujjako omusaayi..

- **Ekifananyi Kya mawugwe:** Bwetunaba tukuba ekifananyi kino tujja kakas nti ebikwatako gamba nga amannyago, nti bijjibwako era tewali ngeli yonna yakukutegera nga osinzira kukifananyi kino.
  Ekifananyi kya mawugwe kino kijja kuterekebwa mungeri enekusifu mukyumakirimagezi nga wetaga olukusa okukiraba.

  Okukebera oba amawugwe go gakola bulungi: Oyinza okukalubilizibwa mu kussa ng'okukeberebwa kukolebwa oba nga kuwedde, naye nga kino tekilwawo. Oyinza okufuna ekifuba, okulumizibwa mu kifuba, n'okukalubizibwa mu kussa, oba oyinza okuwulira ng'agenda okugwamu amanyi.

  Tujja kukuwa eddagala okuyamba enkola yamawugwe go nokumanya oba eddagala likuyamba okusa obulungi.

  Albuterol oba Salbutamol okusinzira kuddagala erinabera liriwo: Eddagala lino liyinza okukuletera okutyemuka omutima, olubugumu bugumu. Oyinza okukayirirwa mukamwa, okukala kwakamwa. Oyinza okuwulira obulumi mukifuba, supidi yentabula y'omusayi eyinza okweyongera oba omutima okulekerawo okukola.

- **Okuuma Ebyama:** Tujja kujjako ebikwatako naye bijja kulabwako obo bokka abakola kukunonyereza kuno. Tujja kozesa ennamba enkusike kubikwatako.
  Ekisumuluzo ky'enamba eno kijja terekebwa mu kifo ekyekusifu. Erinnya lyo terijja kozesebwa kubikwata ku bulamu bwo mukufulumya ebinava mukunonyereza. Abakola kunonyereza bajja kumanya ebikwatako naye tebajja kufulumya bikwatako ng'omuntu eri abantu abalala wadde abanonyereza abalala akozesa ebikujjidwako.
  Okunonyereza ku buzariranwa bwo (Famire), kuyinza okwongera obuzibu singa kukozesebwa okuzuula ebikwata ku by'obulamu bw'omuntu nga tewandiyagadde bimanyibwe. Okuziyiza kino,

tetujja kukiriza kunonyereza ku kintu lirala kyonna okujjako mukenenya (HIV), obulwadde obukwata amawugwe nendwadde ezigwa mukiti ekyo.
Okakasa nti abanonyereza bagoberera enkola enkola z'okunonyereza entuufu, Makerere University Faculty of Medicine Research Ethics Committee, ne Mulago Hospital Institutional Review Board wamu ne UCSF Committee on Human Research, oba Uganda National Council for Science and Technology, bino bye bitongole ebisobola okulaba kubiwandiko ga biraga erinya lyo oba ennaamba yo eyakuwebwa mukunonyereza. Tujja goberera amateeka gonna agakwatagana n'okuuma ebyama byo. Satificate y'okuuma ebyama efunidwa okwongera okutuyamba okukuuma ebikukwatako okuva mu kubuliriza kwa Gavumenti.

- **Okulumizibwa:** Bwoba olumizibwa nga Kivudde kunonyereza kuno, ojja kufuna obujanjabi obwamangu ku bulumi bwonafuna. Otekeddwa okubulira abanonyereza singa owulira okulumizibwa. Osobola ogamba omu kubanonyereza mubuntu oba okuba essimu zabwe ezitereddwa kukiwandiiko kino. Obujjanjabi obwabulijjo obufunibwa mudwaliro e Mulago oba mu dwaaliro eliyitibwa China-Uganda Friendship Hospital-e Naguru bujja kubaawo. Obujjanjabi bujja kuweebwa kubwerere, bwekiba kisoboka nga tukozesa sente zetulina okujjanjaba obuvune obukwatagana ne nkola gyetulina ogobeerera.
  Makerere University nabasasulira okunonyereza kuno, tebalina ngeri ndala yonna gyebakusasulamu ngofunye obuvune.

BYOGANYULWAMU: Okunonyereza kuno kuyinza okuyamba abasawo okweyongera okuyiga ku bireetera amawugwe okulwala era tusubira nti ebinavaamu bijja kuyamba okuzula nokujjanjaba abalwadde b'akafuba (TB) ne lubyamira (Pneumonia)mubiseera ebyomaaso. Okukebera okumu kwakugezesa era tujja kumanya ebivuddemu kunkomerero yokunonyereza kuno. Nolwekyo, ebinavaamu tebijja kubulirwa era tebijja kosa bya bujjanjabi bwo. Wabula, bwoba oyagala okumanya ebyatukibwako mukunonyereza kuno kwonna, tukusaba otutegeze era tujja kumanyisa.

OBWEKUSIFU BWO: Tujja kufunako ebikwaatako ebyomubuntu naye abakozi abakola mu kunonyereza guno bokka beebajja okubirabako. Tujja kkuwa ennamba jetujja okukuuma awekusifu. Tetujja kukozesa linya lyo nga tuwandiika ebivudde mu kunonyereza kuno wadde nga tujja kukozesa ebyobulamu ebikwatako.Wadde nga abakozi abokola mu kunonyereza kuno bajja kuba nebbeetu okumanya ebyobulamu ebikwaatako, tebajja kwaasangunza manya go eri abantu abalala bonna wadde na abanonyerezi abanakozesa sampolo zootuwadde. Okukebera ebya ndaga buttonde yo kuyinza okwongerako emikisa jyokumanyika kwo nebyobulamu bwo byootaalyagadde kumanyika.Okuziyiza kino tetujja kukiriza okunonyereza kulala kwonna okujjako okwo okukwata ku Mukenenya oba amawugwe nendwadde zaago. Okukakasa nti okunonyereza kuno kukolebwa mu ngeri etuukiridde era eya mateeka obukiiko omuli kebayita Makerere University School of Medicine Research Ethics Committee, Mulago Hospital Institutional Review Board, UCSF Committee on Human Research, oba Ugandan National Council for Science and Technology buyinza olumu okutunula ku kopi ze biwandiiko ebiraga amanya ggo oba ennumba yomusomo joonoweebwa. Tujja kugoberera amateeka gonna aga ziyiza okumanyika kwo nebikwaatako. Satifikeeti eziyiza gavumenti oku tuubulirizako ebikukwatako nayo twajifunye.

ENKOLA ENDALA:: Okirizibwa okukebera obulwadde bwo nga bulijjo wadde nga teweetabye mu kunonyereza kuno . Oyinza okubuuza omusawo wo bulwadde ki bwo kirizibwa okukebera wadde nga tonnasalawo kuyingira kunonyereza kuno.

OKUSASULWA OLWOKWETABA MU KUNONYEREZA: Tojja kusasulwa olwokwetaba mu kunonyereza kuno oba kusasulira bisale byo byadwaliro, wabula ojja kuwebwa emitwalo essatu (30,000) buli lwootukiriza enkyaalo zo ezookulondolwa. . Tujja kusindikira enussu enkumi ttaano ku akawunta ye simu yo buli we tunaakubira essimu.

Emitendera eginagobererwa ku buli lukyala mu bufunze

Ekipande ky'omusomo gwa bayina akafuba naye nga tebetabye mu musomo gwookwooza mumawuggwe

Olunaku lwokirizza okweetaba mumusomo	O Olukalala lw'ebibuzo
	Okugibwako omusayi(Miilu 50-65)
	Okutuwa ekikolondolwa
	Okutuwa empitambi(stool)
	Amalusu
	Enkwaaso mu basajja abayina akuwuka ka siliimu(Kino Kya bweyagalile)
Week biri okuva lwewatandika egala lya TB	Okutuwa ekikolondolwa
Emyezi ebiri okuva lwewatandika egala lya TB	Okugibwako omusayi(Miilu 10) Okutuwa ekikolondolwa
Emyezi ena okuva lwewatandika egala lya TB	Okugibwako omusayi(Miilu 10) Okutuwa ekikolondolwa
Okukukubira essimu n'okulondolwa kumyezi ebiri	Okwekenenya obubonero bwakafuba
Olukyala nga omaliriza obujjanjabi bwa kafuba('emyezi nga mukaga bukya webygira mu kunonyereza)	Olukalala lw'ebibuzo
	Okugibwako omusayi(miilu 50-65ml)
	Okutuwa ekikolondolwa
	Okukebera oba amawugwego gakola bulungi
	Diffusion Lung Capacity
	Okupima obuttwa mu Omukka gwossa (PM_{25})
Okukukubira essimu oluvanyuma lwa myezi ebiri nga omaliriza obujjanjabi bwa kafuba(emyezi nga munana bukya webygira mu kunonyereza)	Okwekenenya obubonero bwakafuba
Olukyala oluvanyuma lwamyezi esatu nga omaliriza obujjanjabi bwa kafuba (mwezi nga mwenda bukya webygira mu kunonyereza)	Olukalala lw'ebibuzo
	Okugibwako omusayi (miilu 50-65)
	Okukebera oba amawugwego gakola bulungi
	Diffusion Lung Capacity
	Okutuwa ekikolondolwa
	Okupima obuttwa mu Omukka gwossa (PM_{25})
Okukukubira essimu oluvanyuma lwa myezi enna nga omaliriza obujjanjabi bwa kafuba(emyezi nga kummi bukya webygira mu kunonyereza)	Okwekenenya obubonero bwakafuba
Okukukubira essimu oluvanyuma lwa myezi	Okwekenenya obubonero bwakafuba

mukaaga nga omaliriza obujjanjabi bwa kafuba(emyezi nga kumi ne ebiri bukya webygira mu kunonyereza)	
Okukukubira essimu oluvanyuma lwa myezi munaana nga omaliriza obujjanjabi bwa kafuba(emyezi nga kumi neen bukya webygira mu kunonyereza)	Okwekenenya obubonero bwakafuba
Okukukubira essimu oluvanyuma lwa myezi kumi nga omaliriza obujjanjabi bwa kafuba(emyezi nga kumi namukaaga bukya webygira mu kunonyereza)	Okwekenenya obubonero bwakafuba
Okukukubira essimu oluvanyuma lwa myezi kumi neebiri omaliriza obujjanjabi bwa kafuba(emyezi nga kumi namunaana bukya webygira mu kunonyereza)	Okwekenenya obubonero bwakafuba
Okukukubira essimu oluvanyuma lwa myezi kumi neena omaliriza obujjanjabi bwa kafuba(emyezi nga amakumi abiri bukya webygira mu kunonyereza)	
Olukyala oluvanyuma lw'emyezi kumi neetaano nga omaliriza obujjanjabi bwa kafuba.(Emyezi nga amakumi abiri mu gumu bukya webygira mu kunonyereza)	Olukalala lw'ebibuzo Okugibwako omusayi(miilu 50-65) Okukebera oba amawugwego gakola bulungi Diffusion Lung Capacity Okutuwa ekikolondolwa Okupima obuttwa mu Omukka gwossa (PM_{25})
Okkukubira essimu buliluvanyuma lwamyezi ebiri nga olukyaala olwa buli mwaka telunatuuka	Okwekenenya obubonero bwakafuba
Olukyaala ku dwaliliro lumu buli mwaka okumala enyaaka etaano	Olukalala lw'ebibuzo Okugibwako omusayi(miilu 50-65) Okukebera oba amawugwego gakola bulungi Diffusion Lung Capacity Okutuwa ekikolondolwa Okupima obuttwa mu Omukka gwossa (PM_{25})

Ekipande ky'omusomo gwa abatayina akafuba

Olukyaala	Ebikolwa
Olunaku lwokirizza okweetaba mumusomo	O Olukalala lw'ebibuzo Okugibwako omusayi(Miilu 50-65) Okutuwa ekikolondolwa Okwooza mu mawugge no kuyisa ka bulaasi mu mawugwe Okutuwa empitambi(stool) Enkwaaso mu basajja abayina akuwuka ka siliimu(Kino Kya bweyagalile) Amalusu
Okukukubira essimu n' kumyezi 2 bukya okirizza okweetaba mumusomo	Okwekenenya obubonero bwakafuba
Olukyaala ku dwaliro emyeezi 4 bukya lwokirizza okweetaba mumusomo	O Olukalala lw'ebibuzo Okugibwako omusayi(Miilu 50-65) Okutuwa ekikolondolwa Okukebera oba amawugwego gakola bulungi Diffusion Lung Capacity Okupima obuttwa mu Omukka gwossa (PM_{25}
Okukukubira essimu n'okulondolwa kumyezi 6 bukya okirizza okweetaba mumusomo	Okwekenenya obubonero bwakafuba
Okukukubira essimu n'okulondolwa kumyezi 8 bukya okirizza okweetaba mumusomo	Okwekenenya obubonero bwakafuba
Olukyaala ku dwaliro emyeezi 10 bukya okirizza okweetaba mumusomo	O Olukalala lw'ebibuzo Okugibwako omusayi(Miilu 50-65) Okutuwa ekikolondolwa Okukebera oba amawugwego gakola bulungi Diffusion Lung Capacity Okupima obuttwa mu Omukka gwossa (PM_{25}
Okukukubira essimu n'okulondolwa kumyezi 12 bukya okirizza okweetaba mumusomo	Okwekenenya obubonero bwakafuba
Olukyaala ku dwaliro emyeezi 16 bukya lwokirizza okweetaba mumusomo	O Olukalala lw'ebibuzo Okugibwako omusayi(Miilu 50-65) Okutuwa ekikolondolwa Okukebera oba amawugwego gakola bulungi Diffusion Lung Capacity Okupima obuttwa mu Omukka gwossa (PM_{25}
Okukukubira essimu n'okulondolwa kumyezi 18 bukya okirizza okweetaba mumusomo	Okwekenenya obubonero bwakafuba
Okukukubira essimu n'okulondolwa kumyezi 18 bukya okirizza okweetaba mumusomo	Okwekenenya obubonero bwakafuba
Olukyaala ku dwaliro emyeezi 28 bukya lwokirizza okweetaba mumusomo	O Olukalala lw'ebibuzo Okugibwako omusayi(Miilu 50-65) Okutuwa ekikolondolwa Okukebera oba amawugwego gakola bulungi Diffusion Lung Capacity Okupima obuttwa mu Omukka gwossa (PM_{25}
Okkukubira essimu buliluvanyuma lwamyezi ebiri nga olukyaala olwa buli mwaka telunatuuka	Okwekenenya obubonero bwakafuba
Olukyaala ku dwaliliro lumu buli mwaka okumala enyaaka etaano	Olukalala lw'ebibuzo Okugibwako omusayi(miilu 50-65) Okukebera oba amawugwego gakola bulungi Diffusion Lung Capacity

	Okupima obuttwa mu Omukka gwossa (PM_{25}

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SCHOOL OF MEDICINE
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OKUFUNA OBUVUNE: Bwonofuna obuvune olwo okweetaba mu kunonyereza kuno ojja kufuna obujjanjabi bwobuvune bwo amangu ddala. Oyina okubulila abakozi bo kunonyereza kuno bwoba owulila ofunye obuvune. Oyinza okubabulila mu buntu oba kuba essimu eziri wagulu ku kiwandiiko kino. Obujjanjabi obuweebwa abalwadde Mulago Hospital oba ku China-Uganda Friendship Hospital-Naguru bujja kuberawo ku bwerere oba bwekinaaba nga kisobose tujja kukozesa ku sente ezinaaba ziriwo Makerere University nabateeka ensimbi mu kunonyereza kuno tebatera kusasulira buvune bwonna.

EBIBUUZO: Osobola okwogera nomusawo akola kunonyereza kuno yenna ng'olina ky'omubuuza oba ebyo by'olabye mukunonyereza kuno ng'obakubira ku ssimu ezitereddwa ku muko ogusooka ogwekiwandiiko kino. Bwewabaawo engeri eddembe lyo gy'elikwatibwako olw'okwetaba mukunonyereza kuno, osobola okutukirira Prof. Ponsiano Ocama Ssentebe wa kakiiko keby'okunonyereza aka Makerere University (SOMREC) ku ssimu 0772421190 oba 0414-530-020 mu ddwaliro e Mulago oba Dr Fredrick Nakwagala ssentebbe waakakiiko akakola ku byokunonyereza mu dwaliro lye Mulago (MREC) kussimu 256-41554008 oba akakiiko akakola ku byokunonyereza mubantu (CHR) ak'ettendekero ekulu ely'e California (UCSF). obukiiko buno bukolanga obulondoozi bw'okunonyereza kuno era n'olwekyo bwebukwatibwako okuuma banakyewa mu kunonyereza kuno.

EDDEMBE LYO; Okwetaba mukunonyereza kuno kusalawo kwo. Osobola okulondawo okwetabamu oba obutetabaamu. Bwosalawo okwetabamu, osobola okuvaamu obudde bwonna. Bwosalawo obutetabamu , tewali kibonerezo kyofuna era tojja kufiirwa byolina kufuna muddwaliro. Era tusobola okuwandukulula erinya lyo mukunonyereza ekiseera kyona wadde nga gwe tokirizza. Okuwanduka mu kunonyereza tekijja kukosa byabujjanjabi bwo. Obeera okyasobola okufuna obujjanjabi mu dwaliro e Mulago oba mu dwaaliro eliyitibwa China-Uganda Friendship Hospital-e Naguru. Tojja kusasulibwa oba okusasulwa olwokwetaba mukunonyereza kuno. Tujja kubulira ebipya oba enkyukakyuka eziyinza okosa obulamu bwo oba okiriza okweyongerayo mu kunonyereza. Bwolumizibwa olwokunonyereza kuno, tolina dembe lyo mateeka lyofiirwa okusasulwa olwokuteeka omukono ku kiwandiiko kino.

OKUSAASANYA EBIVUDE MUKONONYEREZA: Tujja kukubulira ebipya ebizuuse wo bwe biba nga biyinza okuyuusa embeera yo byobulamu bwo oba okukyuusa mu kusalawoo ko okwetaba mu kunyonyereza kuno.Bwoba ofunye obuvune olwokwetaba mu kunonyereza kuno tojja kujibwaako ddembe lyo okuliyiribwa olwo kuteeka omukono ku kiwandiko kino.

OKUKIRIZIBWA KWO KUNONYEREZA KUNO: Okunonyereza kuno kukirizidwa mumateeka olukiiko oluyitibwa School of Medicine Research and Ethics Committee (SOMREC) , Mulago Research and Ethics Committee (MREC) ne University of Carlifonia San-Fransisco Committee on Human Research era bano bebajja okukwakitbwaako okulaba nga abeetabye mu kunonyereza kuno tebatusibwako bubi mubugenderevu.

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.....ambulidde byonna ebigenda okukolebwa, ebibi ebiyinza okuvaamu ne ebirungi beyinza okufunamu ne ddembe lyange lyenina elikwatagana no kunonyereza kuno. Ntegedde nti okusalawo kwange okwetaba mukunonyereza kuno tekujja kukyuusa obujjanjabi bwange obwabulijjo. Mukukozesa ebivudde mu kunonyereza amanya gange bijja kukwekebwa. Manyi nti ddembe lyange okusalawo okuva mu kunonyereza kuno. Nkimanyi nti okussa ekinkumu oba omukono gwange ku ka foomu kano tekitegeeza nti mpadeyo eddembe lyange nayye kitegeeza nti nyinyonyoddwa ku kunonyereza kuno kwenzikiriza okwetabamu kyeyagalire. Kopi eya ka foomu kano ejja kumpeebwa

Elinya lya nakyeewa	**Omukono gwo / Ekinkumu**	**Enaku zomweezi**
Erinya ly'omuntu awereddwa	**Omukono gw'omuntu aweredwa**	**Enaku zomweezi**

*. Singa anonyerezebwako aba tasobola kusoma oba okuwandiika, walina okubawo omujulizi atalina luda naga omulwadde annyonyolwa.Oluvanyuma lw'ekiwandiiko kyokukiriza okusomerwa n'okunyonyolwa anonyerezebwako, era nga atadeko omukono oba ekinkumu omujulizu naye alina okutekako omukono era ye kennyini natekako enaku z'omwezi. Mukutekako omukono omujulizi akiriza nti ebiri mukiwandiiko kino nebirala byona ebiwandikiddwamu binyonyoddwa mubutufu era bitegereddwa anonyerezebwako, nti era awadeyo okukiriza kwe mu dembe awatali kukakibwa.

Erinya ly'omujulizi	**Omukono gw'omujulizi**	**Enaku zomweezi**

MAKERERE UNIVERSITY
SCHOOL OF MEDICINE
APPROVED
VALID UNTIL
15 JUL 2021
RESEARCH & ETHICS COMMITTEE
P.O.BOX 7072, KAMPALA

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.....ambulidde byonna ebigenda okukolebwa, ebibi ebiyinza okuvaamu ne ebirungi beyinza okufunamu ne ddembe lyange lyenina elikwatagana no kunonyereza kuno. Ntegedde nti okusalawo kwange okwetaba mukunonyereza kuno tekujja kukyuusa obujjanjabi bwange obwabulijjo. Mukukozesa ebivudde mu kunonyereza amanya gange bijja kukwekebwa. Manyi nti ddembe lyange okusalawo okuva mu kunonyereza kuno. Nkimanyi nti okussa ekinkumu oba omukono gwange ku ka foomu kano tekitegeeza nti mpadeyo eddembe lyange nayye kitegeeza nti nyinyonyoddwa ku kunonyereza kuno kwenzikiriza okwetabamu kyeyagalire. Kopi eya ka foomu kano ejja kumpeebwa

Elinya lya nakyeewa	**Omukono gwo / Ekinkumu**	**Enaku zomweezi**
Erinya ly'omuntu awereddwa	**Omukono gw'omuntu aweredwa**	**Enaku zomweezi**

*. Singa anonyerezebwako aba tasobola kusoma oba okuwandiika, walina okubawo omujulizi atalina luda naga omulwadde annyonyolwa.Oluvanyuma lw'ekiwandiiko kyokukiriza okusomerwa n'okunyonyolwa anonyerezebwako, era nga atadeko omukono oba ekinkumu omujulizu naye alina okutekako omukono era ye kennyini natekako enaku z'omwezi. Mukutekako omukono omujulizi akiriza nti ebiri mukiwandiiko kino nebirala byona ebiwandikiddwamu binyonyoddwa mubututu era bitegereddwa anonyerezebwako, nti era awadeyo okukiriza kwe mu dembe awatali kukakibwa.

Erinya ly'omujulizi	**Omukono gw'omujulizi**	**Enaku zomweezi**

MAKERERE UNIVERSITY
SCHOOL OF MEDICINE
APPROVED
VALID UNTIL
15 JUL 2021
RESEARCH & ETHICS COMMITTEE
P O BOX 7072, KAMPALA

UNIVERSITY YA CALIFONIA, SAN FRANCISCO, MULAGO HOSPITAL, MAKERERE UNIVERSITY, OKUKIRIZA OKWETABA MU KUNOONYEREZA KWA MIND

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OKWANJULA: Kuno kunonyereza kwa kisawo nga kusasulirwa etendekero ekulu elye Makerere University ne university ye Califoniya, San Francisco. Ndi mukwanaganya wa byakunonyereza era nja kunnyonnyola ku kukunonyereza kuno. E'siimu zaffe ziri, 0757247007, 0752900230 ne 0773834149 Tukusaba okwetabemu kubanga olina akawuka ka mukenenya oba oli mukubeza musayi kulaba oba olina akawuka kamukenenya oba olina obubonero bwa lubyamira (obulwadde bwamawwuggwe).
Twala obudde bwo okusaalawo oba onetaba mukunonyereza kuno. Bwoba olina by'obuuza, oyinza okumbuuza oba okubuuza benkola nabo.

EKIGENDERERWA;

- Okunonyereza kuno kukolebwa okusobola okuyiga ku buzibu bwa lubyamira, okusobola okuzula lubyamira amangu ate nokusobola okutebereza ebiva mu ku jjanjaba. Abantu nga 1100 bebajja okwetaba mukunonyereza kuno. Okunonyereza kuno kusasulirwa National Institute of Health mu America. Bwoba okirizza tukujjeko era n'okutereka ebikeberebwa by'o bikozesebwe mukunonyereza mu biseera by'omumaaso, bino wamanga bye bijja okubaawo; Oluvanyuma lwokukebera okwabulijo okwetagisa ku bulamu bwo oba ku lwe ekinonyerezebwako nga kuwedde, ebinaba bisigaddewo ku bikujjidwako bijja kuterekebwa ku lwo kunonyereza okwomumaaso okwa mukenenya,endwadde zomu mawugwe ne ndwadde enddala eziggwa mu kiti e'kyo.Tojja kuffuna binaava mu kunonyereza kuno. Era tujja na kutereka ebikwataku bulamubwo bye tunaba tukujjeko. Tetumanyi kukeberwa kwa ngeri ki okugenda okukolebwa kubi kujjidwako mu kunonyereza kuno okwo mu maaso. Okunonyereza kuno okwomumaaso kuyinza okubaamu okukebera okwebyobuzaliranwa(olulyo). Okunonyereza kuno kuyinza okuletawo okulakulanya ebyamaguzi, omuli eddagala epya, okukebera,obujjanjabi oba ebintu ebirala. Tojja kusasuzibwa ate era tojja kusasulibwa olwe ebintu bino. Abayambako mukunonyereza kuno (Joseph Kovacs, Steven Fischer, ne Henry Masur) bakuggu kubyedembe ly'okunonyereza mu America era bbo bayinza okubiganyurwamu. Gwe, n'aba kujjanjaba tewali ajja kuffuna bivudde mu kunonyereza kuno okwo mumaaso okukoledwa kubikujjidwako okeberebwa.

OKUWAYO NO KUTEREKA SAMPOLO ZE EBIKEKEREBWA

Ebikujjidwako okeberebwa, ne bikukwatako bijja kuweebwa enamba, era biterekebwe mukifo ekyekusifu mu tendekero ekulu elye Makerere ne lye UCSF. Ebikujjidwako okeberebwa bijja kuterekebwa mukifo ekyekusifu ekiyitibwa (Specimen Repository) awaterebwa ebijjidwa kubalwadde abanonyerezebwako ekikolera wansi wa National Institute of Health (NIH) ku lwo kunonyereza okwa mawugwe ne mukenenya era.Ebikujjidwako obikeberebwa bijja kuterekebwa luberera. Bwonosalawo nti toyagala ebikujjidwako nebikukwatako bikozesebwe mu kunonyereza okwe biseera byomumaaso, bambi tubulire esaawa yona, era tujjabisanya'awo. Ebikwata kubulamu bwo, tebijja kwongera kukozesebwa mu kunonyereza nga gwe oba akakiiko aka londozi tekakirizza.Akakiiko kajja kukirizza abantu abatendeke boka okukozesa ebikujjidwako okukeberebwa.

CONFIDENTIALITY: Tuyinza okuwa abononyerezi abalala oba kampani abatakola ku UCSF. en ebikwata ku byobulamu bwo nga (emyaka oba oyina pulesa) naye tetujja kukozesa linya lyo, jyobeera oba enamba yessimu oba ekintu kyonna ekiyinza okukozesebwa okukumanya .Ebinaaba bivudde mu kunonyereza kuno tebijja kukuweebwa.

OBUZIBU OBUYINZA OKUKUTUKAKO OLWO KUWAAYO EBIKEBEREBWA BYO. Okuwayo byetugenda okkebera kiyinza okuvamu okufulumya ebyama, naye'bikukwatako byona tujja bikumira mukyama nga bwe kisoboka. Tujja kuwa enamba enekusifu gye tunateka kubikwata kubyobulamu bwo ne byotuwadde bikeberebwa. Ekisumuluzo wakumirwa enamba eno enekusifu kijja kuteekebwa mukifo ekyekusifu.Amanya go tegajja kozesebwa mu binafulumizibwa byona okuva mukunonyereza kuno nga tukozesa ebikwata kubyobulamubwo, ne byo tuwadde okukeberebwa. Abakozi abanonyereza bo basobola okumanya ebikwatako naye tebajja kufulumya kintu kyona ekikukwatako eri ababala wadde kubanonyereza abakozesa ebikeberebwa byo. Abanalondola okunonyereza kuno abali mu UCSF ne mu ttendekero ekullu elye Makerere bayinza okulaba ebikukwatako okusobola okukakasa nti ebyama byo bikumiddwa bulungi.

BYOGANYULWAMU: Tewali kyoganyulwamu buterevu olwo kukiriza okutereka byotuwadde okeberebwa, ne'bikozesebwa mukunonyereza kwo mumaaso. Wabula, tusubira nti tujja kuyigamu ebintu ebinatuyamba okujjanjaba abalwadde gye bujja. Bewaba nga waliwo ebizuliddwa mu kunonyereza kuno nga biyinza okuba ebyettunzi tojja kuganyula mu bizuulidwa bino.

ENKOLA ENDALA: Bwosalawo obutawaayo ebikeberebwa byo, omusayi ogunasigalawo, amazzi agomumubiri,obunyama obukusaliddwako, obutetagisa mukuzuula bino bijja kusulibwa. Okusalawo obutawaayo ebikeberebwabyo, tekilina we kikosezza bya bujjanjabi bwo.

EBIBUUZO: Osobola okwogera nomusawo akola kunonyereza kuno yenna ng'olina ky'omubuuza oba ebyo by'olabye mukunonyereza kuno ng'obakubira ku ssimu ezitereddwa ku muko ogusooka ogwekiwandiiko kino. Bwewabaawo engeri eddembe lyo gy'elikwatibbwako olw'okwetaba mukunonyereza kuno, osobola okutukirira Ssentebe wa kakiiko keby'okunonyereza aka Makerere University (SOMREC) ku ssimu 0414-530-020 mu dwaliro e Mulago oba akakiiko ake byokunonyereza ku bantu ake UCSF (CHR) ku namba ye simu 0001-415-476-1814.

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.....ambulidde byonna ebigenda okukolebwa, ebibi ebiyinza okuvaamu ne ebirungi beyinza okufunamu ne ddembe lyange lyenina elikwatagana no kunonyereza kuno. Ntegedde nti okusalawo kwange okwetaba mukunonyereza kuno tekujja kukyuusa obujjanjabi bwange obwabulijjo. Mukukozesa ebivudde mu kunonyereza amanya gange gajja kukwekebwa. Manyi nti ddembe lyange okusalawo okuva mu kunonyereza kuno. Nkimanyi nti okussa ekinkumu oba omukono gwange ku ka foomu kano tekitegeeza nti mpadeyo eddembe lyange nayye kitegeeza nti nyinyonyoddwa ku kunonyereza kuno kwenzikiriza okwetabamu kyeyagalire. Kopi eya ka foomu kano ejja kumpeebwa.

Ebikeberebwa/ebinziggidwako byange bisobola okuterekebwa ku lwokunonyereza okusobola okuyiga, okuziyiza no kujjanjaba endwadde zo mu mawugwe.

