Accuracy of the tuberculosis molecular bacterial load assay to diagnose and monitor response to anti-tuberculosis therapy: a longitudinal comparative study with standard-of-care smear microscopy, Xpert MTB/RIF Ultra, and culture in Uganda

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Summary

Background In 2018, the tuberculosis molecular bacterial load assay (TB-MBLA), a ribosomal RNA-based test, was acknowledged by WHO as a molecular assay that could replace smear microscopy and culture for monitoring tuberculosis treatment response. In this study, we evaluated the accuracy of TB-MBLA for diagnosis and monitoring of treatment response in comparison with standard-of-care tests.

Methods For this longitudinal prospective study, patients aged 18 years or older with presumptive tuberculosis (coughing for at least 2 weeks, night sweats, and weight loss) were enrolled at China-Uganda Friendship Hospital Naguru (Kampala, Uganda). Participants were evaluated for tuberculosis by TB-MBLA in comparison with Xpert MTB/RIF Ultra (Xpert-Ultra) and smear microscopy, with Mycobacteria Growth Indicator Tube (MGIT) culture as a reference test. Participants who were positive on Xpert-Ultra were enrolled on a standard 6-month anti-tuberculosis regimen, and monitored for treatment response at weeks 2, 8, 17, and 26 after initiation of treatment and then 3 months after treatment.

Findings Between Nov 15, 2019, and June 15, 2022, 210 participants (median age 35 years [IQR 27–44]) were enrolled. 135 (64%) participants were male and 72 (34%) were HIV positive. The pretreatment diagnostic sensitivities of TB-MBLA and Xpert-Ultra were similar (both 99% [95% CI 95–100]) but the specificity was higher for TB-MBLA (90% [83–96]) than for Xpert-Ultra (78% [68–86]). Ten participants were Xpert-Ultra trace positive, eight (80%) of whom were negative by TB-MBLA and MGIT culture. Smear microscopy had lower diagnostic sensitivity (75% [65–83]) but higher specificity (98% [93–100]) than TB-MBLA and Xpert-Ultra. Among participants who were smear microscopy negative, the sensitivity of TB-MBLA was 96% (95 CI 80–100) and was 100% (95% CI 86–100) in those who were HIV positive. 129 (61%) participants were identified as tuberculosis positive by Xpert-Ultra and these individuals were enrolled in the treatment group and monitored for treatment response. According to TB-MBLA, 19 of these patients cleared bacillary load to zero by week 2 of treatment and remained negative throughout the 6-month treatment follow-up. Positivity for tuberculosis decreased with treatment as measured by all tests, but the rate was slower with Xpert-Ultra. Consequently, 31 (33%) of 95 participants were still Xpert-Ultra positive at the end of treatment but were clinically well and negative on TB-MBLA and culture at 6 months of treatment. Two patients were still Xpert-Ultra positive with a further 3 months of post-treatment follow-up. The rate of conversion to negative of the DNA-based Xpert-Ultra was 3·3-times slower than that of the rRNA-based TB-MBLA. Consequently for the same patient, it would take 13 weeks and 52 weeks to reach complete tuberculosis negativity by TB-MBLA and Xpert-Ultra, respectively. Participants who were positive on smear microscopy at 8 weeks, who received an extra month of intensive treatment, had a similar TB-MBLA-measured bacillary load at 8 weeks to those who were smear microscopy negative.

Interpretation TB-MBLA has a similar performance to Xpert-Ultra for pretreatment diagnosis of tuberculosis, but is more accurate at detecting and characterising the response to treatment than Xpert-Ultra and standard-of-care smear microscopy.

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Introduction

In 2021, 6·4 million people were newly diagnosed with tuberculosis globally, and many more were undiagnosed due to disrupted access to diagnostic services caused by the COVID-19 pandemic.[1,2] Timely diagnosis and treatment reduce tuberculosis-related morbidity and mortality, therefore justifying the demand for fast and accurate molecular diagnostics.

Sputum smear microscopy is the most common tool for tuberculosis diagnosis because it is fast and affordable, with a turnaround time of 1–2 h and a cost of about US$3 per sample, but it requires considerable training to perform well. Moreover, microscopy is dependent on the experience of the operator and the method cannot differentiate between dead and viable *Mycobacterium tuberculosis*, and between *M tuberculosis* complex and non-tuberculous mycobacteria. It also has low sensitivity, especially among people living with HIV and those with low bacterial loads, limiting its applicability.[3–5]

Culture is the optimal confirmatory test for tuberculosis and a reference for other tuberculosis tests because it is sensitive and detects viable bacteria, yet it has several limitations: it is slow and requires a high-containment laboratory, which is expensive to maintain. Indeterminate culture results due to contamination by other bacteria present in patient samples also lead to loss of data.[6,7]

Molecular-based assays have the potential to solve the challenges presented by these conventional tuberculosis diagnostic methods, because they are fast and reproducible, not compromised by contamination, and are highly specific and sensitive.[8] A molecular-based test, Xpert MTB/RIF, was recommended by WHO in 2010 to improve the diagnosis of tuberculosis and rifampicin resistance, but the use of DNA, a molecule that persists long after cell death, compromises the ability to monitor treatment.[9]

Xpert MTB/RIF Ultra (hereafter referred to as Xpert-Ultra), a modified version of the Xpert MTB/RIF, is now recommended as an initial tuberculosis diagnostic and rifampicin resistance test in all adults and children with signs and symptoms of pulmonary tuberculosis. However, trace positive results are not clinically

Research in context

Evidence before this study

We searched the PubMed database for articles in English, published between May 5, 2011, and Dec 31, 2021, using the terms "Xpert Ultra" or "MTB/RIF", "TB-MBLA", "culture", "two-months smear", and "tuberculosis". The search identified eight scientific articles about Xpert MTB/RIF and Xpert MTB/RIF Ultra (Xpert-Ultra) assays, six about tuberculosis-molecular bacterial load assay (TB-MBLA), and one about the 2-month smear microscopy result. Results from these articles showed that Xpert MTB/RIF was sensitive and remained positive in most patients over the course of treatment. A more sensitive version, Xpert-Ultra was being adopted by tuberculosis control programmes as a standard of care for tuberculosis diagnosis. Previous studies also showed that smear microscopy, which is the standard of care for treatment response, has low sensitivity and might not distinguish live from dead bacilli during treatment. The more sensitive Mycobacteria Growth Indicator Tube (MGIT) culture takes a long time to yield results, making it less useful for early treatment decision making. Therefore, we aimed to evaluate TB-MBLA as a potential alternative test for diagnosis and monitoring of tuberculosis treatment response, with the ability to give timely results to inform clinical decisions.

