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Assessment of treatment response by colony forming units, time to culture positivity and the molecular bacterial load assay compared in a mouse tuberculosis model

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ABSTRACT

The aim of the study is to compare counting of colony forming units (CFU), the time to positivity (TTP) assay and the molecular bacterial load (MBL) assay, and explore whether the last assays can detect a subpopulation which is unable to grown on solid media. CFU counting, TTP and the MBL assay were used to determine the mycobacterial load in matched lung samples of a murine tuberculosis model. Mice were treated for 24 weeks with 4 treatment arms: isoniazid (H) - rifampicin (R) - pyrazinamide (Z), HRZ-Streptomycin (S), HRZ – ethambutol (E) or ZES.

Inverse relationships were observed when comparing TPP with CFU or MBL. Positive associations were observed when comparing CFU with MBL. Description of the net elimination of bacteria was performed for CFU vs. time, MBL vs. time and 1/TTP vs. time and fitted by nonlinear regression. CFU vs. time and 1/TTP vs. time showed bi-phasic declines with the exception of HRZE. A similar rank order, based on the alpha slope, was found comparing CFU vs. time and TTP vs. time, respectively HRZE, HRZ, HRZS and ZES. In contrast, MBL vs. time showed a mono-phasic decline with a flat gradient of elimination and a different rank order respectively, ZES, HRZ, HRZE and HRZS. The correlations found between methods reflects the ability of each to discern the general mycobacterial load. Based on the description of net elimination, we conclude that the MBL assay can detect a subpopulation of *Mycobacterium tuberculosis* which is not detected by the CFU or TTP assays.

1. Introduction

Tuberculosis (TB) is a bacterial infection that affects more than ten million people globally every year, with 1.8 million deaths recorded in 2015 and is ranked as a leading cause of death from infectious diseases worldwide [1].

Mycobacterial populations in TB patients are heterogeneous; several subpopulations of bacteria have been identified in the lungs, in different microenvironments [2]. Environmental stress and antibiotic pressure can drive *Mycobacterium tuberculosis* into a non-replicating state [3,4]. As a consequence, extensive and long-term treatment is required to eradicate all these subpopulations of *Mycobacterium tuberculosis* [5]. In order to shorten the treatment duration, new drugs and novel combinations are essential and provide the best opportunity to eliminate all mycobacterial subpopulations.

For the development of new drugs and regimens, it is important at an early stage to determine a drug's and regimen's potency to prevent relapse, which will determine the required treatment duration with this regimen [6].

Monitoring treatment response provides information that can be used to make inferences regarding treatment success. Current methods to monitor treatment response such as sputum smear or sputum serial colony counting have their drawbacks including loss of sensitivity, being laboratory intensive [7], or being sensitive to contamination [8]. As a result, these methods do not predict

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individual success with enough certainty, but also seem to lack precision in predicting the important long-term clinical outcome of a regimen [9]. Furthermore, it is rather unlikely that all different subpopulations of *Mycobacterium tuberculosis* are detected using these methods [10].

In vitro experiments and animal models often use counting of colony forming units (CFU) as a parameter to determine the total bacterial load. It has been shown by Hu et al. that CFU counts are not always representative for the total population present in an *in vitro* system, as well as in the lungs of TB infected mice [11]. Besides the CFU counts found, there was also a population of *Mycobacterium tuberculosis* present that could only be detected when they were resuscitated using resuscitation promoting factors (RPFs), indicating that basic CFU counting is maybe not the most optimal parameter to determine the total bacterial load. Therefore new techniques, which are less dependent on culture viability, are necessary to provide this information.

In the present study, we used matched lung samples of our murine TB model to compare CFU counting, liquid culture/time to positivity (TTP) and the molecular bacterial load (MBL) assay [12]. This MBL assay detects *Mycobacterium tuberculosis* 16S ribosomal RNA with a robust internal control (IC) which normalizes for RNA loss during extraction and the presence of sample inhibitors. By measuring 16S ribosomal RNA the MBL assay is therefore not depending on actively growing mycobacteria.

The aim of the study is to compare CFU counting, TTP and the MBL assay, and explore whether the last assays can detect a mycobacterial subpopulation which is unable to grown on solid media.

