Comparison of the *Mycobacterium tuberculosis* molecular bacterial load assay, microscopy and GeneXpert versus liquid culture for viable bacterial load quantification before and after starting pulmonary tuberculosis treatment

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ABSTRACT

Molecular bacterial load assay (MBLA) rapidly quantifies viable *Mycobacterium tuberculosis* (*Mtb*) and may be useful for monitoring treatment response and treatment efficacy.

We conducted a prospective study in 56 adults with pulmonary tuberculosis from whom 244 sputum samples were collected before and during the first month of treatment. We evaluated MBLA for early monitoring of bacterial burden and investigated bactericidal activities of first-line therapy in patients infected with drug susceptible and resistant isolates.

Mtb loads measured by MBLA and culture were correlated after one-week ($r = 0.56$) and one-month ($r = 0.73$) of treatment. Correlations between culture and GeneXpert or microscopy were weaker during treatment. *Mtb* load by MBLA declined more rapidly than GeneXpert after one-week (2.73 Ct, $P < 0.001$; 0.95 Ct, $P = 0.297$, respectively) and one-month (8.94 Ct, $P < 0.001$; 6.78 Ct, $P < 0.001$). *Mtb* loads in multidrug resistant (MDR) infections were significantly greater than in both sensitive and poly/mono-resistance after one-week ($P < 0.02$) and one-month treatment ($P = 0.001$).

MBLA performed better than GeneXpert and microscopy in comparison to culture for quantifying viable *Mtb* during treatment. It can be used for monitoring bacterial load during TB treatment, facilitating early detection of treatment failure thus improving outcomes.

1. Introduction

Tuberculosis (TB), which is caused by *Mycobacterium tuberculosis (Mtb)*, is ranked as one of the top ten causes of death globally among infectious diseases. Annually, 10.4 million people get active TB, of whom 1.3 million cases result in death [1]. Early diagnosis and proper initiation of anti-TB therapy are important in treatment and control of disease transmission.

Early monitoring of treatment through viable bacterial quantification is important for evaluating treatment efficacy and predicting outcomes. It allows for improved precision in clinical care [2,3]. Monitoring treatment response requires a rapid test to measure viable bacterial

numbers. This is challenging with *Mtb*. Bacterial load in clinical samples from TB patients can be inferred from automatic MGIT culture or GeneXpert. However, culture-based methods are time-consuming and usually at risk of contamination. The presence of non-culturable *Mtb* populations also makes culture less accurate for monitoring treatment efficiency [4]. DNA-based methods are faster than culture but do not reflect the viable bacterial number accurately because the DNA from dead bacteria can persist for a long time [5,6]. Honeyborne et al. has reported that the molecular bacterial load assay (MBLA) based on 16S rRNA has potential to rapidly quantify viable bacteria thereby offering promise in the early detection of poor response to therapy [7–9]. No studies have reported on bactericidal activity of first-line drugs in TB

patients infected with resistant *Mtb*.

We conducted a prospective study in a cohort of 56 pulmonary TB (PTB) patients whose 244 sputum samples were collected before and during the first month of first-line standard treatment. We evaluated the MBLA method by comparing it with Ziehl-Neelsen (ZN) microscopy, GeneXpert and MGIT culture in terms of rapid and accurate detection of viable *Mtb* load. We also used MBLA to investigate the changes of bacterial load during the first month of treatment among isolates having different anti-TB drug resistance phenotypes.

2. Methods

2.1. Human subject's approvals and patient enrollment

Protocols were approved by the Institutional Review Boards of the Hospital for Tropical Diseases in Vietnam and the Oxford Tropical Research Ethics Committee in the United Kingdom. We enrolled 56 adults who had symptoms of PTB and ZN smear-positive with acid-fast bacilli (AFB) from two District TB control units in Ho Chi Minh City, Vietnam between January 2015 and October 2016. Written informed consent was obtained from each participant.

All participants were HIV negative, ≥18 years old, and had new PTB with no history of previous TB treatment and were given supervised first-line anti-TB treatment regimen according to national and WHO guidelines [10,11]. In the 2-month intensive phase of therapy, a fixed-dose combination of rifampicin (10 mg/kg), isoniazid (5 mg/kg), pyrazinamide (25 mg/kg) and ethambutol (15 mg/kg) was given daily. The continuation phase consisted of daily dosages of rifampicin, isoniazid and ethambutol. Treatment regimens were changed if drug susceptibility test (DST) showed multidrug resistance. Approximately, 5 mL of sputum was collected from the participants before treatment (week 0), after week 1, week 2 and week 4 of treatment.

