Out of the

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Evaluation of the performance of Molecular Bacterial Load Assay for TB treatment response in a clinical setting

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Abstract

Background

Tuberculosis (TB) remains a serious global health problem affecting millions of people annually, especially in developing countries. Rapid and quantitative biomarkers to monitor treatment response are considered crucial to control TB and are urgently needed for individual patient management and clinical trials. This work describes the main findings of the evaluation of the performance of Molecular Bacterial Load Assay (MBLA) for TB treatment response conducted in a clinical setting in Mozambique.

Methods

Patients with Xpert-confirmed pulmonary TB were enrolled to the study and provided sputum samples for evaluation of MBLA compared to culture at baseline, weeks 1, 2, 4, 8, 12, 17, 26 and 52. Data were analysed using GraphPad Prism v.6 and IBM SPSS Statistics at 95% confidence interval.

Results

A total of 472 serial cultures and MBLA were done for 58 participants from baseline to week 52. MBLA had highest (61%) rate of positivity compared to MGIT culture (60%) and LJ culture (31%). MGIT time to positivity was inversely correlated to the MBLA, Spearmans $r=-0.67$, $p<0.0001$. The MBLA correlated with respiratory rate, weight, C-reactive protein ($p<0.0001$) and was able to detect viable bacterias in samples treated with Resuscitation-promoting factor and OMNIGene.SPUTUM reagent. The Xpert detected the lowest concentration of bacilli compared to culture and MBLA.

Conclusion

MBLA can be a very powerful biomarker to monitor TB treatment response and has potential to replace culture on the patient management. Further studies to evaluate the relationship between MBLA and clinical parameter are still needed.

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Abbreviations

µl – microliter

16S rRNA – 16S ribosomal Ribonucleic Acid

5-D – 5 day

AFB – Acid fast bacilli

ART - Anti-retroviral treatment

BCG - Bacillus Calmette-Guérin

BSC - Biosafety Cabinet

CD4 – Cluster of differentiation 4

CFU – Colony Forming Unit

cm – Centimeter

CRL - Control

Crl - credible interval

CrP - C-reactive protein

CT - computed tomography

Ct – Cycle threshold

CXR - Chest X-rays

DNA - Desoxiribonucleic acid

DST - Drug Susceptibility Test

eCFU – estimate colony forming unit

GTC – Mixture of Guanidine thiocyanate, Tris-HCL, β-mercaptoethanol and molecular grade water

HBC - High Burden Countries

HIV - Human Immunodeficiecy Virus

IFN-γ - interferon gamma

IGRA - Interferon-Gamma Release Assay

IUATLD – International Union Against TB and Lung Diseases

Kg – Kilogram

LAM - Lipoarabinomannan

LED - Light-emitting diodes

LF-LAM - lateral flow urine lipoarabinomannan assay

LJ - Lowenstein-Jensen

LPA - Line Probe Assay

LTBI - Latent TB infection

MaTuTU – Maputo Tuberculosis Trial Unit

MBLA - Molecular Bacterial Load

MDR-TB - Multidrug-Resistant TB

MGIT - Mycobacterial Growth Indicator Tubes

min – Minute

ml – Mililiter

mm - Milimeter

MRI - magnetic resonance imaging

mRNA - messenger Ribonucleic acid

Mtb or MTB – *Mycobacterium tuberculosis*

MTBC - *Mycobacterium tuberculosis* Complex

NAA - nucleic acid amplification (NAA)

NALC - N-acetyl-L-cysteine

NaOH - Sodium hydroxide

NTM - Non Tuberculosis Mycobacteria

NTP - National TB Program

NTRL – National TB Reference Laboratory

OM-S - OMNIgene.SPUTUM

PANBIOME – Pan African Biomarkers Expansion programme

PANTA - polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin

PB - Phosphate Buffer

PCR - Polymerase Chain Reaction

PPD - purified protein derivative

Rpf - Resuscitation-promoting factor

rpm – Rotation per minute

rRNA - ribosomal Ribonucleic acid

RR-TB - Rifampicin Resistant Tuberculosis

RT-PCR – Reverse transcriptase polymerase chain reaction

RT-qPCR - Reverse transcriptase quantitative polymerase chain reaction

SD - Same-day

SDP - Standard decontamination protocol

TB - Tuberculosis

TB-LAMP - Loopamp MTBC Detection Kit

TST - Tuberculin Skin Testing

TTP - Time to positivity or Time to culture positivity

US$ or USD - United States dollar

WHO - World Health Organization

XDR-TB - Extensively Drug-resistant TB

ZN - Ziehl-Neelsen

1. Introduction

1.1. Global History of Tuberculosis

Tuberculosis (TB) is an airborne infectious disease caused by members of the *Mycobacterium tuberculosis* complex, which primarily affects the lungs, but can also affect any organ of the body[1]. The disease is known as an old problem which affected the humankind since its prehistory [2]. It is documented that even before the identification of *M. tuberculosis* by Robert Koch in 1882, when TB was incurable and incomprehensible, the disease had become epidemic causing deaths of many people [3]. Nowadays, TB remains a serious global health problem which made the World Health Organization (WHO) took the decision of declaring it a global emergency in April 1993[4, 5]. Worldwide in 2017, an estimated 10 million people developed active TB, of whom 1,3 million died among those uninfected by the Human Immunodeficiecy Virus (HIV) and additional 300,000 died among HIV-positive people. Africa, a continent that has 16 out of 30 high TB burden countries in the world, contributed with 25% of the new TB cases [6]. Drug-resistant TB represents another major public health concern in many countries. In 2017, there were an estimated 558,000 incident cases of Rifampicin Resistant Tuberculosis (RR-TB), the most effective first line drug, and of these, 82% (458 000) had Multidrug-Resistant TB (MDR-TB) whilst 8.5% (38,900) of the MDR-TB cases evoluted to Extensively Drug-resistant TB (XDR-TB)[6].

Apart of early, rapid, accurate TB detection as well as early and effective treatment, more rapid and quantitative biomarkers to monitor treatment response is considered crucial to control TB allowing appropriate TB therapy according to the bacterial load of individual patients and the response of the bacteria to specific therapy. This would short unnecessary long treatment in patients driving them to the simplest, most effective and safe regimen. In addition, it would define exact study endpoint in TB treatment trials enabling study designs which are fast, cheap and accurate to define the minimum treatment effect [7].

Currently, the WHO recommends the use of sputum smear microscopy and culture to monitor TB treatment response[8]. Sputum smear microscopy does not allow species identification of mycobacteria, can not permit differentiation between viable and nonviable mycobacteria [9], and generally has low sensitivity compared to culture[10]. Culture-based methods are time consuming, prone to contamination with more rapidly growing bacteria, expensive and is rarely available in high burden settings [11]. The main bottleneck of culture is the slow growth of *M.*

tuberculosis and the time necessary to determine that a patient is truly culture negative, which takes no less than 6 weeks[7]. In addition, is believed that smear positive sputum samples contains dormant *M. tuberculosis* cells that can grow only in the presence of Resuscitation-promoting factor (Rpf), a group of proteins produced by *M. tuberculosis* that act on the bacterial cell wall to stimulate regrowth of dormant and nonculturable bacteria. However, its role to reactivate TB infection in human is uncertain[12].

As alternative to the classical smear microscopy and culture, several molecular methods to detect and quantify mycobacteria nucleic acids have been evaluated as potential markers for monitoring TB treatment. The detection of TB Desoxiribonucleic acid (DNA) using the Xpert® MTB/RIF assay (Cepheid,USA), the rapid test (provide result in less than 2 hours) for diagnosis of TB currently recommended by WHO, simple-to-use system and highly sensitive allowing the detection of *M. tuberculosis* beyond 132 CFU/ml of sputum[13], is one example. However, the positivity rates for sputum *M. tuberculosis* DNA detection with Xpert MTB/RIF assay decline more slowly than those with conventional sputum smear microscopy, solid culture, and liquid culture. In addition, the assay can pick positive results at the end or after TB treatment suggesting that dead TB bacilli or TB DNA fragments are detected by the assay which prevents accurate monitoring[14].

Detection of messenger Ribonucleic acid (mRNA) offers a potentially useful tool for the monitoring of TB treatment efficacy[15]. The challenge in using mRNA as a biomarker is that mRNA has short half-life, relatively unstable and present in smaller concentrations in the infected site than bacterial DNA[16]. Some studies shows that mRNA does not correlate well with the gold standard culture: concentrations of *M. tuberculosis* mRNA decline rapidly after the initiation of treatment turning to negative while the culture remains positive[17]. Due to this limitations, studies has been conducted to evaluate the ribosomal Ribonucleic acid (rRNA) as potential surrogate marker for TB treatment monitoring. The rRNA is described as more stable particulate, with longer half-life than mRNA, present in greater abundance in mycobacterial cells[16], and with an expected half-life shorter than that of DNA.

Recently, the assay named Molecular Bacterial Load (MBLA) was developed and evaluated *in vitro* to detect and quantify viable *M. tuberculosis* by targeting the 16S rRNA. The MBLA declined biphasically as culture in response to treatment and responded rapidly, with a mean decline in bacterial load for 111 subjects of 0.99 log10 after 3 days of treatment[18]. In 2014 a

report from a study which used sputum samples from TB patients under first days of standard WHO treatment for drug-sensitive TB to evaluate the MBLA in comparison with solid agar and liquid culture showed that MBLA correlates well with culture providing early information on the rate of decline in bacterial load and has technical advantages over culture[19]. However, evaluation of this assay under clinical settings evolving long time follow up of the patients under TB treatment is needed to determine the real capacity of MBLA to monitor TB treatment.

1.2. TB disease burden in Mozambique

During the period of 1998 to 2015, the concept of High Burden Countries (HBC), defined as countries that account for 80% of all new TB cases, became familiar and widely used in the context of TB[20]. Mozambique, a country located on the southeastern coast of Africa, bordered by Tanzania in the north, Malawi and Zambia in the northwest, Zimbabwe in the west, and ESwathini (previously known as Swaziland) and South Africa in the south with a population of 28 861 863 and a 2017 GDP per Capita USD 466,18[21]is referred for the first time as one of the 23 HBC in the TB WHO report from 2001[22]. Since then, the country did not move out from this list and according to the last WHO Global TB report[20], nowadays Mozambique belongs to the list of 30 HBC for the period of 2016 – 2020, presenting high burden (about 90% of the global burden) of TB, MDR-TB and TB/HIV.

Data from the National TB Program (NTP) indicates that the TB case notification has been increasing over the last few years. In 2017 the number of all TB forms notified was 86,515 cases. However, the country is still far from reaching the numbers estimated by the WHO, which refers to an estimated incidence of 159,000 cases in 2016, corresponding to a TB incidence rate above 500 cases per 100,000 population (figure 1)[23].

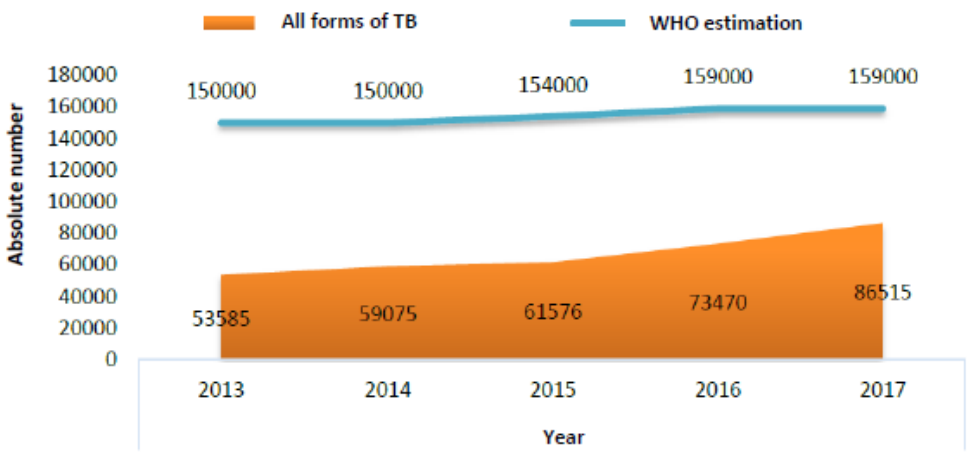


Figure 1. Trends of TB notification in Mozambique. Source: NTP Report 2017

In 2017 the HIV testing rate in TB patients was 97%, the rate of co-infection was 40%, the coverage of Anti-retroviral treatment (ART) in TB/HIV co-infected patients was 95% and the rate of prophylactic treatment with Cotrimoxazole was 97%. The notification of MDR-TB cases in the last 3 years increased from 644 in 2015 to 943 in 2017 (figure 2)[23].

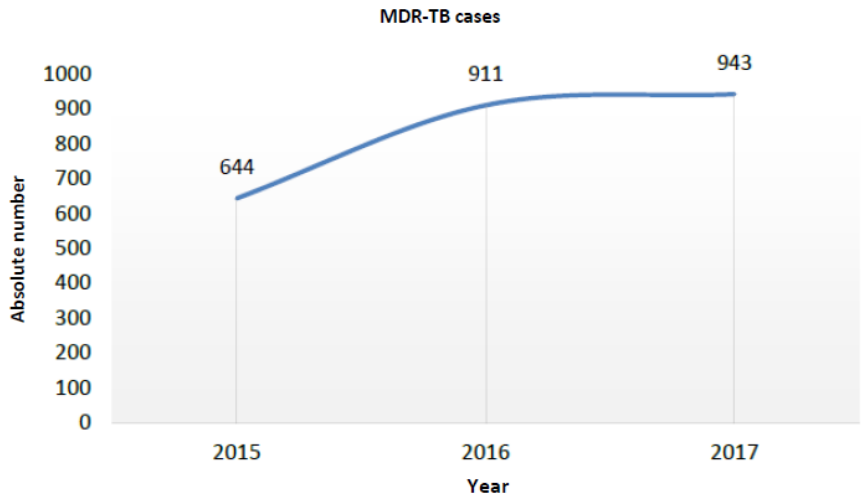


Figure 2. Trends of MDR-TB notification in Mozambique. Source: NTP Report 2017

1.3. Tuberculosis diagnosis and the associated challenges

The choice of a diagnostic tool for TB is related with the purpose of testing which includes detection of latent TB infection, active TB disease or drug resistance[1].

1.3.1. Detection of Latent TB infection

Latent TB infection (LTBI) is defined as subclinical infection with *M. tuberculosis* without any clinical, bacteriological or radiological evidence of the disease[24]. WHO has endorsed two tests for the identification of LTBI: the Tuberculin Skin Testing (TST) and the Interferon-Gamma Release Assay (IGRA). The TST is an immunological test which includes two parts: first, the purified protein derivative (PPD) reagent is injected intradermally into the forearm and, second, the delayed-type hypersensitivity response is monitored 48 to 72 hours post-injection by measuring the diameter of induration (swelling due to inflammation) in milimeters at the site of injection[24, 25]. Conventionally, the test is given on the left forearm to avoid errors in reading. However, right arm may be used in case of any contraindication to use the left arm. The volar aspect of the forearm is the preferred site of test[26].

The results of the TST must be interpreted carefully. The person's medical risk factors determine the size of induration the result is positive (5 mm, 10 mm, or 15 mm). In such cases, 5 mm or more is positive in HIV-positive person, recent contacts of active tuberculosis cases, injectable drug users, residents and employees of high-risk congregate settings (e.g., prisons, nursing homes, hospitals, homeless shelters, etc.), mycobacteriology laboratory personnel, persons with clinical conditions that place them at high risk (e.g., diabetes, prolonged corticosteroid therapy, leukemia, end-stage renal disease, chronic malabsorption syndromes, low body weight, etc.) and infants, children or adolescents exposed to adults in high-risk categories. Fifteen mm or more is positive in persons with unknown risk factors for TB. Reactions larger than 15 mm are unlikely to be due to previous BCG vaccination or exposure to environmental mycobacteria[26]. However, there is no international consensus on what constitutes TST positivity and different cut-offs are used in different countries[24].

The absence of a gold standard to diagnose LTBI makes it difficult to estimate the exact sensitivity and specificity of TST [24, 27]. Although widely used for being less expensive, simpler and more practical in many settings, the TST has limitations: it can generate false negatives results due to the anergy in immunosuppressed patients and can generate false positive results in non infected persons due to the BCG vaccination or contact with other mycobacteria present in the environment. The TST requires two visits to the health facility for tuberculin injection and induration measurementand has to be done by trained personnel[27].In addition, one positive TST result does not distinguish recent from remote infection[28].

Recently two commercial Interferon-Gamma Release Assays (IGRAs) were developed and approved as indirect and adjunct tests for TB infection, in conjunction with risk assessment, radiography and other medical and diagnostic evaluations. These IGRAs are based on the principle that the T-cells of individuals who have acquired TB infection respond to re-stimulation with *Mycobacterium tuberculosis*-specific antigens by secreting interferon gamma (IFN-γ)[29].

The QuantiFERON-TB Gold (QFT-G, Cellestis, Australia) and the newer generation QuantiFERON-TB Gold In-Tube (QFT-GIT, Cellestis, Australia) are whole-blood based enzyme-linked immunosorbent assays (ELISAs) measuring the amount of IFN-γ produced in response to three *M. tuberculosis* antigens (ESAT-6, CFP-10 and TB7.7). In contrast, the enzyme-linked immunospot (ELISPOT)-based T-SPOT.TB (Oxford Immunotec, UK) measures the number of peripheral mononuclear cells that produce INF-γ after stimulation with ESAT-6 and CFP-10[29].

Currently, there are no guidelines for IGRA use in low- and middle-income countries - typically with high TB- and/or HIV-burden, since the majority of IGRA studies have been performed in high-income countries and mere extrapolation to low- and middle-income settings with high background TB infection rates is not appropriate. Systematic reviews have suggested that IGRA performance differs in high- versus low TB and HIV incidence settings, with relatively lower sensitivity in high-burden settings[29]. Pai et al[30] conducted a meta-analysis on the sensitivity and specificity of IGRAs for the diagnosis of LTBI and found that studies of QuantiFERON-TB Gold In-Tube in countries with a high rate of tuberculosis incidence showed lower sensitivity than studies in countries with a low rate of incidence. However, the pooled T-SPOT.TB sensitivity was higher than that of the QuantiFERON-TB Gold and QuantiFERON-TB Gold In-Tube assays. Overall, they found a pooled sensitivity of 78% (95% confidence interval -CI, 73% - 82%) for QuantiFERON-TB Gold and 92% (95% CI, 90% - 93%) for T-SPOT.TB. Nineteen studies conducted in low- and middle-income countries to assess the sensitivity and specificity among 2,067 presumptive TB cases demonstrated a pooled sensitivity of 83% (95% CI, 70% - 91%) and pooled specificity of 58% (95% CI, 42% - 73%) for T-SPOT.TB (8 studies), and a pooled sensitivity of 73% (95% CI, 61% -82%) and pooled specificity of 49% (95% CI, 40% - 58%) for QuantiFERON-TB Gold In-Tube assay (11 studies)[29]. Persons with tuberculosis in high-incidence countries often have advanced disease and are likely to be infected with HIV or malnourished. Like the TST, anergy due to advanced disease, malnutrition, and HIV associated immune suppression may lower the sensitivity of IGRAs[30].

The IGRAs have operational characteristics that should be ideal for serial testing: they require only a single visit to give a sample, are free from observer bias in reading results and provide quantitative results with a single manufacturer-defined cut-off point for a positive test. Unlike the TST, they are ex vivo tests; there is thus no antigen administered to sensitise individuals and affect subsequent tests through boosting of anamnestic responses[31]. The downside is that the incidence of tuberculosis, even in TST or IGRA-positive individuals, is low, suggesting that most TST and IGRA-positive individuals do not progress to tuberculosis disease during follow-up revealing that no available tests for latent *M. tuberculosis* infection have high prognostic value[32].

1.3.2. Detecting active TB disease

Imaging and laboratory based methods are the main technologies available for detection of active TB disease. Among the imaging techniques, chest X-rays (CXR) and computed tomography (CT) are the most commum, while the laboratory based methods includes the classical microbiological assays (such as smear microscopy, culture and detection of antigen) and the new molecular assays (such as DNA and RNA based)[1].

1.3.2.1. Imaging methods

The role of imaging in tuberculosis (TB) has shown exponential growth, as in all spheres of medicine. The possibility of a tubercular etiology is often first suggested on an imaging study, particularly in relatively inaccessible sites[33]. Many TB diagnostic algorithms includes radiography, also called chest X-ray (CXR)[34, 35], although this method is being called into question due to limited diagnostic accuracy, poor film quality and non-expert interpretation, particularly, in low-resource settings. Even with the availability of more sensitive molecular tests for pulmonary TB, such as Xpert MTB/RIF, radiography remains necessary in the evaluation of patients with TB-like symptoms but negative laboratory results[36].

