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Licenciado em Biologia

EVALUATION OF CUTTING-EDGE DIAGNOSTIC AND TREATMENT MONITORING TOOLS IN A COHORT OF TUBERCULOSIS PATIENTS DURING THE FIRST MONTH OF TREATMENT

MESTRADO EM MICROBIOLOGIA MÉDICA

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MESTRADO EM MICROBIOLOGIA MÉDICA

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Evaluation of cutting-edge diagnostic and treatment monitoring tools in a cohort of tuberculosis patients during the first month of treatment

DEDICATORY

I dedicate this work to my children, my wife for the love they offer me to become a better person and to give up my presence with little stress, to friends and family for the good and bad moments in the past that made me the person I am today.

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I would like to thank my esteemed supervisors – Dr. Belen Saavedra and Doctor Diana Machado for them invaluable supervision, support, and tutelage during my master's degree. My gratitude extends to the CISM-TESA for the funding opportunity to undertake my studies at NOVA University of Lisbon. Additionally, I would like to express gratitude to my supervisors for their treasured support, which was influential in shaping my experiment methods and critiquing my results. I also thank Doctor Alberto Garcia-Basteiro for his continuous mentorship. I would like to thank my friends, lab mates, colleagues, and research team – Daniel Otindio Jardel, Claudia Varela, Filipe, Alvaro, IHMT lab mates, CISM Lab colleagues, colleagues from the 9th edition of the master's in medical microbiology for a cherished time spent together in the lab, and in social settings. My appreciation also goes out to my family and friends for their encouragement and support all through my studies. I also appreciate all the support I received from Professor Doctor Jaime Mota and to the scientific committee of the 9th edition of the master's in medical microbiology.

"You cannot teach a man anything; you can only help him discover it in himself."
(Galileo).

ABSTRACT

Drug susceptibility testing (DST) is estimated to be performed only in 58% of previously treated tuberculosis (TB) cases and in 12% of untreated cases. This figure emphasizes the need to evaluate the contribution of molecular-based methodologies for early detection of drug-resistant TB and implementing phenotypic susceptibility methods for drug resistance determination towards a personalized treatment. We evaluated the capacity of the Xpert MTB/RIF Ultra assay for the detection of *M. tuberculosis* complex (MTBc) DNA in clinical samples of patients under therapy for pulmonary TB, through the analysis of respiratory samples taken at four timepoints: 0, 7, 14, and 28 days. The results were compared with those obtained from liquid cultures, used as gold standard. Minimum inhibitory concentration (MIC) determination was used to ascertain drug resistance profiles. The results obtained with the Xpert Ultra throughout the first month of treatment shows a good performance, 97.6% sensitivity and 90% overall agreement (k=0.43), when compared with those obtained with liquid cultures. The performance of the test for the detection of MTBc DNA during the first month of treatment and according to the time points were: 95% sensitivity, visit 1 (day 0); 88%, visit 2 (day 7); 69%, visit 3 (day 14), and 69%, visit 4 (day 28). The isolates studied were susceptible to the four anti-TB drugs tested. No mutation was detected in the genes associated with resistance to these drugs. The results obtained showed that both methodologies complement each other: the Xpert Ultra offers early detection of MTBc DNA (detection of genetic material) and the culture allows performing DST to guide therapy and, at the same time, indicates how effective is being the treatment since, contrary to the Xpert Ultra, it detects viable *M. tuberculosis*. Together, they can improve TB diagnosis, treatment guidance and break the chain of transmission.

Keywords: Xpert Ultra, EUCAST reference method, *Mycobacterium tuberculosis*

RESUMO

Estima-se que os testes de susceptibilidade aos antibióticos (TSA) sejam realizados apenas em 58% dos casos de tuberculose (TB) previamente tratados e em 12% dos casos não tratados. Este número enfatiza a necessidade de avaliar a contribuição de metodologias de base molecular para a detecção precoce da tuberculose resistente aos antibióticos e a implementação de métodos de susceptibilidade fenotípica para a determinação da resistência aos antibióticos, com vista a um tratamento personalizado. Avaliamos a capacidade do Xpert MTB/RIF Ultra para a detecção do ADN do complexo *M. tuberculosis* (MTBc) em amostras clínicas de doentes sob tratamento para TB pulmonar, através da análise de amostras respiratórias obtidas em quatro períodos temporais: 0, 7, 14, e 28 dias. Os resultados foram comparados com os obtidos a partir de culturas líquidas, utilizadas como referência. A determinação da concentração mínima inibitória (CMI) foi utilizada para determinar perfis de resistência aos antibióticos. Os resultados obtidos com o Xpert Ultra ao longo do primeiro mês de tratamento mostram um bom desempenho, 97.6% de sensibilidade e 90% de concordância geral (k=0,43), quando comparados com os resultados obtidos pela cultura líquida. A detecção do ADN de MTBc durante o primeiro mês de tratamento e de acordo com os quatro períodos temporais apresentam 95% de sensibilidade, visita 1 (dia 0); 88%, visita 2 (dia 7); 69%, visita 3 (dia 14), e 69%, visita 4 (dia 28). Os isolados estudados foram susceptíveis aos quatro antibióticos anti-TB testados. Não foi detectada nenhuma mutação nos genes associados à resistência a estes antibióticos. Os resultados obtidos mostraram que ambas as metodologias se complementam: o Xpert Ultra oferece a detecção precoce do ADN de MTBc (detecção de material genético) e a cultura permite realizar o TSA para orientar a terapia e, ao mesmo tempo, indica a eficácia do tratamento uma vez que, ao contrário do Xpert Ultra, detecta *M. tuberculosis* viável. Juntos, podem melhorar o diagnóstico da TB, a orientação do tratamento e quebrar a cadeia de transmissão.

Palavras-chave: Xpert Ultra, método referência EUCAST, *Mycobacterium tuberculosis*

INDEX

LIST OF FIGURES

LIST OF TABLES

ACRONYMS

ART	Anti-Retroviral Treatment
CISM	Centro de Investigação em Saúde da Manhiça
HDM	Manhiça District Hospital
HDSS	Health and Demographic Surveillance System
HIV	Human Immunodeficiency Virus
DSA	Demographic Surveillance Area
DNA	Deoxyribonucleic Acid
DST	Drug Susceptibility Testing
DR-TB	Drug-Resistance Tuberculosis
MDR/RR-TB	Multi-drug Resistance/Rifampicin Resistant Tuberculosis
NTM	Non-Tuberculosis Mycobacteria
TB	Tuberculosis
NTP	National Tuberculosis Control Program
BSL3	Biosafety Level 3
WGS	Whole Genome Sequencing
MTB/MTBc	*Mycobacterium tuberculosis* complex
TTP	Time-To-Positivity
TDM	Therapeutic Drug Monitoring
PK	Pharmacokinetics
MIC	Minimum Inhibitory Concentration
MGIT	Mycobacterial Growth Indicator Tube
NALC	N-Acetyl Cysteine
NaOH	Sodium Hydroxide
WBA	Whole Blood Bactericidal Assay
rRNA	Ribosomal Ribonucleic Acid
RT-qPCR	Reverse transcriptase quantitative real-time polymerase chain reaction
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ECOFF	Epidemiological cut-off
CLSI	Clinical & Laboratory Standards Institute
CCs	Critical Concentrations
Ct	Cycle threshold (the number of cycles before the bacteria is detectable)
CRF	Clinical Research Form
OADC	Oleic acid, Albumin, Dextrose, Catalase
AMR	Antimicrobial Resistance
WHO	World Health Organization

1. INTRODUCTION

1.1. Background

Members of the *Mycobacterium tuberculosis* complex (MTBc) are the cause of human tuberculosis (TB). Tuberculosis is an infectious disease affecting a quarter of the world's population (1) and typically affect the lungs (pulmonary TB). *M. tuberculosis* bacillus is a slow growing *Mycobacterium* with a doubling time of 12–24 h under optimal conditions. A major feature of MTBc is a peculiar cell wall structure (called cord factor), that provides an exceptionally strong impermeable barrier to noxious compounds and drugs and that plays a fundamental role in virulence (2). Usually, TB spread when people who are sick with TB expel bacteria into the air, e.g., by coughing.

Diagnosis of TB is based on clinical, radiological, histological, and bacteriological findings (smear examination, molecular biology, and culture). Nowadays culture remains the reference standard for laboratory confirmation of TB disease and is required for classical drug susceptibility testing (DST). Due to the slow growth of the bacteria, the results usually take several weeks resulting in late diagnosis and delays in susceptibility testing results leading to an increased morbidity, mortality, and ongoing transmission of the disease, and even worse, of drug resistant TB. The rapid and accurate detection of MTBc isolates and methods for detection of drug resistance to first-line drugs are crucial for TB control (3).

Exposure to *M. tuberculosis* can result in the elimination of the pathogen, either because of innate immune responses or because of acquired T cell immunity. Individuals who have eliminated the infection via innate immune responses or acquired immune response without T cell priming or memory can have negative tuberculin skin test (TST) or interferon-γ release assay (IGRA) results (4). Some individuals will eliminate the pathogen but retain a strong memory T cell response and will be positive on the TST or the IGRA. If the pathogen is not eliminated, bacteria persist in a quiescent or latent state that can be detected as positive TST or IGRA results; these tests elicit T cell responses against *M. tuberculosis* antigens (4). Patients with active TB disease experience symptoms such as cough, fever and weight loss, and the diagnosis can usually be confirmed with sputum smear, culture, and molecular tests. Patients with active TB disease might sometimes be negative on the TST or the IGRA because of anergy that is induced

by the disease itself or immune suppression caused by comorbid conditions, such as HIV infection or malnutrition. Individuals with subclinical or active TB disease should receive one of the recommended treatment regimens for active TB disease, which consist of an intensive phase with four drugs, followed by a longer continuation phase with two drugs.

The WHO estimated 9.9 million (range, 8.9–11.0 million) felt ill from TB, 1.3 million (range, 1.2–1.4 million) TB deaths among HIV-negative people, and an additional 214 000 deaths (range, 187 000–242 000) among HIV-positive people in 2020 (1).

Laboratories had critical role on the WHO's End TB Strategy which calls for the early diagnosis of TB and universal DST emphasis to meet the WHO targets. The WHO advocates that rapid TB diagnostics should be available to all persons with signs or symptoms of TB. All bacteriologically confirmed TB patients should be tested for at least for rifampicin, and all patients with rifampicin-resistant TB should be tested at least for fluoroquinolones (FQs) and second-line injectable drugs (SLIDs) (5).
Drug-resistant TB (DR-TB) is defined by concurrent resistance to rifampicin and isoniazid (5) and the relevance to perform DST's assays are to (i) inform individual management (treatment and prevention) of TB cases and (ii) anti-TB drug-resistance surveillance (6). DR-TB is still a public health concern as recently was revealed an increase of 10% (6) of DR-TB from 2018 to 2019 and highlight the concern of rifampicin-resistant TB (RR-TB) that requires treatment with second-line drugs and includes multidrug-resistant TB (MDR-TB) that is resistant to both rifampicin and isoniazid (7).

