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Molecular Bacterial Load Assay, a Culture-Free Biomarker for Rapid and Accurate Quantification of Sputum *Mycobacterium tuberculosis* Bacillary Load during Treatment▽#

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A molecular assay to quantify *Mycobacterium tuberculosis* is described. *In vitro*, 98% ($n = 96$) of sputum samples with a known number of bacilli (10^7 to 10^2 bacilli) could be enumerated within 0.5 $\log_{10}$. In comparison to culture, the molecular bacterial load (MBL) assay is unaffected by other microorganisms present in the sample, results are obtained more quickly (within 24 h) and are seldom inhibited (0.7% samples), and the MBL assay critically shows the same biphasic decline as observed longitudinally during treatment. As a biomarker of treatment response, the MBL assay responds rapidly, with a mean decline in bacterial load for 111 subjects of 0.99 $\log_{10}$ (95% confidence interval [95% CI], 0.81 to 1.17) after 3 days of chemotherapy. There was a significant association between the rate of bacterial decline during the same 3 days and bacilli ml^{-1} sputum at day 0 (linear regression, $P = 0.0003$) and a 3.62 increased odds ratio of relapse for every 1 $\log_{10}$ increase in pretreatment bacterial load (95% CI, 1.53 to 8.59).

Monitoring response to tuberculosis chemotherapy rapidly and cheaply would be of enormous benefit in patient management by identifying those failing therapy (30) and would open up the possibility of shorter, less expensive trials based on its use as a surrogate marker (24). At present, the only widely accepted biomarker of treatment response is culture negativity 56 days after the commencement of therapy (18). Some authors have suggested that measuring the decline in the bacterial load may permit the outcome of treatment to be predicted more accurately (4, 8).

Currently available methods for measuring bacterial load have significant drawbacks. Sputum smears are rapid and cheap but have a sensitivity limit of 10^4 bacteria ml^{-1} sputum (13) and cannot differentiate between live and dead bacilli. Liquid culture methods for sputum bacterial load enumeration require decontamination, and this reduces the mycobacterial count, possibly in an uncontrolled way. The time to positivity of liquid cultures is dependent on the bacterial burden of the sputum inoculum, and therefore those samples negative by smear are likely to take the longest to be determined culture positive. Information is therefore not available quickly enough to aid clinical decision making.

Sputum serial colony counting (SSCC) using selective solid media does not require decontamination but requires extensive laboratory operator time, the *Mycobacterium tuberculosis* colonies grow slowly, and there is difficulty in obtaining single colonies due to bacterial cording. Additionally, we have found that selective media can reduce the colony count by around 1 $\log_{10}$ (our unpublished data). All culture-based methods are affected by contamination with other microorganisms present in the sample and viable noncultivatable *M. tuberculosis* organisms. Publications over the last few years have demonstrated that a significant proportion of viable organisms do not multiply on artificial medium without the addition of resuscitation promotion factors (22, 26).

Mycobacterial DNA can be detected in sputum samples derived from patients that are culture negative (11, 14, 20), whereas studies have found that RNA species have a shorter half-life (11). Previous reports have suggested that abundant mRNA species such as *fbpB-85B* and *icl* may be useful for the detection of live bacilli (5, 9, 10, 15). While the clearance of the *icl* gene showed promise as a biomarker for a fall in CFU at 7 days, it did not match liquid culture sensitivity at day 56. We investigated the use of 16S rRNA due to its cellular abundance and an expected half-life shorter than that of DNA. Data from some studies imply that mycobacterial rRNA would respond rapidly to bacterial cell death (21, 27, 29).