2.2. Sample processing and microbiological examination

Sputum samples were stored for MBLA and processed for ZN smear, MGIT culture and GeneXpert. ZN smears were prepared directly from the sputum to approximately quantify the number of acid-fast bacilli from 100 microscopic fields under 100X objectives. This number then was transformed to $\log_{10}$ values for statistical tests.

The remaining sputum was decontaminated by NALC-NaOH [12] and finally re-suspended in 1 mL of phosphate buffer, equivalent to 1/5 original volume. To quantify bacterial load by MGIT, 200 μL of decontaminated sputum was inoculated in a MGIT tube and incubated in MGIT system for up to 56 days to detect mycobacteria growth. The positive MGIT culture was then used for drug susceptibility testing. Time to detection of positive growth (TTP) in days was automatically recorded and used as a measure of live bacterial load. If there was no detection of *Mtb* growth at day 56, the MGIT culture was deemed negative, and a TTP value of 56 was assigned for data analysis.

For determining bacterial load by GeneXpert, another 200 μL of decontaminated sputum was added into 2 mL of sample reagent and transferred into a test cartridge. The cartridge then was inserted into the test platform of GeneXpert instrument. Threshold Cycle (Ct) values for each of five rpoB probes were recorded [13]. The average Ct value of five probes (excluding any delayed values due to rifampicin resistance) was used to estimate bacterial load. A Ct value of 40 was assigned if GeneXpert was negative for *Mtb* detection.

2.3. RNA extraction and RT-qPCR for MBLA

To store sputum for MBLA, 100 μL of decontaminated sputum, equivalent to 0.5 mL of fresh sputum, was transferred to a Rnase-free tube containing 1000 μL preservative buffer of 4 M guanidine thiocyanate, 0.1 M Tris-HCl, and 1% β-mercaptoethanol, and stored at −80 °C immediately. After collecting all samples, total RNA was extracted as

previously described. *Mycobacterium marinum* (*M. marinum*) at a concentration of $4\log_{10}$ CFU was used as an internal control [14]. In each extraction batch, artificial sputa spiked with $8\log_{10}$ and $4\log_{10}$ *M. bovis* BCG were used as high positive and low positive standards respectively.

Expression of 16S rRNA was measured by reverse transcriptase real-time PCR using LightCycler Multiplex RNA Virus Master (Table S1) [14]. One high and one low BCG positive standard were included in each run. Ct values of MBLA were recorded only if all BCG positive standards and internal controls were passed. According to our optimization, an *M. marinum* internal control at $4\log_{10}$ CFU gave a Ct value between 21.0 and 27.5. Samples with internal controls which yielded Ct values outside that range were excluded from the analysis. The 16S rRNA Ct value of MBLA was used to estimated bacterial load after normalization using the equation 16S rRNA Ct - [(internal control Ct −21.00) x 0.8457] which was constructed based on our data [8] (Fig. S1). $\log_{10}$ bacterial number per milliliter ($\log_{10}$ bacteria/mL) of sputum was converted from normalized Ct value using the standard curve in Fig. S1. To avoid results being affected by cross-reactivity of the internal control and 16S rRNA target, the limit of detection was set to $2\log_{10}$ bacteria/mL. Samples with bacterial loads less than $2\log_{10}$ bacteria/mL were considered negative.

2.4. Drug susceptibility testing

DST of isolates was performed for streptomycin (STR), isoniazid (INH), rifampicin (RIF), ethambutol (EMB) and pyrazinamide (PZA) using BACTEC MGIT 960 SIRE and PZA kits. The critical concentrations of drugs were 1.0 μg/mL for STR, 0.1 μg/mL for INH, 1.0 μg/mL for RIF, 5.0 μg/mL for EMB and 100 μg/mL for PZA.

2.5. Statistical analysis

Statistical analyses were performed using R version 3.3.1 [15] and Graphpad Prism version 6 (Graphpad Software Inc., San Diego, California, USA). The linear relationships of MBLA and other methods to MGIT were analyzed using the Spearman's rank correlation method. To avoid correlation coefficients being affected by a bias or skewing of the data, only samples which gave positive results for all methods were used. To examine bacterial load decline (defined as increase in Ct or TTP value) after one week or one month within a method, the Paired Sample T test was used. To pairwise compare the bacterial load among four drug susceptible and resistant *Mtb* strains during treatment, Wilcoxon rank sum tests were applied. P values of <0.05 were considered statistically significant.