According to the 2020 WHO report, many high TB burden countries fail to reach the 2020 milestones of the End TB Strategy achieving only 9% of the cumulative reduction from 2015 to 2019 (6). Such failure may be due to lack of rapid, sensitive, and deployable TB diagnostic tools hampering the early diagnosis of the disease and early detection of treatment failure (7).

Since the discovery of the Koch bacillus (1882) and the beginning of the 'antibiotic era', the need to develop methodologies able to predict antibacterial sensitivity to specific drugs emerged. In the 1960s, the pioneering experiments of Canetti, at the Pasteur Institute of Paris, lead to the improvement of the "proportion method" (9; 10). The proportion method uses a set of media, each containing the "critical" concentration of a single drug, to test the growth of a strain in comparison with that obtained on a drug-free medium (the growth control) (9).

Despite some improvements, Mozambique is one of the 30 TB/HIV high burden countries ranking third and sixth in TB incidence and mortality (6). Moreover, is one of the seventeen countries that reach the 2020 WHO milestone but it is still among the countries with an estimated ≥1000 MDR/RR-TB incident cases in 2019 (6). The estimated incidence rate for the whole country is 361 per 100,000 inhabitants with 34% of the TB notified cases being HIV positive (6). In 2014 was estimate that 1.5 million lived with HIV and the prevalence of HIV infection was 39.9% eighth highest in the world (10). The available data also reveals that the incidence of the disease in the south of the country might be much higher than that of general country estimates (11). The surveillance for TB is poor and the true magnitude of drug resistance is unknown. DR-TB rates estimated in the country indicated that 3.5 % corresponds to primary resistance while 11.6 % is secondarily acquired. These numbers may be an underestimate because of the lack of laboratory facilities for phenotypic DST in the country, especially for second-line TB drugs (SLDs) (12).

In low-middle income countries, the establishment of phenotypic DST could help to minimize the underestimate magnitude of drug resistance, since this is a method that usually reports the high likelihood of therapeutic success compared to those that are not based on clinical breakpoints, distinguishing samples that are susceptible at the standard dosing regimen (13). Nonetheless, until 2016 no universal reference method for phenotypic DST for *M. tuberculosis* existed, supporting again the needed for an affordable, easy, fast, and accurate phenotypic DST that allows the early detection of resistant strains avoiding transmission (14). This seems challenging when setting clinical reference points for old as well as new drugs, including bedaquiline and delamanid. Around the world is estimated that drug resistance accounts for approximately 30% of the annual deaths. Therefore, accurately diagnosing antimicrobial resistance for TB is crucial not only to select the most effective regimen with the least side effects but also to minimize costs.

Additionally, providing also rapid and accurate genetic information through target-gene sequencing or whole genome sequencing (WGS) is especially important for the End TB strategy, as genome sequencing analysis of *M. tuberculosis* infecting strains, may adequately predict phenotypic susceptibility or resistance (depending on the breakpoints and mutations detected) and consequently the outcomes of the treatment for TB. In this way, genome sequencing analysis is expected to provide a rapid and simple predictive tool (15). Moreover, genome analysis have being considered to be a powerful tool to identify genomic markers that can be correlated to the presence of mutations and their phenotypic expression, especially for new

anti-TB drugs, for which the molecular resistance mechanisms are still not fully understood (14).

1.2. The problematic

Bacterial resistance to antibiotics is the principal obstacle to their successful therapeutic outcome. When resistance develops during a course of treatment, it may deprive an antibiotic of its proper therapeutic effect in the patient being treated (8). Drug susceptibility testing for MTBc is currently based on the proportion method that can be performed (i) using the critical concentration for each drug (a single concentration) and (ii) performing MICs using a range of concentrations, which should include the respective critical concentration, to determine the resistance levels of a strain to an antibiotic (16).
The accuracy of the diagnostic tests and the time taken to provide results were proven to impact TB treatment outcomes (6;16-18). As a result, novel diagnostic tools for monitoring treatment response and early identifying treatment failure are desperately needed (7).

Although the rapid molecular tests are still unable to predict susceptibility or resistance in 100% of cases, they are still able to guide therapy in the high majority of the cases preventing starting potentially toxic therapy or its early discontinuation in cases in which resistance can be predicted (9). In 2010, the development of the Xpert MTB/RIF assay (hereinafter Xpert) was a major step forward, improving the diagnosis of TB by reducing the turnaround time for diagnosis, shortening patient isolation, detecting putative rifampicin resistance, and reducing the time to initiate appropriate TB treatment globally. The Xpert MTB/RIF Ultra assay (hereinafter Ultra) has been developed by Cepheid as a next-generation assay aiming to correlate with the semiquantitative results of the acid fast bacilli (AFB) detection and infection burden, evaluating through this manner the patient transmission potential (19). Although the availability of rapid molecular assays, such Xpert, leading to increasing susceptibility testing to rifampicin, much greater efforts are needed to meet the WHO target of universal drug susceptibility testing (20), since only 22% started TB treatment during 2018 and 2019 (7) what reveal the needed of more efforts. The Ultra assay shows non-inferiority compared to the Xpert for the diagnosis of TB and the detection of rifampicin resistance and can be used as an alternative to the latter in all settings. All patients with *M. tuberculosis* strains harbouring mutations for rifampicin resistance, identified by Ultra should undergo further testing as per current WHO

policy guidance to determine if there is additional resistance to fluoroquinolones and the second-line injectable drugs, the core of the second-line treatment for MDR/RR-TB (21).

The application of molecular assays for the detection of mutations in genes associated with drug resistance in clinical samples, technological advances in targeted WGS have raised the possibility for integration in routine molecular epidemiological investigations in different settings. In particular, the use of WGS in epidemiological investigations has allowed for in-depth resolution of transmission events, compared to traditional epidemiological investigations (22). Currently, the bulk of routine drug resistance testing is undertaken using phenotypic DST. This approach will still be required for a subset of difficult to interpret drug resistance patterns; however, the overarching goal is to detect all variants associated with resistance for comprehensive genome-based resistance profiling. Although the current statistical approach for identifying resistance-associated variants using WGS data is an important step forward for clinical use, a weakness is that phenotype predictions of rare and/or novel genetic variants cannot be assessed (23).

In 2018, the WHO redefined the critical concentrations (CCs) to correspond to epidemiological cut-off values (ECOFFs) and performed an extensive systematic literature review of MIC distributions for WHO-endorsed media (24). This revealed that some CCs had been too high, resulting in misclassification of some resistant strains as susceptible. MICs were often truncated because inappropriate concentration ranges were tested, which precluded a comprehensive assessment of the phenotypically wild-type MIC distributions (23;24). In addition, systematic differences in the MIC distributions from different laboratories that supposedly used the same method became apparent, even for testing on 7H10 despite this method being standardized by CLSI. This had gone largely unnoticed because of the lack of rigorously defined quality control (QC) ranges/targets (14). In general, the quality and quantity of MIC data available for the majority of the anti-TB drugs were insufficient to define ECOFFs according to the criteria adopted by the EUCAST (14).

Since DST was estimated to be performed in only 58% of previously treated TB cases and 12% of untreated cases and is mostly performed in high-income countries where resistance rates are the lowest (5), there is a need to evaluate the contribution of appropriate diagnostic tools as support for monitoring and early detection of MDR/RR-TB and also their implementation as complement to the molecular methods usage of alternative methodologies as MIC determination for DST of *M. tuberculosis* clinical isolates. Additionally, time-to-positivity (TTP), spu-

tum culture conversion, smear conversion, therapeutic drug monitoring (TDM), pharmacokinetics (PK), minimum inhibitory concentration (MIC), and whole blood bactericidal assay (WBA) could facilitate the development of alternative treatment strategies (9). As Cirillo and colleagues (26) said "*In the future, the same investment should be made in training clinician in the interpretation of molecular tests and MIC-based test, that we have devoted to train microbiologists in the use of molecular tests in order to translate into clinical action the information that the technology will allow to collect*."

1.3. Objectives

Main objectives (P):

P01: Evaluate the capacity of the Xpert Ultra on the improve the detection of *M. tuberculosis* complex in respiratory samples from patients in the first month of treatment, at four timepoints (0,7,14,28 days) using liquid culture as gold standard.

Hypothesis: The burden of *M. tuberculosis* DNA detected using Ultra will decrease throughout the first month of treatment and will be comparable to the gold standard. We expect a lower specificity during the first month due to detection of DNA from non-viable mycobacteria.

P02: To evaluate the correlation between treatment outcome, Ultra´s results and Ultra's time to conversion to negative, overall and stratified by HIV status.

Hypothesis 1: There is a correlation between poor treatment outcome and the time in which Ultra results converted into negative due to early detection of mutations related to drug resistance.

Hypothesis 2: The correlation between treatment outcomes, Ultra´s results and Ultra's time to conversion to negative (regarding the number of days in which Ultra converted into negative), will be different regarding HIV status, since the HIV positive patient may have early conversions due to the combination with ART.

Secondary objectives (SO):

SO1: To describe the phenotypic profile of the *M. tuberculosis* isolates collected from patient samples before treatment initiation by MIC determination; assess the agreement between rifampicin resistance and Ultra´s results, overall and stratified by previous TB treatment and HIV status.

Hypothesis 1: The phenotypic resistance profile of *M. tuberculosis* isolates collected before treatment initiation will be concordant with the results on rifampicin resistance obtained with the Ultra assay as proxy of MDR-TB.

2. METHODOLOGY

2.1. Study design and setting

This study consists in a retrospective analysis on the effectiveness of the application of the Ultra for follow up during the first month of treatment and the determination of MICs for *M. tuberculosis* clinical isolates collected from respiratory samples from patients from Manhiça District Hospital (HDM) and Manhiça Health Research Center. These samples belonged to a prospective cohort study conducted at the same institutions mentioned above.

The Manhiça district is a rural area in Southern Mozambique, with high TB and HIV burden. The Health and Demographic Surveillance System (HDSS) was set by the Centro de Investigação em Saúde da Manhiça (CISM; Maputo, Mozambique) founded in 1996 (26;27). In 2019, the demographic surveillance area (DSA) reported 201, 845 inhabitants living in 46,441 households in the entire district, with an expansion area of 2380 km² (27).