Added value of this study

To our knowledge, this is the first study that has compared a ribosomal RNA-based assay (TB-MBLA) against a DNA-based assay (Xpert-Ultra) for pretreatment diagnosis of tuberculosis and monitoring of treatment response over a 6-month period. We show the utility of TB-MBLA for accurate diagnosis of tuberculosis, and provide further evidence of the limitations of Xpert-Ultra and smear microscopy for monitoring tuberculosis treatment response. Our study shows that Xpert-Ultra is more persistently positive than TB-MBLA, with 33% of participants still positive at the completion of treatment, compared with 27% that was reported for the older version, Xpert MTB/RIF. Smear microscopy sensitivity was low and turned negative faster than clinical positivity and other bacteriological measures after the initiation of treatment. At week 8 of treatment, a comparison of participants who were smear microscopy positive versus those who were smear microscopy negative but TB-MBLA positive showed that smear microscopy was insufficient to inform an extra month of intensive treatment. By contrast, TB-MBLA positivity resolved in a manner that is consistent with MGIT culture and clinical signs. Given that TB-MBLA has a laboratory turnaround time of 4 h, it is indicated as a suitable tool for monitoring response to anti-tuberculosis therapy.

Implications of all the available evidence

Xpert-Ultra is a sensitive tool suitable for pretreatment diagnosis of tuberculosis but not for monitoring of treatment response. Smear microscopy is less sensitive, and therefore less dependable for providing treatment response guidance. The current findings support the WHO recommendation not to extend the intensive treatment phase of drug-susceptible tuberculosis based on a smear positive result at the end of the 2-month intensive phase. Furthermore, the current study shows that TB-MBLA closely mirrors MGIT culture in measuring treatment response, and TB-MBLA results are quantitative and available faster than with MGIT culture (in hours rather than days or weeks). We also show that a proportion of patients, particularly those with low pretreatment bacillary load who converted to negative by day 14 and remained negative throughout treatment follow-up, might not need a 6-month treatment course.

conclusive and hence require a second run. In cases whereby the results of the first and second runs are discordant, patient management is delayed.[10]

The tuberculosis-molecular bacterial load assay (TB-MBLA) is a novel RNA-based assay that detects and quantifies viable *M tuberculosis* in sputum.[11] In 2018, WHO recommended TB-MBLA as a molecular assay that could replace smear and culture for tuberculosis treatment response.[12] The TB-MBLA specifically targets the abundant *M tuberculosis* 16S ribosomal RNA and the test is highly sensitive.[13] Previously, TB-MBLA was evaluated as a treatment monitoring tool on samples that were already confirmed to be tuberculosis positive by smear microscopy or Xpert-Ultra (or both), which made it impossible to calculate its specificity and predictive values.

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

Methods

Study design and participants

We conducted a longitudinal prospective study, utilising freshly collected spot sputa, to evaluate the accuracy of TB-MBLA for tuberculosis diagnosis and monitoring treatment response. The current study was nested within the I AM OLD (Inflammation, Aging, Microbes, and Obstructive Lung Disease) study.[14] Between Nov 15, 2019, and June 15, 2022, we screened patients for eligibility at China-Uganda Friendship Hospital Naguru (CUFH-N) in Kampala, Uganda. Adult participants (≥18 years old) who were coughing for at least 2 weeks with or without fever and who had night sweats and weight loss (estimated ≥5% loss of bodyweight as reported by the patient) were enrolled regardless of their HIV status. All participants tested negative for COVID-19 at enrolment and during the tuberculosis treatment follow-up period. Details about patient identification, data collection, and information flow are provided in the appendix (p 2).

The project was approved by the following institutions: University of St Andrews Teaching and Research Ethics Committee (approval code MD14702), Makerere University School of Medicine Research and Ethics Committee (reference number 2006-017), and Makerere University School of Biomedical Sciences Research and Ethics Committee (reference number SBS 529). All participants provided written informed consent for the use of their biological samples and clinical data for this study. Study activities were conducted according to the Good Clinical and Laboratory Practice guidelines.[15]

Study specimens

Participants provided two spot sputa, which were pooled and homogenised using a magnetic stirrer at enrolment (week 0), and again at weeks 2, 8, 17, and 26 after initiation of tuberculosis treatment. Sampling points were modelled along the WHO recommended treatment monitoring points (baseline, week 8 or month 2, and week 26 or month 6) using smear microscopy as a monitoring tool. Homogenised sputa were aliquoted into four 1 mL portions and tested using Xpert-Ultra, smear microscopy, and Mycobacteria Growth Indicator Tube (MGIT) culture tests. The fourth aliquot for TB-MBLA testing was preserved in guanidine thiocyanate and stored at –20°C at point of collection at CUFH-N until the assay was performed at the Medical Molecular Laboratory of Makerere University College of Health Sciences (Kampala, Uganda). Xpert-Ultra was used as the standard-of-care test for tuberculosis diagnosis and a basis for treatment initiation. Xpert-Ultra, smear microscopy, and tuberculosis treatment were done in the study hospital, CUFH-N. Results for Xpert-Ultra and smear microscopy, as well as tuberculosis treatment initiation, were all issued on the day of the first sputum collection. Sputum liquid culture test was perfomed in a College of American Pathologists-accredited Biosafety Level 3 mycobacteriology laboratory, which is located in the Microbiology Department at Makerere University College of Health Sciences.