In a known case of TB, imaging is often requested to assess the extent of disease, evaluate response to therapy, or detect residual infection after completion of therapy[33]. In primary pulmonary TB (TB caused by the first-time exposure to *Mycobacterium tuberculosis*), radiography

remains the mainstay for the diagnosis of parenchymal disease while computed tomography (CT) is more sensitive in detecting lymphadenopathy. In post-primary pulmonary TB (TB that develops and progresses under the influence of acquired immunity, also called reactivation, secondary, or adulthood), CT is the method of choice to reveal early bronchogenic spread. Concerning characterization of the infection as active or not, CT is more sensitive than radiography[37].

Although Chest X-rays remain the basic imaging modality for pulmonary tuberculosis, CT, magnetic resonance imaging (MRI), and nuclear medicine techniques, including positron emission tomography/computed tomography, are extremely helpful in the assessment of both pulmonary and extrapulmonary tuberculosis[33]. For example, MRI is considered superior to CT for the detection and assessment of central nervous system TB, while for abdominal TB diagnosis, lymph nodes are best evaluated on CT[37].

Recently, analysis of currently available literatures on radiological signs associated with pulmonary MDR-TB showed that imaging findings of pulmonary MDR-TB do not differ from those of drug-sensitive TB. Tamhane et al [34] included CXR in their study to evaluate predictors of smear-negative pulmonary TB in HIV-infected patients and they found that the CXR (offsite) was 70% more likely to be abnormal in those with any symptoms and smear positive pulmonary TB cases were significantly more likely to have an abnormal CXR (offsite reading) than smear negative cases. This data suggest that where culture is unavailable, CXR is an important adjunct for diagnosis[34] since it increases correct decisions by doctors allowing rapid treatment initiation of pulmonary TB [35, 36]. Imaging is not meant to compete for TB diagnostics with microbiological and genomics methods, but it may offer significant insight in some cases when sputum is not ideal material for testing, especially at the end of the treatment or for some groups of patients such as children[38].

1.3.2.2. Microscopy

The smear microscopy was developed more that 100 years ago as contribution of many researchers. Franz Ziehl and Neelsen received the credits for improving the bacilli staining method so called "Ziehl-Neelsen" (ZN). Since the cell wall dye complexes of the bacilli are resistant to destaining with mineral acids, mycobacteria are referred to as “acid-fast bacilli”or “AFB”[11].

In the smear microscopy method, the sputum specimens can be smeared directly on to the slides without any processing and subjected to staining or can be liquefied with chemical reagents and then concentrated by centrifugation or sedimentation prior to acid-fast staining[10]. Many different methods of liquefaction and decontamination of sputum specimens exist and each laboratory has to make a choice of the better method to optimize the isolation of mycobacterium[39]. Although has been demonstrated that the liquefaction and decontamination method increases the sensitivity of microscopy, the direct sputum smear microscopy is the most widely used mean for diagnosing pulmonary TB and is available in most primary health-care laboratories at health-centre level in the developing world[40].

The sensitivity and specificity of AFB microscopy is low when compared to culture method. In general, the threshold for detection of AFB in sputum samples under optimal conditions is between 10^4 and 10^5 bacilli per ml[10, 40]. Ziehl-Neelsen microscopy is highly specific (more than 90%), but its sensitivity is considerably variable (20–80%) being more reduced in patients with extrapulmonary TB and in HIV-infected TB patients than in patients with pulmonary TB and HIV negative. It was clearly demonstrated that fluorescence microscopy, which uses Auramine O staining is 10% more sensitive than conventional Ziehl-Neelsen microscopy, and examination of fluorochrome-stained smears are faster taking less time to read[40]. However, its uptake has been limited by high cost, due to expensive mercury vapour light sources, the need for regular maintenance and the requirement for a dark room. In order to offer the benefits of fluorescence microscopy without the associated costs, the Light-emitting diodes (LED) have been developed, and, in 2009 the evidence for the efficacy of LED microscopy was assessed by the WHO. In comparison with conventional mercury vapour fluorescence microscopes, LED microscopes were found to be less expensive, require less power and can run on batteries. Furthermore, the bulbs have a long half-life and do not pose the risk of releasing potentially toxic products if broken, and LED microscopes are reported to perform equally well in a light room[40]. Data from WHO[40] also indicated that LED microscopy are 5% (95% CI, 0–11%) more sensitive and 1% (95% CI, -0.7% - 3%) more specific than conventional fluorescence microscopy. On the basis of these findings, WHO recommended that conventional fluorescence microscopy be replaced by LED microscopy. Its also important to note that proper collection of sputum samples from suspected pulmonary TB patients, the preparation of good, uniform, thin smears and staining of smears with high quality staining reagents and the proficiency to read

smears by the microscopist is prerequisite to achieving accurate results by microscopy[41]. Furthermore smear microscopy cannot distinguish between viable and dead bacilli, which lowers its utility as a treatment response monitoring tool for TB.

1.3.2.3. Culture-based methods

TB culture is traditionally performed on solid egg-based media, such as Lowenstein-Jensen (LJ) media, which is composed of egg proteins, potato flour, salts, and glycerol[11], and on liquid culture system, such as Mycobacterial Growth Indicator Tubes (MGIT, Dickinson and Company, USA), which are the gold standard for TB diagnosis and treatement monitoring [42].

Since mycobacteria are slowly-growing organisms, the contamination of specimens with more rapidly growing bacteria may prevent their detection by culture. Non-sterile respiratory specimens typically contain bacteria that will overgrow any mycobacteria potentially present. Therefore, it is important to process specimens prior to culture in a way that will reduce the burden of contaminating bacteria without adversely affecting mycobacterial viability[11]. In a systematic review, Zingué et al[39] described nine methods of decontaminations of culture. The method using 0.5% N-acetyl-L-cysteine (NALC) with 2% sodium hydroxide(NaOH) is considered to be the best as NALC acts as a strong mucus digester and allows greater concentration of AFB[10, 39]. In addition, NALC-NaOH gives higher rate of mycobacteria recovery, shorter time to positive culture and relatively higher contamination in MGIT system compared with LJ[43-46].

As preventive measure of contaminant growth, cold-chain storage and transport of samples prior culture processing has always been recommended but it is expensive to maintain and often impractical if specimens take a week or longer on the way to the processing laboratory. In order to obviate cold-chain transport by effectively suppress growth of contaminants while maintaining viability of mycobacteria, the company DNA Genotek developed the novel reagent OMNIgene.SPUTUM (OM-S, DNA Genotek, Ottawa, ON, Canada) which liquefies and decontaminates sputum, and preserves *M. tuberculosis* viability during transport. OM-S is simply added at 1:1 ratio to sputum at the point of collection, and the specimen can then be transported at ambient temperature to the processing laboratory for MGIT and LJ culture[47].

In a meta-analysis envolving 10 studies, the MGIT system showed better sensitivity of 81.5% and specificity of 99.6% in detecting mycobacteria with shorter time to detection compared to 67% sensitivity for LJ solid media[48]. However, MGIT system are costly compared to solid cul-

ture[42] estimated at US$12.35 per culture on LJ compared to US$16.62 on MGIT[43]. A market assessment of TB diagnostics methods done in South Africa for 2012–2013 period found an estimated cost of US$14.89 per MGIT culture in public sector and about US$57.02 in the private sector[49]. OM-S is also attempting to solve this limitations since is described as versatile and beneficial product that offer cost reduction improving sample quality for testing and highly stable product requiring no additional preparation in the laboratory [47].

1.3.2.4. Immunological test: TB LAM

Lipoarabinomannan (LAM) antigen is a lipopolysaccharide present in mycobacterial cell walls, which is released from metabolically active or degenerating bacterial cells and appears to be present only in people with active TB disease[50]. The mechanism whereby LAM enters the urine from the systemic circulation is unclear. It seems possible that free circulating LAM could enter the urine readily, but not if present in large immune complexes. LAM antigenuria (presence of LAM antigen in the urine), however, could also potentially result from direct involvement of the renal tract with TB such that antigen may enter the urinary tract directly without passing across the renal glomerular basement membrane[51].

Analysis of urine rather than sputum samples is a very attractive option for TB diagnosis since the urine is simple to collect without generating hazardous bioaerosols, it is safe to handle in the laboratory, it has relatively few bacterial contaminants and sample quality is unlikely to be highly variable[51].

Tests based on the detection of mycobacterial LAM antigen in urine have been developed as potential point-of-care tests for TB[50]. The lateral flow urine lipoarabinomannan assay (LF-LAM, Alere DetermineTM TB LAM Ag, Alere Inc, Waltham, MA, USA) is a commercially available point-of-care test for active TB (pulmonary and extrapulmonary TB). The test detects LAM and is performed by placing urine on one end of a test strip, with results appearing as a line (that is, a band) on the strip if TB is present. It is simple, requires no special equipment, and shows results in 25 minutes[52].

Unlike traditional TB diagnostic methods, LF-LAM has improved sensitivity in TB/HIV co-infection which further increases with lower CD4 counts[50]. The sensitivity of LF-LAM is reported to be around 45-70% and specificity of 98% or more in HIV–tuberculosis-coinfected patients with CD4 counts less than or equal to 100 cells/μL[52, 53].

Its been demonstrated by several studies that detectable LAM in urine is associated with higher mortality rates among HIV positive individuals with low CD4 compared with LF-LAM negative individuals[52-56]. Considering the relative benefits and harms associated with the use of the LF-LAM assay, in 2015 the WHO recommended the use of the LF-LAM to assist in the diagnosis of TB in HIV positive adult or children with signs and symptoms of TB (pulmonary and/or extrapulmonary) who have a CD4 cell count less than or equal to 100 cells/µL, or HIV positive patients who are seriously ill regardless of CD4 count or with unknown CD4 count. "Seriously ill" is defined based on 4 danger signs: respiratory rate > 30/min, temperature > 39°C, heart rate > 120/min and unable to walk unaided. Furthermore, LF-LAM should not be used as a screening test for TB, not even used for pooled urine specimens or other samples than urine (e.g. sputum, serum, plasma, CSF or other body fluids). The implementation of LF-LAM in the targeted patient groups does not eliminate the need for other diagnostic tests - Xpert MTB/RIF, culture or sputum-smear microscopy - as these tests exceed LF-LAM in diagnostic accuracy. As any other test, LF-LAM has some limitations since it does not differentiate between the various species of mycobacterium and cannot be used to distinguish *M. tuberculosis* from other species[50]. Due to its low sensitivity, it is only useful in advanced TB disease cases.

1.3.2.5. Molecular tests

Xpert MTB/RIF assay

The GeneXpert system was launched in 2004 and the development of the XpertMTB/RIF assay for the GeneXpert platform was completed in 2009. In December 2010, WHO recommended the use of the Xpert MTB/RIF assay and since then is considered one of the most important breakthrough in the fight against TB[57]. The Xpert MTB/RIF (Cepheid, Sunnyvale, CA, United States) is a nucleic acid amplification (NAA) assay that targets DNA sequences by utilizing real-time Polymerase Chain Reaction (PCR) methods. Basically, its an automated, cartridge-based system that benefits from ease of use and a closed amplification system that reduces the potential for cross-contamination between specimens. The test is simple for laboratory technicians to perform, can detect *Mycobacterium tuberculosis* Complex (MTBC) directly from patient specimens in as little as two hours with no needs of advanced biosafety equipment and has the added benefit of providing information about potential rifampicin resistance, by detecting mutations in an 81-base pair region of the *rpoB* gene that are responsible for conferring approxi-

mately 96% of rifampicin resistance in MTBC. Its important to note that rifampicin resistance is also a predictor of MDR-TB since the majority of rifampicin-resistant isolates will also be isoniazid-resistant[11].

The Xpert MTB/RIF assay has good sensitivity and specificity for respiratory specimens[11]. The meta-analysis of 22 published studies conducted to evaluate the accuracy of the assay to diagnose pulmonary TB in adults as an initial diagnostic test replacing smear microscopy found that-the Xpert MTB/RIF had comulative sensitivity of 88% (95% credible interval [CrI], 84–92%) for detecting TB, with an increase of case detection of 23% (95% CrI, 15–32%) among culture-confirmed cases, and comulative specificity was 99% (95% CrI, 98–99%). When used to detect rifampicin resistance, Xpert MTB/RIF achieved a comulative sensitivity of 95% (95% CrI, 90–97%) (17 studies) and a cumulative specificity of 98% (95% CrI, 97–99%) (24 studies). Comparing against culture, the overall comulative sensitivity of Xpert MTB/RIF to diagnose pulmonary TB in children was 66% in 10 studies where expectorated sputum or induced sputum was used and the comulative sensitivity was 66% in 7 studies where samples from gastric lavage or aspiration were used. The comulative specificity of Xpert MTB/RIF compared against culture as the reference standard was at least 98%. Finally, the sensitivity of Xpert MTB/RIF to detect rifampicin resistance in specimens from children was 86% (95% CrI, 53–98%). Depending on the type of sample, the sensitivity and specificity of Xpert MTB/RIF varied from 43% for pleural fluid to 83% for gastric lavage/aspirate and 98,1% for gastric lavage/aspirate and other tissues samples to 99,9% for pleural fluid, respectively[58]. However, the Xpert MTB/RIF cannot differentiate between live and non-viable MTBC, so they cannot be used to monitor response to treatment[11, 14] .

Line Probe Assays

Line Probe Assays (LPAs) are a family of novel DNA strip-based tests that uses nucleic acid amplification techniques (e.g. PCR) and reverse hybridization methods for the rapid detection of mutation associated with drug resistance[59]. LPAs also allows the detection of *M. tuberculosis* complex members as well as several commonly encountered Non Tuberculosis Mycobacteria NTM species[11].

In 2008, WHO approved the use of commercial LPAs for detecting MTBC and rifampicin resistance in sputum smear-positive specimens (direct testing) and in cultured isolates of MTBC (in-

direct testing). A systematic review at that time, evaluating the diagnostic accuracy of two commercially available LPAs – the INNO-LiPA Rif.TB assay (Innogenetics, Ghent, Belgium) and the GenoType MTBDR*plus* version 1(Hain Lifescience GmbH, Nehren, Germany) found excellent accuracy for both tests in detecting rifampicin resistance, but their diagnostic accuracy for isoniazid resistance had lower sensitivity, despite excellent specificity. For this reason, these two assays are no longer used in clinical practice[60] and newer versions of LPA technology have been developed, such as the Hain GenoType MTBDR*plus* version 2; and other manufacturers like Nipro (Tokyo, Japan) which developed the Nipro NTM+MDRTB detection kit 2 have entered in the market[61].

LPAs detect rifampicin resistance by identifying mutations in the rpoB gene and isoniazid resistance by identifying mutations in katG and inhA genes[60].

Nathavitharana et al[60]conducted a systematic review and meta-analyses to evaluate the performance of Hain Genotype MTBDRplusV1, MTBDRplusV2 and Nipro NTM+MDRTB and found that in patients with pulmonary TB, the LPAs have high sensitivity and specificity for RIF resistance and high specificity and good sensitivity for INH resistance.

Currently, the WHO recommends the use of commercial molecular LPAs as the initial test instead of phenotypic culture-based Drug Susceptibility Test (DST) to detect resistance to rifampicin and isoniazid in persons with a smear-positive specimen or a cultured isolate of MTBC, from both pulmonary and extrapulmonary sites, although it does not eliminate the need for capacity for conventional culture and DST[61].

The Genotype MTBDR*sl* assay (Hain Lifescience, Nehren, Germany), available in a version 2 since 2015, belongs to a category of rapid molecular genetic tests called second-line line probe assays (SL-LPA) developed to detect the resistance to second-line TB drugs, such as fluoroquinolones (including ofloxacin, levofloxacin, moxifloxacin and gatifloxacin) or injectable drug (including kanamycin, amikacin, and capreomycin). The accuracy of MTBDR*sl* to detect fluoroquinolones and injectable drugs reveales high sensitivity and specifity 72 to 100% [62].

LPAs are technically complex involving DNA extraction from the mycobacteria, preparation of reagents for PCR (pre-amplification), PCR amplification and hybridization, and interpretation of results (post amplification). Its use is indicated for reference or regional laboratory settings under at least biosafety level 2 or 3 conditions with at least, three separate rooms for the differ-

ent molecular steps: DNA extraction, pre-amplification procedures, and amplification and post-amplification processes[63].

TB-LAMP

A commercial molecular assay Loopamp MTBC Detection Kit based on loop-mediated isothermal amplification was developed by Eiken Chemical Company Ltd (Tokyo, Japan) for the detection of *M. tuberculosis* Complex (TB-LAMP). TB-LAMP is a manual assay that requires less than two hours to perform and can be read with the naked eye under ultra violet light. Because of its limited infrastructure requirements and relative ease of use, TB-LAMP is being explored as a rapid, point-of-care diagnostic test for resource-limited settings[64].

In 2016, WHO convened a Guideline Development Group that reviewed the evidence available and found that TB-LAMP technology could be used insettings where conventional smear microscopy is performed with the following recommendations: TB-LAMP may be used as a replacement test for sputum smear microscopy for the diagnosis of pulmonary TB in adults with signs and symptoms consistent with TB. Also, TB-LAMP may be used as a follow-on test to smear microscopy in adults with signs and symptoms consistent with pulmonary TB, especially when further testing of sputum smear negative specimens is necessary. However, TB-LAMP should not replace the use of rapid molecular tests that detect TB and resistance to rifampicin especially among populations at risk of MDR-TB. The TB-LAMP assay may be used in children, based on the generalization of data in adults, while acknowledging difficulties in the collection of sputum specimens from children[64].

1.4. Mycobacterial biomarkers of TB treatment response

Global initiatives have been launched to develop improved TB therapy. The currently recommended treatment is a six-month regimen of four first-line drugs (isoniazid, rifampicin, ethambutol and pyrazinamide) for cases of drug-susceptible TB and 20 months regimen of more expensive and toxic drugs for cases of rifampicin-resistant TB and MDR-TB, although shortened regimens of 9–12 months are now recommended for these patients[20].

Existing markers of treatment outcome (clinical cure and relapse) require prolonged follow-up of patients, therefore, the need for alternative biomarkers or surrogate endpoints predictive of response cannot be more emphasised[65].

Biomarker (biological marker) is the characteristic that is objectively measured and assessed as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention, whereas surrogate endpoint is a biomarker that is intended to substitute for a clinical endpoint (a characteristic or variable that reflects how a patient feels, functions, or survives). A surrogate endpoint is expected to predict clinical benefit (or harm, or lack of benefi t or harm) based on epidemiological, therapeutic, pathophysiological, or other scientific evidence[65].

1.4.1. Sputum smear, sputum culture conversion and time to culture conversion

Sputum smear microscopy and culture are biomarkers very well known for the TB treatment monitoring. The World Health Organization recommends the use of sputum smear microscopy at month 2, 5 and 6 during treatment. If smear is positive at month 2, new smear is necessary at month 3. If smear is again positive, then culture needs to be performed at month 3. Additional culture has to be done at month 5 and 6[8]. However, if resources permit, monthly culture is recommended, given that this has been shown to have the greatest benefit in detecting treatment failure[57].

Although sputum smear microscopy is easy to perform and provides results faster than culture[66], it is less sensitive[9, 67], does not allow species identification of mycobacteria, does not permit differentiation between viable and nonviable mycobacteria [9] and is operator dependent[65]. Therefore, sputum smear is unlikely to be a suitable biomarker for treatment monitoring and drug trials[65].

Sputum culture is time consuming, prone to contamination of rapidly growing microorganisms, takes several weeks to result[65] and, although considered gold-standard for TB diagnostic and treatment monitoring, is poor prognostic marker for individual patients when compared to surrogate endpoints in clinical trials[68]. In addition, is believed that sensitivity of culture decreases due to small proportion of TB bacilli in sputum which are non-culturable without supplementing with resuscitation-promoting factors[12].

The MGIT Time to positivity seems to be a promising area to evaluate TB treatment response as alternative of colony counts in LJ culture. Studies has shown that time to culture positivity for *M. tuberculosis* correlates well with number of colonies forming unit on solid media[69] and with numbers of AFB in sputum smears of patients before and during treatment[66]. However, the

liquid culture system takes readings at intervals through the culture (typically hourly), a discontinuous variable, resulting in a restricted scale of responses compared with colony counts, which measure absolute numbers and are a continuous variable. This restricted scale, and the fact that the time to liquid culture positivity might be affected by the loss of viable organisms during sample decontamination, makes the marker less powerful[65].

1.4.2. Molecular markers

DNA markers

Molecular markers provide a more rapid assessment of mycobacterial burden than culture. Early studies using molecular methods compared DNA detection by PCR with microscopy and culture[65]. Studies conducted to assess whether *M. tuberculosis* DNA could be applicable for monitoring the efficacy of antituberculosis treatment has shown that the inability of PCR to distinguish live and dead organisms precludes DNA amplification from use in treatment monitoring[70, 71].

To determine whether quantitative estimates of *M. tuberculosis* DNA in sputum correlate with the numbers of viable bacilli and thus measure the therapeutic response of patients during treatment, Desjardin et al[72] compared two methods of *M. tuberculosis* DNA quantification using DNA isolated from sputum specimens serially collected during the course of therapy. They found that both PCR systems are reproducible and accurate but the rate of disappearance of both AFB and *M. tuberculosis* DNA did not correlate with the decline in cultivable bacilli in the specimen indicating that PCR DNA-based are not appropriate for monitoring treatment efficacy[72].