YEE	NEDA

Ebikeberebwa/ebinziggidwako byange byembawadde biyinza okozesebwa mu kunonyereza okwobuzalirwana okusobola okuyiga, obuzibu obuli mu ndwadde zo mu mawugwe.

YEE	NEDA

.....	.....	.....
Erinya lya Nnakyewa	**Omukono gwa nnakyewa / Ekinkumu**	**Enaku z'omwezi**

.....	.....	.....
Erinnya ly'omuntu awereddwa okukiriza	**Omukono gwo'muntu awereddwa okukiriza**	**Enaku z'omwezi**

Singa anonyerezebwako aba tasobola kusoma oba okuwandiika, walina okubawo omujulizi atalina luda naga omulwadde annyonyolwa. Oluvanyuma lw'ekiwandiiko kyokukiriza okusomerwa n'okunyonyolwa anonyerezebwako, era nga atadeko omukono oba ekinkumu omujulizu naye alina okutekako omukono era ye kennyini natekako enaku z'omwezi. Mukutekako omukono omujulizi akiriza nti ebiri mukiwandiiko kino nebirala byona ebiwandikiddwamu binyonyoddwa mubututu era bitegereddwa anonyerezebwako, nti era awadeyo okukiriza kwe mu dembe awatali kukakibwa.

.....	.....	.....
Elinya lyo Omujulizi	**Omukono gw'omujulizi**	**Enaku z'omwezi**

(Nnakyewa bwaba tasobola kusoma ne/ oba okuwandiika)

KUKKIRIZA

.....ambulidde byonna ebigenda okukolebwa, ebibi ebiyinza okuvaamu ne ebirungi beyinza okufunamu ne ddembe lyange lyenina elikwatagana no kunonyereza kuno. Ntegedde nti okusalawo kwange okwetaba mukunonyereza kuno tekujja kukyuusa obujjanjabi bwange obwabulijjo. Mukukozesa ebivudde mu kunonyereza amanya gange gajja kukwekebwa. Manyi nti ddembe lyange okusalawo okuva mu kunonyereza kuno. Nkimanyi nti okussa ekinkumu oba omukono gwange ku ka foomu kano tekitegeeza nti mpadeyo eddembe lyange nayye kitegeeza nti nyinyonyoddwa ku kunonyereza kuno kwenzikiriza okwetabamu kyeyagalire. Kopi eya ka foomu kano ejja kumpeebwa.

Ebikeberebwa/ebinziggidwako byange bisobola okuterekebwa ku lwokunonyereza okusobola okuyiga, okuziyiza no kujjanjaba endwadde zo mu mawugwe.

YEE	*NEDA*

Ebikeberebwa/ebinziggidwako byange byembawadde biyinza okozesebwa mu kunonyereza okwobuzalirwana okusobola okuyiga, obuzibu obuli mu ndwadde zo mu mawugwe.

YEE	*NEDA*

.....	.....	.....
Erinya lya Nnakyewa	**Omukono gwa nnakyewa / Ekinkumu**	**Enaku z'omwezi**

.....	.....	.....
Erinnya ly'omuntu awereddwa okukiriza	**Omukono gwo'muntu awereddwa okukiriza**	**Enaku z'omwezi**

Singa anonyerezebwako aba tasobola kusoma oba okuwandiika, walina okubawo omujulizi atalina luda naga omulwadde annyonyolwa. Oluvanyuma lw'ekiwandiiko kyokukiriza okusomerwa n'okunyonyoiwa anonyerezebwako, era nga atadeko omukono oba ekinkumu omujulizu naye alina okutekako omukono era ye kennyini natekako enaku z'omwezi. Mukutekako omukono omujulizi akiriza nti ebiri mukiwandiiko kino nebirala byona ebiwandikiddwamu binyonyoddwa mubututu era bitegereddwa anonyerezebwako, nti era awadeyo okukiriza kwe mu dembe awatali kukakibwa.

.....	.....	.....
Elinya lyo Omujulizi	**Omukono gw'omujulizi**	**Enaku z'omwezi**

(Nnakyewa bwaba tasobola kusoma ne/ oba okuwandiika)

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO, MULAGO HOSPITAL, MAKERERE UNIVERSITY, CONSENT TO PARTICIPATE IN A RESEARCH STUDY

The MIND-IHOP Study

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Background and Rationale of the study: Respiratory infections are a leading cause of death in Africa, especially among Human Immunodeficiency Virus (HIV)-infected patients, and the lack of understanding of host and pathogen biology constitutes a major barrier to developing new management approaches for improving outcomes. Over the past 4 years, rapid, noninvasive tests and strategies have been validated for the diagnosis of tuberculosis (TB), yet substantial improvements in mortality have yet to be realized. This study aims at understanding the biological principles underlying human-microbial interactions in patients with respiratory illness so as to increase possibility of improving current approaches to care.

This is a medical research study, sponsored by Makerere University and the University of California, San Francisco. This study is funded by the National Institute of Health. I am a research officer with this study and will explain the study to you. You can reach us on the following numbers 0757247007, 0752900230 or 0773834149.

PURPOSE: This study is being done to learn about risks for developing pneumonia disease, about diagnosing pneumonia more easily, and about predicting the results of treatment. About 3300 people will take part in this study. We are asking you to participate because you either have or are being tested for HIV and you have symptoms of pneumonia (a lung infection). Take your time to decide whether to participate. If you have questions, you may ask any team member or me.

Participation will take 5years. Participation on the day of enrollment will take about 2 hours 50 minutes and the follow-up phone call will take about 10 minutes. If you chose to come in for follow-up visits, each follow-up visit will last approximately 60 minutes.

PROCEDURES

- **Interview, respiratory exam, and review of your medical record:** We will ask you some questions about your background and about your illness. You may refuse to answer certain questions (if the questions make you feel uncomfortable) and still participate. I will examine your respiratory system, and review your medical record for the results of your physical examination and lab tests.
- **Chest x-ray:** We will take your chest x-ray and after we will photograph your chest x-ray using a digital camera so that we can keep an image of your chest x-ray.

- **Sputum collection:** We will collect sputum samples from you today. Before collecting the sputum sample, we may ask you to rinse your mouth with sterile water. We will check if you have tuberculosis (TB) and we may ask you to breathe a saltwater mist in order to produce sputum. If you are found to have TB, we may invite you to provide additional sputum specimens, if you wish.

- **HIV counseling and testing**: Counselors from the Routine Counseling and Testing Service will visit you and will share your HIV result with you and us.

- **Blood draw:** We will collect 4.5 tablespoons of blood during your hospital admission (3.5 tablespoons at enrollment and one tablespoon if you undergo a clinical bronchoscopy). It will be used to identify causes of lung disease and measure your CD4 T-cell count, which may help your doctors' take better care for you. At follow-up visits, we will collect approximately 3.5-4 tablespoons of blood. If you are found to have TB, we may invite you to provide additional blood specimens, if you wish.

- **Bronchoscopy:** If your doctors decide it is necessary, and if you agree, they will do a test called bronchoscopy to wash out your lungs to diagnose your pneumonia.

  If you chose to undergo a bronchoscopy, you will be treated with medications to help dilate your airways, relax you, and decrease mucous production during the procedure. You will inhale some of these medications while others will be given to you by intramuscular injection.

  On the night prior to bronchoscopy please don't eat or drink after 10PM. If you typically take your medications in the morning, you can do so on the morning of the bronchoscopy with a small sip of water. If you use inhalers regularly to help you breathe, or have a history of asthma or chronic obstructive pulmonary disease, you will be administered intravenous medications to decrease airway inflammation and given additional medications via inhalation to dilate your airways before the procedure. Your vital signs will be measured and monitored before, during, and after the bronchoscopy. Before and during the procedure we will provide you with medications to decrease anxiety and to numb your mouth, throat, and airways.

  During the bronchoscopy we will place a flexible fiber optic bronchoscope in your mouth and advance it into your airways. We will instill sterile salt water into a part of your lungs and then remove the salt water by suction. The instillation of fluid can be repeated up to 5-6 times. We will pass protected tiny brushes along your lower part of your lobe bronchus to pick specimen for testing by the bronchoscopist during the procedure Six times.

  After the bronchoscopy, we will monitor you until the medications wear off and we feel that you are well enough to go home.

- **Oral wash collection**: If you need bronchoscopy we will ask to collect oral wash specimens from you. We will ask you to gargle two teaspoons of salt water for 60 seconds, swish for 5 seconds, and spit it into a laboratory cup. Then we will gently scrape your tongue using a tongue scraper.

- **Stool collection:** We will ask you to provide a stool specimen. We will ask you to place a paper collection device onto your toilet seat just prior to having a bowel movement. Once you deposit the stool into the device, you will wear rubber gloves and collect the sample into a plastic cup using a scoop. Once the stool has been placed in the plastic cup, please take care to seal it tightly. If you agree, we will store your specimen for future tests.

  Semen collection: Our results in our previous studies show that there is an infection called Cytomegalo virus (CMV) among male HIV infected persons which may be responsible for development of abnormal lung function. The CMV is very stable in semen and can be best tested using semen. We will therefore request you if you accept to provide us with semen. We know this procedure is very sensitive and therefore this procedure will be entirely OPTIONAL for all HIV+ males and does not affect your participation in the rest of study procedures. If you qualify and you are okay to provide us with your semen, we will instruct you to collect Semen from home by masturbation after at least 36hours of not having sexual intercourse prior collection of the sample. You

will be requested to collect the sample and bring it to hospital within at least 4hours of collection time. If you anticipate a delay to bring the sample to hospital of more than 4hours, we will request you to refrigerate the specimen and bring it within 4 hours of collecting it from the refrigerator. On day of enrollment our study nurse will give you specific instructions on how to collect the specimen, and there after you will be offered a secure cap tight container which you will go with home before returning with sample on a scheduled visit.

- **Food and GI Questionnaire:** If you choose to provide a stool specimen, we will ask you a short questionnaire about your overall gastrointestinal health, normal dietary habits, and recent food and liquid consumption. This will be used to help us analyze the stool specimen.

- **Follow-up:** If you were diagnosed with TB and did not undergo bronchoscopy on day of enrollment, we will call you by phone or if we cannot reach you by phone, will use an alternative phone number given by you to us at the time of enrollment. We will contact you so that we can assess your status every 2 months from the time of enrollment. If you are diagnosed with TB you will be asked to return to hospital for a follow-up visit in at 2weeks ,2montth and 4month after enrollment, these will coincide the clinic TB drug refills visits and the month you complete treatment (usually six months after your initial treatment). Thereafter, you will be asked to return to hospital for a follow-up visit at 3 months after treatment, (usually nine months from time you start treatment), and 15 months after treatment (usually at 21 months after your initial treatment). Thereafter you will be invited to return for hospital follow up visits once annually for each of the next five years .If you are not diagnosed with TB you will be asked to return to hospital for a follow-up visit at 4 months, 10 months, 16 months and 28 months after enrollment. Thereafter you will be invited to return for hospital follow up visits once annually for each of the next five years We will ask you some questions during these hospital visits and you will asked to undergo the following:

    - **Blood draw:** We will collect several tablespoons of blood on the day of follow-up. If you are diagnosed with TB we will collect approximately 50-65 mL of blood today, for each follow up visit. If you are not diagnosed with TB we will still collect approximately 50 mL of blood today for each follow up visit. If the doctor decides that we should wash your lungs (we call it a clinical bronchoscopy), we will collect another 15mL of blood on the day we do the procedure.

    - **Questionnaires:** You will be asked questions about yourself, your current symptoms and your medical history; similar to those asked for regular medical care. In addition, personal questions about cigarettes, alcohol, and recreational/illicit drug use will also be asked in the questionnaire.

    - **Spirometry:** We will perform a breathing test called spirometry to find out how well you move air in and out of your lungs. You will blow air into a tube connected to a machine (spirometer). The breathing test will be repeated after giving you a breathing medicine (e.g., Albuterol, Salbutamol) to open up your airways to find out if this medicine helps you breathe better. You will be asked to repeat this test several times to get an accurate measure of your breathing. We will do this procedure together with another test which measures ability of your lungs to transfer air you have breathed in to your red blood cells (RBCs). This test is called Diffusion Lung Capacity.
    - **Personal $PM_{2.5}$ exposure:** You will be invited to hospital 48hrs before the scheduled follow up visits and you will be provided with an small device called Ultrasonic Personal Air Samplers to measure air pollution in your home environment. You will be provided with transport refund for both your scheduled visit and any time you are invited to hospital to collect this device. You will be trained on how to wear the personal samplers before going back home with the device. You will be asked to bring back the device at your scheduled study follow up visit date. If You were infected with TB at time of enrollment, you will be undergo the above procedure during follow up visits of end of treatment, 3 and 15 months after TB treatment completion and there after follow up visits of every one year after the last visit for each of the next five years . If You were not infected with TB at time of enrollment, you will be undergo the above procedure during follow up visits at 4 months, 10 months, 16 months and 28 months after enrollment

RISKS AND SIDE EFFECTS

- **Bronchoscopy**: You may cough or gag or become short of breath. You may have a fever a few hours after the procedure. You may get stomach acid into your lungs, bleed from your lungs, or have one of your lungs collapse, but these complications are unlikely. It is also unlikely that you would lose your ability to breathe for yourself or die as a result of this procedure.

- **Stool Collection:** Stool should be handled with gloved hands at all time. Direct contact between stool and ungloved broken skin may lead to infection. Some people, particularly those sensitive to odor, develop nausea and occasionally vomiting.

- **Semen collection**: There are no physical risks to semen collection, but some effects include: embarrassment, nervousness, mild anxiety, increased sweating and increased heart rate.

- **Sputum induction, gargling, tongue scraping, & mouthwash**: You may feel short of breath during these tests. You may gag or feel nauseated. Please tell us if you have side effects during the study.

- **Blood draw:** You may feel some pain from the needle going through your skin. There may be some darkening at the site after, but if you have prolonged pain, warmth, or tenderness tell your doctors. If your blood count is low, you may feel weak after the procedure. We will check your blood count and if your blood count appears low, we will avoid drawing blood.

- **Chest x-ray:** When photographing your chest x-ray we will make sure that personal details such as your name are removed and that there is no way to identify you based on the chest x-ray. The photograph of your chest x-ray will be stored on a password-protected computer.

- **Spirometry:** You might have some difficulty in breathing during and after the procedure, although this is usually temporary. You might also have cough, chest pain, shortness of breath, or you might feel faint.

We will give you a medication for spirometry to find out if this medicine helps you breathe better:

- **Albuterol (or Salbutamol, depending on availability):** This medication may make you feel anxious, flushed or tremulous. You may get a strange taste or feel dryness in your mouth. You may feel pain in your chest and have an increased blood pressure, or a heart attack.

BENEFITS: You will receive some diagnostic tests free of charge that may help your physicians better care for you. Moreover, this study may help doctors learn more about causes of lung infections, and we hope this information will help in the future diagnosis and treatment of patients with HIV and pneumonia. Some of the tests are experimental, and we will only know the results at the end of the study. Therefore, these results will not be communicated to you, and will not affect your care. However, if you wish to know about the overall advances made through this research, please inform us and we will notify you.

CONFIDENTIALITY: We will collect personal information but only the people working on the study will see it. We will assign a code to your information. The key to the code will be stored in a safe place. Your name will not be used in any published reports from research using your health information. Research staff will have access to information about you but they will not release any identifying information about you to others, even to researchers using your specimens. Genetic tests may have additional risks if they are used to identify personal health information that you do not wish to be known. To prevent this, we will not allow studies on topics other than HIV, lung disease, and related diseases. To make sure the project follows good research practices, the Makerere University School of Medicine Research Ethics Committee, the Mulago Hospital Institutional Review Board, the UCSF Committee on Human Research, or the Ugandan National Council for Science and Technology may look at or copy records that show your name or study number. We will comply with all laws that protect your confidentiality. A certificate of confidentiality has been obtained to further protect your information from government inquiries.

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ALTERNATIVES: You may undergo a standard test for your condition without being in this study. You may ask your doctor about your choices before deciding whether to participate in this study.

COMPESATION FOR PARTICIPATION IN THE STUDY:. You will not be financially compensated for participation in the study or your hospital admission, but You will receive 70,000 if we have selected you and accepted to undergo a bronchoscopy and the associated procedures on that day. . You will receive 30,000UGX for completing of scheduled follow-up visits without undergoing a bronchoscopy and the associated procedures. We will transfer 5,000 UGX electronically into your mobile phone account for each phone call. Here is a summary of the procedures per visit:

Longitudinal Follow-Up for TB Infected Subjects Not Undergoing Research Bronchoscopy

Visit	Procedures
Enrollment	Questionnaires Blood draw (50-65ml) Sputum collection Stool and Saliva Semen for HIV+ males (OPTIONAL)
2weeks after enrollment visit	Sputum collection
2 months visit after enrollment	Blood draw(5ml) Sputum collection
4 month	Blood draw(5ml) Sputum collection
Completion of TB therapy (usually at 6 months)	Blood draw (50-65ml) Questionnaires Spirometry/ Diffusion Lung capacity measurement Sputum Measurement of ***Personal $PM_{2.5}$ exposure***
Phone call 2 months after completion of TB therapy (usually at 8 months)	Check to see if you have symptoms of TB
Visit 3 months after completion of TB therapy (usually at 9 months)	Blood draw (50-65ml) Questionnaires Spirometry/Diffusion Lung capacity measurement Sputum Measurement of ***Personal $PM_{2.5}$ exposure***
Phone call 4 months after completion of TB therapy (usually at 10 months)	Check to see if you have symptoms of TB
Phone call 6 months after completion of TB therapy (usually at 12 months)	Check to see if you have symptoms of TB
Phone call 8 months after completion of TB therapy (usually at 14 months)	Check to see if you have symptoms of TB
Phone call 10 months after completion of TB therapy (usually at 16 months)	Check to see if you have symptoms of TB
Phone call 12 months after completion of TB therapy (usually at 18 months)	Check to see if you have symptoms of TB
Phone call 14 months after completion of TB therapy (usually at 20 months)	Check to see if you have symptoms of TB
Visit 15 months after completion of TB therapy (usually at 21 months)	Blood draw (50ml-65ml) Questionnaires

	Spirometry/Diffusion Lung capacity measurement Sputum collection Measurement of ***Personal $PM_{2.5}$ exposure***
Phone Call every after 2 month till the next annual visit	Check to see if you have symptoms of TB
Annual Hospital visit 1year after the previous visit for each of the next five years.	Questionnaires Blood draw(50ml-65ml) Spirometry /Diffusion Lung capacity measurement. Measurement of ***Personal $PM_{2.5}$ exposure***

Longitudinal Follow-Up for Non-TB Infected Subjects

Visit	Procedures
Enrollment	Questionnaires Blood draw (50-65ml) Sputum collection Bronchoscopy (if requested) and bronchial brushes Stool collection Saliva Semen for HIV+ males (OPTIONAL)
Phone call or home visit 2 months after enrollment	Check to see if you have symptoms of TB
4 month visit after enrollment	Blood draw (50ml-65ml) Questionnaires Spirometry/Diffusion Lung capacity measurement Measurement of ***Personal $PM_{2.5}$ exposure***
Phone call 6 month after enrollment	Check to see if you have symptoms of TB
Phone call 8 month after enrollment	Check to see if you have symptoms of TB
10 month visit after enrollment	Blood draw (50ml) Questionnaires Spirometry/Diffusion Lung capacity measurement Measurement of ***Personal $PM_{2.5}$ exposure***
Phone call 12 months after enrollment	Check to see if you have symptoms of TB
16 month visit after enrollment	Blood draw(50ml-65ml) Questionnaires Spirometry/Difussion Lung capacity measurement Measurement of ***Personal $PM_{2.5}$ exposure***
Phone call 18 months after enrollment	Check to see if you have symptoms of TB
Phone call 20 months after enrollment	Check to see if you have symptoms of TB
28 month visit after enrollment	Blood draw (50ml-65ml) Questionnaires Spirometry/Diffusion Lung capacity measurement

	Measurement of ***Personal $PM_{2.5}$ exposure***
Phone Call every after 2 month till the next annual visit	Check to see if you have symptoms of TB
Annual Hospital visit 1year after the previous visit for each of the next five years.	Questionnaires Blood draw(50ml-65ml) Spirometry/Diffusion Lung capacity measurement Measurement of ***Personal $PM_{2.5}$ exposure***

Injuries: If you are injured as a result of being in this study, you will receive immediate treatment for your injuries. You must tell the study team if you feel that you have been injured. You can tell any of them in person or call them at the telephone numbers listed on this consent form. The usual treatments offered at Mulago Hospital or China-Uganda Friendship Hospital-Naguru will be available. Care will be provided free of charge, if possible, for protocol-related injuries using available funds. Makerere University and the study sponsor do not normally provide any other form of compensation for injury.

QUESTIONS: You can talk to any of the study doctors about any questions or concerns you have about this study, by calling them at the telephone numbers listed on the first page of this consent form. If you have any concerns about your rights as a research participant while in this study, you should contact the Prof. Ponsiano Ocama, Chairman of the School of Medicine Research and Ethics Committee (SOMREC), at 0772421190 or 0414-530-020 at Mulago Hospital, Dr Fredrick Nakwagala the Chairman of Mulago Research and Ethics Committee (MREC) at 256-41554008 Mulago Hospital Complex, or the UCSF Committee on Human Research (CHR), at 0001-415-476-1814. SOMREC, MREC and UCSF CHR serve as institutional review boards for this study and are thus concerned with the protection of volunteers in research projects.

STATEMENT OF VOLUNTARINESS: Taking part in this study is your choice. You may choose either to take part or not to take part. If you decide to take part, you may leave the study at any time. If you decide not to take part, there will be no penalty to you and you will not lose any of your regular benefits. We may also terminate your enrollment in the study at any time, even without your consent. Leaving the study will not affect your medical care. You can still get medical care at Mulago Hospital or China-Uganda-Friendship Hospital-Naguru. You will not be charged for taking part in this study, nor will you be paid for being in the study.

DISSEMINATION OF THE STUDY RESULTS: We will tell you about new information or changes that may affect your health or willingness to continue in the study. If you are injured because of this study, you do not lose any of your legal rights to seek payment by signing this form.

ETHICAL APPROVAL: This study has been approved by an accredited School of Medicine Research and Ethics Committee (SOMREC) and Mulago Research and Ethics Committee (MREC) and UCSF CHR and are thus concerned with the protection of volunteers in research projects.

CONSENT

.....has described to me what is going to be done , the risks and benefits involved, and my rights regarding this study. I understand my decision to participate in this study will not alter any usual medical care. In the use of this information, my identity will be concealed. I am aware that I may withdraw anytime. I understand that by signing this form I do not waive any legal rights but merely indicate that I have been informed about the research study in which I am voluntarily agreeing to participate. A copy of this form will be provided to me.

Name of Participant (Print)	Participant's Signature or Thumbprint*	Date
Person Obtaining Consent (Print)	Signature of Person Obtaining Consent	Date

*If the participant is unable to read and/or write, an impartial witness should be present during the informed consent. After the written informed consent form is read and explained to the participant, who has either signed the consent form or provided a thumbprint, the witness should sign and personally date the consent form. By signing, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by, the participant and that informed consent was freely given by the participant.

Person Witnessing Consent (Print)	Signature of Person Witnessing Consent	Date

CONSENT

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UNIVERSITY OF CALIFORNIA, SAN FRANCISCO, MULAGO HOSPITAL, MAKERERE UNIVERSITY, CONSENT TO DONATE SPECIMENS FOR FUTURE RESEARCH

The MIND-IHOP Study

INVESTIGATORS

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Background and Rationale of the study: Respiratory infections are a leading cause of death in Africa, especially among Human Immunodeficiency Virus (HIV)-infected patients, and the lack of understanding of host and pathogen biology constitutes a major barrier to developing new management approaches for improving outcomes. Over the past 4 years, rapid, noninvasive tests and strategies have been validated for the diagnosis of tuberculosis (TB), yet substantial improvements in mortality have yet to be realized. This study aims at understanding the biological principles underlying human-microbial interactions in patients with respiratory illness so as increase possibility of improving current approaches to care .

This is a medical research study, sponsored by Makerere University and the University of California, San Francisco (UCSF). I am a research officer with this study and will explain the study to you. You can reach us on the following numbers 0757247007, 0752900230 and 0773834149. We are asking you to participate because you either have or are being tested for HIV and you have symptoms of pneumonia (a lung infection). Take your time to decide whether to participate. If you have questions, you may ask me or any team member.

PURPOSE. If you agree to let us collect and store your specimens for future research, the following will happen. After all routine tests required for your care or for the proposed research question are finished, we will save leftover specimens for possible future research on HIV, lung disease and other related diseases. You will not receive results from these studies. We will also save medical information that we collect from you.

We do not know which tests will be performed on your samples in these future studies. The future research on your specimens may include genetic testing. Genetic information (also known as genotype data) and the medical record data (also known as phenotype data) may be shared broadly in a coded form for future genetic research or analysis

SAMPLE STORAGE: Your specimens and information will be coded using a study number, and stored in secure facilities at Makerere University and UCSF. Your specimens will also be stored in a secure facility called a specimen repository under contract to the National Institutes of Health (NIH) for the Lung HIV Study and Lung HIV Microbiome Project. Your specimens will be kept indefinitely. If you decide you do not want us to use your specimens and information for future research, please tell us at any time, and we will destroy them. Your health information cannot be used for additional research without approval from either you or a review committee, which will allow only qualified individuals to use your specimens.

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CONFIDENTIALITY: We may give certain medical information about you (for example, diagnosis, blood pressure, age if less than 85) to other scientists or companies not at UCSF, including to a (public or controlled access) government health research database, but we will not give them your name, address, phone number, or any other identifiable information. Research results from these studies will not be returned to you.

RISKS OF SPECIMEN DONATION. Donating your specimens may involve a loss of privacy, but we will handle information about you as confidentially as possible. We will assign a code to your medical information and specimens. The key to the code will be stored in a safe place. Your name will not be used in any published reports from research using your medical information or specimens. Research staff will have access to information about you but they will not release any identifying information about you to others, even to researchers using your specimens. Personnel reviewing the study at UCSF and Makerere University may see information about you to help ensure that your confidentiality is adequately protected.

As with any use of electronic means to store data, there is a risk of breach of data security. Genetic information that results from this study does not have medical or treatment importance at this time. However, there is a risk that information about taking part in a genetic study may influence insurance companies and/or employers regarding your health. Taking part in a genetic study may also have a negative impact or unintended consequences on family or other relationships. It is possible that future research could one day help people of the same race, ethnicity, or sex as you. However, it is also possible through these kinds of studies that genetic traits might come to be associated with your group. In some cases, this could reinforce harmful stereotypes.

BENEFITS. There will be no direct benefit to you from allowing your specimens to be kept and used for future research. However, we hope we will learn something that will help in treatment of future patients. If the data or any new products, tests or discoveries that result from this research have potential commercial value, you will not share in any financial benefits. The research may also lead to the development of commercial products, including new drugs, tests, treatments, or products. There will be no charge to you, but you will not receive any payment from these products. Co-investigators (Joseph Kovacs, Steven Fischer, and Henry Masur) are inventors on US patents for some assays under study and may receive royalties from them. Neither you nor your healthcare provider will receive any results from the future research done on your specimens

ALTERNATIVES. If you choose not to donate your specimens, any leftover blood, body fluids, and/or tissue removed during your surgery that is not needed for diagnosis will be thrown away. Deciding not to donate specimens will not affect your medical care.

QUESTIONS. You can talk to any our study staff Ingvar Sanyu about any questions or concerns you have about this study, by calling them at the telephone numbers listed on the first page of this consent form. If you have any concerns about your rights as a research participant while in this study, you should contact the Prof. Ponsiano Ocama,Chairman of the School of Medicine Research and Ethics Committee (SOMREC), at 0772421190 or 0414-530-020, or Dr Fredrick Nakwagala the Chairman of Mulago Research and Ethics Committee (MREC) at 256-41554008 Mulago Hospital Complex or Uganda National Council of Science and Technology(UNCST) on 041-705500 or the UCSF Committee on Human Research (CHR), at 0001-415-476-1814.

CONSENT

.....has described to me what is going to be done , the risks the benefits involved and my rights regarding this study. I understand my decision to participate in this study will not alter any usual medical care. In the use of this information my identity will be concealed. I am aware that I may withdraw anytime. I understand that by signing this form I do not waive any legal rights but merely indicate that I have been informed about the research study in which I am voluntarily agreeing to participate. A copy of this form will be provided to me. Please answer below.

My specimens may be stored for research to learn about, prevent, or treat infections or lung diseases.

YES	NO

My specimens may be used for genetic studies to learn about risk factors for lung disease.

YES	NO

Name of Participant (Print) Participant's Signature or Thumbprint* Date

Person Obtaining Consent (Print) Signature of Person Obtaining Consent Date

*If the participant is unable to read and/or write, an impartial witness should be present during the informed consent. After the written informed consent form is read and explained to the participant, who has either signed the consent form or provided a thumbprint, the witness should sign and personally date the consent form. By signing, the witness attests that the information in the consent form and any other written information was accurately explained to, and apparently understood by the participant and that informed consent was freely given by the participant.

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My specimens may be stored for research to learn about, prevent, or treat infections or lung diseases.

YES	NO

My specimens may be used for genetic studies to learn about risk factors for lung disease.