Participants who were positive for tuberculosis and without evidence of rifampicin resistance through Xpert-Ultra *rpoB* mutation were enrolled into a treatment follow-up group and treated with the standard 6-month pulmonary tuberculosis regimen (ie, 2 months of isoniazid, rifampicin, ethambutol, and pyrazinamide, followed by 4 months of isoniazid and rifampicin), and monitored for treatment response at weeks 2, 8, 17, and 26 after initiation of treatment. Those who were still Xpert-Ultra positive at week 26 (month 6) of treatment were monitored using telephone calls for a further 3 months post treatment.

Laboratory investigations

For Xpert-Ultra, 1 mL of the homogenised sputa was mixed with 2 mL of the sample reagent buffer (Cepheid, Sunnyvale, CA, USA) and then tested according to the manufacturer's protocol.[16] Results were automatically generated and printed by the Xpert-Ultra platform.[16]

See Online for appendix

For smear microscopy, 1 mL of sample was sedimented at 3000×*g* for 10 min. A smear (1–2 cm in diameter) was prepared from the sediment and stained using auramine O staining (Merck, Darmstadt, Germany). Stained smears were examined using a fluorescent microscope at 400× magnification by the same study personnel as those performing Xpert-Ultra.

For MGIT, 1 mL of sputum was decontaminated using sodium hydroxide-N-acetyl L-cysteine (NALC; ie, fresh 2% solution prepared with 2·9% trisodium citrate and 0·5 g NALC) and neutralised with sterile phosphate-buffered saline (pH 6·8; Becton Dickinson, Sparks, MD, USA). MGIT tubes were inoculated with 500 µL of the

decontaminated sample and incubated at 37°C for a maximum of 42 days. *M tuberculosis*-positive cultures were confirmed by the presence of acid-fast bacilli on Ziehl–Neelsen staining and the presence of MPT64 antigen. The absence of acid-fast bacilli cording and growth on blood agar were recorded as contamination. All results were reported according to the International Union Against Tuberculosis and Lung Disease guidelines.[17]

For TB-MBLA, total *M tuberculosis* rRNA was extracted using the chloroform-phenol method,[18] and then tested at 0·1 dilution. TB-MBLA test was performed based on the duplex reverse transcriptase-real time qPCR principle targeting both *M tuberculosis* complex and the extraction control using a RotorGene 5plex platform (Qiagen, Manchester, UK). Primers and TaqMan dual-labelled probes were manufactured by Eurofin Genomics (Ebersberg, Germany). The qPCR cycling conditions were as reported by Honeyborne and colleagues.[18] Quantification cycle (Cq) readouts were converted to bacterial load using a standard curve that was customised for the site's qPCR platform and recorded as estimated colony forming units (eCFU) per mL.[19] Samples without Cq values, and those with Cq values greater than 30·5 were reported as tuberculosis negative.[19]

For a demonstration of the **TB-MBLA test** see https://www.jove.com/v/60460/a-tuberculosis-molecular-bacterial-load-assay-tb-mbla

Statistical analysis

A complete case analysis approach was followed, leaving out participants who missed their follow-up visits. Differences in baseline continuous variables, including clinical characteristics, Cq values, and TB-MBLA-measured bacterial loads were compared between participants who were positive and negative for pulmonary tuberculosis using the Mann–Whitney *U* test. Correlation analysis of the time to positivity for MGIT culture and Cq values was performed using Spearman's correlation test. Measures of diagnostic performance (sensitivity, specificity, and predictive values) were calculated using Stata version 15.1 with sputum MGIT culture as the reference test. From week 17, calculation of the sensitivity, specificity, and predictive values was not possible because the reference test had gone negative. p<0·05 was considered to indicate statistical significance.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

Between Nov 15, 2019, and June 15, 2022, 236 individuals were screened for pulmonary tuberculosis, of whom 210 fulfilled the study inclusion criteria and were recuited as study participants. Overall, study participants had a median age of 35 years (IQR 27–44) and 135 (64%) were male. The enrolment process, baseline demographics, and clinical characteristics are summarised in figure 1 and table 1. 15 (6%) of the 236 patients objected to returning to the clinic at the scheduled visit points (due to living far away from the clinic), ten (4%) declined to participate in the study, and one (<1%) did not expectorate sputum. Consequently, at baseline, clinical and laboratory data were obtained from 210 (89%) of the screened patients. Of these 210 participants, four (2%) reported a history of cured tuberculosis in the 12 months before enrolment into the study. The gold-standard test (sputum MGIT culture) confirmed pulmonary tuberculosis in 103 (49%) participants. The index test (TB-MBLA) detected tuberculosis in 113 (54%) participants, 28 (25%) of whom were living with HIV. Both MGIT culture and TB-MBLA identified pulmonary tuberculosis in 101 (48%) participants. The standard-of care test, Xpert-Ultra, identified 129 (61%) participants as tuberculosis positive and these individuals were enrolled into the treatment response group. 23 (22%) of the 103 participants that were confirmed to have pulmonary tuberculosis were also co-infected with HIV, with a median CD4 count of 224 cells per µL (IQR 54–340). Six (3%) of the 210 samples assessed by MGIT culture were contaminated. None of the Xpert-Ultra, TB-MBLA, or smear microscopy results were invalid or indeterminate.