Several others studies has been demonstrating that DNA-based assays are not appropriate for monitoring treatment response[73, 74] and that PCR remains positive much longer in patients suffering from extensive disease than in patients with less-extensive disease[71].

Recently, Friedrich and Rachow et al[14] evaluated the Xpert MTB/RIF assay in sputum samples to determine whether this assay can be used as biomarker to replace conventional microbiological tests for monitoring response to TB treatmen. They found that positivity rates for sputum *M. tuberculosis* DNA detection with Xpert MTB/RIF assay decline more slowly than those with conventional sputum smear microscopy, solid culture, and liquid culture. In addition, they found high rates of positive results at the end of the 6 month treatment. For this reason, Xpert

MTB/RIF in its current format cannot be used as a biomarker of disease activity and cannot replace conventional smear and culture for the monitoring of patients under treatment.

RNA markers

Quantitative analysis of messenger RNA (mRNA) offers a potentially useful tool for the monitoring of treatment efficacy[15]. According with Singh et al[75], mRNA can be used to project viability of the organism, treatment efficacy and/or susceptibility to antibacterial agents due to its short half-life.

Singh et al[75] evaluated *M. tuberculosis* mRNA as a surrogate for treatment response in sputum of pulmonary TB patients before starting supervised treatment. They found that reverse-transcriptase PCR (RT-PCR) and culture results were concordant, whereas mRNA declined with time and correlated with culture clearance. In addition, 78% (39/50) patients were smear, culture and RT-PCR negative at 2 months of treatment and the mRNA levels at day 0 had statistically significant correlation with time to culture conversion and drug resistance. Thus, *M. tuberculosis* mRNA quantitation may prove to be of great value for evaluating the response to new drugs under trial[76]. However, Mdivani et al[17] evaluated two real-time PCR assays to detect *M. tuberculosis* specific DNA and mRNA directly in sputum samples of patients under treatment. They found that the concentrations of *M. tuberculosis* mRNA decline rapidly after the initiation of therapy and in 20% (13/65) of the patients it turned negative at the first follow-up time point at week 2, while the culture remained positive.

Ribosomal RNA (rRNA) is more stable, has a longer half-life than mRNA, is present in greater abundance in mycobacterial cells[16], and an expected half-life is shorter than that of DNA. For this reason, Honeyborne et al[18] focused their attention on 16S rRNA to develop the assay termed Molecular Bacterial Load (MBLA).

MBLA is a real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) which uses abundant 16S-rRNA as a target with a robust internal control to normalizes the RNA loss during extraction and the presence of sample inhibitors to quantify viable *M. tuberculosis.* The first report of MBLA showed that using this assay the bacterial load of *M. tuberculosis* declined biphasically as culture in response to treatment. As a biomarker of treatment response, the MBL assay responded rapidly, with a mean decline in bacterial load for 111 subjects of 0.99 log10 after 3 days of chemotherapy[18]. In addition, there was a significant association be-

tween the rate of bacterial decline during the same 3 days and bacilli sputum at day 0. Further study of MBLA conducted to measure viable *M. tuberculosis* in sputum in comparison with solid agar and liquid culture showed that the assay is at least as good as culture for measuring early bactericidal activity during standard tuberculosis therapy to day 14, with higher precision and fewer missing data[19].

Strenghts from MBLA includes the capacity of being unaffected by other microorganisms present in the sample, seldom inhibited and has shorter time to result (24 hours compared to weeks)[18]**.** This assay shows promise as a replacement for culture in future early bactericidal activity trials testing new drugs[19].

2. Rationale and Objectives

2.1. Rationale

Although preventable and curable disease, TB remains a serious public health problem, particularly in high TB burden countries including Mozambique. As described above, the available tools to diagnose TB and monitor the treatment outcome has advantages and limitations that, on one hand, help to detect the cases and guide appropriate treatment, and on the other hand, compromise the diagnosis and treatment monitoring of the disease itself because they are time consuming and less specific. Fast, affordable, patient-accessible and highly sensitive and specific tools are increasingly needed for patient diagnosis and management. In this context, the establishment of Maputo Tuberculosis Trial Unit (MaTuTU study) generated samples to be used in this PhD under the umbrella of Pan-African Biomarker Expansion Programme (PANBIOME) consortium in order to assess the performance of a novel TB treatment monitoring assay, named the Molecular Bacterial Load assay (MBLA), compared to the traditional culture, Xpert MTB/RIF and clinical parameters of patients with pulmonary TB in Maputo, Mozambique. Further, the role of Resuscitation-promoting factor (Rpf) for shortening time to culture positivity and detect dormant bacilli, as well as the utility of MBLA to detect viability of Mtb in samples treated with novel reagent OMNIGene.SPUTUM were also assessed.

2.2. Objectives

2.2.1. General Objective

- To assess the performance of MBLA as molecular biomarker to measure viability of *M. tuberculosis* during TB treatment in patients with pulmonary TB in Mozambique.

2.2.2. Specific Objectives

- Compare the MBLA with culture, Xpert MTB/RIF and clinical parameters of patients with pulmonary TB before and during different time points of treatment.
- Assess the utility of MBLA to measure viability of Mtb in samples treated with OMNIGene.SPUTUM for liquid and solid cultures.
- Determine the limit of detection of MBLA compared to MGIT culture and Xpert MTB/RIF

3. Methods

3.1. The MaTuTU study and PANBIOME sub-study

3.1.1. Study design

MaTuTU was a cohort study where each patient confirmed of having active TB by Xpert MTB/RIF assay was enrolled and followed up at least for 6 months after treatment initiation in order to observe TB therapy and treatment response. Patients on standard anti-TB therapy were treated with Rifampicin, Isoniazid, Pyrazinamide and Ethambtol (RHZE) while Multi-drug resistant (MDR) TB cases were treated with Kanamicin, Levofloxacin, Ethionamide, Cicloserine, Ethambutol and Pyrazinamide (6 Km-Lfx-Eto-Cs-E-Z/18 Lfx-Eto-Cs-E-Z). Sputum samples and clinical data of the participants on the cohort were collected for laboratory assessment before treatment initiation: baseline and during treatment: weeks 1, 2, 4, 8, 12, 17 and 26. A clinical re-assessment was performed at week 52 and in those participants who had any symptom suggestive of TB, sputum sample was collected for further investigation in Mtb culture.

The study sampling was done by convenience as the main objective of the MaTuTU project was to establish a TB clinical trial site in Maputo (Mozambique) by performing a TB cohort study with long enough follow up to investigate the characteristics and outcome of TB patients in Maputo. The initial aim was to enrol 100 patients with Xpert-confirmed pulmonary TB into the cohort study.

The PANBIOME sub-study, which is the main topic of this PhD thesis, used samples from MaTuTU to assess the Molecular Bacterial Load Assay (MBLA), a novel treatment monitoring method of patients on anti-TB therapy. The MBLA was assessed for its performance in measuring treatment response in comparison with culture from baseline, weeks 1, 2, 4, 8, 12, 17, 26 and 52 (if available). The performance of the Resuscitation-promoting factor (Rpf) was also assessed in comparison with culture from baseline, weeks 1, 2, 4, 8 and 12.

3.1.2. Study site and population

The MaTuTU study clinic was established at the Mavalane health center, located in Mavalane area, a deprived suburb of Maputo city with a total estimated population of about 620,000 and high burden of TB. Mavalane is faced with poor sanitation, waste disposal, level of (health) education, and high poverty levels. According to IMASIDA[77] Maputo city has high HIV rate (16.9%)

with prevalence of 21.7% and 11% in women and men, respectively. Sample processing and analysis was done at the National Tuberculosis Reference Laboratory located at Maputo Central Hospital, Maputo city.

Only consenting participants were screened and enrolled into the study. The inclusion criteria were: positive TB result by Xpert MTB/RIF assay, ≥18 years of age and able to give informed consent for study participation, including accepting HIV test. Participants were excluded from the study if one of the following criteria were observed: history of TB treatment in the last 6 months, non-compliance of TB treatment at any time point in the past, suffering from a condition likely to lead to uncooperative behaviour, such as psychiatric illness or alcoholism.

For all study participants, decision to treat was made by the healthcare practitioners at Mavalane health centre in line with the National TB Guidelines.

3.1.3. Study procedures

a) Sample collection, transportation and reception at the National TB Reference Laboratory

Patients were trained on how to produce quality sputum and asked to bring early morning sputum in the morning expectorated after waking up whilst the spot sputum expectorated at the clinic, preferably in the morning and before intake of that day's study drug. Each participant was provided with sterile 30ml plastic sputum container (SAFECAN, ALPHA THERAPEUTICS PVT.LTD, India) for sputum collection in accordance with the national sputum collection instruction. The container was labelled with a sticky paper bearing study ID, date of visit, type of visit and type of sample and patched on the Specimen Request and Transfer form. This ensured that accurate attribution of results to the right sample and participant.

The samples were transported in the cooler box maintained at 2°-8°C. An attached digital thermometer gave continuous temperature measurements of cooler box to ensure that specimens were being transported at the right temperatures. On arrival at the lab, the receptionist read the thermometer and recorded the temperature. The original Specimen Transfer Forms were stored at the laboratory while a copy of each Specimen Request and Transfer Form were sent back to the study clinic as proof of sample reception at the laboratory.

b) Sputum sample evaluation and preservation

After the reception, sputum samples were immediately taken to the high containment TB laboratory for processing. High quality sputum sample were selected for culture, smear microscopy and MBLA, while the other raw sputum aliquoted into cryotubes (Simport, Canada) and immediately stored on ultralow freezer at -80°C (NuAire, USA). The high quality sputum sample was defined as being viscous or mucoid (not composed of saliva only) and not containing considerable amount of blood (not only blood stained) and having volume of ≥3ml. A fraction of this sputum was used for culture and smear while the other fraction (1ml) was diluted in 4ml of the mixture containing 50% Guanidine Thiocyanate (GTC, Promega, UK), 0.1M TrisHCl pH 7.5 and 1% β-mercaptoethanol v/v (SigmaAldrich, UK) then stored at -80°C to preserve the RNA of *M. tuberculosis* until testing. The table 1 shows the volume of sputum sample used for processing and storage.

Table 1. Processing and storage of sputum sample according to different volumes

No table of figures entries found.	**Total volume >8ml**	**Total volume >5ml - 8ml**	**Total volume 3ml-5ml**	**Total volume <3ml**
NALC/NaOH-decontamination, microscopy, culture, storage of pellet	Maximum 5ml	3ml	3ml	all material
GTC-storage for MBLA	2ml	2ml	1-2ml	none
Raw sputum storage (in 1 ml aliquots)	1 to 3 aliquots of 1ml sputum each	1 to 3 aliquots of 1ml sputum each	none	none

c) Culture

After homogenization, inside the biosafety cabinet, sputum samples were processed for solid (Lowestein Jensen - LJ) and liquid (Mycobacteria Growth Indicator Tube - MGIT) culture using a standardized protocol: sputum decontamination using a mixture of 4% NaOH; 1% NALC; 1.45% sodium citrate. The solution of NaOH-NALC sodium citrate solution was added in equal volume to the quantity of sample and mixed by vortexing for about 15-30 seconds. The mixture was allowed to incubate for 15 minutes at room temperature and the reaction stopped with addition of phosphate buffer (pH 6.8) up to 45ml. The 50ml centrifuge tubes were then transferred to biosafety protected centrifuge bucket and centrifuged at 3000g for 15 minutes at4°C (HERMLE Z383k, Germany). The supernatant fluid was carefully decanted into a suitable splash-

proof container containing 10% sodium hypochlorite. The pellet was resuspended in 2ml phosphate buffer (pH 6.8) and 30µl of the suspension was used to prepare smear microscopy to detect acid fast bacilli (AFB). For cultures, 500µl of the suspension was added to 7mL BBL MGIT tube (Modified Middlebrook 7H9 Broth Base and Casein Peptone; Becton, Dickinson and Company, USA) containing 0.8mL of the mixture of MGIT PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin; Becton, Dickinson and Company, USA) and MGIT Growth Supplement (Bovine albumin, Dextrose, Catalase, POES, Oleic acid; Becton, Dickinson and Company, USA) while 200µl was inoculated to LJ slopes.

All MGIT tubes were incubated at 37°C in the BD BACTEC MGIT 960 system (Becton, Dickinson and Company, USA) which detects automatically any growth in the tube by flagging positive and providing a time to positivity (days and hours of incubation) or negative after 42 days of incubation. The LJ slopes were incubated at 37°C in incubators (Memmert GmbH, Germany) and visually checked once a week to detect any growth (positive result or contamination) or no growth (negative result) after 8 weeks of incubation. Positive MGIT tubes were inoculated to the blood agar plate and incubated at 37°C for 48 hours. Growth on the blood agar plate indicated culture contamination. If this occurred within 7 days of incubation, then the remaining decontaminated suspension was treated again as explained above. If contamination was detected after 7 days of incubation then the result was recorded as contaminated. At the same time, Ziehl Neelsen staining was done for all positive MGIT tubes and LJ slopes in order to confirm presence of AFB. The SD BIOLINE MPT64 Ag Rapid test (STANDARD DIAGNOSTICS, INC; Korea) was used to distinguish *M. tuberculosis* complex on all ZN positive MGIT and LJ cultures. The MGIT results and time to positivity were recorded according to the table 2.

Table 2. Possible MGIT result constellations and consequences for time to positivity

Instrument result (MGIT)	**Blood agar**	**Ziehl Neelsen**	**MPT64Ag**	**TTP**	**Culture result**
Positive	Negative	Positive	Positive	Valid	True positive
Positive	Positive	Positive	Positive	Invalid	Positive with contamination
Positive	Positive	Negative	Not done	Invalid	Contamination
Positive	Negative	Negative	Not done	Invalid	Contamination
Negative	Not done	Not done	Not done	Valid	Negative

All solid culture (LJ) results were reported according to the grading outlined in the table 3.

Table 3. Interpretation of solid culture (LJ) results

Colonies seen in the slope	LJ interpretation results
None	Negative
<20 colonies	Record number of colonies
20-100 colonies	+
Innumerable discrete colonies	++
Confluent	+++
Contaminated	Contaminated

Copies of the final result of liquid and solid culture were sent to the study clinic and data entry unit while the original forms were kept at the laboratory.

d) Smear microscopy

The smear microscopy was used to confirm the presence of mycobacteria in sputum samples and in positive cultures from MGIT and LJ.

After decontamination of the sputum samples during culture process, 30µl of the suspension was transferred to a slide and spread to cover an area of 2x1cm diameter circle. Slides were allowed to air dry inside the biosafety cabinet and then heat fixed on the flame fire before staining with Ziehl Neelsen standard protocol. After staining, slides were allowed to dry and examined by binocular optic microscopy.

For positive MGIT cultures, a small amount of sediment was removed directly from the bottom of the BBL MGIT tube using a sterile Pasteur pipette and one drop was added to 30µl of blood plasma in the slide, spread to cover an area approximately of 2x1cm diameter circle and allowed to dry inside the Biosafety Cabinet (BSC) prior Ziehl Neelsen standard staining. On positive LJ slopes the smear was prepared by picking part of the bacterial colony with 10µL loop and emulsify in the slide containing one drop of sterile water. After drying, the slide was heat fixed and stained by Ziehl Neelsen standard protocol.

All microscopy examination were done with 100X oil objective by reading 100 fields of the slide according to the International Union Against TB and Lung Diseases/ WHO (IUATLD/WHO) scaling system outlined in the table 4.

Table 4. Interpretation of smear microscopy results using Ziehl Neelsen staining

	No. of AFBs (average over 100 fields)	Reporting result
Sputum smear microscopy	None	No AFB seen in 100 fields (NS)
	1-9 per 100 fields	scanty/or actual number)
	1-9 per 10 fields,	+
	1-9 per field, read 50 fields	++
	>9 per field, read 20 fields	+++
Positive MGIT and LJ sample	None	Negative
	1 or more AFB	Positive AFB, Atypical/typical, cord/without cord

e) Confirmation of *M. tuberculosis* complex

The confirmation of *M. tuberculosis* complex in cultures was done on all AFB smear-positive MGIT tubes and LJ slopes by using the rapid immunochromatographic identification test SD BIOLINE TB Ag MPT64 Rapid, which detects the antigen MPT64, a mycobacterial protein fraction secreted from M. tuberculosis complex cells during culture. However, the molecular assay GenoType® MTBDR*plus* Version 2 (HAIN Life Science, Germany) was applied on the first positive culture (AFB smear-positive MGIT or LJ slope) of the study patient, not only to confirm the infection of Mtb, but also to quickly screen for Rifampicin and Isoniazid resistance.

f) BACTEC MGIT 960 Drug Susceptibility test

The drug susceptibility test for Isoniazid, Rifampicin, Ethambutol and Pyrazinamide was done according to the standard procedure in BACTEC MGIT 960 system which monitors the fluorescence in the drug-containing tubes (test tube) compared to the fluorescence in the growth control tube containing sensitive *M. tuberculosis* strain (H37Rv, ATCC 27294) to determine susceptibility results. The critical concentration of the antibiotics in the MGIT tubes were 0.1µg/ml for Isoniazid, 0.1µg/ml for Rifampicin, 5.0µg/ml for Ethambutol and 100µg/ml for Pyrazinamide. An isolate was determined resistant if 1% or more of the test population grew in the presence of the critical concentration of the drug. The BACTEC MGIT 960 automatically interpreted the results and reported a susceptible (S) or resistant (R) result for the drugs tested.

The drug susceptibility testing were performed on pre-treatment isolates (screening and/or baseline) and on isolates obtained at week 17 and 26 (causing a suspicion of failure), in order to identify the presence of resistance. If the screening sample (LJ and/or MGIT) was contaminated and pure culture could not be obtained, it was acceptable to use a baseline or week 1 culture, or samples from later time-points as a back up to perform the susceptibility profile.

g) The Molecular Bacterial Load Assay (MBLA)

In order to measure the amount of viable Mtb by counting the 16S ribosomal RNA (16S rRNA) present in the sample, sputum samples and BCG positive control (BCGNCTC5692) preserved in GTC were removed from the -80°C ultra freezer, thawed at room temperature and added 100µl of the internal control prior to ribonucleic acid (RNA) extraction (Vital Bacteria, UK) according to the manufacturers' instructions. The mixture was centrifuged at 3000g for 30 minutes. The supernatant was discarded and the cell sediment was re-suspended in lysis buffer, RNA pro blue solution (MP Biomedicals), and bead homogenized for 40 seconds at 6000rpm using the Precellys 24 (pEQlab, UK). RNA was isolated using FASTprep RNA kit (MP Biomedicals, UK) according to the manufacturer's instructions. Genomic DNA was removed from the extracts by a 1hour DNAase treatment at 37°C using the Ambion Turbo DNase kit (Life Technologies, UK). The reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)(Vital Bacteria UK) was performed in the RotorGene 5plex platform (Qiagen, Germany) using 20µl of the samples mixed with primers and Taqman dual labelled probes targeting Mtb and the internal controlaccording to the manufacturers' instructions. All primers and probes were procured from MWGEurofins, Germany.The optimal PCR cycling conditions included 50°C for 30 minutes (reverse transcription), 95°C for 15 minutes (Taq polymerase activation), 40 cycles of: 94°C for 45 seconds not acquiring, 60°C for 60 seconds acquiring at Green and Yellow cycling. The standard curves for translating cycle threshold (Ct) into bacterial load were also performed according to manufacturer's guideline. A high (10^7eCFU/ml) and low (10^3eCFU/ml) positive control (BCGNCTC5692) in artificial sputum, and negative control of RNase free molecular grade were included in each assay run. Each RNA sample and standard was amplified in duplicate and results interpreted as shown in table 5.

Table 5. Result interpretation and qPCR output data analysis

Target (Mtb/BCG)	Internal control	Result
Positive	Positive	Positive
Positive	Negative	Positive*
Negative	Positive	Negative
Negative	Negative	Invalid

Positive = shown by Cycle threshold (Ct) from the RT-PCR
Negative = shown by no Ct from the RT-PCR
* = The Mtb presence result is positive, but the result cannot be used for quantitative analysis or data normalization.
Invalid = Both target and IC are negative

3.2. The Resuscitation-promoting factor evaluation (Rpf)

Sputum samples of 5 participants from screening up to week 12 after treatment initiation were used to evaluate whether the Rpf-dependent *M. tuberculosis* could be found. The Rpf reagent was received lyophilized from University of St. Andrews, UK and kept in ultralow freezer at -80°C until its use.

a) Preparation of Rpf solution

The 50ml plastic conical tubes of lyophilized Rpf was removed from -80°C ultralow freezer and thawed at room temperature. An aliquot of 10ml sterile distilled water was prepared in 15ml plastic conical tube and kept at 2-8°C for 10 minutes. The lyophilized Rpf was mixed with 10ml of distilled water and left at room temperature for 30 minutes. After this time, the reagent was mixed again inverting the tube 5 times to ensure everything was dissolved. The MGIT PANTA supplement was reconstituted with 15ml MGIT growth supplement as described above. The reconstituted PANTA was added to Rpf solution at ratio of 145µl PANTA/10ml Rpf solution.

b) Sputum sample inoculation

The BBL MGIT tubes were unscrewed the cap and aseptically removed 3.5ml of 7H9 medium using a 100-1000µl automated micropipette. The 3.5ml of 7H9 medium was replaced with 3.5ml of the Rpf-PANTA solution. The sample inoculation was done by adding 500µl of the decontaminated sputum to the Rpf-MGIT tube (see decontamination process above). Immediately the tube was recapped tightly and mixed by inverting 5 times before incubation in the BD BAC-

TEC MGIT 960 system. From the incubation, all downstream procedures of the Rpf-culture were the same with the standard culture described above.

