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Utility of Urinary Lipoarabinomannan (LAM) assays to monitor treatment outcomes in Tuberculosis patients co-infected with Human-Immunodeficiency-Virus (HIV)

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Utility of Urinary Lipoarabinomannan (LAM) assays to monitor treatment outcomes in Tuberculosis patients co-infected with Human-Immunodeficiency-Virus (HIV)

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine.

Division of Molecular Medicine and Haematology

Johannesburg, 2025.

Declaration

I, Thabiso Bridgette Seiphetlo, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine, at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



05th September 2025

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Dedication

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In loving memory of my Father
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1949-2023

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Abstract

Tuberculosis (TB), which is caused by the bacterium *Mycobacterium tuberculosis (M.tuberculosis)*, continues to be the biggest global public health concern, despite advances in treatment regimens and diagnostic tools. Existing diagnostic methods, which involve bacterial culture and nucleic acid amplification tests (NAATs), are limited, particularly for assessing treatment responses. Prolonged time-to diagnosis of TB facilitates the continued dissemination of the disease, increased mortality, and higher health care costs. Consequently, there is a demand for rapid cost-effective diagnostic methods that utilize non-sputum samples. A lipopolysaccharide, lipoarabinomannan (LAM), found in the cell wall structure of *Mycobacterium tuberculosis*, holds potential as a biomarker for monitoring tuberculosis treatment. Urine-based antigen tests, such as rapid strip tests and ELISA, are appealing due to their ease of use, non-invasive nature, and absence of aerosol risks.

This study explores potential of LAM detection in plasma and urine samples as a potential indicator for monitoring TB therapeutic response. The goal was to develop a sandwich ELISA for LAM detection, but an indirect ELISA failed to optimize binding, preventing the sandwich ELISA's development. However, a dot-blot assay successfully confirmed LAM-antibody binding. In the second part of the study, LAM concentrations were measured using the Determine LAM Ag rapid strip test at various time points. At baseline, 72.97% of participants tested positive for urine LAM, with a decline over time: 58.14% at month 1, 51.92% at month 2, and 13.04% at month 6

The average urine LAM grade, which represents the intensity of the test line, was measured on a scale from negative (0) to 4+. Positive samples demonstrated a reduction in the average LAM grade, from 1.4 at baseline to 1.2 at month 6. Among 140 participants, 70% were living with HIV, and this cohort exhibited a significant reduction in LAM grading. Also in this group, participants with CD4 counts ranging from 7 cells/mm^3 to 514 cells/mm^3, had the highest LAM grading at baseline (mean LAM grade 3.67). Considering these findings, it was conclude that urinary LAM seems to be a useful indictor for monitoring TB treatment especially amongst HIV positive individuals.

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Abbreviation List

Abbreviation	Full Name
AFB	Acid-fast bacilli
AI	Artificial intelligence
AIDS	Acquired immunodeficiency syndrome
AMIK	Amikacin
ART	Antiretroviral therapy
BCG	Bacille Calmette-Guérin
BSL 3	Biosafety Level 3
CAD	Computer-aided detection
CAP	Capreomycin
CIs	Confidence intervals
COVID-19	Coronavirus disease
CPSD	Chemiluminescent peroxidase substrate
CSF	Cerebro-spinal fluid
CXR	Chest X-Ray
DC	Dendritic cells
DOTS	Direct observed treatment short-course
DST	Drug susceptibility testing
EBC	Exhaled breath condensate
ELISA	Enzyme Linked Immuno Assay
EMB	Ethambutol
EPTB	Extra pulmonary TB
HHC	Household contacts
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
INH	Isoniazid
KAN	Kanamycin
L-J	Lowenstein-Jenson
LAM	Lipoarabinomannan
LF-LAM	Lateral flow lipoarabinomannan
LPA	Line probe assay

LTBI	Latent TB infection
M. tuberculosis	*Mycobacterium tuberculosis*
mAb	Monoclonal antibody
MDR-TB	Multidrug-resistant tuberculosis
MGIT	BACTECMycobacterial Growth IndicatorTube
MPI	Mannosyl phosphatidyl-inositol
MTBC	Mycobacterium tuberculosis complex
NAATs	Nucleic acid amplification tests
NTB	Nontuberculous Mycobacteria
OD	Optical density
PCR	Polymerase chain reaction
PLWH	People living with HIV
POC	Point-of-care
PTB	Pulmonary tuberculosis
PVDF	Polyvinylidene fluoride
PZA	Pyrazinamide
RIF	Rifampicin
SD	Standard Deviation
SDS-PAGE electrophoresis	Sodium dodecyl sulfate–polyacrylamide gel
SM	Streptomycin
SoHoT	South African Hopkins TB Collaborations
TB	Tuberculosis
UHC	Universal health coverage
XDR-TB	Extensively drug-resistant tuberculosis

Chapter 1: Introduction

1.1 Background

One of the most ancient and long lasting infectious diseases, TB, still affects millions of individuals worldwide each year (1). Although it can infect all organs, *M. tuberculosis,* the causative agent of TB, primarily targets the lungs and even after treatment it may lead to chronic disease (2). Significant regional differences in TB prevalence were noted by the World Health Organisation (WHO) in 2022. The highest burden was found in South-East Asia (46%), Africa (23%), and the Western Pacific (18%), followed by the Eastern Mediterranean (8.1%), the Americas (3.1%), and Europe (2.2%) (3). Of particular concern, eight out of the 30 nations with the highest TB burdens accounted for 87% of all estimated global TB incidences (Figure 1) (3).TB disproportionately affects adults, with a markedly higher annual incidence in men compared to women (3).

The clinical spectrum of TB ranges from asymptomatic infection and latent TB to subclinical and active disease, with the latter often presenting as granulomatous inflammation in the lungs (4). Despite being both preventable and treatable, TB remains a significant worldwide health concern due to multiple factors adding to it persistence (5). These include inadequate treatment adherence, socioeconomic inequalities, undiagnosed TB incidences that exacerbate transmission, and heightened susceptibility to TB infection and disease among people living with HIV (PLWH) especially in nations with high HIV prevalence (5). Furthermore, although effective TB treatment regimens have existed for more than 50 years, treatment results are frequently less than ideal, particularly in cases of drug-resistant TB (DR-TB)(6). Treatment failure, which not only leads to increased morbidity and mortality but also increases the likelihood of disease recurrence, transmission, and the emergence of DR-TB, is a critical concern. According to the WHO, treatment success rates for DR-TB range between 56% and 69% (7,8), while those for drug-susceptible TB (DS-TB) vary from 74% to 91% (9). These relatively low success rates highlight the necessity for more efficient monitoring tools to guide treatment, particularly in the early stages of treatment responses. This is especially critical for the large population of patients undergoing standard therapy for drug-sensitive tuberculosis.

In routine practice, treatment outcome monitoring differs for DS-TB and DR-TB, as outlined in local and WHO guidelines. For DS-TB, follow-up is typically performed using sputum smear microscopy at specific timepoints during therapy (10), while for DR-TB both smear

microscopy and culture are required to document sputum conversion (11, 12). Currently, the gold standard to ascertain TB treatment outcomes is sputum culture which give a readout of time to positivity and also indicates if the result is positive or negative for *M.tuberculosis*. MGIT Culture is time consuming and expensive and it takes 42 days to report negative outcomes under the automated MGIT system - delaying clinical decision-making and complicating patient follow-up (13). Furthermore, sputum collection, which is necessary for these diagnostic methods, presents challenges, especially in at-risk populations such as children and PLWH (14–16). The HIV epidemic has profoundly reshaped the epidemiology of tuberculosis, particularly in Africa, where almost 40% of individuals with active TB are co-infected with HIV (17). In this population, TB often lacks cavities and presents with a low bacillary load, resulting in a high proportion of smear-negative cases (18–21). and up to one-third of patients being unable to produce sputum for diagnostic testing (22). Alternative approaches for sputum collection, such as induction and bronchoscopy, have been investigated in this population, but these are costly, require specialised facilities, and are not widely accessible (14, 23). Furthermore, they involve lengthy processing and often fail to provide same-day diagnosis, a major challenge in high-burden settings where delayed results contribute to loss to follow-up (24). In children, the inability to expectorate sputum poses a major challenge for TB diagnosis, and even when specimens are obtained, microbiological assays have limited sensitivity (25).

The limitations of traditional diagnostic methods to ascertain response to treatment highlight the urgent need for novel treatment monitoring approaches. New diagnostic assays and biomarkers that can provide rapid, accurate predictions of treatment outcomes are critical to improving TB care (23). Ideally, these new tools should use easy- and safe-to-collect specimens, such as blood or urine and exhaled breath condensate (EBC) (24), and require minimal infrastructure or technical expertise, making them inexpensive and more feasible for use in diverse clinical and public health settings. A treatment monitoring test could enhance TB treatment monitoring, reduce diagnostic and decision making delays, and ultimately contribute to better outcomes for patients worldwide (23).

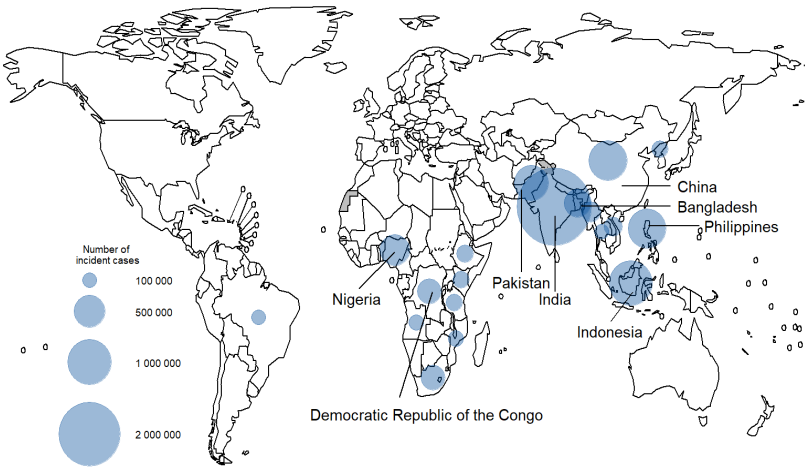


Figure 1. The projected TB incidence in 2022 as reported by WHO. The global map highlighting nations with at least 100 000 reported incidences, with the highest disease burden observed in developing regions such as South-East Asia and Sub-Saharan Africa (3).

1.2 *M. tuberculosis* Cell Wall Components and Virulence Factors

Mycobacterium is a genus of rod-shaped, gram-positive, acid-fast organisms that are part of the phylum Actinobacteria (25). The Actinobacteria phylum, consisting of gram-positive bacteria, exhibits significant diversity, with various species forming distinct symbiotic relationships with a range of hosts, including mammals. Certain *Mycobacterium* and *Nocardia* species are pathogenic, while some *Bifidobacterium* species are recognized as beneficial components of the normal gut microbiota, contributing positively to human health (26,27). Mycobacteria inhabit a range of habitats, such as soil, water, and artificial water systems (28).

Most of the Mycobacterium tuberculosis complex (MTBC) species, are human pathogens with *M. tuberculosis* being causative agent of TB. By contrast *Mycobacterium leprae,* although from the same genus, is not part of the MTBC and is responsible for leprosy (29). *M. tuberculosis* has been studied the most (30). The exceptional molecular complexity of the mycobacterial cell wall is a particularly distinctive characteristic, that distinguishes *Mycobacteria* from other bacteria. Despite being gram-positive, these bacteria possess an envelope that shares similarities with gram-negative cell walls, such as an outer permeability barrier resembling a pseudo-outer membrane (31–33). Figure 2 depicts the complex model of the mycobacterial cell envelope, where the inner membrane's phospholipid bilayer is connected to glycolipids that extend across the periplasmic space. The main structural elements of the cell wall include long-chain mycolic acids, a highly branched arabinogalactan polysaccharide, and a cross-linked

peptidoglycan polymer. The outer membrane consists of lipids that can be extracted by solvents, such as inert waxes and non-covalently associated glycophospholipids, which integrate into the mycolate layer. The lipid- and carbohydrate-rich layers of the cell wall act as a permeability barrier, shielding against hydrophilic substances and playing a crucial role in pathogenesis and survival, the location of the LAM is also highlighted on the cell wall structure (34 – 36). LAM exists in three forms: phospho-myo-inositol that has a capping with LAM (PILAM), mannose that is capping with LAM (ManLAM), and an arabinose LAM that is not capped (AraLAM) (37). The way in which the LAM is capped determines its capacity to regulate immune reactions. ManLAM, commonly known as Lipoarabinomannan, is the predominant type in *M. tuberculosis* and serves as a powerful immunosuppressive agent as well as a key virulence factor (37). It is hypothesized that ManLAM affects various host immunological reactions, such as the neutralization of cytotoxic free radicals, protein kinase C disruption, and interferon gamma transcription blockade in immune components like T-lymphocytes and macrophages. These actions prevent T-cells and macrophages from activating, which helps *M. tuberculosis* survive inside mononuclear phagocytes and disseminate throughout the body. Therefore, ManLAM is a key immunogenic virulence factor with substantial clinical and diagnostic consequences since it causes lower amounts of TNF-alpha than other forms of LAM (38, 39).

1.3 Transmission

By inhaling microscopic drop particles that contain live and viable *M. tuberculosis* bacilli, which range in diameter from 1 to 5 μm, an infection with *M. tuberculosis* can be easily transferred between individuals (Figure 3A) (40). When an individual with active TB of the lungs, referred to as pulmonary TB (PTB), sneezes, coughs, or when mucus is aerosolized during procedures like sputum induction, infectious droplet nuclei can be released into the air (41). Tidal breathing may possibly contribute in the spread of TB (42). Depending on the environmental conditions, these minuscule droplets may survive for several hours as airborne droplets suspended in the atmosphere (43). To acquire TB from an infected person, a susceptible host must be exposed to multiple droplet nuclei during their contact episode (43) There are numerous characteristics that affect probability of infection, including the load of *M. tuberculosis* bacteria present in the source individual, the closeness of persons, airflow in indoor spaces, and the duration of exposure and risk of the uninfected person being exposed (41, 44). When *M. tuberculosis* is transmitted, several variables influence its pathogenesis,

including the strain type, drug resistance, treatment adherence, access to healthcare, and the host's co-morbidities. Among these, HIV is the most significant determinant for the development of TB, along with the infection site (pulmonary or extrapulmonary) (44). These variables affect the likelihood of a person contracting TB (TB infection), whether the infection remains contained (latent TB), and whether it is ultimately cleared by the host's immune response or progresses to symptomatic TB disease which is known as having active TB disease (45).

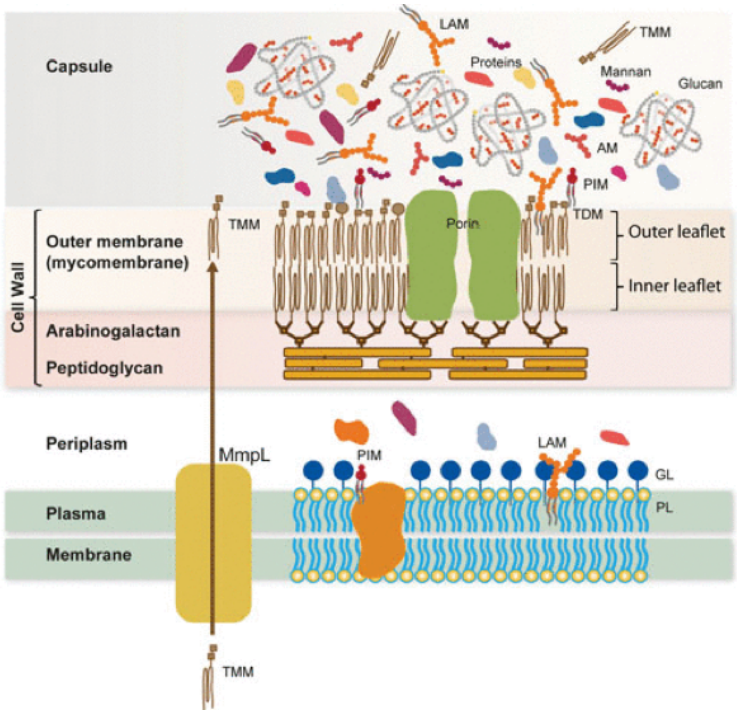


Figure 2. Mycobacterial cell wall model.A schematic illustration of the main components of the mycobacterial cell wall, showing the glycolipids (phosphatidyl-myo-inositol mannosides, mannosylated lipoarabinomannan), peptidoglycan, arabinogalactan, and mycolic acids. The acyl lipids (such as sulfoglycolipid, diacyltrehalose, polyacyltrehalose, trehalose monomycolate, trehalose dimycolate, and phthiocerol dimycocerosate) are intercalated into the mycolate layer. Image taken from https://doi.org/10.1128/microbiolspec.gpp3-0027-2018 (46).

1.4 Pathogenesis of Active TB

TB disease occurs when *M. tuberculosis* replicates and escapes elimination by the immune system of the host (1, 47). A key characteristic of active TB infection is the formation of granulomas, which are an attempt to create an immunological microenvironment that controls the infection (48). Granulomas are made up of various types of cells, with alveolar

macrophages as the main cell type accountable for granuloma creation (49). Upon pathogen detection, phagocytosis in alveolar macrophages is initiated. Following phagocytosis, an inflammatory reaction is initiated, prompting the secretion of chemokines and cytokines that recruit immune defense cells, to surround the macrophage and create a granuloma (50, 51). Successful infection by *M. tuberculosis* requires its ablity to survive by regulating the immunological reaction triggered by alveolar macrophages (52).

M.tuberculosis sustains a balance between cytokines that both promote and reduce inflammation, which are generated during the immune reaction process to limit or regulate bacterial replication (53). Chemokines generated during the infection attract the initial influx of immune cells, including circulating macrophages, neutrophils, natural killer (NK), and T lymphocytes, to the infected alveolar macrophages. These cells collaborate to initiate inflammation and tissue remodelling (54). After the first inflammatory cells are present around the infection site, T-cells are poised to develop into effector T-cells (55). Within the developing granuloma, mycobacterial growth is prevented by these poised pathogen-specific T-cells by stimulating the macrophages (Figure 3B) (56). The organisation of the granuloma is caused by the activated T-cells, by attracting *M. tuberculosis*-infected macrophages to its centre where they are encircled by lymphocytes (56, 57). The immune system and the pathogen engage in a battle inside the granuloma, that results in cell death, the acquisition of additional immune cells, as well as alterations in blood vessels and tissue structure (54). When macrophages around a cellular area undergo degeneration and cell death and necrosis, a caesium is created, forming caseous granulomas (56, 57). When a cavity forms in the caesium that erodes into an airway, the bacteria inside are released into the airways, and then transported via the bronchial treat into the environment through coughing (56, 58). Granulomas function as growth regulators for intracellular *M. tuberculosis* and as barriers to the transmission of bacilli to other lung regions and limit disseminated TB (51). The likelihood of reactivation or advancement to active TB is higher in situations when the immune system of the host is weakened, by conditions such as in co-infection with HIV/AIDS and TB (59). When the granuloma encapsulates the pathogen and stops tissue damage but allows bacterial viability, the resulting condition is known as latent TB infection (59).

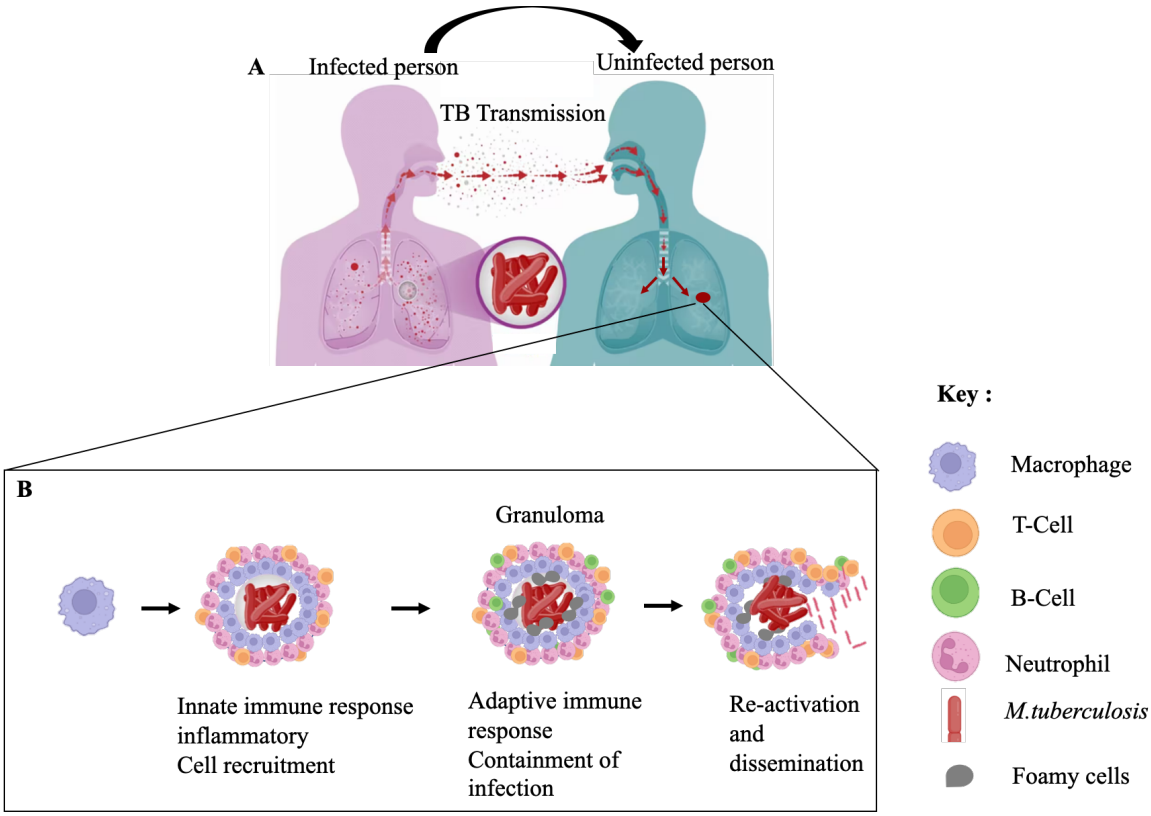


Figure 3. Transmission and development of the granuloma in human following M. tuberculosis infection. (A) M. tuberculosis is transmitted via coughing and sneezing from infected to uninfected individuals. (B) The host immune system enlists immune cells, including neutrophils, T cells, macrophages, and B cells, in response to infection in order to eradicate or confine the invasive bacteria by granuloma formation. Disruptions in immune signaling or secondary infections can compromise granuloma integrity, leading to disease reactivation.This Figure was adapted from (60).

1.5 Latent TB and reactivation

LTBI refers to immunological sensitivity to *M. tuberculosis* antigens without clinical TB symptoms. *M. tuberculosis* is assumed to persist in this phase of infection in a non-proliferating (dormant) form, with the bacteria primarily confined within oxygen-deprived granulomatous lesions in the lungs (61). The length of latent infection depends on an individual's health status. Although most people can carry the bacteria for life, reactivation can happen 5–10% of the time, usually within the first two years after infection (62). Immune impaired individuals, such as those with unmanaged HIV infection have a 10% higher annually likelihood of advancement to TB disease (63). Individuals who are mostly affected ranging between 90 % and 95% can eradicate the acute illness or either manage the acute illness and transition into a dormant (latent) state (64). The immunological response of the host and the metabolism of the bacteria must coexist in a delicate balance for the infection to remain dormant. Infection will reactivate

and symptoms will appear if this equilibrium is disturbed (65). French and German physicians first introduced the term TB, or Phthisis (the term previously used for TB), in the early 1800s, as the field of pathological anatomy was emerging in medicine, using it to describe abnormalities observed in the lungs of individuals who had not shown signs of TB prior to death during autopsies (66). Additionally, the latent state was observed in the cultured tissues of healthy individuals who had died from other causes and showed no clinical signs of active TB (67). As a result of these and similar observations, the term "LTBI" became widely accepted in both clinical and literary contexts.

Recent evidence has refined this traditional view of TB as simply “latent” or “active.” TB infection is now recognized as existing on a continuum, or spectrum, of stages ranging from sterilizing immunity to incipient TB, subclinical TB, and active TB (45, 68–71). Within this framework, LTBI represents one point along the spectrum, characterized by immune control of bacilli without clinical symptoms. Some individuals may exist in earlier stages such as incipient TB, where viable bacilli are present but disease is not yet clinically detectable, or subclinical TB, in which infection is detectable through microbiological or imaging tests despite absent or minimal symptoms (45, 68–71).

1.6 Active TB progresstion from LTBI

Progression from latent to active TB is now understood to occur through intermediate stages rather than as a simple phased transition. Individuals may pass through incipient TB, characterized by bacterial replication and heightened risk of clinical disease, or subclinical TB, where infection is detectable but symptoms are minimal or absent(42, 65–68). Those individuals with LTBI are at an increased risk of developing active TB. People with healthy immune systems that can prevent bacterial replication are at lower risk (51, 69). However, individuals with weakened immune systems, including those co-infected with HIV, organ transplant recipients, patients undergoing tumor necrosis factor (TNF-α) therapy, those receiving treatment for inflammatory diseases, and individuals with autoimmune conditions, face a greater risk of developing active TB (42, 70, 71). Due to the increased prevalence of HIV co-infection in South Africa, the chance of reactivation illness is raised (72, 73). Bacterial burden, host resistance, immunological activation, and inflammation are some of the variables that add to the broad spectrum of disease presentation (54, 74, 75).

1.7 HIV and TB comorbidity.

Among the countries with the highest TB burden, South Africa ranks in the top 20 (1). The highest rates of TB and HIV co-infection and related deaths are found in Sub-Saharan African regions (75). More than 60% of newly discovered TB cases in South Africa are found in PLWH (79). Their compromised immune function has a substantial role in increasing susceptibility to TB infection and TB disease in co-infected patients (73, 77). In PLWH, *M. tuberculosis* infection may disseminate to organs beyond the lung such the lymph nodes, liver, spleen and kidneys (73, 78). In these patients, low CD4 T-cell counts impair the formation of granulomas, which restrict bacterial growth in the lungs (53, 79). This leads to enhanced bacterial growth, borader mycobacterial dissemination, and extensive tissue damage (83). These effects can be attributed to both general and interconnected processes, including (i) a reduction in the overall number of CD4 T cells due to an increased viral load within the affected tissue, (ii) impaired macrophage function, (iii) disturbance of *M. tuberculosis*-specific T cell activity, and (iv) resulting functional and harmful changes within granulomas (83). Additionally, the TB-HIV pandemic in South Africa is fueled by the country's poor socioeconomic conditions, low patient compliance to both TB treatment and Antiretroviral therapy (ART), as well as a high prevalence of undetected infection (71, 81). HIV-associated TB is often negative in sputum tests and unlikely to exhibit with the typical radiological and clinical features of active TB, resulting in delays in diagnosis and treatment (85).