2. Materials and methods

2.1. Mouse TB model

Specified pathogen-free female BALB/c mice were obtained from Charles River (Les Oncins, France). At the day of infection, animals were 13–15 weeks old and weighed 20–25 g.

Four groups of mice were infected with *Mycobacterium tuberculosis* as described previously [13]. In short, mice under anesthesia were infected by intra-tracheal instillation of a suspension (40 μL) containing 1.3×10^5 CFU ($0.7–1.7 \times 10^5$) of the Beijing VN 2002-1585 genotype strain [14], followed by proper inhalation to ensure the formation of a bilateral TB infection.

2.2. Antibiotic therapy

Four treatment arms, each with a total length of 6 months (24 weeks) were designed, as part of the research conducted within the PreDiCT-TB consortium: HRZ (2HRZ/4HR), ZES (2ZES/4E), HRZE (2HRZE/4HR) and HRZS (2HRZS/4HR). All treatment arms started 2 weeks after infection.

Human equivalent doses of antibiotics were used: isoniazid (H) 25 mg/kg, rifampicin (R) 10 mg/kg, pyrazinamide (Z) 150 mg/kg, streptomycin (S) 200 mg/kg and ethambutol (E) 100 mg/kg [15]. All drugs were dissolved in water and given orally (0.2 mL) as a bolus, 5x/week, except for S, which was administered subcutaneously.

2.3. Determination of the mycobacterial load in infected lungs

To determine the mycobacterial load in infected lungs, mice were sacrificed by CO_2 exposure at the start of therapy and after 1, 2, 4, 8, 12 and 24 weeks of therapy ($n = 3$). To prevent carry-over of TB-drugs, therapy was stopped 72 h before sacrificing the mice.

The lungs were removed aseptically and homogenized within 5 min in M-tubes with the gentleMACS Octo Dissociator using the RNA program (Miltenyi Biotec BV, Leiden, the Netherlands) in 2 mL Phosphate Buffered Saline (PBS).

2.4. Colony forming units counts

From each tissue homogenate 10-fold serial dilutions were performed and samples of 200 μL were cultured on drug-free 7H10 Middlebrook agar containing activated charcoal (0.4%), and incubated for 28 days at 37 °C with 5% CO_2 to perform colony counting. Activated charcoal was added to the agar to inhibit the antibiotic residue from the tissue samples and prevent carry-over [16]. Lower limit of quantification is 10 CFU per lung.

2.5. Time to positivity

From each tissue homogenate 200 μL was added to a mycobacterial growth indicator tube (BBL MGIT; Becton, Dickinson and Company, MD, USA) in combination with 800 μL OADC enrichment. Tubes were incubated in the Bactec MGIT 960 (Becton, Dickinson and Company, MD, USA) and TTP was automatically recorded.

2.6. MBL assay

From each tissue homogenate 300 μL was directly preserved in guanidine thiocyanate (GTC) containing 1% ß-mercaptoethanol and stored at −80 °C. MBL assay was performed as previously described by Honeyborne et al. [12]. In short, following thawing, 50 ng of the internal control (IC), a 1957-bp RNA molecule that contain parts of the *sol* gene from potato tuber (*Solanum tuberosum*) [12], was spiked into each tissue sample. The IC normalizes for RNA loss during extraction and the presence of sample inhibitors. To assign cycle threshold values to bacterial loads, a standard curve was prepared. Naïve lung homogenate in GTC was spiked with serial diluted concentrations of a *Mycobacterium tuberculosis* stock ranging from 1.3×10^7 down to 1 CFU/mL, including the IC (Fig. 1).

Lung homogenate in GTC plus IC was centrifuged (14,000 x *g*;20 min) at room temperature, supernatant was discarded and 1 mL of RNApro (FastRNA pro Blue kit; MP Biomedicals) was added to the pellet. RNA extraction was performed according to the manufacturer's instructions. DNase I (Turbo DNA-fee kit; Ambion) was used to remove contaminating DNA.