The Rpf-culture results were compared with the same sample submitted to MGIT culture without Rpf in order to compare the effect of Rpf. All Rpf-culture results were not used to decide about TB treatment at the clinic study.

3.3. Evaluation of OMNIGene.SPUTUM on sputum liquefaction, decontamination and preservation of *M. tuberculosis*

This sub-study was conducted between October 2015 and August 2016 to evaluate the novel reagent OMNIgene.SPUTUM (OM-S, DNA Genotek, Ottawa, ON, Canada) for sputum liquefaction, decontamination and preservation of *M. tuberculosis* viability. Smear positive sputum from 139 new TB cases or cases having received less than 1 month anti-TB treatment were assigned to one of two study arms: the same-day (SD) arm (samples processed within 7 hours of reaching the National TB ReferenceLaboratory) to test short-term exposure to OM-S, and the 5-day (5-D) arm (samples incubated for 5 days at room temperature prior to processing at National TB Reference Laboratory) to test long-term exposure to OM-S. In SD arm, samples were homogenized and splited in two equal fraction. One had an equal volume (1:1) of OM-S added to it on sample recruiting site, and the other (control sample destined for standard decontamination protocol) was not treated. OM-S-treated samples were transported to the National TB Reference Laboratory at ambient temperature, and a refrigerated cold box was used for control samples. On other hand, raw samples on 5-D arm were transported to the same laboratory where were homogenized and splited in two equal fraction: one had an equal volume (1:1) of OM-S added and the other (control) was not treated. Both samples were then incubated at room temperature for 5 days, after which the control sample was subjected to standard decontamination protocol (SDP) and OM-S sample submitted to Omnigene protocol.

The SDP is described above in the section 3.1.3. line c). Prior the processing, 1ml of each samples was preserved in 1:4 guanidine thiocyanate (GTC) (Promega, Southampton, UK) solution with 1% β-mercaptoethanol v/v (Sigma Aldrich, Gillingham, UK) and stored at −80°C for MBLA as described in the section 3.1.3. line g). The processing of OM-S-treated sample envolved addition of equal volume of Phosphate Buffer (PB), centrifugation of the sample at 3000×g for 15

minutes to remove OM-S and resuspension of the sediment in 1ml PB. The sediment was used to inoculate MGIT and LJ cultures[47].

3.4. The assessmentof reproducibility and limit of detection of the MBLA, culture and Xpert MTB/RIF assay

The limit of detection of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

Sample constituted of the reference strains of *M. tuberculosis* (H37Rv, ATCC 27294) was aseptically subcultured on 10 BBL MGIT tubes and incubated at 37°C in the BD MGIT 960 system. After 5 days of incubation, the tubes flagged positive. To allow multiplication of *Mtb* population the tubes were incubated for more 10 days at 37°C in the incubator (Memmert GmbH, Germany). Prior the assessment, all samples were confirmed to be not contaminated by inoculating in blood agar as described above.

For assessment, 10 samples were divided in two groups of 5 tubes. Each sample group (total volume approximately 35ml) were mixed into a 50ml plastic conical tube. The tube was then closed tightly and centrifuged for 15 minutes at 3000g speed to concentrate the *Mtb* colonies. After centrifugation, the samples were allowed to stand for 15 minutes and carefully 15ml of the supernatant fluid were removed from each tube using a 100-1000µL automated micropipette. The two fractions of remaining 20ml of Mtb colonies were ressuspended by vortexing 10-15 seconds and then pooled to have total volume of 40ml. The sample was then inverted five times to ensure the same population in the tube. The 40ml sample was divided in 4 equal fractions of 10ml into a 50ml plastic conical tube. At this point two arms were created:

- **Treated sample arm:** two 50ml plastic conical tubes were treated for culture with the standard procedure of NALC-NaOH sodium citrate, as described above. The pellet in each tube was re-suspended with 3ml of sterile phosphate buffer solution (PB) at pH 6.8 using an automated 100-1000µl micropipette and then pooled to achieve 6ml of the final working sample.

- **Untreated sample arm:** Each two 50ml plastic conical tubes were filled up to 50ml with sterile phosphate buffer solution at pH 6.8, centrifuged at 3000g for 15 minutes, discarded the supernatant and the pellet of colonies ressuspended with 3ml of PB using an

automated 100-1000µl micropipette. The samples were pooled and to achieve 6ml of the final working sample.

The 6ml samples in each arms were divided in equal volume for culture (2ml), MBLA (2ml) and Xpert MTF/RIF assay (2ml). For each assay within the arms, samples were diluted 10 times in a v/v ratio of 1mL sample/9mL distilled water from dilution $1x10^{-1}$ up to $1x10^{-9}$ and then processed for comparison in triplicate (figure 3).

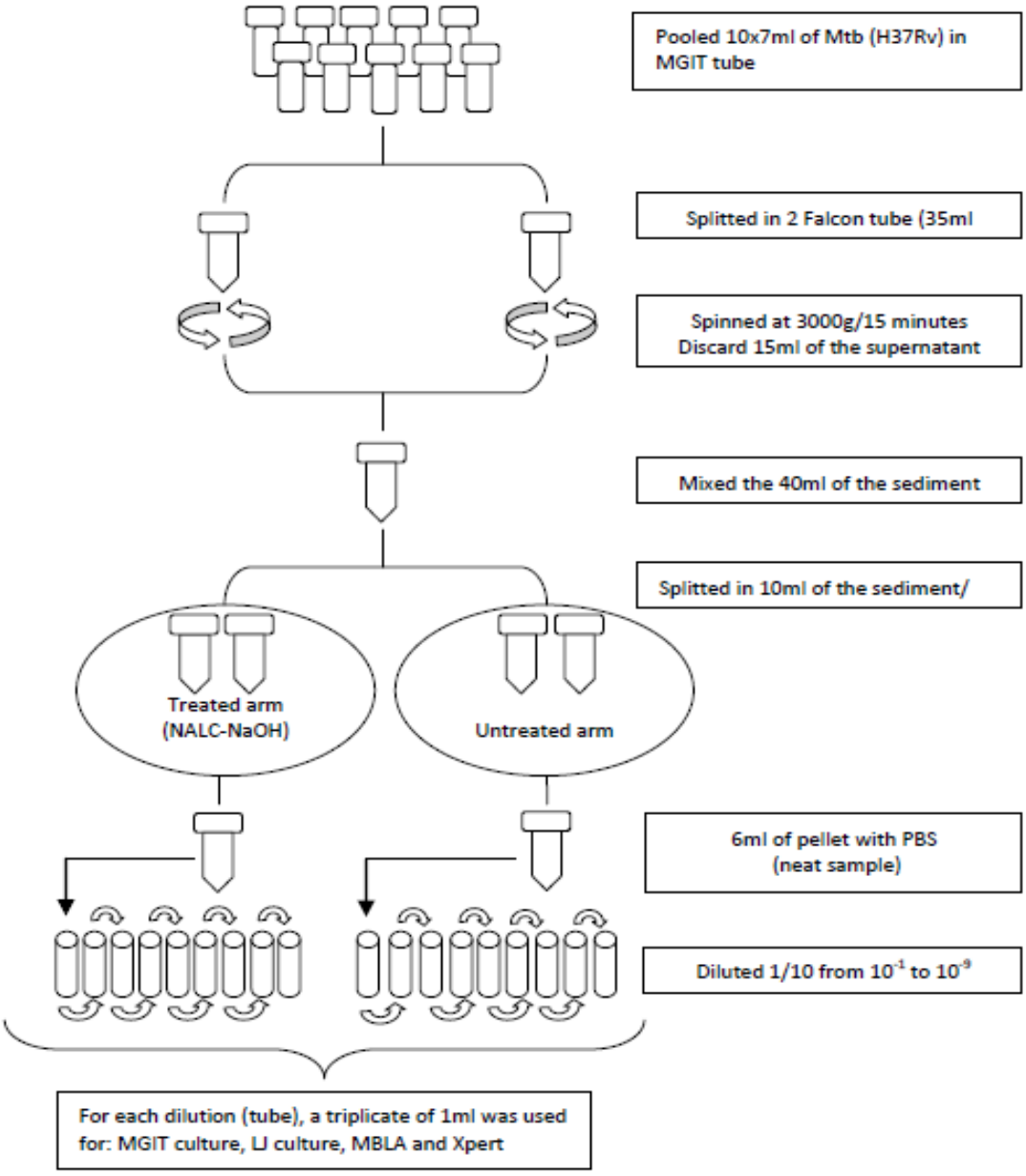


Figure 3. Diagram describing the steps of sample preparation to assess the reproducibility and limit of detection of culture, MBLA and Xpert assay

3.5. Study definitions

Baseline is the (first) day which the patient was included into the study.

Time to positivity or Time to culture positivity (TTP) was defined as the number of days from time of sample inoculation into MGIT vial to the first positive culture result that the instrument flagged positive. The valid TTP does not include results of contaminated cultures[78].

Cycle threshold (Ct) corresponds to the number of PCR cycles required to detect MTB. Each subsequent cycle represents approximately 50% less starting material than the last, thereby providing a semi-quantitative result of bacillary burden, with higher Ct results reflecting lower bacillary burden[79].

3.6. Data analysis

A database was created on MS Excel for statistical analysis. The Spearman's coefficient to determine the correlation and the Mann-Whitney U test to determine the differences between variables was done using GraphPad Prism v.6 (GraphPad Software, La Jolla, CA, USA) at 95% confidence interval. The General linear model and linear regressions were done on IBM SPSS Statistics program Version 20. Bacterial load is presented in mean with standard deviation at $\log_{10}$.

3.7. Ethical considerations

The study protocol and informed consent was approved by the Mozambique's National Review Board (Ref 274/CNBS/13) and the Ethics Commission of Ludwig-Maximilians Universität, Munich. At the study clinic, each participant received written information about the study and the consent form that was signed and dated by the patient and the member of staff administering the consent.

The sub-study of OMNIGene.SPUTUM evaluation was approved by Instituto Nacional de Saúde institutional review board and national ethics committee for Mozambique (97/CNBS/16) and by the medicine ethics committee on behalf of the university teaching and research ethics committee for the University of St Andrews (MD11983).

Good clinical laboratory practice rules were followed in line with the World Medical Association Declaration of Helsinki.

All patient information was treated as confidential and personal data were anonymized by unique study ID number. All files were stored at a secure, locked place to which only authorized study staff had access. Paper case report forms were entered into a secured clinical data management system.

The risks related to study participation were considered low as sputum collection is painfulness and the instructions to collect were done in concordance with the national guidelines. Study patients were not paid for their participation in the study.

4. Results

4.1. Study participants and demographic characteristics

From June 17th 2014 to May 28th2015, a total of 103 patients were screened, and 69 who met inclusion criteria and were enrolled into the MaTuTU study. A cohort of 58 participants had all samples for each study visit submitted simultaneously to both culture and MBLA, and were included in this PhD project and data analysis (figure 4).

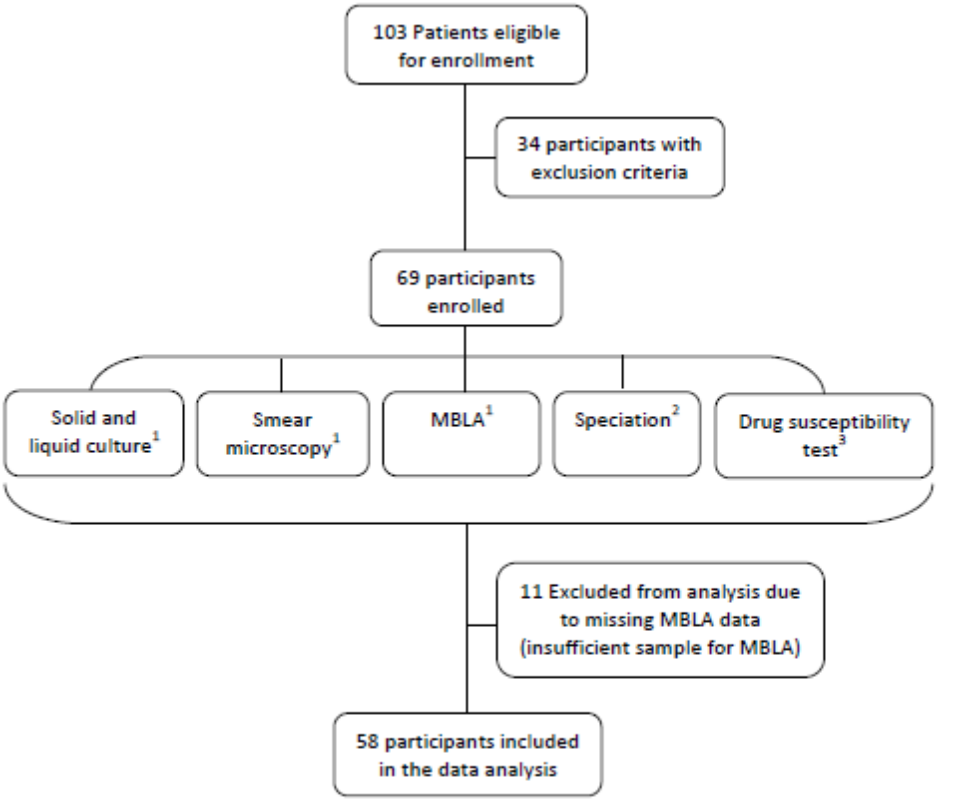


Figure 4. Participant flow and sample testing

Of the 58 participants, 38 (65.5%) were male and 39 (67.2%) were HIV positive. The median age was 30 (IQR: 18 – 56) and only 3 (5.2%) participants reported previous history of having TB. Majority of them had drug susceptible TB (89.7%) (table 6).

Table 6. Demographic and clinical characteristics of the study participants

Demographic or clinical characteristics	N=58
Age, years - median (IQR)	30 (18 - 56)
Male - n/N (%)	38/58 (65.5%)
Race (African) - n/N (%)	58/58 (100%)
HIV Positive - n/N (%)	39/58 (67.2%)
TB history - n/N (%)	3/58 (5.2%)
Resistance	**N=58**
Susceptible - n/N (%)	52/58 (89.7%)
Polyresistant to Isoniazid and Pyrazinamid - n/N (%)	2/58 (3.4%)
MDR - n/N (%)	4/58 (6.9%)

4.2. Comparison of the performance of culture and MBLA

4.2.1. The Liquid (MGIT) & solid (LJ) culture and MBLA results over the study visits

Overall, a total of 472 serial cultures, both solid (LJ) and liquid (MGIT), and MBLA were done for 58 participants from baseline to week 52. Only 11 (19%) participants provided a sputum sample at week 52 study visit. Over the study, 281 (60%) samples were MGIT TB positive of which 146 (31%) samples generated valid Time to Positivity (TTP) (i.e contamination-free and *M. tuberculosis* positive culture) and 135 (29%) presented *M. tuberculosis* growth in the presence of contaminants, thus, the TTP for these culture results could not be used in the further analysis. Fifty-eight (12%) samples grew contaminants and could not be defined as *M. tuberculosis* positive or negative. As the TB treatment progressed, the rate of MIGIT positivity decreased, while culture negativity and contamination increased. The LJ culture generated 144 (31%) positive results and 22 (5%) samples were contaminated making the LJ contamination rate lower than MGIT culture. In contrast, 289 (61%) samples were MBLA positive and contamination-free (table 7).

Table 7. MGIT, LJ and MBLA results per study visit

		RESULTS				
ASSAY	**VISIT**	**M.tb pos: % (n/N)**	**M.tb pos + Cont: % (n/N)**	**Cont**	**Negative**	**Missing**
MGIT	Baseline	55% (32/58)	43% (25/58)	2% (1/58)	0% (0/58)	0% (0/58)
	Week 1	45% (26/58)	38% (22/58)	3% (2/58)	12% (7/58)	2% (1/58)
	Week 2	53% (31/58)	29% (17/58)	5% (3/58)	7% (4/58)	5% (3/58)
	Week 4	48% (28/58)	19% (11/57)	10% (6/58)	17% (10/58)	5% (3/58)
	Week 8	21% (12/57)	19% (11/57)	9% (5/57)	51% (29/57)	0% (0/57)
	Week 12	9% (5/57)	14% (8/57)	25% (14/57)	44% (25/57)	9% (5/57)
	Week 17	9% (5/57)	19% (11/57)	33% (19/57)	39% (22/57)	0% (0/57)
	Week 26	9% (5/58)	45% (26/58)	14% (8/58)	31% (18/58)	2% (1/58)
	Week 52	18% (2/11)	36% (4/11)	0% (0/11)	36% (4/11)	9% (1/11)
LJ	Baseline	62% (36/58)	0% (0/58)	0% (0/58)	36% (21/58)	2% (1/58)
	Week 1	57% (33/58)	0% (0/58)	3% (2/58)	40% (23/58)	0% (0/58)
	Week 2	50% (29/58)	0% (0/58)	2% (1/58)	47% (27/58)	2% (1/58)
	Week 4	36% (21/58)	0% (0/58)	9% (5/58)	55% (32/58)	0% (0/58)
	Week 8	23% (13/57)	0% (0/57)	11% (6/57)	67% (38/57)	0% (0/57)
	Week 12	11% (6/57)	0% (0/57)	0% (0/57)	88% (50/57)	2% (1/57)
	Week 17	5% (3/57)	0% (0/57)	4% (2/57)	91% (52/57)	0% (0/57)
	Week 26	5% (3/58)	0% (0/58)	10% (6/57)	84% (49/58)	0% (0/58)
	Week 52	0% (0/11)	0% (0/11)	0% (0/11)	91% (10/11)	9% (1/11)
MBLA	Baseline	100% (58/58)	0% (0/58)	0% (0/58)	0% (0/58)	0% (0/58)
	Week 1	97% (56/58)	0% (0/58)	0% (0/58)	3% (2/58)	0% (0/58)
	Week 2	95% (55/58)	0% (0/58)	0% (0/58)	5% (3/58)	0% (0/58)
	Week 4	83% (48/58)	0% (0/58)	0% (0/58)	17% (10/58)	0% (0/58)
	Week 8	68% (39/57)	0% (0/57)	0% (0/57)	32% (18/57)	0% (0/57)
	Week 12	42% (24/57)	0% (0/57)	0% (0/57)	58% (33/57)	0% (0/57)
	Week 17	7% (4/57)	0% (0/57)	0% (0/57)	93% (53/57)	0% (0/57)
	Week 26	7% (4/58)	0% (0/58)	0% (0/58)	93% (54/58)	0% (0/58)
	Week 52	9% (1/11)	0% (0/11)	0% (0/11)	91% (10/11)	0% (0/11)

Pos= positive (for M.tb); Cont= contamination

4.2.2. Concordance and discordance of MGIT culture and MBLA results

The comparison of the performance of MGIT culture with MBLA is shown in figure 5. At baseline, all 58 (100%) participants had a MBLA positive result including quantitative eCFU/ml count. In contrast, 57 (98%) were MGIT culture TB positive, of whom 32 (55%) were MGIT culture positive and free of contamination, 25 (43%) MGIT culture positive but contaminated and one (2%) participant had undefined result (contaminated, neither positive nor negative). In subsequent visits as treatment progressed the number of participants that were both MBLA and MGIT posi-

tive decreased. In parallel there was an increase in number of MGIT negatives from 2 (4%) participants at week 1 to 19 (33%) at both week 17 and 26. Additionally, an increase of samples contaminated in MGIT was observed in the late stages of anti-TB treatment. After week 12, there were only 6 (5%) participants that were both MBLA and MGIT positive but continuously contaminated, rendering an invalid TTP result. Taking all study visits together, 44 (10%) samples were positive on the MBLA but were negative on MGIT (potential MGIT false negative), whereas only 20 (4%) samples were negative on MBLA but positive on MGIT (potential MBLA false negative).