3. Results

Sputum samples were collected from 56 PTB participants before treatment. All were AFB microscopy positive, 54/56 (96%) were positive by MGIT. Of two MGIT-negative cases, one was scanty by microscopy but negative for both MBLA and GeneXpert, suggesting a very low number of bacteria. One was AFB grade 1 and positive for both MBLA and GeneXpert, suggesting non-culturable *Mtb*. These two cases were excluded from this study. Baseline characteristics of the remaining 54 participants are given in Table 1.

3.1. Correlation between bacterial load measured by MBLA and culture before and during treatment

Of the 224 samples collected before and during treatment, 168 positive samples (defined as positive in all four methods) were included in correlation analysis. Spearman rank correlation revealed a relatively strong correlation of *Mtb* load between the MBLA and MGIT (r = 0.66; 95% CI [0.56, 0.74]). GeneXpert and microscopy were less correlated with MGIT (r = 0.57; 95% CI [0.46, 0.67]; and r = −0.50; 95% CI [-0.61, −0.37], respectively) (Fig. 1A).

In the 54 samples before anti-TB treatment, the correlation of MGIT

Table 1
Baseline characteristics of 54 participants by drug sensitivity phenotypes.

Characteristics	Total (n = 54)	Sensitive (n = 27)	EMB-PZA R (n = 10)	INH R (n = 12)	MDR (n = 5)
Age (year), median [IQR]	46.0 [35.0; 54.0]	43.0 [33.5; 56.5]	43.5 [36.0; 56.0]	46.0 [41.0; 50.5]	48.0 [43.0; 52.0]
Sex n (%)					
Male	46 (85.2%)	21 (77.8%)	9 (90.0%)	12 (100%)	4 (80.0%)
Female	8 (14.8%)	6 (22.2%)	1 (10.0%)	0 (0.00%)	1 (20.0%)
Duration of illness (days), median [IQR]	7.00 [7.00; 10.0]	7.00 [5.00; 7.00]	10.0 [7.00; 10.0]	7.00 [6.00; 8.50]	10.0 [7.00; 10.0]
Weight loss n (%)					
Yes	13 (25.0%)	5 (18.5%)	2 (22.2%)	3 (27.3%)	3 (60.0%)
No	39 (75.0%)	22 (81.5%)	7 (77.8%)	8 (72.7%)	2 (40.0%)
Fever n (%)					
Yes	33 (63.5%)	19 (70.4%)	6 (66.7%)	4 (36.4%)	4 (80.0%)
No	19 (36.5%)	8 (29.6%)	3 (33.3%)	7 (63.6%)	1 (20.0%)

with MBLA (r = 0.70; 95% CI [0.50, 0.83]) was stronger than that with GeneXpert (r = 0.65; 95% CI [0.46, 0.79] or microscopy (r = −0.54; 95% CI [-0.71, −0.31]) (Fig. 1B). However, after one week of treatment, the correlation between MGIT and MBLA dropped to 0.56 (95% CI [0.33, 0.73]), while it was 0.51 (95% CI [0.27, 0.70]) for GeneXpert and −0.32 (95% CI [-0.57, −0.03]) for microscopy (Fig. 1C). After two weeks of treatment, the correlations were not very different to those seen after the first week (Fig. 1D). However, after one month, the correlation of MBLA with MGIT improved and was as high as before treatment (r = 0.73; 95% CI [0.44, 0.89]), whereas correlations of MGIT with GeneXpert and microscopy remained relatively low (r = 0.37; 95% CI [-0.08, 0.69]; and r = −0.21; 95% CI [-0.61, 0.27]) (Fig. 1E). Overall, sputum bacterial loads measured by culture and MBLA showed a strong relationship both before and during treatment.

We analyzed the decline of bacterial load measured by MBLA and GeneXpert, and compared it with the load measured by MGIT during the first month of treatment. Bacterial loads measured by all three methods declined during the first month of anti-TB treatment. However, the rates of decline were different. After one week of treatment, bacterial load measured by MBLA had significantly declined at rate of 2.73 Ct per week (95% CI [1.39, 4.09]; P < 0.001), whereas GeneXpert did not show a significant change, with a rate of 0.95 Ct (95% CI [-0.86, 2.45]; P = 0.297). The bacterial load measured by MGIT had also significantly decreased, at a rate of 7 days TTP per week (95% CI [3.62, 10.39]; P < 0.001) (Fig. S2). After one month of treatment, the rate of bacterial decline for MBLA was 8.94 Ct per month (95% CI [7.38, 10.50], P < 0.001); but for GeneXpert, it was slower at 6.78 Ct per month (95% CI [4.39, 9.16], P < 0.001) (Fig. S2). These results show that unlike MGIT, GeneXpert is not sufficiently sensitive to monitor bactericidal decline, whereas MBLA rapidly measures the decline in the first week and the first month of treatment.