Expression of 16S-ROX and IC-JOE was measured using multiplex reverse transcriptase-quantitative PCR (RT-qPCR). RT-qPCR

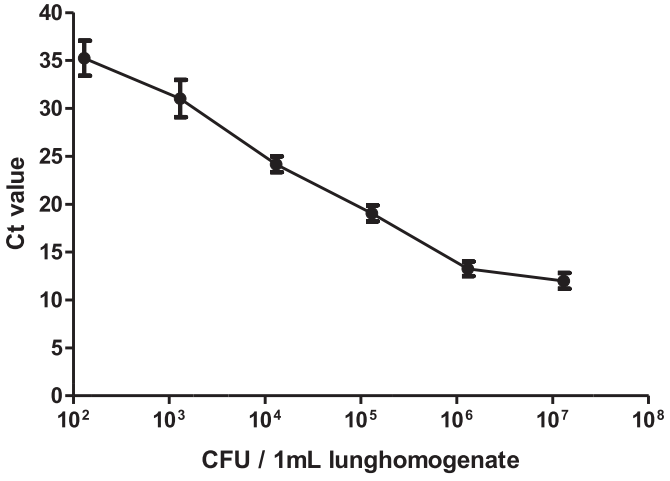

Fig. 1. Standard curve of CFU and CT values obtained from a serially diluted *Mycobacterium tuberculosis* stock spiked in naïve lung homogenate.

was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Bleiswijk, the Netherlands) using a one-step qRT-PCR MasterMix for probe Assay Low ROX (Eurogentec, Maastricht, the Netherlands). Primers and dual-labelled probes were also purchased from Eurogentec.

2.7. Data analysis

Data 0–24 weeks of treatment (2–26 weeks following infection) were used to investigate elimination of bacteria under the four therapeutic conditions (HRZ, ZES, HRZE, HRZS). Contaminated CFU (n = 2) and TTP (n = 11) samples were excluded. Correlations between measurements obtained by CFU, TTP and MBL assay over time collectively were evaluated by Spearman's Rank ($p < 0.017$ was considered statistically significant at the 5% level incorporating a Bonferroni correction for 3 pairwise comparisons for each treatment). Given that data from 3 distinct mice were available at each time point, naïve pooling was undertaken for analysis [17]. Mathematical models to describe the profiles of elimination of bacteria under treatment were fitted by nonlinear regression to the observed profiles of bacterial load vs. time as measured by CFU, 1/TTP, and MBL (TTP in broad terms being inversely proportional to the number of bacteria in the system). Parameter estimates for this modelling analysis were determined using the nonlinear least squares optimization function "lsqnonlin" as part of the "pracma" package in R (www.r-project.org). Standard errors were calculated using previously described methods by Landaw et al. with the Jacobian of model parameter sensitivities estimated using a numerical central difference method [18]. Exponential growth and death of bacteria cannot be differentiated with this data (the growth and death processes may occur simultaneously across the bacterial population as a whole) therefore exponential rate constants describing the "net" rate of bacterial growth or death were estimated from the quantitative timecourse profiles of bacterial load as