1.8 TB Diagnosis

Several methods are used in clinical environments for TB detection. Before the broad adoption of molecular diagnostics, standard medical laboratories relied on smear microscopy to detect acid-fast bacilli for TB testing. Since then, several nucleic acid amplification tests (NAATs) and improved culture-based techniques have been implemented (83, 84). Bronchial secretions, sputum, blood, gastric lavage, blood, biopsies, cerebrospinal fluid, or urine are amongst the several biological samples from which *M.tuberculosis* can be detected or isolated (86). Despite the widespread roll-out of NAATs, in many limited resource settings microscopy by sputum smear testing and possibly sputum culturing are the standard (88). Smear microscopy exploits the acid-fast characteristic of *Mycobacterium tuberculosis*, allowing selective visualization of bacilli using Ziehl–Neelsen or auramine-based fluorescent stains, and demonstrates high analytical specificity(85). Test methods that can be used to detect and diagnose *M. tuberculosis* infections, are described below.

1.8.1 Clinical diagnosis

The pulmonary parenchyma is typically the initial site of *M. tuberculosis* infection. Chest imaging is therefore, a crucial initial step in achieving an early diagnosis of the condition in clinical settings (89).

1.8.1.1 Chest radiography and Computed Tomography (CT) imaging

Individuals displaying the following symptoms: a cough that lasts for two weeks or longer, have an undiagnosed chronic fever, and/or weight loss should be tested for TB (90). The usual radiologic evaluation of suspected or confirmed pulmonary TB is a chest X-ray. Although the radiological manifestation of TB can vary, in many cases it is quite characteristic. Although informative, chest X-rays are not specific for identifying pulmonary TB and can be normal even in the presence of TB disease or a positive sputum MGIT culture (91, 92). Chest CT, with or without contrast enhancement, can help characterize radiographic findings more accurately by identifying changes that are not detectable on normal radiographs or by helping to differentiate between past dormant and active disease (93).

In PLWH, radiographic manifestations of pulmonary TB vary depending on the degree of HIV-related immunodeficiency (94). In the early stages of HIV, when individuals are not yet immunocompromised, the radiographic pattern typically resembles that seen in HIV-negative individuals, with more common lesions such as upper lobe infiltrates, with or without cavities. (94). Intra-thoracic or mediastinal lymphadenopathy, extrapulmonary involvement , lower lobe infiltrates, and miliary TB become increasingly prevalent as immunosuppression advances (94). Moreover, the x-ray presentation in PLWH differs from seronegative patients with TB (95). Subclinical illness, which is prevalent in patients with advanced HIV immunosuppression, cannot be independently diagnosed with radiographic testing due to variations in how the disease presents, discrepancies in interpreting the data, and the overlap of patterns to those seen in other non-TB conditions (96). Additionally, non-TB conditions can have radiographic findings that are similar especially in young children with HIV-related lung disease (89,97). Chest imaging is therefore a crucial initial step in achieving an early diagnosis in clinical settings (93). Reading and interpreting chest X-rays is often based on judgment and typically requires the expertise of multiple trained physicians to ensure accurate and reliable outcomes (97). Tests using radiography cannot be utilised as a stand-alone diagnostic method

(98). Small, portable, battery-operated digital radiography instruments and computer-aided detection (CAD) software driven by artificial intelligence (AI) have been made possible by digital imaging and software technology (99). CAD using computer algorithms and machine learning is massively increasing the sensitivity of chest x-rays (100). CAD software use has been approved by the WHO since 2021 for screening people who are 15 years of age or older (101). These software systems utilize AI to replace the human presentation of chest radiographs and generate abnormality scores that indicate the likelihood of TB (99).

1.8.2 Laboratory based TB diagnosis

In the laboratory, methods which are most commonly used to diagnose active TB include MTB culture, smear microscopy, molecular based assays like the GeneXpert® MTB/RIF test (102).

1.8.2.1 Culturing

M. tuberculosis can be detected in sputum samples using a variety of diagnostic methods, but culture remains the gold standard (75). Initially *M. tuberculosis*, was cultured using the traditional method of inoculating on solid medium, such as Lowenstein-Jenson (L-J) medium slants or Middlebrook medium, which is a sensitive but a slow method, with no growth reported as a negative result 6-8 weeks of incubation (95, 103). To address this, the BACTEC Mycobacterial Growth Indicator Tube (MGIT) (Becton Dickinson), a more accurate and speedy liquid-based detection test, was developed (104, 105). The MGIT is a system based on liquid culture, with a silicone tube with a fluorescent chemical incorporated at the bottom of the tube (106). As oxygen is consumed, the fluorescent chemical becomes activated, and as respiring organisms consume the oxygen, they allow UV light to detect the fluorescence signal (106). A positive signal may appear within one to five weeks after testing. However, if no signal is detected by 42 days, the result is reported as negative, indicating the absence of bacterial growth. It is still necessary to speciate a a specimen that is MGIT positive to ensure that it is indeed *M. tuberculosis*, since other actively growing microorganisms in sputum may result in false positives. This is done by ZN staining a small amount of the culture from the MGIT tube using a sterile loop, and if acid-fast bacilli (AFB) are seen, speciation with NAAT is performed to confirm or refute presence of *M. tuberculosis* (74, 106).

Once *M. tuberculosis* is confirmed, phenotypic drug susceptibility testing (DST) is often performed to guide therapy. The commonly used phenotypic DST is a proportion method that attempts to culture *M. tuberculosis* in preset concentrations of first-line TB medications,

including rifampicin (RIF), ethambutol (EMB), and isoniazid (INH). This approach determines the proportion of bacilli resistant to each drug and helps inform effective treatment regimens (107).

1.8.2.2 Microscopy

For individuals with pulmonary TB and bacterial burdens of over 5000 bacilli per ml of sputum, the smear microscopy method is quick and accurate (74). Microscopy using a smear test, however, is not sensitive and can be challenging for individuals with lower bacterial concentrations and LTBI (108). Ziehl-Neelsen staining was initially used to stain the bacteria, but the auramine staining method was later adopted to detect bacteria in the sputum sample due to its greater sensitivity and lower cost (108, 109). The Ziehl-Neelsen stain contains carbol fuchsin, which is absorbed by all cells. During the destaining process with acid-alcohol, only acid-fast organisms (like the mycobacteria complex) retain the red color, while non-acid-fast organisms become blue or green. The Auramine stain works on a similar principle, with the dye attaching to the mycolic acids in the cell wall of the mycobacteria complex. The acid-alcohol then removes the stain from non-acid-fast organisms. Auramine staining has several drawbacks, including its lack of specificity and can identify other mycobacteria besides *M. tuberculosis*, which can lead to incorrect diagnoses. The consistency of the sputum as well as the precision with which the slides are prepared and interpreted determine the reliability of smear microscopy (109). As a result, to obtain accurate results from this diagnostic test, laboratory staff must be skilled, trained, and subject to routine quality inspections (75, 109). Many countries with high TB prevalence are moving away from testing using smear microscopy in favor of molecular diagnostics because smear are less sensitive in cases with minimal bacterial loads.

1.8.2.3 Molecular diagnosis

The continuous effort to produce better and faster diagnostic methods has advanced significantly with the creation of Nucleic Acid Amplification Tests (NAATs). Polymerase chain reaction (PCR) is used by NAATs to amplify the DNA of bacteria found in sputum (95,103). The Xpert MTB/RIF Ultra (Cepheid) system is now the most widely used NAAT technology for diagnosing TB (95, 105). This molecular diagnostic tool was developed to advance diagnostic methods by speeding up the diagnosis and to identify extrapulmonary TB (110). The Xpert Ultra can identify both *M. tuberculosis* and rifampicin (RIF) resistant bacteria

in respiratory samples (95, 105). The GeneXpert technique was enhanced more recently through the introduction of a GeneXpert Ultra cartridge, which can diagnose patients with minimal bacterial loads by detecting as few as 10 organisms per ml of sputum (111). The effectiveness of treatment and limiting TB transmission have been improved because of these modern molecular-based diagnostic methods (104, 112).

1.8.2.4 Urinary lipoarabinomannan assay

For antigen-based detection, immunoassay methods like the fast lateral flow point of care strip test are ideal. Antigen-based TB assays have recently been focused on detecting lipoarabinomannan (LAM) in TB-infected people's urine.(113). As of 1997, a urine test for detecting LAM has been evaluated as a diagnostic marker for the testing of TB. Chemogen Inc. (South Portland, OR, USA) created a urine-based Enzyme Linked Immuno Assay (ELISA) for LAM detection in 2003 (114), which Inverness Inc. subsequently made available for purchase as the lateral flow Clearview TB ELISA assay. Determine TB LAM Ag lateral flow assay (LFA) "Alere LAM" was the first quick point-of-care (POC) test created by Inverness, who rebranded themselves as Alere in 2010 (113). Abbott Diagnostics (Lake Bluff, USA) purchased Alere in 2017. Alere Determine LAM Ag is a straightforward immunoassay that can be used at the patient's bedside (115). Several studies demonstrated an increased sensitivity of the test for TB detection among patients living with HIV, with the additional caveat that their CD4 count should be low (CD4 count $\leq$ 200) (116). It is unclear why this is the case, however it could be related to a high bacillary and antigen load in people who have TB and a low CD4 count, increased glomerular permeability resulting in high antigen levels in urine, or TB of the genitourinary tract, given that disseminated/extra pulmonary TB (EPTB) is common in this patient group (116, 117). ELISA assays are used to measure urinary LAM for diagnosing TB, especially in individuals with lower CD4 counts and disseminated disease. It also serves as a prognostic indicator of the risk of death in PLWH (117). However, currently the Alere LAM Lateral flow lipoarabinomannan (LF-LAM) assay is the sole TB POC test that is commercially available and has been approved by the WHO for the diagnosis of TB in HIV-positive people who have TB symptoms and who are extremely immunocompromised and/or very unwell (1). More advanced and seemingly more sensitive LAM assays are currently in development. One such innovation is the Fujifilm SILVAMP TB LAM (FujiLAM), a novel point-of-care urine test designed to detect lower concentrations of LAM compared to Alere LAM test. This test utilizes high-affinity monoclonal antibodies that specifically target *M. tuberculosis*-associated LAM epitopes. Studies from South Africa and Ghana indicate that FujiLAM exhibits higher

sensitivity than Alere LAM test in PLWH. In the Southern Africa, FujiLAM achieved a sensitivity of 70% compared to 42% for Alere LAM, while in Ghana, the sensitivity was 74% for FujiLAM versus 53% for Alere LAM. However, FujiLAM demonstrated slightly lower specificity, with 91% compared to 95% in South Africa and 89% versus 96% in Ghana (118, 119).

With small cohorts of HIV patients (70 and 68 individuals, respectively), historical research conducted in Nigeria and a prospective study conducted in Zambia have shown results that are similar. (120, 121). Refer to Table 1 below for update on the LAM assay diagnosis.

Table 1: Overview of modifications implemented based on suggestions from the World Health Organization (WHO) policy update (2019), on the LF-LAM assay for the diagnosis of active TB in people living with HIV (122).

Clinical setting	**Previous guidance**	**WHO recommendations**	**Current recommendation for South African context**
Inpatient setting	LF-LAM is recommended for critically ill patients with advanced HIV in hospital settings, including those undergoing medical evaluation in the emergency department or admitted to medical wards, regardless of TB suspicion or CD4 count.	• Regardless of symptoms and signs of TB (pulmonary or extrapulmonary) and with a CD4 cell count of 200 mm^3 or lower (≤ 200mm^3). • With advanced HIV disease, including WHO Stage 4 or severe illness, regardless of CD4 count	• The guidelines adopts recommendations to include the use of LF-LAM to assist in the diagnosis of active TB in HIV-positive patients irrespective of whether TB is suspected or not (i.e., irrespective of signs and symptoms of TB) and irrespective of the patient's CD4 count, and irrespective of whether Advanced HIV Disease (AHD) is present or not. • A molecular TB test using sputum (e.g., GeneXpert) should be conducted simultaneously.
Outpatient setting	For outpatients receiving care at community health centres, primary healthcare facilities, day hospitals, or ART initiation clinics, LF-LAM should only be conducted when: 1. TB is presumed based on the presence of symptoms and/or clinical signs, and 2. The CD4 count is below 100 cells.	• Patients presenting with signs and symptoms of tuberculosis (pulmonary and/or extrapulmonary) or those who are severely ill. • Regardless of TB signs and symptoms, individuals with a CD4 cell count of less than 100 cells/mm^3.	The guidelines adopts recommendations for utilizing LF-LAM to support the diagnosis of active tuberculosis in individuals living with HIV who have: 1. Signs and symptoms indicative of TB (pulmonary and/or extrapulmonary). 2. A CD4 count of 200 cells/mm^3 or lower (≤ 200 cells/mm^3), advanced HIV disease (AHD) classified as WHO stage 4, or those presenting with severe illness.

1.9 TB prevention and treatment

1.9.1 TB prevention

Reducing the disease burden in public health systems requires preventing TB infection and disease progression to an active state. There are now three clinical strategies available to prevent TB, namely: (1) providing the Bacillus Calmette-Guérin (BCG) vaccine to children, (2) treatment for LTBI individuals, and (3) controlling the transmission of *M. tuberculosis* through infection prevention strategies aimed at TB incidences (123). BCG effectively prevents both miliary TB, which is the spread of the bacteria throughout the body via the bloodstream, and TB meningitis, which is brought on by *M. tuberculosis* penetrating the membranes surrounding the brain and spinal cord in neonates, young children, and adolescents (1). The WHO advises using strategies like administrative, environmental, and personal protection measures to stop the spread of active TB and manage its infection.

1.9.2 Treatment for active TB and latent TB

Rifampicin (RIF), isoniazid (INH), ethambutol (EMB), and pyrazinamide (PZA) are the four standard first-line chemotherapy drugs routinely used in conjunction to treat active pulmonary drug-susceptible TB for a period of six months (44). The standard regimen comprises an intensive 2-month phase with all four first-line drugs, followed by a 4-month continuation phase with INH and RIF only (124). The WHO currently recommends four different treatment regimens for LTBI. These include a four-month regimen of daily RIF, six months of daily INH three months of weekly rifapentine combined with INH, and six months of weekly INH doses (124).

The 6-month therapy plan can effectively treat about 85% of affected people (125). Misdiagnosis, excessive medication use, or patient non-compliance with the recommended course of therapy may lead to drug-resistant forms of TB (44). In addition to contributing to high TB morbidity and mortality, delayed TB diagnosis and inadequate DOTS (Direct Observed Treatment Short) program implementation and administration also encourage the development and spread of drug resistance in communities (126). This is because the DOTS strategy has been the cornerstone of international TB control, in lowering TB incidence (127) Delays in diagnosis and treatment are also frequently caused by stigma towards infected individuals, which makes patients reluctant to disclose symptoms (128).

The evolution of drug resistance presents the greatest threat to TB eradication, particularly in cases of multidrug resistance, where the bacteria responsible for the disease are resistant to multiple medications. According to the World Health Organization's 2022 TB report, South Africa not only has one of the highest rates of TB but also one of the nations most affected by drug-resistant TB (125). First-line treatment regimens for TB are no longer effective due to the development of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of the disease (129). Strains of MDR-TB are linked to resistance to INH and RIF, while XDR-TB is linked to MDR strains that are also resistant to injectable drugs and fluoroquinolones, which are commonly utilized in MDR treatment (8).

1.10 TB treatment response and monitoring

The mainstay of TB control is early diagnosis and immediate treatment initiation. Rapid identification of treatment response and altering treatment regimens accordingly plays a critical role in preventing the emergence of drug resistant TB (130). It is mandatory for all on anti-tubercular treatment to be monitored to evaluate their response to therapy (130). Regular monitoring, during and immediately after cessation of TB treatment, of weight gain, symptom resolution and X-ray improvements, is aimed at identifying patients who are not responding to treatment; either because they are not adherent to the drug therapy, or the patient may have drug resistant TB (130). Due to concurrent illness, clinical evaluation of treatment response can be difficult. Radiographic resolutions also lag clinical improvement for it to be utilized for immediate TB treatment response monitoring (130). WHO advises using routine sputum microscopy and culture for treatment monitoring, as well as molecular and phenotypic drug susceptibility testing to determine the drug resistance profile of MTBC and modify/ customize the treatment plan accordingly (75, 131). In the latter instance, consecutive negative cultures taken towards the end of treatment indicate a successful treatment outcome (86). However, there are disadvantages to using culture-based methods which include, higher expenses, contamination from other oral microbiota, delayed detection as a result of the the slow growth of *M .tuberculosis*, as well as requirement of specialized Biosafety Level 3 (BSL 3) facilities with trained staff (86). Smear microscopy is a crude means of monitoring treatment as it does not distinguish between dead and live *M. tuberculosis,* is not specific for *Mtb* only and also lacks sensitivity (132). Whilst these methods for assessing TB treatment response are robust, they perform poorly in certain sensitive populations such as children and PLWH (133,134). Genexpert detects *M. tuberculosis* DNA which can be present for years after viable (culturable

bacilli) were present, thus rendering molecular assays unsuitable for treatment monitoring (135). The most crucial aspect of all these methods, is that they all require a sputum sample, the collection of which creates an aerosol of infectious material when collected and in the absence of pulmonary lesions, is a less reliable diagnostic specimen (117) Moreover, sputum is often challenging to collect from patients after two months of treatment, at a time when it may be possible to change treatment plans. Additionally, sputum samples from individuals with extra-pulmonary TB and/or HIV co-infection are frequently paucibacillary. Paucibacillary refers to sputum samples being microbiologically confirmed with pulmonary TB with negative results on AFB smear of the initial respiratory specimens (136). Aspects impeding the control of the TB epidemic is the difficulty of precisely monitoring and predicting treatment outcomes in a timely, effective, dependable, and cost-effective manner (21, 137, 138). There is an urgent need for the development of an affordable, simple, non-sputum-based TB diagnostic test that is highly sensitive and specific, using readily available biological specimens such as blood and/or urine (139, 140). The monitoring of TB treatment is critical to making clinical decisions and in this context, host biomarkers exhibit an important role (141). Additionally, host biomarkers can be used to detect and differentiate between active TB and LTBI in addition to being used in disease prediction and therapy monitoring (142).

1.11 Biomarkers and treatment response

Biomarkers that can effectively indicate treatment progress both during the early stages of therapy and at the end of treatment could significantly improve the assessment of response to TB therapy (142). Biomarkers have been suggested as a promising approach to identify, track, and predict therapy response in active TB additionally, this would facilitate more rapid TB medication development and individualized treatment plans (143). Urine has been assessed for the detection of TB using several *M. tuberculosis* antigens (144). From these assessed *M.tuberculosis* antigens, LAM is the most thoroughly assessed and shows the greatest promise in detecting TB (144).

1.11.1 The detection of the LAM biomarker for monitoring TB treatment response.

LAM, a significant, heat stable, glycolipid found in TB cell walls, may be a helpful pathogen biomarker for evaluating the effectiveness of anti-TB treatment, especially in adult HIV-positive patients (145). Up to 15% of the total bacterial weight is composed of LAM, the main carbohydrate antigen of *M. tuberculosis* (146). With a branching arabinan polymer at the centre

of its mannan core, LAM also has motifs that are capping at the ends of its arabinan branched chains in certain species. When the arabinan termini in *M. tuberculosis* are modified with oligomannoside-based caps, molecules known as ManLAM are formed. The mannan core of LAM is covalently bonded to a lipid moiety called mannosyl phosphatidyl-inositol (MPI) that contains residues of stearic, palmitic, and tuberculostearic acids, as depicted in Figure 4. In some TB patients, lipoarabinomannan is expelled in their urine, and can be detected by polyclonal antibodies in a simple sandwich ELISA assay (147). Less invasive specimens like urine and blood can be easily collected from admitted patients or those visiting the hospital, or even outside the hospital (148). Urine is an attractive alternative biological sample, easily obtainable and does not generate dangerous aerosols during collection, thus reducing the risk of nosocomial transmission of TB to healthcare and laboratory workers (115). Urine, unlike sputum, does not typically generate aerosols under normal conditions. However, laboratory processing steps such as pipetting, vortexing, or centrifugation may lead to droplet formation, and appropriate biosafety precautions are therefore required (149 – 151).

Immunodeficiency is the result of HIV's onslaught on the human immune system. Due to this, *M. tuberculosis* can multiply widely within the human body, which results in increased amounts of LAM in circulation. Immune suppression not only encourages *M. tuberculosis* replication, but also decreases the production of antigen–antibody complexes, increasing the amount of free LAM in circulation (152). An increase in filtered LAM is caused by reduced podocyte activity in late-stage HIV patients (153). Consequently, patients who have both HIV and TB have much higher levels of LAM in their urine. Most HIV-negative individuals have healthy immune systems, which prevent *M. tuberculosis* replication and encourage the development of antigen-antibody complexes, and as a result there is a decrease in LAM concentrations in the urine (154).

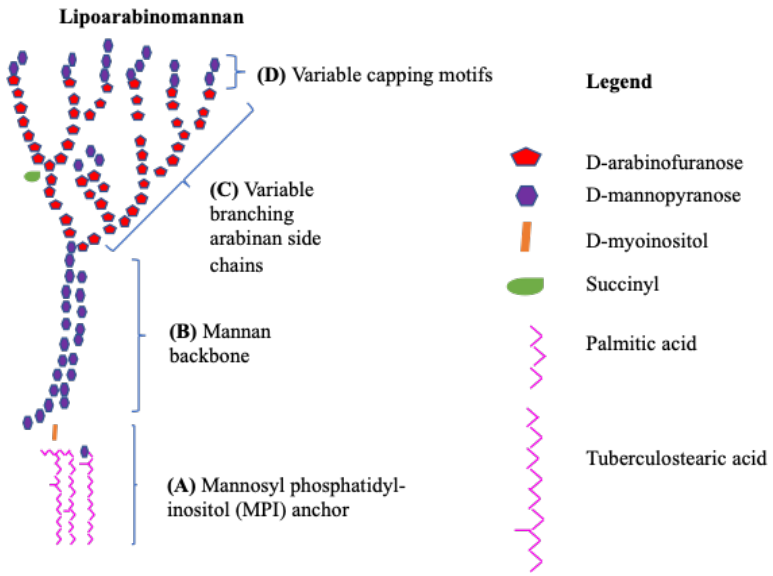


Figure 4. Diagrammatic representation of Mycobacterium spp. LAM structure. Four domains characterise the heterogeneous structure of LAM. (A) The phosphatidyl-myoinositol forms the basis of (B) the mannan backbone and is attached to the plasma membrane, which is then followed by the (C) arabinofuranose backbone and the (D) capping motifs of extra-mannoses, methylthio-D-xylose, or arabinofuranosides. Figure adapted from (134).

1.11.2 LAM detection in urine

Urine contains the heat-stable LAM glycolipid in an antigenically intact state since it goes through the circulation and the renal filtration barrier without undergoing significant alterations, as shown in Figure 5 (155–157).

According to a study by Hamasur and associates, polyclonal antibodies were employed to develop an ELISA detection technique (145). Early research demonstrated that LAM could be detected in the urine of mice given an intraperitoneal injection of purified *M. tuberculosis* cell wall extract. The techniques exhibited high detection capability, identifying LAM at concentrations as low as 1 ng/ml and 5 pg/ml (145). The Clearview® TB ELISA, a commercially available and standardized lipoarabinomannan (LAM) antigen detection assay developed by Inverness Medical Innovations, USA, has replaced the pre-commercial prototype MTB LAM ELISA Test® (Chemogen, Portland, USA), which was initially evaluated in Tanzania in 2005 (145).

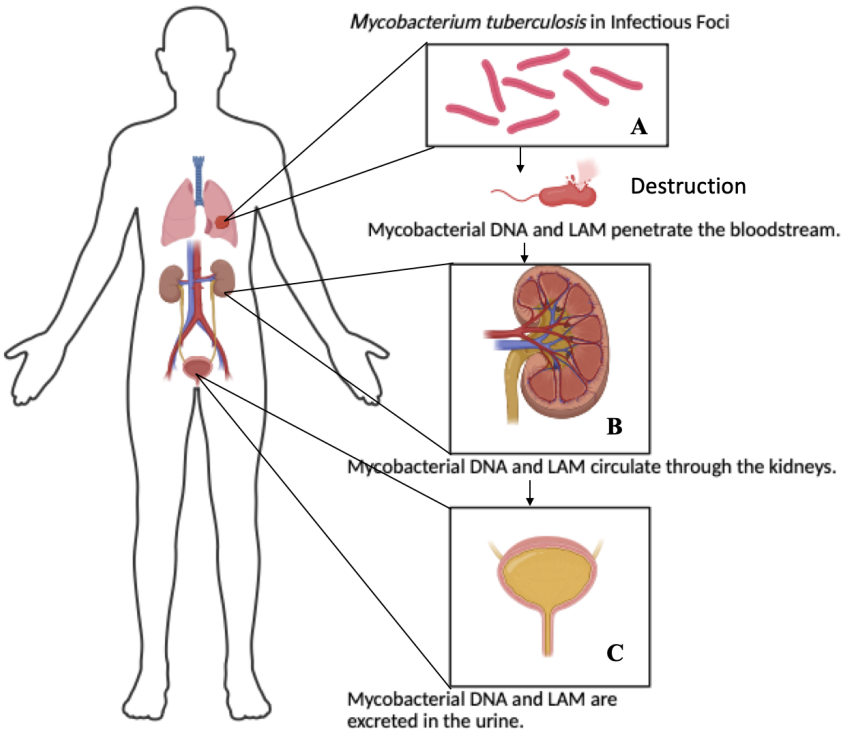


Figure 5. Schematic representation depicting the movement of lipoarabinomannan antigen from the site of infection into the urine. (A) The release of LAM during a TB infection from metabolically active or breaking down bacterial cells. (B) Filtration of the LAM through the kidneys. (C) Mycobacterial DNA and LAM contained in the urine. Adapted from Peter et al (158). Created in https://BioRender.com.

1.11.3 LAM detection in Exhaled breath condensate

Since 2015, urine tests based on the identification of LAM (Figure 4), have been advised by the WHO 2016, to aid in the diagnosis of tuberculosis in patients who are very ill with HIV (113,159,160). However, the sensitivity of urinary tests is not the most ideal, which may limit their ability in TB screening. The liquid phase of exhaled air, known as the exhaled breath condensate (EBC), can be sampled by cooling and is one possibility for TB testing (161–163) The EBC is accessible and, like blood and urine, deserves more research as a possible fluid sample for TB detection (24). EBC collection is a straightforward, cost-effective, and non-invasive technique that does not require specialized personnel. As EBC reflects the composition of the airway lining fluid, it has the potential to contain biomarkers specific to respiratory diseases, which may be associated with the presence of infectious agents or alterations in host tissue resulting from disease processes (161, 163).

1.11.4 Test performance of LAM ELISA

The diagnostic utility of the TB LAM ELISA-kit has been thoroughly assessed. Urine is the preferred biological sample in the majority of studies used to assess performance (158)

However, there has been limited success in research studies examining the clinical potential of the LAM detection using the ELISA platform on various bodily specimens, such as sputum, serum, cerebro-spinal fluid (CSF) and pleural fluid (164 – 167). With the exception of the early research by Boehme and Tessema (155, 167), the urine LAM ELISA's overall sensitivity was reported to be relatively low, with values ranging between 13-51%, for the standard diagnosis of TB (156, 167 – 169). Urine LAM ELISA sensitivity does, however, improve in PLWH with advanced immune deficiency (i.e low CD4 T-cell levels) and in patients hospitalized with an increased disease severity (158). Detection of LAM using the ELISA platform in patients co-infected with TB and HIV, with a CD4 count of less than 50 cells/mm³ had an 85% sensitivity, according to a South African hospitalized cohort study conducted by Shah *et al* 2009 (114). Furthermore, in this same research, and of particular clinical significance, amongst the sputum smear-negative individuals there was a 56% urinary LAM sensitivity (114).