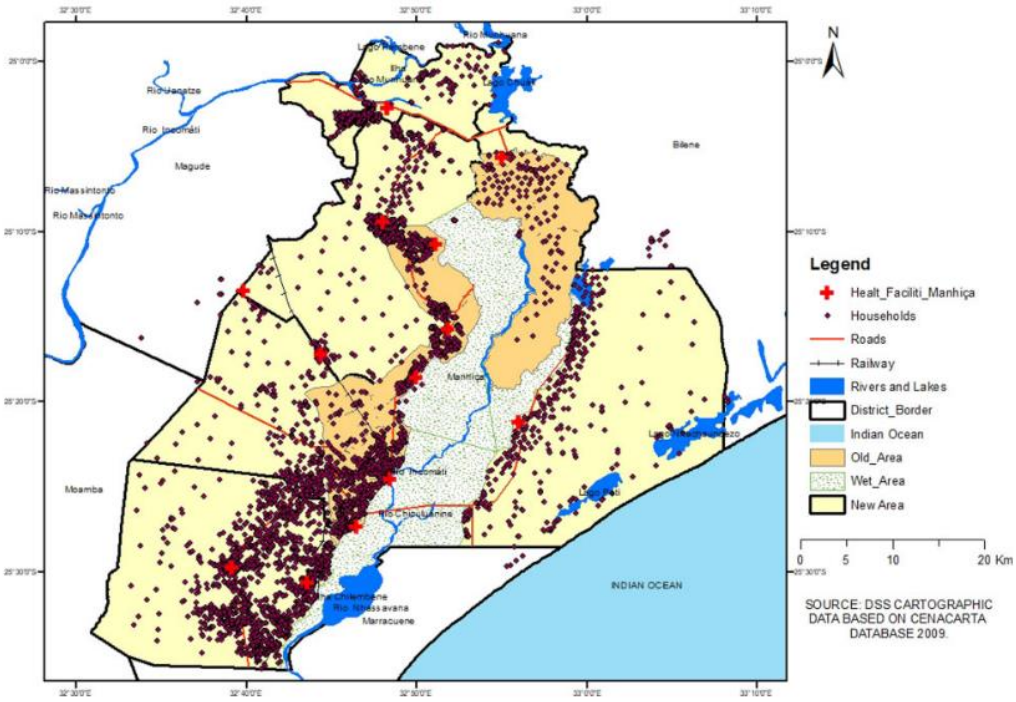


Figure 1: Map of Manhiça district showing the old and the new HDSS areas (28)**.**

All provided samples were processed in the biosafety level-3 laboratory established at the CISM.

2.2. Study participants

The study participants were identified at the National Tuberculosis Control Program (NTP) office when they come to start treatment. The inclusion criteria included all TB cases confirmed by Xpert MTB/RIF cartridge that belonged to the study area. The patients who agreed to participate were recruited consecutively and were asked to provide sputum before starting TB treatment (day 0) and during follow-up study visits at day 7, day 14 and day 28. During the study, a subset of participants was identified with "marking events" due to the presentation of the following characteristics:

1- Having be indicated as retreatment:
2- Having a positive microscopy result in the follow-up visits in months 2 and 6 and/or;
3- Have been indicated to change treatment from susceptible to the multi-drug resistant scheme.

2.3. Study Diagnostic Flow

Sputum sample from all TB cases: A 'spot' specimen was obtained on the day that the participant TB cases started treatment (day 0). Subsequently, an early morning sample was requested to be provided for the following visits at day 7, day 14 and day 28. Patients were properly instructed on how the samples need to be collected to ensure that the specimen is of sputum, not saliva, and transport occurs in optimal conditions. Specimens were transported and processed at CISM BSL3 laboratory on the following day.

Culture: For culture preparation, the samples were digested and decontaminated with solution containing NALC solution combined with 2% NaOH and 2.9% sodium citrate. They were incubated at room temperature for 20 min, then added phosphate buffer until 45 ml in 50 ml Falcon tube. After, the specimens were centrifuged at 3000xg, and supernatant fluid discarded slowly into a container with 5% phenol solution. The pellet was suspended in 2 ml sterile phosphate buffered saline.

Two hundred microliters of the treated sputum solution were used for inoculation into 7 ml MGIT tubes an incubated into the BACTEC MGIT 960. The 7ml MGIT tubes were enriched with supplement (oleic acid-albumin-dextrose-catalase) and antibiotics (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin) prior to inoculation. The MGIT cultures were monitored continuously through the automated system. *M. tuberculosis* complex identifications process was done by performing an *M. tuberculosis* rapid ID test, Ziehl-Neelsen staining, and blood agar plate from all MGIT positive tube.

Processing Xpert Ultra cartridges: Retrospectively, frozen stored samples from day 0, 7, 14 and day 28 were recovered and processed using the GeneXpert system to evaluate the performance of Ultra assay for the detection of MTBc in respiratory samples from patients in the first month of treatment. The processing method follow the manufacturer's guidelines by adding a ratio of 1:2 mL of sample-to-sample reagents, incubation at room temperature for 15 min (vortexing each 5 min interval), 1 mL of the mixture added to Ultra cartridge and analysed in the Xpert system according to manufacturer's guidelines (Figure 2).

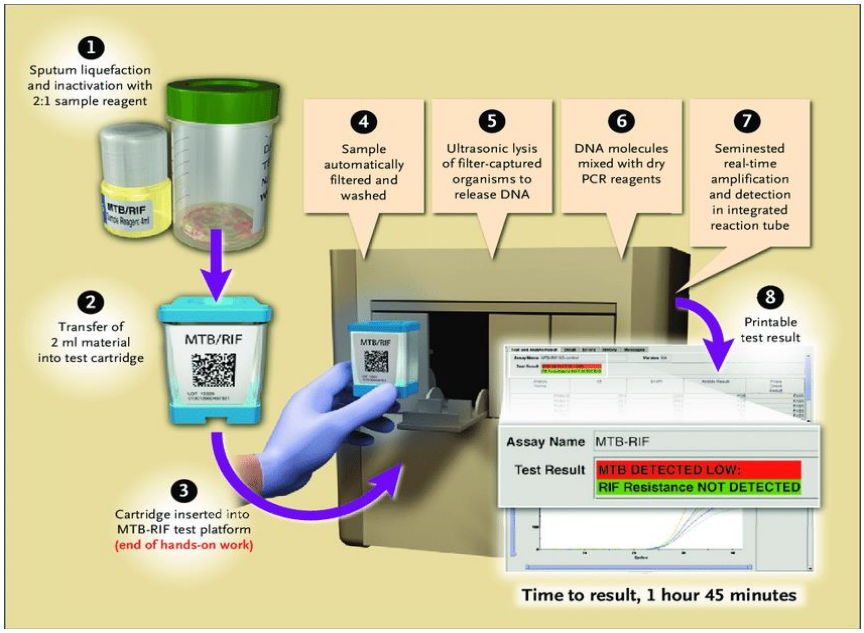


Figure 2: Summary flow for assay procedure for the Xpert cartridge test (adapted (29)**).**

Phenotypic characterization of *M. tuberculosis* complex isolates: The phenotypic susceptibility profile was obtained by the determination of MICs for all isolates obtained on visit 0 (before starting the treatment). We use the EUCAST reference method for MIC procedure (7H9 AMST in-house - appendix 01) to obtain the MICs for all clinical isolates for selected drugs: streptomycin, isoniazid, rifampicin, ethambutol, levofloxacin, and amikacin. For calibration, initially we tested the protocol using *M. tuberculosis* H37Rv ATCC 27294.

The EUCAST reference method is a method based on broth microdilution using Middlebrook 7H9 medium (7H9) culture media supplemented by a mixture of oleic acid, albumin, dextrose, and catalase (OADC) performed in U 96-well microtiter plates. In summary, the EUCAST reference method consist in three steps:

a) Preparation of broth and anti-tuberculous agents: consisted of stock preparation of anti-tuberculosis agents and assay anti-tuberculosis agents; preparation of assay plate with 7H9 medium.

b) Inoculation of broth, incubation of plates and MIC determination: consisted of the addition of 7H9 medium (7H9) + OADC, anti-tuberculosis agents to the plate, selection of isolates, preparation of inoculum and controls.

c) Incubation and MIC determination: consisted of cover the plates with a plastic lid, then put them in O_2-/CO_2- permeable plastic bags and incubation at 36°C±1°C and reading the plates using an inverted mirror.

Whole genome sequencing: A set of 11 isolates was sent to the Instituto de Biomedicina de Valencia, in Spain to for WGS. After inactivation, samples were used to prepare WGS libraries. Sequencing libraries were constructed with a Nextera XT DNA library preparation kit (Illumina), following the manufacturer's instructions (Illumina, Inc). Sequencing was performed on an Illumina MiSeq instrument. Sequence analysis was carried out following a validated previously described bioinformatics pipeline (30). Resistant phenotypic profiles were compared with genetic markers and the results of rifampicin results obtained with the Xpert Ultra and MIC determination.

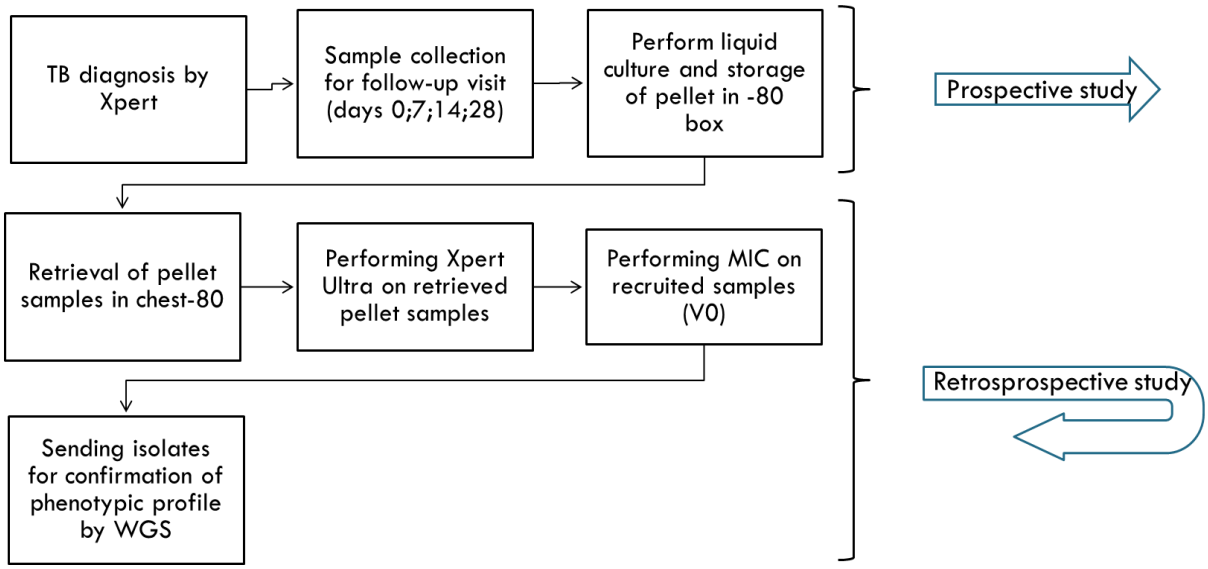


Figure 3: Study flow summary diagram.