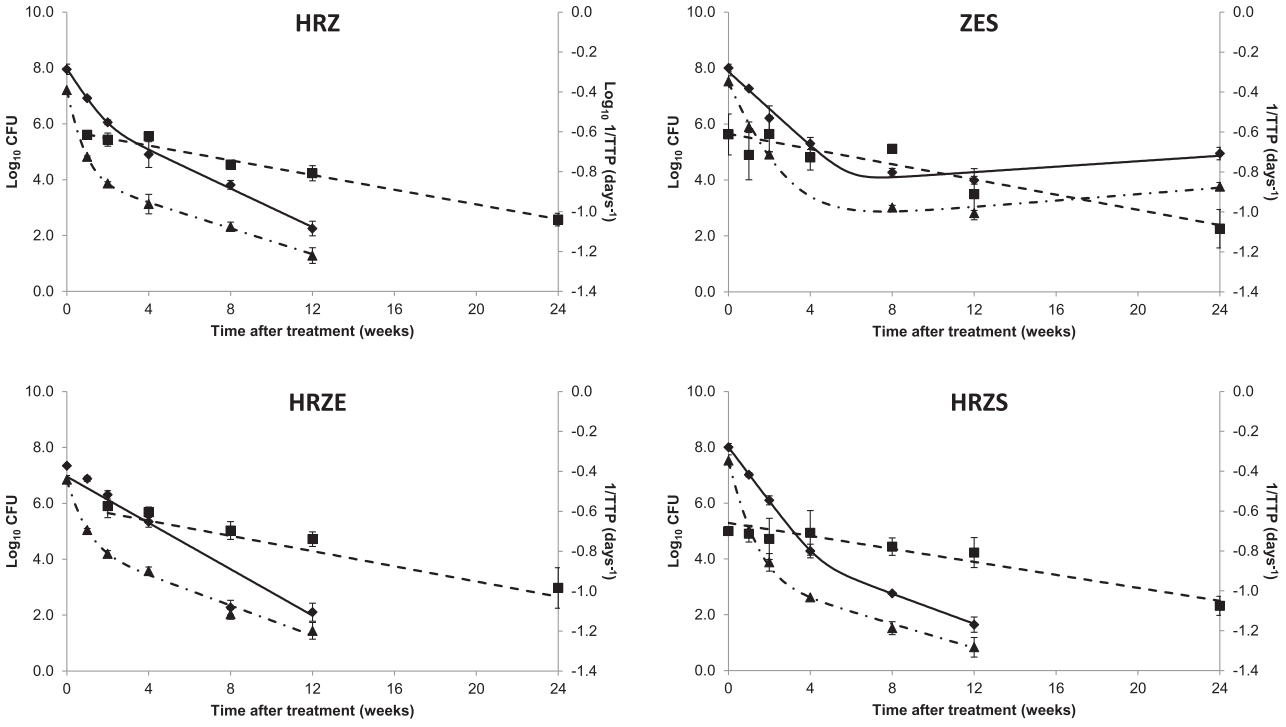

Fig. 2. Mouse lung CFU, MBL and TTP over 24 weeks post-treatment (26 weeks post-*Mycobacterium tuberculosis* infection) with HRZ, ZES, HRZE and HRZS. Data are presented as mean value (n = 3 mice per time point) ± SD with CFU and MBL on the left y-axis and 1/TTP on the right. The solid and dashed lines represent the modelled fit to the data.

determined by the 3 assays. These net rate constants take a negative value corresponding to net elimination, and a positive one to describe net growth.

3. Results

The data of lung CFU, MBL and TTP (TTP data is converted to 1/TTP) over 24 weeks of treatment are illustrated (Fig. 2) and statistical relationships between assays are summarized (Table 1). Overall, inverse correlations were observed between TTP with CFU and MBL and positive associations between CFU and MBL with statistical significance for all regimens with the exception of TTP *vs.* MBL for ZES and HRZE and CFU *vs.* MBL for HRZS (Table 1). For HRZ, HRZE and HRZS, MBL consistently reported lower than CFU between 2 and 8 weeks post-infection and higher than CFU to 14 weeks and 26 weeks post-infection (Fig. 2).

3.1. Modelling of CFU vs. time

For CFU profiles over time (t) from the lung while under therapy the best description was given by a bi-exponential decline of the form $CFU(t) = A^{*}10^{(-\alpha^{*}t)}+B^{*}10^{(-\beta^{*}t)}$ with the exception of HRZE for which a mono-phasic decline was supported [$CFU(t) = A^{*}10^{(-\alpha^{*}t)}$] (Fig. 2). The first order exponential rate constants alpha (α) and beta (β) (in broad terms the "slopes" of the phases of the profile vs. time when plotted on a semi-logarithmic scale) are summarized (Table 2). Based on the β exponential rate constant, governing the 2nd phase of a multiexponential profile (if 2 exponential phases are present, and governing the only phase if the profile is mono-phasic) a rank order HRZE > HRZ > HRZS > ZES was shown, i.e. HRZE produced the steepest gradient on a logarithmic scale corresponding to a more rapid rate of elimination of bacteria, in contrast to ZES which actually showed signs of regrowth of bacteria during the second phase of its profile, though at a slow rate close to stasis over the 12–24 week period of the study (β of −0.414 vs. +0.049; Table 2). The β exponential rate constant is potentially a more interesting parameter of comparison as it is likely to reflect more the activity of treatment against a slower dying, less drug susceptible mycobacterial subpopulation similarly though, based on α slope, rate of eliminations were in the order of HRZ > HRZS > ZES (Table 1) with HRZE for unknown reasons showing only a monophasic decline in this experiment.