1.11.5 The Urinary LAM strip test

A simplified version of the Clearview TB LAM ELISA, using the same polyclonal antibody conjugated to gold particles in a strip format, was developed by the Alere Medical Innovations (22, 170). The Determine-TB LAM Ag strip test (Alere, USA) is the only commercially available LAM test and was the first true point-of-care TB test. The test only needs 60 µl of raw urine and could be conducted at the bedside of the patient without the need for electricity or specialized laboratory training, and high costs (22). The test is read using the reference guide card provided by manufacturer to evaluate the intensity of the band after 25 minutes of urine being dispensed onto the loading platform (Alere Determine LAM Ag Lateral Flow Assay package insert). The World Health Organisation recommends it exclusively for the detection of TB in people with HIV (122). Alere LAM's sensitivity however, differs depending on the patient. The sensitivity percentage increases to 45% in individuals with CD4+ T cell counts below 200 cells^3/µl, whereas it is only 16% in those with levels above 200 cells^3/µl (119). Alere LAM has a sensitivity rate of 42% in the HIV-positive population (119) and just 18% in the HIV-negative population (153). The urine LAM test cannot serve as a stand-alone diagnostic test due to its limited sensitivity; however, it has value as an adjunct within the diagnostic algorithm. When combined with other methods, such as sputum microscopy, it enhances overall diagnostic sensitivity (22, 170) A valid test is indicated by the presence of a band in the control window. The colour intensity of the band in the patient window is then compared to

the reference card and graded accordingly (Figure 6). In environments with limited resources and a high TB HIV burden, this basic test has a lot of potential for detecting TB.

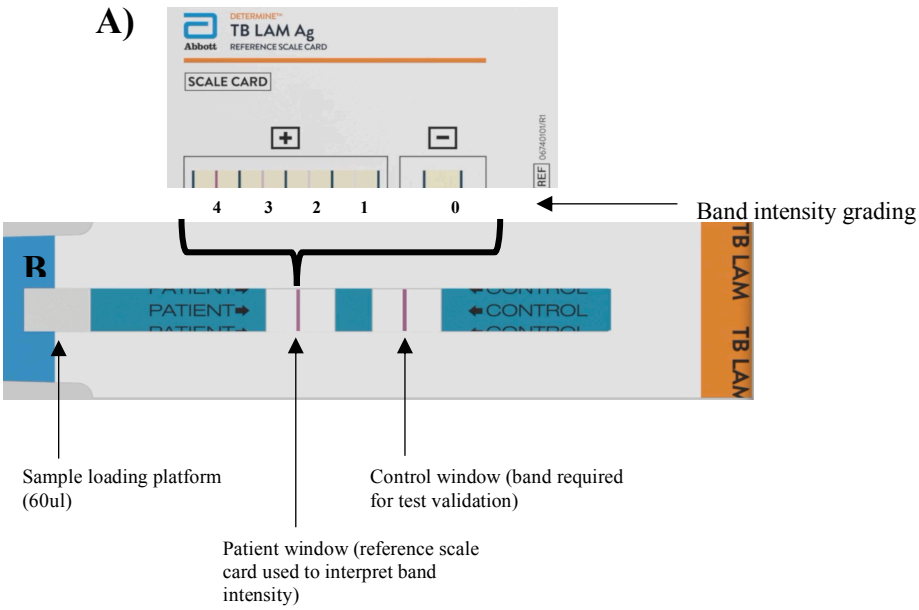


Figure 6. Interpretation of TB LAM Ag LFA outcomes in urine (A) Reference scale card, with band intensity grading the "-"represents negative samples whilst the "+" represents positive samples**. (B)** Determine LAM Ag strip test, with test pad, control and patient window for testing.image adapted from (22).

1.12 Current study Rationale

1.12.1 Hypotheses:

1. We hypothesize that the concentration of free LAM in urine of TB-HIV co-infected individuals will reduce over time-on-treatment and that quantifying LAM concentration over the course of treatment will allow assessment of response to TB treatment. In this context, LAM could serve as a biomarker for response to TB treatment.
2. We hypothesize that purified LAM and anti-LAM antibodies can be used to construct a laboratory assay (ELISA) to detect LAM.
3. We hypothesize that quantifying the LAM analyte band intensity of the Determine LAM strip test, and calibrating this with known standard, will allow for constructing a semiquantitative method of LAM detection. This method can then also be used to assess the effectiveness of TB response.
4. We hypothesize that LAM concentration in plasma will be lower than that detected in urine.

5. We hypothesize that as the urine LAM concentration decreases over the 6 month period of taking anti-tubercular medication, the time to positivity over-time will also be longer.

1.12.2 Aim:

Herein, we aim to use specimens from a prospective cohort study to assess the changes in LAM concentrations of urine and plasma over a 6-month period of receiving antitubercular treatment. Additionally, stored plasma samples will be used to measure LAM concentration as an exploratory measure. The data from urine and plasma will be compared to ascertain if a similar reduction of LAM is observed in both samples, indicative that plasma could serve as an alternate sample for TB treatment monitoring.

1.12.3 Objectives:

1) To detect, grade and quantify the concentrations of LAM in stored urine samples using both the rapid POC strip test and a lab-based ELISA test at various time points (baseline, month 1, month 2 and month 6) during the treatment period.
2) To quantify the concentrations of LAM in stored plasma samples using a lab-based ELISA test at various time points (baseline, month 1, month 2 and month 6 visits) during the treatment period.
3) To correlate results longitudinally from both the LAM ELISA and the Determine LAM Ag POC test at each time point to determine if LAM detection can be used to monitor treatment response**.**
4) To compare both ELISA results from plasma and urine to ascertain if the same changes will be observed, and that plasma could serve as a proxy in cases where LAM detection in urine is low due to renal dysfunction.
5) To correlate LAM concentrations with other methods of monitoring treatment response, such as liquid culture MGITs (including time to positivity), and to compare them with GeneXpert results (acknowledging that GeneXpert is not suitable for monitoring response but included here for comparative analysis), as well as with ELISA and rapid POC strip test outcomes.

Chapter 2: Methods and materials

2.1 Introduction

This study aimed to detect and quantify LAM levels in human urine and plasma samples from TB patients, both with and without HIV. To achieve this, the study initially intended to develop a sandwich ELISA assay for LAM detection in urine, while also testing plasma samples to assess whether similar results could be obtained. Additionally, urine and plasma samples were analyzed using the Determine LAM Ag test, a POC test, to evaluate LAM detection in both sample types. However, the development of the sandwich ELISA assay could not be completed, as validation of LAM detection using indirect ELISA was unsuccessful. As a result, ELISA data were not available for comparison with the POC test results. Before attempting to establish the assay, purified LAM from *Mycobacterium tuberculosis* H37Rv and anti-LAM monoclonal antibodies were tested to confirm their integrity. This involved performing SDS-PAGE, followed by indirect ELISA and dot blot assays based on established protocol to evaluate the binding capacity between LAM and the antibodies, as well as to determine the limit of detection. LAM detection using the POC test was conducted in two patient groups: Group 1 included patients with confirmed TB who were MGIT culture-positive at baseline, with a positive HIV status, while Group 2 consisted of MGIT culture-negative patients at baseline, with a positive HIV status status. Refer to Figure 7 for the participant disposition flow chart.

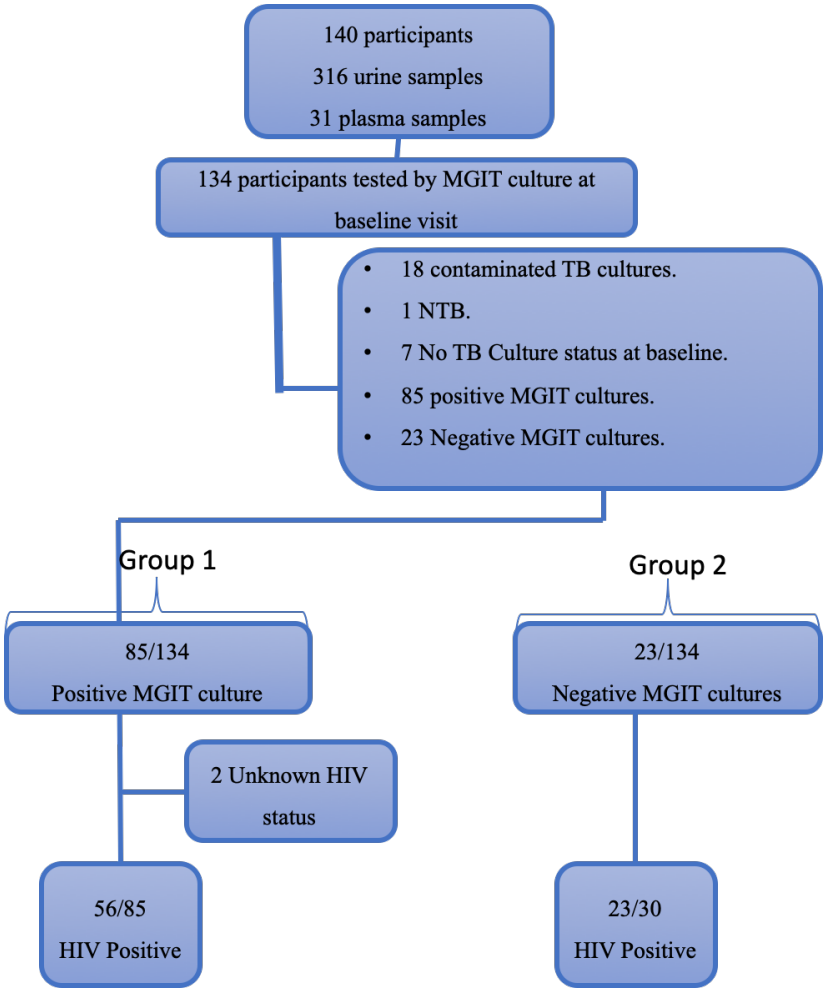


Figure 7. Participant disposition flow chart. Group 1 and group 2 patient cohort, illustrating the stratification based on TB diagnosis and HIV status.

Anonymized urine and plasma samples were obtained from the South African-Hopkins TB collaboration study (SoHoT Study). Samples were collected between 2017 and 2019 from a total of 140 participants, from the two selected health districts in South Africa: Matlosana, and Capricorn Health District in Limpopo Province. **Cohort A were individuals with confirmed TB**, both PLWH and HIV seronegative individuals who provided a total of 316 urine samples and 31 plasma samples . **Cohort B, consisted of adult household contacts (HHC**) of Cohort A participants, all of whom were confirmed TB-negative. From this cohort, 20 urine samples were collected, with four samples used for spiking with purified LAM and for generating a standard curve.

At baseline, 134 participants were tested using MGITculture assay. Among these, 85 participants were MGIT culture-positive for *M.tuberculosis*, 30 were MGIT culture-negative, and 18 samples showed contamination. Additionally, one participant tested positive for

nontuberculous mycobacteria (NTB) at baseline. Within the MGIT culture-positive subset (n = 85) of Cohort A, 56 participants were PLWH, and two participants had an unkown HIV status.

Urine and plasma samples were stored in a -18°C freezer for future research use and consent for storage was granted by the recruited and enrolled individuals. Ethics approval was obtained for the use of these samples in this study, the ethics clearance approval can be found under Appendix D (ethics clearance number 160413). Figure 8 presents a summary of the methods applied in this research.

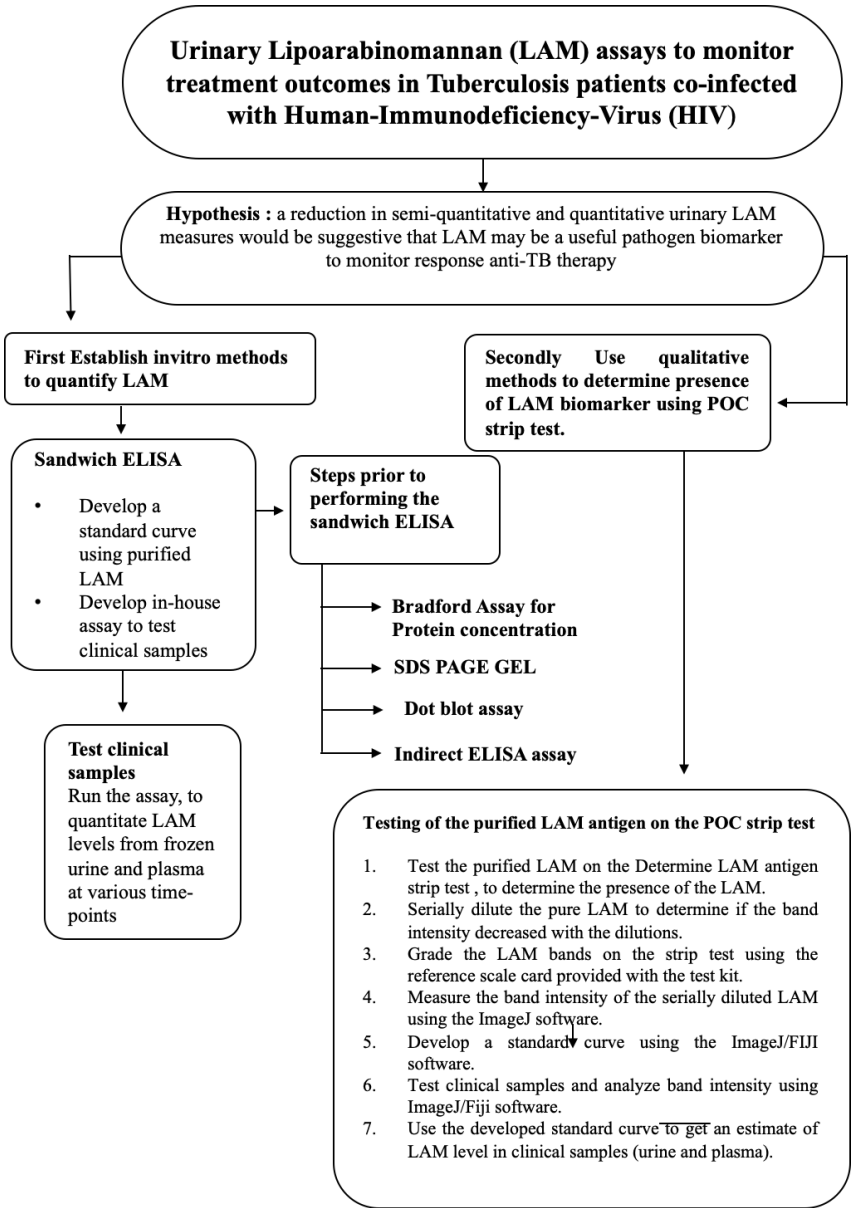


Figure 8. Overview of methodology used in the study.

2.2 Materials

The reagents and solutions used in this study are listed in Appendix A.
The inclusion and exclusion criteria for samples collected from Cohort A patients with TB is outlined below:

Biological specimens were collected from individuals either prior to, or shortly after, the initiation of standard rifampicin-based 6-month multidrug TB therapy. This enabled comprehensive analysis of biological signals and their modulation in relation to treatment response. Participants included individuals with presumptive but not yet confirmed active pulmonary TB (either newly diagnosed cases or relapses). If active pulmonary TB was not confirmed, such individuals were excluded from the active TB cohort. However, their specimens (or a subset thereof) were retained for use as control samples. There were no age restrictions for study enrollment.

2.3 Methods

The following methods were employed to assess the pure LAM concentrations and validate antibody interactions. The Bradford assay was first performed to determine the concentrations of LAM and antibodies, ensuring accurate sample loading for subsequent analyses on the SDS PAGE gel.

2.3.1 Bradford assay for assessing protein concentration.

The Bradford reagent (AMRESCO, Biotechnology grade) was diluted 1:5 in dH2O and filtered using Whatman filter paper into a 50 ml Falcon tube. Duplicate dilutions of Bovine serum albumin (BSA), 10.0, 5.0, 2.5 and 1.25 µg/ml was prepared in 800 µl of dH2O. Upon the addition of 200 µl of the prepared Bradford reagent to each BSA dilution, the mixture was left at room temperature for five minutes. The spectrophotometer was used to determine and record the absorbance of each dilution at 600 nm. The duplicate values for each dilution was averaged and a standard curve was plotted showing the relationship between BSA absorbance (OD_{600nm}) and concentration in (µg/ml). The concentration of each antibody and LAM antigen was then extrapolated from the standard curve.

2.3.2 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

The SDS-PAGE gel was performed to confirm the presence and integrity of the LAM antigen and the anti-LAM monoclonal antibodies. This step was crucial to ensure that both the antigen and antibodies were intact and suitable for use in subsequent assays, such as the indirect ELISA, where accurate binding interactions needed to be established for reliable results.

An acrylamide gel with a concentration of 15% was used for the separation of the LAM and respective antibodies, along with the corresponding protein weight marker. Two gels were prepared (15 % resolving gel and 4 % stacking gel). The resolving gel was prepared by dissolving 5.7ml of 40% *bis-acrylamide* (Bio-Rad), 4.0 ml 1.5 M Tris-HCl (pH 8.8), 160 µl of 10% sodium dodecyl sulphate (Sigma Aldrich) and 10% ammonium persulfate (Bio- Rad) and lastly 16 µl TEMED (Bio-Rad) into 6.1 ml of sterile water. The stacking gel was prepared by dissolving 0.5 ml of 40% *bis-acrylamide* (Bio-Rad), 1.25 ml 1.5 M Tris-HCl (pH 8.8), 50 µl of 10% sodium dodecyl sulphate (Sigma Aldrich) and 10% ammonium persulfate (Bio- Rad) and lastly 5 µl TEMED (Bio-Rad) into 3.15 ml of sterile water. The ingredients were mixed in this precise order ensuring that there are no air bubbles formed. The glass plates were assembled carefully into the cassette and water was used to ensure the plates sealed properly. The resolving gel was gently poured 10 mm below the edge of the glass plates in the cassette, leaving some space for the stacking gel. The stacking was poured gently on top of the resolving gel with an appropriate size comb inserted at the top, to create wells into which samples will be loaded. After 30 min, once the gel solidified, it was transferred to the gel tank containing a buffer (1X TAE buffer) (40 mM Tris-acetate, 1 mM). Based on the concentrations obtained from the Bradford assay (section 2.3.1), the appropriate amounts of the LAM antigen and corresponding antibody samples were prepared up to a volume of 10 µl, to which 2 µl of the 6X gel loading dye (composed of 30% glycerol, 0.25% xylene cyanol, and 0.25% bromophenol blue) (Biolabs) was added. The samples were loaded into the wells within the stacking gel. A molecular weight marker (PageRuler Plus prestained protein ladder, ranging from 10 to 250 kDa from Thermofisher), which also served as the positive control, was added in the first lane of the gel. The gel was run for 90 minutes at 90V at room temperature, using the PowerPacTM High-Current Power Supply (BioRad). The SDS-PAGE gels were visualised by staining with Coomassie blue dye. The integrity and quality of the LAM antigen and respective antibodies were captured and visualized using the Syngene G: Box equipped with the Syngene GeneSnap version 7.12.

2.3.2.1 Silver staining SDS PAGE gel

The initial SDS-PAGE gels were stained with the Coomassie Blue stain to detect the LAM antigen and 4 monoclonal antibodies. No signal was detected with this stain so the repeat gel was stained with silver stain as an alternative method to enhance the sensitivity and successful visualization of the LAM antigen on the gel. Procedure is described further below.

The gel was run as described in section 2.3.2, after which it was carefully removed from the glass plates in the cassette and transferred to a glass container. The gel was fully submerged in the 1st Fix solution and placed on a shaker for 45 minutes. After this incubation, the 1st Fix was decanted, and a 0.7% periodic acid solution (0.7 g of periodic acid in 100 ml of 1st Fix) was prepared and added to the gel in the container. The gel was then placed back on the shaker for 5 minutes. Following this, the periodic acid was discarded, and the 2nd Fix solution was added to the container. The gel was incubated with the 2nd Fix on the shaker for 5 minutes. Following that, enough 2% glutaraldehyde solution was added to cover the gel, and the gel was incubated on the shaker for 5 minutes. The glutaraldehyde solution was then discarded, and the gel was washed with sterile water for 10 minutes on the shaker, repeating this wash process three times. After the final wash, the water was removed, and 5 mg of dithiothreitol was dissolved in 200 ml of sterile water and added to the container with the gel. The gel was incubated with this solution on the shaker for 5 minutes before the dithiothreitol solution was discarded. A 0.1% silver nitrate solution (0.1 g of $AgNO_3$ in 100 ml of sterile water) was prepared and added to the gel, which was placed on the shaker for 5 minutes. The silver mixture was discarded, and the gel was rinsed briefly three times with sterile water. The developer solution was prepared by dissolving 6 g of Sodium carbonate ($Na2CO_3$) and 3-6 drops of formaldehyde in 200 ml of sterile water. Approximately one-third of the developer solution was added to the gel, and the container was gently rocked until the liquid turned yellow. The remainder of the developer was then added, and the gel was placed on the shaker to develop until the desired bands were visible. Once the bands appeared, the reaction was stopped by adding a stop solution prepared by 10 % ethanol and 5 % acetic acid solution for 2 minutes. The stop solution was replaced with water. A white sheet was placed under the glass container to enhance the visibility of the bands of interest.

2.3.3 DOT BLOT Assay.

The dot blot assay was performed to assess the binding capacity between the LAM antigen and the anti-LAM monoclonal antibodies. This method allowed for the rapid and efficient detection of antigen-antibody interactions, providing preliminary confirmation of the specificity and sensitivity of the antibodies before proceeding to the indirect ELISA assay. The Dot blot assay was performed to determine the antibody/ antigen binding capacity**.** Four rectangular pieces of polyvinylidene fluoride (PVDF) membrane were used to test the binding capacity of the LAM antigen against the four monoclonal antibodies. A grid with 10 small square was drawn with a pencil onto each of the 4 PVDF membrane pieces, to allow for loading of individual samples without cross contamination. Thereafter, 2 µl of the dilutions of the pure *M. tuberculosis* H37Rv LAM samples were blotted onto the PVDF membrane at the center of each square within the grid. The remaining binding surface on the grid after the LAM antigen was blotted, was blocked by soaking each of the PVDF membrane pieces in TBS-T containing 5% BSA for 1 hour at room temperature (RT), in a 10 cm Petri dish. This was performed to prevent non-specific binding of the antibodies. Each of the four primary antibodies namely mouse IgG3 anti- LAM antibody known as CS-35, CS-40, Clone 906 and Clone 908, (these antibodies were selected because they are well-validated monoclonal antibodies with high specificity for *M. tuberculosis* LAM, recognizing different epitopes) supplied by BEI Resources, were diluted in the range of 1: 100, 1:1000 and 1:10 000 in TBS in a Falcon tube. The membrane was then subsequently incubated with the primary antibody for 30 minutes at RT. Each of the 4 PVDF membrane pieces was washed 3 times with TBS-T (3 x 5 min) using a 10 cm petri dish on a shaker. The secondary antibody conjugated with Horseradish peroxidase (HRP), goat anti-Human (IgG-HRP produced in goat SIGMA), was used at a dilution of 1:10 000 as per manufacterers guide. The secondary antiibody was used to subsequently incubate the PVDF membrane pieces using a petri dish for 30 minutes at RT. The membrane was washed with TBS-T wash buffer 3 times for 15 minutes(each wash step was 5 minutes) using a 10 cm petri dish on a shaker, followed by a single wash with TBS for 5 min. The membrane was then incubated with 1ml Chemiluminescent Peroxidase Substrate (CPSD) for 1 min, covered with plastic pocket sleeve (to remove excessive solution from the surface), and exposed to an X - ray film (Thermo Scientific CL-XPosure film) in a cassette, in the dark room. The blot was initially exposed to the X-ray film in the cassette for 30 minutes overnight. After exposure, the X-ray film was developed by preparing four plastic containers: 1) fixing solution (Axim), 2) water, 3) developing solution (Axim), and 4) water. These solutions were used in sequence to visualize the signal. After the exposure time, the X-ray film was removed from the cassette, in

the dark room, and placed into the developing solution for 30 seconds, removed and placed in water for 30 seconds, removed and placed into fixer for 30 seconds, then lastly in was placed in water for 30 seconds. The film was dried by attaching the x-ray film with pegs to the string in the darkroom and the lights were then switched on to visualize the signals. See figure 9 for method flow.

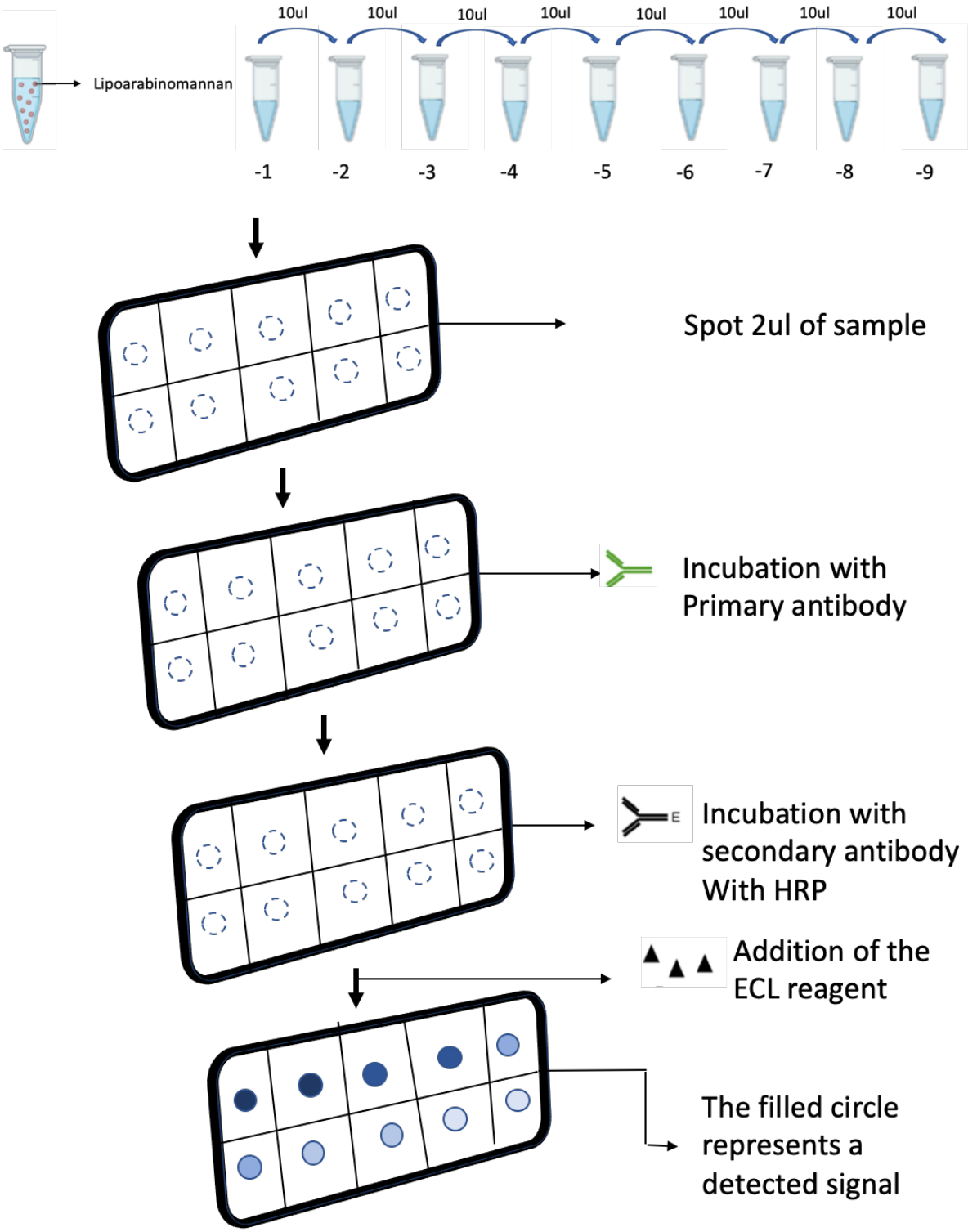


Figure 9. Schematic representation of the DOT blot assay. This figure illustrates the procedure of the DOT blot assay, the serially diluted LAM antigen was spotted onto a membrane, followed by incubation with a primary antibody and secondary-HRP conjugated antibody. The reaction was then visualised by adding a chemiluminescence substrate to identify the presence and relative amount of the target biomolecule in this context the LAM.