Data analysis

For carrying out the analysis proposed, we defined the following datasets:

- Dataset 1: The clinical, demographic, and potential risk factors. Data was collected from the clinical database (beginning and end of treatment - CRF enrolment and end of treatment study MULTIDRUG with the reference 555/CNBS/178, RedCAP).
- Dataset 2: Laboratory data containing Ultra results information (burden and rifampicin resistance based on mutations in the *rpoB* gene) and liquid culture results (category and positivity time) were gathered from laboratory data base.
- Dataset 3: Laboratory data from MTBc strains collected from routine bacteriologic procedures to confirm presence of BAAR. Time to positivity (TTP) was gathered from the laboratory database designated as Servolab 4.
- Dataset 4: Laboratory data on MIC was collected from the results of the EUCAST broth microdilution-based reference method for *M. tuberculosis*.
- Dataset 5: Results of WGS data was gathered from Valencia Institute. Information on genetic markers processed and interpreted by the Instituto de Biomedicina de Valencia

Statistical analysis

Descriptive statistical analyses were performed by using Rstudio Version 1.0.143 - © 2009-2016 and included the calculation of counts and relative frequencies for nominal variables and measures of central tendency and dispersion, and minima and maxima for numeric scale variables. Comparative analyses were performed using contingency table and Kappa agreement parameter. Sensitivity and specificity (estimations based on Clopper-Pearson) was performed on epitools webpage (**https://epitools.ausvet.com.au/**) to evaluate Ultra performance.

3. RESULTS AND DISCUSSION

3.1. Clinical and demographic characteristics

From June 2019 to April 2020, a total of 91 participants were enrolled in the study, and 88 (95.7%) completed all the scheduled visits. One participant died after the enrolment; two participants withdrew from the study just after being enrolled.

Sixty-one out of 88 participants were men (69.32%), the median age was 39 years (interquartile range (IQR) 33-49). There were 81 new cases (92.05%) and 7 (7.96%) retreatment cases. HIV status was available for all participants: 55 (62.5%) were HIV positive and 41(74.55%) were taken ART. Among the HIV participants, 50 (90.01%) were present to clinic in stage III WHO grade of the disease, 32 (58.18%) received co-trimoxazole (CTX) as prophylaxis and 1 (1.82%) received prophylaxis with isoniazid (INZ).

Table 1. Overall clinical and sociodemographic data: relative frequencies of TB cases and HIV status (n=88).

Gender		**Age**	
Male	61 (69.32%)	Median	39
Female	27 (30.68%)	IQR	33-49
Type of TB case		**Chest X-ray**	
New	81 (92.05%)	Suggestive of TB	5 (5.68%)
Retreatment	7 (7.96%)	Not suggestive of TB	83 (94.32%)
Bacillary burden		**Prophylaxis with INZ in past**	
High	44 (50)	Yes	1 (1.82%)
Medium	36 (40.91%)	No	54 (98.18%)
Low	8 (0.09%)		
Contact with a TB patient		**TB cases in contacts with children under 5 years old**	
Yes	5 (5.68%)	Yes	16 (18.18%)
No	83 (95.32%)	No	72 (81.82%)
HIV Status		**ART**	
Negative	33 (37.5%)	Yes	41 (74.55%)
Positive	55 (62.5%)	No	14 (25.45%)
WHO stage		**Prophylaxis with CTX**	
I	2 (3,64%)	Yes	32 (58.18%)
IV	3 (5.45%)	No	23 (41.8%)
IV	3 (5.45%)		

Table 2. Overall clinical and sociodemographic: potential risk factor for acquiring TB (n=88).

Smoker	
Yes	5 (5.68%)
No	77 (87.50%)
Ex-smoker	6 (6.82%)
Former prisoner	
Yes	4 (4.55%)
No	84 (95.45%)
Work in a farm	
Yes	4 (4.5%)
No	84 (95.45%)
Worked or lived outside Mozambique	
Yes	26 (29.55%; South Africa)
No	65 (70.45%)
Sector of work when outside Mozambique	
Bricklayer	8 (30.76%)
Not specified	1 (3.85%)
Mines	10 (38.46%)
Driver	1 (3.85%)
Domestic	1 (3.85%)
Scrap Collector	1 (3.85%)
Farm worker	3 (11.54%)
Trader	1 (3.85%)

Regarding the potential risk factors for TB, only 5 (5.68%) and 6 (6.82%) TB cases were smokers and ex-smokers respectively, and 4 (4.55%) had been previously in prison. A total of 26 (29.55%) cases had lived in South Africa and most of them had worked in mines (Table 2). Factors such as smoking, being a prisoner as well as working for mining are indicated as risk factors for developing TB. In case of miners and other workers in stone have increased risk through silicosis of the lung (31).

Description of the sociodemographic characteristics of the population, stratified by HIV status is displayed Table 3. Univariate analysis by using Pearson's chi-square teste did not revealed any association between the serological status and the evaluated covariates.

Table 3. Sociodemographic and risk factors based on HIV status.

	HIV negative	HIV Positive	*p*-value*
Gender			
Male	24	37	0.6402
Female	9	18	
Age, mean (SD)	45.45 (±18.6)	40.5 (±10.8)	-
BMI, mean (IQR)	54.4 (10.80)	60.2 (11.55)	-
TB type			
New	32	49	0.4965
Retreatment	3	6	
Xpert MTB/RIF bacillary burden			
Low	1	7	0.2132
Medium	16	20	
High	16	28	
Smoking habit			
Yes	1	4	0.8821
No	30	47	
Ex-smoker	2	4	
History of being in prison			
Yes	2	2	0.6287
No	31	53	

The HIV-positive status represents the group who had a higher number of cases, which 28 (50.9%) with high bacillary load and 4 (7.3%) with smoking habits, but a higher percentage of former prisoners was observed in the HIV-negative group (6.1%).

Culture results

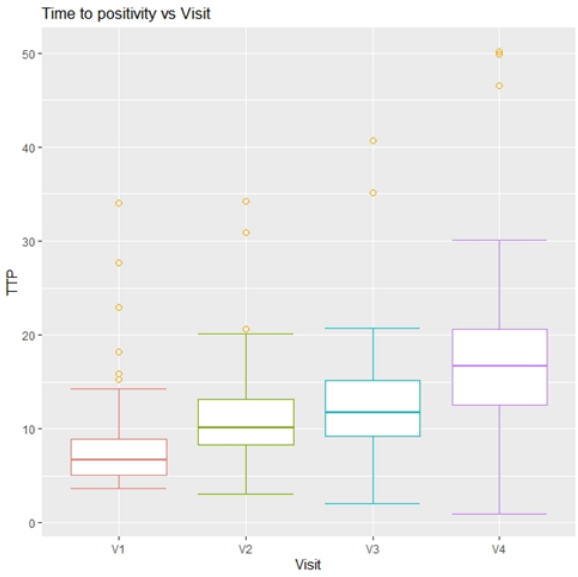
Altogether, 338 samples were obtained during the study. For 14 samples were not obtained since patients miss the visit (six samples from visit 1, three from visit 3 and five from visit 4). Among all, 80.8% (273/338) samples were identified as MTBc by liquid cultures; 3.3% (11/338) identified as non-tuberculous mycobacteria and 10.9% (37/338) had negative liquid culture results. It was also observed that 4.1% (14/338) of the cultures were contaminated and for 0.9% (3/338) of the cultures it was not possible to obtain a result due to insufficient amount of sample to perform culture. As part of diagnostic workflow, all the samples were first submitted to Xpert MTB/RIF and after that, culture was performed, and the remains stored.

Table 4 Overall culture result per study visit.

	MTBc	NTM	Negative	Contaminated	Insufficient sample	*p value*
Visit 1	83 (24.5%)	1 (0.3%)	4 (1.2%)	0	0	0.003
Visit 2	73 (21.5%)	2 (0.6%)	6 (1.8%)	0	1 (0.3%)	
Visit 3	61 (17.9%)	3 (0.9%)	13 (3.8%)	7 (2.1%)	1 (0.3%)	
Visit 4	56 (16.5%)	5 (1.5%)	14 (4.1%)	7 (2.1%)	1 (0.3%)	

NTM, non-tuberculous mycobacteria; **MTBc**, *Mycobacterium tuberculosis* complex.

Stratification of culture results per visits denotes a decrease in the numbers of MTBc isolates obtained (83, 73, 61 and 56 on visit 1, visit 2, visit 3, and visit 4, respectively) and an increase of negative cultures (4, 5, 13 and 14 on visit 1, visit 2, visit 3, and visit 4, respectively) (see Table 4). The median of the distributions of the TTP per study visit is reflected on Figure 4. The TTP increases from visit 1 to visit 4, suggesting that there is a reduction of viable mycobacterial cells along the treatment period.



	Median	IQR	SD
Visit 1	**6.22**	**3.35**	**5.38**
Visit 2	**10.00**	**4.08**	**5.33**
Visit 3	**12.19**	**5.80**	**6.88**
Visit 4	**16.80**	**7.99**	**9.99**

Figure 4. Overall time to positivity in liquid culture per visit. (V1= visit 1; V2= visit 2; V3= visit 3; V4= visit 4.)

For decades, culture has been used as a routine microbiological tool for monitoring DR-TB treatment response (32). Culture is regarded as the best available reference standard for active TB disease and is the reference standard for TB (31; 32).

3.2. Performance of Xpert MTB/RIF Ultra assay

Two hundred and eighty-five stored samples from TB patients belonged to the Multidrug study, were retrospectively selected to be tested by Ultra, although 25 of them did not have enough quantity to be tested. Therefore, a total 260 were included in the analysis. MTBc was detected in 240 of the samples (including traces) and 20 gave negative for MTBc (Table 5). When stratifying Ultra's results through bacillary burden and type of visit, most samples from visit 1 presented high bacillary load (16.1%). Likewise, on visit 2 the most common results were medium (6.3%) and low on visits 3 and 4 (6.7% and 7%, respectively). No mutations suggesting rifampicin resistance were detected in any of the samples with a known result however, nine samples had an indeterminate result with traces bacillary load.

Table 5. Overall Ultra results and TB bacillary burden per study visit.

	High	Medium	Low	Very Low	Trace	Mtb not detected	Insufficient sample	*p-value*
Visit 1	46 (16.1%)	15 (5.3%)	12 (4.2%)	3 (1.1%)	2 (0.7%)	5 (1.8%)	2 (0.7%)	0.001
Visit 2	16 (5.6%)	18 (6.3%)	11 (3.9%)	6 (2.1%)	3 (1.1%)	3 (1.1%)	8 (2.8%)	
Visit 3	18 (6.3%)	16 (5.6%)	19 (6.7%)	1 (0.4%)	1 (0.4%)	7 (2.5%)	6 (2.1%)	
Visit 4	12 (4.2%)	13 (4.6%)	20 (7.0%)	5 (1.8%)	3 (1.1%)	5 (1.8%)	9 (3.2%)	

Like the time of positivity, the detection of MTBc by Ultra during the follow-up visits decreased, indicating a reduction in the bacterial population. These results were expected since the patients were under the intensive phase of treatment. The contingency table of results for the Ultra detection of MTBc (Table 6) shows 90% agreement with fair kappa value (0.43) when compared to liquid culture.

Table 6: Summarized contingency Table comparing Ultra for the detection of MTBc to liquid culture.