Table 1
Summary of spearman rank correlation coefficients evaluating the relationships between assays for measurement of *Mycobacterium tuberculosis* in mouse lung following 24 weeks treatment with HRZ, HRZE, HRZS, ZES (analyses from week 2–26 post- *Mycobacterium tuberculosis* infection). P values highlighted in **bold** type represent statistically significant correlations with Bonferroni correction of 3 per drug combination for multiple comparisons ($p < 0.017$).

Treatment	Comparison	Rho[a] (95% CI)	P value
HRZ	TTP vs. CFU	−0.993 (−0.998 to −0.978)	<**0.0001**
	CFU vs. MBL	0.846 (0.529–0.956)	**0.0008**
	TTP vs. MBL	−0.874 (−0.964 to −0.602)	**0.0003**
HRZE	TTP vs. CFU	−0.766 (−0.911 to −0.452)	**0.0005**
	CFU vs. MBL	0.797 (0.412–0.941)	**0.0027**
	TTP vs. MBL	−0.382 (−0.799 to −0.283)	0.248
HRZS	TTP vs. CFU	−0.965 (−0.989 to −0.890)	<**0.0001**
	CFU vs. MBL	0.507 (−0.0069 to 0.809)	0.054
	TTP vs. MBL	−0.753 (−0.922 to −0.345)	**0.0044**
ZES	TTP vs. CFU	−0.982 (−0.994 to −0.952)	<**0.0001**
	CFU vs. MBL	0.492 (0.0888–0.757)	0.010
	TTP vs. MBL	−0.457 (−0.762 to 0.0120)	0.028

CFU: Colony Forming Units; MBL: Molecular Bacterial Load; TTP: Time To Positivity; H: isoniazid; R: rifampicin; Z: pyrazinamide; S: streptomycin; E: ethambutol; CI: confidence interval.
[a] Spearman's rank correlation co-efficient.

3.2. Modelling of MBL vs. time

Profiles of MBL over time during therapy were described by mono-phasic declines (Fig. 2). The gradients of the elimination slopes on the logarithmic scale for each combination therapy were relatively flat and similar numerically suggesting slow bacterial elimination for all therapies (Table 2).

3.3. Modelling of TTP vs. time

Given the inverse relationships observed between TTP with CFU and MBL a bi-exponential elimination model was fitted to 1/TTP vs. time data in order to determine a comparable elimination rate for TTP (Fig. 2) (1/TTP being a more directly comparable measure of bacterial load). Based on the β rate constant, rate of bacterial decline was of an identical order to that observed for CFU (HRZE > HRZ > HRZS > ZES) with ZES also showing potential regrowth (i.e. a positive β rate constant) in the 12–24 week period as it did in its CFU profile.

4. Discussion

Identification of subpopulations of *Mycobacterium tuberculosis* is important for drug development, refinement of existing or novel drug combinations to ultimately achieve therapeutic success in a clinical setting. Animal models can be particularly useful to evaluate potential methods of quantifying different populations of *Mycobacterium tuberculosis* [11,19]. One of the benefits of an animal model is the ability to study the total population of *Mycobacterium tuberculosis* in the lung, including the more hidden bacteria in granuloma like formations, whereas in sputum from TB patient only the secreted bacteria can be studied [20]. However, animal studies from the past using CFU counts as a parameter are maybe not predictive enough because it has been shown by Hu et al. that CFU counts are not representative for the total population present in an *in vitro* systems as well as in the lungs of TB infected mice, using RPFs to resuscitate a persistent and non-growing population [11].

We have presented for the first time the comparison of CFU, MBL and TTP methods using matched lung samples from a mouse TB model and explored the use of mathematical modelling to further summarize and scrutinize the data, describing the elimination of *Mycobacterium tuberculosis* over time for each method under different therapeutic conditions.