3.2. Mtb load changes by drug resistance during the first month of treatment

We hypothesized that patients infected by drug-resistant *Mtb* would show higher bacterial loads during treatment than those with drug-sensitive *Mtb*. The relationships between bacterial load and *Mtb* drug susceptibility phenotypes before and during early treatment were examined, with four phenotype groups: sensitivity to all drugs used in treatment (S, n = 27); EMB and/or PZA resistance (EMB-PZA R, n = 10); INH resistance (INH R, n = 12); and multidrug resistance (MDR, n = 5).

Baseline characteristics of these groups are given in Table 1.

We used MBLA for bacterial quantification by converting the normalized Ct values into $\log_{10}$ bacteria/mL of sputum (Fig. S1). There was no difference in pre-treatment bacterial load by drug susceptibility (Fig. 2A). After the first week of treatment, median bacterial loads were 5.81 (IQR 4.61, 6.83), 5.94 (IQR 4.74, 7.30), 5.04 (IQR 4.05, 6.93) and 7.75 (IQR 7.21, 7.86) $\log_{10}$ bacteria/mL for the sensitive, EMB-PZA R, INH R and MDR groups, respectively. Bacterial load in the MDR group was significantly greater than in the sensitive, EMB-PZA R and INH R groups (P = 0.002, P = 0.019 and P = 0.005, respectively; Fig. 2B and D). There was no significant difference in bacterial loads among the sensitive, EMB-PZA R and INH R groups. After one month of intensive treatment, bacterial load in MDR remained very high, 6.67 $\log_{10}$ bacteria/mL (IQR 5.30, 7.69), which is still significantly higher than in the other three groups, separately or combined (P < 0.05, Fig. 2C and D). In MDR, bacterial load appears to be unchanged in the first two weeks of treatment (P = 0.054), decreases in week 2 then increases from week 2 to week 4 (P = 0.022, Fig. 2D). The dynamic responses of bacterial load quantified by MBLA concurred with that measured by MGIT TTP, particularly after one month of treatment (Fig. S3).

4. Discussion

In our study, MBLA performed better than GeneXpert and microscopy in comparison to MGIT culture for quantifying viable bacteria during the first month of treatment. Before treatment, when bacterial loads were high, all three methods had high correlations with culture. Once treatment started, the correlations were strongly affected by the sensitivity of the methods in detecting viable bacteria. Although the correlation of MBLA and culture slightly reduced after the first and second weeks of treatment, it recovered by the fourth week; whereas GeneXpert and microscopy showed low correlations with culture throughout. The bacterial load measured by MBLA showed a significant drop after one week of treatment while GeneXpert values remained almost unchanged. MBLA based on 16S rRNA detection could more rapidly reflect the number of viable bacteria in comparison with GeneXpert based on DNA detection of *Mtb* [6,8]. MBLA had advantages over conventional methods in our study. It agrees with other data [4,7,8] suggesting that MBLA is comparable to liquid and solid culture but much faster, and is more accurate than DNA-based quantitative methods. As such it could be used to monitor viable bacterial load during TB treatment.

All patients in this study received first-line drug regimen following the guidelines of Vietnam's national TB program [10]. Monitoring bacterial load, together with identifying baseline drug-susceptibility phenotypes, allowed us to understand the effect of *Mtb* drug resistance on bactericidal activity of these first-line drugs. Drug resistance was not associated with pre-treatment bacterial load, suggesting that drug resistance did not affect the ability of bacteria to survive and multiply before treatment. INH is a key drug in TB treatment with a strong bactericidal effect in the first few days of treatment [16]. However, INH R did not influence bacterial load after the start of treatment. Our findings indicate that poly/mono-resistance to INH, EMB and PZA does not change bactericidal activity of the first-line regimen in the first month and that the regimen still works effectively in these drug resistant strains during early treatment.

MDR-TB in this study showed consistent associations with higher bacterial load in comparison with sensitive and other drug-resistant *Mtb* groups. Therefore early diagnosis of new MDR TB cases for precise treatment helps reduce treatment failure and MDR TB transmission.