In the treatment response group (n=129), 106 (82%) people completed treatment successfully, five (4%) were lost to follow-up, four (3%) defaulted on treatment (ie, did

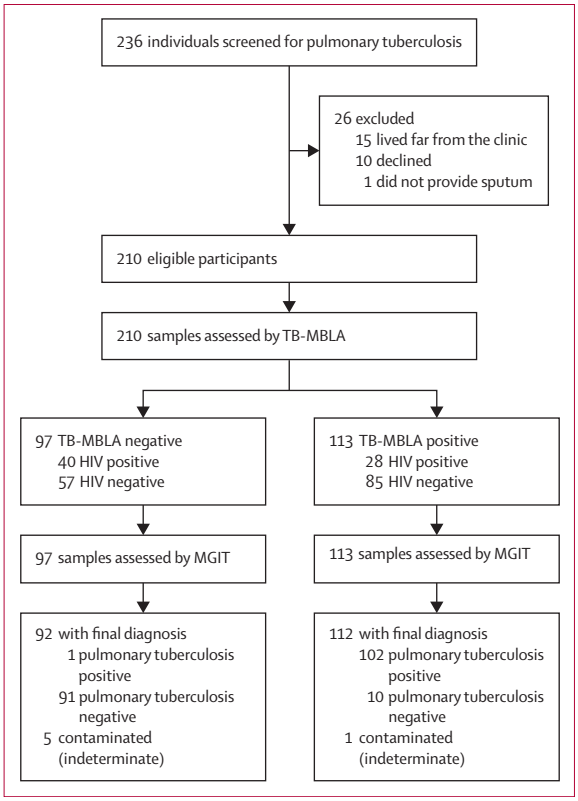

Figure 1: **Participant enrolment and sputum collection**
MGIT=Mycobacteria Growth Indicator Tube. TB-MBLA=tuberculosis-molecular bacterial load assay.

	Participants with indicated pulmonary tuberculosis status*				p value‡
	Overall (n=210)†	Positive (n=103)	Negative (n=101)	Contaminated (n=6)	
Age, years	35 (27–44)	32 (26–43)	35 (30–47)	37 (30–48)	0·60
Sex					
Female	75 (36%)	25 (24%)	47 (47%)	3 (50%)	..
Male	135 (64%)	78 (76%)	54 (54%)	3 (50%)	..
Evening fevers	176 (84%)	92 (89%)	77 (76%)	5 (83%)	..
Weight loss of over 5%: n (%)	161 (77%)	86 (83%)	67 (66%)	6 (100%)	..
Cough >2 weeks	208 (99%)	102 (99%)	98 (97%)	6 (100%)	..
Haemoptysis	54 (26%)	28 (27%)	25 (25%)	1 (17%)	..
Heart rate, beats per min	98 (85–112)	104 (88–114)	93 (79–104)	104 (66–123)	0·27
Respiratory rate, breaths per min	22 (20–28)	24 (20–28)	21 (20–26)	20 (18–23)	0·27
Oxygen saturation, SpO_2	96% (94–98)	96% (94–97)	97% (94–98)	98% (97–98)	0·82
Living with HIV or AIDS	72 (34%)	24 (23%)	44 (44%)	4 (67%)	..
Antiretroviral therapy use§	35 (49%)	7 (7%)	25 (25%)	3 (50%)	..
CD4 count, cells per µL§	222 (54–381)	227 (57–345)	183 (52–601)	514 (113–922)	0·44
CD8 count, cells per µL§	585 (413–874)	589 (459–872)	585 (410–897)	631 (466–883)	0·68
CD4–CD8 ratio§	0·28 (0·11–0·52)	0·24 (0·12–0·51)	0·33 (0·11–0·92)	0·62 (0·25–1·12)	0·51
BMI, kg/m^2	19 (17–22)	20 (17–24)	19 (17–21)	24 (18–27)	0·20
Body temperature, °C	37 (36–37)	37 (36–37)	37 (36–37)	37 (37–37)	0·81

Data are median (IQR) or n (%). MGIT=Mycobacteria Growth Indicator Tube. *Bacteriologically confirmed positive or negative tuberculosis on the gold-standard reference test (sputum MGIT culture). †n=210 refers to all participants who were positive or negative for pulmonary tuberculosis on sputum MGIT culture. ‡Comparison between participants who were positive and negative for pulmonary tuberculosis. §Measured for participants with HIV only (n=72).

Table 1: **Demographic data and clinical characteristics at baseline**

not follow treatment as scheduled), six (5%) transferred to a different treatment centre, seven (5%) died, and one (1%) person experienced treatment failure. 95 (90%) of 106 were thus able to provide adequate sputum volume to perform all the diagnostic tests.

We assessed agreement across all the tests and noted that TB-MBLA and Xpert-Ultra detected more positive tuberculosis cases than MGIT culture at the baseline visit, with ten (5%) of 210 positive on TB-MBLA and 26 (12%) of 210 positive on Xpert-Ultra. These participants had a TB-MBLA-measured mean bacterial load of 3·6 log_{10} eCFU per mL (SD 1·4; median Cq 25 [IQR 26–32]) and Xpert-Ultra median Cq of 26 (24–32). TB-MBLA and MGIT culture concurred on the negativity of the 15 (7%) cases that were positive by Xpert-Ultra, indicating consistent specificity between the two assays. Three (50%) of the six indeterminate culture results were positive with Xpert-Ultra and two (33%) were positive with TB-MBLA.

Trace calls are a new category of results on the Xpert-Ultra platform that can be used to make clinical decisions. A comparison of Xpert-Ultra trace-positive results with other test results is shown in the appendix (p 3). At baseline, ten (8%) of the 129 Xpert-Ultra positive results were trace positive and, as recommended by WHO, all of these participants were placed on tuberculosis treatment. Four of the ten trace-positive participants reported a history of cured tuberculosis disease. Eight (80%) of the ten trace-positive results were negative on both MGIT and TB-MBLA and nine (90%) were negative on smear microscopy at baseline; these remained negative throughout the tuberculosis treatment period (appendix p 3).

TB-MBLA had a high pretreatment diagnostic sensitivity (99% [95% CI 95–100]; 102 of 103) and specificity (90% [83–96]; 91 of 101). Although the pretreatment diagnostic sensitivity of Xpert-Ultra (99% [95–100]) was similar to that of TB-MBLA, the specificity of Xpert-Ultra was lower (78% [68–86]; 77 of 101). TB-MBLA and Xpert-Ultra were both more sensitive than smear microscopy (75% [65–83]; 77 of 103), but the specificity of smear microscopy (98% [93–100]; 99 of 101) was higher than that of TB-MBLA and Xpert-Ultra. Positive predictive values (PPV) were 92% (85–96; 102 of 112) for TB-MBLA, 82% (74–86; 102 of 126) for Xpert-Ultra, and 98% (91–100; 77 of 79) for smear microscopy. Negative predictive values (NPV) were 99% (95–100; 91 of 92) for TB-MBLA, 99% (95–100; 77 of 78) for Xpert-Ultra, and 79% (71–86; 99 of 125) for smear microscopy. Among smear negative participants, sensitivity, specificity, PPV, and NPV for TB-MBLA were 96% (80–100), 92% (84–96), 76% (58–89), and 99% (94–100), respectively. In those with HIV co-infection, TB-MBLA sensitivity, specificity, PPV, and NPV were 100% (86–100), 93% (81–99), 89% (71–98), and 100% (91–100), respectively (table 2).