2.3.4 Indirect ELISA Assay.

The indirect ELISA was performed not only to validate the specificity and sensitivity of the anti-LAM monoclonal antibodies but also to determine the optimal antibody-antigen binding concentration. Additionally, it was used to establish the limit of detection for the LAM antigen, ensuring that the assay could reliably detect low concentrations of LAM in the samples. An overview of the principle and procedure of the LAM ELISA is provided below.

The LAM antigen was diluted to a final concentration of 2 µg/ml in 1x PBS (Thermofisher, Dulbecco′s Phosphate Buffered Saline), of which 100 µl was used to coat the plate 96 well high binding plates (Corning® 96-well EIA/RIA Clear Flat Bottom Polystyrene High Bind Microplate) or (NUNC MAXISORP FLAT-BOTTOM microplate). The plate was sealed with a clear plate sealer and incubated in a 4°C fridge overnight. After the overnight coating with LAM, the next day, the plate sealer was removed from the plate and the wells washed 3 times with 250 µl wash buffer (wash buffer composed of 1x PBS, 0.05% Tween 20 in distilled water) per well using an automated plate washer (BioTek 50 TS Microplate Washer). After washing the plate, 200 µl of 5% blocking buffer (buffer was composed of 2,5 g skim milk powder into 50 ml wash buffer) was added per plate to all wells using a multi-channel pipette. The plate was incubated at RT, for a minimum of 1 hour after adding blocking buffer to the plates. After the 1 hour incubation, the plates were washed 3 times with 250 µl per well of wash buffer using an automated plate washer. The primary antibodies (IgG3 anti-LAM antibodies produced in mouse) were diluted to 5 µg/ml. After the wash step, 150 µl of the 5ug/ml diluted antibodies (CS-35, CS-40, 906.41 and 908.41) were added in duplicate in row A1- 8 of the 96 well plate. Then, 100 µl of assay buffer (PBS) was added to rows B-H of the plate. Following this, a 3-fold serial dilution was performed by taking 50 µl of row A and adding it to row B and mixing by pipetting up and down, and continuing with the 50 µl transfer to row H. The last 50 µl was discarded to have a final volume of 100 µl per well. The plate was gently tapped to allow the liquid to completely cover the bottom of each well, see Figure 10. The plates were then incubated at RT for a minimum of 1 hour after the serial dilution step. Whilst the plate was incubating with the primary antibody, the secondary-HRP conjugated anti-human antibody was diluted to a 1:500 concentration initially then increased to 1:3000 concentration. Then, 100 µl of the diluted secondary antibody was added per well of the 96 well plates and incubated for 1 hour at room temperature. The plates were washed 3 times with 250 µl per well of wash buffer using an automated plate washer.

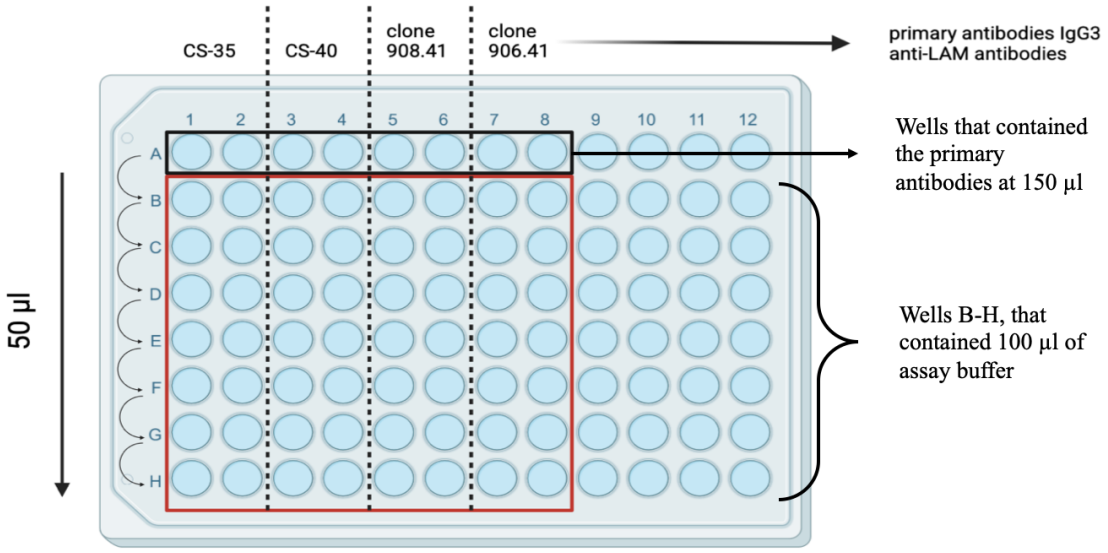


Figure 10. 96 well plate annotating ELISA primary antibody dilution flow. Primary IgG3 anti-LAM antibodies were prepared at a concentration of 5 µg/ml and added to row A1-8 of the 96-well plate (150 µl in duplicate). Assay buffer (PBS) was then added to rows B-H of the plate (100 µl per well). A 3-fold serial dilution was performed by transferring 50 µl from row A to row B and mixing by pipetting up and down. This process was repeated across rows B-H, completing the dilution series. The last 50 µl was discared to achieve a final volume of 100 µl in each well.

Upon the wash step, in a dark biological safety cabinet, 100 µl of the 1-step TMB substrate (Thermofisher scientific) was added to all the wells in the plates, the plates were covered in foil and incubated, for 5 minutes until the blue colour was observed. The reaction was stopped by adding 50 µl 1M Sulphuric acid H_2SO_4 (Thermofisher scientific) to all the wells of the 96 well plates. The plates were immediately read at a wavelength of 450 nm on the plate reader (Molecular Devices SpectraMax ABS Plus). Figure 11 below displays a flowchart that outlines the steps involved in the LAM ELISA assay.

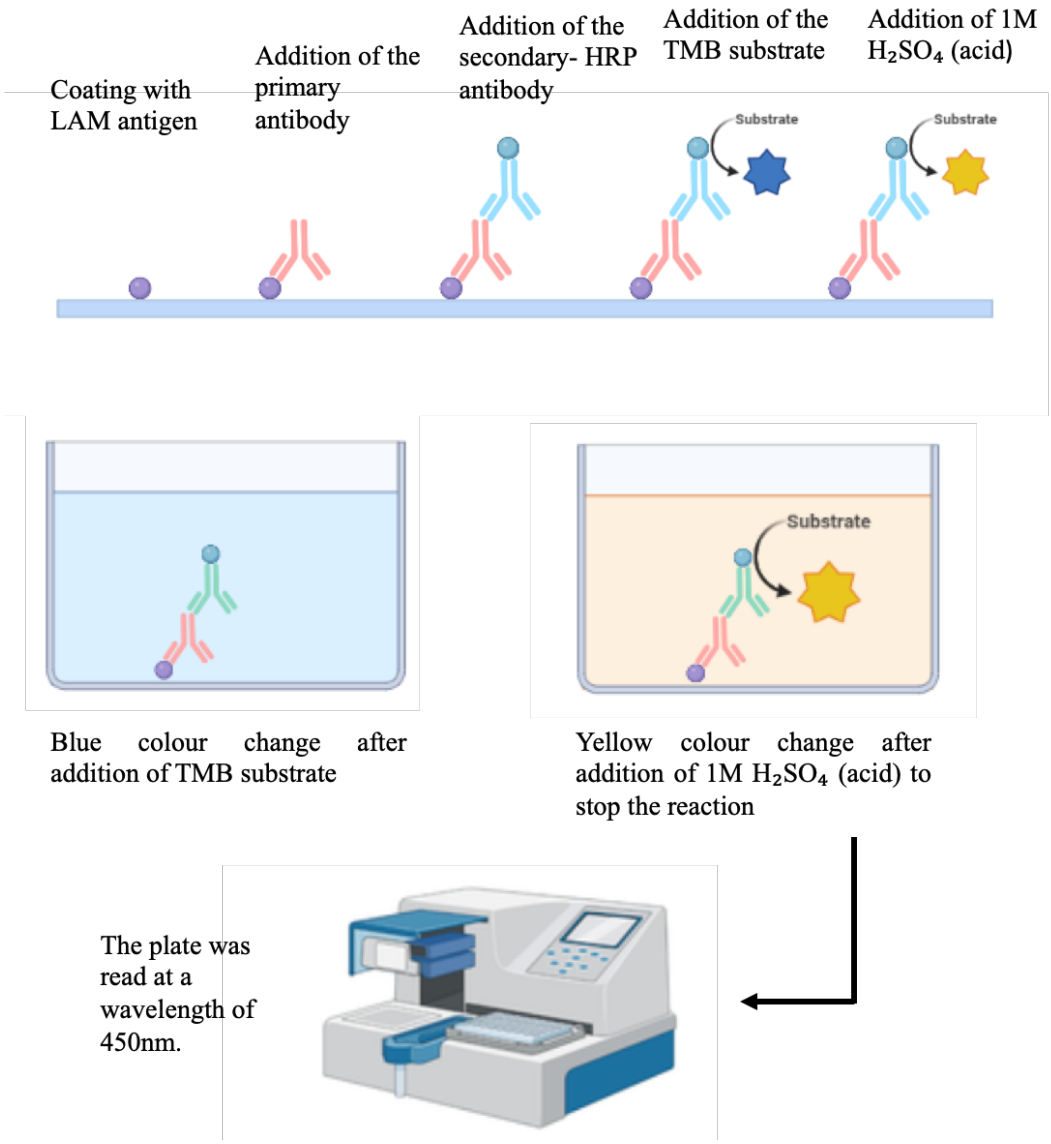


Figure 11. Schematic representation of Indirect ELISA flow. The 96 well high binding microplate was coated with the LAM antigen, followed by incubation with primary anti-LAM antibody. The secondary HRP conjugated antibody was then added. After incubation with the secondary antibody, the TMB substrate was added, which catalyses a reaction that produces a detectable signal (blue colour) the colour also correlates with amount of antibody present. The sulphuric acid was added to stop the reaction, indicated by the yellow colour change. The colour development was subsequently measured at a wavelength of 450nm using the plate reader.

2.3.5 Validation of the purified LAM on the Determine LAM Point of care (POC) strip test.

LAM purified from *M. tuberculosis* H37Rv (obtained from BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv, NR- 14848), was initially tested to determine its integrity, using the Determine TB LAM test (Abbott) in accordance with the manufacturer's

specifications. Briefly, the purified LAM was thawed on ice, and 60 µl was carefully transferred onto the test strip using a sterile pipette. Sterile water was used as a negative control. The test was allowed to develop and the results were interpreted after 25 minutes. The test strips were then photographed to allow for analysis on the Fiji software.

2.3.6 Development of a standard curve to determine the LAM concentration.

Due to the inability to establish a standard curve using the ELISA assay, an alternative approach was implemented to develop a standard curve for the quantification of the LAM. This involved using control (cohort B) urine samples. Four cohort B urine samples were spiked with varying concentrations of LAM (1:10, 1:100, 1:1000 and 1:10 000 dilution) and assessed on the Determine LAM Ag test strip. The results were analysed on the Fiji software to develop the curve, see detailed process below.

The cohort B urine samples were spiked with 500 ug/ml purified LAM from *M. tuberculosis* H37Rv, the LAM was diluted using the following concentration ranges (1:10, 1:100, 1:1000 and 1:10 000 respectively) to obtain a concentration range for the establishment of a standard curve. Rapid LAM testing was carried it out on the LAM dilutions using the Determine TB LAM test in accordance with the manufacturer's specifications. The Fiji software was used to analyse and measure the band intensity on the test strips, the result were then plotted to develop the standard curve of the urine spiked with LAM. This standard curve was subsequently used for the semi-quantitative determination of LAM levels in clinical samples See Figure 12A, for method flow.

2.3.7 Analysis of frozen urine and plasma samples for LAM detection using the Determine TB LAM Ag rapid test.

Clincal samples (urine and plasma) that were stored and frozen, were tested on the Determine LAM antigen test following the suppliers guidelines. Urine samples and frozen plasma were briefly thawed for an hour at room temperature and centrifuged at a speed of 10 000 g for five minutes. A sterile pipette and tip was used to apply 60 µl of the clear supernatant to the test pad on the test. The test was allowed to develop and the results were interpreted within 25 minutes. Positive results were categorized into four grades, 1+ being the lowest intensity and 4+ being the highest intensity. This grading was based on the manufacturer's reference guide. See Figure 12B, for method flow.

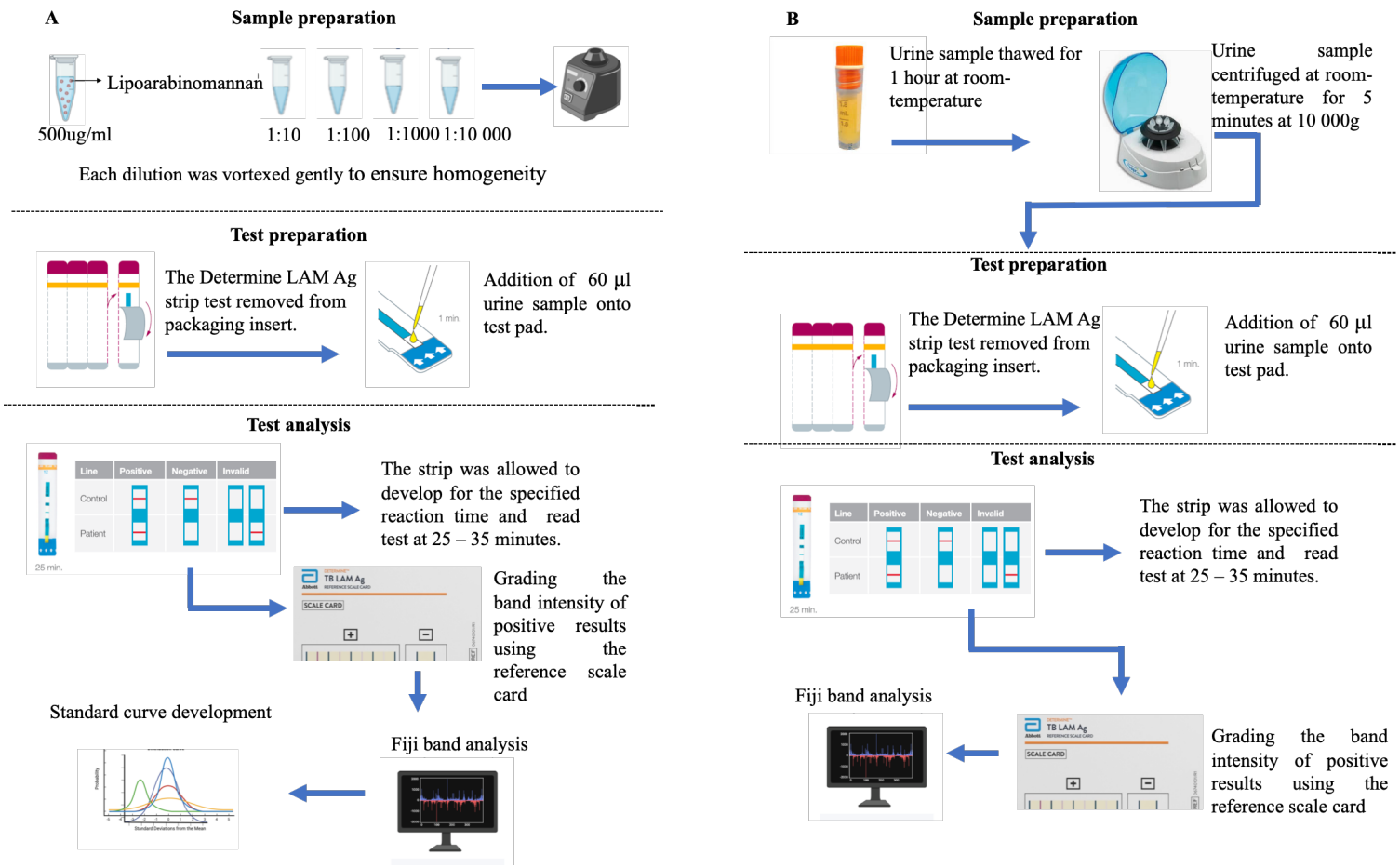


Figure 12. The flow chart outline for the steps in both the development of the standard curve for the semi-quantification of LAM and the testing of clinical urine and plasma samples using the Determine LAM rapid strip test. (A) Cohort B urine samples were prepared by spiking with varying LAM dilutions, then tested on the strip test and graded acoordingingly. The resultant data was analyzed using Fiji software to develop the standard curve. (B) Clinical urine and plasma samples were centrifuged and tested on the strip test. Samples were graded accordingly using the reference scale card. The strip test was photographed, and the bands were analyzed on Fiji software to quantify band intensity, which was used to determine LAM concentrations.

2.3.8 Rapid LAM testing band analysis on Fiji software

To analyse the bands generated by the Determine LAM rapid strip test, the Fiji software was utilized as a proxy for determining LAM concentrations. The software allowed for precise quantification of the band intensity, which was then correlated to known concentrations of LAM, as outlined in section, 2.3.7. This method enabled the semi-quantitative analysis of LAM levels in clinical samples based on band intensity measurements.

2.3.8.1 Sample preparation

Each of the participants urine sample image to be assessed was merged to the one of the standard images see appendix B (in this case the 2nd replicate standard image) prior to analysing on the Fiji software. The /Fiji programme was downloaded and installed. The image to be analysed was converted to 8-bit using Fiji software function (Image→Type→8-bit) and subjected to analyses detailed below.

2.3.8.2 Fiji software process

The measurement criteria was set by selecting set measurements under the "analyse" tab on the Fiji software, and under the checkbox, only the "Grey Mean value" was checked. The JPEG image was uploaded onto the file menu. For each band across the test strips a single region of interest was defined by drawing a frame around the band of interest, using the Fiji square selection tool to highlight the first lane (this was achieved by pressing Ctrl and 1 to set the first lane). Upon adjusting the frame size properly, a second lane was created by clicking on the centre of the 1st square and dragging it across to the next lane, (this was achieved by pressing Ctrl and 2 to set the next lane).

For the last lane, the above procedure was repeated but Ctrl and 3 was pressed to designate the last lane. Upon creating the last lane a window with lane plots appeared on the screen. Lines were drawn to eliminate the lane background from the calculations, by using the line tool from the tool bar. The line was drawn where the peak begins and ends for each peak. The magic wand from the Fiji software tool bar was used to highlight the area underneath the peak for each lane. The report for each PID then appeared automatically on the screen .

Chapter 3: Results

This section presents the results of the validation assays and clinical sample testing performed in this study. The integrity of the LAM antigen and the corresponding antibodies was first assessed through a series of validation experiments, including SDS-PAGE gel electrophoresis and dot blot assays, both of which successfully confirmed the integrity of the LAM antigen and its binding capacity with the antibodies. In contrast, the indirect ELISA did not demonstrate reliable validation of the antigen-antibody interaction. Based on the assays that showed successful validation, clinical samples were subsequently tested using the rapid POC test to evaluate its practical application for detecting LAM in urine and plasma samples. Each of these results are outlined below.

3.1 SDS Page outcomes of the LAM antigen and antibodies.

The SDS page gel was performed on the LAM and four monoclonal anti LAM antibodies to determine sample integrity as per sections 2.3.2. The outcomes of the SDS PAGE gels, are depicted in figure 13(a), the LAM antigen did not stain whilst the four monoclonal antibodies stained. In figure 13(b), the gel was repeated, however, the LAM antigen did not stain again, whilst the four monoclonal antibodies stained. In figure 13(c) the gel was repeated only for the LAM antigen but a different stain was used, the silver nitrate stain was used instead of coomasie blue stain. Staining with silver nitrate enabled visualisation of the band corresponding to the LAM antigen. LAM migrates as broad bands approximately 30 kDa in size, as determined by protein molecular weight standards. This finding confirms the presence of the LAM antigen in the sample.

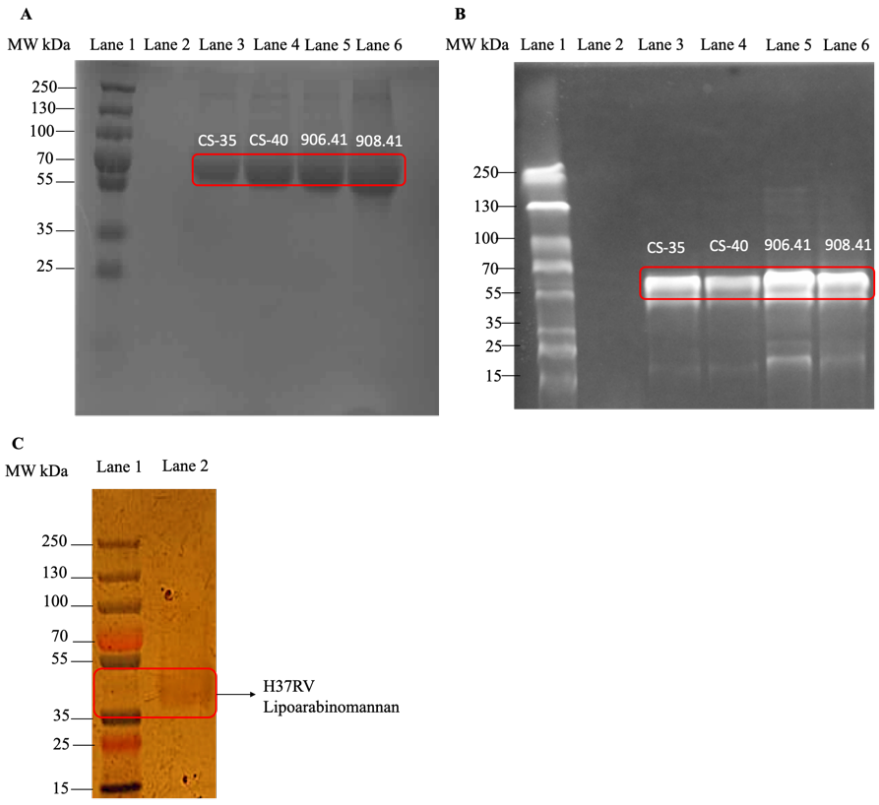


Figure 13. SDS-PAGE analysis of M. tuberculosis LAM and four monoclonal antibodies to confirm integrity thereof. (A) Lane 1: PageRuler Plus prestained protein ladder, Lane 2: H37Rv Lipoarabinomannan, Lane 3: CS-35 MAb, Lane 4: CS-40 MAb, Lane 5: Clone 906,41 MAb, Lane 4: Clone 908 MAb). **(B)** Repeat SDS-PAGE analysis of M. tuberculosis LAM and four monoclonal antibodies to assess purity thereof, prior to performing dot blot assay and indirect ELISA assay. Lane 1: PageRuler Plus prestained protein ladder, Lane 2: H37Rv Lipoarabinomannan, Lane 3: CS-35 MAb, Lane 4: CS-40 MAb, Lane 5: Clone 906,41 MAb, Lane 4: Clone 908 Mab. **(C)** SDS-PAGE analysis of M. tuberculosis LAM to confirm its integrity. Lane 1: PageRuler Plus prestained protein ladder. Lane 2: H37Rv LAM.

3.2 DOT BLOT assay outcomes

The dot blot assay was explored to determine the binding capacity of the LAM to the antibodies as outlined in section 2.3.3. The dot blot was performed on the PVDF membrane and as shown in figure 14A-D, all the primary monoclonal antibodies were diluted to 1: 100, and the secondary anti-mouse-HRP antibody was diluted to 1:10 000 as per manufacturers guide, however, no signal was observed on the blot for the target protein (LAM) under the experimental conditions used.

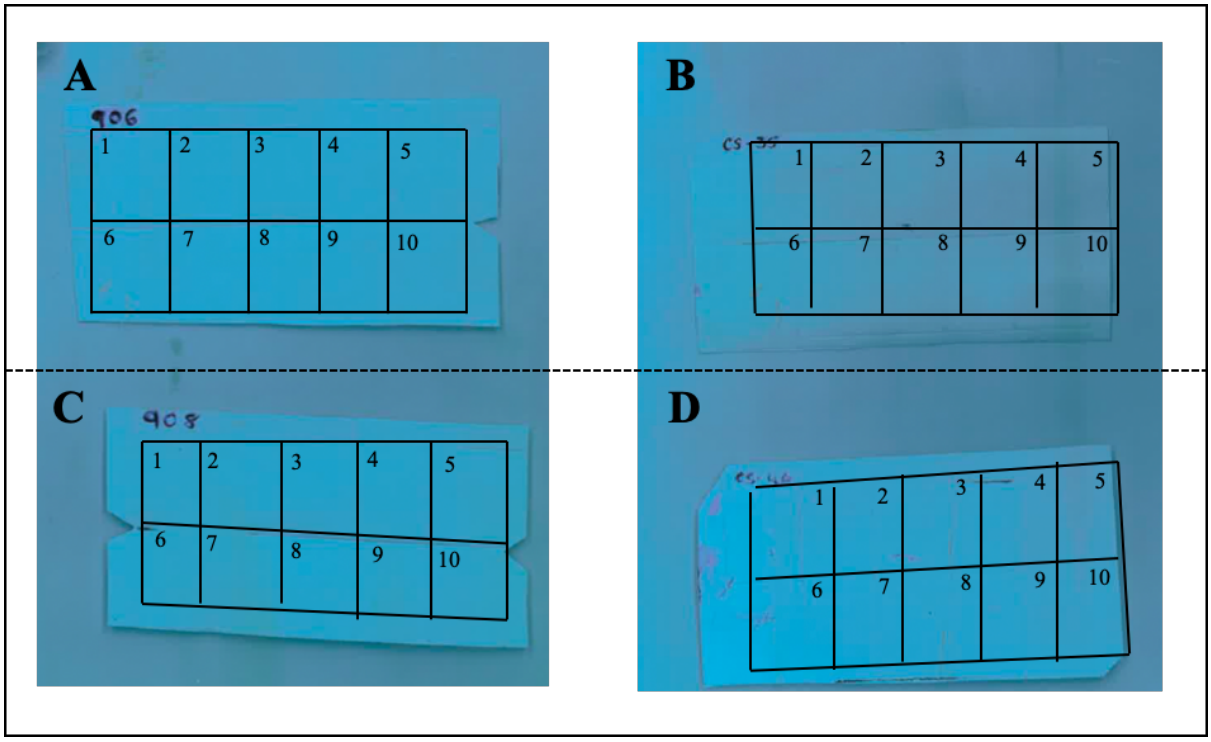


Figure 14. Analysis of the binding capacity testing by Dot blot assay on the LAM and 4 monoclonal antibodies. (A) Clone 906.41 MAb, **(B)** CS-35 MAb, **(C)** Clone 908.41 MAb and **(D)** CS-40 MAb. 2 µl of serially diluted LAM was blotted onto the center of each PVDF membrane u piece using four different monoclonal primary antibodies. Each membrane was incubated with its respective primary antibody at 1:100 dilution.