YES	NO

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Person Witnessing Consent (Print) Signature of Person Witnessing Consent Date

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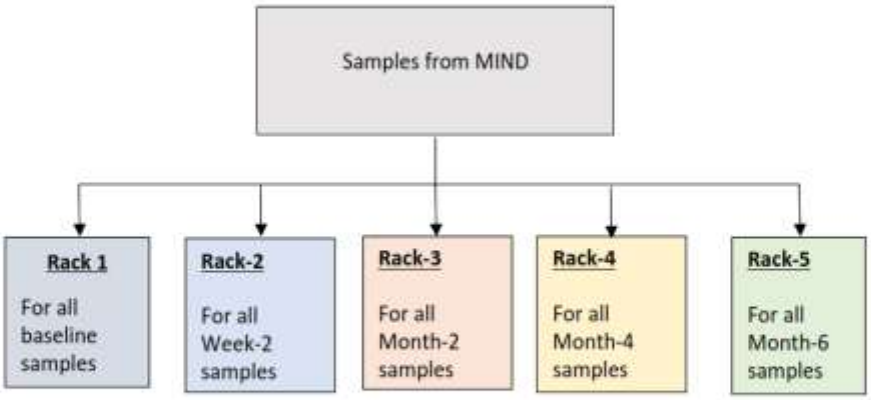
Appendix II: Bench top protocols and data capture forms

SHORT PROTOCOL FOR EXTRACTION OF Mtb. RNA



NOTES

- Pick samples from the same rack for the day
- Confirm visit (i.e. baseline, week-2, month-2, month-4 or month-6)
- List the selected IDs in the table below

#	Extraction Date	Rack Number	Id	Staff Initials	Comments
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
15					
16					
17					
18					
19					
20					

1

Short version of Mtb RNA extraction protocol
Emmanuel Musisi
10th July 2020

Checklist for Mtb. RNA extraction

- ☐ Biosafety cabinet
- ☐ Thawed samples
- ☐ Thawed internal controls
- ☐ Tissue paper (Kitchen towel)
- ☐ Permanent marker
- ☐ Lab book
- ☐ DNAse enzyme, Turbo DNA-free, AM1907M, Invitrogen
- ☐ Chloroform
- ☐ RNAse away (10666421, Fisher Scientific)
- ☐ Absolute ethanol (99-100%)
- ☐ Ice cold 70% ethanol
- ☐ TB disinfectant (Tristle Fuse),
- ☐ Molecular grade water (RNAse and DNAse free, eg. 13138533, Fisher Scientific)
- ☐ Sterile RNase-free microtubes (1.5ml),
- ☐ FASTRNA Pro blue kit with homogenization beads from MP biomedicals
- ☐ Vortex (eg Megafuge 16, Heraeus)
- ☐ Refrigerated microcentrifuge for 1.5ml and 2 ml homogenization tubes
- ☐ Homogeniser (Precelleys 24, Peqlab set at 6000rpm, 40s)
- ☐ Thermomixer with thermal block for 1.5 ml microtubes
- ☐ Freezer (-20^{0}C)
- ☐ Freezer (-80^{0}C)
- ☐ Pipettes and sterile filtered pipette tips that are DNAse and RNAse free, Range: P1000, P200, P10 and P2
- ☐ Sterile pasture pipettes (2 ml)
- ☐ Safety goggles that are chemical resistant
- ☐ Chemical waste discard jar
- ☐ 500 ml plastic containers
- ☐ Racks for 1.5 ml and 2 ml microtubes and for 15 ml falcon tubes
- ☐ Electronic pipette with matching pipette tips (10ml tips)

RNA extraction Protocol (DAY-1). Tick the left box for each step done

- ☐ Thaw samples and extraction control (EC) on ice
- ☐ Label same number of homogenization tubes with glass beads. Label both lids and tubes
- ☐ Add 100µl of EC directly into the sample, mix by inverting 20 times. Change tips/sample
- ☐ Spin at 3000g for 30 minutes, carefully tip off GTC into 15 ml falcon tube for recycle
- ☐ Use a fine pipette tip to remove all the GTC. Avoid the pellet as much as possible
- ☐ Resuspend pellet in 950 µl lysis buffer. Use P1000
- ☐ Transfer the resuspended pellet to lysing matrix beads. Use P1000
- ☐ Bead beat at 6000rpm for 40 s (Homogenization)
- ☐ Spin at 12,000g for 5 minutes at RT
- ☐ Incubate tube at RT for 5 minutes
- ☐ Transfer all the sup to 300 µl of chloroform in microcentrifuge tube. Use fine pipette
- ☐ Vortex for 10 s
- ☐ Incubate at RT for 5 minutes
- ☐ Spin at 12,000g for 5 minutes
- ☐ Transfer sup to labelled microcentrifuge tubes. Use 200 µl filter tip
- ☐ Add 500 µl of ice-cold absolute ethanol
- ☐ Keep at -20°C for overnight

Continuation of RNA extraction short protocol (Day-2)

- ☐ Chill the microcentrifuge at 4°C
- ☐ Spin at 13000g for 20 minutes
- ☐ Discard the sup using a fine pipette tip
- ☐ Add 500 µl of ice-cold absolute 70% ethanol
- ☐ Spin at 13000g for 10 minutes at 4°C
- ☐ Discard the supernatant
- ☐ Dry RNA at 50°C in a heat-hot block for 30 minutes
- ☐ Dissolve the extracted RNA in 100 µl of RNAase free water
- ☐ Re-suspend by vortexing for 5s
- ☐ Can store at -80°C

DNAse treatment on the same day 2

- ☐ Prepare master mix as follows:
- ☐ Add 10 µl (10X) DNase buffer, and 2 µl DNAse enzyme to a sterile eppendorf. Eg, for a 20-samples master mix will contain 200 µl (10X) buffer and 40 µl DNase enzyme
- ☐ Add 12 µl of DNAse master mix to each sample
- ☐ Vortex for 5 s
- ☐ Spin at 13,000g for 10s
- ☐ Incubate at 37°C for 30 minutes in the heat hot block
- ☐ Add 1 µl of DNAse enzyme to each tube. Pipette up and down three times
- ☐ Incubate at 37oC for 30 minutes in the heat hot block
- ☐ Thaw DNase inactivation buffer (10 minutes before)
- ☐ Add 10 µl of DNAse inactivation reagent
- ☐ Vortex three times every after five minutes
- ☐ Spin at 13,000g for 2 minutes
- ☐ Transfer 110 µl of the supernatant to a new clean centrifuge tube
- ☐ Store at 4°C for same day, or -80oC for long-term

Preparation of probes and primers notes

- Primers and probes are lyophilised and stable at room temperature
- Make stock primer per manufacturers instruction
- Make stock probe per manufacturers instruction

Preparation of Primer mix for the Internal Control (total volume=200µl)

Given	**Use the formula; $V_1C_1=V_2C_2$**
Stock concentration = 100µM Final concentration = 10µM Final volume = 200µl Stock volume of the **Internal Control** primers = Y **volume**	Y. 100µM = 200µl. 10µM Y = 200µl. 10µM / 100 µM **Y= 20 µl < ------- Stock**
So; Add 20µl of **Internal Control** stock **forward primer** **20**µl of **Internal Control** stock **reverse primer** **160** µl of RNAse free molecular grade water	

Preparation of Primer mix for the Mtb 16s rRNA (total volume=200µl)

Given	**Use the formula; $V_1C_1=V_2C_2$**
Stock concentration = 100µM Final concentration = 10µM Final volume = 200µl Stock volume of the **Mtb 16s rRNA** primers = Y **volume**	Y. 100µM = 200µl. 10µM Y = 200µl. 10µM / 100 µM **Y= 20 µl < ------- Stock**
So; Add 20µl of **Mtb 16s rRNA** stock **forward primer** **20**µl of **Mtb 16s rRNA** stock reverse **primer** **160** µl of **RNAse free molecular grade water**	

Preparation of the probes for the Internal Control

Given	**Use the formula; $V_1C_1=V_2C_2$**
Stock concentration = 100µM Final concentration = 20µM Final volume = 100µl Stock volume of the **Internal Control** probe = Y **volume**	Y. 100µM = 100µl. 20µM Y = 200µl. 10µM / 100 µM **Y= 20 µl < ------- Stock**
So; Add 20µl of stock **Internal Control** probe **80** µl of **RNAse free molecular grade water**	

1

Preparation of the probes for the Internal Control

Given	**Use the formula; $V_1C_1=V_2C_2$**
Stock concentration = 100µM Final concentration = 20µM Final volume = 100µl Stock volume of the **Mtb 16s rRNA** probe = Y **Y= 20 µl < ------- Stock volume**	Y. 100µM = 100µl. 20µM Y = 200µl. 10µM / 100 µM
So; Add 20µl of stock **Mtb 16s rRNA** probe **80** µl of **RNAse free molecular grade water**	

Preparation of RT-qPCR master mix (We use QuantiTec multiplex RT-PCR NR kit (QT))

- This mix contains reverse transcriptase enzyme
- Aliquot 200 µl of the mix into 1.5 ml RNAse free microcentrifuge tubes, store at -25^{0}C

PCR MASTER MIX PREPARATION (Y=Total number of reaction)

Master Mix	**Volume per RT+ reaction**	**Volume per RT- reaction**	**Total vol. for Y number of RT+ reactions**	**Total vol. for Y number of RT- reactions**
Quantitec mix	10	10	--	--
Mtb 16S primer mix (F+R)	0.4	0.4	--	--
IC primer mix (F+R)	0.4	0.4	--	--
Mtb 16S-FAM probe	0.2	0.2	--	--
IC probe	0.2	0.2	--	--
RT enzyme	0.2	0	--	--
RNAs free water	4.6	4.8	--	--
Total volume	**16µl**	**16µl**	**--**	**--**

Extra volumes

Number of samples		
Reactions per sample		
Additional volume		
Positive control		
Negative control		

REAGENTS PREPARATION ROSTA

PREPARATION DATE	VOLUME	STAFF INITIALS

TB-MBLA MICROBIOLOGY SMEAR RESULTS LOG

	BASELINE			WEEK-2			MONTH-2			MONTH-4			MONTH-6		
MIND-ID	MICRO ID	Date	Result	MICRO ID	Date	Result	MICRO ID	Date	Result	MICRO ID	Date	Result	MICRO ID	Date	Result
4780															
4781															
4782															
4783															
4784															
4785															
4786															
4787															
4788															
4789															
4790															
4791															
4792															
4793															
4794															
4795															

		TB-MBLA MGIT RESULTS						
		BASELINE						
MIND-ID	MICROB ID	ZN	BA	ID	TT	Final Result	Final Rslt date	Comments
4719								
4720								
4721								
4722								
4723								
4724								
4725								
4726								
4727								
4728								
4729								
4730								
4731								
4732								
4733								
4734								
4735								
4736								
4737								

		TB-MBLA MGIT RESULTS						
		WEEK-2 FOLLOW-UP						
MIND-ID	MICROB ID	ZN	BA	ID	TT	Final Result	Final Rslt date	Comments
4719								
4720								
4721								
4722								
4723								
4724								
4725								
4726								
4727								
4728								
4729								
4730								
4731								
4732								
4733								
4734								
4735								
4736								

		TB-MBLA MGIT RESULTS						
		MONTH-2 FOLLOW-UP						
MIND-ID	MICROB ID	ZN	BA	ID	TT	Final Result	Final Rslt date	Comments
4719								
4720								
4721								
4722								
4723								
4724								
4725								
4726								
4727								
4728								
4729								
4730								
4731								
4732								
4733								
4734								
4735								
4736								
4737								

		TB-MBLA MGIT RESULTS						
		MONTH-4 FOLLOW-UP						
MIND-ID	MICROB ID	ZN	BA	ID	TT	Final Result	Final Rslt date	Comment(s)
4719								
4720								
4721								
4722								
4723								
4724								
4725								
4726								
4727								
4728								
4729								
4730								
4731								
4732								
4733								
4734								
4735								
4736								
4737								

		TB-MBLA MGIT RESULTS						
		MONTH-6 FOLOOW-UP						
MIND-ID	MICROB ID	ZN	BA	ID	TT	Final Result	Final Rslt date	Comments
4710								
4711								
4712								
4713								
4714								
4715								
4716								
4717								
4718								
4719								
4720								
4721								
4722								
4723								
4724								
4725								
4726								
4727								
4728								

	TB-MBLA LJ CULTURE RESULTS			
	BASELINE			
ID	MicroB ID	Date P/N	Rslt	Comments
4397				
4398				
4399				
4400				
4401				
4402				
4403				
4404				
4405				
4406				
4407				
4408				
4409				
4410				
4411				
4412				
4413				
4414				
4415				

	TB-MBLA LJ CULTURE RESULTS			
	WEEK 2			
ID	MicroB ID	Date P/N	Rslt	Comments
4397				
4398				
4399				
4400				
4401				
4402				
4403				
4404				
4405				
4406				
4407				
4408				
4409				
4410				
4411				
4412				
4413				
4414				
4415				

	TB-MBLA LJ CULTURE RESULTS			
	MONTH 2			
ID	MicroB ID	Date P/N	Rslt	Comments
4397				
4398				
4399				
4400				
4401				
4402				
4403				
4404				
4405				
4406				
4407				
4408				
4409				
4410				
4411				
4412				
4413				
4414				
4415				
4416				

	TB-MBLA LJ CULTURE RESULTS			
	MONTH 4			
ID	MicroB ID	Date P/N	Rslt	Comments
4397				
4398				
4399				
4400				
4401				
4402				
4403				
4404				
4405				
4406				
4407				
4408				
4409				
4410				
4411				
4412				
4413				
4414				
4415				

	TB-MBLA LJ CULTURE RESULTS			
	MONTH 6			
ID	MicroB ID	Date P/N	Rslt	Comments
4397				
4398				
4399				
4400				
4401				
4402				
4403				
4404				
4405				
4406				
4407				
4408				
4409				
4410				
4411				
4412				
4413				
4414				
4415				

MIND STUDY-TB-MBLA

FORM 3.6 TB-MBLA SPECIMEN COLLECTION DATA & RESULTS (TMBSCD) FORM

1. Which visit is this?	0 Days (0) 2Weeks (2) 2 Months (3) 4 Months (4) 6 Months (5) 9 Months (6)				**1.**	
2. TB-MBLA Sample (SSM-B) Collected?	No (0) Yes, Expectorated (1) Yes, Induced (2)				**2a.**	
b. Date of collection	DAY	MONTH			**2b.** YEAR	
c. Time of collection (Hrs.: Min)					**2c.** |_|_|_|_|_|	
d. Sputum characteristic	Salivary (0) Mucoid (1) Purulent (2) Blood stained (3)				**2d.**	
e. Time sputum added to GTC					**2e** |_|_|_|_|_|	
f. DFM Smear Results	Negative (0) Positive scanty (1-29 AFB/30 fields) (1) Positive 1+ (30-299AFB per 30 fields) (2) Positive 2+ (10-100 AFB per field) (3) Positive 3+(>100 AFB per field (4) NOT DONE (9)				**2f.**	
3a. Paxgene Sample (PAX-1) collected	Yes (0) No (1)				**3a.**	
b. Date of paxgene collection	DAY	MONTH			**3b.** YEAR	
c. Time of Paxgene collection **(Hrs.: Min)**					**3c.** |_|_|_|_|_|	
(Trial 1 is filled in a; Do Trial 2 if Trial 1 is indeterminate, and is filled in b)					**Trial 1**	**Trial 2**
4.Xpert MTB result	MTB Negative (0) MTB Positive (1) Indeterminate (invalid/error/no result) (2) Not Done (3)				**4a.**	**4b.**
5. Semi-quantitative result	Trace (0) Very low (1)		Low (2) Medium (3) High (4)		**5a.**	**5b.**
6. Xpert Rifampin resistance result	No Resistance (0) Resistance (1) Indeterminate (2)				**6a.**	**6b.**
7. CT Values	**7a.** SPC	**7b.** IS1081	**7c.** IS6110	**7d.** rpoB1	**7e.** rpoB2	**7f.** rpoB3	**7g.** rpoB4
8. Specify Error type, if any	**8a.**	**8b.**	**8c.**	**8d.**	**8e.**	**8f.**	**8g.**

1

Form 3.6 Version 1.
By: Musisi Emmanuel

TBMBLA HIV test results

9. Date of blood collection (for HIV tests)	DAY	MONTH	**9.** YEAR
b. If HIV test done, what was the result? (If Negative, STOP)	Negative (0) Positive (1)		**9b.**
10. CD4 Count (only if patient HIV-positive)			**10.**
11. CD8 Count (only if patient HIV-positive)			**11.**

TB-MBLA CFM and MGIT results

12. CFM results date	DAY	MONTH	**12.** YEAR
13. CFM FM Smear Result	Negative (0) Positive Scanty (1-19 AFB/length) (1) Positive 1+ (20-199 AFB/length) (2) Positive 2+ (5-50 AFB/field) (3) Positive 3+ (>50 AFB/field) (4) Not Done (9)		**13.**
14. Final MGIT result date	DAY	MONTH	**14.** YEAR
15. MGIT result (If Negative or Not done, STOP)	Negative (0) Positive (1) NTM (2) Contaminated (6) Not done (9)		**15.**
a. ZN smear result	Negative (0) Positive (1) Not done (9)		**15a.**
b. Capilia result	Negative (0) Positive (1) Not applicable (2) Not done (9)		**15b.**
c. SD Bioline -MPT64 result	Negative (0) Positive (1) Not applicable (2) Not done (9)		**15c.**
d. Blood agar result	Negative (0) Positive (1) Not done (9)		**15d.**
16. Positive MGIT result time (by machine)	Time |__|__|:__|__| days hrs.		**16.**

TB-MBLA SPUTUM LJ CULTURE RESULT

17. Result Date:	DAY	MONTH	**17.** YEAR
18. Result	NEGATIVE (0) +/-: 1-9 CFU (1) 1+: 10-100 CFU (2) 2+: >100-200 CFU (3) CONFLUENT GROWTH: > 200 CFU (4) CONTAMINATED (6) NTM (7) NOT DONE (9)		**18.**
a. If +/- (1-9 CFU), specify exact number of colonies.	_____CFU		**18a.**

Accuracy of Tuberculosis Molecular Bacterial Load Assay to diagnose and monitor response to anti-tuberculosis therapy: a longitudinal comparative study with standard-of-care smear microscopy, Xpert MTB/RIF Xpert-Ultra, and culture

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Running title: TB-MBLA is an accurate tool for monitoring TB treatment response.

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5. School of Chemistry, University of St Andrews, Scotland, UK
6. Medical And Molecular Laboratories (MML), Kampala, Uganda
7. Kilimanjaro Clinical Research Institute, Kirimanjaro, Tanzania
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41 **Abstract**

42 **Background:** Tuberculosis (TB) takes long to treat, and thus requires effective tools to monitor
43 treatment response and guide clinical decision making. Tuberculosis-Molecular Bacterial Load
44 Assay (TB-MBLA), a ribosomal RNA based test was evaluated for accuracy to diagnose and
45 monitor treatment response.

46 **Methods:** Presumptive TB cases were enrolled into diagnostic arm and tested for TB by TB-
47 MBLA in comparison with Xpert MTB/RIF Ultra (Xpert-Ultra) and smear microscopy (SM)
48 using Mycobacteria Growth Indicator Tube (MGIT) culture as a reference test. Xpert-Ultra-
49 positive cases were enrolled on anti-TB therapy and monitored for treatment response at
50 weekly-monthly intervals until month-6 and then three months after treatment.

51 **Findings:** 210 participants, median age 35 years (IQR: 27-44), 135 (64%) males, 72 (34%)
52 HIV positive were enrolled. The sensitivities of TB-MBLA and Xpert-Ultra were similar, 99%
53 but different specificities, 91% and 78% respectively. Ten participants were Xpert-Ultra trace-
54 positive, 80% of whom were negative by TB-MBLA and MGIT culture. SM had lower
55 diagnostic sensitivity, 75% but higher specificity 98%. Among SM-negative and HIV-positive
56 participants, TB-MBLA and Xpert-Ultra sensitivities were 92% and 100% respectively. 129
57 participants were enrolled into the treatment follow-up arm. Positivity for TB dropped with
58 treatment in all tests, but the rate was slower with Xpert-Ultra. Consequently, 33% of
59 participants were still Xpert-Ultra positive at the end of treatment but were clinically well at 3-
60 months post-treatment. Rate of conversion-to-negative of DNA-based Xpert-Ultra was 3·3-
61 fold slower than the rRNA-based TB-MBLA. TB-MBLA-measured bacillary load, among
62 month-2 SM-positive cases who received extra month of intensive treatment was not different
63 from SM negative cases.
64

Interpretation: TB-MBLA is consistent with Xpert-Ultra for pre-treatment diagnosis of TB but is more accurate for treatment response monitoring than Xpert-Ultra and standard-of-care SM.

Funding: European & Developing Countries Clinical Trials Partnership, Makerere University Research and Innovation fund, National Institutes of Health.

Research in context

Evidence before this study: Through PubMed database search, we used the terms "Xpert Ultra" or "MTB/RIF", "TB-MBLA", "culture", "two-months smear", and "tuberculosis" to identify eight scientific articles about Xpert MTB/RIF and Xpert MTB/RIF Ultra (Xpert-Ultra) assays, six about TB-MBLA, and one about the two months smear result. Reviewed articles were published in English between 2011 and 2020. Results from the reviewed articles showed that Xpert MTB/RIF was sensitive and remained positive in most patients over the course of treatment. Furthermore, a more sensitive new version of the Xpert-Ultra was released and it was being adopted by the TB control programmes as a standard of care for TB diagnosis. Literature further revealed that smear microscopy (SM) which is the standard-of-care for treatment response has low sensitivity and may not distinguish live from dead bacilli following initiation of treatment. The more sensitive culture takes long to yield results making it less useful for early treatment decision making. We, therefore, set out to evaluate TB-MBLA as a potential accurate alternative for the diagnosis and TB treatment response monitoring, with ability to give timely results to inform clinical decisions.

Added value: This is the first study that has compared, a ribosomal RNA-based assay (TB-MBLA) against a DNA-based assay (Xpert-Ultra) for pre-treatment diagnosis of TB and monitoring treatment response over a six-months period. As a result, we have demonstrated

89 the utility of TB-MBLA for accurate diagnosis of TB, and further confirmed the limitation of
90 Xpert-Ultra, and SM for monitoring TB treatment response. Our study has revealed that Xpert-
91 Ultra is even more persistently positive with 33% of the cases still positive at completion of
92 treatment compared to 27% reported for the older version, Xpert MTB/RIF. On the other hand,
93 SM sensitivity was low and turned negative faster than clinical positivity and other
94 bacteriological measures following the initiation of treatment. Analysis of the month-2 SM
95 positive- versus SM negative- cases but positive with TB-MBLA revealed that SM was
96 insufficient to inform an extra month of intensive treatment. In contrast, TB-MBLA positivity
97 resolves in a manner that is consistent to MGIT culture and clinical signs. This makes TB-
98 MBLA with laboratory turn-around time of four hours, a more suitable tool for monitoring
99 response to anti-tuberculosis therapy.

100 **Implications of all the available evidence:** Xpert-Ultra is a very sensitive tool suitable for
101 pre-treatment diagnosis of TB but not for treatment response monitoring. SM is less sensitive
102 hence less reliable in providing treatment response guidance. This finding adds more evidence
103 to support the new WHO strong recommendation not to extend intensive treatment phase of
104 drug susceptible TB based on smear positive result at the end of the 2-month intensive phase.
105 Furthermore, the study has shown that TB-MBLA closely mirrored MGIT culture in measuring
106 treatment response, but more importantly TB-MBLA results are quantitative and available
107 faster (in hours) to inform clinical decision-making compared to days/weeks by culture. We
108 have further shown that a proportion of patients, 23% (30/129), particularly with low pre-
109 treatment bacillary load converted-to-negative by day-14 and remained negative throughout
110 treatment follow-up, an indication some patients may not need a six-month treatment course.

111

112

Introduction

Globally, 10 million people were estimated to have developed TB in 2020, and 30% of them were projected to have missed diagnosis or not notified to the national authorities.[1] In 2018, the United Nations high-level meeting on tuberculosis, the political heads at local and international levels committed to support endeavours towards improving TB diagnosis and treatment.[1] Subsequently, a 9% reduction in TB incidence and a 14% drop in TB-related deaths were noted between 2015 and 2019, but this progress was too slow to achieve the set goals.[1] Timely diagnosis and treatment to reduce TB-related morbidity and mortality therefore justify the demand for more suitable tools.

Smear microscopy is the most common tool for TB diagnosis because it is fast and affordable, but it requires significant training to perform well. Morever, it is observer dependent, and cannot differentiate between dead and viable *Mycobacterium tuberculosis* (Mtb), and between Mtb complex and non–Mtb. Smear microscopy is associated with low sensitivity, especially among people living with HIV and those with low bacterial loads such as those on treatment hence limiting its applicability.[2–5] Culture is considered to be the optimal confirmatory test for TB diagnosis and a reference for other TB tests, yet it has several limitations: it is slow and requires an expensive high containment laboratory.[6,7] Molecular-based assays have the potential to solve the challenges that are experienced by these conventional TB diagnostic methods since they are usually fast and reproducible, are not compromised by contamination, and are proven to be highly specific and sensitive for Mtb.[8,9]

A molecular-based test, Xpert MTB/RIF was recommended by WHO to improve the diagnosis of TB and rifampicin resistance, but Xpert MTB/RIF targets DNA which is not cleared rapidly

after Mtb is killed, which means that there is a high chance of giving false positive results among patients on treatment.[10]

Xpert MTB/RIF Xpert-Ultra (Xpert-Ultra), a modified version of the Xpert MTB/RIF, is now recommended as an initial TB diagnostic and rifampicin resistance test in all adults and children with signs and symptoms of pulmonary TB. However, trace call positive cases are not clinically conclusive since rifampicin resistance is usually indeterminate hence requiring a second run. In cases where the results of the first and second runs are discordant, clinical decision making becomes difficult and potentially delay clinical decisions.[11]

Tuberculosis-Molecular bacterial load assay (TB-MBLA) is a novel RNA-based assay that detects and quantifies viable Mtb bacilli in sputum.[12] In 2018, TB-MBLA was noted to be a biomarker with potential to replace smear and culture for monitoring TB treatment response by the WHO.[13] The TB-MBLA targets the abundant Mtb16S ribosomal RNA specifically and the test is highly sensitive.[14] Previously, TB-MBLA was evaluated as a treatment monitoring tool on samples that were already confirmed TB positive by other tests such as SM and or Xpert-Ultra, which made it impossible to calculate its specificity, and predictive values. TB-MBLA has potential for routine TB testing but supporting data is needed.

In this study, we evaluated TB-MBLA for its diagnostic performance and monitoring response to treatment among adult individuals who were presumed to have pulmonary TB in comparison with standard-of-care tests for tuberculosis.

METHODS

Ethics: The project was approved by the the University of St Andrews Teaching and Research committee (UTREC) approval [Approval code: MD14702], Makerere University School of Medicine Research and Ethics committee [REC REF No. 2006-017] and Makerere University School of Biomedical Sciences Research and Ethics Committee [REC REF No: SBS 529] respectively. Before enrolment, participants consented to utilising their biological samples and clinical data for this study. All study related activities were conducted according to the guideline outlined in the Good Clinical and Laboratory Practice manual.[15]

Participants and study design: We conducted a longitudinal prospective study utilising spot sputa that were obtained from individuals who were being investigated, and later treated for pulmonary tuberculosis (PTB). This sub-study was nested within the I AM OLD (Inflammation, Aging, Microbes, and Obstructive Lung Disease) cohort of persons with pneumonia at Naguru Referral Hospital, Kampala, Uganda for the period between 2019 and 2022.[16] Adult participants (≥18 years of age) who were coughing for at least two weeks with/without fever, night sweats, and weight loss; with/without symptoms of extrapulmonary tuberculosis were enrolled. For details on patient identification, data collection and information flow (supplementary Figure S1)

Study specimens: All participants were invited for visits to the study and requested to provide two spot sputa, which were pooled and homogenised at enrolment (week 0), and then at weeks 2, 8, 17 and 26 after the initiation of TB treatment. Homogenised sputa were aliquoted into 4 portions, each 1 mL and tested using Xpert MTB/RIF Xpert-Ultra (Xpert-Ultra), smear microscopy (SM), and Mycobacteria Growth Indicator Tube (MGIT) culture tests. Aliquots for TB-MBLA were preserved in Guanine thiocyanate and stored at -20°C until the assay was

performed. Xpert-Ultra was used as the standard of care for tuberculosis and a basis for treatment initiation.

Importantly, we note that Xpert-Ultra, SM results and treatment initiation were issued on the day of the first sputum collection.

Participants who were positive for tuberculosis and without evidence for rifampicin resistance were treated with the standard 6-months pulmonary tuberculosis regimen (i.e., 2 months of isoniazid, rifampicin, ethambutol, and pyrazinamide followed by 4 months of isoniazid and rifampicin).

Laboratory investigations

Xpert MTB/RIF Xpert-Ultra (Xpert-Ultra): One mL of the homogenised sputa was mixed with 2 mL of the sample reagent buffer (Cepheid, Sunnyvale, CA, USA) and then tested according to the manufacturer's protocol.[17] Results were automatically generated semi-quantitatively by the Xpert-Ultra platform. Average of the quantification cycle (Cq) vales of the Xpert-Ultra was calculated from the Cqs of Mtb-specific probes including the insertions sequences.

Sputum smear microscopy (SM): One mL of each homogenised sputum sample was sedimented at 3000g for 10 minutes and a smear (1-2 cm diameter) was prepared from the sediment and stained using auramine-O-staining technique guidelines (Merck, Darmstadt, Germany). Stained smears were examined by the same study personnel using a fluorescent microscope at x 400 magnification.

Sputum culture: Sputa for liquid and solid culture were decontaminated using NaOH/N-acetyl L-cysteine (NALC) (i.e., fresh 2% solution prepared with 2·9% trisodium citrate and 0·5 g NALC) and neutralized with sterile Phosphate-Buffered Saline (PBS; pH 6·8; Becton

Dickinson, Sparks, MD, USA). MGIT tubes were inoculated with 500 µL of the decontaminated sample and incubated at 37°C for a maximum of 42 days. MTB-positive cultures were confirmed by the presence of acid-fast bacilli (AFB) on Ziehl–Neelsen staining and presence of MPT64 antigen. Absence of acid-fast bacilli cording, and growth on blood agar was recorded as contamination. All results were reported according to the International Union Against Tuberculosis and Lung Disease guidelines.[18]

TB-MBLA: Total Mtb rRNA was extracted using a method described elsewhere,[19] and then tested for pulmonary TB using TB-MBLA at 0.1 dilution. TB-MBLA test was performed basing on the duplex reverse transcriptase-real time qPCR principle targeting both *Mycobacterium tuberculosis* complex and the extraction control using a RotorGene 5plex platform (Qiagen, Manchester, UK). Primers and TaqMan dual-labelled probes were manufactured by MWG Eurofins, Germany. The qPCR cycling conditions were as reported by Honeyborne, *et. al*.[19] Quantification cycles (Cq) readouts were converted to bacterial load using a standard curve that was customized for the site's qPCR platform and recorded as estimated colony forming units per mL (eCFU/mL).[20] Samples without Cq values, and those with Cq values above 30·5 were reported as TB negative.

Statistical analyses: Mean and median were calculated. Differences in baseline clinical characteristics were compared using Mann-Whitney U-test for continuous variables, respectively. Quantification cycles for Utra and TB-MBLA, and the TB-MBLA bacterial loads between HIV negative and HIV positive participants were compared using Mann Whitney U test. Correlation of the time-to-positivity for MGIT culture and quantification cycles was performed using Spearman's correlation test. Measures of diagnostic performance (sensitivity, specificity, negative predictive value, and positive predictive value) were calculated using STATA version 15·1 (StataCorp, College Station, Texas, USA) using sputum MGIT culture

as the reference test. From week 17, calculation of sensitivity, specificity and predictive values was not possible because the reference test had gone negative. Statistical significance was considered at probability value less than 0·05.