Of the 124 smear-negative sputum samples at baseline, 26 (21%) were confirmed to have *M tuberculosis* by MGIT culture, 34 (27%) were positive on TB-MBLA (with mean bacterial load 3·4 eCFU per mL [SD 1·3]), 48 (39%) were positive on Xpert-Ultra; and 24 (19%) were positive on both MGIT culture, TB-MBLA, and Xpert-Ultra (appendix p 6). Among the smear negative participants without HIV co-infection, TB-MBLA sensitivity, specificity, PPV, and NPV were 96% (80–100), 92% (84–96), 76% (58–89), and

	TB-MBLA				Xpert-Ultra				Smear microscopy			
	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV
Overall participants												
Week 0 (n=204)	99% (95–100)	90% (83–96)	92% (85–96)	99% (95–100)	99% (95–100)	78% (68–86)	82% (74–86)	99% (95–100)	75% (65–83)	98% (93–100)	98% (91–100)	79% (71–86)
Week 2 (n=121)	87% (77–93)	92% (79–98)	96% (87–99)	77% (62–88)	98·8% (93–100)	33% (19–50)	76% (67–84)	93% (66–100)	74% (64–83)	92% (79–98)	95% (87–99)	63% (49–76)
Week 8 (n=113)	67% (35–90)	88% (80–94)	40% (19–64)	96% (89–99)	92% (62–100)	26% (18–36)	13% (7–22)	96% (81–100)	50% (21–79)	93% (86–97)	46% (19–75)	94% (87–98)
Overall	84% (69–94)	90% (81–96)	76% (64–86)	91% (82–96)	97% (83–100)	46% (35–57)	57% (49–64)	96% (80–100)	66% (50–82)	94% (86–98)	80% (66–91)	79% (69–87)
Smear microscopy negative												
Week 0 (n=124)	96% (80–100)	92% (84–96)	76% (58–89)	99% (94–100)	96% (84–100)	78% (69–86)	54% (39–69)	99% (93–100)	..	..	..	..
Week 2 (n=48)	67% (43–85)	94% (81–99)	88% (62–98)	83% (68–93)	95% (76–100)	33% (19–51)	46% (30–61)	92% (64–100)	..	..	..	..
Week 8 (n=98)	33% (4–78)	87% (79–93)	14% (2–43)	95% (89–99)	84% (36–100)	28% (19–39)	7% (2–16)	96% (81–100)	..	..	..	..
HIV positive												
Week 0 (n=68)	100% (86–100)	93% (81–99)	89% (71–98)	100% (91–100)	100% (79–100)	81% (63–90)	72% (53–86)	97% (85–100)	54% (33–74)	98% (87–100)	93% (66–100)	79% (65–89)
Week 2 (n=32)	67% (38–88)	100% (81–100)	100% (69–100)	77% (55–92)	93% (68–100)	47% (23–72)	61% (39–80)	89% (52–100)	67% (38–82)	94% (71–100)	91% (59–100)	76% (53–92)
Week 8 (n=28)	75% (19–99)	86% (67–97)	50% (12–88)	96% (77–100)	100% (40–100)	57% (35–77)	29% (8–58)	100% (75–100)	50% (7–93)	96% (79–100)	67% (9–99)	92% (74–99)

Data are percentage (95% CI). Data for sensitivity, specificity, PPV, and NPV were calculated against sputum MGIT culture. TB-MBLA=tuberculosis-molecular bacterial load assay. Xpert-Ultra=Xpert MTB/RIF Ultra. PPV=positive predictive value. NPV=negative predictive value. MGIT=Mycobacteria Growth Indicator Tube.

Table 2: **Diagnostic performance of TB-MBLA, Xpert-Ultra, and smear microscopy**

99% (94–100), respectively. TB-MBLA sensitivity, specificity, PPV, and NPV among smear negative participants with HIV co-infection were 100% (72–100), 95% (84–99), 85% (55–98), and 100% (91–100), respectively (appendix p 5).

Overall, before treatment initiation, mean TB-MBLA-measured bacterial load was 4·8 $\log_{10}$ eCFU per mL (SD 1·5). The mean bacterial load was lower among participants who were HIV positive than among those who were HIV negative (3·8 $\log_{10}$ eCFU per mL [1·6] *vs* 5·2 $\log_{10}$ eCFU per mL [1·3]; p=0·0002). Median MGIT time to positivity was 7 days (IQR 5–10) and correlated with both Xpert-Ultra and TB-MBLA (r=0·5, p=0·021).

All the tests showed response to treatment as demonstrated by a decrease in test positivity. At week 2, 32 participants tested negative by TB-MBLA, 12 by Xpert-Ultra, 24 by smear microscopy, and 21 by MGIT culture. 19 (59%) of 32, five (42%) of 12, 13 (54%) of 24, and 15 (71%) of 21 participants remained consistently negative throughout treatment, respectively. The average baseline bacillary load of the participants who consistently remained negative was 4·2 $\log_{10}$ eCFU per mL (SD 1·4).

Similarly, test positivity decreased from baseline to completion of treatment. For example, at 8 weeks (n=113), positive results were 84 (74%) with Xpert-Ultra, 20 (18%) with TB-MBLA, 13 (12%) with smear, and 12 (11%) with MGIT culture. The decrease in test positivity was markedly slower for Xpert-Ultra; consequently, by the end of treatment (week 26), 31 (33%) of 95 were still positive on Xpert-Ultra compared with six (6%) on smear microscopy and none on TB-MBLA or sputum MGIT culture (figure 2, table 3). Of the 31 (33%) participants who were Xpert-Ultra positive at the end of treatment, 13 (42%) were positive at week 8, whereas 18 (58%) turned positive after week 8.