NALC-NaOH decontamination of sputum samples can cause a loss of viable bacteria. This has been measured by MBLA up to $0.66 \pm 0.21 \log_{10}$ bacteria/mL in high bacteria burden samples [17]. In this study, MBLA was performed on decontaminated sputum, thus the bacterial loads reported could be slightly lower than the actual numbers. However, using the same source of sputum for all our three methods gives consistency
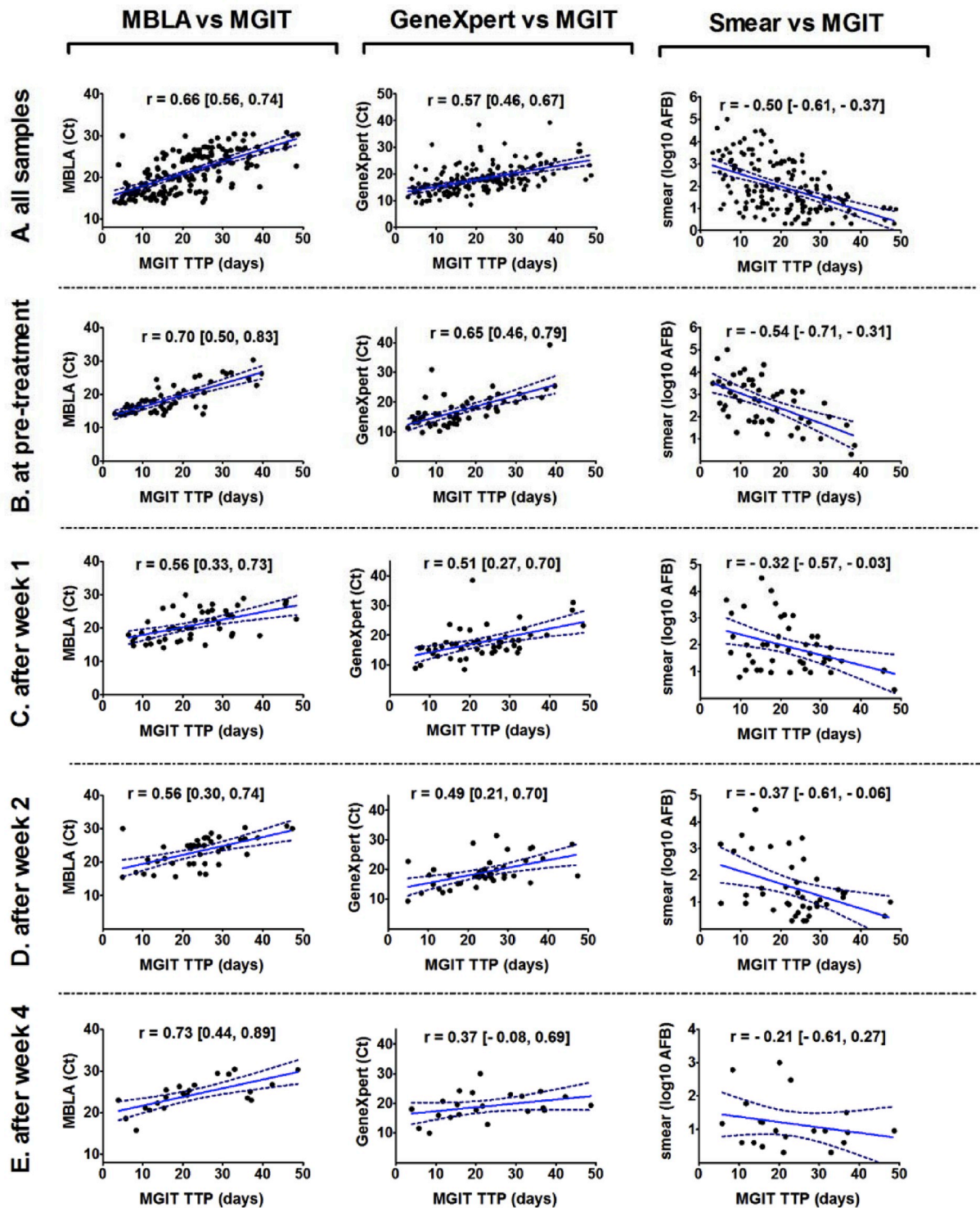
Fig. 1. Correlations of *Mtb* load measured by MGIT versus MBLA, GeneXpert and microscopy. Plots of MGIT TTP (days) against MBLA (Ct), GeneXpert (Ct) and microscopy ($\log_{10}$ AFB) in **(A)** all samples (n = 168), **(B)** before treatment (n = 54), **(C)** after week 1 (n = 50), (D) after week 2 (n = 42) and **(E)** after week 4 of treatment (n = 22). TTP, Ct and $\log_{10}$ AFB number were used to calculate correlations using the Spearman method. The analysis included all drug-sensitive and drug-resistant bacteria samples from patients. Only samples with detected values of bacterial load were included in analyses (n = 168). Spearman's correlation coefficients (r) and 95% confident interval are presented in the figure. Best-fit linear regression lines are shown with 95% confidence intervals. TTP: time to positivity; Ct: threshold cycle value.