		Liquid culture (MGIT)					
		HIV (-)	HIV (+)	Overall	HIV (-)	HIV (+)	Overall
		Positive			Negative*		
Ultra	Positive**	87 (82.9%)	120 (77.4%)	207 (79.6%)	16 (15.2)	17 (10.9%)	33 (17.7%)
	Negative	1 (0.95%)	4 (2.6%)	5 (2.1%)	1 (0.95%)	14 (9%)	15 (4.5%)
	Overall *p*-value			P<0.001			
	Overall sensitivity			96.6% (94.6%-99.2%)			
	Overall specificity			31.3% (18.7% -46.3%)			
	Negative likelihood ratios			0.0755			
	Positive likelihood ratios			1.4			

*NTM and contaminated samples were considered negative; **Positive include trace results.

Sensitivity and specificity estimation based Clopper-Pearson (exact) shows a 97.6% (95% CI: 94.6%-99.9%) and 31.3% (95% CI: 18.7% – 46.3%) values, respectively. Positive and negative likelihood ratios by comparison with the reference (liquid culture) test showed in Table 6.

Table 7. Comparison between Ultra and liquid culture results stratified per HIV group.

	HIV (+)	HIV (-)
Sensitivity	0.968 (0,99-0.96)	0.9886 (0.99-0.94)
Specificity	0.45 (0.64-0.27)	0.0588 (0.28-0.0015)
Kappa	0.43	
p	0.008	

The sensitivity and specificity trade-off is expected to vary by setting (35). We found a good sensitivity (97%) on Ultra´s performance, as described in other studies. These results might be explained by the detection of *M. tuberculosis* DNA by the Ultra assay based on the amplification of the IS*6110* and IS*1081* that are present in multiple copies thought the *M. tuberculosis* genome, allowing a better sensitivity than that obtained with the Xpert assay. The latter is based only on the amplification of the *rpoB* gene (36). For diagnosing pulmonary TB, the Ultra must have higher sensitivity and lower specificity than the Xpert, especially in smear-negative participants and people living with HIV (35).

Several authors reported poorer specificity when Ultra is used to test subjects with a recent history of TB due to the presence of persistent dead bacilli DNA, because this assay is capable of

detecting very low amounts of MTBc DNA (36). The low specificity of Ultra compared to culture can be explained by the occurrence of false positives due to the ability of Ultra to detect DNA regardless of whether the bacillus remains viable or non-viable, whereas culture detects only viable bacilli. The decreasing specificity over the study visits supports the previous statement, since even though there is less and less viable bacilli (observed by an increasing TTP) in the cellular environment.

Table 8. Contingency Table for Ultra agreement during follow-up study visit.

		Liquid culture (MGIT)							
		Pos.*	**Neg.**	**Pos.***	**Neg.**	**Pos.***	**Neg.**	**Pos.***	**Neg.**
Utra	**Pos.**	76 (91.6%)	3 (3.6%)	49 (85%)	1 (1.8%)	42 (67.7%)	6 (9.7%)	40 (68.9%)	0
	Neg**	2(2.4%)	5 (6%)	4 (7%)	3 (5.3%)	13 (20.9%)	7 (11.3)	13 (22.4%)	5 (8.6%)
		Visit 1		Visit 2		Visit 3		Visit 4	
Sensitivity		96% (89%-99%)		98% (89%-100%)		98% (87%-99%)		100% (91%-100%)	
Specificity		50% (6%-93%)		29% (3%-70%)		32% (13%-57%)		27.8% (1%-53%)	
Kappa		0.413		0.352		0.355		0.346	
Agreements		93%		89%		77%		77%	

Pos, positive; Neg, negative; ** NTM and contaminated samples were considered negative; **Positive include trace results.

The detection of MTBc by Ultra during the treatment visits decreases, indicating that there is a reduction in the bacterial population. These results are expected since the patients are in the intensive phase of treatment. A reduction in viable bacterial load is the most vital currently available marker for TB treatment response. So far, no specific molecular method has been recommend as a tool for monitoring TB treatment response, necessitating the continued use of phenotypic methods (7). Because sputum sample must be pre-treated with propidium monoazide, a chemical substance previously known to bind the DNA of dead bacilli, the Xpert MTB/RIF cannot distinguish viable from non-viable DNA of MTBc. Therefore, DNA-based assays are not suitable for monitoring TB treatment response (32).

Graphic representation of Xpert Ct mean in MTBc detection and culture growth time (TTP) along the study visits is shown in Figure 5. There is a dispersion of Ultra´s Ct along the time on visit 4

while on visits 1 and 2 there is a concentrated trend (Figure 4). Statistical analyses on the correlation between Ct media and TTP revealed a lack of correlation between both parameters (*p*-value = 0.4677 (CI: -0.09-0.18)). Although not statistically significant, a positive correlation was observed, like results found in other studies. Ultra´s semi-quantitative results were related to the time to MGIT growth detection, demonstrating that Ultra is influenced by the bacillary load of the clinical specimens (37). This data indicates that these measurements were correlated with viable *M. tuberculosis* load in samples (36). This correlation may be interesting in several aspects such as monitoring the adequacy between the sample's MTBc DNA load (provided by the result of the Xpert MTB/RIF Ultra) and also because the delay to culture positivity could be used as a quality indicator of the whole MTBc culture process in a clinical laboratory, including the delicate step of sample decontamination; it could also be used to assess the contagiousness of patient (36).

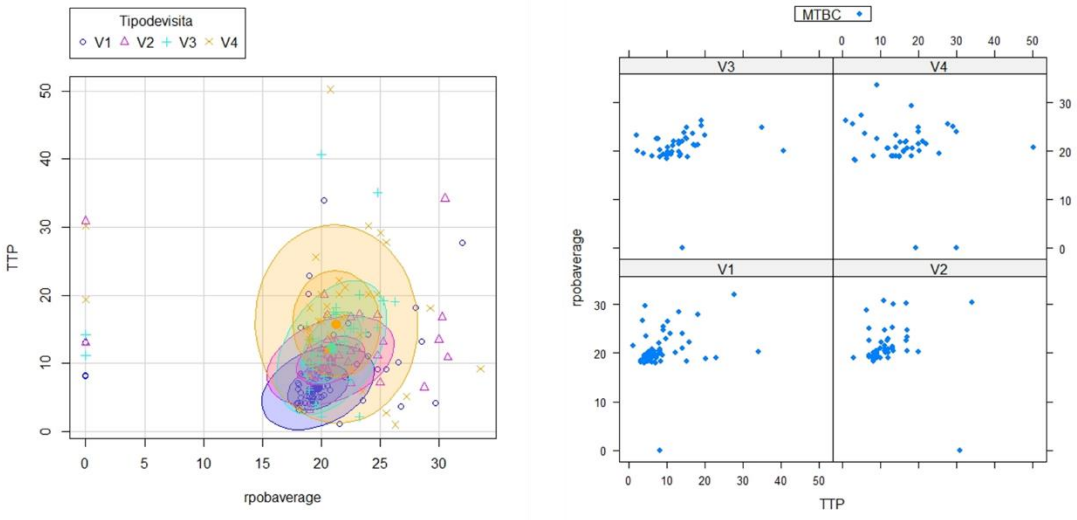


Figure 5. Graphic representation of the distribution of the average Ct of the *rpoB* gene and the TTP of liquid cultures.

Despite all participants were bacteriological confirmed at time of diagnosis, when they provided the baseline sample (for recruitment), twenty samples resulted negative by Xpert Ultra. The negative results may be explained by the physiological mechanism of dispersion of microorganisms, as the unclear understood relationship between bacterial populations in granulomas and those in the sputum. However, it is widely accepted that granulomas with access to airways serve as the

source of bacteria in the sputum. As a result, the transition of MTBc from the granuloma to sputum is likely to be a stochastic process (38) and for this reason "early morning" samples are considered to be the best for screening. Other explanation for the negative results could be the presence of the amplification inhibitors. It is reported that the performance of NAAT can be compromised under several clinical conditions including inadequate specimens, laboratory contamination, inappropriate processing, and the presence of an amplification inhibitor (32).
When mycobacterial cells are killed by anti-TB drugs, there is a decrease in rRNA amount and thus easily estimates the number of viable cells in a patient's sputum sample. A decline in rRNA has been defined as a surrogate biomarker of microbial viability and bactericidal activity for the anti-TB regimen, due to a cellular abundance of 16S rRNA and half-life being shorter than that of DNA (7).

Nowadays, sputum smear microscopy remains the most commonly used test for diagnosis and monitoring of treatment, despite being less sensitive and non-specific for MTBc, while culture, as being identified and applied as the gold standard for TB diagnosis, has the disadvantage of providing results after a substantial period (3–4 weeks) (7). Authors recommend the use of reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) of 16S rRNA detection tests that quantifies TB bacillary load and is used as a marker of treatment response for patients on anti-TB therapy in contrast to DNA-based techniques like Xpert MTB/RIF which is also a quick method for identifying bacilli but that do not offer information on viable bacterial load (7).
The time of conversion from positive to negative was possible to evaluate in three patients in which two converted to negative after 14 days, and one only converted to negative after 28 days. The remaining patient's conversion could not be retrieved from Ultra results. The conversions described above corroborated the results of the liquid culture in the respective the visits.