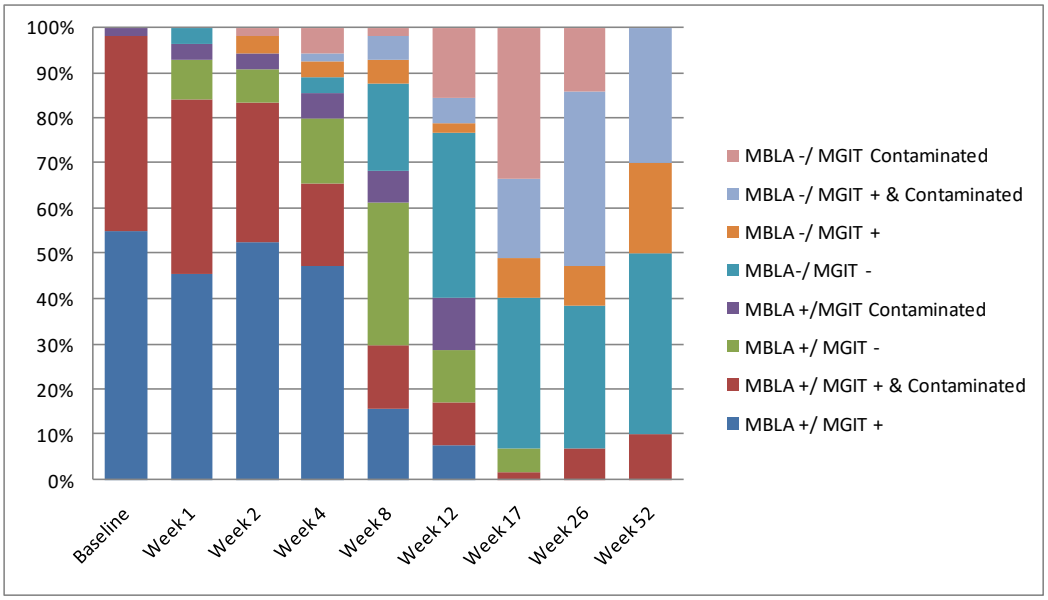


Figure 5. Comparison of MBLA and MGIT results during TB treatment

Like MGIT culture, the LJ also showed a decrease in the number of both MBLA and LJ positive from 34 (60%) participants at baseline to 5 (9%) at week 12. Again, this decrease was parallel to increase of MBLA and LJ negative results. The overall LJ contamination rate was lower compared with MGIT culture. Only 13 (23%) samples were lost to contamination when MBLA was positive and 9 (16%) samples when MBLA was negative. From baseline to week 26 of visit, 146 (32%) samples generated positive MBLA and negative LJ (potential LJ false negative). On other hand, only 14 (3%) samples were negative for MBLA and positive for LJ (potential MBLA false negative)(figure 6).

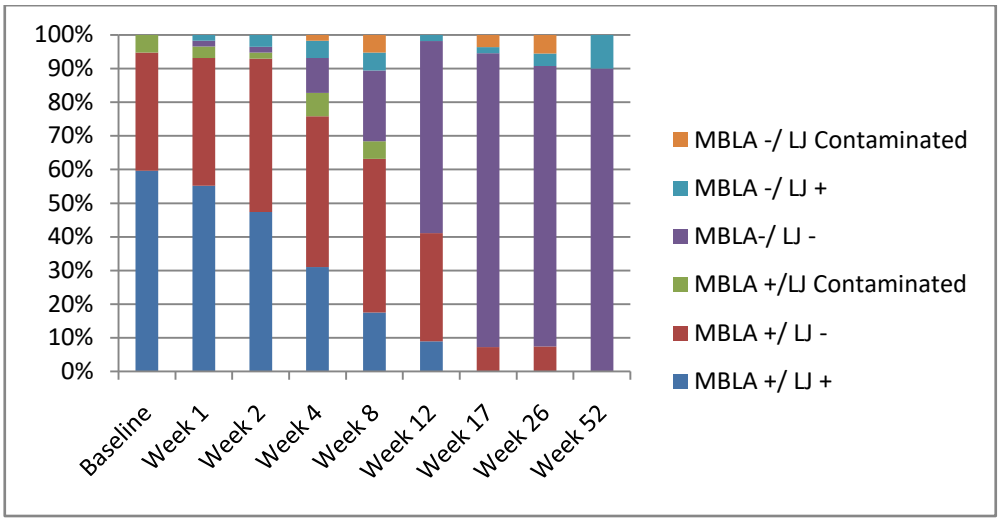


Figure 6. Comparison of MBLA and LJ results during TB treatment

4.2.3. Comparison between the MGIT Time to Positivity and bacterial load (log_{10}eCFU/ml)

Valid MGIT TTP was determined as time to culture positivity in absence of contamination of which 144 MGIT samples had valid TTP, 13.11±11.7 days. MGIT TTP was inversely correlated to the bacterial load measured by MBLA, Spearmans r=-0.67, p<0.0001. This TTP increased from 4.11±2.6 days at baseline (n=32) to 15.7±13.4 days at week 26 (n=5). MGIT culture contamination meant that most of the samples were lost to contamination or had no valid TTP as treatment progressed. The overall mean of bacterial load of the 144 samples was 4.19±2.04log_{10}estimate colony forming units (eCFU)/ml. Bacterial load declined from 6.18±1.07log_{10}eCFU/ml at baseline (n=32) to 0±0eCFU/ml at week 26 of treatment (n=5). Figure 7 shows the correlation of MGIT TPP and Bacterial load by MBLA.

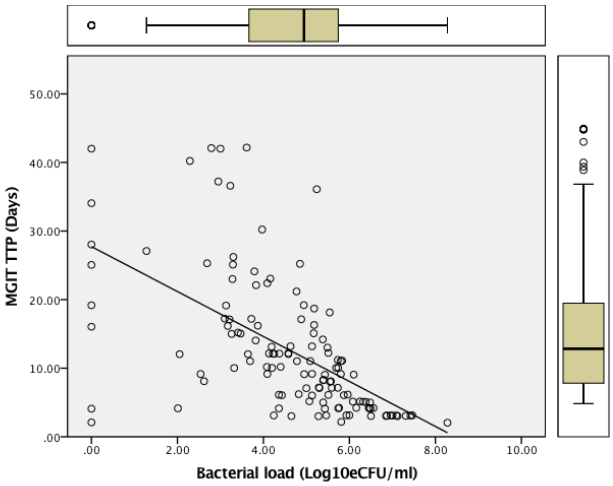


Figure 7. Global correlation between MGIT TTP and Bacterial load. MGIT TTP had higher variation from the median (vertical Box plot) compared to MBLA (horizontal box plot).

When assessed weekly, the correlation between MGIT TPP and bacterial load could no longer hold after two weeks of treatment. The relationship was very strong at baseline, r= -0.72, p<0.0001; reducing to r= -0.68, p=0.0001 at week 2; then r = -0.06, p=0.76 at week 4; and r = -0.64, p=0.12 at week 8. The dwindling correlation corresponded with an increasing reduction of valid MGIT TTP, which reduced the number of pairs to be tested: N = 31, 31, 25, 7 at baseline, week 2, week 4 and week 8, respectively. Correlations at weeks 12, 17, 26 and 52 could not be tested as there were two or less valid MGIT TTP results available at these weeks (Figure 8A, B, C & D).

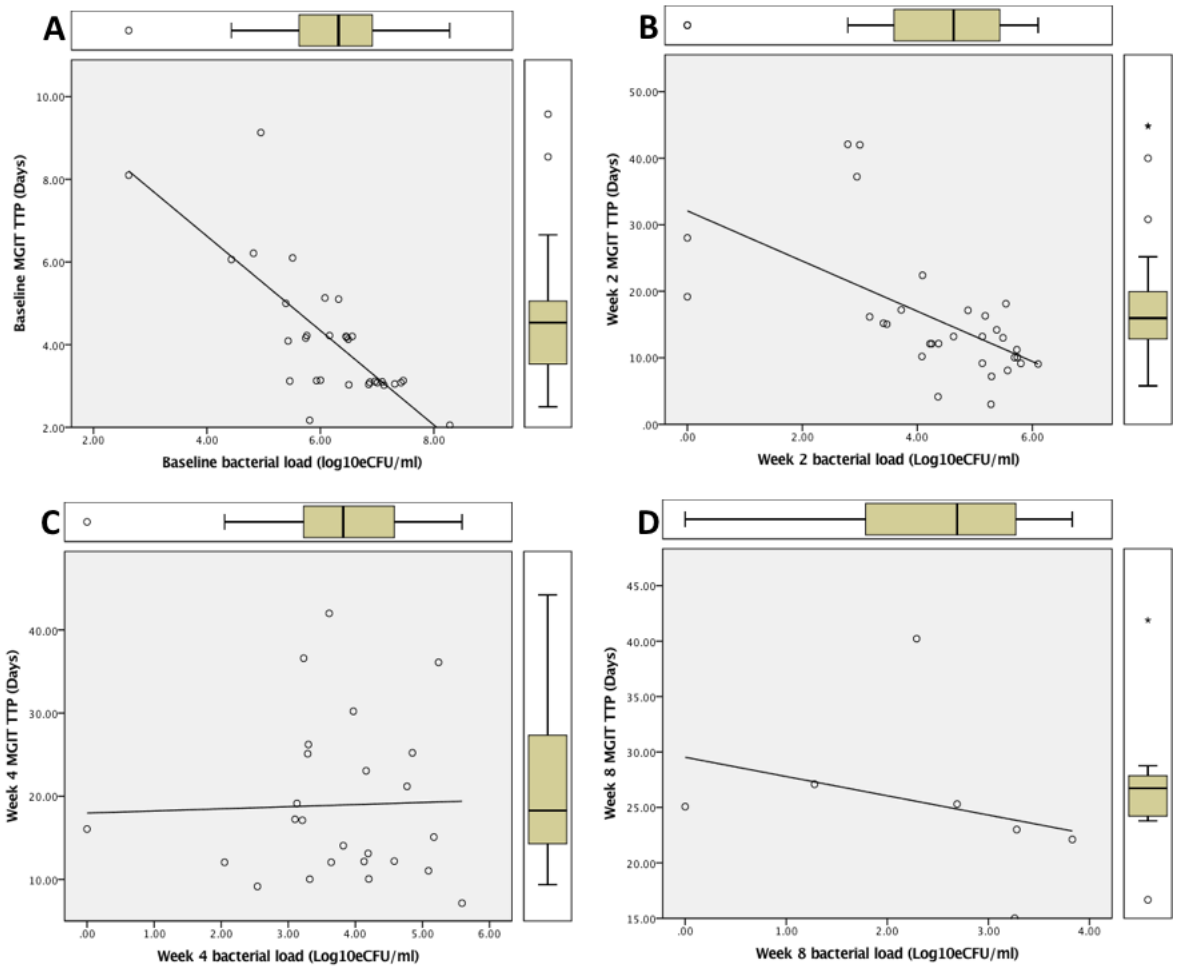


Figure 8. Week by assessment of the correlation between MGIT TTP and Bacterial load. A) Correlationat baseline, B) correlation at week 2, C) correlation at week 4 and D) correlation at week 8. Box plots are medians and interquartile ranges from the median.

4.3. Comparison of culture and MBLA to define treatment outcome

This study also explored the utility of MBLA to define treatment outcome in relation to MGIT, LJ and smear. We assumed that patient could be defined as treatment failure if results from MGIT, LJ and smear were consecutively positive at week 17 and 26. The data showed that of the 56 patients, 16 (29%) had MGIT positive and 4 (7%) had MBLA positive at week 17. Only 1 (2%) patient had both MGIT and MBLA positive result at this study visit. Around 18 (32%) MGIT had no result during this study visit due to contamination. At week 26, the number of MGIT positive increased to 31 (55%) patients whilst the number of patients with MBLA positive remained the same, and interestingly this 4 (7%) patients with positive MBLA were also MGIT positive. Assessment of positivity at week 17 and 26 showed that 10 (18%) patients were consecutively

MGIT positive, and thus could be defined as treatment failure. None of the 56 (100%) patients had consecutive MBLA positive results at week 17 and 26, meaning that all patients would be considered cured by this assay.

For LJ, out of 57 patients, 1 (2%) was LJ positive at week 17 and 3 (5%) at week 26. None of them were consuctively LJ positive at week 17 and 26. Likewise, 4 (7%) patients were MBLA positive at week 17 and 26 but none of them were consecutively positive for both weeks. Eight patients, 2 (4%) at week 17 and 6 (10%) at week 26, had no LJ results due the contamination. Overall, and according to our assumption, no patients were ascribed as failed treatment based on LJ and MBLA. According to smear results, 4 (7%) patients were positive (scanty AFB) at week 17 and no smear positive were observed at week 26. Taken together, all these patients could be declared as cured based on smear microscopy and MBLA (table 8).

Table 8. Treatment outcome of the patients based on the MGIT, LJ, smear and MBLA.

Week 17		Week 26		n (%)	Outcome by MGIT	Outcome by MBLA
MBLA	MGIT	MBLA	MGIT			
Negative	Positive	Negative	Positive	8 (14%)	Failure	Cured
Negative	Positive	Positive	Positive	2 (4%)	Failure	Cured
Negative	Negative	Negative	Negative	7 (13%)	cured	cured
Negative	Negative	Negative	Positive	7 (13%)	cured	cured
Negative	Positive	Negative	Negative	3 (5%)	cured	Cured
Positive	Negative	Negative	Negative	1 (2%)	cured	Cured
Positive	Negative	Negative	Positive	2 (4%)	cured	Cured
Negative	Negative	Positive	Positive	1 (2%)	cured	Cured
Negative	Contaminated	Negative	Negative	5 (9%)	Unknown	Cured
Negative	Contaminated	Negative	Contaminated	2 (4%)	Unknown	Cured
Negative	Contaminated	Negative	Positive	10 (18%)	Unknown	Cured
Negative	Negative	Negative	Contaminated	4 (7%)	Unknown	Cured
Negative	Positive	Negative	Contaminated	2 (4%)	Unknown	Cured
Negative	Contaminated	Positive	Positive	1 (2%)	Unknown	Cured
Week 17		Week 26		n (%)	Outcome by LJ	Outcome by MBLA
MBLA	LJ	MBLA	LJ			
Negative	Negative	Negative	Positive	3/57 (5%)	Cured	Cured
Negative	Negative	Negative	Negative	38/57 (67%)	Cured	Cured
Positive	Negative	Negative	Negative	4/57 (7%)	Cured	Cured
Negative	Positive	Negative	Negative	1/57 (2%)	Cured	Cured
Negative	Contaminated	Positive	Negative	1/57 (2%)	Unknown	Cured
Negative	Contaminated	Negative	Negative	1/57 (2%)	Unknown	Cured
Negative	Negative	Positive	Negative	3/57 (5%)	Cured	Cured
Negative	Negative	Negative	Contaminated	6/57 (11%)	Unknown	Cured
Week 17		Week 26		n (%)	Outcome by Smear	Outcome by MBLA
MBLA	Smear	MBLA	Smear			
Negative	Negative	Negative	Negative	46/57 (81%)	Cured	Cured
Positive	Negative	Negative	Negative	3/57 (5%)	Cured	Cured
Negative	Positive	Negative	Negative	3/57 (5%)	Cured	Cured
Negative	Negative	positive	Negative	4/57 (7%)	Cured	Cured
Positive	Positive	Negative	Negative	1/57 (2%)	Cured	Cured

4.4. 4.4. Evaluation of relationship of bacterial load and Clinical diagnostic paramaters

4.4.1. Relationship between bacterial load and percentage of lung affected

The study explored the relationship between bacterial load and percentage of lung area affected at baseline. The X-ray showed that among 58 participant, the extent of the lung area affected varied from 5 to 45%. At 5% significance level, we found no significant Spearman correlation between bacterial load and percentage of lung affected, r=0.1613 (-0.1114 to 0.4114; p=0.23) (figure 9).

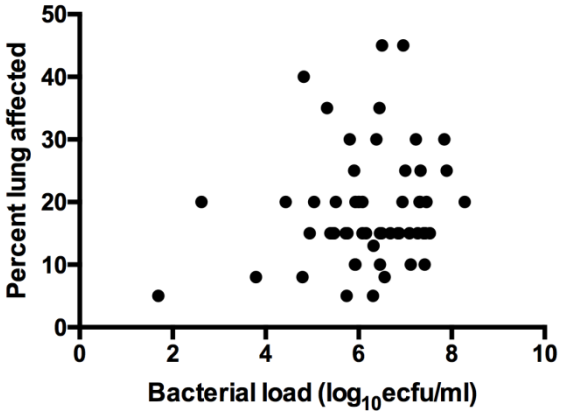


Figure 9. Correlation between MBLA and percentage of lung affected by TB at baseline visit

In addition the study did not find correlation between bacterial load and number of cavities in the lungs at baseline among 57 participants, Spearman's correlation r=0.054 (-0.216 - 0.3181), (p=0.69) at 5% significance level (figure 10).

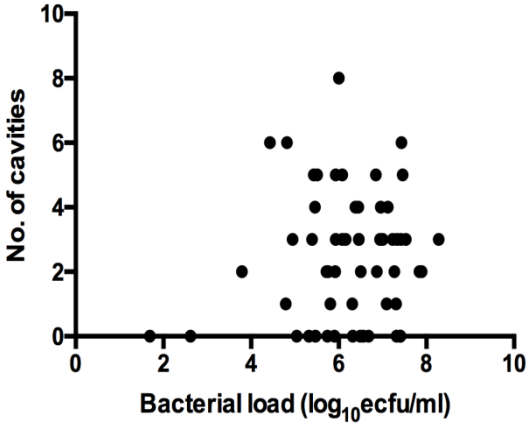


Figure 10. Correlation between bacterial load and number of lung cavities at baseline visit

4.4.2. Relationship between bacterial load and respiratory rate

The study asked if there was correlation between bacterial load and respiratory rate (number of breath per minute) at 5% significance level from baseline to week 26 of treatment. A positive correlation was found between the two variables, bacterial burden and respiratory rate r=0.2856 (0.1977 – 0.3690), p<0.0001(figure 11).

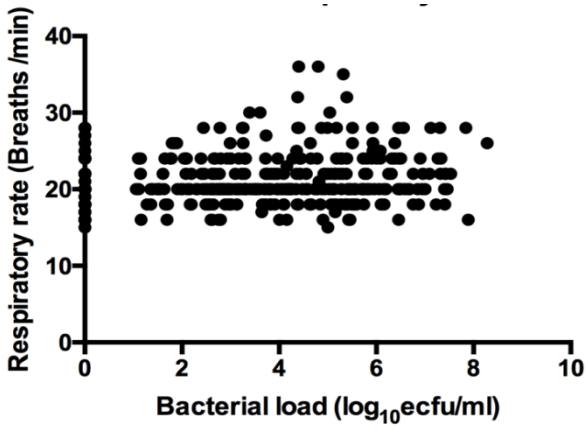


Figure 11. Correlation between bacterial load and respiratory rate

4.4.3. Effect of the weight of the patients in bacterial load

Weight is a clinical diagnostic parameter for tuberculosis and was measured at every patient visit in this study. We explored whether there is correlation between bacterial load and weight. At 5% significant level, weight had negative correlation with bacterial load, Spearmans r=-0.2395 (-0.3255 to -0.1497), p<0.0001 (figure 12).

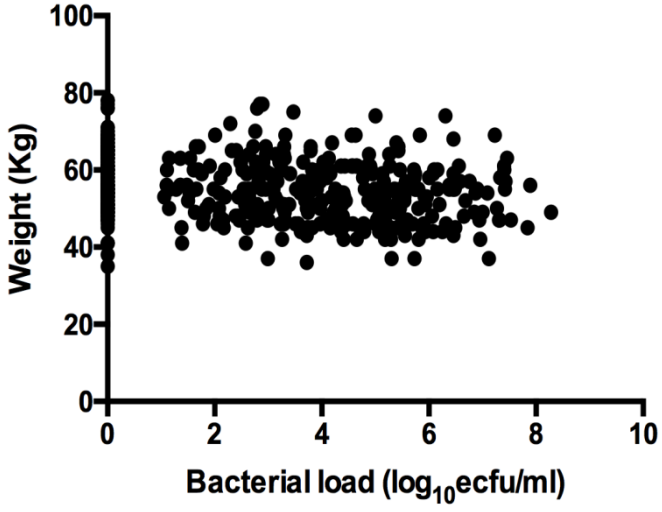


Figure 12. Correlation between bacterial load and weight

4.4.4. Relationship between lung function with bacterial load, weight and respiratory rate

The study explored the relationship between lung function with bacterial load, weight of the participants and their respiratory rate. In 413 measurements of lung function, 214 (51.8%) times were classified as normal and 199 (48.2%) were abnormal. The mean of TB bacterial load, weight and respiratory rate correspondent to both respiratory evaluations showed that participants with abnormal lung function had higher bacterial load (3.07±2.11log_{10}eCFU/ml), lower weight (53.54Kg) coupled with higher respiratory rate (21.29 breaths/min) (Table 9).