The decreases in positivity were supported by reductions in bacterial load. For example, relative to the bacterial load at baseline, mean bacterial load measured by TB-MBLA reduced by 1·4 $\log_{10}$ eCFU per mL at week 2, 2·3 $\log_{10}$ eCFU per mL at week 8, 2·6 $\log_{10}$ eCFU per mL at week 17, and reached zero $\log_{10}$ eCFU per mL at

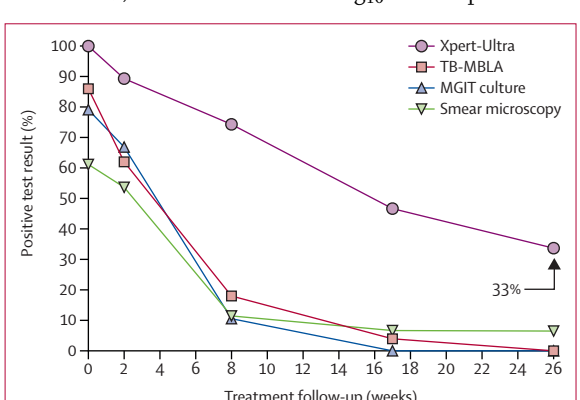

Figure 2: **Tuberculosis test positivity rates across 6 months of treatment**
MGIT=Mycobacteria Growth Indicator Tube. TB-MBLA=tuberculosis-molecular bacterial load assay. Xpert-Ultra=Xpert MTB/RIF Ultra.

	Week 0	Week 2	Week 8	Week 17	Week 26
TB-MBLA positive	113/210 (54%)	70/121 (58%)	19/112 (17%)	6/106 (6%)	0/95
TB-MBLA quantification cycles	20 (17–30)	26 (23–29)	28 (26–30)	30 (29–30)	36 (35–36)
TB-MBLA bacterial load (log_{10} eCFU per mL)	4·9 (3·6–6·1)	3·5 (2·6–4·4)	2·6 (2·2–3·3)	2·3 (2–2·3)	0
Xpert-Ultra positive	129/210 (61%)	110/121 (91%)	84/112 (75%)	49/106 (46%)	31/95 (33%)
Xpert-Ultra quantification cycles	20 (19–23)	20 (19–23)	25 (21–28)	26 (23–30)	26 (21–30)
Smear microscopy positive	79/210 (38%)	65/121 (54%)	13/112 (12%)	7/106 (7%)	6/95 (6%)
Smear microscopy grade, median (range)	3 (2–3)	2 (2–3)	2 (2–2)	1 (1–2)	1 (1–2)
MGIT culture positive	102/210 (49%)	81/121 (67%)	12/112 (11%)	0/105	0/95
MGIT culture time to positivity, days	7 (5–9)	13 (11–16)	23 (12–27)	..	..

Data are n/N (%) or median (IQR), unless otherwise specified. TB-MBLA=tuberculosis-molecular bacterial load assay. eCFU=estimated colony-forming units. Xpert-Ultra=Xpert MTB/RIF Ultra. MGIT=Mycobacteria Growth Indicator Tube.

Table 3: **Changes in test positivity rate and bacterial load**

week 26. Xpert-Ultra semi-quantitative grading varied among the 31 results that were positive at week 26. 15 (48%) were graded by Xpert-Ultra as low positive, ten (3%) as very low positive, five (16%) as trace positive, and one (3%) as medium-positive; however, clinically, none of these participants exhibited tuberculosis-like symptoms at this stage of treatment. At 12 weeks after the end of treatment, two (6%) of the 31 Xpert-Ultra positive participants remained positive but without clinical symptoms, seven (23%) were negative, and 21 (68%) were clinically well and did not provide sputum because their cough had cleared. Moreover, samples tested at 12 weeks after the end of treatment were negative on TB-MBLA, MGIT culture, and smear microscopy.

TB-MBLA overall sensitivity reduced with treatment to 84% (95% CI 69–94), whereas specificity remained the same with a value of 90% (81–96). Similarly, smear microscopy sensitivity decreased to 66% (50–82), whereas specificity reduced from 98% (93–100) at baseline to 94% (86–98). By contrast, the overall sensitivity of the Xpert-Ultra remained high at 97% (83–100) but specificity decreased to 46% (35–57), indicating a delayed conversion of the Xpert-Ultra. Sensitivity, specificity, and predictive values for participants who were HIV positive were similar to the overall population, as were those among participants who were negative on smear microscopy at the baseline visit and 8 weeks after treatment initiation (table 2).

To understand why Xpert-Ultra positivity remained high at the end of treatment, we estimated *M tuberculosis* DNA and rRNA in-vivo elimination rates by modelling the time variation of the PCR Cq values using an