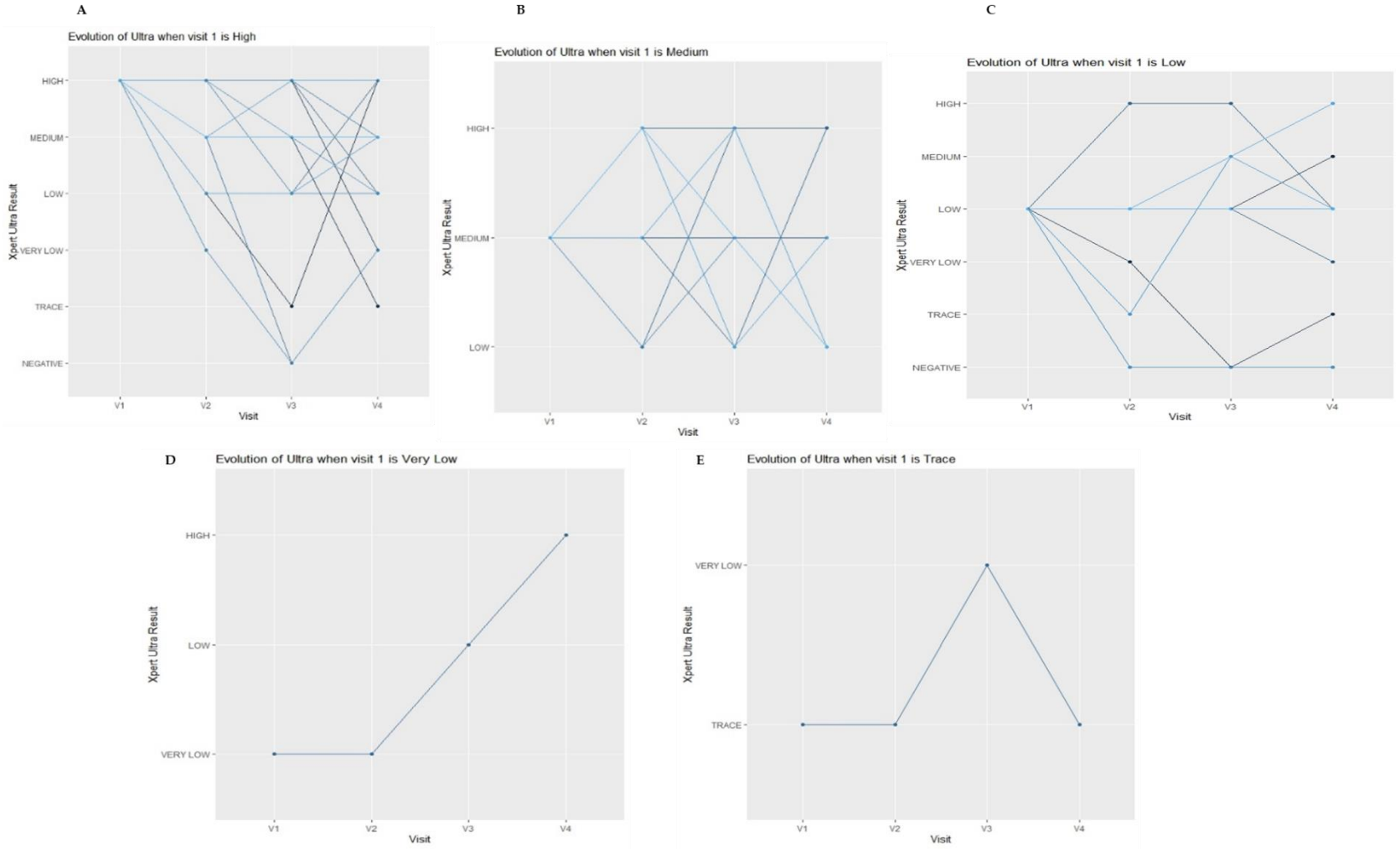


Figure 6. Evolution Ultra MTBc detection during follow-up visit. A represents evolution when Ultra semiquantitive result was high on Visit 1; **B** represents evolution when Ultra semiquantitive result was medium on Visit 1; **C** represents evolution when Ultra semiquantitive result was lown on Visit 1; **D** represents evolution when Ultra semiquantitive result was very low on Visit 1; **E** represents evolution when Ultra semiquantitive result was trace on Visit 1.

3.3. Treatment follow-up and outcomes

All participants who were included in the study were followed up according to national guidelines. Most patients recruited that had as result of treatment being cured, only two started the treatment for MDR. During the follow-up, 12 participants were identified as "mark events[1]" whose clinical-demographic characteristics are described in Tables 7 and 8. Among the subgroup of participants with "mark events", four (33%) were women, the mean age of the subgroup was 43 (34-63) and nine (75%) were HIV positive. For the patients diagnosed with pulmonary TB, seven (58.3%) were retreatments and the remaining, new cases (Table 9).

During the follow-up visits in month 2, four participants (all new cases, two seropositive and two seronegative) had positive microscopy results, suggesting non or late-conversion after the end of the intensive phase of treatment. The first patient had a low bacillary load, which was maintained until visit 3, and at visit 4 had a medium bacillary load without detection of resistance-conferring mutations. He did not convert to negative at month 6, even though the WGS did not report the presence of resistance-conferring mutations and this participant end up death. The second patient presented a medium bacillary load at visit, and this was maintained until visit 4 but at month 6 there was a conversion, and the result of treatment was cure. In the other two patients it was not possible to evaluate the evolution of the Ultra due to insufficient samples to carry out the tests to evaluate the progression of the disease. One of the participants died early after the start of treatment (*post-mortem* not available).

[1] a subset of participants with the following characteristics a) retreatment; b) positive microscopy result in the follow-up visits and c) change treatment regimen from drug susceptible to drug resistance

Table 9. Clinical characterization of participant subset "mark events".

Study ID	Age	Sex	Type of case	Cough >15 days	Fever > 15 days	Night sweats	Weight loss	Lack of appetite	Smoker	Was arrested	Contact with a TB patient	Status HIV	Treatment outcome
1093	38	F	Retreat-ment	Yes	No	Yes	Yes	No	No	No	No	Positive	Cured
1069	42	M	Retreat-ment	Yes	No	Yes	Yes	No	No	Yes	No	Positive	Cured
1013	47	M	Retreat-ment	Yes	Yes	Yes	Yes	Yes	No	No	No	Positive	Cured
1009	40	M	Retreat-ment	Yes	Yes	Yes	Yes	Yes	Ex-Smoker	No	Yes	Positive	Cured
1008	49	F	Retreat-ment	Yes	Yes	Yes	Yes	Yes	No	No	No	Positive	Cured
1078	49	M	Retreat-ment	Yes	No	No	No	No	No	No	No	Negative	Cured
1003	63	M	Retreat-ment	Yes	No	Yes	No	Yes	Ex-Smoker	No	No	Positive	Cured
1005	34	F	New case	Yes	Yes	Yes	Yes	Yes	No	No	Yes	Positive	Died
1050	36	F	New case	Yes	Yes	Yes	No	No	No	No	No	Positive	Cured
1027	35	M	New case	Yes	Yes	Yes	Yes	No	No	No	No	Negative	Cured
1041	42	M	New case	Yes	No	Yes	No	No	No	No	No	Negative	Cured
1002	39	M	New case	Yes	No	Yes	Yes	No	No	No	No	Positive	Died

F=female; M=male; TB=Tuberculosis, HIV= Human Immunodeficiency Virus

Table 10. Laboratory results of participant subset "mark events".

Study ID	Age	sex	Type of case	Baseline MTBc bacillary burden	Visit 1 Xpert Ultra	Visit 2 Xpert Ultra	Visit 3 Xpert Ultra	Visit 4 Xpert Ultra	Month 2	Month 6	Treatment outcome
1093	38	F	Relapse	Medium	Very low	No result	No result	No result	Negative	Negative	Cured
1069	42	M	Relapse	High	High	Medium	Medium	Medium	Negative	Negative	Cured
1013	47	M	Relapse	High	High	No result*	High	Medium	Negative	Negative	Cured
1009	40	M	Retreatment	Medium	High	No result	Low	Low	Negative	Negative	Cured
1008	49	F	Retreatment	High	Medium	No result	Mtb not detected	Mtb not detected	Negative	Negative	Cured
1078	49	M	Relapse	High	High	No result	No result	No result	Negative	Negative	Cured
1003	63	M	Retreatment	Medium	Mtb not detected	Medium	Medium	Medium	Negative	Negative	Cured
1005	34	F	New case	Medium	Low	Low	Low	Medium	Positive	Positive	Died
1050	36	F	New case	Low	Mtb not detected	Very low	Mtb not detected	No result	Positive	Positive	Cured
1027	35	M	New case	High	Medium	Medium	Medium	Medium	Positive	Negative	Cured
1041	42	M	New case	High	High	Mtb trace detected	No result	Low	Positive	Negative	Cured
1002	39	M	New case	Medium	Very low	No result (died a week after start treatment)				Positive	Died

F=female; M=male; TB=Tuberculosis, HIV= Human Immunodeficiency Virus

In the subgroup identified as “marked events” it was not possible to identify a relationship between the results of Ultra at visit 1 and the results of treatment. When submitted to Fisher's test the differences were not statistically significant (p=0.1364). The evolutionary profile of Ultra in most cases was static, i.e., if the initial bacillary load result was high, then in the subsequent visit the bacillary load could be reduced to medium or low and remain stable until visit 4. In general, no “evolutionary” pattern of Ultra was observed to describe the progressive reduction of bacillary load in the samples at the follow-up visits during the first month.

Patient-important outcomes are especially relevant to patients, decision-makers, and the wider TB community. Ultra´s results did not show a statistically significant effect on all-cause mortality, though the direction of effect was towards mortality reduction. Early detection of TB and rifampicin resistance may not lead to improved patient outcomes if the test result is not linked to appropriate treatment and other healthcare services (33).

3.4. Phenotypic profile (MIC)

The broth microdilution EUCAST reference method results of the calibration with H37Rv reference strains reveal the following range of MICs: 0.031mg/L (0.25-0.031) for streptomycin; 0.078mg/L (0.5-0.0078); isoniazid, 0.0078 (0.5-0.0078), rifampicin; 0.0625 (0.5-0.0313), ethambutol; 0,0313 (0.25-0.0313), amikacin; and 0.0625 (2-0.0313) for levofloxacin.
Phenotypic profile based on broth microdilution based on EUCAST reference method showed low-level MICs, revealing that we are on the presence of susceptible strains both for drug that define MDR or extensively drug resistant TB, according to (39). For three strains were observed a high MIC’s values due to presence of more than one microorganism (1 contaminated and 2 with NTM).

The most frequent MIC values were 0.013mg/L and 0.065mg/L for streptomycin; 0.031mg/L for isoniazid and rifampicin, 0.125mg/L for ethambutol and < 0.0625mg/L for levofloxacin (Figure 5). The rifampicin susceptibility profile observed in the study was concordant with Xpert Ultra findings and treatment outcomes. The lowest MIC values were observed in HIV positive participants (Figure 6).

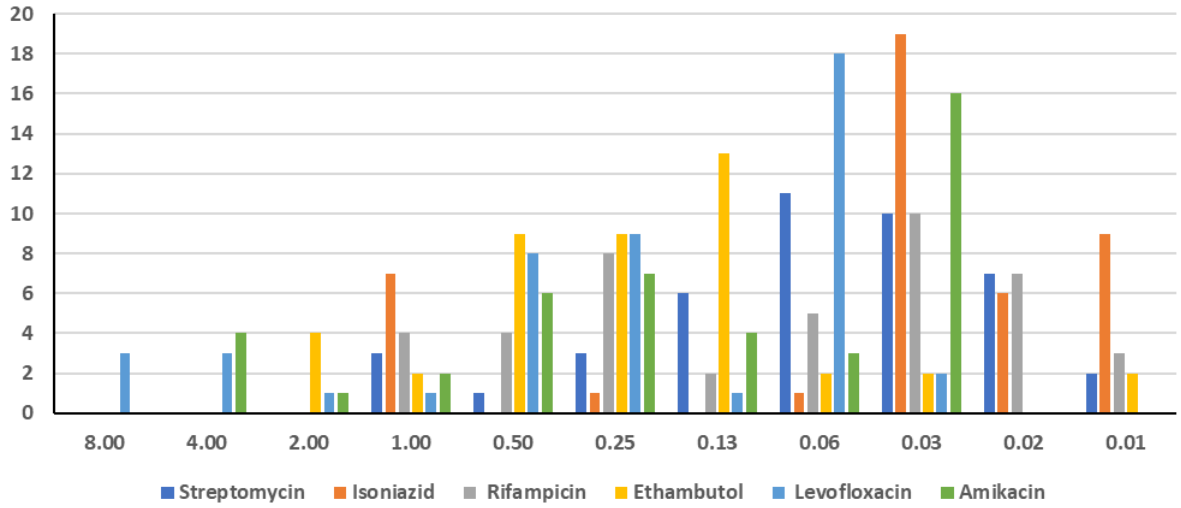


Figure 7: Frequencies of minimal inhibitory concentrations for clinical strains distribution in overall study. y axis: frequency of strains; x axis: MIC values in mg/L for strains.