No binding or interaction between the LAM and all 4 antibodies was observed. In an attempt to address this more dilutions of the primary antibodies were prepared in the range (1: 100, 1:1000 and 1: 10 000) to determine if binding will be observed. However, as depicted in figure 15, for all 4 antibodies CS35, CS-40, 908.41 and 906.41, even with the adjusted concentrations, no binding or specific signal was detected, suggesting that there was no interaction between LAM and the antibodies under the experimental conditions used.

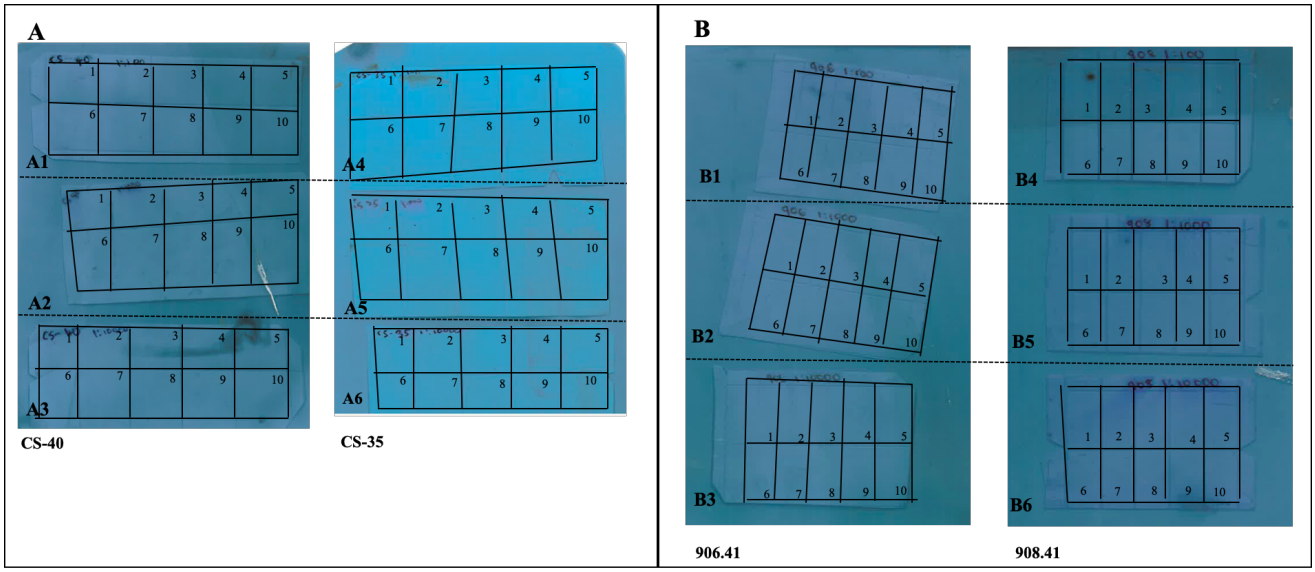


Figure 15. Analysis of the binding capacity of the LAM and 4 monoclonal antibodies using Dot blot assay with increased dilutions ranges. (A1-A3) CS-40 MAb and (A4-A6) CS-35 MAb, (B1-A3) 906.41 MAb and (B4-A6) 908.42 MAb. 2µl of serially diluted LAM was blotted directly onto the center of each PVDF membrane piece. Each membrane piece was incubated with its respective primary antibody at 1:100, 1:1000 and 1:10 000 dilution.

After repeated attempts to detect LAM on dot blots, the lack of any signal led us to hypothesize that there might be methodological flaws. To test this hypothesis, we used an in-house protein, Rv1776c, which was generated in a previous study (this was the only in-house protein that didn't require a specific primary anti-body to bind to) and was predicted to bind to the secondary antibody. This protein was employed as a control to validate the method. However, even with the use of this control protein, no binding was observed after overnight exposure, as shown in Figure 16. This result further suggested that either the experimental conditions were unsuitable for detecting LAM, or that the interaction between LAM and the antibodies did not occur under

the tested conditions.

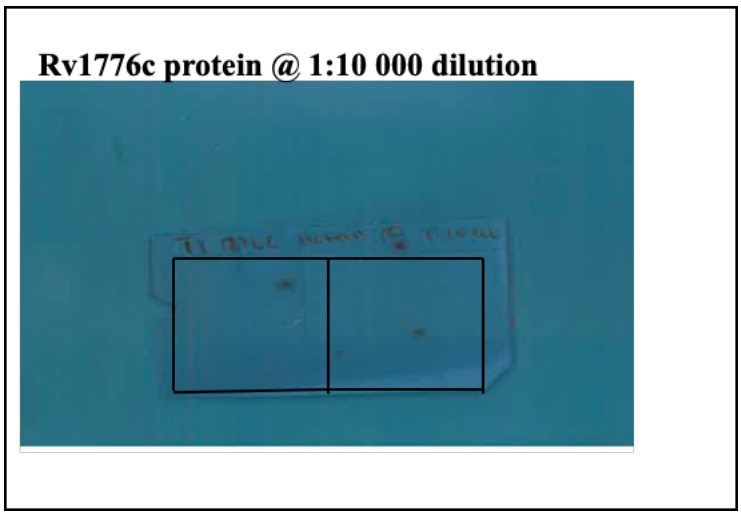


Figure 16. Assessment of the in-house anti-HIS protein (Rv1776c) and secondary HRP-conjugated antibody binding using the dot blot assay for the detection of potential methodological flaws.

We further hypothesized that the membrane used in our assay, which was PVDF, could be contributing to the lack of signal, and that nitrocellulose membranes might be more effective for detecting LAM. To explore the possibility that the PVDF membrane was not optimal and to improve detection, the LAM antigen and antibody binding capacity was tested on a nitrocellulose membrane. Nitrocellulose membranes have a higher protein binding capacity and offer better sensitivity with lower background noise, making them ideal for high-sensitivity assays like Western and dot blots. Under these experimental conditions, only two monoclonal antibodies, CS-35 and CS-40, were tested at dilutions of 1:1000 and 1:10 000. Despite these adjustments, no signal or specific blots were observed after overnight exposure as shown in Figure 17A-B.

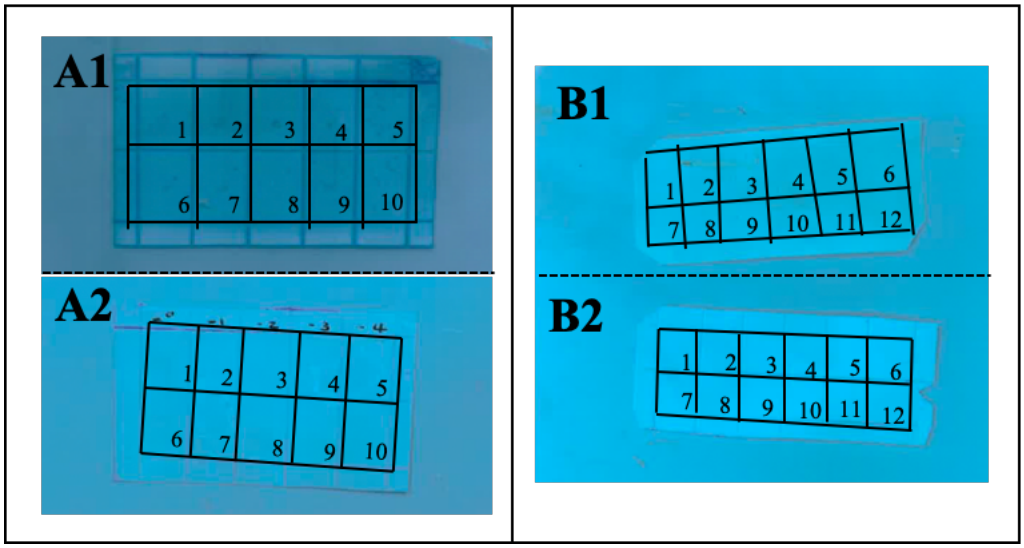


Figure 17. Analysis of the binding capacity testing by Dot blot assay on the LAM and 2 monoclonal antibodies at dilution range of (1:1000 and 1:10000). (A1-2). 2ul of serially diluted LAM blotted directly on the centre of a nitrocellulose membrane using CS-35 MAb at a 1:1000 dilution. In panel (A1) a CPSD substrate and in (A2), a TMB substrate was used to reveal signal. (B1-2) 2ul of serially diluted LAM blotted directly on the centre of a nitrocellulose membrane using (B1) CS-35 MAb and (B2) CS-40 MAb at 1:10 000 dilution.

Thus far, we confirmed that the LAM antibodies (figure 13A and B) and that the LAM antigen (figure 13C) were intact. Given the negative interaction results between the LAM and the different antibodies, suggested that the secondary antibody(which was anti-human-HRP) used in the assays may have not been appropriate. To address this, the dot blot assay was repeated as per section 2.3.3, however the secondary antibody was changed. A 1: 200 dilution of the primary anti LAM monoclonal antibody (CS-35) was prepared. An anti-mouse secondary-HRP antibody produced in sheep was used at a dilution of 1:2500. The antibody and antigen interaction was now observed, with a signal detected after 30 minutes exposure, as shown in Figure 18.

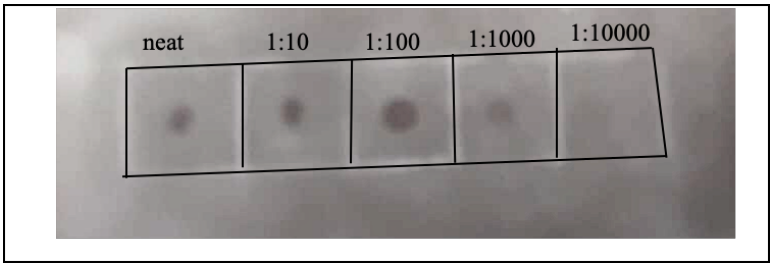


Figure 18. Analysis of the binding capacity of the LAM and 1 monoclonal antibody at a dilution of (1:200) using the dot blot assay. 2 ul of serially diluted LAM blotted directly on the centre of a Nitrocellulose membrane using CS-35 MAb at a titer of 1:200 and anti-mouse secondary HRP antibody at a titer of 1: 2500 (4 ul of secondary HRP into 10 ml dilution buffer).

3.3 Indirect ELISA outcomes.

As we were able to confirm the interaction between the antibody and the antigen, we now sought to develop an ELISA test to detect LAM in clinical specimens. An indirect ELISA assay was performed to determine LAM and antibody binding capacity and to also determine which antibody has the highest binding capacity to the LAM antigen. The absorbance was read at 450 nm (Absorbance is read at 450 nm in ELISA because it corresponds to the optimal wavelength for detecting the color change of the substrate product) and a cut-off value for the sensitivity and specificity was set at an optical density (OD) of 0.4 which was set by testing three known negative samples against the antigen of interest, the OD result for the known negative samples was averaged. The standard deviation (SD) of these three negative samples was then calculated and the resultant SD OD was then multiplied by 3. The OD reading of 0.4 was set at the lowest limit of detection. An OD reading of >0.4 was then determined as the positive threshold.

The microplate was coated with 2 µg/ml of LAM and incubated with a 3-fold titration series of primary monoclonal antibody, starting at a concentration of 5 µg/ml. An anti-human secondary-HRP monoclonal antibody (mAb) was used at a 1:500 dilution. Two different high-bindng ELISA plates, **(19A)** Corning® 96-well EIA/RIA Clear Flat Bottom Polystyrene High Bind Microplate and **(19B)** (NUNC MAXISORP FLAT-BOTTOM),were used. However, the results of the assay, as shown in Figure 19 were inconsistent, as evidenced by fluctuating optical density (OD) readings and the inability to generate a coherent binding curve. These irregular results suggest that there was no significant interaction between LAM and the antibodies under the conditions tested.

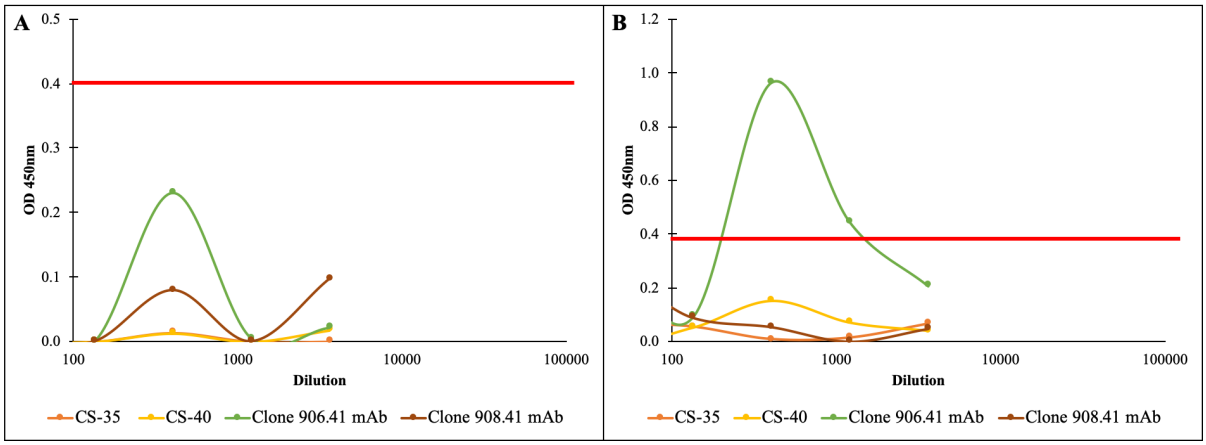


Figure 19. ELISA titration curve of monoclonal antibodies, CS-35, CS-40, Clone 906.41 and Clone 908.41 directed against the LAM antigen,using two different high binding plates. (A) Corning® 96-well EIA/RIA Clear Flat Bottom Polystyrene High Bind Microplate. (B) NUNC MAXISORP FLAT-BOTTOM high binding microplate.

In an attempt to address the non specific binding observed, the primary antibodies were diluted.The microplate was coated with 2 µg/ml of LAM and incubated with primary antibodies at a concentration of 5 µg/ml. A three-fold titration series, starting at a 1:50 dilution, was performed in an attempt to enhance antibody-antigen binding capacity. A monoclonal anti-human secondary-HRP antibody was used at a 1:500 dilution. However, as shown in Figure 20, the assay results were inconsistent, with fluctuating optical density (OD) readings and an inability to generate a coherent binding curve. These irregularities suggest a lack of significant interaction between LAM and the antibodies under the tested conditions.

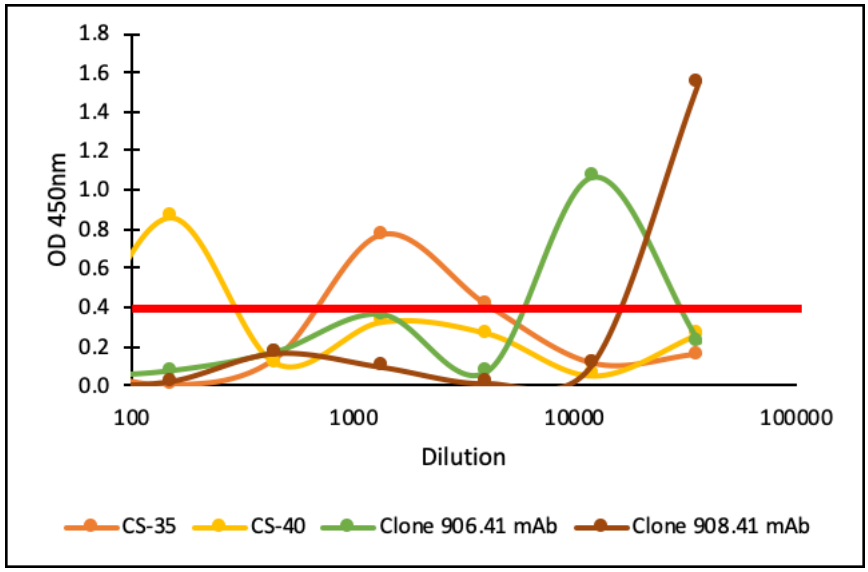


Figure 20. ELISA titration curve of monoclonal antibodies, CS-35, CS-40, Clone 906.41 and Clone 908.41 directed against the LAM antigen 2ug/ml using the NUNC MAXISORP FLAT-BOTTOM high binding microplate. The red line shows the lower limit of accurate detection.

To address this we next opted to change the plate set-up that was used previosuly for the ELISA and performed a 3 fold titration starting at a 1:100 dilution of the 5 µg/ml primary monoclonal antibodies using two high binding plates **(21A)** Corning® 96-well EIA/RIA Clear Flat Bottom Polystyrene High Bind Microplate and **(21B)** NUNC MAXISORP FLAT-BOTTOM, high binding microplate. The secondary anti-human-HRP mAb was at a 1: 3000 dilution. Considering the results thus far, we thought to increase the amount of LAM adsorbed onto the ELISA plate prior to performing the assay. Both the plates were coated overnight with 10 µg/ml of LAM. This resulted in fluctuating OD readings, and thus, no significant binding between antibody and antigen was observed, shown in Figure 21. The red line shows the threshold of accurate detection.

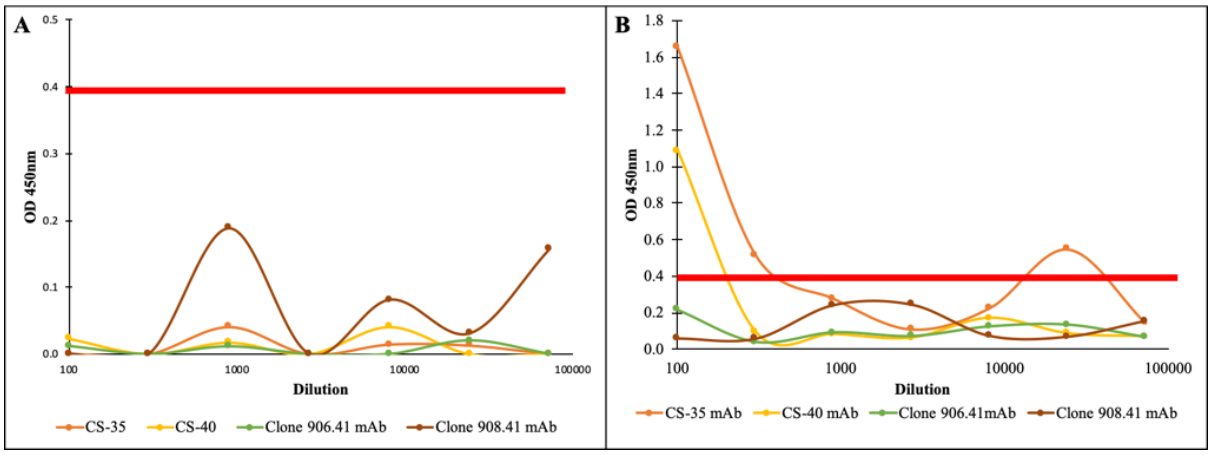


Figure 21. ELISA titration curve of monoclonal antibodies, CS-35, CS-40, Clone 906.41 and Clone 908.41 directed against the LAM antigen at 10 µg /ml using two different high binding plates. (A) Corning® 96-well EIA/RIA Clear Flat Bottom Polystyrene High Bind Microplate. (B) NUNC MAXISORP FLAT-BOTTOM high binding microplate.

These OD readings were promising when LAM was coated at 10 µg/ml instead of 2µg/ml, as seen in Figure 21B. For antibodies CS-35 and CS-40, signals above the detection threshold were observed as seen with higher OD readings but this fell below the limit of detection after the first and second dilution respectively. Despite this decline the results were more encouraging than those obtained previously. Based on this, we reasoned that further increasing the LAM concentration in the plate may yield more promising results.

The NUNC MAXISORP FLAT-BOTTOM high binding microplate was coated overnight with 25µg/ml LAM, the starting concentration of the primary antibodies was 25 µg/ml. A 3 fold titration starting at a 1:100 dilution of the 50 µg/ml primary monoclonal antibodies. Only 3 monoclobal mAb, were used. The secondary anti-human-HRP mAb was used at a titer of 1: 3000.

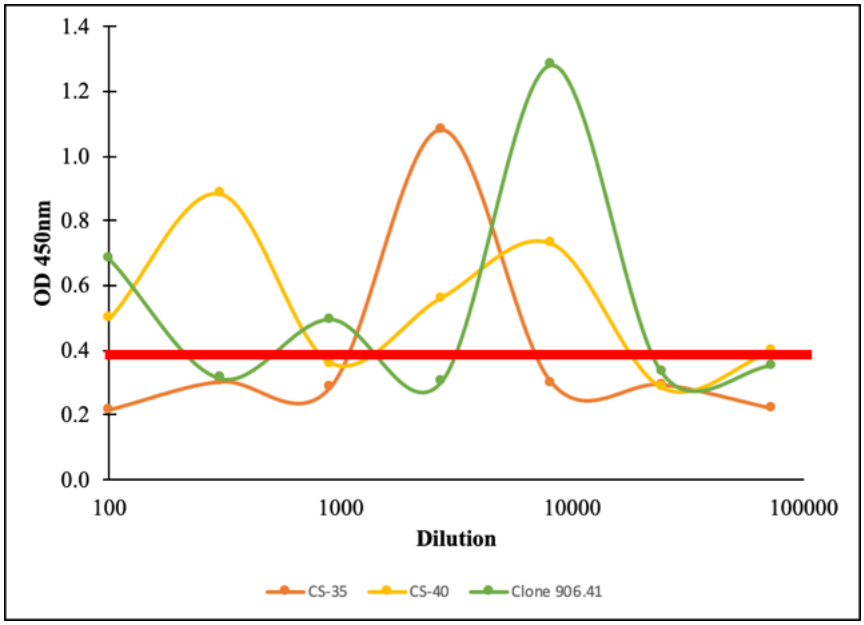


Figure 22. ELISA titration curve of monoclonal antibodies, CS-35, CS-40 and Clone 906.41 against the LAM antigen at 25 µg/ml, using the (NUNC MAXISORP FLAT-BOTTOM high binding microplate.

However, whilst signal above the threshold for detection was observed, there was no quantitative association. Based on these data, we surmised that the antibodies we had in hand were not suitable for the development of a LAM ELISA. We sought to set this assay to monitor LAM levels in response to therapy however, our collective results suggested that accurate quantification of LAM with reagents we had was not possible. As a result, we opted to shift to a semi-quantitative approach to detecting LAM in clinical samples. For this, we used the Alere Determine TB LAM Ag test strip test and determined band intensity with a set of calibrated controls to create a standard curve. This standard curve could then be used to determine the LAM concentration in clinical samples by extrapolating band intensity. The results from these efforts are reported in the next section.

3.4 Standard curve development outcomes.

The standard curve for LAM concentrations was developed using the Alere Determine TB LAM Ag test strips, as described in section 2.3.6. Three sets of standards, consisting of control urine spiked with varying amounts of LAM (starting with neat LAM at 500 µg/ml, a 10-fold dilution series was performed), were applied to the test strips. The band intensity was observed to decrease with progressively lower amounts of LAM applied to the strips. The results of this experiment are presented in Figure 23.

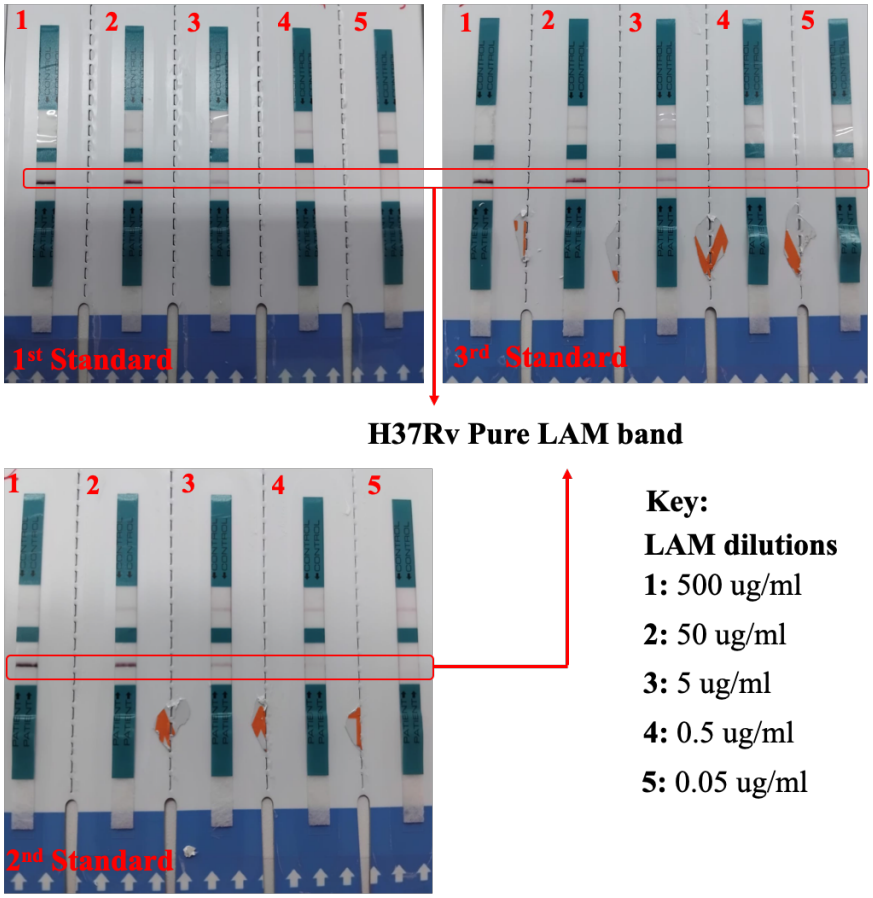


Figure 23. Control urine LAM standards. Shown are three replicates for the dilutions of LAM used on strip tests. The red text indicates number of replicates and concentration of LAM used. The rectangular selection shows the position of the band of the LAM.

After running the strip tests with known concentrations of LAM, the Fiji software was used to quantitate LAM and plot a standard curve where density/intensity of the band was plotted against the concentration of LAM spotted on the strips. The standard curve, shown in figure 24, displayed a good linear relationship with LAM concentration and could be used to infer the concentration of LAM in clinical samples.