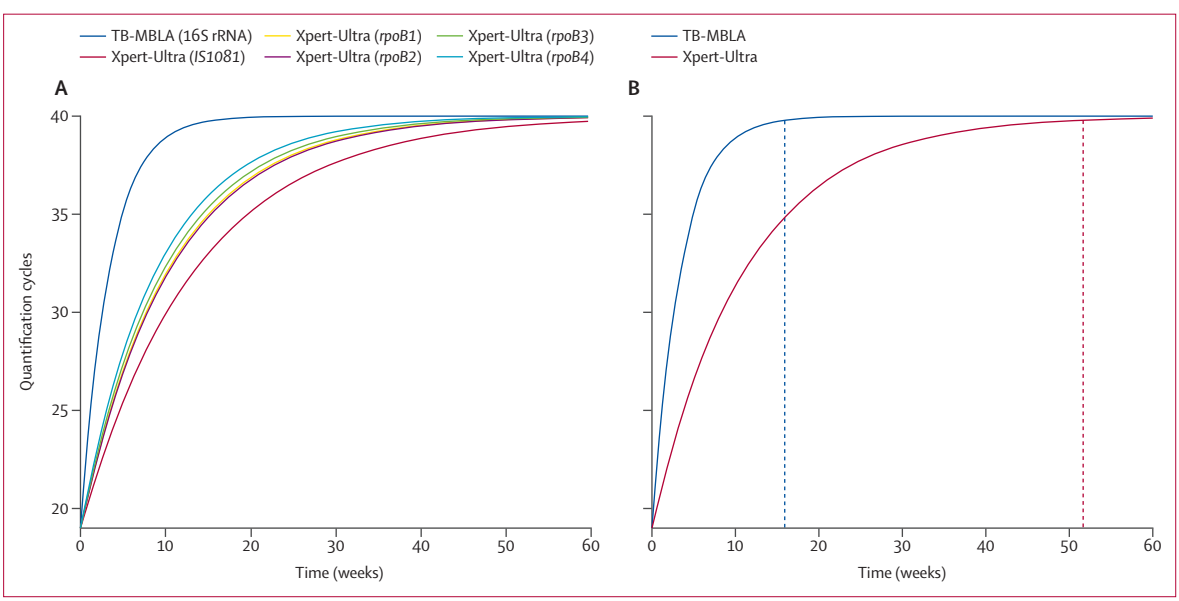

Figure 3: **Rate of increase of quantification cycles (reduction of rRNA or DNA) measured by TB-MBLA and Xpert-Ultra**
(A) Individual genes measured by Xpert-Ultra *vs* 16S rRNA measured by TB-MBLA. (B) Median of the genes measured by Xpert-Ultra *vs* 16S rRNA measured by TB-MBLA. Overall, the median time to attain Cq 40 (limit of quantification) was 15·7 weeks for TB-MBLA 16S rRNA (blue dashed line) and 51·6 weeks for Xpert-Ultra genes (red dashed line). The starting baseline Cq was taken to be 19, which is close to the median of the measured Cq_0 values for both TB-MBLA and Xpert-Ultra. When the concentration of the 16S rRNA or DNA was less than the limit of detection, a Cq of 40 was assigned. Cq=quantification cycle. TB-MBLA=tuberculosis-molecular bacterial load assay. Xpert-Ultra=Xpert MTB/RIF Ultra.

exponential saturation function.[20] When the concentration of DNA or rRNA was less than the limit of detection, a Cq value of 40 was used in the analysis (appendix pp 6–7). The $T_{99\%}$ values corresponded to the time required to reach 99% Cq 40, equivalent to the lowest concentration of quantifiable rRNA by TB-MBLA and DNA by Xpert-Ultra. A lower $T_{99\%}$ was observed for most samples by TB-MBLA compared with Xpert-Ultra, indicating that *M tuberculosis* rRNA degrades more rapidly than DNA. Consequently, the proportion of patients who attained $T_{99\%}$ within 26 weeks of treatment follow-up was 74% by TB-MBLA and 18% by Xpert-Ultra (appendix p 8). The Cq values for TB-MBLA increased about 3·3-times faster than those for Xpert-Ultra. Overall, on the same sample for which TB-MBLA was negative (Cq 40), Xpert-Ultra took 3 more weeks to attain the same result (figure 3, appendix p 8).

A positive smear microscopy test result after week 8 of tuberculosis treatment is recognised as a predictor of unfavourable outcome and a negative test result as a predictor of a favourable outcome. We assessed the association between the smear microscopy test results at week 8 and at the end of treatment outcome (appendix p 9). At 8 weeks (n=113), 20 (18%) participants were positive with TB-MBLA (median Cq value 28 [IQR 26–30]; mean bacterial load 2·7 $\log_{10}$ eCFU per mL [SD 0·6]) and 84 (74%) were positive with Xpert-Ultra (median Cq value 23 [21–28]).

Six (30%) of the 20 participants who were positive with TB-MBLA, and 13 (15%) of the 84 participants who were positive with Xpert-Ultra were also positive by smear microscopy. The mean bacterial load for participants who were both TB-MBLA and smear microscopy positive (2·6 $\log_{10}$ eCFU per mL [SD 0·8]) was lower than for those who were TB-MBLA positive but smear microscopy negative (2·8 $\log_{10}$ eCFU per mL [0·6]), but the difference was not statistically significant (p=0·44). Furthermore, 100 (88%) of the 113 participants were smear microscopy negative at 8 weeks and 14 (14%) of these were positive with TB-MBLA, with low mean bacillary load (2·8 $\log_{10}$ eCFU per mL [0·8]). Only participants who were smear microscopy positive at 8 weeks received 1 extra month of the intensive phase treatment before switching to the continuation treatment phase. Despite this discordance, the treatment success rate was similar (appendix p 9). Telephone follow-up at 3 months after the end of treatment showed that none of the participants with successful treatment outcome had tuberculosis-like symptoms. Test performance discordance is summarised in the appendix (p 9).

Discussion

The findings of this study indicate that TB-MBLA and Xpert-Ultra have similar pretreatment diagnostic sensitivity, but TB-MBLA has higher specificity, regardless of HIV status. Unlike Xpert-Ultra, TB-MBLA specificity remained high during treatment follow-up, with positivity similar to that of MGIT culture. Although the sensitivity of Xpert-Ultra was higher and specificity was lower than that reported elsewhere for diagnostic accuracy,[21] we confirm the unsuitability of Xpert-Ultra and smear microscopy for monitoring tuberculosis treatment response. Consequently, we point to TB-MBLA as the most accurate alternative test for monitoring tuberculosis treatment response. We also confirm that 8-week sputum smear microscopy is insufficient to inform the decision to extend the intensive treatment phase. This observation is in line with the WHO guideline that discourages use of a 2-month sputum smear microscopy positive result as the basis to extend the intensive treatment phase of drug-susceptible tuberculosis.[22]

The high sensitivity and specificity of TB-MBLA before and after treatment initiation gives it a comparative advantage over smear microscopy and Xpert-Ultra. When Xpert-Ultra was used for monitoring response to treatment, specificity substantially reduced, due to accumulation of DNA from dead bacilli, because PCR might remain positive even after successful therapy. However, before treatment initiation, Xpert-Ultra appears to reflect DNA mostly from viable bacilli. This is demonstrated in baseline Xpert-Ultra and TB-MBLA Cq values, which were similar and correlated with MGIT time to positivity.