Levofloxacin and amikacin median MIC had high values in HIV positive participants than HIV negative as shown in Figure 7.

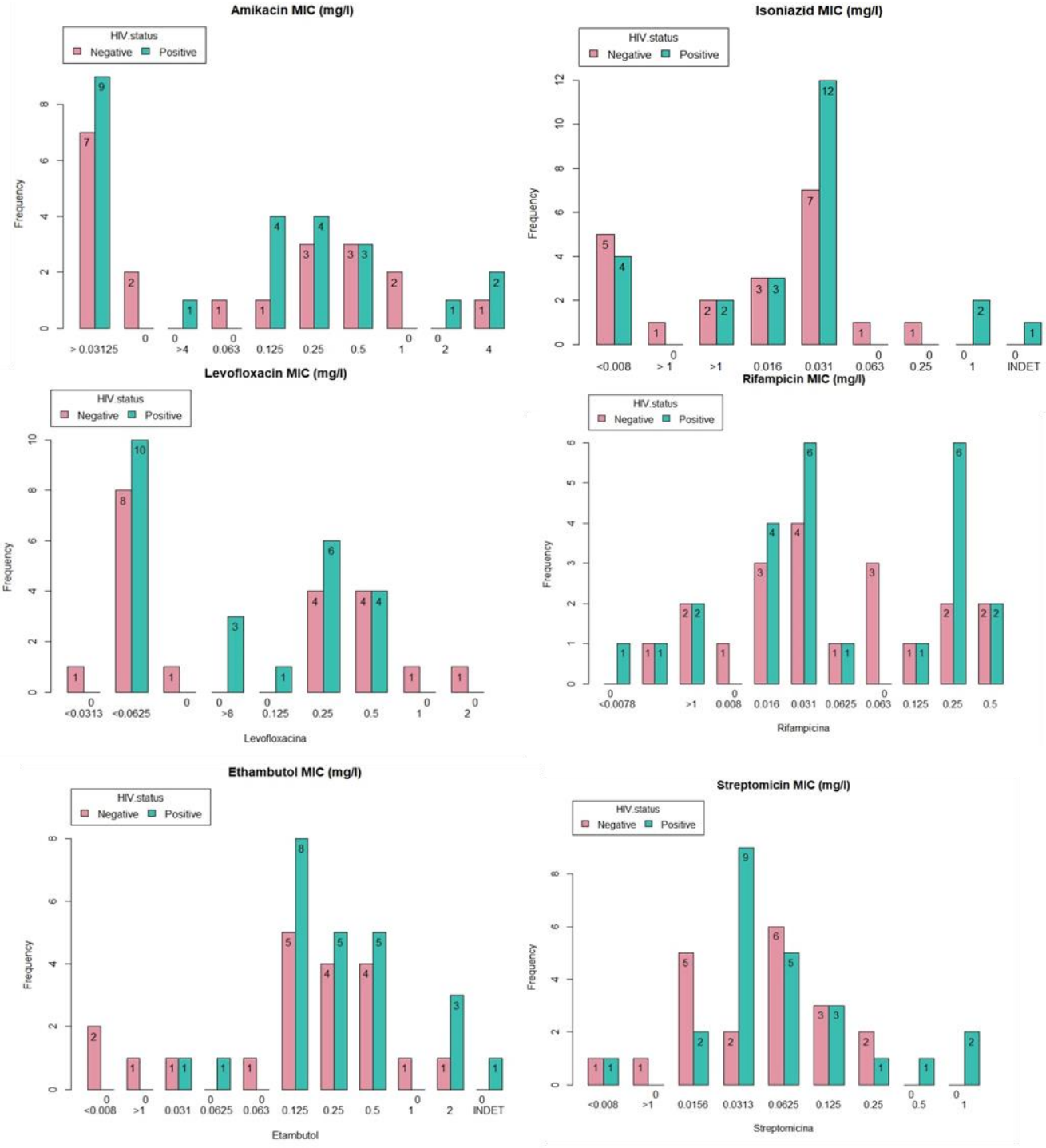


Figure 8: MIC profiles stratified by HIV status.

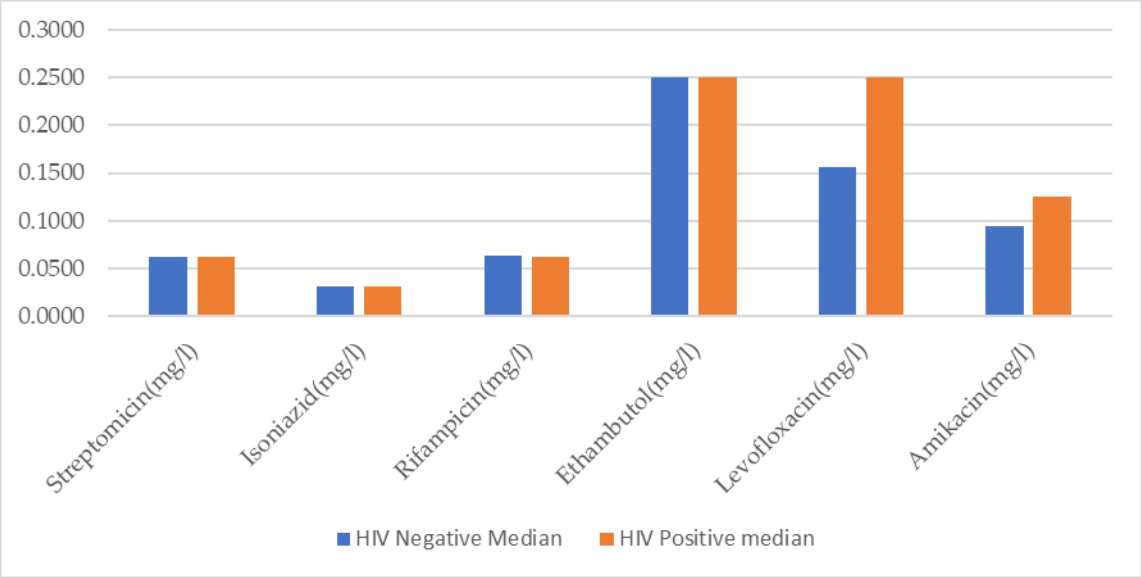


Figure 9: Median of MIC stratified by HIV status.

3.5. Whole genome sequencing

The WGS results confirm the absence of drug resistant mutations on most of the study samples interrogated according to the selected anti-TB drugs (Table 11), except in two, in which a mutation to isoniazid was found suggesting monoresistance to this drug. The isoniazid monoresistant patient was a relapse case. This situation is well explained by (40) and (9) who states that the risk of drug resistance is higher among TB patients who have previously received TB treatment, and there has been a widespread belief that MDR-TB is due primarily to the acquisition of resistance during inadequate treatment. We were not able to achieve our secondary objective, which was focused on early identification of mutations on the strains collected during first month of treatment. The assumption is supported by (40) hypothesis that resistance is much more likely to occur in the induction phase, with high bacterial loads, than after successful induction and follow-up treatment in the consolidation phase.

In the sub-set of strains submitted to WGS, we observed a higher frequency of lineage 4, followed by lineage 1 and a low frequency of lineage 2. Isoniazid resistance genes were detected in lineage 1 (two samples). Curiously, a polyclonal isolate involving lineage 4 and 2 was observed. The geographical spread of MTBc lineages differs markedly in some lineages exhibiting a global distribution and others a strong geographical restriction. Lineage 2 (also known as East-Asian lineage and includes the Beijing family of strains) and lineage 4 (Euro-American

lineage) occurs worldwide. Lineage 1 (also known as Indo-Oceanic or EAI) occurs all around the Indian Ocean (9).

Our findings confirm what many authors report: WGS can now characterize profiles of susceptibility to first-line anti-tuberculosis drugs with a degree of accuracy sufficient for clinical use (41). The genomic approach could be used to guide the choice of which drugs to prescribe and not just which drugs to avoid, in a way similar to phenotyping (41).

Table 11. Results from whole genome sequencing:

Study code	Streptomycin	Isoniazid	Rifampicin	Ethambutol	Fluoroquinolones	Amikacin	Phylogeny
TBMDR-01005	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.3.2
TBMDR-01006	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.3.4.2
TBMDR-01012	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.3.2.1
TBMDR-01013	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 1.2.2
TBMDR-01015	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 1.2.2
TBMDR-01017	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Polyclonal 4.4+2.2
TBMDR-01023	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 2.2
TBMDR-01027	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.10
TBMDR-01028	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.3.2.1
TBMDR-01031	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.3.3
TBMDR-01034	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.3.4.2.1
TBMDR-01041	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.4.1.1
TBMDR-01046	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.3.4.2
TBMDR-01069	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 4.1.2
TBMDR-01078	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Lineage 1.2.2

4. CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

4.1. Conclusion

A comparison between Xpert Ultra and culture offers an interesting perspective on the differences between the two forms of detection (viable mycobacteria and genetic material), but they still offer the same diagnosis and treatment guidance. Our evaluation of Xpert Ultra throughout the first month of treatment shows a good performance with 97.6% of sensitivity and 90% overall agreement (k=0.43) with liquid culture. Time points detection of MTBc at first month of treatment the performance was 95%, 88% for visit 1(day 0), visit 2(day 7) and 69% for visit 3 (day 14) and 4 (day 28).

The phenotypic profile of samples collected before treatment initiation by MICs reveal low levels to drug concentrations and points to the presence of fully drug susceptibility strains which has an agreement with the genotype profile presented by the Xpert Ultra.

4.2. Limitations

Time constraints did not allow to perform the phenotypic DST from the strains belonging to the follow up visit where we expected to get the variations of MIC as signal of effective anti-tuberculosis drugs against the strains and maybe get a resistance profile from the patient that change to the treatment regimen to MDR.

Despite the presence of fair agreement between the Xpert Ultra and liquid culture, the absence of a profile with decrease of bacillary burden it remains in doubt about which would be the best periodicity to perform the Ultra assay to obtain information on the evolution of the treatment suggesting the realization of studies with a longer follow-up period and sample collection.

Also due to lack of resources and time we were not able to perform WGS to all samples from the study so we do not get a complete genotypic DST profile.

4.3. Recommendation

Xpert Ultra showed a good MTBc detection performance, although higher Xpert Ultra detection does not imply treatment failure at that moment, as the technique detects non-viable bacilli. Therefore, Ultra cannot yet be recommended as a tool for monitoring treatment progress and further research on the kinetics of Ultra positivity is recommended to obtain more data on which to base treatment failure indicators.

Further studies applying other molecular tests, such as the TB molecular bacterial load assay (TB-MBLA) that is a reverse transcriptase quantitative real-time polymerase chain reaction (RT-qPCR) of 16S rRNA should be planed as an alternative to the molecular profile of treatment evolution and bacillary reduction.

The broth microdilution EUCAST reference method showed to be user-friendly. The protocol is easy to follow and could be used in a research microbiology laboratory allowing MIC determination. However, its implementation for routine use at a clinical microbiology laboratory should be very well evaluated in terms of capacitated human resources and assay requirements as it requires long periods of bench work, is influenced by accurate pipetting skills and inoculum preparation, and the need to be adapted to the conditions of each laboratory.