Table 9. Relationship between lung function with bacterial load, weight and respiratory rate

Lung function	N	Mean bacterial load (Log10ecfu/ml)	Mean Weight (kg)	Mean respiratory rate (breaths/min)
Normal	214	1,45	57,62	19,72
Abnormal	199	3,07	53,54	21,29

4.5. Relationship of bacterial burden with demographic factors and HIV co-infection

4.5.1. Effect of sex of participants in bacterial load

The study population was constituted of 38 (66%) male and 20 (34%) female. Overall bacterial load from baseline to end of treatment was higher in males (2.83±2.40log_{10}eCFU/ml) than in females (2.67±2.45log_{10}eCFU/ml), p=0.03. In the group of male, the bacterial load decreased from 6.27±1.32log_{10}eCFU/ml at baseline to 0.27±0.60log_{10}eCFU/ml at week 26 with a time to conversion of 119 days where the bacterial load was 0.12±0.42log_{10}eCFU/ml. In the group of female, the bacterial load decreased from 6.15±1.00log_{10}eCFU/ml at baseline to 0.00±0log_{10}eCFU/ml at week 26 with time to conversion of 84 days where bacterial load was 0.86±1.21log_{10}eCFU/ml (figure 13).

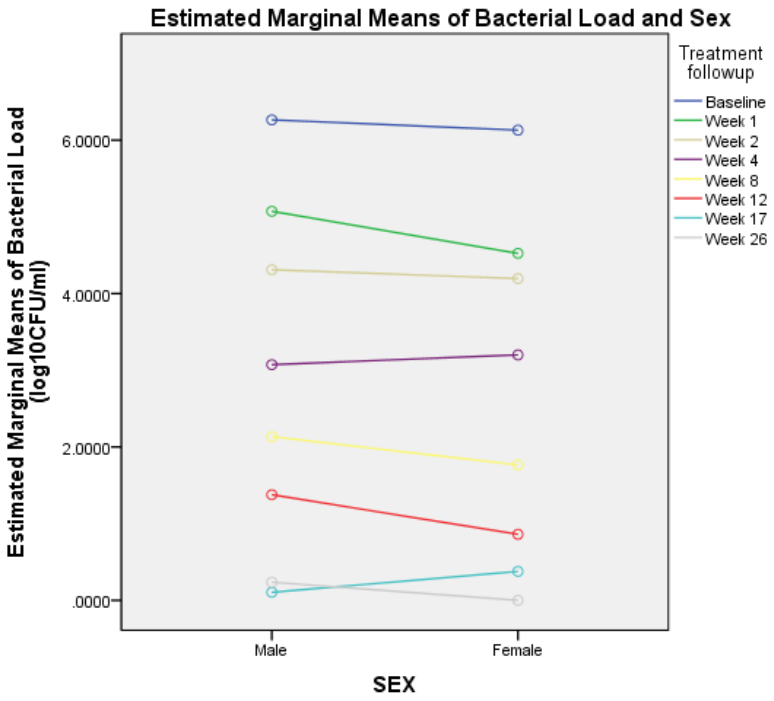


Figure 13. Relationship between sex and bacterial load

4.5.2. Effect of age of participants in bacterial load

The study further asked if age has an impact of bacterial load and its clearance during treatment. Participants were divided in two groups: below median age (<30 years) and those above the median age (≥30 years). Twenty-four (41.4%) participants had <30years and their overall bacterial load was 2.52±2.37log_{10}eCFU/ml while 34 (58.6%) participants had ≥30 years with overall bacterial load 2.89±2.41log_{10}eCFU/ml. The bacterial load difference between the two age groups was not statistically significant, p=0.51. In participants below 30 years, bacterial load decreased from 6.03±1.33log_{10}eCFU/ml at baseline to 0.24±0.58log_{10}eCFU/ml at week 26 of treatment, with time to conversion of 119 days when bacterial load was 0.30±0.75log_{10}eCFU/ml. In participants with and above 30 years, the bacterial load decreased from 6.36±1.13log_{10}eCFU/ml at baseline to 0.13±0.45log_{10}eCFU/ml at week 26 with similar time to conversion, 119 days and bacterial load of 0.13±0.50log_{10}eCFU/ml (figure 14).

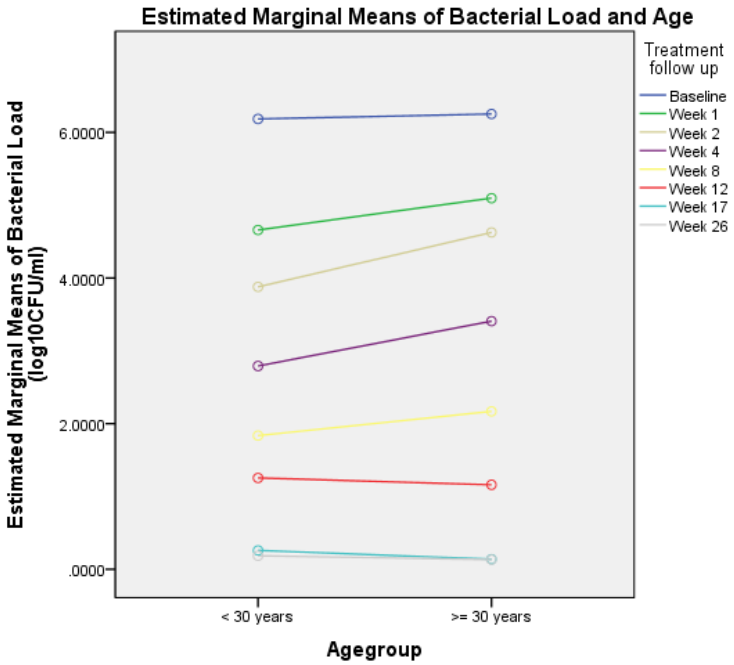


Figure 14. Relationship between bacterial load and age group

4.5.3. Effect of HIV co-infection and bacterial load

HIV co-enfection has been shown to compromise immunity and cause reactivation of tuberculosis. The study asked if underlying HIV co-infection has effect on patient TB bacterial load and its clearance during treatment. Of the 58 participants, 39 (67.2%) were HIV positive and 19 (32.8%) were HIV negative. The bacterial load decreased from 6.30±0.98log_{10}eCFU/ml at baseline to 0.17±0.52log_{10}eCFU/ml at week 26 of treatment among HIV+ participants. Time to conversion in the group of HIV+ participants was 120±49days at an average bacterial load of 0.22±0.59log_{10}eCFU/ml. In HIV- participants, the baseline bacterial load was 6.07±1.61log_{10}eCFU/ml decreasing to 0.20±0.47log_{10}eCFU/ml at week 26. The HIV- group had shorter time to conversion of 118±50 days and a slight lower bacterial load, 0.17±0.50log_{10}eCFU/ml at time of conversion. The difference between the bacterial loads of two groups was not statistically significant, p=0.85 (Unpaired t Test) (figure 15).

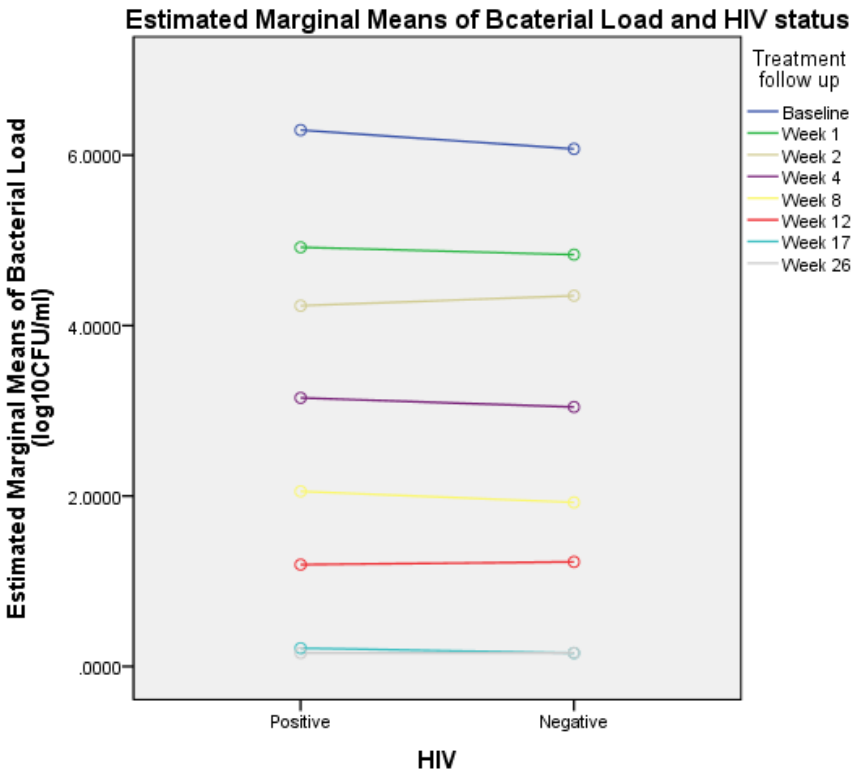


Figure 15. Relationship between bacterial load and HIV status

4.5.4. Relationship between CD4 count and bacterial load among patients with HIV+

The CD4 count was measured in two time points during the study visit: at baseline and week 26. To compare the relationship between CD4 count and bacterial load, the 39 participants with HIV+ were divided in two groups: with CD4 <300 and with CD4≥300. At baseline, 24 (62.0%) had CD4<300 and 15 (38.0%) had CD4≥300. The average bacterial load of the group with CD4<300 was 2.61±2.31log_{10}eCFU/ml while in the group with CD4≥300 was 3.10±2.51log_{10}eCFU/ml. The difference in the the bacterial load of the two groups was not significant, p=0.31 (figure 16).

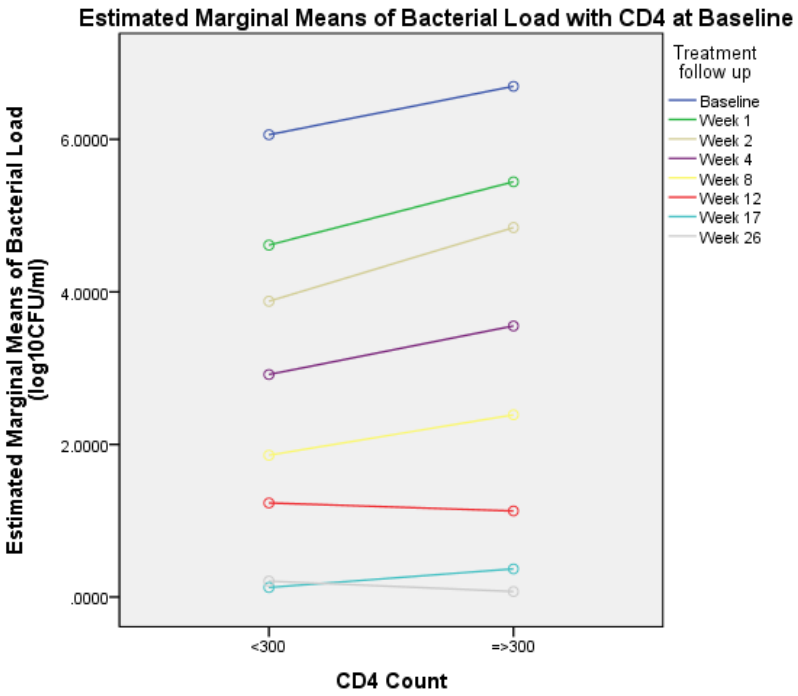


Figure 16. Relationship between CD4 count and bacterial load at baseline

At week 26 of treatment, 28 (72.0%) participants had record of CD4 measurement. Of the 28, 12 (43%) had CD4<300 and 16 (57.0%) had CD4≥300. At this treatment point, the bacterial load of the CD4<300 group, 0.45±0.84$\log_{10}$eCFU/ml was higher than the group with CD4 ≥300, 0.07±0.29$\log_{10}$eCFU/ml, however the difference was not statistically significant, p=0.29 (figure 17).

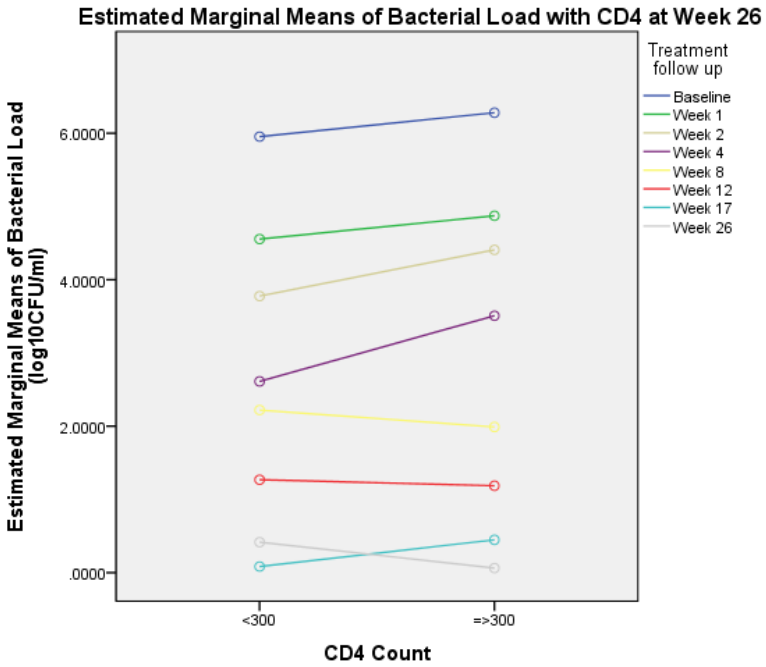


Figure 17. Relationship between CD4 count and bacterial load at week 26

Changes of CD4 count in the two HIV groups (CD4<300 and CD4≥300) was observed over the treatment follow-up: the 24 participants with CD4<300 at baseline had CD4=172±56.12cells/mm^3 at this study visit while 15 participants with CD4≥300 had CD4=492.47±200.84 cells/mm^3. Twenty-one (87.5%) out of 24 participants with CD4<300 at baseline were measured the CD4 at week 26 and had 261.33±148.89cells/mm^3, showing an increase of 89.33 cells/mm^3. Similarly, 8 (53.3%) participants of the group of participants with CD4≥300 at baseline had CD4=552.50±201.97 cells/mm^3 at week 26, showing an increase of 59.73 cells/mm^3.

4.5.5. Relationship between bacterial load and C-reactive protein

C-reactive protein (CrP) is an inflammatory marker commonly used clinically as marker of infection. Studies have shown that CrP declines as infection subsides following treatment. In this study C-reactive protein was measured for each participant at baseline, week 8 and week 26 and we explored correlation with bacterial load at this treatment points. There was strong positive correlation, Spearmans r=0.7314 (0.6467 – 0.7983), (p<0.0001) at 5% level of significance, indicating that the higher the bacterial burden, the higher the C-reactive protein (figure 18).

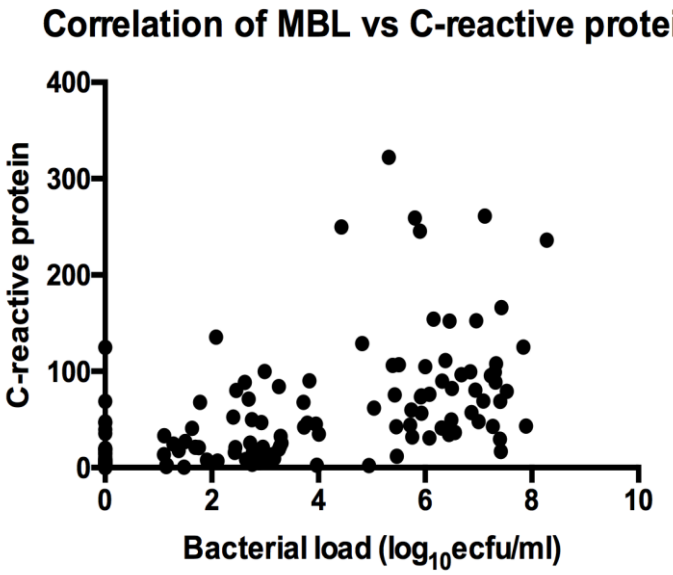


Figure 18. Correlation between bacterial load and C-reactive protein

The figure 19 shows the relationship between the TB bacterial load and C-reactive protein during the time point measured: both biomarkers decline in response to anti-TB therapy, the higher bacterial load , the higher the CrP and vice-versa.

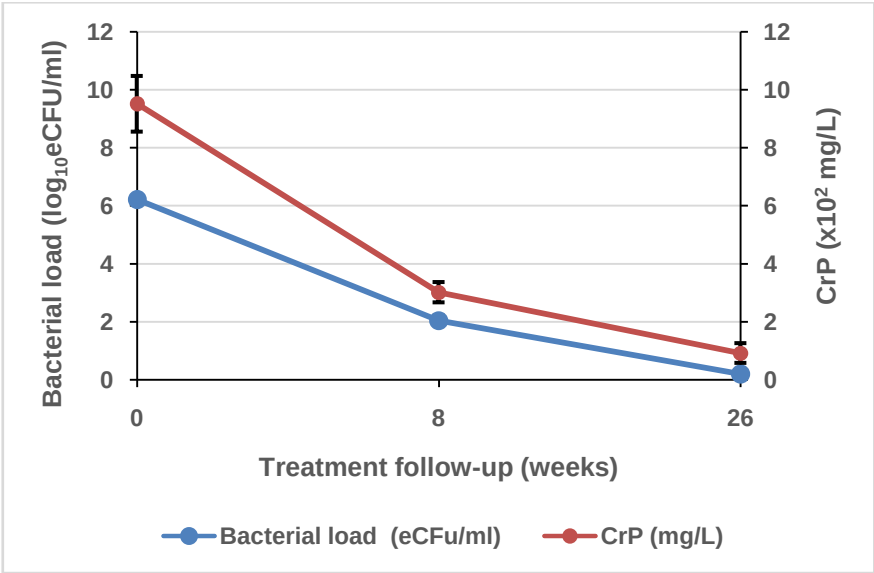


Figure 19. Relationship between the bacterial load and C-reactive protein. Error bars are standard error of the mean.

4.5.6. Relationship between TB resistance pattern and bacterial load

Drug susceptibility test were done for all 58 participants to direct to adequate treatment, of which 52 (89.7%) were classified as susceptible for all drugs, 4 as TB MDR and 2 as TB polyresistant. The overall bacterial load for each group of patients was 2.74±2.42log_{10}eCFU/ml for the TB drug sensitive, 3.29±2.38log_{10}eCFU/ml for the TB MDR and 2.72±2.44log_{10}eCFU/ml for the TB polyresistant. The figure 20 shows the decline in bacterial load according to the TB resistance pattern during the study.

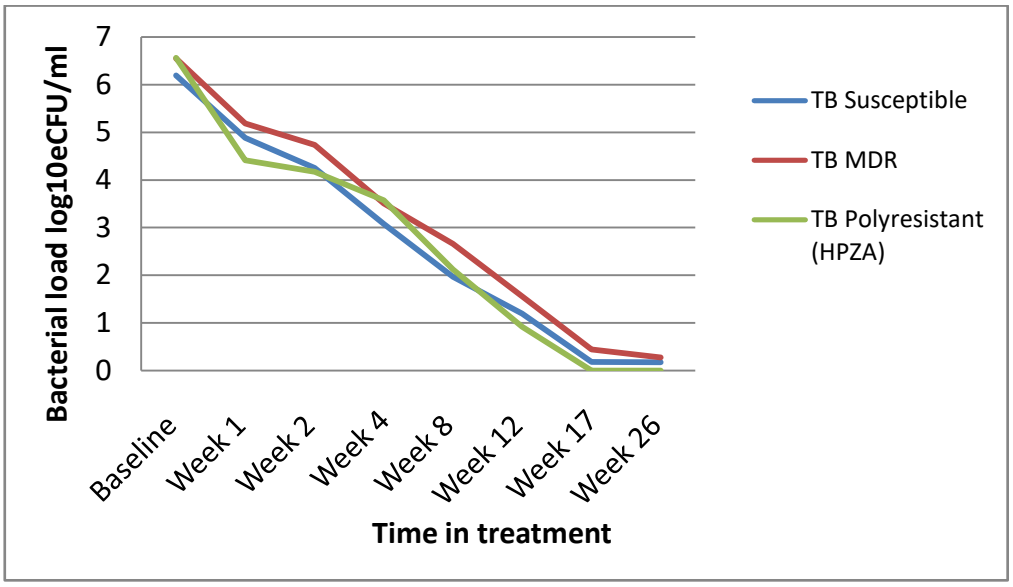


Figure 20. Decline in bacterial load according to the TB resistance pattern.

4.6. Resuscitation-promoting factor

4.6.1. Recovery and detectability of *M. tuberculosis*

A total number of 35 samples from 5 TB patients (samples from 7 study visits each, from screening to week 12) were inoculated with and without the Rpf supplement to evaluate whether the Rpf has influence to recover *M. tuberculosis* (M.tb) on cultures before and after anti-TB treatment initiation. The data showed that in the presence of Rpf supplement, 34 (97%) sputum samples were culture positive presenting growth of Mtb with contaminants (AFB positive + blood agar positive) and 1 (3%) sample had no growth (culture negative). The Rpf-culture negative was seen at week 8 in one patient that had all culture positive.