Like smear microscopy, Xpert-Ultra cannot distinguish viable from dead bacilli.[10,23] By modelling, we have shown that Xpert-Ultra Cq values change slowly (3·3-times less than for TB-MBLA per week), reflecting slow degradation of DNA from dead bacilli. This could explain in part why 33% of the 95 patients at month 6 of treatment were still positive on Xpert-Ultra but negative on TB-MBLA and MGIT culture. These findings corroborate the 2013 study by Friedrich and colleagues, which showed that 22 (27%) of 83 patients were still Xpert MTB/RIF positive at 6 months.[24] The slightly higher positivity rate for the Xpert-Ultra observed in our study might be explained by the higher sensitivity of Xpert-Ultra compared with the Xpert MTB/RIF assay.[25] Indeed, five (16%) of the 31 Xpert-Ultra positive results at the end of treatment were trace positive. Friedrich and colleagues also demonstrated that the positivity rate of Xpert MTB/RIF decreased linearly as opposed to the non-linear (biphasic) form of resolution shown by smear microscopy, MGIT culture, and, currently, TB-MBLA. Similar to the study by Friedrich and colleagues, we note that an increase in sensitivity is achieved at the expense of specificity.[24]

Here, we show that TB-MBLA specificity was 13 percentage points higher than that of the Xpert-Ultra. We hypothesise that the high number of trace-positive results, inconsistent with the reference test (MGIT), were responsible for reducing the specificity score of Xpert-Ultra. Eight (80%) of the ten pretreatment trace-positive results were negative with both MGIT and TB-MBLA. These findings might point to overdiagnosis when treatment is based on a trace-positive result. It remains unclear whether all trace-positive results are

from viable or dead bacilli, given that two of the trace-positive participants were TB-MBLA and MGIT positive and responded to treatment. Additionally, 40% of trace-positive participants at baseline had a history of cured tuberculosis disease, which points to the possibility of residual DNA from previously killed bacilli. Future studies should investigate such trace-positive results and explore the time taken for patients who are Xpert-Ultra positive but culture or TB-MBLA negative to also become Xpert-Ultra negative.

Accurate early markers of poor prognosis to minimise overtreatment are still needed. Over time, an 8-week sputum smear has had a positive impact on cure but with several challenges.[26] Our findings that smear microscopy missed 14 participants who were otherwise positive with TB-MBLA further confirms its low sensitivity and inadequacy to inform extension of the intensive phase treatment. In 2018, WHO cited TB-MBLA as a potential replacement of smear and culture for treatment monitoring.[13] This study shows that TB-MBLA is fully quantitative and measures viable *M tuberculosis*; hence, using 2-week TB-MBLA to monitor treatment response would reveal the actual bacterial load in samples. We show that TB-MBLA provides useful information for clinical treatment decisions at the end of the intensive phase treatment.

More than 80% of the patients who converted to negative at week 2 and remained negative until the end of treatment were detected by TB-MBLA, which suggests its prognostic utility and applicability in personalised management of tuberculosis. A recent study has shown the ability of TB-MBLA to predict relapse earlier than MGIT culture.[27] Studies to further justify its prognostic utility in short-term and long-term treatment outcomes are highly recommended. Nevertheless, in its current state, TB-MBLA would benefit from protocol streamlining by automation to shorten hands-on time for users in settings with a small workforce. The next steps include seeking WHO endorsement supported by cost-effectiveness and implementation studies.

The strength of the current study is that it was nested in the larger, longitudinal I AM OLD study,[16] with an experienced team who guided on collection of high-quality data coupled with a high retention rate of participants in tuberculosis care. Additionally, no data were lost due to invalid or indeterminate Xpert-Ultra tests and the culture contamination rate was 2·5-times below the average (8%) at the study laboratory. The low contamination rate was attributed to the careful decontamination protocol and clear sample collection instructions to patients.

The main limitation of the current study is the dependence on culture as the reference test for the molecular-based assays. Future studies could benefit from using sensitivity and specificity correction methods or a composite reference. Another limitation is the sample size of 210 participants enrolled at one site, which could have underpowered the analyses to detect large differences. Nevertheless, the sample size was consistent with that used to evaluate the diagnostic utility of Xpert-Ultra among patients with tuberculosis meningitis at a single site in Viet Nam (n=205).[28]

In conclusion, this study has shown that TB-MBLA has pretreament diagnostic accuracy of tuberculosis consistent with that of Xpert-Ultra. In terms of monitoring treatment response, the accuracy of TB-MBLA is consistent with MGIT culture and superior to Xpert-Ultra. Of note, TB-MBLA results are quantitative and are available within hours compared with days or weeks for culture. Therefore, TB-MBLA is indicated to be a better tool for assessing efficacy of tuberculosis medication both in routine practice and therapeutic clinical trials.

Contributors

EM, WSs, SW, SHG, LH, WW, MJ, and WSa designed the study and the protocols. SKasi, AS, IS, SKasw, JZ, PB, PK, DK, WM, DTT, KD-MS, NW, and EK participated in data collection and curation. SHG, WSs, EM, WSa, and LH obtained the funds that supported the study. EM, WSa and SHG wrote the first and the final draft. EM, WSs, and WSa accessed and verified all the data reported in this study. MBL and ED contributed to modelling of clearance of *M tuberculosis* rRNA measure by TB-MBLA compared with *M tuberculosis* DNA measured by Xpert-Ultra. All authors had final responsibility for the decision to submit for publication.

Declaration of interests

WSa and SHG provide pro bono advice for LifeArc, a company that is developing TB-MBLA for clinical use. All other authors declare no competing interests.

Data sharing

Deidentified individual participant and aggregated data are available at the University of St Andrews database. Requests to access the data can be sent to ws31@st-andrews.ac.uk and will be assessed on a case-by-case basis as to whether they comply with the available participant consent.

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