Results

Figure 1 and Table 1 show the enrolled participants, sputum samples that were obtained and participants' demographics and clinical characteristics data. Fifteen (6·4%) of the 236 patients that were screened objected to the prospects of returning to the clinic at the scheduled visit points, while ten (4·2%) declined to participate in the study, and one (0·4%) patient failed to expectorate sputum. Consequently, at baseline, clinical and laboratory data were obtained from 210 (89%) of the screened patients. The gold standard test (sputum MGIT culture) confirmed pulmonary tuberculosis in 103 (49%) participants whereas TB-MBLA and Xpert-Ultra identified 111 (53%) and 129 (61·4%) participants, respectively. Twenty-three (22·3%) of the 103 participants that were confirmed to have pulmonary TB were also HIV-coinfected with median CD4 cell counts 224 cells/µl (IQR: 54-340) (Table 1). MGIT culture contamination rate among the 210 tested samples was six (2·9%) and none of the Xpert-Ultra results was invalid or indeterminate. Out of the 129 TB participants that were monitored for treatment monitoring, 107 (82·9%) completed treatment of which 95 (88·8%) were to provide adequate sputum volume to perform all the diagnostic tests (Table 1).

We assessed agreement across all the tests and noted that TB-MBLA and Xpert-Ultra detected 11/210 (5·2%) and 22/210 (10·5%) more pulmonary TB positive cases, respectively, than MGIT culture at baseline visit. These participants had a TB-MBLA-measured mean bacterial load 3·6 $\log_{10}$eCFU/mL (median Cq 25) and Xpert-Ultra median Cq 26 that were potentially missed by MGIT culture. TB-MBLA and MGIT culture concurred on the negativity of the 15/210 (7·4%) cases that were positive by the Xpert-Ultra signifying consistent specificity between the two assays. Three (50%) of the six indeterminate culture results were positive with Xpert-Ultra and two (33·3%) indeterminate results were positive with TB-MBLA.

253 Trace-positive result is a new category of results on the Xpert-Ultra platform that can be used
254 to make clinical decisions. Table 2 shows the comparison of Xpert-Ultra Trace-positive results
255 with other tests results. At baseline, 10 (7·8%) of the 129 Xpert-Ultra positive results were in
256 the Trace-positive. Four out of the 10 trace-positive participants reported a history of cured TB
257 disease. Most of the Trace-positive results were negative, 8/10 (80%) for both MGIT and TB-
258 MBLA and 9/10 (90%) by SM at baseline, and remained negative during TB treatment. The
259 two Trace-positive results that were positive by both TB-MBLA and MGIT culture at baseline
260 had a relatively high bacterial load, 4·6log$_{10}$ eCFU/mL, and median MGIT time to positivity of
261 10-days. Although all Trace-positive results had indeterminate rifampicin resistance results,
262 we observed that most of them converted to negative by all other tests except two participants
263 who remained Xpert-Ultra positive at the end of treatment (Table 2). Drug and sensitivity test
264 for the two positive samples was not done but it is important to note that these participants were
265 clinically well, and their sputum samples were negative by SM, TB-MBLA MGIT culture.

266 **Diagnostic performance and prediction of binary out come**

267 We observed that TB-MBLA had a high pre-treatment diagnostic sensitivity 99% (95%CI: 95-
268 100) and specificity 91% (95%CI: 83-96). Although TB-MBLA pre-treatment diagnostic
269 sensitivity (99%) was similar, Xpert-Ultra specificity 78% (95%CI: 68-86) was 13% lower.
270 Compared with smear, TB-MBLA and Xpert-Ultra were both more sensitive (99% versus
271 75%), but specificity was comparable with that of the TB-MBLA (91% versus 98%). Positive
272 predictive values were 92%, 82%, 98% for TB-MBLA, Xpert-Ultra and SM respectively.
273 Negative predictive values were 99% for both-MBLA and Xpert-Ultra, and 79% for SM.
274 Sensitivity, specificity and predictive values followed a similar trend in participants living with
275 or without HIV co-infection (supplementary Tables S1).

276

Baseline smear negative participants

Out of the 124 smear-negative sputum samples, 26 (21·0 %) were confirmed to have Mtb by MGIT culture, 34 (27·4 %) were positive on the TB-MBLA (with mean bacterial load ± SD; 3·38 ± 1·33 eCFU per mL), 48 (38·7 %) were positive on the Xpert-Ultra; 24 (19·4 %) were positive by MGIT, TB-MBLA, and Xpert-Ultra (Figure 2). Sensitivity at 96% was similar for Xpert-Ultra and TB-MBLA but specificity at 92% was higher for TB-MBLA than 78% for the Xpert-Ultra. Although positive predictive values dropped among the smear negative samples, it was still higher for TB-MBLA at 76% compared to 54% for the Xpert-Ultra. Negative predictive value remained high at 99% for both Xpert-Ultra and the TB-MBLA. Sensitivity, specificity and predictive values followed a similar trend in participants living with HIV (supplementary Table S3)

Mtb bacillary load quantification

Overall, before treatment initiation, mean ± SD TB-MBLA measured bacterial load of the cohort was 4·8 ± 1·5$\log_{10}$ eCFU per mL. The mean ± SD bacterial load 3·8 ± 1·6 $\log_{10}$ eCFU per mL among the HIV-TB positive participants was lower than 5·2 ± 1·3$\log_{10}$ eCFU per mL for those who were HIV negative (p=0·0002). The median MGIT time-to-positivity before treatment initiation was 7 days (IQR: 5-10), and was correlated with both Xpert-Ultra and TB-MBLA (r = 0·5, p= 0·02)

Treatment response monitoring

All the tests showed response to treatment as demonstrated by drop in test positivity. At week 2, a proportion of participants tested negative by different tests. These include 32, 12, 24, and 21 by TB-MBLA, Xpert-Ultra, SM and MGIT culture respectively. We assessed whether these cases remained negative throughout treatment and found 19, 5,13, and 15 were consistently negative by TB-MBLA, Xpert-Ultra, SM and MGIT culture respectively at month-6 of

301 treatment. The average baseline bacillary load of the participants who consistently remained
302 negative was 4·2 ± 1·4$\log_{10}$ eCFU/mL and 10 of them were TB-MBLA negative.

303 Similarly, positivity rate and bacterial burden measured by tests changed differently from
304 baseline to completion of treatment. We noted that the positivity rate reduced occurred
305 remarkably slower for the Xpert-Ultra. For instance, at 8 weeks (n=113), positive results were
306 84 (74·3%) with Xpert-Ultra, 20 (17·7%) with TB-MBLA, 13 (11·5%) with smear, 12 (10·6%)
307 with MGIT culture and 7 (6·2%) with solid culture. Consequently, by end of treatment at
308 month-6, 31/95 (32·6%) were still Xpert-Ultra positive compared to six (6·3%) with SM and
309 none for TB-MBLA, and culture tests. Out of the 31 participants who were Xpert-Ultra positive
310 at end of treatment, 13 (41·9%) were positive at week 8, while 18 (58·1%) turned positive after
311 week 8, indicating an increasing trend of Xpert-Ultra positive results as treatment progresses
312 (Figure 3 and Table 3).
313 Decrease in the positivity rate was corroborated by the reduction in the bacterial load. For
314 example, relative to the bacterial load at baseline, mean bacterial load measured by TB-MBLA
315 reduced by 1·4logs, 2·3logs, 2·6logs to zero at week 2, 8, 17, and 26 of treatment respectively.
316 Similar trend was observed for the time to positivity of liquid and solid culture grade, except
317 for smear grade, and quantification cycles for Xpert-Ultra which remained nearly constant at
318 weeks 8, 17 and 26 (Table 3). We also performed both passive (using phone calls) and active
319 follow-up at 12 weeks after the end of treatment. We noted that two (6·5%) of the 31 Xpert-
320 Ultra positive participants remained positive but with no clinical symptoms, seven (22·6%)
321 were Xpert-Ultra negative while 21 (67·7%) were clinically well and did not provide sputum.
322 Semiquantitative grading varied among the 31 results that were positive by Xpert-Ultra results
323 at week 26. Fifteen (48·4%) were graded by Utra as low-positive, ten (3·1%) very low-positive,
324 five (16·1%) trace-positive and one (3·2%) medium-positive but none exhibited TB-like
325 symptoms at this stage of treatment.

TB-MBLA overall sensitivity reduced with treatment to 67%, a reflection of fall in TB bacterial burden whereas specificity increased with an overall value of 97%. The same trend was observed for SM at 66% sensitivity and 96% specificity. In contrast, the overall sensitivity of the Xpert-Ultra was high at 90%, but the specificity dropped to 65% indicating a delayed conversion of the Xpert-Ultra. Sensitivity, specificity, and predictive values followed similar trend among the HIV positive as well as among the smear negative participants at the baseline visit and 8 weeks after treatment initiation (Tables 3 and 4).

Deciphering Xpert-Ultra- and TB-MBLA- test positivity rates

To understand why Xpert-Ultra positivity remained high at the end of treatment, we estimated Mtb DNA and rRNA in vivo elimination rate by modelling the time variation of the PCR Cq values using an exponential saturation function. When the concentration of DNA or rRNA was below the limit of detection, a Cq value of 40 was used in the analysis (supplementary text 1). The $T_{99\%}$ values corresponded to the time required to reach 99% Cq 40, equivalent to lowest concentration of quantifiable rRNA and DNA by TB-MBLA and Xpert-Ultra respectively. Lower $T_{99\%}$ was observed for most samples by TB-MBLA compared to Xpert-Ultra indicating that the Mtb- rRNA degrades more rapidly than DNA. Consequently 74% and 18% of the patients attained $T_{99\%}$ within 26 weeks of treatment follow-up by TB-MBLA and Xpert-Ultra respectively (Figure 4). The Cq values for TB-MBLA increased about 3·3-fold faster than those for the Xpert-Ultra. Overall, on the same sample where TB-MBLA was negative (Cq 40), Xpert-Ultra took three more weeks to attain the same result (Table 5 and Figure 5).

Reliability of the 2 months smear for monitoring treatment response

Table 6 shows the relationship between results at week 8 and end of treatment outcome. At 8 weeks (n=113), twenty participants (17·7%) were positive with TB-MBLA (median Cq value

28; mean bacteria load $2{\cdot}7 \pm 0{\cdot}6\log_{10}$ eCFU/mL) and eighty-four (74·3%) were positive with Xpert-Ultra (median Cq value 22·7).

Six (30%) out of the 20 participants who were positive with TB-MBLA, and thirteen (15·5%) of the 84 participants who were positive with Xpert-Ultra were also positive by SM. Mean bacterial load $2{\cdot}64 \pm 0{\cdot}8\log_{10}$ eCFU/mL for participants who were both TB-MBLA-SM positive was 0·16log less than $2{\cdot}8 \pm 0.6$ $\log_{10}$eCFU/mL for those who were TB-MBLA positive but SM negative however the difference was not statistically significant (p=0·44). Further, we observed that 100 (88·4%) of the 113 participants were SM negative at 8 weeks and 14 (14%) of these were positive with TB-MBLA with a relatively high mean bacillary load $2{\cdot}8 \pm 0{\cdot}8$log eCFU/mL indicating low sensitivity of SM. Only participants who were SM positive at 8 weeks received one extra month of the intensive phase treatment before switching to the continuation treatment phase. Despite this discordance, treatment success rate was comparable. A phone call follow-up at three months after the end of treatment revealed that none of the participants with successful treatment outcome had TB-like symptoms.

Discussion

Our findings indicate that TB-MBLA and Xpert-Ultra have similar pre-treatment diagnostic sensitivity, but TB-MBLA is more specific among the smear negative and people living with HIV. While our findings show that the sensitivity of the Xpert-Ultra was higher, and that specificity was lower than those reported elsewhere for diagnostic performance[21], we confirm the unsuitability of Xpert-Ultra and SM for monitoring TB treatment response and point to TB-MBLA as the most accurate alternative. The findings further reveal that 2-month sputum smear microscopy is insufficient to inform decision to extend intensive treatment phase, in line with the recent WHO guideline discouraging use of month-2 sputum smear positive result as basis to extend intensive treatment phase of drug susceptible TB.[22] We have further shown that a good proportion of patients convert-to-negatve early in treatment and remain so until end of treatment, implying that not all TB patients may require 6-month treatment course.

The high sensitivity and specificity of TB-MBLA before and after initiation of treatment gives it a comparative advantage over SM (standard-of-care for treatment monitoring) and Xpert-Ultra. When Xpert-Ultra was used for monitoring response to treatment, specificity significantly reduced, most likely due to accumulation of DNA from dead bacilli, which take long to be eliminated by DNases within the host enviroment. This leaves Xpert-Ultra more suitable for pre-treatment TB diagnosis and less suitable for treatment monitoring. Before treatment is initiated, Xpert-Ultra appears to reflect DNA mostly from viable bacilli. This is demonstrated in baseline Xpert-Ultra and TB-MBLA quantification cycles which were similar and correlated with MGIT time-to-positivity.

Like SM, Xpert-Ultra fails to distinguish viable from dead bacilli.[23,10] By modelling we have shown that the Xpert-Ultra quantification cycles change slowly, 3·3-fold less than TB-MBLA

per week, reflecting slow degradation of DNA from bacilli that may have been killed by therapy. This renders Xpert-Ultra less specific as patients progress on treatment and may explain why 33% of the 95 patients at month six of treatment were still positive for Xpert-Ultra but negative for TB-MBLA and MGIT culture. These findings corroborate the 2013 Friedrich et al study which showed that 27% of patients were still Xpert MTB/RIF positive at six months, and concluded that Xpert MTB/RIF was an unsuitable treatment response biomarker.[24] The slightly higher positivity rate for Xpert-Ultra observed in our study may be explained by the higher sensitivity of Xpert-Ultra compared to Xpert MTB/RIF assay as reported by other studies.[25,26] Indeed, 16% of the 31 Xpert-Ultra positive results at the end of treatment were trace-positive. The 2013 Friedrich et al study further demonstrate that positivity rate of Xpert MTB/RIF declined in a linear fashion as opposed to the non-linear (biphasic) form of resolution shown by SM, culture and, currently TB-MBLA. Compared with the 2013 Friedrich et al study, we noted it is often the case that an increase in sensitivity is achieved at the expense of specificity.[24]

Here we show that TB-MBLA specificity was 13% higher than Xpert-Ultra's. We hypothesise that the high number of the trace-positive results inconsistent with the reference test, MGIT were responsible for reducing the specificity score of Xpert-Ultra. Eight (80%) of the10 pre-treatment trace-positive results were negative with both MGIT and TB-MBLA. Much as these findings may point to over diagnosis when treatment is based on trace-positive result, it remains unclear whether all Trace-positive results are from viable- or dead- bacilli given the fact two Trace-positive participants were TB-MBLA and MGIT positive and responded to treatment. Furthermore we note that 40% of trace-positive participants at baseline had a history of cured TB disease, which points to the possibility of leftover DNA from previously killed bacilli. Future studies should investigate such trace-positive results and explore the time taken for

414 Xpert-Ultra positive but culture or TB-MBLA negative patients to also become Xpert-Ultra
415 negative.
416
417 Accurate early markers of poor prognosis to minimise over treatment are still needed.
418 Overtime, a 2-month sputum smear has had a positive impact on cure but with several
419 challenges which limit its impact on treatment success rate.[27] Our findings that smear missed
420 fourteen participants who were otherwise positive with TB-MBLA further points to its low
421 sensitivity and inadequacy to inform extension of the intensive phase treatment. In 2018, WHO
422 cited TB-MBLA as a potential replacement of smear and culture for treatment monitoring.[13]
423 This study confirms this early promise. TB-MBLA is fully quantitative and measures viable
424 Mtb in a shorter time hence using 2-month TB-MBLA to monitor treatment response would
425 reveal the actual bacterial load present in the sample. Informative results from TB-MBLA
426 provide useful information for prognosis and informing treatment clinical decisions at the end
427 of the intensive phase treatment.
428
429 The strength of the current study is that it the first to compare head-to-head rRNA-based TB-
430 MBLA and DNA-based Xpert MTB/RIF technology. Most importantly, the study was nested
431 in a larger long-term longitudinal Inflammation, Aging, Microbes, and Obstructive Lung
432 Disease study with experienced research team who guided on collection of high-quality data
433 coupled with high retention rate of the participants into TB care. Additionally, no data was lost
434 due to invalid/indeterminate Xpert-Ultra tests, yet culture contamination rate of 2·9% was 2·5
435 times below average (8%) of the laboratory where the study was conducted. The low
436 contamination rate was attributed to good sputum collection and processing practices including
437 training patients to expectorate properly, and careful decontamination protocol.
438

439 The main limitation of the study is the dependence on culture as the reference test for the
440 molecular-based assays. Culture is an imperfect surrogate and may have distorted the results
441 of the diagnostic performance of the molecular tests. Secondly, our findings are further limited
442 by the small sample size and being undertaken at only one site. Nevertheless, the sample size
443 of 210 is consistent with 205-patient sample size used to evaluate diagnostic utility of Xpert-
444 Ultra among TB mengitis patients at a single site in Vietnam [28]
445

446 We have also shown that TB-MBLA has potential as both a diagnostic and treatment
447 monitoring tool. Over 80% of the patients who converted-to-negative at week-2 and remained
448 negative until end of treatment were detected by TB-MBLA, increasing its prognostic utility
449 and potential application in personalised management of TB. A recent paper has shown TB-
450 MBLA's ability to predict relapse earlier than MGIT culture, thus more studies to further justify
451 its prognostic utility in short- and long- term treatment outcomes are highly recommended.[29]
452 Nevertheless, in its current state, TB-MBLA would benefit from protocol streamlining or
453 automation to shorten hands-on-time for potential users in settings with limited workforce.
454

Figures

Figure 1

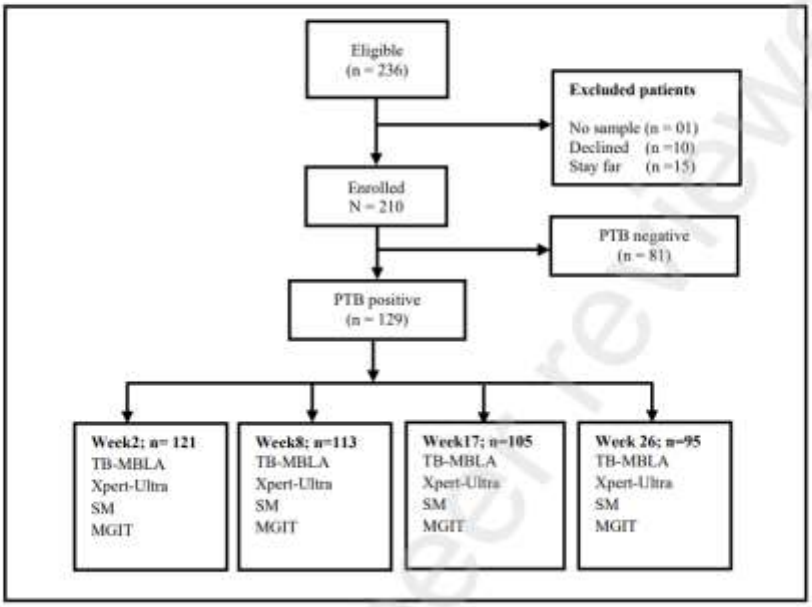
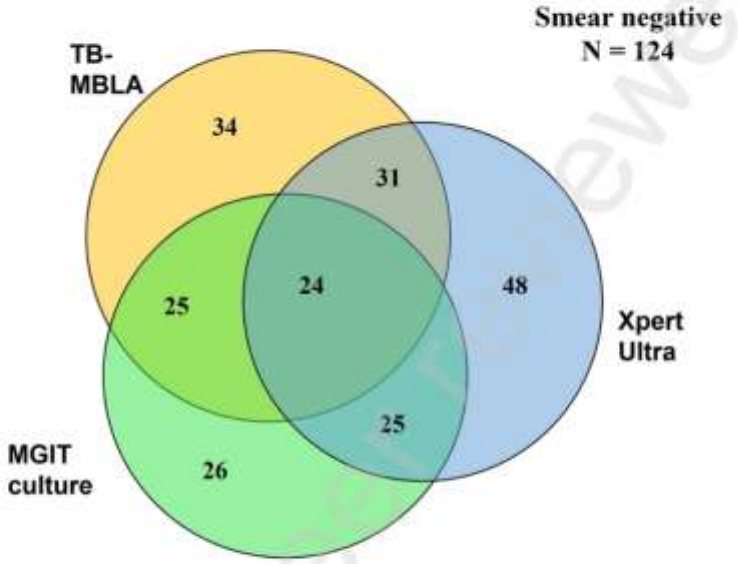


Figure 1: Participant enrolment and sputum collection. Patients who stay far were excluded because it would be hard to monitor them during treatment. One patient was unable to provide sputum sample. Ten declined to participate in the study. At week 8, one-person defaulted treatment, three had died, one was lost to follow-up, one was transferred out of the clinic, two defaulted, and three had travelled. Out of the 129 participants who were enrolled into the treatment follow -up arm, 107 completed treatment. Six participants were lost to follow-up, three more had defaulted, and two-declined to provide sputum sample. and 95/107 provided sample volume that was enough to perform all the tests.

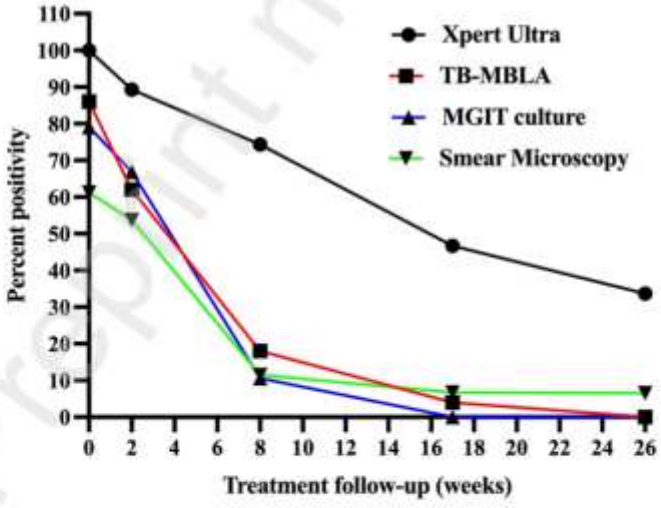
477

478 **Figure 2**



479
480 **Figure 2: Comparison of the positive cases detected by TB-MBLA, Xpert-Ultra and MGIT culture among**
481 **the smear negative samples**. Out of the 26 (21·0 %) samples that were confirmed PTB positive by the MGIT
482 culture, 25 (20·2 %) were also positive on both TB-MBLA and Xpert-Ultra. Overall, molecular assays detected
483 more Mtb positive samples than MGIT culture. Twenty-four (19·4 %) samples were positive on all the three
484 assays. Majority 31 (25 %) of the samples that were positive by TB-MBLA were also positive by Xpert-Ultra.

485 **Figure 3**



486

Figure 3: Treatment response demonstrated by test positivity across treatment follow-up period. Positivity rates reduced for all tests but remarkably slower for Xpert-Ultra. For instance, at 8 weeks (n=113), positive results were 84(74·3%) with Xpert-Ultra, 20(17·7%) with TB-MBLA, 13(11·5%) with smear, 12(10·6%) with MGIT culture, and 7(6·2%) with solid culture. At week 26 (n=95), positive results were 31(32·6%) for Xpert-Ultra, 6(6·3%) for smear, and none for TB-MBLA, liquid and solid culture. Out of the 31 results that were Xpert-Ultra positive at week 26. Fifteen (48·4%) were graded as 'Low', ten (3·1%) were 'very low', five (16·1%) were 'trace calls', and one (3·2%) was graded 'median'.

Figure 4

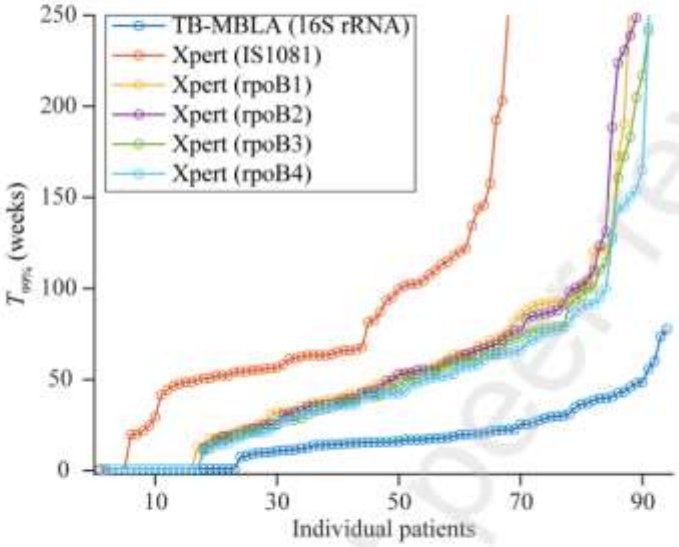
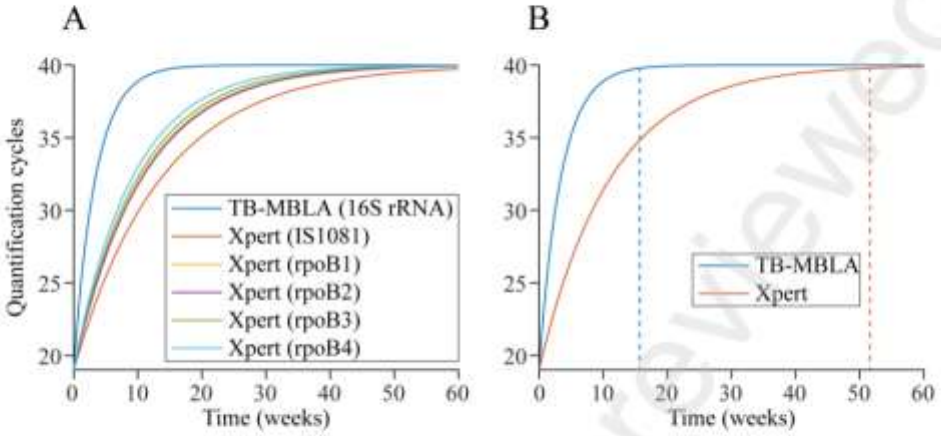


Figure 4: Time required to achieve 99% reduction of bacillary load by rRNA-based TB-MBLA versus DNA-based Xpert-Ultra in individual patients. The rate varies between individuals. Since the ratio of the viable and killed Mtb in a clinical sample is independent of the measurement technique, these results indicate that free bacterial rRNA degrades more rapidly than free bacterial DNA. 74% and 18% of patients would reach $T_{99\%}$ by TB-MBLA Xpert-Ultra respectively within 26 weeks of treatment.

512 **Figure 5**



513

514 **Figure 5: Rate of increase of quantification cycles (reduction of rRNA or DNA) measured by TB-MBLA**
515 **versus Xpert-Ultra.** A) Presentates individual genes measured by Xpert Ultra versus 16S rRNA measured by
516 TB-MBLA. B) Presents the median of the Xpert-Ultra genes versus TB-MBLA. Overall the median time to attain
517 Cq 40 (limit of quantification) was 15·7 weeks for TB-MBLA 16S rRNA compared to 51·6 weeks for Xpert-Ultra
518 genes. The starting baseline quantification cycle was taken to be 19, which is close to the median of the measured
519 Cq_0 values for both TB-MBLA and Xpert Xpert-Ultra. When the concentration of the 16S rRNA or DNA was
520 below limit of detection, a quantification cycle value of 40 was assigned.

521

522 **TABLES**

523 **Table 1: Demographic data at baseline**

524

	Overall (N^1=210)	Participants with indicated pulmonary TB status [a]			P-value [b]
		Positive (n= 103[49%])	Negative (n=101[48%])	Contaminated (n=6[3%])	
				37(30-48)	0·6
Age (Years)	35(27-44)	32(26-43)	35(30-47)		
Male	135(64)	78(58)[a]	54(40)[b]	3(2)	..
Evening fevers	176(84)	92(52·3)	77(44)	5(3)	..
Weight loss>5%	161(77)	86(53)	67(42)	6(4)	..
Cough >2weeks	208(99)	102(49)	98(47)	6(3)	..
Haemoptysis	54(27)	28(52)	25(46)	1(2)	..
Heart rate (%)	98(85-112)	104(88-114)	93(79-104)	104(66-123)	0·27
Respiratory rate (%)	22(20-28)	24(20-28)	21(20-26)	20(18-23)	0·27
Oxygen saturation (%)	96(94-98)	96(94-97)	97(94-98)	97·5(97-98)	0·82
Living with HIV/AIDS	72(34)	24(33)	44(61)[b]	4(6)	..
Antiretroviral therapy use	35(48·6)	7(20)	25(71)	3(9)	..
CD4(cells/μL)[c]	222(54-381)	227(57-345)	183(52-601)	514(113-922)	0·44
CD8(cells/μL)[c]	585(413-874)	589(459-872)	585(410-897)	631(466-883)	0·68
CD4/CD8 [c]	0·28(0.1-0.5)	0·24(0·1-0·5)	0·3(0·1-0.9)	0.6(0·25-1·1)	0·51
Body mass index	19(17-22)	20·1(17-24)	18·8(17-21)	24(18-27)	0·2
Body temperature	37(36-37)	36·6(36·4-37)	36·5(36-37)	37(37-37)	0·81
End of treatment outcome (N^2=129)					
Successful at our clinic		106(88·3)	..	..	..
Lost to follow-up		5(3·9)	..	..	..
Dead		7(5·4)	..	..	..
Defaulted on treatment		4(3·1)	..	..	..
Failure		1(0·8)	..	..	..
Transferred out		6(4·7)	..	..	..