In the standard MGIT culture (control group, free of Rpf supplement) 34 results were available for analysis, of which 25 (74%) presented growth for Mtb, 5 (15%) had no growth and 4 (12%)

samples were contaminated. Of the 25 samples positive for Mtb, 19 (76%) presented growth of contaminants (AFB positive + blood agar positive). The 5 MGIT cultures negative were observed in four different patients: first at weeks 8 and 12, second at week 2, third at week 12 and fourth at week 8.

As the MBLA were processed in the samples from baseline to week 12 (6 study visits), 30 results were available of which, 26 (87%) were positive and 4 (13%) negatives. The MBLA negatives were observed in two patients: one at week 8 and another at weeks 4, 8 and 12 (figure 21).

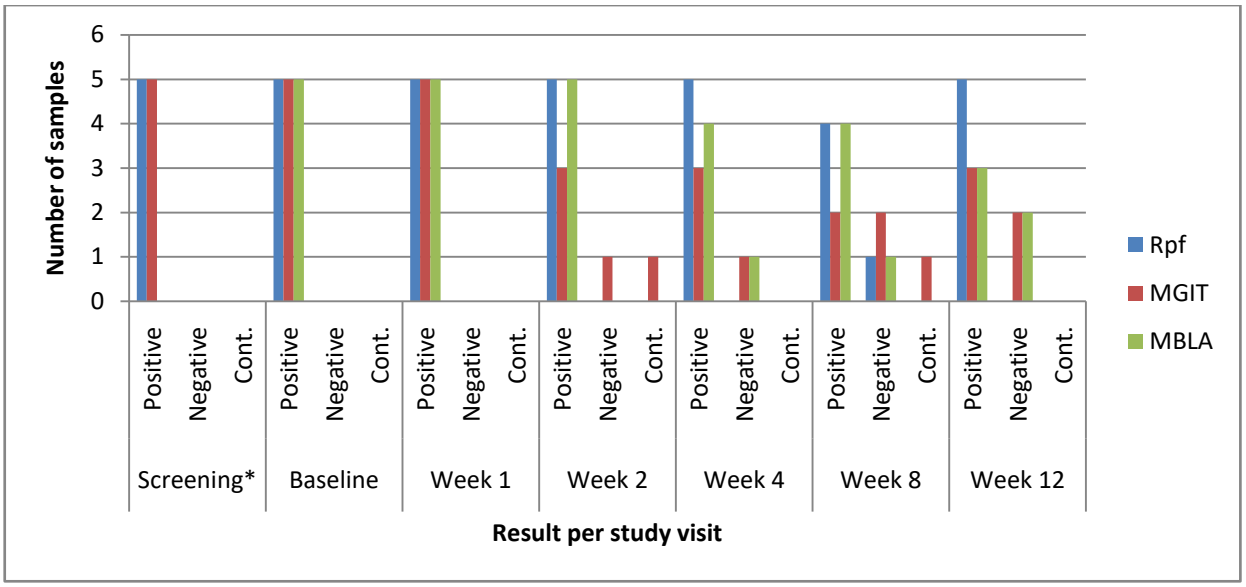


Figure 21. Results of cultures with and without Rpf and MBLA

*MBLA not done on this study visit

4.6.2. 4.6.2 Measurements of time to culture positivity in Rpf supplemented cultures

Regarding the Time to Positivity (TTP), 34 (97%) samples treated with Rpf were positive for Mtb and all had no valid Time to Positivity (TTP) due to growth of contaminants. On the standard MGIT cultures, 7 (21%) samples had valid TTP. Despite the TTP was not valid to Rpf cultures, we used this data to compare with the standard MGIT and found that the median TTP of the Rpf treated samples were 12 hours whereas the standard MGIT were 16 days and 8 hours.

4.7. Detection of viability of *M. tuberculosis* in liquid and solid cultures treated with OMNIGene.SPUTUM

A total number of 270 samples were enrolled to this evaluation. Of them, 156 (78 pairs, 65%) were processed in the same-day (SD) arm and 114 (57 pairs; 42%) were processed after 5 days (5D) arm. Main finding is shown below.

4.7.1. Recovery rate of *M. tuberculosis* and decontamination efficiency

The same day arm showed that a recovery rate of *M. tuberculosis* was 31% (24/78) in OMNI-Gene.Sputum (OM-S) MGIT whereas de standard procedure (SDP) MGIT was 83% (65/78). In the OM-S LJ, the recovery rate of Mtb was 79% (62/78) whereas the SDP LJ recovered 69% (54/78) of the bacteria. On the 5-day arm the recovery rate of OM-S MGIT was 47% (27/57) whereas the SDP MGIT recovered 75% (43/57) of *M. tuberculosis* in OM-S. The OM-S LJ had a recovery rate of 84% (48/57) compared to only 28% (16/57) of Mtb recovered by the SDP LJ. Interestingly, on the same day arm the MBLA showed high rate of *M. tuberculosis* detectability varying from 96% (30/31) in OM-S to 98% (55/56) in SDP treated samples. On 5-day arm MBLA detected 100% of *M. tuberculosis* on samples treated with SDP (54/54) and OM-S (34/34), respectively[47]. Furthermore, decontamination efficiency of MGIT and LJ was assessed by measuring the contamination rate in SDP and OM-S treated samples on same day arm and 5-day arm. The data shows that for MGIT culture, OM-S was much efficient than SDP providing lower contamination rate of 9 and 2% on same day arm and 5-day arm, respectively. Contamination rate was higher on samples treated with SDP resulting in 17 and 21% on same day arm and 5-day arm, respectively. For LJ culture, contamination rate of OM-S were 14 and 4% on same day arm and 5-day arm, respectively. The SDP LJ showed a contamination rate of 13 and 32% same day arm and 5-day arm, respectively (table 10)[47].

Table 10. Valid results of MGIT and MBLA by treatment group in each study arm

	Same day arm (n=78)			5 Day arm (n=57)		
	Positive	**Negative**	**Cont.**	**Positive**	**Negative**	**Cont.**
MGIT						
SDP	83%	0%	17%	75%	4%	21%
OM-S	31%	60%	9%	47%	51%	2%
LJ						
SDP	69%	18%	13%	28%	40%	32%
OM-S	79%	6%	14%	84%	12%	4%
MBLA						
SDP[#]	98%	2%	0%	100%	0%	0%
OM-S[¶]	96%	4%	0%	100%	0%	0%

Cont: contamination; #: n=56 and n=54 for same-day and 5-day arms, respectively; ¶: n=31 and n=34 for same-day and 5-day arms, respectively.

4.7.2. Measurements of viable *M. tuberculosis* using MGIT TTP and MBLA

Apart of giving more negative results on same day arm (SD) and 5-day arm (5D) (table 6), OM-S provided positive MGIT results with longer median TTP compared to standard decontamination procedure (SDP). On the same day arm, the median (range) MGIT TTP of samples treated with SDP was TTP 5(2–12) days whilst the samples treated with OM-S had MGIT TTP of 9 (1–40) days (p=0.007). On the 5-day arm, media (range) MGIT TTP of samples treated with SDP was 7 (1–29) days whilst samples treated with OM-S had MGIT TTP of 17 (4–35) days (p<0.0001). Differences of MGIT TTP in each study arm were statistically significant (figure 22)[47].

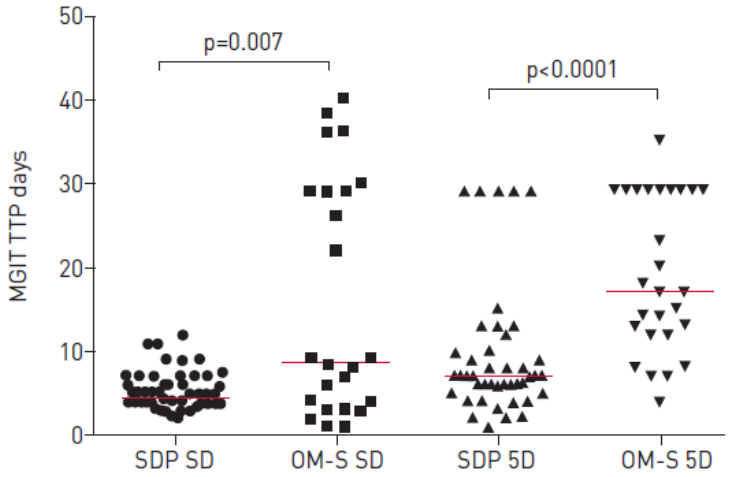


Figure 22. MGIT TTP of samples treated with SDP and OM-S for the two study arms. Bars represent median values. SDP: standard decontamination procedure; SD: same-day arm; OM-S:OMNIgene.SPUTUM procedure; 5D:5-day arm[47].

Unlike MGIT TTP, there were no difference in the measurements of bacterial load on the same day arm (SD) where samples treated with SDP presented bacterial load of 6log10eCFU/ml (range: 3–8) and the samples treated with OM-S also had 6log10 eCFU/ml (range: 3–7) (p=0.3). However, this condition was different on the 5-day arm where samples treated with SDP presented bacterial load of 6log10 eCFU/ml (range: 3–8) and samples treated with OM-S had 1 log lower bacterial load presenting 5log10eCFU/ml (range: 2–7) (p<0.0001) (figure 23)[47].

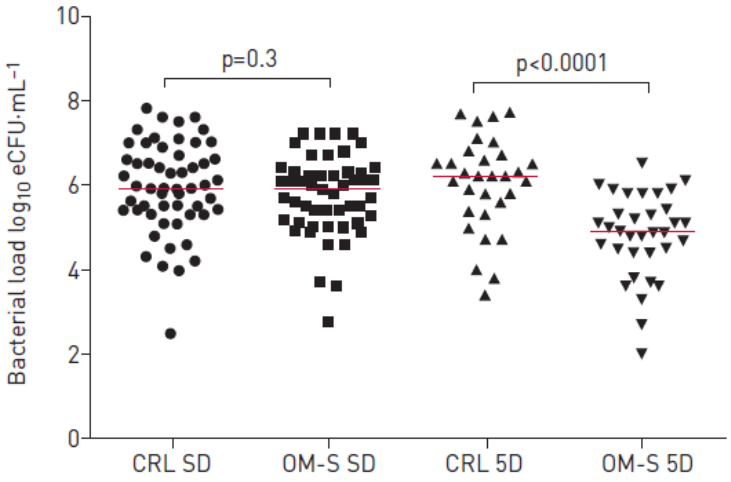
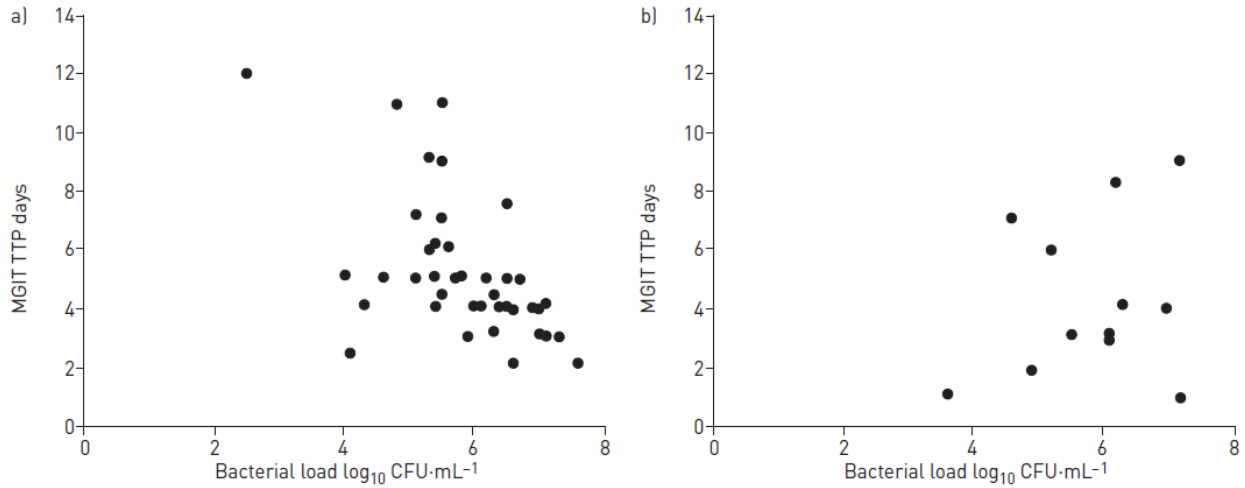


Figure 23. Differences in viable molecular bacterial load for the two groups in each study arm. Bars represent median values. CRL: control; SD: same-day arm; 5D: 5-day arm; OM-S: OMNIgene.SPUTUM procedure.

Considering that many MGIT negative results were generated in OM-S treated samples and this could be related with loss of *M. tuberculosis* viability, the study tested the correlation between SDP/OM-S MGIT TTP and bacterial load among same day samples. We found strong correlation between SDP MGIT TTP and bacterial load (Spearman's r=−0.6,95% CI −0.8–−0.4; p<0.0001). In contrast, such correlation between OM-S MGIT TTP and bacterial load was not found (r=−0.1, 95% CI −0.4–0.5; p=0.8) (figure 24)[47].



1. Figure 24. The correlation between MGIT TTP and bacterial load. a) standard decontamination procedure-treated samples and b) OMNIgene.SPUTUM-treated samples.

4.8. Evaluation of limit of detection of MBLA, Xpert and MGIT culture

4.8.1. Recovery of M.tb and Time to Positivity

A total number of 168 samples were processed from 2 neat samples, 27 (9 triplicates) samples of treated arm and 27 (9 triplicates) samples of untreated arm, resulting in 56 MGIT culture, 56 Xpert and 56 MBLA.

Of the 28 treated samples processed in MGIT culture, 12 (43%) were positive and no contamination was observed. All this positive samples were from dilution $1x10^{-1}$ up to $1x10^{-4}$ including the neat sample. The negative cultures were observed in all samples diluted from $1x10^{-5}$ up to $1x10^{-9}$ including only one sample from dilution $1x10^{-4}$. On other hand, the 28 untreated sample revealed 13 (46%) cultures positive with no contamination. As the treated samples, all positive samples were from dilution $1x10^{-1}$ up to $1x10^{-4}$ including the neat sample. The negative cultures were observed in all samples diluted from $1x10^{-5}$ up to $1x10^{-9}$.

Overall, the positive cultures showed an increase of Time to Positivity (TTP) as the sample dilution also increased in both arms, treated and untreated. The neat treated sample had TTP of 3:19 (days:hours), followed by the average TTP of 5:9, 8:19, 12:4 and 14:7 in the samples diluted at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, respectively. The neat untreated sample had TTP of 2:6 (days:hours), followed by the average TTP of 3:15, 5:4, 7:8 and 12:3 in the samples diluted at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, respectively. According to this data, samples from untreated arm was 2:6 (day:hours) faster than treated arm to appear as positive culture.

4.8.2. Detectability of Xpert

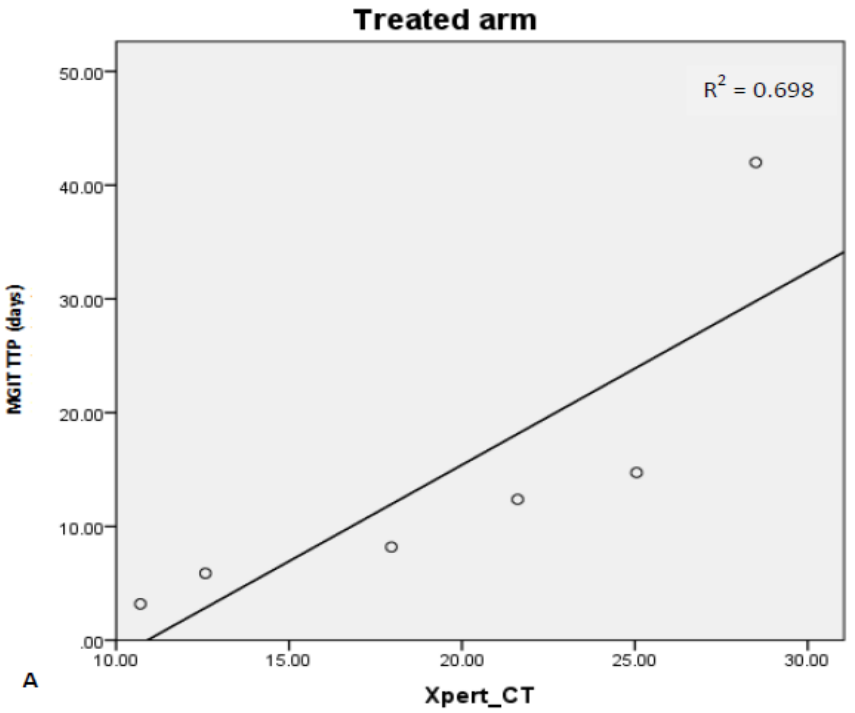
Of the 28 treated samples processed for Xpert, 15 (54%) were positive from dilution 10^{-1} to 10^{-5} including the neat sample. One sample from dilution 10^{-4} had error and the result could not be used for analysis. The remaining 12 samples from dilution 10^{-5} up to 10^{-9} were all negatives. The 28 samples processed without treatment resulted in 13 (46%) Xpert positive from dilution 10^{-1} to 10^{-4} (triplicate of each dilution) including the neat sample. None sample from dilution 10^{-5} to 10^{-9} was found as positive or with error result as in the treated arm.

4.8.3. Detectability of MBLA

The MBLA done on 28 treated samples showed that the neat and samples from dilutions 10^{-1} to 10^{-4} were all positive. Positive-negative borderline results were found between samples ditued at 10^{-5} to 10^{-7} whereas the samples from dilution 10^{-8} were positive and samples from dilution 10^{-9} were negatives. On 28 untreated samples the neat and samples from dilutions 10^{-1} to 10^{-4} were all positive. Samples from dilution 10^{-5} to 10^{-6} resulted in MBLA positive-negative borderline. The samples from dilution 10^{-7} were all positive for MBLA while samples diluted at 10^{-8} and 10^{-9} were all negative.

4.8.4. Comparison between MGIT Time to positivity with Xpert and MBLA

Results of Xpert and MBLA from each sample dilution were compared with the gold standard MGIT to analyse the correlation on NALC-NaOH treated and untreated arm. The data demonstrated that on treated arm, a strong positive correlation was found between the Xpert and MGIT TTP ($r=0.83$, $p=0.03$) whereas strong negative correlation was found between MBLA and MGIT TTP ($r=-0.84$, $p=0.001$): the MGIT TTP increases when the Cycle threshold (ct) in Xpert also increases and the MGIT TTP decreases when MBLA increases (figure 25 A and B).



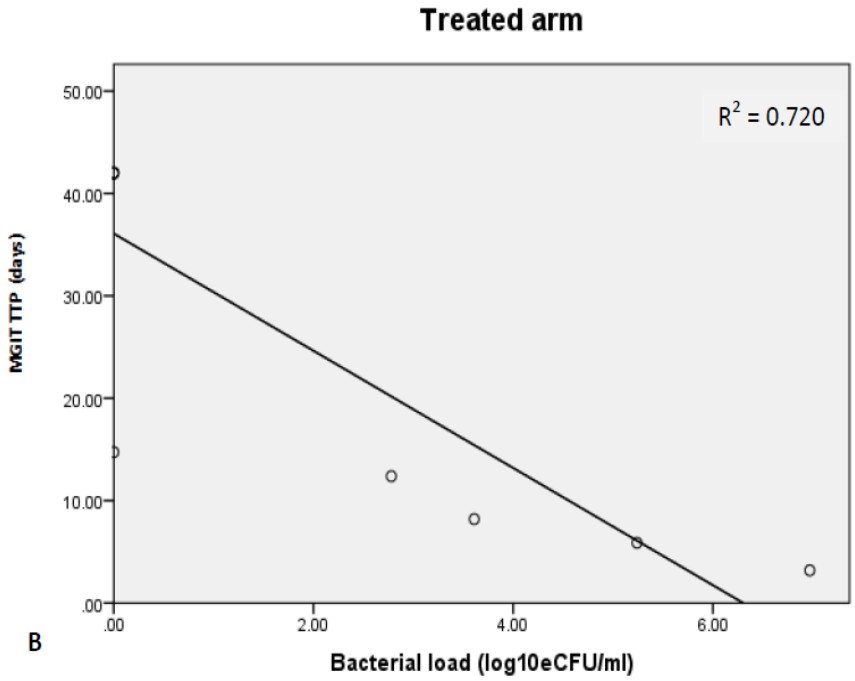


Figure 25. A) Correlation between Mycobacterium growth indicator tube (MGIT) time to culture positivity (TTP) and Cycle threshold (ct) of Xpert on neat samples and dilutions 10^{-1} to 10^{-9}, treated arm. B) The correlation between MGIT TTP and bacterial load count by molecular bacterial load assay (MBLA), treated arm.

Likewise, the untreated arm showed strong positive correlation between MGIT TTP and Xpert (r=0.9; p=0.01) and strong negative correlation between MGIT TTP and MBLA (r=-0.92; p<0.05):

the MGIT TTP increases when the ct in Xpert also increases and the MGIT TTP decreases when MBLA increases (figure 26 A and B).