The ability to detect different *Mycobacterium tuberculosis* populations in our mouse TB model using the three methods can be explained by the principles of the techniques. In contrast to DNA based assays like the GeneXpert® MTB/RIF assay, the MBL assay detects the presence and amount of 16S rRNA and is therefore less hampered by the presence of genomic material of already dead cells [21]. The 16S rRNA is much more unstable compared to DNA and degrades much faster [22]. It has been shown that RNA, in particular mRNA, correlated with solid culture but more closely with growth in liquid culture [23]. However, for culture on solid media or in liquid media actual growth is still required, thereby presenting only the fittest mycobacteria which were capable of growing. In contrast, for the MBL assay a particular population of *Mycobacterium tuberculosis* should be alive, and have metabolic activity, but actual growth is not required.

Statistically significant correlations between assays were evident for the majority of regimens tested. Lack of association between TTP *vs.* MBL for ZES and HRZE and CFU *vs.* MBL for HRZS was possibly due to small sample size in these groups. Unsurprisingly, TTP was inversely related to CFU and MBL as a shorter time to positivity would be expected with high bacterial load measured

Table 2
Parameter estimates and associated percentage relative standard errors (%RSE) for α and β exponential rate constants describing net bacterial growth/elimination for each treatment regimen between weeks 2–24 of infection. Data analysed by fitting a bi or mono-exponential model as appropriate.

Treatment	α (wk^{-1})	%RSE	β (wk^{-1})	%RSE
CFU *vs* time				
HRZE[a]	–	–	**−0.414**	**9.4**
HRZ	−1.127	30.4	**−0.346**	**7.6**
HRZS	−0.971	11.2	**−0.270**	**19.0**
ZES	−0.664	9.8	**+0.049**	**25.6**
MBL *vs* time				
ZES[a]	–	–	**−0.136**	**11.4**
HRZ[a]	–	–	**−0.132**	**4.5**
HRZE[a]	–	–	**−0.130**	**10.0**
HRZS[a]	–	–	**−0.116**	**10.9**
(1/TTP) *vs* time				
HRZE	−0.605	19.7	**−0.038**	**10.4**
HRZ	−0.718	14.2	**−0.032**	**9.2**
HRZS	−0.526	12.6	**−0.030**	**17.8**
ZES	−0.276	7.6	**+0.008**	**18.6**

CFU: Colony Forming Units; MBL: Molecular Bacterial Load; TTP: Time To Positivity; H: isoniazid; R: rifampicin; Z: pyrazinamide; S: streptomycin; E: ethambutol; %RSE: percentage Relative Standard Error of parameter estimate.
In bold the no. on which the ranking is based on.
[a] Mono-exponential fitting.

with CFU as well as with the MBL assay. Conversely, if the viable mycobacterial count CFU was zero, the MGIT will not measure fluorescence as a result of oxygen consumption and time to positivity would be infinite, because there are no growing mycobacteria present.

Mathematical modelling allowed estimation of the elimination rate of bacteria under each of the four drug combinations. The rates of elimination of bacteria for HRZ, ZES, HRZE and HRZS as measured by CFU and TTP were of a similar rank order and were both typically bi-exponential in this study. This suggests that CFU and TTP are measuring similar bacterial populations that show net elimination under chemotherapy. A study conducted by Diacon et al. showed that CFU can be substituted with TTP in early bactericidal activity (EBA) studies [7], but on the other hand, a sample taken at the end of treatment can take longer to signal positive in MGIT compared to a sample taken early with a similar CFU count as shown by Bowness and colleagues [24]. They showed that the relationship between CFU and TTP changes over time in early response to treatment (14 days). These observations imply that MGIT detects an extra subpopulation of *Mycobacterium tuberculosis* and that this subpopulation decreases in number during drug exposure and treatment. A similar analysis to Bowness and colleagues was not feasible however with the data in our study due to the limited number of samples per time point (or groups of time-points). We therefore cannot conclude that CFU and TTP are detecting different subpopulations of *Mycobacterium tuberculosis* in our mouse model.