525 N^1 = 210 refers to all PTB positive and PTVB negative per the gold standard reference test (sputum MGIT culture)
526 N^2 =129 refers to all baseline PTB positive per the standard of care test (sputum Xpert-Ultra).
527 Binary data are n/N (%).
528 Quantitative data are median(IQR)
529 [a] Bacteriologically confirmed positive or negative TB cases using sputum MGIT; [b]Comparison between
530 pulmonary-TB-positive and -negative participants. [c] Measured for HIV infected only (n = 72). Successful outcome
531 = Participants with resolved clinical symptoms with negative sputum MGIT results; Patient who failed was
532 restarted on treatment; Post-mortem details of the participants that died was not available by the time of the
533 manuscript write-up
534
535

536

537 **TABLE 2: Comparison of 'Trace call' results**

	Xpert-Ultra		TB-MBLA		SM		MGIT	
	Positive	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**	**Positive**	**Negative**
Week0	10	..	2/10	8/10	1/10	9/10	2/10	8/10
Week2	5/9	4/9	..	9/9	1/9	8/9	..	9/9
Week8	3/9	6/9	1/9	8/9	..	9/9	..	9/9
Week17	2/9	7/9	..	9/9	1/9	8/9	..	9/9
Week26	2/6	4/6	..	6/6	..	6/6	..	6/6

538
539 Seven (70%) of these ten participants were co-infected with HIV; all were treated for tuberculosis without
540 repeating the test and all had indeterminate rifampicin results. At week 2, one (10%) failed to expectorate. At
541 weeks 8 one participant had died while at week 26, one more participant died, one failed to expectorate; one was
542 transferred out of the study on request. Importantly, only one participant was positive throughout the treatment
543 period on Xpert-Ultra. Across the treatment period, Xpert-Ultra grading ranged between trace call and very low.
544 Generally, all other tests agreed on the negativity of the results that were otherwise trace positive with Xpert-
545 Ultra.
546

547 **Table 3: Changes in positivity rate and bacillary load**
548

	Week 0	Week 2	Week 8	Week 17	Week 26
TB-MBLA, positivity results	111/210 (52·9%)	70/121 (57·9%)	19/112 (16·9%)	6/106 (5·7%)	0/95 (0%)
TB-MBLA quantification cycles	20 (17-30)	26 (23-29)	28 (26-30)	30 (29·6-30·4)	36·2 (35·1-36·1)
TB-MBLA, bacteria load (Log_{10} eCFU/mL)	4·9 (3·6-6·1)	3·5 (2·6-4·4)	2·6 (2·2-3·3)	2·3 (2-2·3)	0
Xpert-Ultra, positivity results	129/210 (61·4%)	110/121 (91·0%)	84/112 (75·0%)	49/106 (46·2%)	31/95 (32·6%)
Xpert-Ultra, quantification cycles	20 (19-230	20·2 (18·6-23)	25 (21-28)	26·2 (23-30)	26 (21-30)
Smear microscopy, positivity rate	79/210 (37·6%)	65/121 (53·7%)	13/112 (11·6%)	7/106 (6·6%)	(6/95) (6·3%)
Smear microscopy, median grade	3 (2-3)	2 (1·5-3)	2 (2-2)	1 (1-2)	1 (1-2)
MGIT culture positive results	102/210 (48·6%)	81/121 (66·9%)	12/112 (10·7%)	0/105 (0%)	0/95 (0%)
MGIT culture, TTP (days)	7 (5-9)	13 (11-16)	22·5 (12-27)	..	..

549
550 Binary data are n/N (%).
551 Quantitative data are median (IQR)
552

553 **Table 4: Diagnostic performance of TB-MBLA; Xpert Xpert-Ultra and smear**
554

	TB-Molecular Bacterial Load Assay				Xpert-Ultra				Smear microscopy			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
OVERALL participants												
Week0 (n=202)	99% (95-100)	91% (83-96)	92% (85-96)	99% (94-100)	99% (95-100)	78% (68-86)	82% (74-86)	99% (93-100)	75% (65-83)	98% (93-100)	98% (91-100)	79% (71-86)
Week2 (n-121)	87% (77-93)	92% (79-98)	96% (87-99)	77% (62-88)	98.8% (93-100)	33% (19-50)	76% (67-84)	93% (66-100)	74% (64-83)	92% (79-98)	95% (87-99)	63% (49-76)
Week8 (n=113)	67% (35-90)	88% (80-94)	40% (19-64)	96% (89-99)	92% (62-100)	26% (18-36)	13% (7-22)	96% (81-100)	50% (21-79)	93% (86-97)	46% (19-75)	94% (87-98)
Week17 (n=105)	14% (0-58)	96% (90-99)	20% (0.5-72)	94% (88-98)	71% (29-96)	56% (46-66)	10% (3.4-22)	97% (88-100)	..	..	..	..
Week26 (n=92)	..	100%	..	100%	..	67%	..	100%	..	100%		100%
Overall	67 (52-85)	93% (66-97)	62 (48-83)	93 (67-97)	90 (70-99)	65 (30-68)	45 (38-54)	97 (66-100)	66 (50-82)	96 (65-99)	80 (66-91)	84 (52-90)
Smear negative participants												
Week0 (n=123)	96% (80-100)	92% (84-96)	76% (58-89)	99% (94-100)	96% (84-100)	78% (69-86)	54% (39-69)	99% (93-100)	..	..	..	..
Week 2 (n=48)	67% (43-85)	94% (81-99)	88% (62-98)	83% (68-93)	95% (76-100)	33% (19-51)	46% (30-61)	92% (64-100)	..	..	..	..
Week 8 (n=98)	33% (4-78)	87% (79-93)	14% (2-43)	95% (89-99)	84% (36-100)	28% (19-39)	7% (2-16)	96X% (81-100)	..	..	..	..
HIV positive participants												
Week0 (n=66)	100% (86-100)	93% (81-99)	89% (71-98)	100% (91-100)	100% (79-100)	81% (63-90)	72% (53-86)	97% (85-100)	54% (33-74)	98% (87-100)	93% (66-100)	79% (65-89)
Week2 (n=32)	67% (38-88)	100% (81-100)	100% (69-100)	77% (55-92)	93% (68-100)	47% (23-72)	61% (39-80)	89% (52-100)	67% (38-82)	94% (71-100)	91% (59-100)	76% (53-92)
Week 8 (n=28)	75 (19-99)	86% (67-97)	50% (12-88)	96% (77-100)	100% (40-100)	57% (35-77)	29% (8-58)	100% (75-100)	50% (7-93)	96% (79-100)	67% (9-99)	92% (74-99)

555
556 Data are percentage (95% CI)
557 Data for sensitivity, specificity, positive predictive value, and negative predictive value were calculated against
558 the MGIT culture, TB-MBLA; Tuberculosis-Molecular Bacterial Load Assay, Xpert-Ultra; GeneXpert MTB/RIF
559 Xpert-Ultra, PPV; Positive Predictive Value, NPV; Negative Predictive Value. Smears were stained using
560 Auramine-O-staining technique.
561

Table 5: Median values of the elimination rates for 16S rRNA and DNA

Method	$T_{99\%}$ (weeks)
TB-MBLA	15.7
Xpert-Ultra – gene IS1081	63.2
Xpert-Ultra – gene rpoB1	48.4
Xpert-Ultra – gene rpoB2	49.3
Xpert-Ultra – gene rpoB3	46.0
Xpert-Ultra – gene rpoB4	42.1
Xpert-Ultra (overall)	51.6

TB-MBLA; Tuberculosis-Molecular Bacterial Load Assay; $T_{99\%}$; $T_{99\%}$ corresponds to the timepoint when the bacterial load is decreased by 99% during treatment. The overall central value for Xpert Xpert-Ultra, the median was calculated from all the $T_{99\%}$ parameters including all five genes.

576 **TABLE 6: Treatment outcome at six months among positive cases at two Months**
577

	TB-Molecular Bacteria Load Assay			Xpert Xpert-Ultra		
	Overall (N=19)	Treated for extra month (n=6 [30%])	Not treated for extra month (n=14 [70%])	Overall (N=84)	Treated for extra month (n=13 [15.5%])	Not treated for extra month (n=71 [84.5%])
Successful	17(85·5)	6(100)	12(85·7)	70(83·3)	9(69·2)	61(86)
Failure	None	None	None	1(1·2)	1(7·7)	None
Mortality	1(5)	None	1(7·1)	2(2·4)	1(7·7)	1(1·4)
Lost to follow-up	None	None	None	4(4.8)	1(1·7)	3(4·2)
Others	1(5)	None	1(7·1)	7(8.3)	1(1·7)	6(8·4)

578
579 Data are n/N (%).
580 Success refers to a negative sputum MGIT culture result with no clinical symptoms, Failure refers to positive
581 sputum MGIT culture with/out clinical TB symptoms. Others include those who declined/failed to give a sputum
582 sample, and those who opted out of the study. When the participants who belonged to 'others' and those who were
583 lost to follow-up were considered, success rate was 100% for TB-MBLA-with treatment extension, 92% for TB-
584 MBLA-without treatment extension; and 92% for Xpert-Ultra-with treatment extension, and 98% for Xpert-Ultra-
585 without treatment extension. Passive phone call follow-up at three months after the end of treatment revealed that
586 none of the participants with successful treatment outcome had TB-like symptoms.
587

588

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Declaration of interests: Wilber Sabiiti and Stephen Gillespie provide a *pro bono* advice for a company that is developing TB-MBLA for clinical use. All other members declare a no conflict of interest. No contributing author (s) declared him/herself to be (a) medical writer(s) or editor(s)

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Early depressing effect on abundancy and diversity of sputum microbiome followed by recovery during anti-tuberculosis treatment: a multi-regimen analysis of the High-Rifampicin II- and Multi-Arm-Multi-Stage clinical trials

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Running title: Anti-tuberculosis therapy does not limit recovery of sputum microbiome

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Abstract

Background: Respiratory tract microbiota has been described as the gate keeper for respiratory health. Using new drug combinations, attempts have been made to shorten treatment of tuberculosis (TB), but the impact of such drug combinations on the respiratory microbiome have not been studied.

Methods: Using total RNA and V3-V4 16S rRNA gene sequencing, sputum microbiome of participants under the HIGHRIF2 and Multi-Arm-Multi-Stage clinical trials were analysed across a 3-month treatment period. Participants were treated with standard TB regimen Isoniazid(H)-Rifampicin-($R_{600mgor10mg/kg}$)-Pyrazinamide(Z)-Ethambutol(E) in comparison to investigational regimens containing fixed dose, R_{900mg} and R_{1200mg} under HIGHRIF2 and/or differing doses of rifampicin (10mg/kg, 20mg/kg, 35mg/kg including novel combinations replacing ethambutol with SQ109(Q) or moxifloxacin(M) in the Multi-Arm-Multi-Stage study.

Findings: Sequence data was obtained from 397 pre- and post-treatment samples across seven treatment regimens. Pre-treatment microbiome (n=206 genera) was dominated by firmicutes, 44%, and *Streptococcus*, 36% at phyla and genus levels, respectively. All regimens had a depressing effect on microbiome abundancy and diversity in the first two weeks of treatment, but this was only significant under the $HR_{20mg/kg}ZM$ and $HR_{35mg/kg}ZE$, Shannon diversity index p=0.003 and p=0·03 respectively. Gram negative bacteria were the most sensitive to bactericidal activity of treatment with the highest number of species suppressed being under the moxifloxacin regimen. By week-12, microbiome had recovered to pre-treatment level except with the $Rif_{35mg/kg}$ regimen and genus *Mycobacterium* that did not show recovery across all regimens. TB culture conversion-to-negative by week-8 of treatment was associated with clearance of genus *Neisseria*, 98% reduction of the pre-treatment level.

Interpretation of the findings: Within the first-line regimen, changing rifampicin dose alone required as high as 35mg/kg to significantly reduce microbiome diversity, an outcome achieved by less rifampicin-20mg/kg supplemented with moxifloxacin without limiting microbiome recovery. An effective anti-TB regimen to shorten treatment may be achieved without harming the commensal microbiota.

Funding: European and Developing Countries Clinical Trials Partnership and German Ministry of Education and Research.

Research in context

Evidence before this study

Tuberculosis disease is treated by combinations of antibiotics in first- and second- line regimens depending on the drug sensitivity profile of the tuberculosis bacteria. We asked what impact these antibiotics, particularly the new clinical trial regimens, have on the respiratory microbiome and treatment outcome. This followed the completion of the PanACEA MAMS TB-01 study and HIGHRIF2 study clinical trials where high dose rifampicin plus novel combinations including moxifloxacin and SQ109- for PanACEA MAMS TB-01 study were assessed for efficacy in treating tuberculosis. These studies showed high dose rifampicin was safe and efficacious whilst including moxifloxacin increased early mycobactericidal activity. Prior to the current study in 2019, we searched Pubmed, Medline and EMBASE using key terms: 'respiratory microbiome', 'microbiota', 'anti-tuberculosis antibiotics', 'tuberculosis treatment response', 'antibiotic impact on microbiome'. The available literature described the microbiome of healthy versus TB patients and effect of the first-line anti-TB regimen (HRZE) on microbiome. No information was available on the new combinations of antibiotics to treat TB. We, therefore, assessed the impact of the new regimens on the sputum microbiome in comparison with the standard first line regimen.

Added value of this study

In contrast to most microbiome studies, this study presents data drawn from RNA, a molecule that reflects cell viability, and is eliminated soon after cell death. This implies that the measured microbiome is a closer reflection of the live microbiome present pre- and post- treatment initiation. Consequently, we have shown that suppression of microbiome occurs early in treatment in a manner that mirrors the early bactericidal activity of anti-TB antibiotics on *Mycobacterium tuberculosis*. The magnitude of the impact varies under different regimens, and this study has revealed that significant reduction of alpha diversity was achieved by regimens containing $Rif_{20mg/kg}$-Moxifloxacin and Rif_{35mg} up to eight and twelve weeks of treatment, respectively. Unlike the $Rif_{35mg/kg}$, recovery of the microbiome abundancy and diversity was achieved by week 12 of treatment. Whilst other taxa recovered, members of genus *Mycobacterium* never recovered under all regimens across the treatment follow-up period. The reduction of *Mycobacterium sequences* was consistent with the TB bacillary load reduction measured in the same samples by the novel tuberculosis Molecular Bacterial Load Assay (TB-MBLA). Early conversion to TB culture negative was associated with suppression of genus *Neisseria* to sub-detectable level.

Implications of all evidence available

The most effective anti-TB regimens were also responsible for significant reduction of the microbiome in the first two months of treatment followed by recovery in the $Rif_{20mg/kg}$-moxifloxacin group by month-3 of treatment. Evenness (distribution of species) was less affected by all regimens, implying the action of antibiotics had a balancing effect, that is, reducing the number of dominant taxa to the level of less dominant taxa in the microbial community. The established view is that broad spectrum antibiotics kill most bacteria, however, this study shows that *Mycobacterium* is selectively eliminated while other members of the microbiome recover.

111 Our finding increases the assurance that high dose- shorter -term TB treatment regimens could
112 be achieved without causing irreversible damage of the beneficial host microbiome. A question
113 for further investigation is whether recovery of the microbiome while still under antibiotic
114 pressure means replenishment from dietary sources or acquisition of antibiotic resistant genes.
115

Introduction

Respiratory microbiota is a gate keeper of respiratory health responsible for modulation of host immunity and resistance of colonisation by pathogens.[1] Different disease states, and exposure to antibiotics have been shown to cause dysbiosis of microbial community.[2] Caused by *Mycobacterium tuberculosis*, tuberculosis (TB), a chronic disease that requires six months of a combination of four antibiotics to treat if drug susceptible and longer for drug resistant TB.[3] It has been shown that treatment with standard first-line anti-TB regimen, 2HRZE/4HR does not perturb overall microbiome diversity but depletes some immunologically significant commensal bacteria, an outcome that may have long-term consequences on individual's health .[4] Short-term TB regimen courses could shorten length of microbiome exposure to antibiotics and reduce the risk of long-term damage of the microbiome. Accordingly, novel short-term TB regimen courses are widely being investigated including the 4-month rifapentine-moxifloxacin containing regimen that was recently recommended by WHO.[5]

Human lung is non-sterile even in health individuals. The generally diverse core composition of the human lung microbial population (microbiota) differs in relative abundance and prevalence.[6] Microbiome may vary based on age (neonates, infants versus youth and adults), diet and/or disease.[7] Diseases such as, chronic bronchial sepsis, lung cancer and TB have been associated with changes in microbiome characterised by increase or decrease of some taxa. The lung is the predilection site for *Mycobacterium tuberculosis* (Mtb) -infection and its microbiota is associated with various states of TB. This means an intervention like antibiotic treatment adds extra level of pressure on the microbiome and that may further alter the composition and function of the lung microbiota.[8] The impact of TB regimens on lung microbiota is likely to be more associated with antibiotics that have activity against a wide range of Gram-positive and Gram-negative bacteria.[9]

It is thus necessary to investigate the impact of the novel TB regimen courses especially those incorporating broad spectrum-high dose antibiotics.[10–12]

Different sample types including bronchoalveolar lavage (BAL), Bronchial washing, Lung tissue, Protected brush, Tracheal aspirate, and sputum, of which sputum is the most used in adult microbiome studies.[13] To this end, we used sputum as proxy for the lung to investigate the effect of standard-of-care and investigational TB regimen courses on the lung microbiota and implications on treatment outcome. We show that anti-TB antibiotics cause an early microbiome depression that mirrors that of early bactericidal activity against *Mycobacterium tuberculosis*. Interestingly this depression is followed by recovery, which for all regimens except the Rifampicin 35mg/kg was back to pre-treatment microbiome levels by week-12 of treatment. This increases confidence in the possibility of high dose short course regimens to treat tuberculosis without irreversibly damaging beneficial microbiome to the host.

METHODS

Study site, participants, and samples

This study is a retrospective analysis of the impact of anti-tuberculosis drugs on the sputum microbiome utiised samples that were collected during the Multi-Arm-Multi-Stage randomised controlled trial (PanACEA MAMS TB-01; project NCT identifier: **NCT01785186**) and the HIGHRIF2 study (project NCT identifier: **NCT00760149**) clinical trials. conducted between the period of 2012 and 2015.

The PanACEA MAMS TB-01 trial was a 5-arm trial assessing four experimental treatment regimens including the following: $HR_{10mg/kg}ZE$, $HR_{10mg/kg}ZQ$, $HR_{20mg/kg}ZQ$, $HR_{20mg/kg}M$, and $HR_{35mg/kg}$ ZE (supplementary Table S1).

The trial was conducted in three sites in Tanzania, and four sites in South Africa, and is described elsewhere in more detail. For the current study, only samples from Mbeya, Tanzanian participants were included in the analysis. All patients who responded well clinically and/or had at least one negative culture towards the end of treatment provided the basis for this study. Patients were followed up until 6 months after end of treatment by telephone calls or on-site visits if participants were unwell. The current study utilised samples taken weekly up to week 8. An outcome of cure, no information, or relapse was assigned at the end of follow-up. Bacterial load was measured by MGIT Time To Positivity (TTP) and by TB-MBLA as published in prior study [14]

The HIGHRIF2 study (NCT identifier: **NCT00760149**) was conducted to investigate the pharmacokinetics, tolerability, and bacteriological response of Rifampicin administered at varying doses. Details of the study design, sites, and selection of the study participants were as published by Aarnoutse et al.,[15] Briefly, the study was conducted between 2010 and 2013 in Kilimanjaro and Dar es Salaam, constituting north-southeast regions of Tanzania. Participants were treated with a fixed-dose of standard TB drugs containing rifampicin at 600-mg ($HR_{600mg}ZE$), 900-mg ($HR_{900mg}ZE$), or 1,200-mg ($HR_{1200mg}ZE$) daily (Supplementary Table S2). Study medications were taken together once daily in the morning for 7 days per week with a glass of water. After the intensive treatment phase, all patients were treated according to Tanzanian guidelines for 4 months. The current study utilised banked RNA samples which were extracted from patient sputa collected over 12 weeks of treatment were investigated to establish the impact of the novel and standard anti-tuberculosis regimens on abundance and diversity of the sputum microbiome.

Study samples

Sputum was collected once per week up to week 12, and at weeks 14, 17, 22, and 26 during treatment course. Sputum total RNA was extracted as described by Sabiiti et al.,[16]

For the current study, banked RNA samples extracted from patient sputa collected over 12 weeks of treatment from the PanACEA MAMS TB-01 and HIGHRIF2 studies were investigated to establish the impact of anti-TB antibiotics on respiratory microbiome and how the effect on the respiratory microbiome affects patient treatment outcome. We only included RNA samples which had traceable corresponding laboratory data from the the PanACEA MAMS TB-01 and HIGHRIF2 clinical trial studies' data bases.

RNA transcription and Amplification of complementary DNA

Total RNA was reverse transcribed to complementary DNA (cDNA) using QuantiTect Reverse transcription Kit supplied by Qiagen, United Kingdom. Reverse transcription reaction mixture was prepared by adding reverse transcription enzyme (1 µl), transcription buffer (4 µl), and transcription primer mix (1 µl) to the thawed RNA sample before incubation at 95°C for 3 minutes. Resulting cDNA was quantified using high sensitivity single stranded DNA Qubit assay. The cDNA was diluted with nuclease free water to ensure the starting template is not too high to inhibit PCR.

Primers targeting the V3-V4 region of the 16S rRNA gene were used to amplify the DNA as described by Klindwort et al., [17] Briefly, 2·5 µl of cDNA were added to 17·7 µl of the amplicon PCR master mix containing Taqmix (9·5 µl), forward primer (4 µl), and reverse primer (4 µl) supplied by Qiagen, United Kingdom. The Amplicon PCR conditions were set up as follows: 95°C for 3 minutes, 35 cycles at 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and then 72°C for 5 minutes. The amplicons were cleaned up using AMPure XP reagent supplied by the Beckman coulter, United Kingdom, and 10 mM Tris; pH 8·5 and ethanol(Supplementary text 1).[18]

Library preparation

Index PCR was used to label the amplicons using variable DNA adapters following the illumina metagenomic sequencing protocol. Briefly, 10 µl of cDNA amplicons were added to the index PCR master mix containing KAPA Hifi hot start ready mix (25 µl) (Sigma Aldrich) and PCR grade water (10 µl). Index PCR products were cleaned up (Supplementary text 1), quantified using Qubit assay, and then labelled by adding unique indices.

Amplicon concentration was normalised to ensure equal concentration before pooling into one library. Amplicon quality and specificity was assessed using gel electrophoresis using 2.5% agarose gel, SYBRsafe dye and 50 bp ladder. A single band of 600 bp was obtained indicating the specificity of the amplicons in the pooled library (Supplementary Figure S1). The concentration of the pooled library was measured and found to be 2·47 ng/µl. Background or cross contamination was checked by running negative control samples, nuclease free water and master mixes for cDNA synthesis and amplicon PCR. These controls were run all the way from in vitro cDNA synthesis to sequencing. Absence of detectable sequences was confirmation of no background contamination.

DNA sequencing and analysis

High throughput amplicon sequencing was performed at the Integrated Microbiome Resource (IMR), Edinburgh Genomics centre, United Kingdom on HiSeq platform. Before sequencing, a bioanalyzer quality check confirmed sufficient material for sequencing with no appreciable adapter dimers. Raw reads were filtered, trimmed and dereplicated, paired reads merged, then denoising and chimeras removed using the DADA2 pipeline within QIIME2 v2020.2. Taxa were allocated to amplicon sequence variants (ASVs) using the SILVA 132 database. Sequences assigned to eukaryotes and archaea were removed. The analyses were then performed on this dataset for total bacteria, and then sequences assigned to photosynthetic

cyanobacteria extracted and analysed separately. Alpha-diversity metrics: Amplicon Sequence Variant richness (taxa relative abundancy), Faith's Phylogenetic Diversity (summation of length and number phylogenetic tree units), Shannon's Diversity (number of species scaled by their distribution in the community) and Pielou's Evenness (distributions of different species in the community) were calculated for treatment medians within QIIME2 at a rarefaction of 1500 reads, after ensuring all samples had reached the rarefaction curve plateau. Sequence data were deposited and are publicly available in the NCBI Sequence Read Archive (SRA) under the BioProject ID PRJNA729425.

Statistical analysis

Participants were divided based on their region of origin and the difference in their microbiome diversity tested using Mann Whitney (MW) test. Similarly, MW was applied to calculate difference of pre-treatment and post treatment alpha diversity under different regimens was tested using Mann Whitney. Spearman's rank correlation was used to calculate the correlation between *Mycobacterium* relative abundancy and tuberculosis bacterial load measured by tuberculosis Molecular Bacterial Load Assay. One way analysis of variance was applied to test the variation in taxa richness over 8 weeks of treatment among those whose TB culture was negative, positive, or indeterminate. Indeterminate was defined as culture whose TB status could neither be called positive nor negative. All calculations were done in Prism version 6 and statistical significance was accepted at $p<0{\cdot}05$.

Results

Participants and Samples

Of the 397 samples, 122 (30·7%), 40 (10·1%), 48 (12.1%), 52 (13·1%), 45 (11·3%), 42 (10·6%) and 45 (11·3%) came from patients treated with $HR_{600}ZE$, $HR_{600}ZQ$, $HR_{900}ZE$, $HR_{1200}ZE$, $HR_{1500}ZE$, $HR_{1200}ZQ$ and $HR_{1200}ZM$ treatment regimens, respectively. Demographic data for the participants who provided sputa that were included in the current study are summarised in the supplementary material (Supplementary text 2).

Pre-treatment alpha and beta diversity of the microbiome

A total of 6500 Amplicon Sequence Variants (ASVs) were analysed. Pre-treatment taxa was dominated by Firmicutes (44%) followed by Bacteroidetes (18%), Proteobacteria (13%), and Actinobacteria (8%). When the microbiome was divided by region of participant origin, Firmicutes remained dominant at 42% and 53% in southwest and north-southeast Tanzania, respectively. The proportion of pre-treatment actinobacteria was over three times higher in north-southeast, 19% compared to southwest 5% vis-à-vis Bacteroidetes, 18% and Proteobacteria, 15% that were proportionally more abundant in southwest than north-southeast (Figure 1)

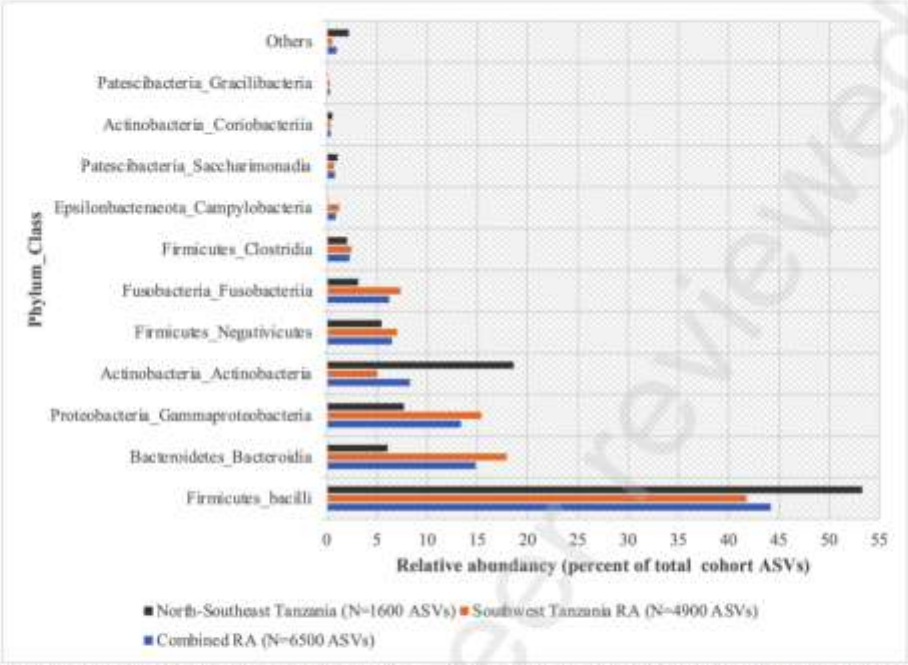
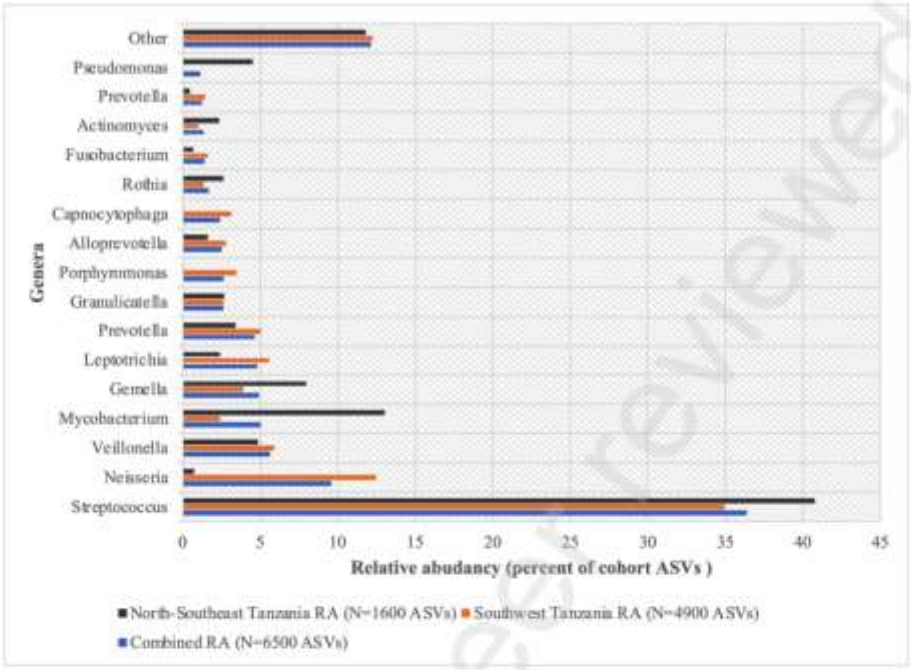


Figure 1: Pre-treatment microbiome diversity at phylum and class level divided by the region of origin. North-southeast Tanzania (black bars), Southwest Tanzania (orange bars) and combination of the 2 regions (blue bars). There was an over representation of Firmicutes and Actinobacteria in North-southeast region.

At genus level, the four most abundant phyla were represented by *Streptococcus* (36%), *Neisseria,* (10%), *Veillonella* (6%,) and *Mycobacterium* (5%). When divided by sample origin, *Mycobacterium* (13%) was 2nd most dominant genus in north-southeast Tanzania surpassing *Neisseria* and *Veillonella* (Figure 2).



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283 **Figure 2: Pre-treatment microbiome diversity at genus level divided by region of origin. North-southeast**
284 **Tanzania (black bars), Southwest Tanzania (orange bars) and combination of the 2 regions (blue bars).**
285 **There was an over representation of genus *Mycobacterium* in North-southeast region.**
286

287 Before treatment, the alpha diversity of the whole cohort was median (range) 81 (17-205)
288 sample richness, 7·9 (2-12) Faith phylogenetic diversity (Faith PD), 4·7 (1.6-6·2) Shannon
289 diversity index and 0·7 (0·3-0·9) Pielou evenness. Evenness was comparatively lower than
290 Shannon and Faith PD suggesting unevenly distributed microbiome in which some taxa
291 dominate over the others (Figure 3).