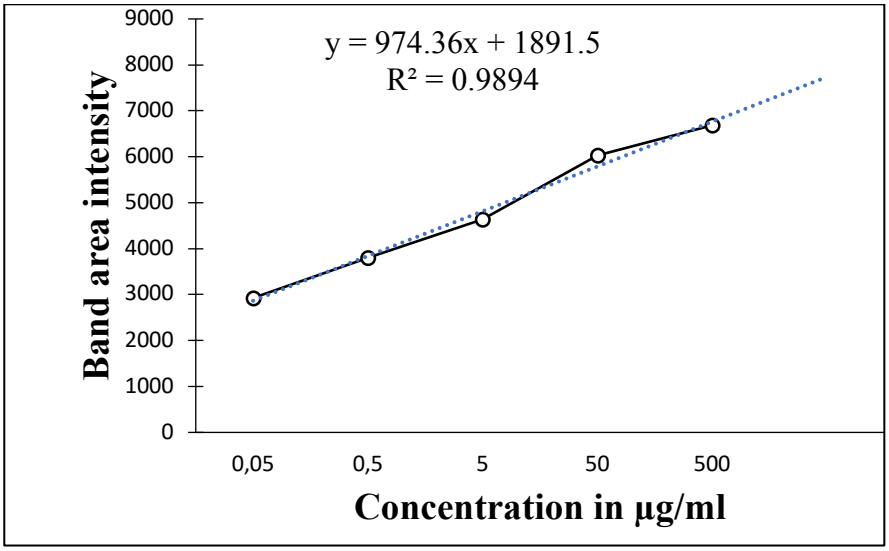


Figure 24. Standard curve derived from the testing of control urine LAM standards on the rapid urine LAM test.

3.5 Rapid urine LAM testing on clinical samples outcomes.

A total of 140 participants were enrolled in the study, with a mean age of 38.86 years (SD = ±13.4 years), and 54 participants (43.90%) were female (see Table 2). At baseline, the average body mass index (BMI) was 19.64 kg/m² (SD = ±4.70 kg/m²). Regarding TB-related symptoms, 62 participants (50.41%) exhibited approximately 4-7% of TB-related symptoms, 54 participants (43.90%) displayed 1-3% of symptoms, and 7 participants (5.69%) reported no TB-related symptoms. HIV status was assessed in 135 participants, of whom 98 (72%) tested positive for HIV. Among these, the median count of the CD4 cells was 177 cells/mm³ (IQR 75–519 cells/mm³). Of the HIV-positive individuals, 47 participants had a CD4 count of <100 cells/mm³, while 39 participants had a CD4 count >100 cells/mm³ and 12 participants had missing CD4 count data. Participants who tested positive for rapid urine LAM at baseline exhibited a considerably reduced median CD4 count compared to those who tested negative for rapid urine LAM (Table 2).

Additionally, of the 140 Cohort A participants 135 participants' sputum specimens (96.43%) were assessed using MGIT culture to confirm PTB as part of the original SOHOT study. In total 316 urine samples were analysed using the rapid LAM antigen assay., of which the baseline visit, 132/140 participants were tested for urinary LAM: 95 participants (72%) tested positive for the urinary LAM, 37 were negative, and 8 had no baseline sample available. When

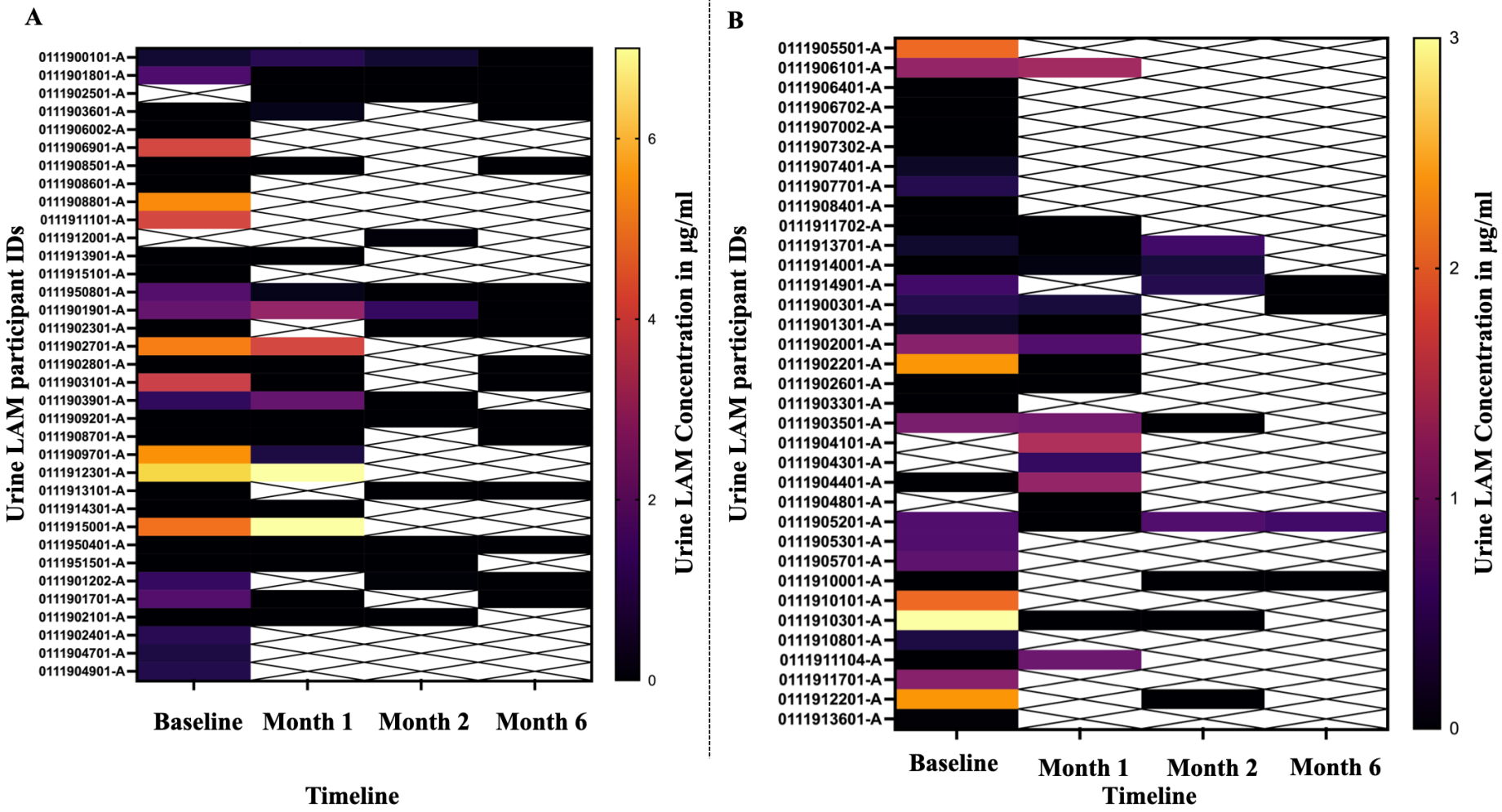
compared to sputum culture, the urine LAM assay demonstrated a sensitivity of, 72.22% (95% CI: 61.88%–82.57%) and 25.81% (95% CI: 10.40%–41.21%).

Table 2: Demographic overview of the study population

Variables	Overall	LAM positive	LAM negative	P Value
Age in years				
Mean age(SD)	38.86(13.398)	38.13(12.54)	40.97(15.13)	0.29
Median age (IQR)	35(29-49.5)	35.5(29-47)	36(27-55)	0.39
Body Mass Index				
Mean BMI(SD)	19.64 (4.70)	19.85(4.94)	19.28(4.0)	0.55
Median BMI(IQR)	18.64(16.34-21.95)	18.88(16.64-22.05)	19.11(16.14-22.05)	0.91
Gender				
Female (%)	54(43.90)	39(44.32)	15(42.86)	0.88
Male (%)	69(56.10)	49(55.68)	20(57.14)	
Symptoms				
Fever %	47 (38.21)	35 (39.77)	12 (34.29)	0.5720
Cough %	100 (81.30)	72 (81.82)	28 (80.00)	0.8155
Cough blood %	7 (7.00)	6 (8.33)	1 (3.57)	0.4020
Weight loss %	93 (76.86)	66 (76.74)	27 (77.14)	0.9624
Fatigue %	55 (45.08)	38 (43.68)	17 (48.57)	0.6232
Night sweats	72 (58.54)	49 (55.68)	23 (65.71)	0.3082
Chest pains	49 (39.84)	36 (40.91)	13 (37.14)	0.7003
Number of TB symptoms				
0 %	7 (5.69)	6 (6.82%)	1 (2.86)	0.6957
1-3 %	54 (43.90%)	38 (43.18)	16 (45.71)	
4-7%	62 (50.40%)	44 (50.00)	18 (51.43)	
HIV status				
Negative (%)	35(29.17)	27(31.76)	8(22.86)	0.34
Positive (%)	85(70.83)	58(68.24)	27(77.14)	
CD4 count				
Mean	319.06(311.94)	310.83(296.67)	411.31(358.52)	0.81
Median(IQR)	177(75-519)	195(76.5-498)	299(137-638)	0.21
Culture results				
Negative (%)	28(27.18)	20(27.78)	8(25.81)	0.84
Positive (%)	75(72.82)	52(72.22)	23(74.19)	
Sensitivity and Specificity for urine LAM by culture				
Sensitivity %	**Specificity %**	**Positive predicted value %**	**Negative predicted value %**	
72.22 (61.88-82.57)	25.81(10.40-41.21)	69.33(58.90-79.77)	28.57(11.84-45.30)	

A heat map was used to represent the rapid urine LAM results based on the band intensity of each test strip as shown in Figure 25A-D. In the heatmap, each cell corresponds to the relative strength of the LAM antigen detected in each urine sample. Darker colours (black to dark purple) indicating low levels of LAM and the lighter colours (orange to yellow) indicating high levels of the LAM antigen. At baseline, most urine samples displayed lighter shades, consistent with higher LAM concentrations prior to treatment initiation. Over time, a progressive shift towards darker shades was observed, particularly by the 6-month visit, reflecting the expected reduction in urinary LAM concentration as TB treatment took effect.

Urine samples that were visibly concentrated (dark yellow in appearance) were tested using LAM test strips. In these cases, the strips developed a yellow background (see Appendix C), which interfered with the analysis. Although most of these samples tested positive for LAM at a grade 1 score (indicating low LAM concentration), the yellow discoloration caused the Fiji software to overestimate the true LAM levels. This effect is visible in the heat map (Figure 26), where many baseline samples appear as green to yellow, indicating artificially high LAM levels, similar to the trend observed in Figure 25. As treatment progressed, the colours gradually shifted to dark purple and black, reflecting the expected decline in LAM concentration. The colour gradient in the heat map therefore illustrates how urine appearance (degree of concentration) can influence the accuracy of LAM measurement, particularly in darker, concentrated urine samples.


A
Urine LAM participant IDs
0111900101-A
0111901801-A
0111902501-A
0111903601-A
0111906002-A
0111906901-A
0111908501-A
0111908601-A
0111908801-A
0111911101-A
0111912001-A
0111913901-A
0111915101-A
0111950801-A
0111901901-A
0111902301-A
0111902701-A
0111902801-A
0111903101-A
0111903901-A
0111909201-A
0111908701-A
0111909701-A
0111912301-A
0111913101-A
0111914301-A
0111915001-A
0111950401-A
0111951501-A
0111901202-A
0111901701-A
0111902101-A
0111902401-A
0111904701-A
0111904901-A
Baseline
Month 1
Month 2
Month 6
Timeline
Urine LAM Concentration in µg/ml
0
2
4
6
B
Urine LAM participant IDs
0111905501-A
0111906101-A
0111906401-A
0111906702-A
0111907002-A
0111907302-A
0111907401-A
0111907701-A
0111908401-A
0111911702-A
0111913701-A
0111914001-A
0111914901-A
0111900301-A
0111901301-A
0111902001-A
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0111903501-A
0111904101-A
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0111904801-A
0111905201-A
0111905301-A
0111905701-A
0111910001-A
0111910101-A
0111910301-A
0111910801-A
0111911104-A
0111911701-A
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Baseline
Month 1
Month 2
Month 6
Timeline
Urine LAM Concentration in µg/ml
0
1
2
3

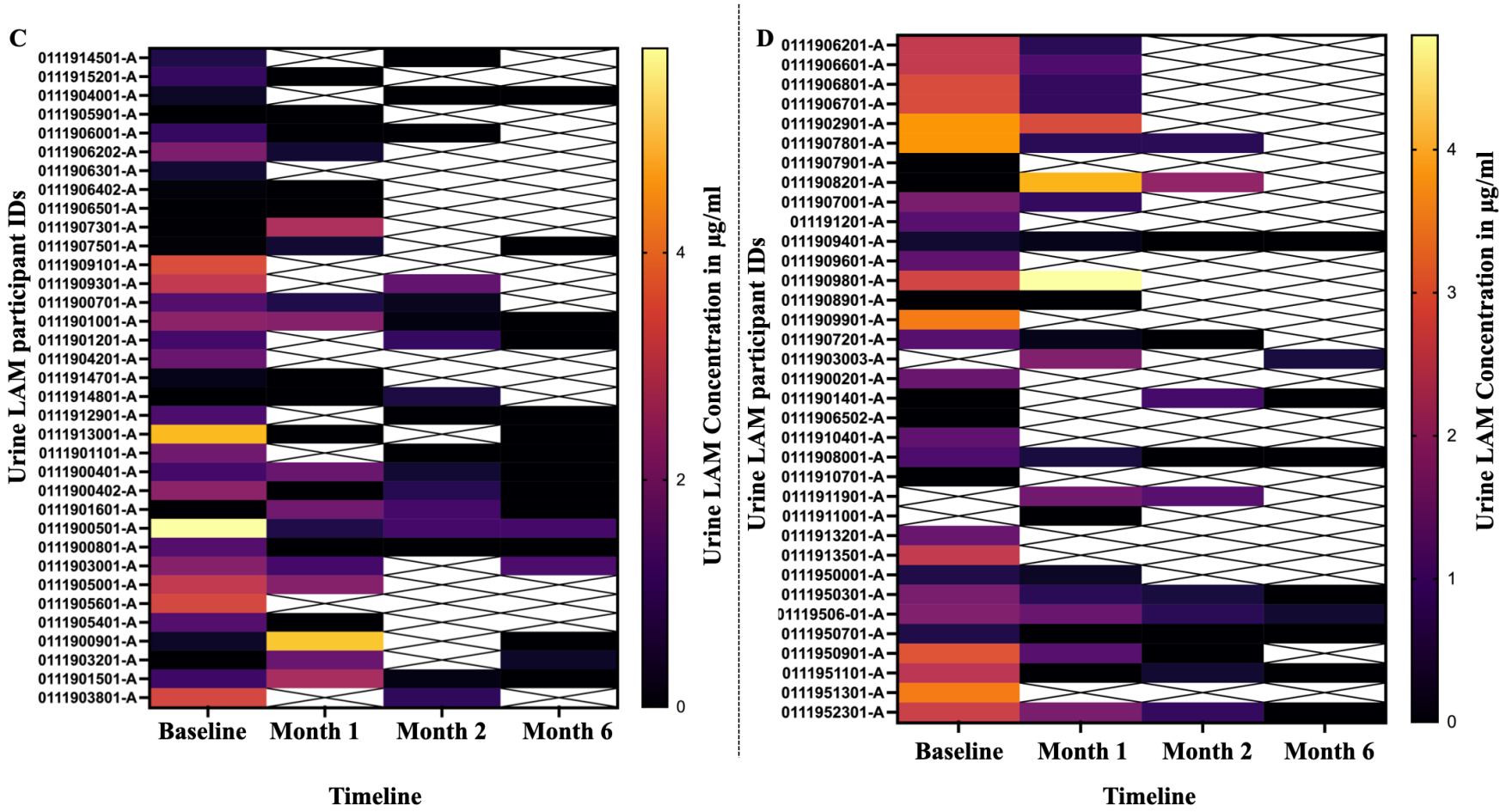


Figure 25. Heatmap representing the concentration of LAM in urine clinical samples, concentration across samples analyzed using the Fiji Software. (A) represents samples 1-35, (B) represents samples 36-70, (C) represents samples 71-105 and (D), represents samples 106-140. The intensity of the color corresponds to the relative concentration of LAM, with lighter shades indicating higher concentrations and darker shadesindicating lower concentrations. The scale ranges from 0 which is the lowest concentration to 6 which is the highest concentration. Indicative of the highest detected LAM concentration). The "X" in some of the cells denotes the absence of a sample for a specific visit.

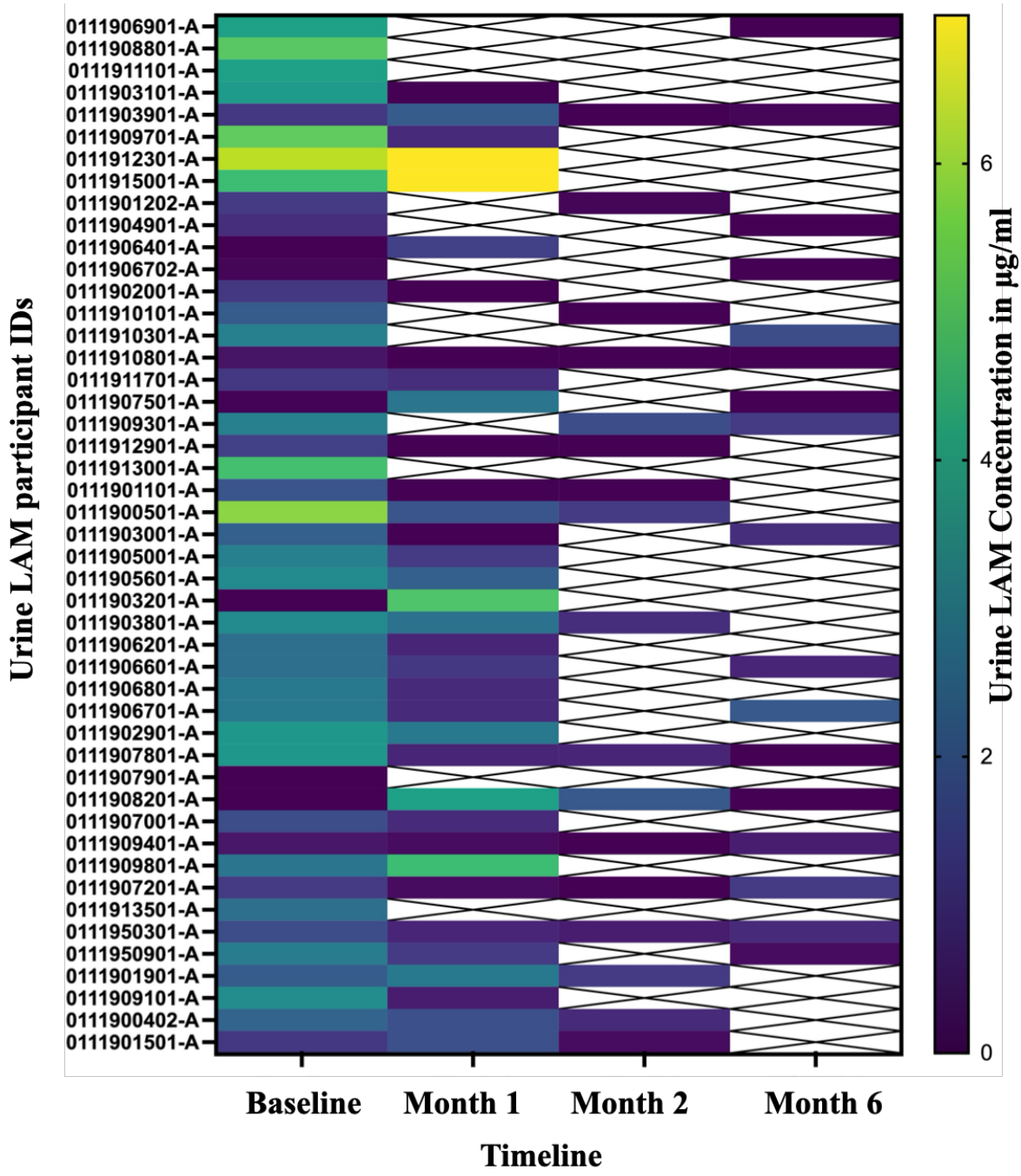


Figure 26. Heatmap representing the LAM concentrations in urine samples , with a focus on samples exhibiting high urine concentration that caused a color change in the test strip. In this heatmap, lighter shades correspond to higher LAM concentrations, while darker shades indicate lower concentrations. The "X" in some of the cells denotes the absence of a sample for a specific visit.

Throughout the duration of receiving TB therapy, the proportion of participants testing positive for urine LAM significantly decreased between the baseline and the month 2 follow-up visits (72% to 21.9%) and the decrease was also observed between the baseline and the month 6 visits

(72 % to 4.5%). At baseline, 30 of the 95 (31.57%) LAM-positive participants converted to being negative at the month 2 visit (p = 0.0106). In this context, testing positive refers to the presence of the LAM antigen in the sample, whereas testing negative refers to its absence. Consequently 29 of the 95 (30.5%) paticipants who had a positive urinary LAM test result at the baseline visit converted to being negative for urinary LAM at month 6 visit of receiving TB therapy (p = <0.0001). The one participant, that was initially negative on both the rapid LAM test and MGIT culture at the baseline visit, became positive on rapid LAM at the visit for month 2. After receiving TB treatment for 6 months, there were only four participants that remained positive for urinary LAM. See table 3 below.

Table 3: Results of the rapid urine LAM test over the 6-month period of receiving TB treatment.

	Baseline N=132	**Month 2 N= 52**	**p Value (Baseline vs Month 2)**	**Month 6 N=46**	**P Value (Baseline vs Month 6)**
Rapid urine LAM positive					
All participants	95	28*	0.0106	7 ⌘	<0.0001
HIV positive	65	17	0.0204	3	<0.0001
HIV positive + CD4 ≤ 100 cell/ mm³	23	5	0.0025	2	<0.0001
HIV positive + CD4 ≥ 100 cell/ mm³	34	12	0.6	1	<0.0001
Rapid urine LAM grade	Mean	Mean		Mean	
All participants	0.9924	0.6078	0.0113	0.1333	<0.0001
HIV positive	1.516	0.68	0.0004	0.1429	<0.0002
HIV positive + CD4 ≤ 100 cell/ mm³	0.7933	0.2409	0.2148	0.03333	0.0014
HIV positive + CD4 ≥ 100 cell/ mm³	1.556	0.5833●	0.02229	0.1818	0.0014
Rapid urine LAM concentration	Mean	Mean		Mean	
All participants	1.341	0.3783	<0.0001	0.09565	<0.0001
HIV positive	1.738	0.9226	0.0818	0.22364	<0.0001
HIV positive + CD4 ≤ 100 cell/ mm³	1.476	0.4077	0.0253	0.1455	0.0025
HIV positive + CD4 ≥ 100 cell/ mm³	0.9733	0.2409	0.009	0.03333	0.01
*A culture-negative participant with a negative baseline rapid urinary LAM result tested positive at the 2-month follow-up visit					
⌘A Culture-positive participant who had a negative baseline rapid urinary LAM result subsequently tested positive at the 6-month follow-up visit.					
● A culture-positive participant who had a baseline rapid urinary LAM result of 1+ demonstrated an increase to a 2+ grade at the 2-month follow-up visit. This participant initially had a negative LAM result at baseline					

The proportion of positive samples decreased, from 72% at baseline to 15.2% at month 6 and the proportion of the negative samples on the rapid urine LAM assay increased from 28% at baseline to 84.78% at month 6 (Figure 27).

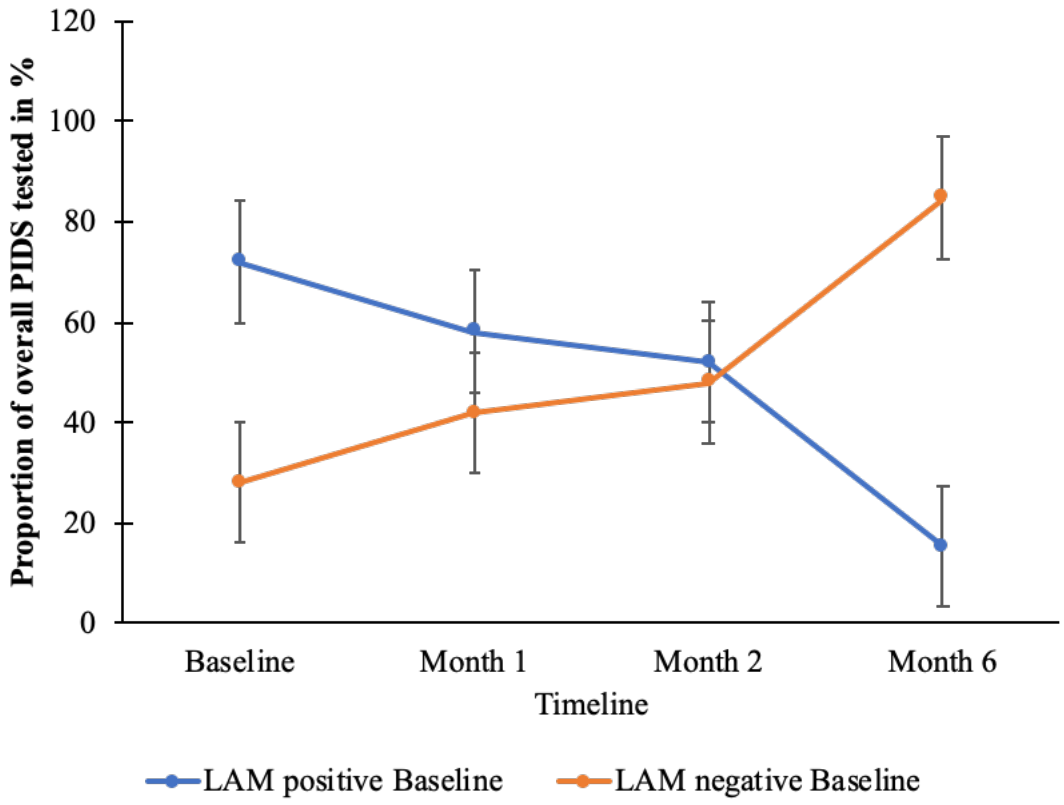


Figure 27. The binary outcomes of LAM testing, where participants were classified as either positive or negative for LAM. The proportions represent the percentage of participants who tested positive versus negative during the 6 months of taking anti-tubercular treatment.