Compared to the bi-phasic decline of CFU and TTP over time (with the exception of HRZE), description of MBL was mono-phasic for all four treatments with gradients on the logarithmic scale close to zero, but MBL counts remained positive during treatment in all four treatment arms, suggesting the presence of a mycobacterial subpopulation which decreases slowly under treatment, but cannot be detected by TTP and CFU counting. However, elimination rate constants derived from MBL data cannot arms tested. All four treatment arms have MBL elimination rate constants of similar magnitude, whose differences cannot be considered statistically significant. In contrast to the mono-phasic elimination in the present study, a bi-phasic decline with the MBL was observed by Honeyborne et al. [12]. However, the study reported an EBA measured over 14 days with the first 3 days showing a steeper gradient compared to EBA 3–14 days. The difference in observations between the Honeyborne study and the present study may be a result of the richer initial time course studied by Honeyborne et al. compared to sampling 1 week after start treatment in our study. Thereby potentially failing to capture a bi-phasic decline of the first 3 days, which is a limitation of the current study.

At week 12, MBL numbers were higher compared to CFU and TTP (with the exception of ZES) and at week 24 CFU counts were negative for HRZ, HRZE and HRZS but the MBL numbers were still positive. Because rRNA is downregulated in bacteria entering dormancy [25], we could speculate this could infer that the MBL assay maybe even underestimates the mycobacterial load during the continuation phase of treatment. This could for instance explain the failure of the MBL assay to differentiate between the efficacy of ZES and the HRZ-based regimens at the end of treatment.

In the present study, MBL also measured lower than CFU in the early phase of treatment, indicating a less sensitive assay to detect high numbers of actively replicating *Mycobacterium tuberculosis*. This was also shown by van der Vliet et al. [26], demonstrating that conversion of 16S rRNA to CFU must be optimized. The present study also shows that the starting material is of influence on the sensitivity of the assay, especially in the high range of present bacteria. Therefore, the MBL assay must be further optimized when *Mycobacterium tuberculosis*-infected tissue is the starting material.

Also notable, is the disparity between CFU and MBL counts after 12 weeks with rifampicin and isoniazid containing regimens (HRZ, HRZE and HRZS), compared with the observation of CFU and MBL after 12 weeks of the ZES treatment where minimal difference is found between the two. This observation is in keeping with a pattern where during the early phase of treatment the metabolically active *Mycobacterium tuberculosis* are eliminated by isoniazid, while rifampicin is one of the drugs responsible for the elimination during the sterilizing phase [27]. The positive MBL counts at later time points which are not counted by the CFU and TTP assay possibly represent a subpopulation of *Mycobacterium tuberculosis*, which are in a dormant state. To add on to this, Malherbe et al. recently showed that mRNA can still be found in a bronchoalveolar lavage samples at the end of treatment in cured patients, also indicating that there is a subpopulation of mycobacteria which cannot be detected on solid media but is still present in the lungs [28]. This suggest an important complementary role for the hosts immune system to maintain a disease-free state and shows that sterilizing or host-directed therapies are needed for the development of shortened and improved treatment regimens [28].

A study by de Steenwinkel et al., aiming for a shortened treatment regimen found that increasing the rifampicin dose up to 80 mg/kg/day reduced the treatment duration by four months, without relapse of infection [29]. To add on to this, Hu et al. recently found that increasing the rifampicin dose eventually also eliminate the non-culturable population in their model [11]. However, Kayigire et al. showed that rifampicin significantly reduced the CFU counts, but also significantly changed the proportions of non-growing, lipid body-containing mycobacteria an viable mycobacteria, indicating that treatment can drive mycobacteria in a non-culturable state [30].

Therefore, additional investigation of the use of the MBL assay with high doses of rifampicin in combination with RPFs is warranted to further characterize the non-culturable subpopulation.

In conclusion, we show strong correlations between CFU, TTP, and the MBL assay reflecting the ability of each to determine the mycobacterial load in our mouse TB model. The findings also show that the MBL assay tells “a different story” to that of CFU counts and TTP, by detecting a subpopulation of *Mycobacterium tuberculosis* which is not detected by CFU or TTP assays.

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Ethical approval

Experimental protocols adhered to the rules specified in the Dutch Animal Experimentation Act and are in concordance with the EU animal directive 2010/63/EU. The Institutional Animal Care and Use Committee of the Erasmus MC approved the present protocols (117-12-08 and 117-12-13).

Transparency declarations

None to declare.

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