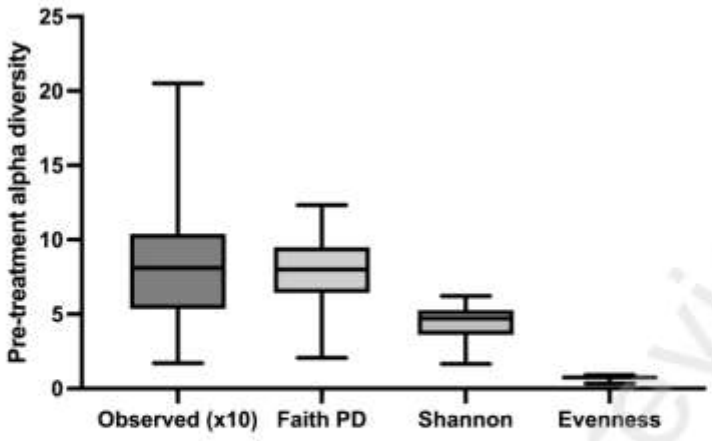


Figure 3: The pre-treatment microbiome richness, distribution, and diversity. Some taxa were more represented than others as indicated by low evenness. The scale of observed sequence variants (sample richness) was x10 of the y-axis value. Faith PD = Faith Phylogenetic Diversity.

Compared to North southeast Tanzania, there was a higher microbial diversity in southwest Tanzanian participants as demonstrated by significantly higher richness, Shannon diversity index and Faith PD. However, there was no significant difference in taxa evenness (Table 1).

	North Southeast Tanzania, n=16 (Median)	Southwest Tanzania, n=50 (Median)	Difference in medians	P Value
Richness	40	93	53	<0·0001
Shannon diversity	3·77	4·92	1·16	0·0005
Pielou evenness	0·72	0·75	0·034	0·1458
Faith Phylogenetic diversity	4·97	8·57	3·61	<0·0001

Table 1: Statistical difference in regional diversity. The Southwest Tanzania sample was more diverse than North Southeast.

Depression and recovery of alpha diversity over the anti-tuberculosis treatment course

After establishing the pre-treatment alpha and beta diversity, we then explored how this diversity changed under different regimens post initiation of treatment. In all regimens, the highest reduction in abundancy and diversity occurred in the first two weeks of treatment after which it begun to recover, achieving pre-treatment level by week-8 of treatment in most of the regimens. There was a fall and rise pattern of alpha diversity induced differently by different regimens. $HR_{10mg/kg}ZQ$ was responsible for the smallest reduction of the alpha diversity across all measures (Figure 4A-D). In figure 4D, under the $HR_{1200mg}ZE$, there was no reduction in evenness in the first two weeks but continued to rise across the treatment period. The moxifloxacin-rifampicin containing regimen ($HR_{20mg/kg}ZM$) followed by high dose rifampicin regimen $HR_{35mg/kg}ZE$ caused the largest depression of alpha diversity (Figure 4A-D).

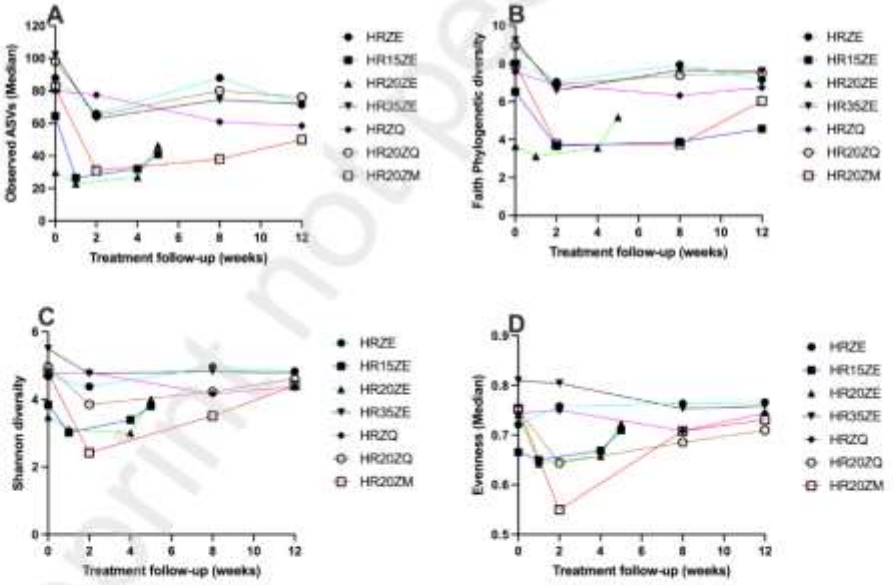


Figure 4: Change in alpha diversity under different regimens following initiation of treatment. The highest fall in alpha diversity occurred in the 1st two weeks of treatment after which it begun to recover. HRZE= $HR_{600mg or 10mg/kg}ZE$), HR15ZE=$HR_{900mg}ZE$, HR20ZE=$HR_{1200mg}ZE$, HR35ZE=$HR_{35mg/kg}ZE$, HRZQ=$HR_{10mg/kg}ZQ$, HR20ZQ=$HR_{20mg/kg}ZQ$ and HR20ZM=$HR_{20mg/kg}ZM$.

Using Mann Whitney test, we assessed whether the change in alpha diversity was significantly different between pre-treatment and post-treatment microbiome across the treatment course. Evenness significantly increased in the standard regimen HRZE from 0·72 at baseline to 0·80, p = 0·03, 0.02 and 0·03 at week 2, 8 and 12 of treatment, respectively. Furthermore, the standard regimen did not cause significant reduction of richness, Faith's phylogenetic diversity (Faith PD) and Shannon index across the treatment period. The high dose rifampicin regimen, HR35ZE reduced richness only at week 2, p=0·03, while Faith's PD and Shannon index were reduced across the follow-up period, p=0·01, 0·02, 0·01 and p=0·03, 0·02, 0·01, at week 2, 8 and 12, respectively.

There was no significant reduction of evenness with HR35ZE across the treatment course. The rifampicin-moxifloxacin regimen, HR20ZM had an early effect on all alpha diversity measures, reducing richness, p = 0·001, 0·002; Faith PD, p = 0·01, 0·003; Shannon diversity index, 0·004; 0·02; and evenness, p = 0·02 at week 2 and 8 of treatment, respectively. While there was an early hit by HR20ZM, microbiome recovery was observed in all diversity measures at week 12 and from week 8 of treatment for evenness. HR15ZE only had reduced richness at week one, p = 0·03 whereas HR20ZQ reduced evenness at week two, p = 0·03 of treatment. No significant reduction or increase of was observed with HR20ZE and HRZQ in all alpha diversity indices across the treatment period (Table 2).

Regimen	Mann Whitney test (Pre-treatment versus post-treatment alpha diversity)											
	Observed ASVs			Faith PD			Shannnn			Evenness		
$HR_{600mg or 10mg/kg}ZE$												
Time on treatment	W2	W8	W12	W2	W8	W12	W2	W8	W12	W2	W8	W12
Baseline (median), n=19	88	88	88	8·02	8·02	8·02	4·68	4·68	4·68	0·72	0·72	0·72
Follow-up (median), n=16-26	66	88	71·5	7·04	7·94	7·16	4·38	4·97	4·83	0·76	0·76	0·77
P value	0·09	0·92	0·61	0·09	>0·99	0·50	0·97	0·27	0·50	**0·03**	**0·02**	**0·03**
$HR_{600mg}ZE$												
Time on treatment	W1	W4	W5	W1	W4	W5	W1	W4	W5	W1	W4	W5
Baseline (median), n=6	64·5	64·5	64·5	6·52	6·52	6·52	3·84	3·84	3·84	0·67	0·67	0·67
Follow-up (median), n=7-14	26·5	32	41	3·66	3·88	4·58	3·02	3·39	3·79	0·65	0·67	0·71
P value	**0·03**	0·25	0·33	0·09	0·18	0·35	0·09	0·23	0·85	0·90	>0·99	0·28
$HR_{1200mg}ZE$												
Time on treatment	W1	W4	W5	W1	W4	W5	W1	W4	W5	W1	W4	W5
Baseline (median), n=7	30	30	30	3·67	3·67	3·67	3·48	3·48	3·48	0·74	0·74	0·74
Follow-up (median), n=6-11	23	27	46·5	3·13	3·58	5·19	3·12	3·01	4·01	0·64	0·66	0·72
P value	0·20	0·29	0·63	0·29	0·37	0·95	0·15	0·21	0·95	0·33	0·25	0·73
$HR_{35mg/kg}ZE$												
Time on treatment	W2	W8	W12	W2	W8	W12	W2	W8	W12	W2	W8	W12
Baseline (median), n=9	102	102	102	9·23	9·23	9·23	5·49	5·49	5·49	0·81	0·81	0·81
Follow-up (median), n=8-9	63	74·5	72	6·58	7·66	7·6	4·76	4·83	4·78	0·8	0·75	0·76
P value	**0·02**	0·07	0·09	**0·006**	**0·015**	**0·014**	**0·03**	**0·02**	**0·01**	0·54	0·17	0·06
$HR_{10mg/kg}ZQ$												
Time on treatment	W2	W8	W12	W2	W8	W12	W2	W8	W12	W2	W8	W12
Baseline (median), n=7	80	80	80	7·55	7·55	7·55	4·75	4·75	4·75	0·74	0·74	0·74
Follow-up (median), n=8	77·5	61	58·5	6·88	6·33	6·75	4·79	4·16	4·37	0·75	0·71	0·74
P value	0·63	0·29	0·52	0·34	0·39	0·61	0·96	0·78	0·87	0·39	0·78	0·55
$HR_{35mg/kg}ZQ$												
Time on treatment	W2	W8	W12	W2	W8	W12	W2	W8	W12	W2	W8	W12
Baseline (median), n=8	98	98	98	8·96	8·96	8·96	4·96	4·96	4·96	0·75	0·75	0·75
Follow-up (median), n=7-9	65	80	76	6·92	7·39	7·5	3·85	4·22	4·61	0·64	0·69	0·71
P value	0·16	0·15	0·34	0·09	0·08	0·19	**0·014**	0·06	0·24	**0·009**	0·14	0·33
$HR_{35mg/kg}ZM$												
Time on treatment	W2	W8	W12	W2	W8	W12	W2	W8	W12	W2	W8	W12
Baseline (median), n=9	82	82	82	7·96	7·96	7·96	4·77	4·77	4·77	0·75	0·75	0·75
Follow-up (median), n=7-9	31	38	50	3·76	5·08	6·032	2·42	3·51	4·41	0·55	0·71	0·73
P value	**0·001**	**0·002**	0·29	**0·008**	**0·003**	0·41	**0·004**	**0·015**	0·41	**0·02**	0·24	0·76

Table 2: Mann Whitney test of the difference between pre- and post- treatment microbiome under different regimens. Except upward trend of evenness under the HRZE regimen, in most cases microbiome took a downward trend that was significant in the R_{1500mg} and R_{1200mg}M regimens.

Taxa with an abundancy of above 1% remained relatively stable

We sought to identify which taxa and how they changed under the treatment regimens that induced significant change of alpha diversity measure. Taxa were sorted based on their relative abundancy (abundancy) with those above one percent were named whilst the ones with abundancy between 0-0·9% were grouped under others. Along the course of treatment, some of the over one percent1 abundant species were reduced to below one percent and were replaced by those from the others group whose relative abundancy rose above one percent. In general, the most abundant species never fell below one percent abundancy across the treatment period in all regiments.

Under standard regimen HRZE, there were 11 genera with ≥1% abundancy in the pre-treatment microbiome of which *Streptococcus,* 47% was the most abundant, followed by the other group, 16%, *Neisseria* 10% and *Gemella* 6%. At week 2 of treatment, the first three retained their positions but with a drop in RA to 43%, 10%, 9·4% whilst *Gemella* fell to 3·8% and was replaced by *Veillonella*, 9·1% in the 4th position. *Streptococcus* dropped to 34%, others group rose to 20%, *Veillonella* rose to 13% taking over *Neisseria*, and *Prevotella* became the 4th abundant genus, 8% by week 8 of treatment. By week 12 of treatment, *Streptococcus* dropped to 33%, *Neisseria* bounced back to 20% taking over others group, 12% and *Veillonella* at 11%. Although the other group changed from 16% at baseline to 10%, 20% and 12% by week 2, 8 and 12 respectively, they retained 2nd most abundant position except week 12 where they dropped to 3rd position. *Mycobacterium*, 2% abundancy at baseline dropped below one percent and never recovered till end week 12 (Figure 5).

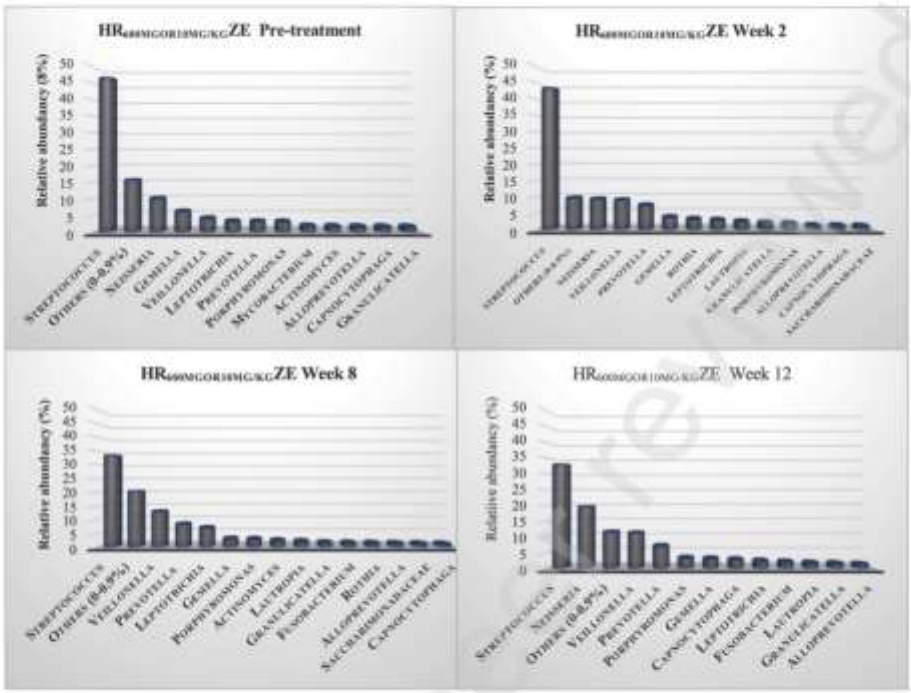


Figure 5: The taxa and how they changed under treatment with first-line regimen HR$_{600mgor10mg/kg}$ZE. The 'others' group retained 2nd position of abundancy until week 8 of treatment.

In the highest dose rifampicin regimen, HR35ZE, pre-treatment microbiome was comprised of 16 named genera that had ≥1% abundancy. The most abundant pre-treatment genera were *Streptococcus* 28%, *Veillonella* 12%, and *Prevotella* 11·4% plus the others group at 11·1%. By week two of treatment, the genera with ≥1% abundancy had dropped to 11 of which *Streptococcus* 35% and *Veillonella* 14% increase in abundancy while *Prevotella* was replaced by *Neisseria* 9%.

Although the 'Other' group retained the 4th position throughout treatment with this regimen, its abundancy changed from 11% to 9%, 8% and 11% by week two, eight and 12 of treatment. *Porphyromonas* 7% in the 5th position dropped to 10th position with 2% abundancy by week

2 of treatment but recovered to 6th position with 7% and 6% abundancy by week 8 and 12 of treatment (Figure 6).

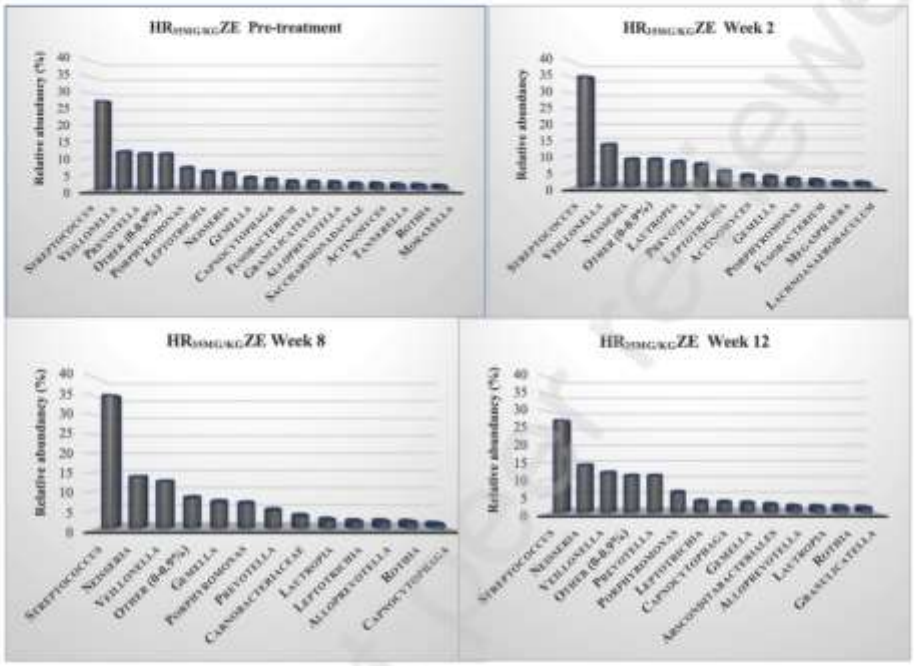


Figure 6: The taxa and how they changed under treatment with high dose rifampicin regimen $HR_{35mg/kg}ZE$. The 'others' group retained 4th position of abundancy until week 12 of treatment whilst Porphyromonas was significantly reduced in the first 2 weeks of treatment.

For the high dose rifampicin-moxifloxacin regimen, the ≥1% pre-treatment microbiome was comprised of 12 genera plus the others group. The most abundant was *Streptococcus*, 40% followed by *Neisseria*, 21%, the others group, 13% and *Rothia* at 5%. By week 2 of treatment, the total number of genera with ≥1% abundancy reduced to 9. Streptococcus dropped to 25% RA and was replaced by *Prevotella*, 27% abundancy in the first position.

Neisseria was replaced by *Streptococcus*, in the 2nd most abundant position while *Gemella* 13% abundancy and *Veillonela* 10% replaced the others group and *Rothia* in the 3rd and 4th position, respectively. The others group dropped 7% abundancy moving to 6th position while *Rothia*

dropped to below 1% RA and only recovered to 2% abundancy by week 12 of treatment. *Streptococcus* recovered to 35%- and 33%- RA by week 8 and 12 respectively but not to pre-treatment level. In contrast *Neisseria* dropped to 7% and 4% RA by week 2 and 8 respectively but recovered to 21% RA consistent with pre-treatment level by week 12 of treatment. *Mycobacterium* that was detectable at 1·1% RA at week 2 of treatment, dropped to <1% and never recovered until of end of treatment follow-up (Figure 7).

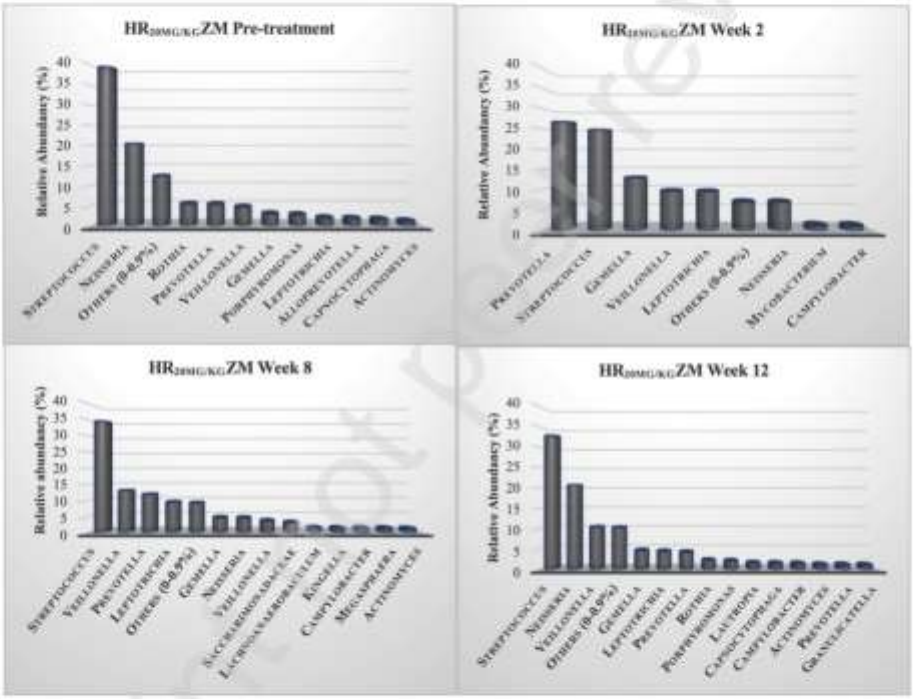


Figure 7: The taxa and how they changed under treatment with high dose rifampicin-moxifloxacin regimen $HR_{20mg/kg}ZM$. The others group was highly suppressed in the 1st two weeks of treatment. Unlike other regimens, *Streptococcus* abundancy was significantly reduced and never recovered to pre-treatment level over the treatment follow-up period.

Distinctive regimen induced taxa changes were observed in the community with <1% abundancy

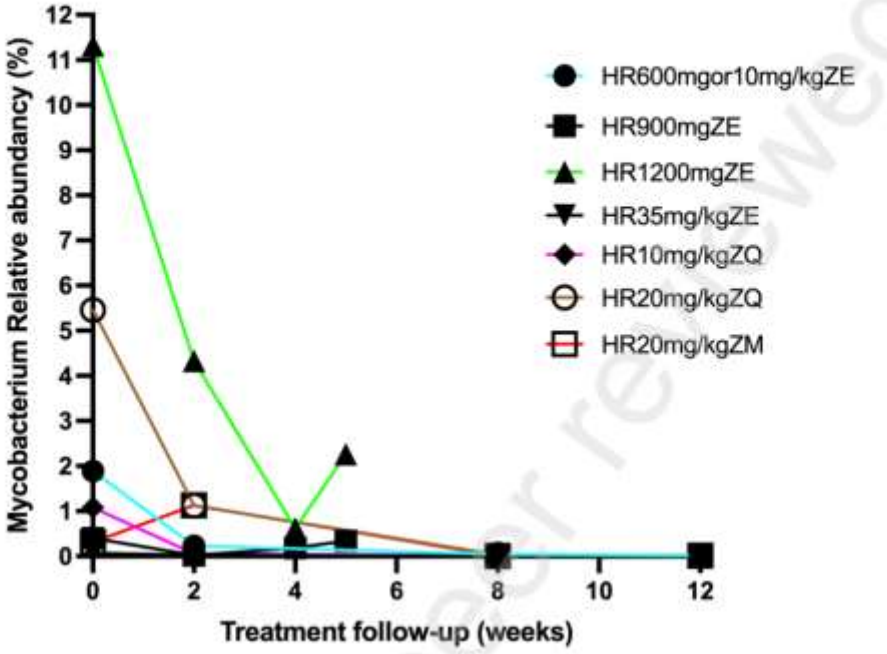
Given the changes in the over 1% abundant taxa were less distinctive between regimens, we analysed the less abundant taxa, 0.01 – 0.99 relative abundancy. In first line regimen, HRZE, 50 taxa were below 1% RA before treatment of which 14% (7/50) were reduced to undetectable level by week two of treatment. In the experimental regimens, HR35ZE and HR20ZM, there were 56 and 62 pre-treatment taxa below 1% RA of which 45% (24/53) and 53% (32/62) were undetectable by week two of treatment, respectively. Gram negative genera were the most represented in the taxa cleared by regimens, $HR_{600or10mg/kg}ZE$ 71% (5/7), $HR_{35mg/kg}ZE$ 50% (12/24) and $HR_{20mg/kg}ZM$ 59% (19/32) compared to Gram positive 28% (2/7), 21% (5/24) and 19% (6/32) cleared by the same regimens respectively (Table 3).

HRZE Taxa<1% RA at baseline = 50			HR35ZE Taxa <1 RA% at baseline = 53			HR20ZM Taxa <1% RA at baseline = 62		
Detected at baseline but not at week 2	**Gram stain**	**Detected at week 2 but not at baseline**	**Detected at baseline but not at week 2**	**Gram stain**	**Detected at week 2 but not at baseline**	**Detected at baseline but not at week 2**	**Gram stain**	**Detected at week 2 but not at baseline**
Bacillus	Positive	*Butyrivibrio*	*Actinobacillus*	Negative	*Alloprevotella*	Absconditabacteriales (SR1)	ND	*Bifidobacteriaceae*
Enterobacter	Negative	*Corynebacterium*	*Aggregatibacter*	Negative	*Granulicatella*	*Actinobacillus*	Negative	*Bifidobacterium*
Haloglobus	Negative	Defluviitaleaceae UCG-011	*Bergeyella*	Negative	*Tannerella*	*Actinobacillus porcinus*	Negative	*Bradyrhizobium*
Klebsiella	Negative	*Dialister*	*Butyrivibrio*	Negative		*Bergeyella*	Negative	*Capnocytophaga*
Lactococcus	Positive	*Filifactor*	*Candidatus Saccharimonas*	N/D		*Cardiobacterium*	Negative	*Enterococcus*
Serratia	Negative	*Lautropia*	*Dialister*	Negative		*Carnobacterium*	Positive	*Finegoldia*
Acinetobacter	Negative	Lentimicrobiaceae	*Dolosigranulum*	Positive		*Catonella*	Negative	*Roseburia*
		Moraxellaceae	*Filifactor*	Positive		*Comamonas*	Negative	*Staphylococcus*
		Mycoplasma	*Johnsonella*	Negative		*Corynebacterium*	positive	
		Odoribacter	Lachnospiraceae	ND		*Dialister*	Negative	
		Peptococcus	*Lactobacillus*	Positive		*Eikenella*	Negative	
		Rothia	*Lautropia*	Negative		*Escherichia-Shigella*	Negative	
		Staphylococcus	*Moraxella*	Negative		*Flexilinea*	Negative	
		Actinobacillus	*Oceanivirga*	Negative		*Johnsonella*	Negative	
		Actinomycetales	*Olsenella*	Negative		Lentimicrobiaceae	ND	
		Clostridiales	Paludibacteraceae	ND		*Moraxella*	Negative	
			Parvimonas	Negative		Muribaculaceae	ND	
			Peptococcus	Positive		*Mycoplasma*	ND	
			Propionibacteriaceae	ND		*Oceanivirga*	Negative	
			Rikenellaceae RC9 gut group	ND		*Olsenella*	Positive	
			Roseburia	Positive		Paludibacteraceae	ND	
			Ruminococcaceae UCG-014	ND		*Parvimonas*	Negative	
			Staphylococcus aureus	Positive		*Peptococcus*	Positive	
			Streptobacillus	Negative		*Peptoniphilus*	Positive	
						Rikenellaceae RC9 gut group	ND	
						Roseburia	Positive	
						Ruminococcaceae	ND	
						Serratia	Negative	
						Stenotrophomonas	Negative	
						Streptobacillus	Negative	
						Tannerella	Negative	
						Treponema	Negative	
14% (7/50) taxa reduced to sub-detectable level	**71% (5/7) Gram negative**		**45% (24/53) taxa reduced to sub-detectable level**	**50% (12/24) Gram negative**		**53% (32/61) taxa reduced to sub-detectable level**	**59% (19/32) Gram negative**	

Table 2: The under 1% RA taxa that were reduced to sub-detectable levels with the 1st two weeks of treatment and those that replaced them within the same period of treatment. ND =Gram stain status not defined

Members of genus *Mycobacterium* did not recover to pre-treatment level in all treatment regimens

The relative abundancy continuously reduced and did not recover in all regimens. The reduction in RA of *Mycobacterium species* was positively correlated with the *M. tuberculosis* bacillary load measured by the ribosomal RNA-based reverse transcriptase quantitative PCR test, Tuberculosis Molecular Bacterial Load Assay (TB-MBLA) across the period of treatment, r = 0·74, p<0·0001 (95CI 0·49-0·87) (Figure 8).



Regimen	0	2	4	5	8	12
$HR_{600mgor10mg/kg}ZE$	1·89	0·23			0·048	0·014
$HR_{900mg}ZE$	0·4	0·019	0·18	0·34		
$HR_{1200mg}ZE$	11·32	4·33	0·62	2·27		
$HR_{35mg/kg}ZE$	0·07	0·03			0·003	0·004
$HR_{10mg/kg}ZQ$	1·08	0·057			0·011	0·004
$HR_{20mg/kg}ZQ$	5·46	1·12			0·031	0·012
$HR_{20mg/kg}ZM$	0·31	1·13			0·007	0·018

Figure 8: The relative abundancy of *Mycobacterium* over treatment course. There was no recovery to pre-treatment level in all regimens.

Microbiome changes and association with treatment outcome

Participants were grouped according to their week-8 TB liquid culture results: culture negative (early conversion), culture positive (non-converters) and indeterminate (culture grows a contaminant but TB cannot be definitively ruled in or out). We observed that the effect of the regimen-microbiome interaction was associated with culture test outcome (Supplementary text 3; Figure S2)Contingency plots of their microbiome at phylum-class and family-genus levels

revealed Proteobacteria of class Gammaproteobacteria distinctively changed between the treatment outcome groups. Gammaproteobacteria include most implicated pathogenic bacteria: *Neisseria*, *Pseudomonas*, *Moraxella,* and *Haemophilus*. By week eight of treatment, abundancy of Gammaproteobacteria was substantially reduced of which genus *Neisseria* was decreased by 98% among the culture negative (early conversion group). In the non-converted (culture positive) group, Gammaproteobacteria abundancy stayed relatively stable including a 2% increase of genus *Neisseria*. The indeterminate group had a small pre-treatment abundancy of Gammaproteobacteria that remained small but was dominated by genus *Neisseria* which increased by 25% from baseline to week 8 of treatment (Figure 9A and B).