for comparisons.

In summary, MBLA can be used for early monitoring of bacterial load during TB treatment and for assessing treatment response. Resistance to drugs other than rifampicin did not influence declines in bacterial load; whereas MDR had a significant impact, indicating the urgent need for early MDR detection and appropriate treatment.

Conflict of interest statement

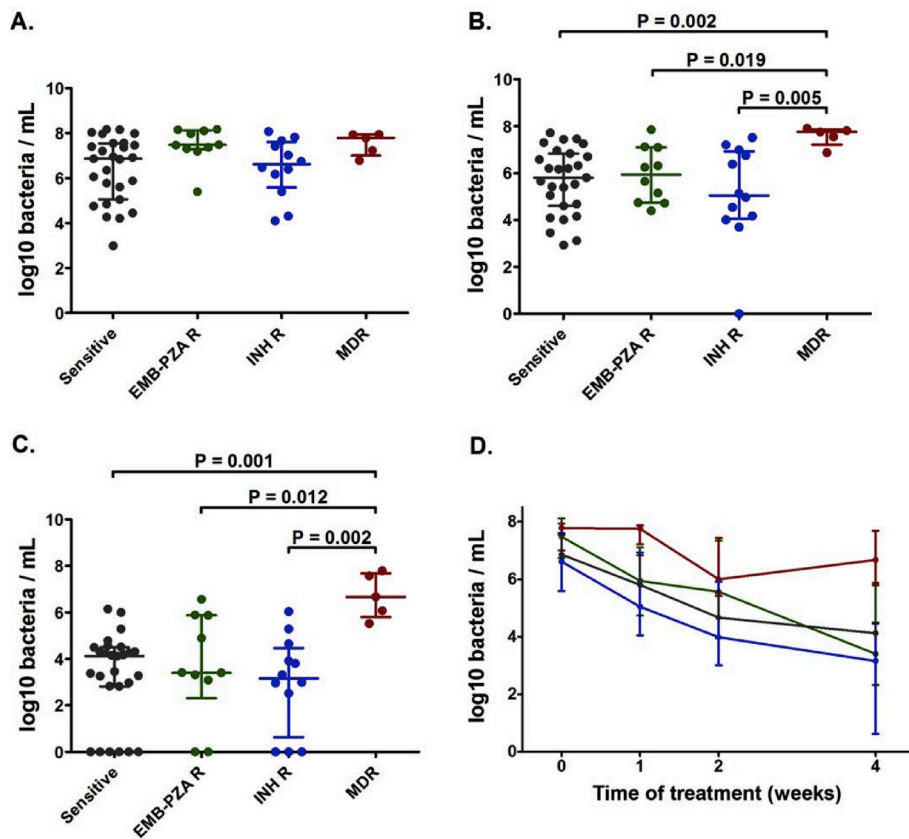The authors declare that the research was conducted in the absence

Fig. 2. *Mtb* load quantified by MBLA in drug sensitive and resistant *Mtb*-infected patient groups before and during the first month of treatment. Bacteria loads were measured by MBLA in four *Mtb* drug-susceptibility phenotype groups: the sensitive group (black, n = 27), EMB-PZA R group (green, n = 10), INH R group (blue, n = 12) and MDR group (red, n = 5). Bacterial loads in the four groups were compared **(A)** before treatment, **(B)** after week 1 and **(C)** after week 4 of treatment. **(D)** Bacterial loads in the four groups were plotted against time during the first month of TB treatment. Error bars in plots represent medians and interquartile ranges. Statistical comparisons were made using Wilcoxon rank sum test. Only significant results are indicated with P values. Sensitive: sensitive group; EMB-PZA R: EMB and/or PZA resistant group, INH R: INH resistant group, MDR: multidrug resistant group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.101864.

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