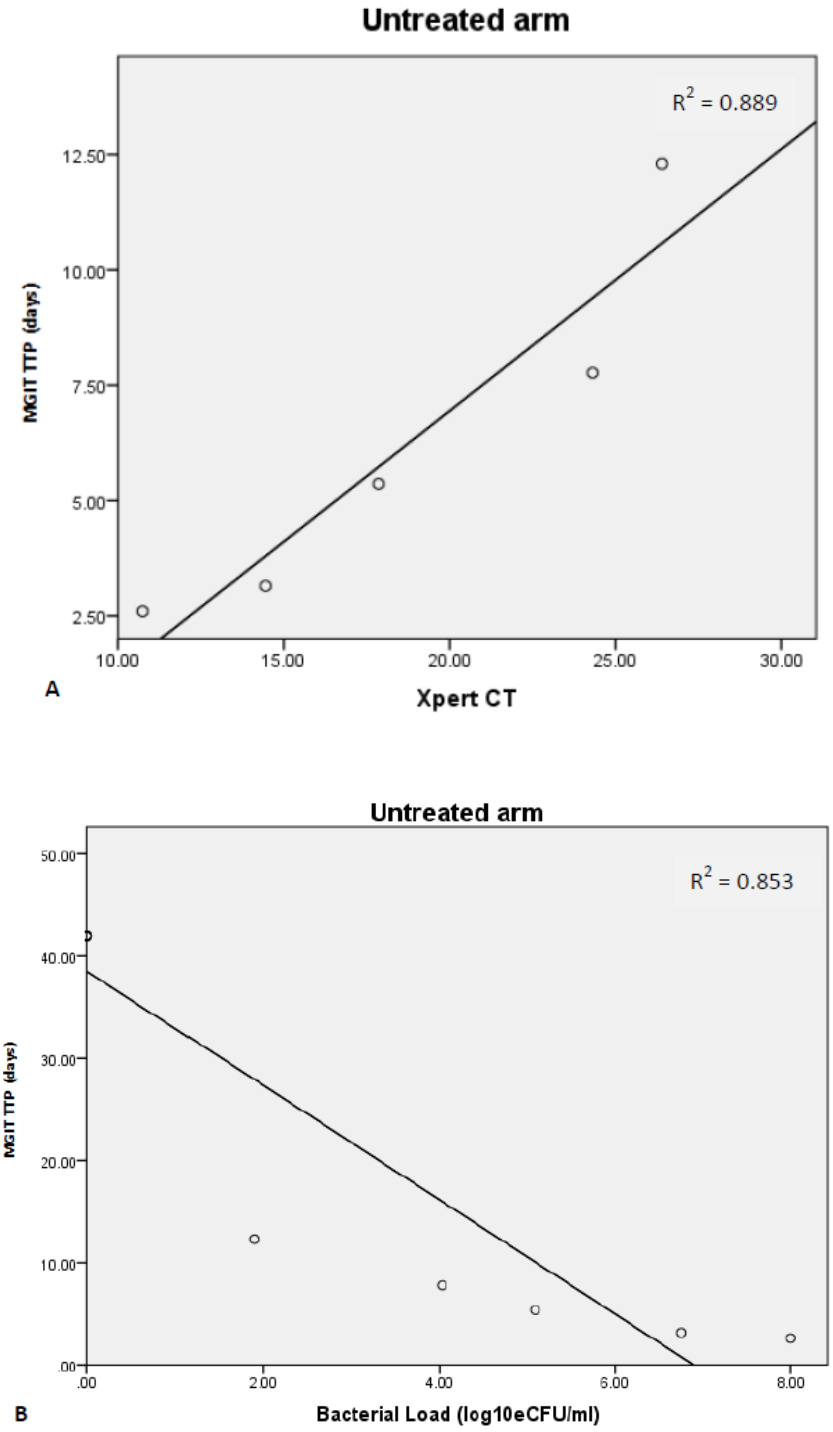
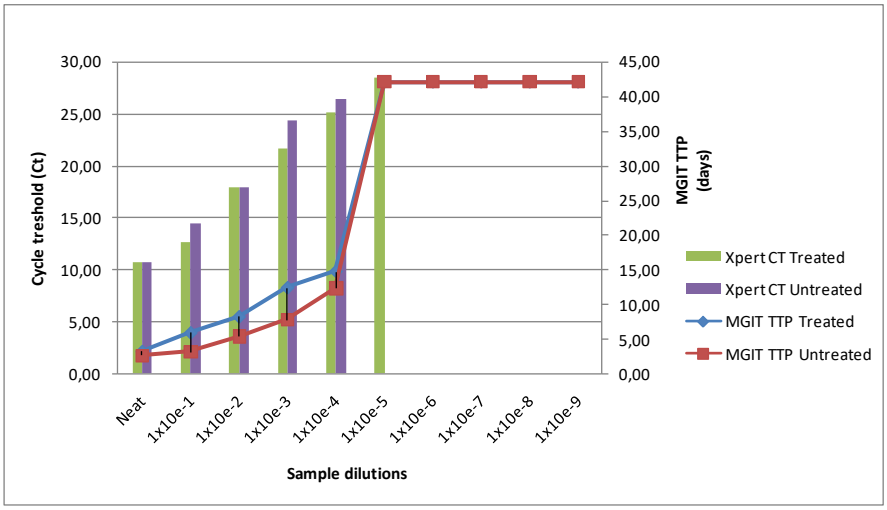
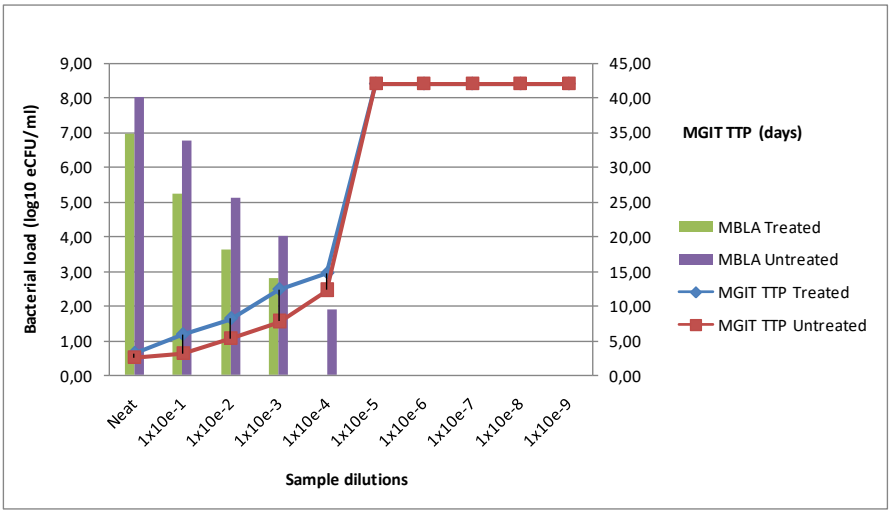


Figure 26. A) Correlation between MGIT TTP and Cycle threshold (ct) of Xpert on neat samples and dilutions 10^{-1} to 10^{-9}, untreated arm. B) The correlation between MGIT TTP and bacterial load count by molecular bacterial load assay (MBLA), untreated arm.

The comparison of quantitative data between MGIT TTP and Ct from Xpert or results of MBLA showed that on samples treated with NALC-NaOH, the average MGIT TTP was 14.73 days at dilution 10^{-4} whilst the average Ct for Xpert was 28.5 at dilution 10^{-5} and 2.78log_{10}CFU/ml for MBLA at dilution 10^{-3}. In untreated samples, the average MGIT TTP was 12.3 days at dilution 10^{-4} whilst the average Ct for Xpert was 26.4 at dilution 10^{-4} and 1.89 log_{10}CFU/ml for MBLA at dilution 10^{-4}. Thus, untreated samples were 2.43 days (MGIT TTP) faster than treated samples. The Ct of Xpert in treated samples was 2.1 better than in untreated samples. Finally, untreated MBLA was 0.89 logs higher than in treated samples (figure 27 A e B).



A



B

Figure 27. A) Comparison of quantitative data between MGIT TTP and Ct from Xpert. B) Comparison of quantitative data between MGIT TTP and Ct from Xpert.

5. Discussion

5.1. Performance of MBLA compared to different parameters

This is part of the first study which evaluates the performance of MBLA for TB treatment response using sputum samples in clinical setting with long follow up of patients. We have demonstrated that at the beginning of treatment, sputum samples are usually positive with high bacterial load when measured by the MBLA, MGIT and LJ. However, the positivity rate measured by the three assays decreases during treatment. Overall, we found very few discrepant results between MGIT, LJ and MBLA. These findings concur with Honeybourne et al[19] who also observed declines in bacterial load on liquid culture, solid culture and MBLA.

While MBLA was not affected by contamination and all the results were available for analysis, culture based-methods (MGIT and LJ) showed an increase of contamination rate along the treatment, although LJ was less prone to contamination than MGIT. Sample culture contamination leads to invalid results, meaning that the MGIT TTP or readouts of the results (e.g. two month culture positivity) are lost and clinicians can not monitor the treatment. According to Honeybourne et al[18], MBL is a robust assay which can account with less than 1% of samples with no valid result due to inhibition of process, whilst culture, specially the liquid, can generate 4 to 9% of contamination rate leading to invalid results[48].

Comparing MBLA with MGIT TTP as biomarkers for treatment response, we found an inverse correlation between these two assays. Although with higher variation, MGIT TTP tended to increase when bacterial load decreased. Data from this study showed clearly that at the beginning of treatment, when usually bacterial load is high, the MGIT TTP is low (short). With treatment, the bacterial load decreases due to the effect of therapy that kills the bacteria and the patients starts getting better. Thus, when the bacterial load decreases, the MGIT TTP becomes high (long). This finding concur with results of other studies which also found that in TB treatment studies the MGIT TTP takes long period when sputum bacillary load becomes low[18, 80]. Mukamulova et al[12] demonstrated that many TB bacilli can only grow and be detected by liquid culture after addition of resuscitation-promoting factors. As MBLA detects rRNA present in all mycobacteria, it is possible that MBLA detects only the viable mycobacteria also detected by MGIT and misses the ones that grows in liquid culture with the addition of rpf. Our data showed strong relationship between MBLA and MGIT TTP over the first 2 weeks of treatment, suggesting that such phenomena may not affect the MBLA-MGIT relationship during early

treatment at least. However, the significance of this event remains uncertain, since we found that the correlation during treatment was affected by the reduced number of pairs to be compared.

We have demonstrated that according to MBLA and smear microscopy, all the patients from this study could be defined as cured, since we did not find any positive results of smear or MBLA, consecutively at week 17 and 26. Curiously, we found 10 patients whom could be classified as treatment failure by MGIT, but cured by MBLA. Probably the reason for this discrepancy was due to possible cross-contamination of the samples during culture processing which yielded serial positive samples at weeks 17 and 26 consecutively. We observed that majority of these patients had previous negative cultures and were declared cured at the clinic since they were physically well and without symptoms of TB at the end of 6 month of treatment (data not shown). This data is in line with Phillips et al[81] who conducted a recent study to compare liquid and solid culture for determine relapse and cure in phase III TB trials using new regimens. They found that patients with favourable TB treatment outcome and no need of retreatment presented positive MGIT cultures. In some cases, this positive MGIT cultures were preceded and followed by negative MGIT cultures. Similarly with us, the authors of this study also indicates laboratory cross-contamination as the probable cause of this result[81].

Considering that clinical information can predict risk of treatment failure in TB patients, we measured and compared different parameters with MBLA, such as percentage of lung area affected and presence of cavities in the lungs. The study did not find significant correlation between the bacterial load and percentage of lung area affected, nor even between bacterial load and number of cavities in the lungs. Differently, Palaci et al[82] studied the relationship between sputum bacterial loads and cavitary disease in adults with newly diagnosed TB and found that higher bacterial load are associated with presence of cavitary disease. It should be emphasized that sputum samples collected from patients with cavities in the lungs may present high bacterial load in a state of rapid division[18]. In addition, studies has been demostrated that bacterial load in the lung have an influence on treatment response[18, 83].

It was notable in this study that patients with higher bacterial load were associated with higher respiratory rate, abnormal lung function and lower weight. Recently, Ravimohan et al[84] conducted a systematic review on TB and lung function and found that the disease itself is a risk factor for long-term respiratory impairment. Its still unclear how specific host and pathogen

factors causes lung impairment, although it may be because of the host immune responses to the damage occured in the lung during TB[84].

Our study indicates that bacterial load was higher in males than in females and age was not found as having influence in bacterial load. Although not evaluated in this study, it can be possible that male presented high bacterial load due to delays to seek medical assistance to diagnose TB and start the treatment. Saifodine et al[85] demonstrated that in Beira City, Mozambique, TB is prevalent in male and the delays in TB diagnosis and treatment are caused both by patients and the health care system. The delay from the patient and health system was 150 and 61 days, respectively. We suggest that delays to start treatment of TB may contribute to development of severe disease which is also characterized by high bacterial load in the affected organ of the body.

In the present study we did not find difference between bacterial load and HIV status. This data is consistant with Theron et al[86] who used MGIT TTP as surrogate of bacterial load and found it lower in patients with HIV negative than in patients with HIV positive, meaning that TB bacterial load is higher in patients with HIV negative than in patients with HIV positive. However, they observed that pooled data revealed the same bacterial load in both groups of patients. Its well known that is difficult to diagnose TB in HIV patients and their risk of increased mortality is high because usually there sputum is scarce, they have difficulties to collect it and are paucibacillary [87]. Concerning the bacterial load and CD4, our finding was different from Theron et al[86] who found differences in TTP according to CD4 count. Its important to consider that our threshold was 300 cells/ml while they used 200 cells/ml.

Interestingly, this study found that C-reactive protein (CrP) had strong association with MBLA, suggesting that CrP can be a surrogate biomarker of bacterial load to monitor TB treatment response. It was notable that CrP and bacterial load declined in response to the treatment. Lisboa et al[88] also found that CrP decrease during treatment patients receiving appropriate antibiotic for pneumonia and it correlates with bacterial load. Yoon et al[89] found that CrP has high sensibility and moderate specificity to diagnose active pulmonary TB in patients with HIV positive and recommended more studies in other high-risk groups of patients.

5.2. Performance of Rpf in MGIT and MBLA to measure viability on OMNIGene. SPUTUM

The present study demonstrated that Rpf can stimulate mycobacterial growth. We enphasize that almost all the samples processed with Rpf showed growth of *M. tuberculosis.* However, the growth of *M. tuberculosis* was accompanied with gowth of other bacterias (contaminants) resulting in low MGIT TTP of about 12 hours. The standard MGIT generated more negative and purely contaminated results than Rpf. The valid MGIT TTP was longer (16 days and 8 hours) than that with Rpf. This data is in line with Mukamulova et al[12], whom demonstrated in their study that 80–99% of *M. tuberculosis* was detected only by treating the samples with Rpf. Mukamulova et al[12] suggest that there are population of *M. tuberculosis* cells that cannot grow in culture media without addition of Rpf, and probably this population increases during treatment. We believe that the Rpf does not boost the *M. tuberculosis* growth only, but also that of a very few amount of other microorganisms present in the sputum. This may be the reason of getting contamination in all our positive cultures processed with Rpf. We know that the shorter MGIT TTP of samples with Rpf is not valid due to presence of contaminats. Cruciane et al[48], demonstrated in meta-analysis that MGIT TTP can vary from 7 to 16 days, depending on the species and bacterial load (smear results). Our present data showed that MBLA was positive in 87% of samples which were also Mtb positive (plus contaminated) in culture after treatment with Rpf.

When used to measure the viability of *M. tuberculosis* in samples treated with OMNIGene.SPUTUM (OM-S) compared with NALC-NaOH standard procedure, the study showed that in MGIT, samples treated on the same day with both methods had no difference in bacterial load, however, samples incubated for 5 days in contact with OM-S prior processing showed lower bacterial load than in those incubated without OM-S and, further, processed with NALC-NaOH. This low bacterial load corresponded with the long MGIT TTP seen in samples treated with OM-S than in NALC-NaOH, in the 5 day arm of the study. Differently, the samples treated with OM-S produced more positive LJ cultures than the samples treated with NALC-NaOH, both on the same day and 5-days arm. We hypothesise that this is not due to loss of *M. tuberculosis* viability, but due to inhibition by leftover reagent that diffused into the MGIT following inoculation. We believe that LJ agar absorbs and neutralises the leftover OM-S, leaving bacilli to grow free of the reagent. The absence of correlation between OM-S TTP and bacterial load supports our hypothesis[47].

5.3. Limit of detection of MBLA, Xpert and MGIT culture

This study showed that the trend of positivity rate decreases in MBLA, MGIT culture and Xpert MTB/RIF during the processing of samples diluted in series. When compared to MGIT, we found association between the Xpert and MGIT TTP and between MBLA and MGIT TTP on treated and untreated arm, indicating that MGIT TTP increases when the Ct in Xpert also increases and the MGIT TTP decreases when MBLA increases. This results concur with previous studies[18, 80] that found inverse correlation between MBLA and MGIT TTP. In addition, the association found in the present study between the Xpert and MGIT TTP are in agreement with Theron et al[86] who found similar positive and strong correlation between these two variables using pulmonary samples.

Overall, we demonstrated that NALC-NaOH increases the TTP, indicating loss of bacilli killed during sample decontamination. Thus, NALC-NaOH has impact on the limit of detection of MGIT. The Xpert has the lowest limit of detection. This findings concur with Beynon et al[79] who found that higher Ct on Xpert means low bacterial load and vice-versa. We consider the fact that Xpert is a DNA based assay and, thus, Mtb DNA or fragments of DNA can be detected by this assay even after TB treatment regimen in cured patient[78].

6. Conclusion

We conclude that MBLA can be very powerful biomarker to monitor TB treatment response and has potential to replace culture on the patient management, since it provides real-time data to guide the treatment, is a quantitative based-method, unaffected by contamination and rapid to provide results. However, as many other molecular biology methods, MBLA has limitations such as the need of different rooms in the laboratory (at least three separed one), is non-automated, needs laboratory technicians highly trained and the costs of equipments and reagents are expensive compared to culture and smear microscopy.

The interesting correlation between MBLA and other clinical parameters found in the present study, like C-reactive Protein, for instance, opens floor for further investigations as potential biomarkers to diagnose TB and monitor treatment response.

7. References

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8. Annex

8.1. List of Publications

1. **Azam K**, Cadir N, Madeira C, et al. OMNIgene.SPUTUM suppresses contaminants while maintaining Mycobacterium tuberculosis viability and obviates cold-chain transport. ERJ Open Res 2018; 4: 00074-2017

2. Viegas SO, **Azam K**, Madeira C, et al. Mozambique's journey toward accreditation of the National Tuberculosis Reference Laboratory. Afr J Lab Med. 2017; 6(2), a491.

3. W. Sabiiti, B. Mtafya, D. Kuchaka, **K. Azam**, S. Viegas, A. Mdolo, E. C. W. Farmer, M. Khonga, D. Evangelopoulos, I. Honeyborne, A. Rachow, N. Heinrich, N. E. Ntinginya, N. Bhatt, G. R. Davies, I. V. Jani, T. D. McHugh, G. Kibiki, M. Hoelscher, S. H. Gillespie. Optimising molecular diagnostic capacity for effective control of tuberculosis in high-burden settings. Int J Tuberc Lung Dis 2016; 20(8): 1004–1009

4. Saifodine A, Fyfe J, Sievers A, Coelho E**, Azam K**, Black J. Genetic diversity of Mycobacterium tuberculosis isolates obtained from patients with pulmonary tuberculosis in Beira city, Mozambique. International Journal of Mycobacteriology. 3 (2014) 94-100.

8.2. Statement on Pre-release and Contribution

The present study was conducted under the establishment of Maputo Tuberculosis Trial Unit (MaTuTU study), a TB project from University of Munich and Instituto Nacional de Saúde (INS) de Moçambique. I worked in the study as TB Laboratory Coordinator and Manager of the National TB Reference Laboratory of Mozambique. My responsabilities included preparation of the study protocol, training of the laboratory technicians, monitoring of study progress, procurement of reagents and materials for the laboratory, sample processing, analysis of data and laboratory indicators, as well as publication.

In collaboration with my supervisors I analysed the data described in the chapter 4. I contributed as second co-author to write the main manuscript of the Molecular Bacterial Load Assay which will be submitted to a peer journal. At the same time, I played important role coordinating (also processing the samples) the evaluation of MBLA to measure viability of *M. tuberculosis* in samples treated with OMNIGene.SPUTUM. Within this evaluation we published the manuscript entitled "OMNIgene.SPUTUM suppresses contaminants while maintaining *Mycobacterium tuberculosis* viability and obviates cold-chain transport" which I am the first author[47]. This manuscript will be submitted to the CIH PhD Coordination together with this monograph as my accompanying scientific article.

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block 2 and I saw him for the first time when he was already 2 months old. Many times I had to leave you alone to devote myself to studies and laboratory work, even knowing that you would be sad for my absence. I hope this sacrifice was not in vain and I hope one day to see my children reaching this academic degree. I love you so much.