Subsequently the total urinary LAM grade (grade in this context refers to the level of LAM antigen detected in the sample, based on the intensity of the test line) and LAM concentration decreased between the 6 months duration of receiving TB treatment . The total average urine LAM test grade decreased between the baseline and month 2 visit from (grade 1.0 at baseline to grade 0.60 at 2 months), and further to 0.13 at the 6-month visit ($P = 0.0113$) and ($p < 0.0001$) respectively. The total average urine LAM concentration exhibited a decrease from 1.34 µg/ml at the baseline visit to 0.38 µg/ml at the month 2 visit and further down to 0.09µg/ml at month 6 visit (both $p < 0.0001$). see Figure 28

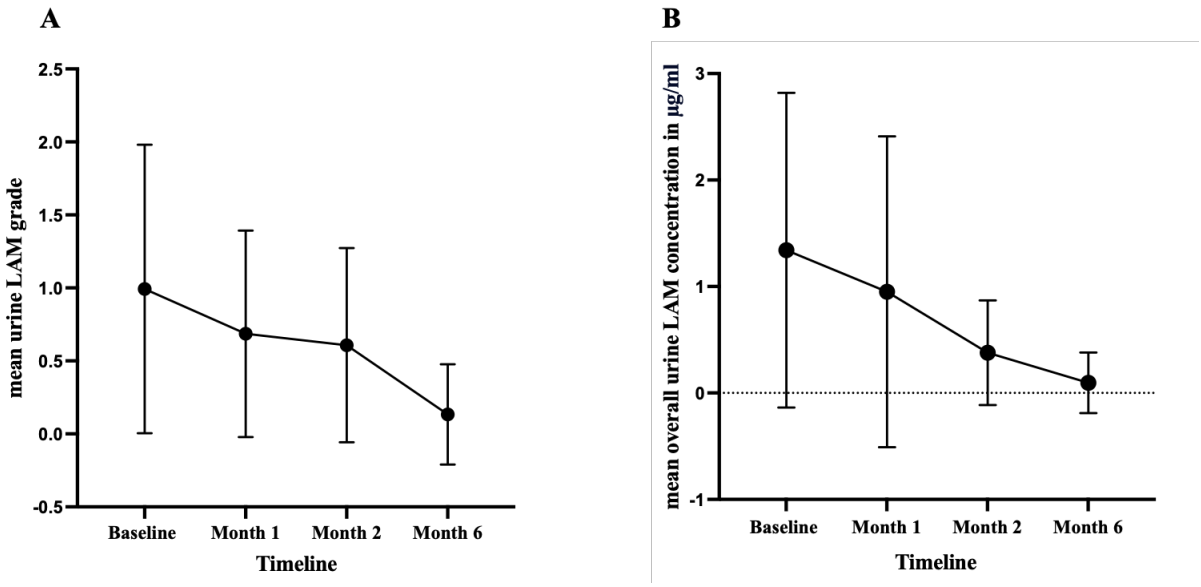


Figure 28. Mean rapid urine LAM concentration and grading Over a 6-month period for participants that tested LAM positive. The concentrations were derived from the developed standard curve. (**A)** Depicts a decrease in the overall mean LAM grading, (**B)** depicts the reduction in the overall mean LAM concentration (µg/ml) over time.

Comparable results were observed in the PLWH cohort, including when categorised based on CD4 count above or below 100 cells/mm³ (Figure 29). Amongst this group, there was one individual with a CD4 count of 3 but the LAM test and culture were negative at baseline, culture was contaminated but was LAM negative at month 2. In those participants that had a positive LAM result at the baseline visit , the average urine LAM grade decreased from 1.9 initially at the baseline visit to 0.4 at the month 2 visit, and further down to 0.2 at the month 6 visit see (Figure 29A). Subsequently the urine LAM concentration from the same group decreased (Figure 29B). Among participants who were negative for the urinary LAM at baseline, no significant change in the urinary LAM grade nor concentration was observed over the 6 months of being on the TB treatment. However, there were five participants that had a urine LAM test grade of 1+ at the month 2 visit, and one participant that had a score grade 1+ at the month 6 visit.

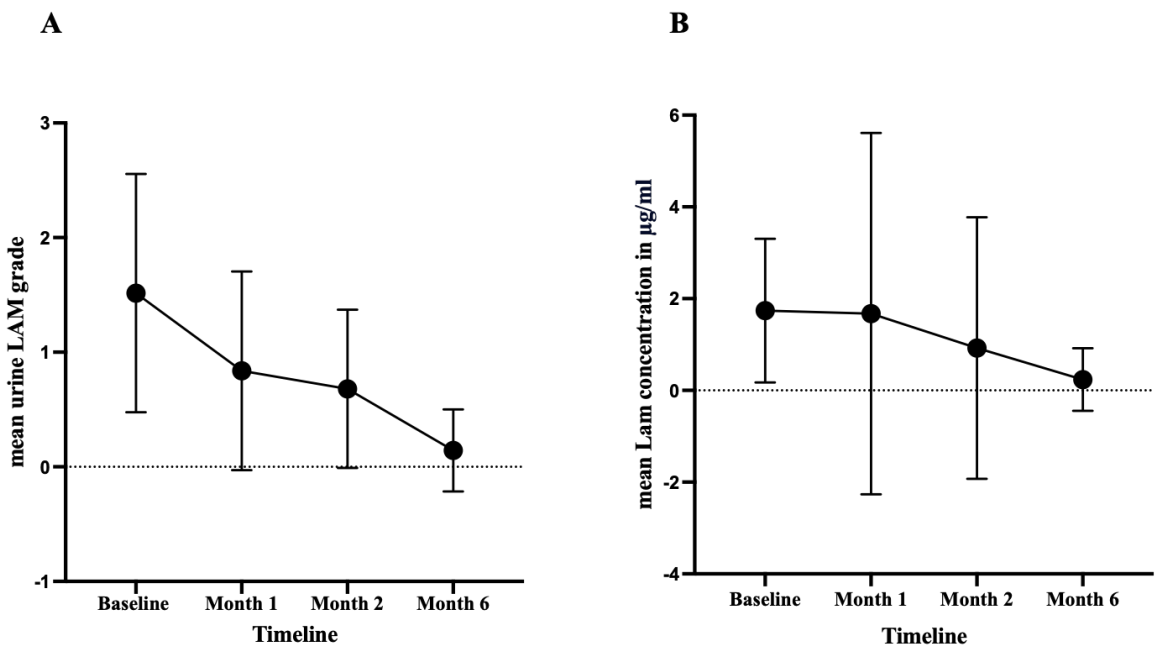


Figure 29. Overall mean rapid urine LAM concentration and grading over 6 months period on the PLWH group. (A) Depicts the decrease in the mean overall LAM grading. (B) Depicts the decrease in the mean overall concentration in ug/ml during the period of 6 month of taking anti-tubercular treatment.

Among the participants that had a positive result for urinary LAM at the baseline visit , a decrease was observed in the average urine LAM grade from 1.4 to 0.6 at the month 2 visit and further down to 0.1 at the month 6 visit (Figure 30A). Similar changes were observed for the mean urine LAM concentration (Figure 30B). Within this cohort, just two participants who had a positive result for urinary LAM at the baseline visit experienced an increase in the grading of the urinary LAM grade during the period of receiving TB therapy (grade 1 increased to grade 2 at the month 2 visit); these participants had negative results for urinary LAM at the month 6 visit. Among the participants that test negative of urinary LAM at the baseline visit , there was no significant observations in the LAM grade during 6 months of being on TB treatment. However, there were five participants that had a grading of 1+ at the month 2 visit, and one participant had a grading of 1+ at the month 6 visit.

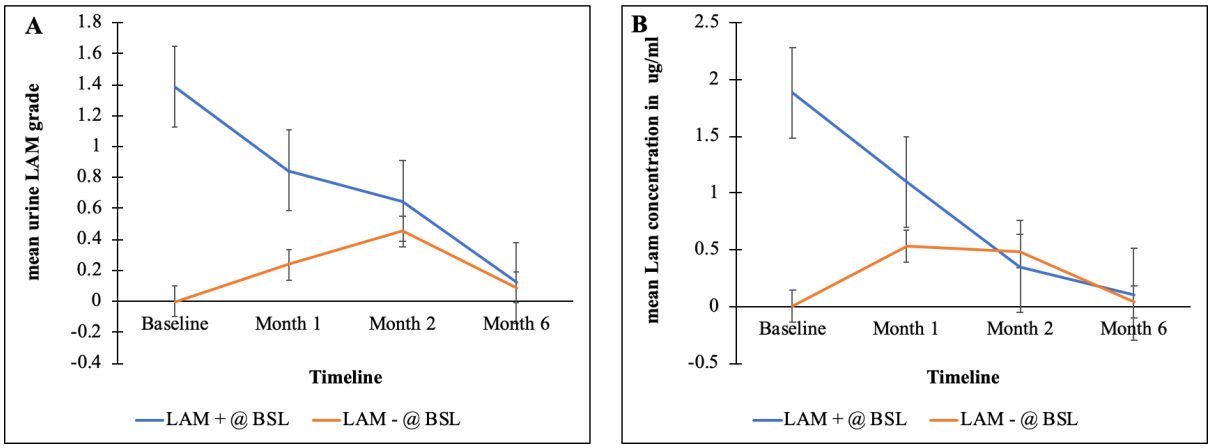


Figure 30. Urinary LAM changes in the grade and concentration between the six months period of receiving TB treatment for participants with positive and negative outcomes at the baseline visit. 95% confidence intervals (CIs) are displayed by error bars.(A) represents the decrease in average urine LAM grade, (B) represents decrease in the average LAM concentration over the 6 months period.

Among patients with culture-confirmed pulmonary tuberculosis, both the overall mean urine LAM concentration and LAM grading decreased over the 6-month study period, as shown in Figure 31A-B**.** At the baseline visit, those participants who were MGIT culture-positive, the proportion of those testing positive for urinary LAM, declined from 63% at the baseline visit to 39% at the month 2 visit, and further to 14% at the month 6 follow-up visit. Additionally, in this group, there was a decrease observed in the average LAM grade from 1.5 at the baseline visit to 0.6 at the month 2 visit**,** and further down to 0.2 at 6 months 6 visit **(**Figure 31A**).** Similarly, the average urine LAM concentration dropped from 1.8 µg/ml at baseline to 0.3 µg/ml at 2 months**,** and further to 0.08 µg/ml at 6 months **(**Figure 31B). These findings indicate a progressive decline in urine LAM levels among the MGIT culture-confirmed TB patients over time.

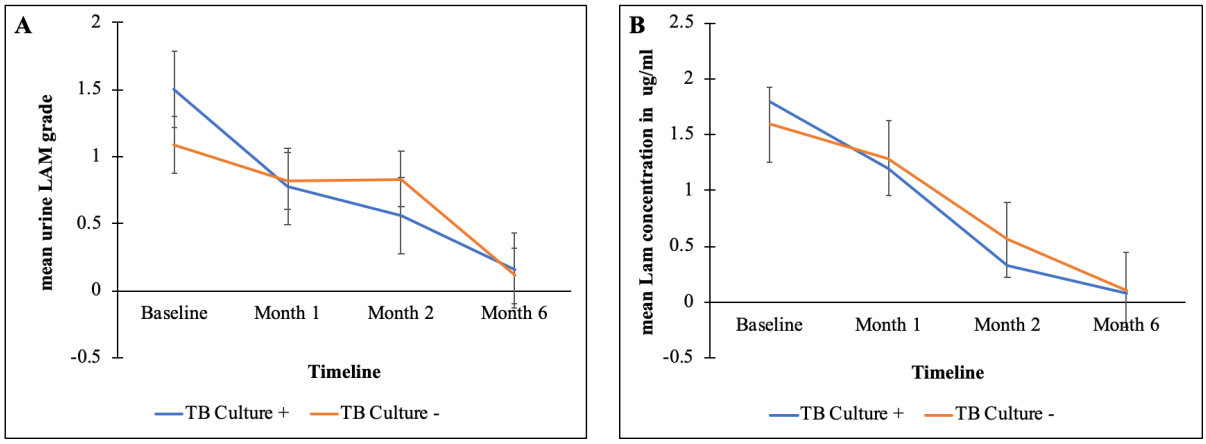


Figure 31. Average urine LAM grade and concentration between the six months period of receiving TB therapy amongst participants with MGIT culture results that were positive and negative at baseline visit for pulmonary TB. Error bars indicate the 95% Cls.

3.6 Statistical analysis

Baseline characteristics were compared between rapid urine LAM positive and negative participants using Fisher's exact test. Urine LAM test results at follow-up visits were compared with baseline results in terms of the percentage of participants testing LAM-positive, mean LAM grade, and mean LAM concentration, using McNemar's test for paired binary outcomes and paired Student's t-tests for continuous outcomes; all analyses were performed in Prism 10 software

3.7 Rapid plasma LAM outcomes.

In addition to the urine samples tested, 23 participants were tested for LAM detection in plasma samples using the rapid strip test. Only 14 (60.9%) participants tested positive for LAM at baseline, for both month 1 and month 2, only 8 samples were tested for each visit and all samples were positive for LAM (see Figure 32). There were no follow-up samples for the month 6 visit due to patients being lost to follow-up. There was also no significant changes observed in the mean LAM grading in the plasma samples. The overall mean plasma grading for baseline visit was grade 1.133, grade 1.125 for month 1 and grade 1.200 for month 2 respectively.

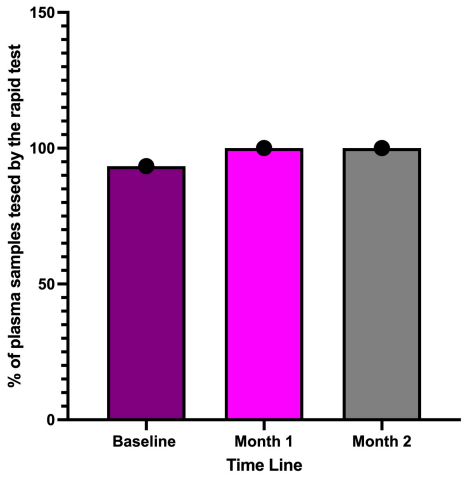


Figure 32. Proportion of positive plasma samples tested using the rapid Lateral Flow Assay.

LAM detection was performed on both plasma and urine samples. In samples that tested positive for LAM, a comparison of the grading scores revealed that, for the same patient, the urine sample had higher grading, while the plasma sample had a lower grading, see highlighted section on table 4. These results are suggestive that the concentration or level of LAM in plasma is lower than in urine for the same individual.

Table 4: Results of the rapid plasma LAM test over the 6-month period of receiving TB treatment.

Subject ID	HIV Status	Urine_LAM Baseline	Plasma LAM Baseline	Urine_LAM Month 1	Plasma LAM Month 1	Urine LAM Month 2	Plasma LAM Month 2
119-01-A	Negative	No sample	No sample	Positive (1+)	No sample	Positive (1+)	Positive (1+)
120-01-A	Positive	No sample	No sample	No sample	Positive (2+)	Positive (1+)	No sample
130-01-A	Negative	Positive (1+)	Positive (1+)	No sample	No sample	No sample	No sample
132-01-A	Negative	Positive (1+)	Positive (1+)	No sample	No sample	No sample	No sample
509-01-A	Positive	Positive (1+)	Positive (1+)	Positive (1+)	No sample	Positive (1+)	No sample
511-01-A	Positive	Positive (1+)	Positive (1+)	Negative	No sample	Negative	Positive (1+)
513-01-A	Positive	Positive (4+)	Negative	No sample	No sample	No sample	No sample
515-01-A	Positive	Negative	Positive (4+)	Negative	Positive (1+)	Negative	No sample
111-01-A	Positive	Positive (2+)	Positive (1+)	No sample	No sample	No sample	No sample
092-01-A	Positive	Negative	No sample	Negative	No sample	Negative	positive (2+)
506-01-A	Positive	Positive (2+)	Positive (1+)	positive (2+)	Positive (1+)	posititve (3+)	No sample
110-01-A	Negative	No sample	Positive (1+)	Positive (1+)	Positive (1+)	No sample	Positive (1+)
108-01-A	Positive	Positive (1+)	Positive (1+)	Negative	Positive (1+)	Negative	Positive (1+)
107-01-A	Negative	Negative	Positive (1+)	No sample	No sample	No sample	No sample
103-01-A	Positive	Positive (1+)	Positive (1+)	No sample	Positive (1+)	No sample	No sample
508-01-A	Not done	Positive (1+)	Positive (1+)	Positive (1+)	Positive (1+)	Positive (1+)	Positive (1+)
507-01-A	Positive	Positive (1+)	No sample	Negative	No sample	Negative	Positive (1+)
092-01-A	Positive	Negative	No sample	Negative	No sample	Negative	Positive (1+)
091-01-A	Positive	Positve (2+)	No sample	Positve (1+)	No sample	No sample	positive (2+)
104-01-A	Negative	Positive (1+)	No sample	No sample	No sample	No sample	Positive (1+)
143-01-A	Positive	Negative	Positive (1+)	Positve (1+)	No sample	No sample	No sample
145-01-A	Positive	Positive (4+)	Positive (1+)	No sample	No sample	Negative	No sample
503-01-A	Positive	Positive (1+)	No sample	Positive (1+)	Positive (1+)	Positive (1+)	No sample

3.8 Correlation amongst methods used for TB testing.

The study aimed to compare urine LAM testing outcomes with MGIT culture and GeneXpert outcomes on sputum samples that was performed as part of the original SOHOT study. The comparison outocmes are presented using a Venn diagram to illustrate the overlap. Among the tested samples, 85 were positive by MGIT culture, 95 by urine LAM, and 37 by GeneXpert. Only 7 samples tested positive across all three methods, while 52 were detected by both MGIT and urine LAM, 9 by urine LAM and GeneXpert, and 11 by MGIT and GeneXpert, see Figure 33. These results highlight the variability in detection among the different diagnostic approaches.

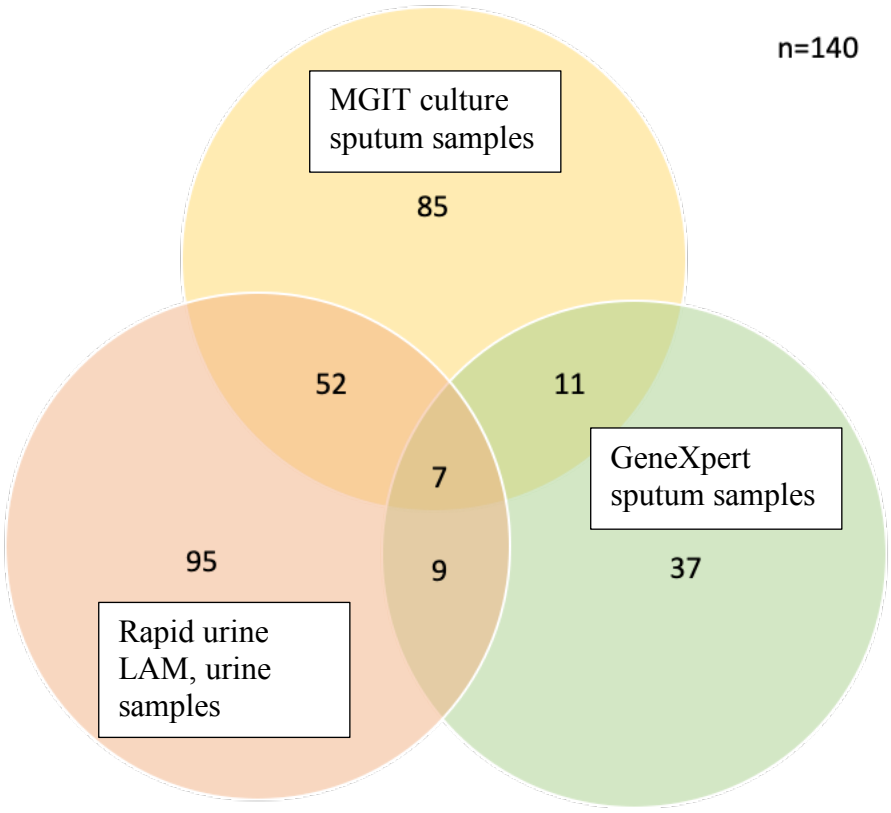


Figure 33. Venn diagram showing the overlap between MGIT culture, urine LAM, and GeneXpert testing for the baseline visit.

The results suggest that no single diagnostic method is sufficient on its own for detecting (TB), as there is significant variability in detection across MGIT culture, urine LAM, and GeneXpert. The low overlap between all three methods **(**only 7 samples tested positive by all**)** indicates that each test identifies different subsets of TB cases.

Futhermore the time to positivity for those positive MGIT cultures and urine LAM samples was compared to the urine LAM concentrations. As hypothesized the comparison between the time to positivity in days for the MGIT cultures and the urine LAM concentrations revealed an inverse relationship over time. As the urine LAM concentration decreased, as determined by the band intensity analysis of the test strips using Fiji software, the for the MGIT cultures increased. This trend suggests that as the bacterial load and LAM levels decline during treatment, the time required for the MGIT cultures to test positive also becomes longer see Figure 34. This indicates that lower LAM concentrations, which correspond to redu time to positivity ced bacterial activity, may be associated with a delayed time to positivity in MGIT cultures.

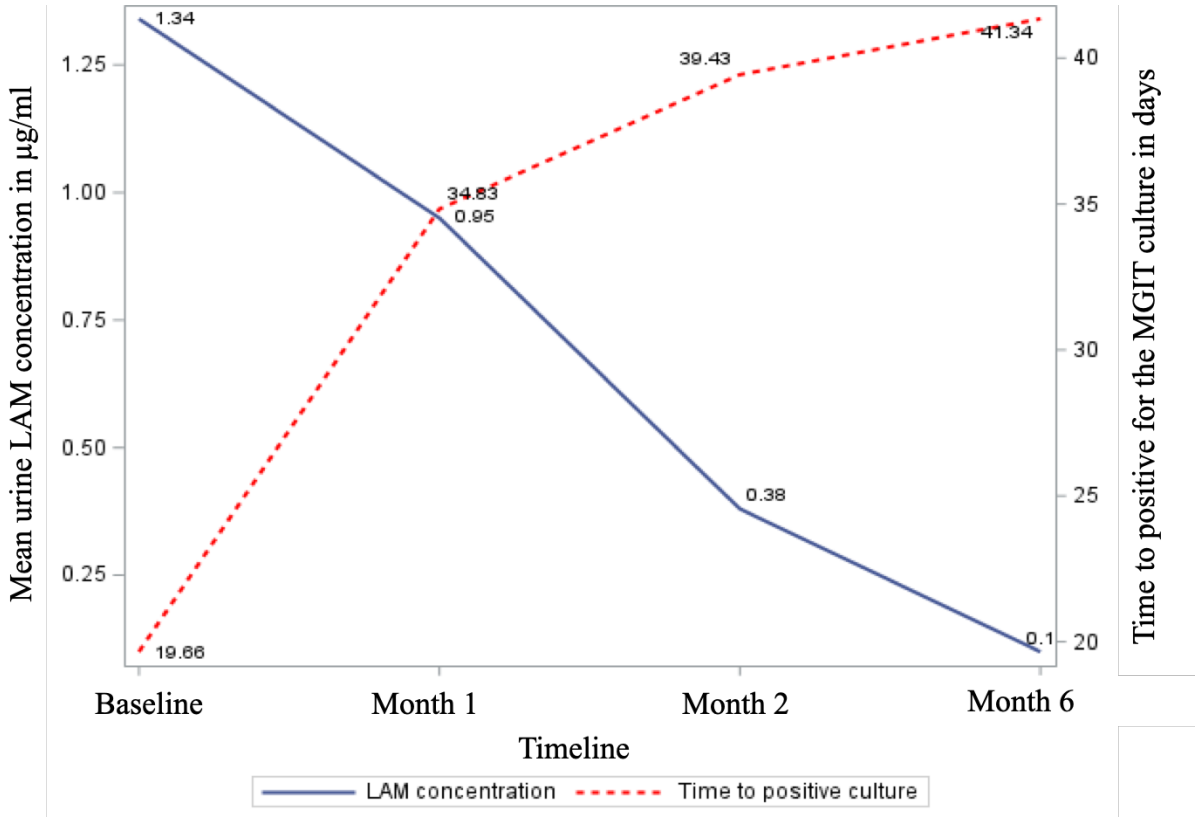


Figure 34. Comparison of time to positivity (in days) for MGIT cultures and urine LAM concentrations over time. The graph shows the inverse relationship between the two variables, with decreasing urine LAM concentrations (measured through band intensity analysis using Fiji software) associated with an increase in the time to positivity for MGIT culture.

4 Discussion

This study aimed to detect and quantify LAM in urine and plasma samples and evaluate its potential for monitoring TB treatment response. Objective 1 was partially met, with the rapid POC test successfully detecting and grading LAM in urine, but the ELISA could not quantify LAM, highlighting challenges in assay development. Objective 2 was not achieved as LAM could not be quantified in plasma using ELISA, though the POC test was effective in grading plasma LAM. The difference in LAM concentrations between urine and plasma suggests that urine may provide higher sensitivity for LAM detection. Objective 3 could not be assessed due to the lack of reliable ELISA data, but comparisons with MGIT culture and GeneXpert indicated that LAM detection in urine complements these methods in diagnosing TB. Objective 4 suggested that plasma could serve as a proxy for urine in cases of renal dysfunction, although this remains unconfirmed. Objective 5 was not fully met, but the Venn diagram indicated that urine LAM detection could be valuable for monitoring treatment alongside other diagnostic methods. The time to posive for the MGIT cultures was correlated to the LAM concentrations that were obtained by assessment on the Fiji software. As the concentration decreases the time to positivity by MGIT culture also increase during the course of taking TB treatment. Further below methods attempted in the development of the ELISA are discussed.

4.1 SDS PAGE GEL

In this study, SDS-PAGE was used to assess the integrity of the LAM antigen and four monoclonal anti-LAM antibodies before performing the indirect ELISA. Coomassie Blue staining revealed that while the antibodies were clearly visible, LAM itself did not stain, which can be attributed to LAM's unique glycolipid structure that does not bind well to Coomassie Blue, a stain designed for proteins (171). This is consistent with the known difficulty of staining glycolipids with Coomassie Blue. In contrast, when the LAM antigen was stained with periodic silver stain, it showed successful staining, indicating the presence and integrity of the glycolipid. Silver staining, known for its high sensitivity to carbohydrates and glycolipids, proved to be more effective for detecting LAM, aligning with its known ability to interact with carbohydrate moieties (172).

4.2 Dot blot assay

A Dot Blot assay was conducted to assess the binding of LAM antigen to specific anti-LAM monoclonal antibodies. The assay was initially performed on a PVDF membrane using primary antibodies diluted to 1:100, and a secondary anti-mouse antibody at 1:10 000. However, no binding was detected under these conditions. The experiment was repeated with increased primary antibody dilutions (1:1000 and 1:10 000), while maintaining the secondary antibody dilution at 1:10 000, but again, no binding was observed. Subsequently, the assay was performed on a nitrocellulose membrane using the same antibody concentrations, but no binding signals were observed.

Both PVDF and nitrocellulose membranes are commonly used in Western and Dot Blot assays, with their protein-binding characteristics influencing assay outcomes. Nitrocellulose membranes, with high protein-binding capacity, may still yield poor results if antigen-antibody interactions are weak or if membranes are improperly pre-treated or activated. In contrast, PVDF membranes, known for superior protein retention, may exhibit high background noise if the blocking step is insufficient or if non-specific binding occurs (173).

Additionally, factors such as conformational changes in the antigen, epitope accessibility, or poor solubility may have hindered the antigen-antibody interaction. The use of an internal control protein in the Dot Blot failed to produce binding, suggesting that the issue might lie with the experimental setup itself rather than the specific interaction between LAM and the antibodies. This could indicate problems with antigen preparation, blocking procedures, or antibody recognition capacity, potentially contributing to non-specific binding and obscuring the expected signal.

In an effort to optimize conditions, the experimental design was revised. A single monoclonal antibody (CS-35) specific to LAM, produced in mice, was paired with a secondary anti-mouse antibody produced in sheep. The secondary antibody dilution was reduced to 1:2500. This modification successfully resulted in the detection of binding, with clear signals observed on the nitrocellulose membrane. This prompted further efforts to develop an ELISA to quantify LAM antigen levels.