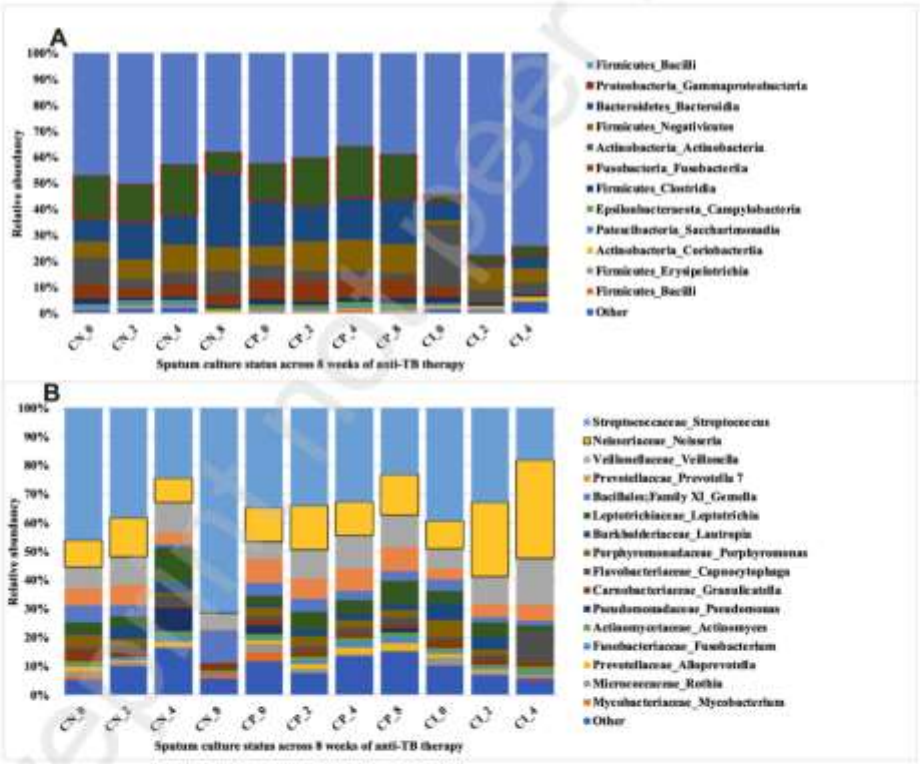


Figure 9: Association of taxa change and treatment outcome at month two of treatment. A)Phylum-Class level plot showing change in Proteobacteria-Gammaproteobacteria in culture negative (CN), culture positive (CP) and culture indeterminate (CI) participants. B) Genus level association showing changes in

genus *Neiserria* among CN, CP and CI participants. Neisseria was reduced by 98% among participants who culture converted by month-2 of treatment.

DISCUSSION

Our study systematically assessed the effect of different combinations of anti-TB antibiotics on respiratory microbiome and implication to treatment outcome. Unlike most studies that use DNA, the microbiome was drawn from RNA, enabling us to accurately assess effect of antibiotics on microbiome viability over the course of treatment. We show that once exposed to anti-TB antibiotics, the microbiome takes a fall and rise pattern akin to that of predator-prey relationship, reflecting sensitivity and resilience of the microbiome under antibiotic pressure. In a longitudinal follow-up of 12 weeks, a significant reduction in microbiome diversity was observed in the first two weeks of treatment, recapitulating the early bactericidal activity of anti-TB antibiotics. The moxifloxacin- and rifampicin-35mg/kg- containing regimens were responsible for the largest reduction of microbiome diversity that was significantly different from pre-treatment levels. While most of the taxa recovered, genus *Mycobacterium* did not show recovery, suggesting a unique sensitivity to anti-TB antibiotics. The trend of *Mycobacterium* elimination concurred with that measured by the tuberculosis Molecular Bacterial Load Assay (TB-MBLA), a reverse transcriptase qPCR-based assay that specifically quantifies viable bacilli from the *M. tuberculosis* complex.[16] It is important to note that despite causing TB disease, genus microbiome was never the most abundant taxon in any of the patients. Future studies may need to explore the existence of *Mycobacterium tuberculosis* as a commensal particularly in asymptomatic/healthy individuals and threshold or trigger by which *M. tuberculosis* becomes pathogenic.

Pre-treatment microbiome was more diverse in the southwest of Tanzania than in north-southeast, suggesting a geographical influence on host microbiome. Geographical location which encapsulates environment and the kind of diet one eats has been shown to shape the microbiome acquired.[19] Participants in both regions, however, had uneven microbiome, dominated by Firmicutes (*Streptococcus*), Bacteroidetes (*Veillonella*), Proteobacteria

485 (*Neisseria*), and Actinobacteria (*Mycobacterium*). Dominance of *Streptococcus*, *Viellonela* and
486 *Neisseria* in respiratory microbiota have been reported by other studies [28,29], while high
487 abundancy of *Mycobacterium* is explained by the fact that the participants were
488 bacteriologically confirmed TB patients. The first two weeks of antibiotic exposure were the
489 most significant as pertains to the effect on microbiome.

490 While all regimens exhibited reduction in richness, diversity, and evenness, this was significant
491 under Rif_{1200}mg-moxifloxacin and Rif_{1500}mg regimens, which had effect in over across alpha
492 diversity metrics. While moxifloxacin regimen effect was strong until week 8, the effect of
493 $Rif_{35mg/kg}$ on taxa diversity was sustained up to 12 weeks of treatment, suggesting a long-term
494 impact of rifampicin. These observations are in line with the reported rapid action and
495 sterilising effect of moxifloxacin and rifampicin respectively. [20,21] Of the two regimens, only
496 moxifloxacin regimen reduced evenness in the first two weeks of treatment plus the
497 Rif_{1200mg}SQ109 regimen which had effect on phylogenetic diversity and evenness in the same
498 two-week period. In contrast, there was gain in evenness by taxa under the standard $HR_{600mg\ or}$
499 $_{10mg/kg}$ZE regimen from week two through to week 12 of treatment. The insignificant reduction
500 of evenness by $Rif_{35mg/kg}$ and gain exhibited by $HR_{600mg\ or\ 10mg/kg}$ZE is an indication that
501 rifampicin bactericidal effect may be evenly spread across taxa, which consequently evens out
502 taxa distribution rather than elimination. The effect may be different at a rifampicin dose higher
503 than $Rif_{35mg/mg}$. The PanACEA MAMS TB-01 trials found the $Rif_{35mg/mg}$ regimen more
504 effective at causing statable culture conversion and recommended it for phase three studies.
505 However, considering the longer suppression of microbiome observed in this study, it is
506 important that such phase three studies include assessment of long-term impact on microbiome
507 and patient post-treatment health. $Rif_{20mg/kg}$-moxifloxacin regimen, which was found to have
508 modest efficacy on culture, has been shown in this study to have stronger early microbiome
509 suppression but allows recovery to occur faster.

Since the HIGHRIF2 study found fixed dose R_{1200mg} not superior to standard R_{600mg} at causing culture conversion, it is plausible to speculate that the effect we have observed in the $Rif_{20mg/kg}$-moxifloxacin regimen is most likely contributed by moxifloxacin.

A few taxa, 12 had relative abundancy above 1% leaving the majority under 1% (0.1-0.99%). The over 1% abundant taxa remained fairly, stable experiencing minimal reduction by antibiotic action. Notable reduction was under the moxifloxacin regimen where *Streptococcus* was reduced by 15% and displaced by *Prevotella* to 2nd position of abundancy in; *Neisseria* 14% reduction displacing it to 7th position taking up week 12 to recover to 2nd position of abundancy. In contrast, there was a 7% and 4% increase in *Streptococcus* and *Neisseria* respectively under the Rif_{1500mg} regimen in the first two weeks of treatment, and only 3% and 1% reduction of the same genera by the $HR_{600mg}ZE$ regimen. This again demonstrates the strong early bactericidal effect of the moxifloxacin containing regimen. This implies that supplementing rifampicin with moxifloxacin achieves strong and faster action than just increasing rifampicin dose alone.

The relatively stable over 1% abundant taxa could not explain the reduction in richness and diversity observed in first two weeks of treatment. A zoom into the less than 1% abundant taxa revealed the members of community that were reduced to below detection by week two of treatment. Over a half of the taxa reduced to sub-detectable levels were gram negative bacteria, 57% (36/63) of which 53% (19/36) were under the moxifloxacin regimen. It has long been demonstrated that fluroquinolones such as moxifloxacin have strong action against gram negative bacteria including *Neisseria meningitidis* and gram-positive *Streptococcus pnuemoniae* and generally anaerobic bacteria.[22–24]

While the rest of the taxa recovered during treatment, members of genus *Mycobacterium* did not recover across all regimens, suggesting some form of selective elimination. A similar

reduction of *Mycobacterium* vis-à-vis recovery of other taxa was reported by Katete *et al*., in TB patients treated with standard $HR_{10mg/kg}ZE$ regimen.[25] The sustained elimination of *Mycobacterium* may be explained by constituents of the anti-TB regimen: isoniazid, pyrazinamide, and ethambutol that act specifically on *Mycobacterium* possibly enhanced by broad spectrum rifampicin or moxifloxacin.[26,27] Rifampicin was implicated as a main driver of dysbiosis in a mouse model of TB infection and treatment.[28]

We explored whether antibiotic-microbiome interaction had impact on TB treatment outcome. Proteobacteria genus *Neisseria* was reduced to sub-detectable level in patients who converted to TB culture negative by week 8 of treatment. In contrast, there was a 2% and 25% in patients who remained culture positive and indeterminate (neither TB positive nor negative but grew contaminants) respectively by week 8 of treatment. Further studies should investigate the robustness of this association and its implications to treatment outcome of patients.

Study limitations

The sample size is small when divided by the number of analysed regimens. It is important to note, however, that the pre-treatment core microbiome covered in this study is consistent with and representative of the respiratory microbiota composition published by other studies conducted in East Africa and outside Africa[29,30] This implies that covering 100s to 1000s of participants may not necessarily increase the taxa in the core metagenome. Secondly the study did not examine from which source the recovering taxa came from. Further studies should investigate whether recovery was due to regrowth of suppressed taxa or due to replenishment from dietary sources, and impact on long-term clinical and health outcomes.

In summary, we have shown that different anti-TB regimens and dosages have different effects on the sputum microbiome. The standard first-line regimen, HRZE appeared soft on microbiome, causing increase in taxa evenness and no significant reduction of diversity. Within the backbone of the standard regimen, increasing the dose of rifampicin alone required up to 35mg/kg to achieve significant reduction of microbiome, which did not recover to pre-treatment level by month-3 of treatment follow-up. A less rifampicin dose of 20mg/kg supplemented with 400mg moxifloxacin achieved significant reduction of microbiome diversity but recovered to pre-treatment level by month-3 of treatment. The drug SQ109 appeared to have added no significant value added to the performance of anti-TB regimen as well as effect on microbiome. Most importantly, *Mycobacterium tuberculosis* did not show recovery across regimens, an effect implies the promise that novel optimal anti-TB regimens to shorten treatment course achieved without irreversible damage of the beneficial respiratory microbiome. Further large-scale longitudinal studies will be needed to ascertain whether it is only *M. tuberculosis* that is eliminated and what implications this has on the recovering microbiome and treatment outcome.

Contributors: EM, WS, and SHG designed the study and the protocols. EM, SE, WS, AW, ED, BM, NEN and GSK participated in data collection and curation. EM, WS and AW analysed the data. WS and AM drew the figures. WS, SHG, NH, MH, and MB obtained the funds that supported the study. EM, WS and SHG wrote the first and the final draft. All authors participated in writing the manuscript

Declaration of interests: Wilber Sabiiti and Stephen Gillespie provide a *pro bono* advice for a company that is developing TB-MBLA for clinical use. All other members declare a no conflict of interest. No contributing author (s) declared him/herself to be (a) medical writer(s) or editor(s)

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RESEARCH ARTICLE

High *Mycobacterium tuberculosis* Bacillary Loads Detected by Tuberculosis Molecular Bacterial Load Assay in Patient Stool: a Potential Alternative for Nonsputum Diagnosis and Treatment Response Monitoring of Tuberculosis

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ABSTRACT Not all patients produce sputum, yet most available TB tests use sputum. We investigated the utility of a novel RNA-based quantitative test, the tuberculosis molecular bacterial load assay (TB-MBLA), for the detection and quantification of *Mycobacterium tuberculosis* in stool. Stools from 100 adult individuals were treated with OMNIgene-sputum reagent and tested using Xpert MTB/RIF ultra (Xpert ultra), auramine O smear microscopy (smear), mycobacterial growth indicator tube (MGIT), and Lowenstein-Jensen (LJ) cultures. The remaining portions were frozen at −20°C and later tested by TB-MBLA. MGIT sputum culture was used as a TB confirmatory test and reference for stool tests. Sixty-one of 100 participants were already confirmed TB positive by MGIT sputum culture, 20 (33%) of whom were HIV coinfected. TB-MBLA detected *M. tuberculosis* in 57/100 stool samples, including 49 already confirmed for TB. The mean bacterial load measured by stool TB-MBLA was 5.67 ± 1.7 $\log_{10}$ estimated CFU (eCFU) per mL in HIV-coinfected participants, which was higher than the 4.83 ± 1.59 $\log_{10}$ eCFU per mL among the HIV-negative participants ($P = 0.04$). The sensitivities (95% confidence intervals [CI]) of stool assays were 80% (68 to 89) and 90% (79 to 98) for TB-MBLA and Xpert ultra, which were both higher than the 44% (32 to 58), 64% (51 to 76), and 62% (45 to 77) for smear, MGIT, and Lowenstein-Jensen (LJ) stool cultures, respectively. The specificity (95% CI) of stool assays was highest for smear, at 97% (87 to 100), followed by Xpert ultra at 91% (76 to 98), TB-MBLA at 79% (63 to 90), LJ at 80% (64 to 91), and MGIT at 62% (45 to 77). Twenty-six percent of MGIT and 21% of LJ stool cultures were indeterminate due to contamination. Detection and quantification of viable *M. tuberculosis* bacilli in stool raises its utility as an alternative to sputum as a sample type for TB diagnosis.

IMPORTANCE This paper highlights the value of stool as a sample type for diagnosis of tuberculosis. While other studies have used DNA-based assays like the Xpert MTB/RIF and culture to detect *Mycobacterium tuberculosis* in stool, this is the first study that has applied TB-MBLA, an RNA-based assay, to quantify TB bacteria in stool. The high microbial density and diversity in stool compromises the specificity and sensitivity of culture-based

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tests due to overgrowth of non-*M. tuberculosis* flora. Consequently, TB-MBLA becomes the most sensitive and specific test for the detection and quantification of viable TB bacteria in stool. Most crucially, this study raises the possibility of a nonsputum alternative sample type for diagnosis of TB among people who have difficulty in producing sputum.

KEYWORDS molecular bacterial load assay, molecular diagnostics, *Mycobacterium tuberculosis*

Tuberculosis (TB) is a persistent global health challenge (1). In 2020, an estimated 9.9 million people fell ill due to TB. In the same year, an estimated 1.3 million deaths, up from 1.2 million in 2019, occurred among HIV-negative people, and an additional 214,000 deaths occurred among HIV-positive individuals (1). In 2020, WHO reported a sharp reduction in TB case notifications in several high-TB-burden countries, partly due to the COVID-19 pandemic (2). Rapid case detection and treatment initiation is critical to minimizing TB-related morbidity and mortality, especially among vulnerable groups, such as young children and people living with advanced HIV disease. However, diagnosis of TB in young children, the terminally ill, and the immunocompromised, as well as neurologically impaired patients, may be challenging due to inability to provide an adequate sputum sample, leading to low case detection and high mortality rates (3–5). Consequently, bacteriological confirmation of pulmonary TB with microscopy, culture, and Xpert MTB/RIF (Xpert) assay in such groups of individuals may benefit from the use of alternative sample types, such as gastric and nasopharyngeal aspirates (6–9). However, some of these sample collection methods are invasive and have low diagnostic yield (10). Importantly, such sampling procedures are not routinely used in low-resource, high-burden settings. WHO recommends the use of the Xpert MTB/RIF ultra (Xpert ultra) on sputum, nasopharyngeal aspirate gastric aspirates, or stool for the diagnosis of TB in children aged below 10 years (1).

Often, people swallow sputum, which ends up in the gut, and hence, stool has been suggested as an alternative or additional sample type for bacteriological confirmation of pulmonary TB (11–14). The sensitivity and specificity of the Xpert MTB/RIF evaluation of stool varies depending on the population and laboratory processing methods (11, 15–20). The new version, the Xpert MTB/RIF ultra (Xpert ultra), has been shown to have better sensitivity (21); however, Xpert ultra may detect DNA from dead bacilli (22). On the other hand, mycobacterial growth indicator tube (MGIT) stool culture is associated with low yield on stool samples (23).

The tuberculosis molecular bacterial load assay (TB-MBLA) was developed to utilize the abundant cellular 16S rRNA as a marker for the detection and quantification of viable *Mycobacterium tuberculosis* bacilli in sputum samples. Like viral-load monitoring in HIV-positive individuals, TB-MBLA monitors the TB treatment response by measuring changes in the *M. tuberculosis* bacillary load over the course of treatment (24–29). Consequently, TB-MBLA potentially offers both diagnostic and treatment response monitoring advantages in real time to inform clinical decision making. A recent multisite study of adult TB patients showed that TB-MBLA is more sensitive than MGIT sputum culture and that its results are reproducible in different settings (30). Additionally, TB-MBLA was proven to differentiate the treatment outcomes of different TB regimens (31). Although TB-MBLA may detect and quantify *M. tuberculosis* in stool samples, its utility in stool has not been assessed. Having a sensitive quantitative test that can be used on an easily accessible sample may improve TB diagnosis and treatment response monitoring in patients who do not provide suitable sputum samples easily.

In this study, we assessed the ability of TB-MBLA to detect and quantify viable *M. tuberculosis* bacilli in archived stool samples from presumptive pulmonary TB patients. We show that *M. tuberculosis* in patient stools was detectable by TB-MBLA with high sensitivity and specificity and that stool could be an alternative sample type for nonsputum diagnosis and treatment response monitoring of TB.

RESULTS

Study participants. Most of the study participants, 54/100, were female adults aged between 33 and 36 years. Of the 100 stool samples tested, 61 samples had already

TABLE 1 Demographic and clinical characteristics of the participants who provided stool samples

Characteristic[a]	Median value (IQR) or no. (%)			
		Participants with indicated pulmonary TB status[b]		
	Overall (n = 100)	Positive (n = 61 [61%])	Negative (n = 39 [39%])	P value[c]
Age (yr)	34 (25–42)	33 (25–41)	36 (26–45)	0.72
Female	53 (53)	32 (52.5)	21 (53.9)	0.80
HIV positive	36 (35)	20 (33)	16 (41)	0.27
ART use	20 (38)	10 (16.4)	10 (26)	0.31
CD4 cells/μL[d]	110 (44–228)	71 (26–171)	170 (66–254)	0.03
BMI	20 (18–22)	19.7 (18–23)	19 (17–21)	
Alcohol use	66 (66)	42 (69)	24 (61)	0.63
Smoking	21 (21)	12 (20)	9 (23)	0.73
Fever	79 (79)	48 (78.7)	31 (79)	0.86
Wt loss of >5%	87 (87)	54 (88.5)	33 (84)	0.78
Cough for >2 wks	100 (100)	61 (100)	39 (100)	0.46
HR	100 (84–111)	100 (84–111)	101 (81–111)	
RR	22 (20–26)	22 (20–26)	24 (20–27)	

[a]HIV, human immunodeficiency virus; ART, antiretroviral therapy; BMI, body mass index; HR, heart rate; RR, respiratory rate.
[b]Bacteriologically confirmed positive or negative TB cases.
[c]Comparison between pulmonary-TB-positive and -negative participants.
[d]Measured for HIV-infected adults only (n = 36).

been confirmed positive for pulmonary tuberculosis using MGIT sputum culture. Among the participants who were confirmed positive for pulmonary tuberculosis, 20 (33%) were living with HIV infection, with a median CD4 cell count of 70.5 cells/μL, including 5 (8%) who were on HIV treatment. All participants reported at least one of the following symptoms prior to enrollment: unexplained persistent fever, weight loss, and cough (Table 1).

Stool *M. tuberculosis* bacillary load quantification. Measured as the conversion of quantification cycle (C_q) values to CFU per mL, the overall mean bacterial load (mean ± standard deviation [SD]) in stool was 5.1 ± 1.59 $\log_{10}$ estimated CFU (eCFU) per mL. Stool from participants living with HIV had a mean bacterial load of 5.67 ± 1.7 $\log_{10}$ eCFU per mL, which was higher than the 4.83 ± 1.59 $\log_{10}$ eCFU per mL in stool of HIV-negative participants (P = 0.04). In line with previously published data for sputum TB-MBLA (25), the C_q values and the molecular bacterial loads measured by the stool TB-MBLA were inversely related (r = −0.99) (Fig. 1). To compare the quantification between stool TB-MBLA and stool Xpert ultra, samples that were positive by both assays were considered. The mean cycle threshold (C_T) values of all the stool Xpert ultra probes were calculated and compared with the C_q values of the stool TB-MBLA. A Mann-Whitney test showed that the median C_q value of 20.3 (interquartile range [IQR], 15.4 to 24.8) for the stool TB-MBLA was significantly lower than the median C_q value of 25.1 (IQR, 22 to 28) for stool Xpert ultra (P < 0.00001; n = 45). Importantly, we note that equal volumes of stool sample were used for TB-MBLA and Xpert

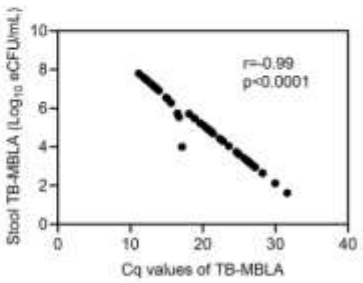

FIG 1 Correlation between the TB-MBLA C_q and bacterial load values in stool samples. The molecular bacterial loads ($\log_{10}$ CFU/mL) and the C_q values showed a strong negative correlation (r = −0.99).

TABLE 2 Results for analysis of accuracy of stool assays

Test[a]	Mean value (95% CI) for[b]:			
	Sensitivity	Specificity	PPV	NPV
TB-MBLA[c]	80 (68–89)	79 (63–90)	86 (74–93)	72 (56–85)
Xpert ultra	90 (79–98)	91 (76–98)	86 (70–95)	86 (70–95)
FM smear	44 (32–58)	97 (87–100)	96 (82–100)	53 (41–65)
MGIT culture	64 (51–76)	62 (45–77)	52 (37–67)	52 (37–67)
LJ culture	44 (32–58)	80 (64–91)	48 (35–61)	48 (35–61)

[a]TB-MBLA, tuberculosis molecular bacterial load assay; Xpert ultra, Xpert MTB/RIF ultra; FM, fluorescence microscopy; MGIT, mycobacterial growth indicator tube; LJ, Löwenstein-Jensen.
[b]MGIT sputum culture was used as the reference. PPV, positive predictive value; NPV, negative predictive value.
[c]Done using stool frozen at −20°C for 18 months.

ultra. A Spearman's correlation analysis showed a nonsignificant positive correlation between the stool TB-MBLA and stool Xpert ultra C_T values ($r = 0.17$, $P = 0.25$).

Sensitivity and specificity of stool assays with reference to MGIT sputum culture. Using MGIT sputum culture as the reference test, the sensitivity (95% CI) of stool assays was 80% (68 to 89) for TB-MBLA and 90% (79 to 98) for Xpert ultra, and both were higher than the 44% (32 to 58) for smear, 64% (51 to 76) for MGIT, and 62% (45 to 77) for LJ stool cultures. The specificity (95% CI) of stool assays was highest for smear at 97% (87 to 100), followed by 91% (76 to 98) for Xpert ultra, 79% (63 to 90) for TB-MBLA, 80% (64 to 91) for LJ, and 62% (45 to 77) for MGIT (Table 2).

Concordance between stool assays and MGIT sputum culture. Sixty-one of 100 of the tested participants were confirmed positive for pulmonary tuberculosis (PTB) by MGIT sputum culture. TB-MBLA detected and quantified *M. tuberculosis* in 57/100 participants, 49 of whom were among the 61 participants that had already been confirmed PTB positive by MGIT sputum culture. Stool Xpert ultra identified *M. tuberculosis* in 55/100 of the tested samples, of which 51/100 had been confirmed by MGIT sputum culture. Stool LJ culture detected the lowest number, 27 of the 61 MGIT sputum-positive cultures (Fig. 2). The overall percentages of concordance of stool positivity and negativity to MGIT sputum culture results were higher with molecular assays than with nonmolecular assays. Using kappa analysis, the two molecular assays strongly agreed with MGIT sputum culture, at 81% ($\kappa = 0.6$) for TB-MBLA and 87% ($\kappa = 0.72$) for Xpert ultra. Among the nonmolecular assays,

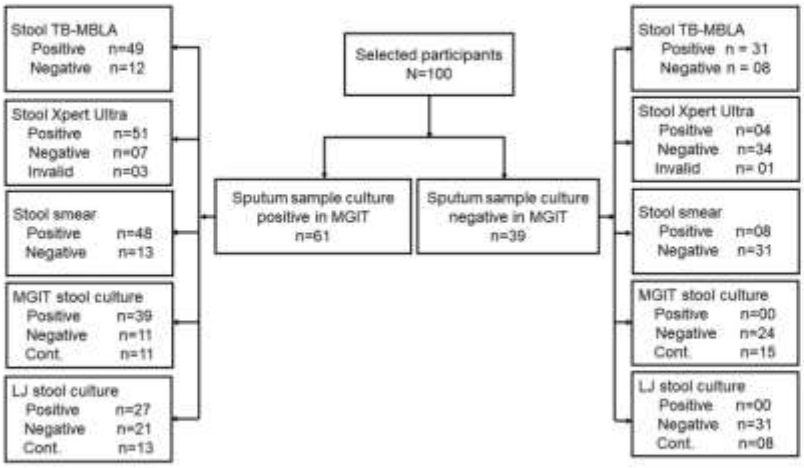

FIG 2 Flow chart showing the numbers of patients, samples, and test results for stool samples. TB-MBLA, tuberculosis molecular bacterial load assay; Xpert, Xpert MTB/RIF ultra; smear, smear fluorescence microscopy; MGIT, mycobacterial growth indicator tube; LJ, Löwenstein-Jensen culture.

TABLE 3 Results for analysis of the concordance of stool assays and MGIT sputum culture results

Test[a]	% agreement			κ statistic	Strength
	Positive	Negative	Overall		
TB-MBLA	82	89	84	0.67	Substantial
Xpert ultra	85	90	87	0.72	Substantial
MGIT culture	62	60	61	0.2	Poor
LJ culture	43	80	56	0.19	Poor
FM smear[b]	42	97	61	0.31	Fair

[a]TB-MBLA, tuberculosis molecular bacterial load assay; Xpert ultra, Xpert MTB/RIF ultra; MGIT, mycobacterial growth indicator tube; LJ, Löwenstein-Jensen; FM, fluorescence microscopy.
[b]Done using stool frozen at −20°C for 18 months.

stool smear had the highest concordance with the MGIT sputum culture, at 63% ($\kappa = 0.34$), followed by MGIT stool at 46% ($\kappa = 0.2$) and LJ stool at 42% ($\kappa = 0.2$), respectively (Table 3).

Concordance within TB-positive stool assays. Using a Venn diagram, we illustrated the levels of concordance within stool assays. Considering the samples detected as TB positive by each test, we found that only 14/100 were consistently positive by all five stool tests. Concordance was highest between the two molecular tests, stool TB-MBLA and Xpert ultra, with agreement for 45/100 samples tested. Molecular tests and combined stool MGIT and LJ cultures showed the 2nd highest concordance, with agreement for 21/100 samples tested. Concordance was only 14/100 in a combination of stool TB-MBLA, Xpert ultra, smear, and LJ culture (Fig. 3).

Indeterminate stool culture. We noted that 26% and 21% of MGIT and LJ stool cultures, respectively, were contaminated, and their results were indeterminate (neither positive nor negative status) (Table 4). Twelve (46%) of the indeterminate MGIT stool

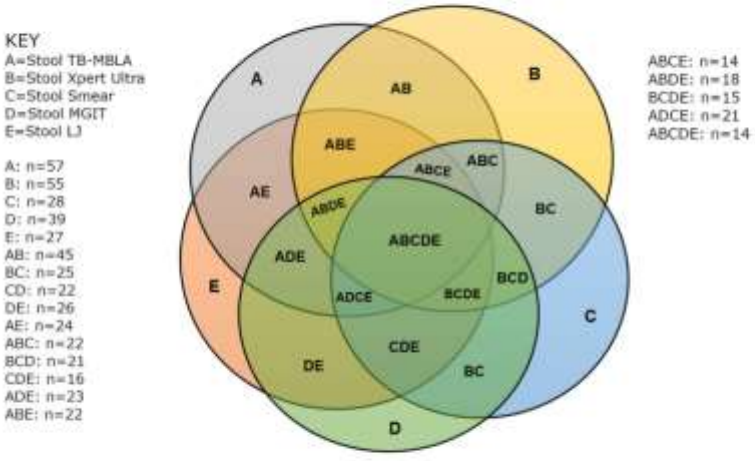

FIG 3 Venn diagram for positive results for stool assays. A, TB-MBLA ($n = 57$); B, Xpert ultra ($n = 55$); C, smear ($n = 28$); D, MGIT culture ($n = 39$); E, LJ culture ($n = 27$); AB, TB-MBLA and Xpert ultra ($n = 45$); BC, Xpert ultra and smear ($n = 25$); CD, smear and MGIT culture ($n = 22$); DE, MGIT culture and LJ culture ($n = 26$); AE, TB-MBLA and LJ culture ($n = 24$); ABC, TB-MBLA, Xpert ultra, and smear ($n = 22$); BCD, Xpert ultra, smear, and MGIT culture ($n = 21$); CDE, smear, MGIT culture, and LJ culture ($n = 16$); ADE, MGIT culture, LJ culture, and TB-MBLA ($n = 23$); ABE, TB-MBLA, Xpert ultra, and LJ culture ($n = 22$); ABCE, TB-MBLA, Xpert ultra, smear, and LJ culture ($n = 14$); ABCD, TB-MBLA, Xpert ultra, smear, and MGIT culture ($n = 18$); BCDE, Xpert ultra, smear, MGIT culture, and LJ culture ($n = 15$); ABDE, TB-MBLA, Xpert ultra, MGIT culture, and LJ culture ($n = 21$); ABCDE, TB-MBLA, Xpert ultra, smear, MGIT culture, and LJ culture ($n = 14$). It is apparent that molecular assays detect more TB-positive cases than do smear and culture tests and that combining molecular assays with stool culture or stool smear microscopy does not increase the number of identified TB cases, meaning that one molecular test is sufficient for a clinician to make a clinical decision.