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A
APPENDIX

A.1 A Reference protocol for MIC determination of anti-tuberculous agents against isolates of the *Mycobacterium tuberculosis* complex in Middlebrook 7H9 broth

Version 6.1. 4th of July 2019.

1. First step: Preparation of broth and anti-tuberculous agents:

1. A 96-well U-bottom-shaped polystyrene plate should be used. Plates or tubes made of polypropylene or other plastic material should not be used. When the plates have been prepared, they should be used as soon as possible (within the same day).
2. For each agent, MIC determination should be done by testing at least 8 concentrations in separated wells to cover the full range of potential MIC values (outlined in Table 1 and Appendix 1).
3. Prepare Middlebrook 7H9 medium (7H9) from the base, according to the manufacturer's instructions. After the medium is autoclaved, allow to cool to 50°C in a pre-warmed water bath before adding 10% OADC which should be pre-warmed to room temperature (RT; 18-22°C). For each 96-well plate, 10 mL of ready-made 7H9/OADC broth is needed.
4. A stock solution should be prepared as outlined in Table 1, by dissolving the active agent in its solvent as recommended in the ISO-20776-1 standard or if not listed, per recommendation by the manufacturer. As an example, if a stock solution of 10,240 mg/L is needed, 102.4 mg will be dissolved in 10 mL of the solvent if the potency of the agent is 100%.
5. The stock solution is then aliquoted into 0.2 mL/vials and may be stored at -80ºC for a maximum of 12 months unless otherwise specified by the manufacturer. Thawed vials should not be reused. Record ordering and batch number of all agents as well as date of stock solution preparation.
6. Prepare a 4X working solution in two dilution steps in 7H9/OADC from an aliquot of a stock solution as outlined in Table 1 (example for isoniazid, levofloxacin, and amikacin).
7. Add 0.1 mL 7H9/OADC to all wells, except the peripheral wells, which will be filled in by sterile distilled water, as described in Appendix 1, to prevent desiccation during the incubation time.

8. Add 0.1 mL of the 4X working solution to the wells corresponding to the highest concentration of each agent (C1 in Appendix 1). Make sure not to add any agent to the negative and growth control (GC) wells.
9. Use a multichannel pipette to make 1:2 dilutions by adding 0.1 mL of the antibiotic solution present in the highest concentration row to the following row and finally discard the last 0.1 mL of the last row/wells. Use the plate outline in Appendix 1. It should be noted that this step is not adequate for some agents, especially when the solvent should be kept at the same minimum concentration (e.g., 1% DMSO). In this case, the agent working solutions should be diluted separately and then each dilution added one by one.

For all the following steps including the MIC reading, biosafety measures recommended for handling cultures of *M. tuberculosis* including working in safety cabinets must be carefully followed.

2. Second step: Inoculation of broth, incubation of plates and MIC determination

1. Make sure that broth and plates are at 18-22°C prior to inoculation. Isolates of the *M. tuberculosis* complex to be tested should be grown on solid media (7H10 or 7H11 Middlebrook agar, LJ, or other egg-based solid media) and sampled from fresh cultures (within 2 weeks from visible growth). The reference strain *M. tuberculosis* H37Rv ATCC 27294 should be included in each testing round and the same lot should not be used beyond five passages.
2. Bacterial colonies should be sampled from several morphologically similar colonies (when possible, to avoid selecting an atypical variant) at approximate 1 mg (4 loops of 1µl or a full 3 mm loop). Emulsify the colonies along the inside wall of the tube using an applicator stick or plastic loop. It is important to avoid scraping off medium.
3. First add colonies in a 10-15 mL sterile screw-cap glass tube containing 5-10 sterile glass beads (3mm) then vortex at least 2 minutes after careful closing of the cap. When clumps are well dispersed, add 5 mL fresh sterile distilled water. Close the cap tightly and homogenize the tube's content by vigorously vortexing the tube to swirling for at least 2 minutes. Wait 30 min for remaining clumps to settle.
4. Adjust the turbidity of the supernatant in a new glass tube to McFarland 0.5 by sterile dH2O. Vortex for 30 s. If the suspension density is above McF 0.5, add dH2O until it is reached. If the suspension density is below McF 0.5, it is required to start again from 2.4, otherwise colonies will not be sufficiently dissociated. The turbidity of the suspension should be determined by using a densitometer (suspension turbidity meter).
5. Prepare a 1:100 dilution of the bacterial suspension in 7H9/OADC broth by two steps of tenfold dilutions. The volume of bacterial suspension required for one test plate is

10 mL. Prepare a 10^{-1} suspension by adding 1 mL of the 0.5 McF bacterial suspension to 9 mL of 7H9/OADC and vortex until swirling is obtained for at least 30 seconds. For the 10^{-2} inoculum, add 1 mL of the 10-1 suspension to 9 mL of 7H9/OADC.

6. Additionally, from the 10^{-2} suspension (growth control (GC100%), a 10^{-4} (GC1%) suspension should be prepared in two dilution steps: 1+9 mL (10^{-3}), then 1+9 mL (10^{-4}). This will be used as a GC for checking the inoculum and to assess the MIC values.
7. Check the bacterial quantity in the inoculum by CFU counting on Middlebrook 7H10 agar: plate 10 µl of 10^{-2} (equivalent to 500-5000 CFU, i.e., confluent growth), 10 µl of 10^{-3} (50-500 CFU) and 10 µl of 10^{-4} (5-50 CFU) dilutions and read after 14-21 days incubation at 36°C±1°C. The results should be recorded, and the target is $1x10^5$ CFU/mL from the 10^{-2} dilution of 0.5 McF with an acceptable range from $5x10^4$ to $5x10^5$ CFU/mL for a valid test. The results should be recorded.
8. Add 0.1 mL of the 10^{-2} inoculum of 0.5 McF to antibiotic containing wells as outlined in the Appendix 1 starting by the lowest dilution where 0.1 mL of antibiotic-dilutions were pre-added earlier using sterile tips (may be facilitated by using a disposable inoculum reservoir and an 8-channel micropipette with the outer channels corresponding to dH2O removed).
9. Growth controls (GC100% and GC1%) should then be inoculated as outlined in the Appendix 1. The GCs consist of a 1:100 dilution of the 10^{-2} inoculum of 0.5 McF (i.e., 1% of the inoculum present in antibiotic containing wells; GC1%), and the same inoculum (10^{-2} suspension of 0.5 McF (i.e., 100% of the inoculum present in antibiotic containing wells; GC100%).

3. Third step: incubation and MIC determination

1. After inoculation, cover plates with a plastic lid and then put them in O2-/CO2- permeable plastic bags or boxes and incubate at 36°C±1°C. A maximum of 3 plates can be stored on top of each other.
2. Read the plates using an inverted mirror, first after 7 days of incubation and then at 14 days of incubation (visual growth of the GC1% is mandatory for reading and more often present at the day 14 reading). The negative control should show no growth for the test to be valid. If the GC100% is positive, check the GC1%. If the GC1% also shows visible growth (usually weaker positivity than GC100%), MIC can be determined as the lowest concentration of the agent where no visible growth is observed. If there is still insufficient growth of the GC1% after 14 days, incubate until a maximum of 21 days.
3. Report the MIC value in mg/L

Table A1. Examples of preparation of anti-tuberculous agents that were evaluated using the reference protocol by EUCAST-AMST against *M. tuberculosis* H37Rv ATCC 27294.

Antimicrobial	Sigma No	Solvent	Stock conc** (mg/L)	Dilution:1 (7H9)***	Dilution:2 (7H9/OADC)	Working conc. in 7H9/OADC (mg/L) 1mL=10 plates	Final concentration in 7H9 broth (mg/L)
Isoniazid	I3377	dH_20	10 240	1:64	1:40	4	1-0.008
Amikacin	A1774	dH_20	10 240	1:64	1:5	32	8-0.06
Levofloxacin	28266	*	10 240	1:64	1:10	16	4-0.03

*Add powder to 50% dH_20 of the total volume and then 1 mol/L NaOH dropwise to dissolve.Then add dH_20 to the final volume.

**Calculate the amount of drug to dissolve in 10 mL according to potency: m = V*p/P. m=mass of the antimicrobial agent (powder) in g; p=concentration of the stock solution inmg/L; P=potency of the antimicrobial agent (powder) in mg/g (ie 67% potency means 670mg/g or and 100% potency 1000 mg/g); V=volume of diluent in Liter.

***The addition of OADC is not necessary in this step as it is for further dilution only.

A.2 Plate outline (7H9 AMST In-house)

	1	2	3	4	5	6	7	8	9	10	11	12
A	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20
B	negative control	GC 100%	AA1 (10-2) C8	AA1 (10-2) C7	AA1 (10-2) C6	AA1 (10-2) C5	AA1 (10-2) C4	AA1 (10-2) C3	AA1 (10-2) C2	AA1 (10-2) C1	GC 1%	200ul dH20
C	negative control	GC 100%	AA2 (10-2) C8	AA2 (10-2) C7	AA2 (10-2) C6	AA2 (10-2) C5	AA2 (10-2) C4	AA2 (10-2) C3	AA2 (10-2) C2	AA2 (10-2) C1	GC 1%	200ul dH20
D	negative control	GC 100%	AA3 (10-2) C8	AA3 (10-2) C7	AA3 (10-2) C6	AA3 (10-2) C5	AA3 (10-2) C4	AA3 (10-2) C3	AA3 (10-2) C2	AA3 (10-2) C1	GC 1%	200ul dH20
E	negative control	GC 1%	AA4 (10-2) C8	AA4 (10-2) C7	AA4 (10-2) C6	AA4 (10-2) C5	AA4 (10-2) C4	AA4 (10-2) C3	AA4 (10-2) C2	AA4 (10-2) C1	GC 100%	200ul dH20
F	negative control	GC 1%	AA5 (10-2) C8	AA5 (10-2) C7	AA5 (10-2) C6	AA5 (10-2) C5	AA5 (10-2) C4	AA5 (10-2) C3	AA5 (10-2) C2	AA5 (10-2) C1	GC 100%	200ul dH20
G	negative control	GC 1%	AA6 (10-2) C8	AA6 (10-2) C7	AA6 (10-2) C6	AA6 (10-2) C5	AA6 (10-2) C4	AA6 (10-2) C3	AA6 (10-2) C2	AA6 (10-2) C1	GC 100%	200ul dH20
H	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20	200ul dH20

AA1-AA6	Antituberculous agent 1-6 (May be expanded to 2 rows/agent depending on target MIC range)
GC	Growth control
GC100%	Same inoculum as in the drug containing wells
GC1%	Hundredfold diluted inoculum compared to drug containing wells

Negative Ctrl	200µl 7H9-OADC
dH20	sterile distilled water

2021 EDSON TERESO MAMBUQUE EVALUATION OF CUTTING-EDGE DIAGNOSTIC AND TREATMENT MONITORING TOOLS IN A COHORT OF TUBERCULOSIS PATIENTS DURING THE FIRST MONTH OF TREATMENT