4.3 Indirect Elisa

The indirect ELISA resulted in fluctuating OD readings, which is often a sign of non-specific binding. Non-specific binding occurs when molecules other than the intended target antigen (in this contex LAM) bind to the microplate, the antibody, or the enzyme-conjugated secondary antibody, resulting in false positives, inconsistent results, or fluctuating measurements.This could have been due to several reasons including the high binding plates that were used, the blocking steps, and antibody and antigen pairing. Blocking is a crucial step that helps in filling the unoccupied sites with a reagent that doesn't participate in the assay's immunochemical responses (174). It obscures some protein locations and disrupts immunoreactivity, which further raises the background noise and reduces the immunosignal (175). When developing ELISA assays, microplate selection is essential. Negatively charged high-binding surfaces are made to bind to the medium-sized molecules ranging between (10 kDa–20 kDa) via hydrophobic and ionic interactions. In contrast, medium-binding or untreated surfaces are hydrophobic and have the ability to passively absorb bigger molecules with a size of (>20 kDa), like antibodies. Medium-binding microplates can bind up to 500 ng/cm of mouse IgG, whereas high-binding microplates can bind 100–200 ng/cm². While a higher binding capacity may enhance assay performance, it can also increase the risk of non-specific binding, necessitating the use of protein blockers to mitigate this issue. Using anti-mouse primary antibodies alongside anti-human secondary HRP could have also resulted in non-specific binding. Based on repeated attempts, we surmised that development of an ELISA test would not be possible with the reagents we had in hand. To advance the proposed research, we set out to develop a semi-quantitative method of detecting LAM in clinical samples to determine if LAM concentrations can be used to assess the effectiveness of TB therapy treatment.

4.4 Standard curve development

The standard curve could not be developed using the ELISA, however this was developed with the pure LAM dilutions and testing thereof on the rapid urine LAM test, the bands were analyzed on the Fiji software and the curve was developed which yielded an equation which could be extrapolated to semi-quantitate the clinical samples.

4.5 Rapid urine LAM testing

The levels of urine LAM decreased during the 6-month period of taking TB treatment in patients with culture-positive tuberculosis. Assessment on the urinary LAM test provided outcomes within a period of 25 minutes without the use of additional equipment nor specialised reagents, or exceptionally skilled laboratory professionals. The test strips were also photographed and analysed on the Fiji/Image J software which is also easy to download. The bands of each PID were assessed and this could be used to semi quantitate the LAM in the clinical urine sample and also corelate it to the LAM grading. The findings were indicative that the rapid urine LAM assays could serve as a convenient and accessible tool for monitoring the response to anti-TB therapy. Several studies that are hospital-based have documented the diagnostic performance of the urinary LAM test (22,176,177). In a past study of hospitalized HIV-infected TB participants in the Western Cape, the urinary LAM test demonstrated a sensitivity and specificity of 66%, which increased to 90% when a control group without TB was used (22). Among hospitalized HIV-infected individuals in South Africa and Uganda, the rapid urine LAM test showed diagnostic sensitivities ranging from 62% to 63% and specificities between 78% and 88% (177,178). While these cohorts predominantly consisted of ill hospitalized participants, the documentetd diagnostic performance characteristics have been generally consistent across studies. The results suggests that LAM detected in urine could be a useful biomarker for assessing the effectiveness of anti-TB therapy in patients receiving treatment for pulmonary TB. While MGIT culture has traditionally been used for assessing TB treatment and as an alternate marker for approving drugs, its not very useful in treatment failure or disease relapse (179,180). For instance a research study investigating short treatment durations had to be terminated prematurely because participants who converted to negative MGIT culture at 2 months later experienced relapse, underscoring its limitation in assessing long term treatment efficacy (181). Nonetheless, MGIT remains an established reference method for monitoring early bacteriological clearance and continues to serve as a benchmark against which novel biomarkers can be compared. Previous research has evaluated the reduction in viable bacilli in sputum during therapy by using colony counts on agar, time to positivity in liquid culture, or by measuring TB antigen 85 and 85B RNA levels in sputum. However, these methods are not currently suitable for routine, rapid application in clinical settings (182–185).

The use of a point-of-care lateral flow assay to measure a pathogen biomarker in urine offers multiple benefits over traditional sputum microscopy and MGIT culture, such as a reducing

the risk of transmission to healthcare workers. Participants who had a positive urinary LAM test at the baseline visit had most TB-related symptoms and a lower CD4 count levels, all of which are a sign of a higher TB disease burden. However, the observed decrease in urine LAM levels may reflect a change in the relationship between LAM released from degenerating bacilli and the restricted excretion by the kidneys.

A positive result for urine LAM after the intensive phase of TB treatment may warrant more clinical investigations, including the evaluation of drug-resistant TB and/or medication adherence. Given that endogenous TB relapse may arise from the persistence of metabolically less active bacilli located away from cavity sources, urine LAM testing may also be particularly useful for detecting extrapulmonary bacilli (186).

4.6 Rapid plasma LAM.

The observed difference in LAM grading between plasma and urine samples is intriguing and supports the hypothesis that LAM concentrations may vary across different biological fluids. Urine, being a common sample type in TB diagnostics, could provide higher sensitivity for detecting LAM, as it might accumulate higher concentrations of the antigen compared to plasma. The higher LAM concentrations in urine could be attributed to the excretion of the antigen by the kidneys or the fact that urine may act as a more concentrated repository for substances filtered from the blood.

It is also important to consider that plasma and urine are different biological matrices with distinct dynamics in the way they process and excrete substances. Plasma, as part of the circulatory system, represents the antigen in circulation, which could explain the lower concentration observed in plasma compared to urine, where substances may accumulate more readily.

4.7 Correlation by different methods and variables.

The results highlight the potential of urine LAM testing for treatment monitoring due to its ability to reflect changes in bacterial load over time. In this study, decreasing urine LAM concentrations correlated with increasing time to positivity in MGIT cultures. Although MGIT is not a perfect predictor of long-term outcomes, its use here provides a valuable reference point, demonstrating that urinary LAM trends mirror recognised indicators of bacterial clearance. This supports the potential role of urinary LAM as a non-invasive biomarker for

tracking treatment response. Unlike MGIT, which is time-consuming and resource-intensive, urine LAM testing offers a faster and simpler approach, particularly important in resource-limited settings where culture facilities are not readily available.

5. Limitations.

The study had several limitations that could impact the interpretation of its findings. One key limitation was the use of thawed stored urine samples for rapid LAM testing, which may not accurately reflect the performance of the test on fresh urine due to potential degradation or changes in urine composition during storage. This could have affected the quality of the results, particularly the band intensity in the strip test, which may have influenced the accuracy of the Fiji software analysis. Additionally, the use of Fiji software was limited in concentrated urine samples, where the yellow color of the urine interfered with the software's ability to correctly analyse the LAM test bands, possibly leading to misinterpretation of LAM positivity.

The LAM detection using the Determine LAM Ag test also had limitations in terms of their sensitivity and specificity. The LAM test demonstrated promising detection capabilities but lacked sufficient sensitivity and specificity for reliable quantification, which may have been influenced by variations in sample quality or assay conditions. These limitations highlight the need for further optimization of the assays to improve their diagnostic accuracy. Furthermore, the patient cohort, which includes HIV-infected outpatients in a TB-endemic area, may restrict the applicability of the findings to other populations with varying TB incidence rates.

Other study limitations included an insufficient sample size for baseline GeneXpert comparisons, which hindered the ability to detect significant clinical differences. Plasma samples for follow-up visits were also limited, and not all urine samples tested for LAM had corresponding plasma samples, leading to missing data due to patient loss to follow-up. These factors, along with the challenges related to sensitivity and specificity, underscore the need for larger, more diverse cohorts and optimized assay conditions in future studies to enhance the reliability and applicability of LAM detection in TB diagnostics.

6. Conclusion

In conclusion, while not all objectives were fully met, this study provided valuable insights into the potential of LAM detection for both TB diagnosis and treatment monitoring. The rapid POC test showed promising results for detecting LAM in urine and plasma, but further optimization of the ELISA assay is necessary for reliable quantification of LAM. Despite challenges in quantifying LAM in plasma using ELISA, the POC test proved effective in detecting and grading LAM, and future studies should explore the utility of plasma LAM detection as a proxy for urine, particularly in patients with renal complications.

Urine LAM testing could be a useful method for evaluating the effectiveness of anti-TB treatment, especially in resource-limited, HIV-endemic settings. We successfully developed a semi-quantitative approach for detecting LAM based on band intensity scanning and a standard curve, which correlated well with other clinical measures of treatment response. This approach holds promise for larger studies and should be further validated in broader populations.

Additionally, discrepancies observed between LAM concentrations in plasma and urine warrant further investigation, particularly regarding the kinetics of LAM excretion and its diagnostic utility. Exploring the relationship between LAM concentrations in these two fluids throughout the stages of TB disease and treatment could provide deeper insights into its role as a biomarker for disease progression and therapeutic response. Overall, our findings suggest that urine could be a more reliable sample for LAM detection in TB patients, with significant implications for improving diagnostic methods and optimizing the monitoring of TB treatment outcomes.

7. Future work

Future studies should aim to further optimize LAM detection assays to improve their sensitivity and specificity. This could include testing alternative antibody combinations, evaluating advanced platforms such as nanoparticle-based or digital ELISA systems, and refining sample pre-treatment methods to enhance LAM recovery from urine and plasma. Standardizing sample processing and storage conditions would also be valuable for ensuring reproducibility and reliability across different laboratories and settings.

linically, larger longitudinal studies involving diverse patient groups, including those with HIV co-infection, drug-resistant TB, pediatric TB, and extrapulmonary disease, are needed to

validate urine LAM as a biomarker of treatment response. Correlating LAM concentrations with additional biomarkers such as cytokines, C-reactive protein, or cell-free DNA could provide a multi-marker approach with greater predictive value. Furthermore, assessing the performance of urine LAM testing in cases of extrapulmonary or disseminated TB would provide insights into its broader diagnostic utility.

From a public health perspective, future work should investigate the feasibility of integrating rapid urine LAM testing into routine TB treatment monitoring, particularly in resource-limited settings where non-invasive and rapid biomarkers are most needed. Implementation research focusing on cost-effectiveness, usability at point-of-care, and acceptability among healthcare workers and patients will be crucial for determining its potential impact. In addition, alternative non-invasive specimens such as **exhaled breath condensate (EBC)** could be explored for LAM detection, as this sample type may reflect respiratory tract involvement and provide another accessible matrix for biomarker-based monitoring. Finally, mechanistic studies exploring how LAM is released and cleared in the host could advance our understanding of its role as a biomarker and support the development of next-generation assays.

8. Appendices

8.1 Appendix: A List of reagents and solutions

Reagents prepared for the Bradford assay

10% Bovine serum Albumin solution: (5g BSA in 50 ml sterile water)

Reagents used for the SDS PAGE and Dot Blot:

Running Buffer: Dissolve 303 g of Tris, 144 g of glycine, and 10 g of SDS in sterile water, adjusting the final volume to 1 L.

Coomassie Blue Staining Solution: Combine 2 g of Coomassie dye with 400 ml of ethanol and 100 ml of acetic acid. Add sterile water to reach a total volume of 1 L.

De-staining Solution: Mix 400 ml of ethanol and 100 ml of acetic acid, then add sterile water to achieve a final volume of 1 L.

Transfer Buffer: Prepare a solution containing 3 g of Tris, 14.4 g of glycine, 1 g of SDS, and 200 ml of methanol, then adjust the volume to 1 L with sterile water.

Blocking Solution: Dissolve 5 g of non-fat dry milk (Cell Signaling) in TBST, bringing the total volume to 100 ml.

CPS Reagent (Sigma): Chemiluminescent peroxidase substrate.

5% Bovine Serum Albumin (BSA) Solution: Prepare by dissolving 2.5 g of BSA in 50 ml of sterile water.

Tris-Buffered Saline (TBS): Dissolve 1.21 g of Tris and 8.77 g of NaCl in sterile water, adjusting the final volume to 1 L.

Tris-Buffered Saline with Tween (TBS-T): Combine 1.21 g of Tris, 8.77 g of NaCl, and 0.5 ml of Tween 80, then add sterile water to make a final volume of 1 L.

1M Tris-HCl Buffer: Dissolve 60.56 g of Tris in distilled water, adjust the pH to 8.0 using HCl, and bring the total volume to 500 ml.

10% SDS Solution: Dissolve 50 g of sodium dodecyl sulfate (SDS) in distilled water to a final volume of 500 ml.

TAE Buffer: Prepare by dissolving 242 g of Tris base in 100 ml of 0.5 M EDTA and 57.1 ml of glacial acetic acid. Adjust the pH to 8.0 and bring the total volume to 1 L with sterile water.

Reagents used for the Silver staining SDS PAGE gel

First Fixing Solution: Mix 500 ml of sterile water, 400 ml of methanol, and 100 ml of glacial acetic acid.

Second Fixing Solution: Combine 880 ml of sterile water with 50 ml of methanol and 70 ml of glacial acetic acid.

0.7% Periodic Acid Solution: Dissolve 0.7 g of periodic acid in 100 ml of the first fixing solution.

0.1% Silver Nitrate Solution: Prepare by dissolving 0.1 g of silver nitrate ($AgNO_3$) in 100 ml of sterile water.

Developing Solution: Dissolve 6 g of sodium carbonate (Na_2CO_3) in 200 ml of sterile water and add 3–6 drops of formaldehyde.

Stop Solution: Prepare a mixture containing 10% ethanol and 5% acetic acid.

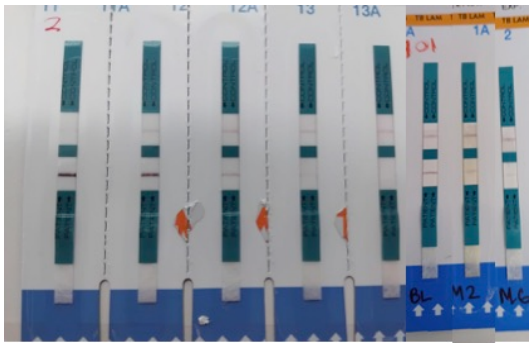
Reagents used for ELISA:

5% blocking buffer: (buffer was composed of dissolving 2,5 g skim milk powder into 50 ml wash buffer)

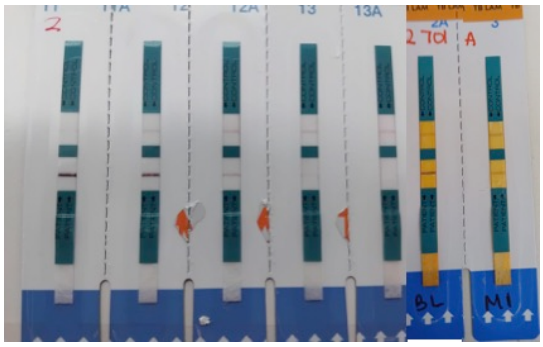
Assay buffer: (PBS)

Wash buffer: (wash buffer composed of 1x PBS, 0.05% Tween 20 dissolved distilled water up to the 1L mark**)**

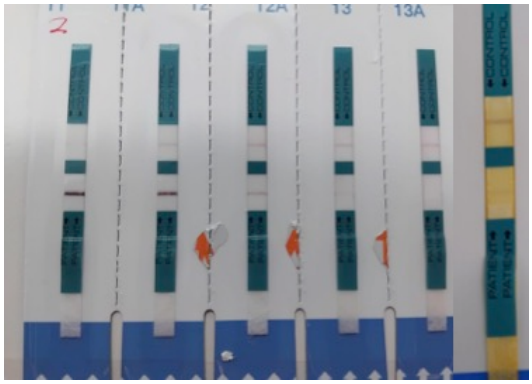
8.2 Appendix B: Rapid urine test images merged to standard curve image for Fiji analysis.



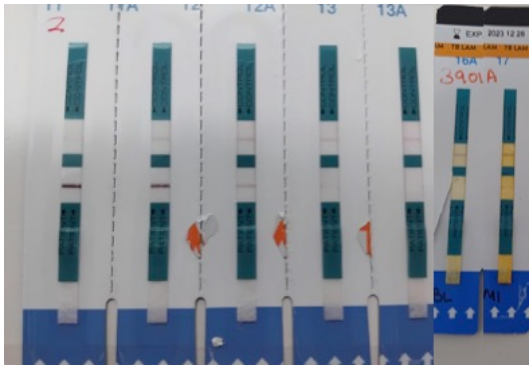
011-19-019-01-A



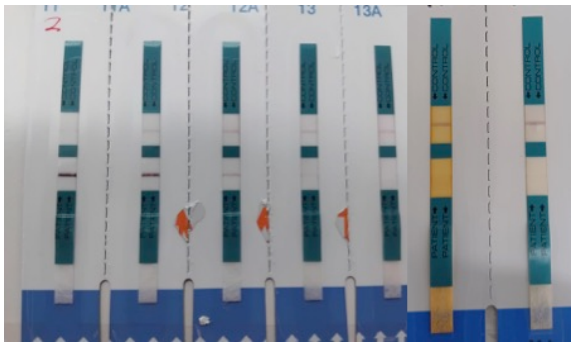
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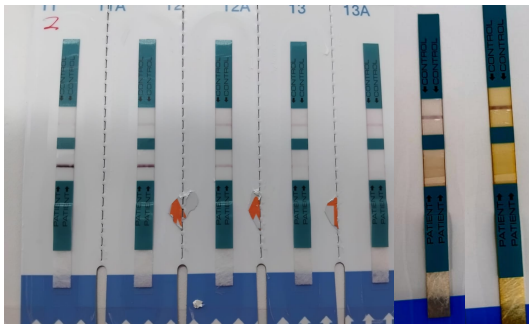
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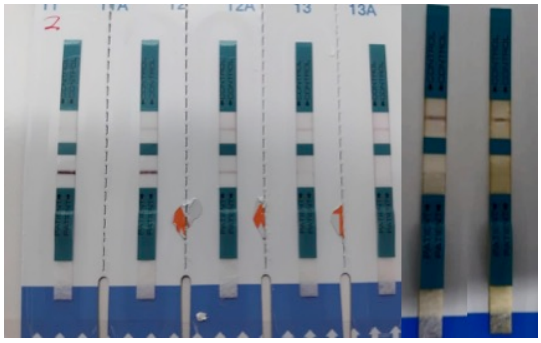
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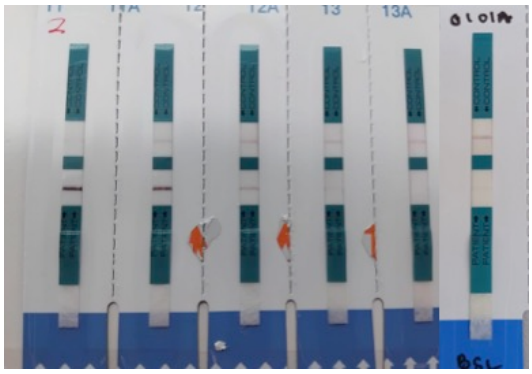
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011-19-123-01-A

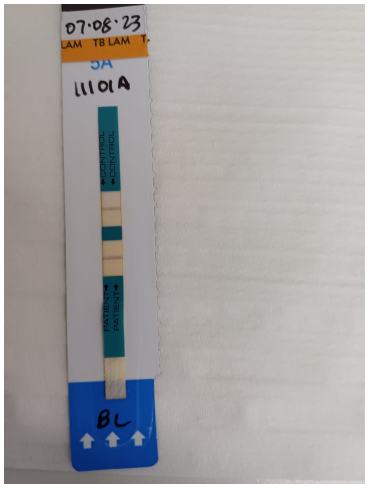


011-19-150-01-A



011-19-001-01-A_BSL

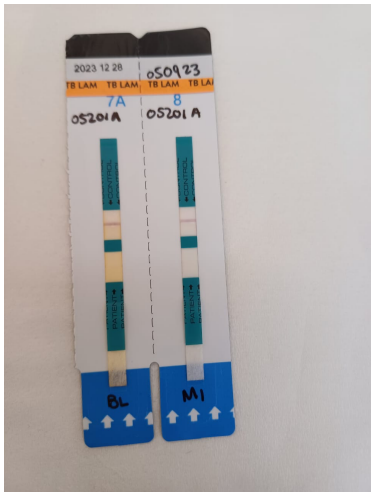
8.3 Appendix C: A subset of those urine samples with the most concentrated urine, which gave an over estimation when analysed on the Fiji software.



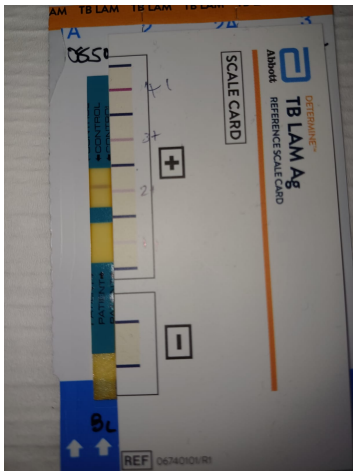
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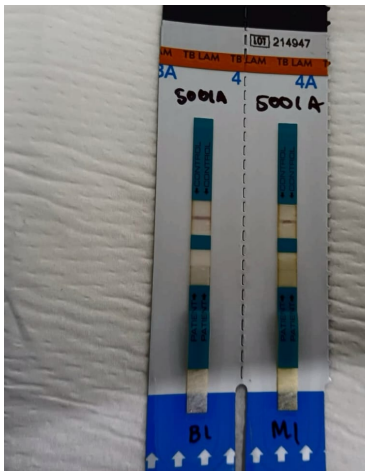
01119088-01-A



01119052-01-A



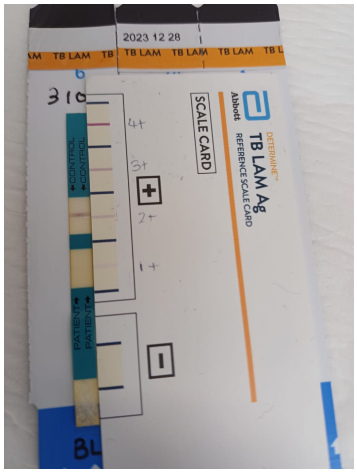
01119085-01-A



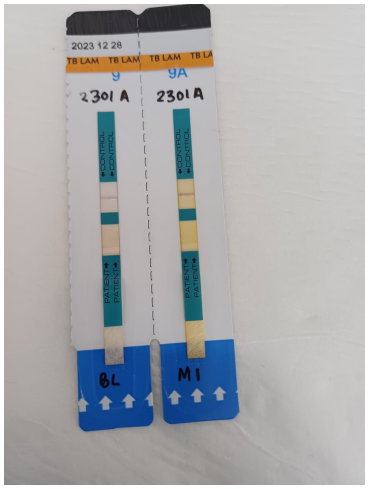
01119500-01-A



01119043-01-A



01119131-01-A



01119023-01-A



01119055-01-A

8.4 Appendix D: Approval to use samples from the SOHOT parent Study

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Human Research Ethics Committee: (Medical)
FWA Registered No IRB 00001223

SECRETARIAT: Suite 189, Private Bag x2600, Houghton 2041, South Africa Tel: +27-11-274 9200 Fax: +27-11-274 9281

Miss P Bhana

EMAILED

Regulatory Consultant
Perinatal HIV Research Unit
PO Box 114
Diepkloof
1864

22 August 2022

Email: bhanap@phru.co.za

Dear Miss Bhana,

PROTOCOL: SOHOT001 - TB IN HOT AND COLD SPOTS IN SOUTH AFRICA: RESEARCHING INDEX CASES AND THEIR HOUSEHOLDS: A SOUTH AFRICA - HOPKINS TB COLLABORATION (THE SOHOT COLLABORATION)

ETHICS REFERENCE NO: 160413

RE : APPROVAL FOR USE OF STORED URINE SAMPLES

We acknowledge receipt of your letter dated 11 August 2022 with the following documentation pertaining to the above-captioned trial.

Amendment Date:	Amendment Version:	Storage Amendment Application
Amendment Number:	Received Date:	11-Aug-2022

The following has been approved by the Wits Human Research Ethics Committee: (Medical):

* Storage amendment application form signed by Principal Investigator, Prof Neil Martinson on 01 August 2022

The following has been received:
* Proposal for Ms Thabiso Bridgette Seiphetlo
* Participant Informed Consent Form for Stored Samples (Cohort A & B), Version 3.0 dated 25 June 2018
* Parent/Legal Guardian Informed Consent Form for Stored Samples (Cohort B), Version 3.0 dated 25 June 2018
* Participant Informed Assent Form for Stored Samples (Cohort B), Version 3.0 dated 25 June 2018

Noted:
MOTIVATION:
The study will be conducted at the Centre of Excellence for Biomedical TB Research, University of the Witwatersrand and the National Health Laboratory Service under the supervision of Prof Kana and co-supervised by Dr B. Gordhan and Prof Neil Martinson.

A prospective study cohort of TB patients on treatment was established with all follow up completed approximately two years ago, to assess novel TB biomarkers. Participants were index patients with

laboratory confirmed TB and their consenting household contacts, the latter were recruited shortly after the index patient recruitment and all were followed up prospectively; the TB patients to ascertain responses to TB treatment and household contacts, to identify those that had TB at baseline or progressed to TB while in follow up. In addition, urine and blood (plasma) specimens from each of these patients was collected and stored in a repository to assess for novel TB biomarkers. These studies have not been conducted yet and due to budgetary constraints to store specimens in the CLS repository, an assessment of this study's repository specimens was conducted.

This offer has provided the CBTBR with a valuable opportunity to use these stored urine and plasma samples for detection low concentrations of urine LAM to measure TB infection. This is of particular interest in patients co-infected with HIV who are generally unable to provide sputum for diagnosis.

This study will increase our understanding in measuring TB treatment responses that will allow for changes and/or improved interventions.

Ethics Approval Date: 22 August 2022

The above has been noted for the Ethics Committee information and records.

KINDLY FORWARD TO THE RELEVANT INVESTIGATORS / CRA / STUDY CO-ORDINATORS

Regards,



PROF CLEMENT PENNY

For and on behalf of the Human Research Ethics Committee: (Medical)

Processed by: Thashin Reddy - Tel: 011 274 3353

8.5 Appendix E: Ethics clearance certificate for further analysis of stored samples from the SOHOT study.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Ms T Seiphethlo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M240623

NAME: Ms T Seiphetlo **DEGREE:** MSc (Med)
(Principal Investigator)

DEPARTMENT: School of Pathology
CoE in Biomedical TB Research
Faculty of Health Sciences

TITLE: *Utility of urinary lipoarabinomannam (LAM) assays to monitor treatment outcomes in Tuberculosis patients co-infected with Human Immunodeficency Virus (HIV)*

DATE CONSIDERED: 21 June 2024

DECISION: Approved unconditionally

CONDITIONS:

NOTE: If contact information regarding student study participants is required, please contact the Registrar's office <Nicoleen.Potgieter@wits.ac.za>

SUPERVISORS: Professor B Kana

APPROVED BY:
Professor P Ruff, Chairperson, HREC (Medical)

DATE OF APPROVAL: 7 August 2024 **EXPIRY DATE:** 6 August 2029

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report** in the format available at https://wits.ac.za/research/researcher-support/research-ethics/ethics-committees/. This report is due annually, for the duration of the Clearance Certificate, beginning on the first anniversary of Date of Approval above. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

08 August 2024
Date

8.6 Turnitin Report

Thabiso Bridgette Seiphetlo_MSc_Dissertation_1388245.docx

ORIGINALITY REPORT

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