TABLE 4 Results for analysis of the indeterminate results in stool samples that were resolved by TB-MBLA and Xpert ultra

Test[a]	No. (%) with indeterminate culture result[b]	No. (%) with indicated result in[c]:					
		TB-MBLA		Xpert ultra		Smear	
		Positive	Negative	Positive	Negative	Positive	Negative
MGIT culture	26 (26)	12 (46)	14 (54)	12 (46)	14 (54)	2 (8)	24 (92)
LJ culture	21 (21)	14 (67)	7 (33)	12 (57)	9 (43)	5 (24)	16 (76)

[a]MGIT, mycobacterial growth indicator tube; LJ, Lowenstein-Jensen.
[b]Contaminated culture results that were neither positive nor negative.
[c]TB-MBLA, tuberculosis molecular bacterial load assay; Xpert ultra, Xpert MTB/RIF ultra.

cultures were positive by both stool TB-MBLA and stool Xpert ultra. Considering the LJ stool cultures, 14 (67%) of the indeterminate samples were positive by stool TB-MBLA, while 12 (57%) were positive by stool Xpert ultra. Overall, indeterminate results that were resolved by stool TB-MBLA and stool Xpert ultra were concordant with MGIT sputum culture at 81% ($\kappa = 0.54$) and 85% ($\kappa = 0.73$), respectively, suggesting that they were true positives (Table 4). In contrast, we observed weak positivity and negativity concordance of the results that were resolved by stool smear and MGIT sputum culture, at 43% ($\kappa = 0.2$) and 62% ($\kappa = 0.2$), respectively, indicating the possibility of false positivity by stool smear microscopy.

Stool TB-MBLA-positive but sputum MGIT-negative participants. Eight patient stool samples were positive by TB-MBLA, but their corresponding MGIT sputum cultures were negative. Further analysis revealed that 6 of these 8 stool samples had at least one positive corresponding sputum test: 4 of them were positive by both sputum smear and sputum Xpert ultra and 2 were positive by only sputum smear but negative by sputum Xpert ultra. The remaining 2 stool samples were negative by all the investigated sputum tests.

Stool TB-MBLA-negative but sputum MGIT-positive participants. Twelve patient stool samples tested negative by TB-MBLA, but their corresponding MGIT sputum cultures were positive. Compared with the corresponding stool Xpert ultra-tested samples, 6 samples were negative, 5 were positive and detected as "low" ($n = 4$) and "trace" ($n = 1$), and 1 was invalid. The mean MGIT time to positivity (TTP) of the sputa that corresponded to the stool samples that were negative by both stool TB-MBLA and stool Xpert ultra was 11 days, indicating a moderately high bacillary load in sputa. The mean C_q value of the extraction control (±SD) in stool samples that were positive by stool TB-MBLA was 24.1 ± 1.9, and for the negative stool samples, it was 24.1 ± 2.3, not a significant difference ($P = 0.48$), potentially indicating absence of inhibition. Additionally, the average C_q value for 7/12 stool samples was 0, indicating the absence of any amplification by TB-MBLA, but 5 of the samples showed late amplification with an average C_q value of 32.8, well above the limit of detection of 30 C_q.

Time to positivity and C_q values. Overall, the mean MGIT TTP ± SD of 12.3 ± 6.4 days in stool samples was higher the 7.1 ± 3.1 days in sputum samples. Prior work on sputum TB-MBLA (25) showed a strong, direct correlation between C_q values and MGIT TTP. We thus investigated whether this was replicated in stool TB-MBLA and found that the mean C_q values for both stool TB-MBLA and stool Xpert ultra did not significantly correlate with the MGIT sputum TTP. Similarly, there was no significant relationship between C_q values and MGIT stool culture TTP (Fig. 4A and B).

DISCUSSION

Stool is an easy-to-obtain sample and could enhance TB diagnosis in individuals who cannot provide adequate sputum samples, and yet, MGIT culture, which is the current confirmatory test for TB, has low yields for stool. In this study, we assessed the ability of a molecular-based fully quantitative assay, the TB-MBLA, to detect and quantify viable *M. tuberculosis* bacilli in frozen stool samples.

We show that TB-MBLA for stool is sensitive and specific, signifying its potential utility for clinical decision making. Our results provide evidence for the presence of high bacillary loads in stool samples both in people living with HIV and those without, indicating its potential broad applicability in multiple settings. It is not clear whether the higher bacillary

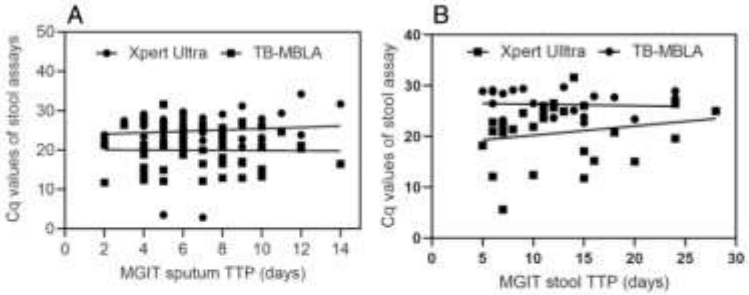

FIG 4 (A) Relationship between the C_q values of stool TB-MBLA or the C_q values of stool Xpert ultra and the MGIT sputum culture time to positivity (TTP) (days). The Spearman regression R^2 values were 0.000 and 0.13 for stool TB-MBLA and stool Xpert ultra, respectively. (B) Relationship between the C_q values of stool TB-MBLA or the C_q values of stool Xpert ultra and the MGIT stool culture TTP (days). The Spearman regression R^2 values were 0.02 and 0.04 for stool TB-MBLA and stool Xpert ultra, respectively. Overall, we did not find a significant correlation between C_q values and MGIT TTP.

loads observed in stool samples of HIV-positive patients are due to higher dissemination of bacilli from the respiratory tract to the gut. A larger-scale study would establish the robustness of this difference and what mechanisms underlie it.

Although TB-MBLA had significantly lower C_q values (potentially higher bacterial loads detected), Xpert ultra had higher sensitivity and specificity than TB-MBLA. The lower TB-MBLA C_q values could be explained by the fact that TB-MBLA detects the more abundant rRNA compared to the DNA detected by Xpert MTB/RIF. *Mycobacterium tuberculosis* has been shown to contain 700 ribosomes per cubic micrometer of cytoplasm, implying a larger amount of rRNA available for detection (32). Ideally, this would mean higher sensitivity of TB-MBLA than of Xpert ultra, but the reverse was true. One reason could be the use of multicopy *M. tuberculosis* gene targets in Xpert ultra, enabling it to detect fewer bacilli in a sample and to have a lower limit of detection (33). Furthermore, unlike Xpert ultra, specimen processing for TB-MBLA, particularly the initial centrifugation step to harvest *M. tuberculosis* cells from sputum, has been shown to cause a substantial loss of viable *M. tuberculosis* bacilli, which do not easily sediment, and consequently reduces assay sensitivity (34). Third, loss of *M. tuberculosis* cell viability due to prolonged storage at −20°C may have compromised the sensitivity of TB-MBLA.

In principle, there should be a correlation between rRNA and DNA detected in the same cell. However, our analysis showed a nonsignificant correlation between stool TB-MBLA and Xpert ultra *Cq* values. This requires further analysis in a large-scale study ensuring all analyses are done on freshly collected samples to eliminate the confounder of loss of viability caused by storage conditions. Second, the average C_q value for Xpert MTB/RIF should be derived from probes that detect *M. tuberculosis* DNA and not those of the non-TB internal controls of the assay.

Overall, our results concur with findings from other studies where molecular assays were shown to have higher diagnostic accuracy than stool culture assays when MGIT sputum culture was used as the reference test (35, 36). The low diagnostic accuracy of stool culture may partly be attributed to overgrowth of non-*M. tuberculosis* flora due to the rich microbial population in the gut, while loss of *M. tuberculosis* viability during transition in the gut is plausible but requires further investigation, since substantial numbers of the contaminated stool cultures were detected as *M. tuberculosis* positive by TB-MBLA, which is known to detect viable *M. tuberculosis* bacilli. *M. tuberculosis* growth inhibition and/or reduction of viable bacillary load due to the killing effect of the decontamination methods could be a factor in reducing the productivity of culture and further highlights the importance of sample processing methods (26).

In this study, stool samples were processed using OMNIgene-sputum (OM-S) reagent. OM-S suppresses contaminants, but it delays MGIT sputum time to culture positivity

(TTP) (37, 38). It is plausible that the same effect occurred in our stool samples; hence the absence of correlation between MGIT TTP and the C_q values of the molecular tests. Other studies have shown inverse correlation of MGIT TTP and C_q values (24, 27, 39).

We noted that 12 samples from PTB cases confirmed by MGIT sputum culture were not amplified by stool TB-MBLA. Whether this was due to inhibition could not be ascertained, because the spiked extraction control was efficiently recovered, as demonstrated by quantitative PCR (qPCR) amplification. However, we hypothesize that loss of *M. tuberculosis* viability during stool storage at −20°C, compounded by some loss of RNA during extraction, may have led to nondetection by TB-MBLA (34).

We also observed that in 5 of the 12 TB-MBLA-negative samples, the Xpert ultra positivity results were low/trace, implying that there were few bacilli in the samples prior to storage at −20°C. Some stool samples showed late amplification, and these reported as negative based on the cutoff value of 30. Whether the C_q cutoff of 30 wrongly placed these samples in the "TB-negative" category could not be ascertained. Future studies need to investigate the C_q cutoff value for stool.

Using molecular-based assays for stool has the potential to increase TB case detection in both adults and children, because they use *M. tuberculosis*-specific primers and probes that are not affected by non-*M. tuberculosis* flora present in stool. In this study, we show that 26% of the tested stool samples were contaminated (grew non-TB flora) on MGIT stool culture and 21% on LJ stool culture. All contaminated culture samples had a definitive (positive or negative) result by TB-MBLA. To confirm the validity of these results, the percentages of agreement with the reference standard test were calculated and found to range between 81 and 85%, implying that over 80% of the resolved results were valid and could be used to inform clinical decision making. Unlike Xpert ultra, which may detect nonviable *M. tuberculosis* bacilli due to persistent DNA after cell death, TB-MBLA has been shown to be sensitive to agents that reduce cell viability (30, 40).

The RNA extraction part of TB-MBLA is manual and takes a substantial amount of hands-on time and, thus, would benefit from automation of the process to increase its ease of implementation in the clinical laboratory setting. It is important to note that even in its current form, TB-MBLA is more rapid than culture. Shortening the TB-MBLA protocol will increase its potential for real-time application in the real-time clinical setting.

Diagnosis in children and immunocompromised individuals is difficult, usually depending on the unreliable clinical symptoms. Using stool samples and TB-MBLA could improve the confidence of clinicians in deciding to initiate or withhold treatment. Placing patients on an appropriate treatment early enough minimizes the risk of TB transmission and associated mortality. Smear microscopy is the fastest and the most accessible test, making it the most common TB diagnostic in resource-constrained settings (41). However, as in sputum (42), we noted that its sensitivity in stool was lower than that of molecular tests and that it might not give reliable yields if performed for a sample that had less than 10,000 bacilli/mL, as also reported elsewhere (43, 44). The detection of substantial bacillary loads in both HIV-negative and -positive stool samples is a testament to the sensitivity of stool as a sample type and makes it worth considering among sample types for primary diagnosis of TB. Further studies should investigate the mechanism underlying the higher bacillary loads in HIV-positive than in HIV-negative patients.

Using stool samples might provide an easier way to enhance diagnosis of gastrointestinal TB. Gastrointestinal TB is a life-threatening form of TB that control programs have not accorded due attention, perhaps because it is less transmissible and is challenging to diagnose (45). We noted that TB-MBLA detected and quantified *M. tuberculosis* in 8 stool samples among the MGIT sputum-negative participants, 2 of which did not have any corresponding positive sputum test. Whether these cases represented gastrointestinal TB or not could not be ascertained in this study because we lacked data about the relevant descriptors, such as colitis symptoms and Bristol stool chart scales. Future evaluations to unravel the use of stool specimens to diagnose gastrointestinal TB are urgently necessary.

Limitations. The main limitations of this study are the small sample size and the use of stored samples for TB-MBLA. Storing samples at −20°C for 18 months could have

degraded some rRNA, hence lowering the sensitivity of TB-MBLA. Nevertheless, we demonstrate the ability of TB-MBLA to detect and quantify *M. tuberculosis* in stool with a high level of accuracy, indicating its potential role in enabling clinical decision making. Besides, TB-MBLA is rapid and will enable rapid detection and quantification of *M. tuberculosis* in clinical specimens. Molecular testing of stool may facilitate TB diagnosis in patients unable to produce sputum and in screening of high-risk groups.

Future studies will explore the diagnostic accuracy and treatment response monitoring utility of TB-MBLA in a larger sample size of fresh stool samples from patients who are unable to provide sputum, including children. These investigations will benefit from modifying the current sample-processing methods to make it shorter and also to minimize the loss of bacilli caused by centrifugation steps. Future studies will also explore the role of TB-MBLA for revealing the mycobactericidal effect of TB regimens in patient populations where sputum production is often problematic, including children.

MATERIALS AND METHODS

Ethics. The parent and current studies were approved by the Makerere University School of Medicine Research and Ethics Committee (REC reference no. 2006-017) and the Makerere University School of Biomedical Sciences Research and Ethics Committee (REC reference no. SBS 529), respectively. All patients consented to the use of their stored stool samples and data for future TB investigations. Good clinical practice guidelines were observed (46).

Study site and design. We conducted a cross-sectional, laboratory-based study utilizing banked stool samples collected between 2018 and 2019. The study was nested in a cohort of persons with pneumonia (47–49) at Naguru Referral Hospital, Kampala, Uganda.

Specimen collection and processing. At enrollment, participants provided spot stool (~12.0 g) in a prelabeled sterile wide-mouthed container (Sarstedt, Australia). Six grams of each stool sample was homogenized and incubated at ambient temperature in OMNIgene-sputum reagent (OM-S; DNA Genotek, Inc., Ottawa, Canada) for 10 min. OM-S reagent decontaminates TB samples while preserving *M. tuberculosis* viability (50). The resulting suspension was pelleted twice in OM-S at 3,000 × *g*, for 20 and 10 min, respectively. The resulting pellet was resuspended in 6 mL of phosphate-buffered saline before being aliquoted into four different portions of 1 mL each to be tested by auramine O smear microscopy (smear), Xpert MTB/RIF ultra (Xpert ultra), MGIT, and LJ cultures. The remaining portions were banked at −20°C and tested by TB-MBLA in a batch on all specimens at once. OM-S reagent is compatible with TB molecular assays, smear, and solid and liquid culture tests (51, 52). In addition to stool samples, sputum samples were collected and tested on the same day using MGIT sputum culture and Xpert ultra. We note that sputa were not processed in OM-S but, rather, decontaminated using 2% NaOH/*N*-acetyl L-cysteine.

Selection of banked stool samples. By the time of this study, there were 600 stored stool samples, 597 of which had a valid corresponding MGIT sputum culture result as the reference comparator and all of which had valid Xpert results on sputum as the standard-of-care test. A systematic random sampling selection method (52) was conducted to obtain 100 archived stool samples. A sampling interval was calculated as the ratio of total banked stool samples to the target sample size. Samples without matching clinical data, sputum Xpert ultra, and MGIT sputum culture results were excluded. We note that selection of the samples was not based on any stool-related results.

TB-MBLA. Total RNA was extracted by adopting a method described elsewhere (24, 25, 27). Homogenized stool samples (1 mL) were thawed at ambient temperature, spiked with 100 μl of the extraction control, and then centrifuged for 15 min at 3,000 × *g*. RNA was isolated using the Fast Prep RNA pro blue kit (MP Biomedicals, Santa Ana, CA, USA). Duplex reverse transcriptase qPCR targeting both *Mycobacterium tuberculosis* and the extraction control was performed on a Rotor-Gene 5plex platform (Qiagen, Manchester, UK). Primers and TaqMan dually labeled probes were manufactured by MWG Eurofins, Germany. qPCR cycling conditions were as reported by Honeyborne et al. (24). Quantification cycle (C_q) values were converted to bacterial loads using a standard curve customized for the site's qPCR platform and recorded as estimated CFU per mL (eCFU/mL) (25). The standard curve was constructed using *M. tuberculosis* rRNA that was extracted from *M. tuberculosis* culture of a known concentration (CFU/mL). Stool samples without C_q values and those with C_q values above 30 were reported as TB negative.

Stool Xpert ultra. One milliliter of the homogenized stool was mixed with 2 mL of the sample reagent buffer and tested according to the Cepheid protocol. The same Xpert ultra machine was used for all the samples. Results were automatically generated in the categories negative, trace call, very low, low, medium, or high MTB (*M. tuberculosis*) positive by the Xpert ultra platform.

Smear microscopy. One milliliter of the homogenized stool was sedimented, and a smear (1 to 2 cm) was prepared and air dried. The dried smear was stained for 15 min using a 0.5% solution of auramine O (Merck, Darmstadt, Germany), decolorized for 2 min in 3% acid alcohol, and counter stained for 1 min using 0.5% potassium permanganate solution. Smears were examined within 1 h under a fluorescence microscope at ×400 magnification.

Liquid and solid cultures. MGIT tubes were inoculated with 500 μL of the homogenized stool samples and incubated at 37°C for a maximum of 42 days. LJ slants were inoculated with 200 μL of the resuspended stool sediments and incubated at 37°C for a maximum of 56 days. TB-positive cultures were confirmed by the presence of acid-fast bacilli upon Ziehl-Neelsen staining and the presence of antigen MPT64. The absence

of acid-fast-bacillus cording and growth on blood agar was recorded as contamination. Results were reported according to International Union Against Tuberculosis and Lung Disease guidelines (53). Contaminated cultures with no definitive positive or negative result for TB were categorized as indeterminate. We note that similar MGIT and LJ culture protocols were followed.

Statistical analyses. Differences in baseline clinical characteristics were compared using Fisher's exact test and Mann-Whitney U test for categorical and continuous variables, respectively. All bacterial load results (eCFU/mL) were log transformed before statistical analyses. Negative, positive, and overall (kappa scores) percentages of concordance between tests and MGIT sputum cultures were calculated using STATA version 15.1 (StataCorp, College Station, TX, USA). Sensitivity and specificity were calculated at the 95% confidence interval by using STATA version 15.1, using sputum MGIT culture as the reference test as was reported elsewhere (54). A sensitivity analysis method was used in calculation of specificity and sensitivity to minimize the interpretation bias due to contamination (54). Statistical significance was considered to be shown at a probability value of less than 0.05. Contaminated stool culture results could not be interpreted as either positive or negative. The status of these results was assessed for positivity or negativity using stool TB-MBLA, stool Xpert ultra, and stool smear, and thereafter, we categorized them as "resolved." To ascertain whether the status of the "resolved" result was valid, we investigated the agreement with the corresponding MGIT sputum culture using kappa statistics.

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TABLE 1 Demographic and clinical characteristics of study participants

Characteristic[a]	Data for participants with PTB status of[b]: Overall ($n = 100$)	Positive ($n = 61$)	Negative ($n = 39$)	P[c]
Age (median [IQR]) (yr)	34 (25–42)	33 (25–41)	36 (26–45)	0.72
Female (no. [%])	53 (53)	32 (52.5)[d]	21 (53.9)[e]	0.8
HIV-positive (no. [%])	36 (35)	20 (33)[d]	16 (41)[e]	0.27
ART use (no. [%])	20 (38)	10 (16.4)[d]	10 (26)[e]	0.31
CD4$^+$ cell count (median [IQR]) (cells/mm^3)[f]	110 (44–228)	71 (26–171)	170 (66–254)	0.03

[a]IQR, interquartile range; ART, antiretroviral therapy.
[b]Bacteriologically confirmed positive or negative cases.
[c]Comparison between PTB-positive and PTB-negative participants.
[d]Percentage of bacteriologically confirmed TB cases.
[e]Percentage of bacteriologically confirmed TB-negative cases.
[f]Measured for HIV-infected participants only ($n = 36$).

fact that the TB-MBLA was performed on stool samples that had been stored at −20°C for more than 1 year, conditions under which the *M. tuberculosis* RNA-preserving ability might have been lower than that of OM-S. This means that PBS-processed stool samples might achieve similar sensitivity, compared to OM-S-processed samples, if TB-MBLA is performed with freshly prepared stool samples.

OM-S was previously shown to be a strong preservative of *M. tuberculosis*, as well as suppressing non-*M. tuberculosis* contaminants (5). However, TB-MBLA uses primers and probes specific to *M. tuberculosis* and is not affected by non-*M. tuberculosis* contaminants found in patient sputum (7). This eliminates the need to use decontaminating reagents to process stool samples or other samples for TB diagnosis using molecular tests such as TB-MBLA; we previously showed that such processes reduce the viable count by 0.6 $\log_{10}$ CFU/mL on average (8). Based on these findings, we think that PBS may be an effective and inexpensive alternative for the preparation of stool samples for TB-MBLA and other molecular applications in both resource-rich and resource-limited settings. Larger studies are needed to verify the performance of PBS in recovering viable *M. tuberculosis* bacilli from both fresh and frozen stool samples, compared to the established RNA-preserving reagents.

Data availability. Raw data will be available at the University of St Andrews upon request and meeting of the ethical requirements according to which the samples were collected.

TABLE 2 Comparative performance of TB-MBLA and MGIT culture with PBS-processed versus OM-S-processed stool samples

Parameter	Data for: OM-S-processed stool samples ($n = 100$)	PBS-processed stool samples ($n = 100$)	P
Confirmed PTB by MGIT sputum culture (no. [%])	61 (61)	61 (61)	
Positive by stool TB-MBLA only (no. [%])	8 (8)	4 (4)[a]	
Positive by both MGIT sputum culture and stool TB-MBLA (no. [%])[b]	49 (49)	47 (47)[a]	
Bacterial load (mean ± SD) ($\log_{10}$ estimated CFU/mL)[c]	5.1 ± 1.59	4.28 ± 0.95	0.003
Threshold cycle (median [IQR])	20 (15–25)	22 (21–25)	0.002
Stool contamination by MGIT culture (no. [%])	26 (26)	69 (69)	
Stool contamination by MGIT culture but TB-MBLA positive (no. [%])	12 (46)	35 (51)	
Sensitivity (% [95% CI])	80 (68–89)	77 (65–87)	
Specificity (% [95% CI])	79 (63–90)	90 (76–97)	
Positive predictive value (% [95% CI])	86 (74–93)	92 (81–98)	
Negative predictive value (% [95% CI])	72 (56–85)	71 (57–83)	

[a]Forty-seven samples were sputum MGIT culture-stool TB-MBLA positive, while 4 samples were stool TB-MBLA positive only. Overall stool TB-MBLA positivity was 51% (51/100 samples) or 77% (47/61 samples) considering sputum MGIT culture as the gold standard.
[b]Sputum MGIT was used as the gold standard and reference test for TB-MBLA.
[c]Bacterial load values were log transformed before the mean was calculated.

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Policy brief submitted to Makerere University

Title: *New molecular RNA-based test simplifies diagnosis and assessment of the efficacy of anti-tuberculosis medicine*

Key findings: In a study evaluating the accuracy of a new molecular RNA-based test, tuberculosis Molecular Bacterial Load Assay (TB-MBLA) in Kampala, Uganda, we found that:

- TB-MBLA accurately detected and quantified tuberculosis (TB) bacteria in patients before and during treatment, thereby providing advantage over current standard-of-care tests.
- Not all TB patients require 6-month treatment course because TB-MBLA showed that irrespective of pre-treatment bacterial load, a proportion of patients who were positive before start of treatment tested negative (no TB) at week two of treatment and remained so for the rest of the 6-month treatment period.
- Current standard-of-care Smear microscopy and GeneXpert MTB/RIF Ultra are unsuitable for monitoring response to TB treatment (measuring efficacy of anti-TB medicine) because they cannot distinguish between TB bacteria that are killed- or not yet killed by the anti-TB medicine.
- Two-month smear microscopy result is inaccurate and may be insufficient to inform extension of the intensive TB treatment phase.

Recommendation: Support operational research to accelerate adoption of TB-MBLA by the National Tuberculosis and Leprosy Programme for the benefit of TB patients in Uganda.

Background: The global and national TB case detection, treatment success and case notification rates are still lower than the expected targets, in part, because of the less effective diagnostic tools currently in use[1]. Drug resistant TB is increasing partly because of patients overstaying on inappropriate medication or being discharged prematurely before TB bacteria are cleared. Current standard-of-care tests are either inaccurate, failing to distinguish live from dead bacteria or take long for results to be realised. Therefore, the need for tests that can accurately assess efficacy of anti-TB treatment and give timely results to guide doctors and nurses on how to manage the patients cannot be more emphasised. Following several multi-site evaluations, the WHO recognised TB-molecular bacterial load assay (TB-MBLA) as a potential laboratory test[2] which may replace smear microscopy and culture for monitoring response to TB treatment [3]. TB-MBLA is a new RNA-based diagnostic tool that detects and rapidly quantifies live TB bacteria in patient sample, and measures whether the anti-TB medicine is killing these bacteria or not as the patient progresses on treatment.

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With funds from the Government of Uganda through Makerere University Research and Innovations Fund (MakRIF), and the University of St Andrews (UK), researchers at the Department of Biochemistry & Sports Science, College of Natural Sciences and colleagues from the College of Health Sciences, Makerere University in collaboration with researchers at University of St Andrews, evaluated the accuracy and usefulness of the TB-MBLA in comparison with the current standard-of-care tests.

Methodology: The study was conducted at the Uganda-China friendship Hospital, Naguru Kampala and had two arms: A cross sectional arm to evaluate diagnostic accuracy of TB-MBLA among presumptive cases of TB and longitudinal arm to assess accuracy of TB-MBLA in measuring treatment response. In each arm, each consented participant provided three sputum samples which were pooled together and tested for TB using TB-MBLA in comparison to smear microscopy and GeneXpert MTB/RIF Ultra. MGIT liquid culture was used as the reference test to determine the sensitivity, specificity, positive and negative predictive values of TB-MBLA vis-à-vis smear microscopy and GeneXpert MTB/RIF Ultra. Participants who tested positive for TB using GeneXpert MTB/RIF Ultra were enrolled into a longitudinal treatment arm and assessed for treatment response at 14 days-, month two-, four- and six- of TB treatment. Treatment response was measured as either fall in number of TB bacteria measured by TB-MBLA or change from positive to negative by all tests including smear microscopy, GeneXpert MTB/RIF Ultra and culture.

Findings:

Diagnostic accuracy among 210 presumptive cases of TB:

- The sensitivity of TB-MBLA was 99%, like GeneXpert MTB/RIF Ultra but higher than 75% for smear microscopy.
- TB-MBLA specificity was 91%, compared to 78% and 98% of GeneXpert MTB/RIF Ultra and smear microscopy, respectively.
- In a sub-population of smear microscopy-negative and HIV-positive participants, TB-MBLA sensitivity was 92%.

Treatment response measuring accuracy among 129 TB positive cases:

- Positivity for TB dropped with treatment in all tests, but the rate was slower with GeneXpert MTB/RIF. Consequently, 33% of participants were still Xpert-Ultra positive at the end of treatment but were clinically well at 3-months post-treatment (*Figure 1*).
- TB-MBLA remained specific at 93% as patient TB bacillary load reduced and was consistent with the gold standard test, MGIT culture at 94% agreement.
- TB-MBLA-measured bacillary load, among month-2 smear microscopy positive cases who received extra month of intensive treatment was not different from smear microscopy negative cases.

References:

1. Global Tuberculosis Report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.
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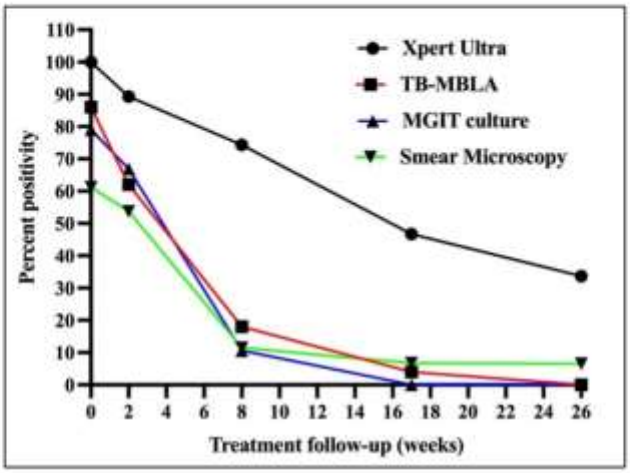

Figure 1: Change in proportion of patients who tested TB positive by TB-MBLA (red curve) compared to smear microscopy (green curve), GeneXpert MTB/RIF Ultra (black curve) and MGIT culture (blue curve) before- and during- treatment.

Implications of the study findings:

- GeneXpert MTB/RIF Ultra is a sensitive tool, suitable for pre-treatment diagnosis of TB, but not for treatment response monitoring.
- Smear microscopy is cheaper, but it is less sensitive, and quite subjective in result interpretation, making it less reliable in measuring treatment response.
- TB-MBLA closely mirrored MGIT culture (gold standard TB test) in measuring treatment response. Since it takes shorter time (four hours) to get results from the time of sample collection, TB-MBLA provides a rapid method for monitoring response to TB treatment.
- Clinical signs disappear faster or stay longer than bacteriological positivity, yet culture underestimates patient TB bacterial burden and the results take too long to come out which delays clinical decisions.
- DNA based molecular tests such as GeneXpert MTB/RIF Ultra overestimate patient TB bacterial burden during treatment which may lead to unnecessary prolonged treatment. RNA based molecular tests like TB-MBLA may be the answer.

References:

1. Global Tuberculosis Report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.
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Way forward: To date, TB-MBLA technology has evolved through a series of developmental levels (*Figure 2*). The next step will focus on evaluating TB-MBLA in the operational environment. The feedback from such experiments will guide, modify, or simplify technology followed by undergoing thorough standard manufacturing to ensure GMP compliance. This will be followed by Technology Readiness level (TRL) 8 and 9 – regulatory approval & licensing and subsequently adoption by the National TB Programmes for application in routine management of tuberculosis.

Figure 2: TB-MBLA is at technology readiness level 7.
Figure adapted from TWI Global. (https://www.twi-global.com/technical-knowledge/faqs/technology-readiness-levels)

What were the immediate benefits of the study to Uganda?

During this study, human resource capacity to adopt the technology at Makerere University was developed. Researchers at MSc and PhD levels were trained and equipped with knowledge to perform this assay (*Figure 3*). The trained personnel will not only perform the assay, but also lead in the manufacturing and commercialising the new technology. Ugandan researchers can take advantage of the presidential initiative to make Uganda a hub for scientific research and development (R&D). Researchers will be involved in replicating the current study in a bigger sample size and also investigate the applicability of TB-MBLA in samples other than sputum.

References:
1. Global Tuberculosis Report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.
2. World Health Organization. Global tuberculosis report 2018. World Health Organization; 2018.
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Figure 3: Training of volunteer Biochemistry graduates at the Molecular laboratory under the Department of Immunology & Molecular Biology, College of Health Sciences, Makerere University, Uganda.

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- Medical & Molecular Laboratory, Mulago Hospital
- Makerere Biomedical Research Centre

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