This study reports the development and evaluation of an assay detecting *M. tuberculosis* 16S rRNA with a robust, novel

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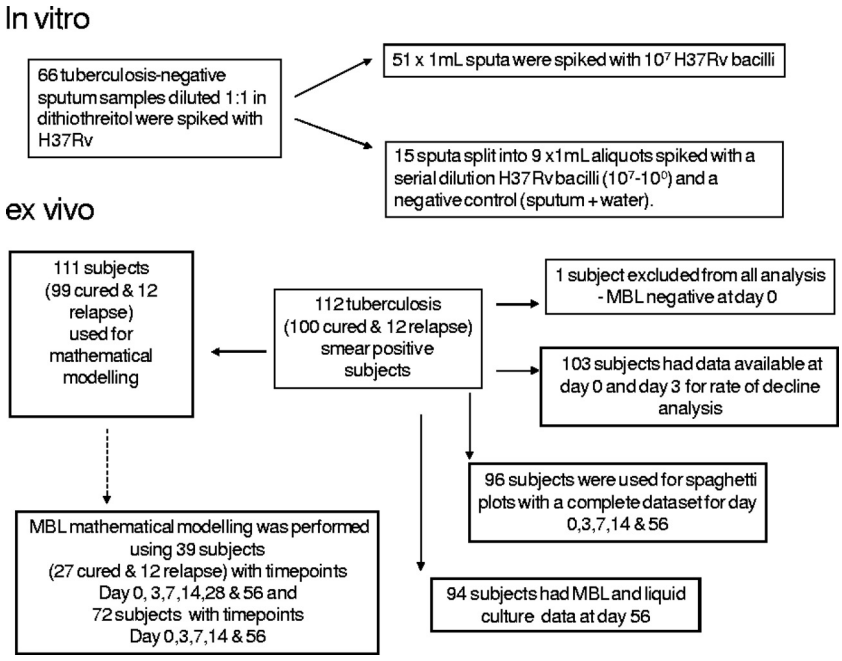


FIG. 1. Flowchart of *in vitro* and *ex vivo* sputum samples used in this study.

internal control (IC) which normalizes for RNA loss during extraction and the presence of sample inhibitors. The method, which we have termed the molecular bacterial load (MBL) assay, could be used to monitor tuberculosis treatment response in the clinic and the clinical trials setting.

MATERIALS AND METHODS

Preparation of internal control. A 1,957-bp internal control (IC) was prepared using *in vitro* transcription. For further details, see Table S1 and Data S1 in the supplemental material. Serial dilutions of the internal control were performed and spiked into sputum in a one-off experiment to ascertain the optimal concentration.

Correlation between novel IC and *M. tuberculosis* 16S rRNA for sputum samples spiked with a known concentration of *M. tuberculosis*. Fifty-one *M. tuberculosis*-negative sputum samples collected at the Royal Free Hospital, NHS Trust, London, were used. These were already diluted with an equal volume of Sputasol (Oxoid), and 1-ml aliquots were spiked with 10^7 *M. tuberculosis* bacilli from an exponential culture of H37Rv (NTCC 7416). *M. tuberculosis* was cultured in 7H9 medium (BD) containing 0.2% Tween 80 during incubation (cultures were passed 5 times through a needle, prior to use, to reduce clumping). Bacteria were quantified by a modified Miles and Misra method as described previously (17). These spiked sputa were treated with 5 ml of 5 M guanidine thiocyanate (GTC) containing 0.7% β-mercaptoethanol and kept at room temperature for 2 h before being frozen at −80°C. Samples were kept frozen until RNA was extracted.

Determining the detection range for the MBL assay range using sputum samples spiked with concentration known of tuberculosis. Fifteen tuberculosis-negative sputa were obtained at the Royal Free Hospital, NHS Trust, London. One-milliliter aliquots (obtained diluted with an equal volume of Sputasol, Oxoid) of each were spiked with a dilution series (10^7 to 10^0) of *Mycobacterium tuberculosis* strain H37Rv (grown as detailed above). A negative control where 100 μl of sterile water was added instead of bacterial culture was also included. Samples were fixed in GTC as detailed below.

Patient cohort. Subjects with active pulmonary tuberculosis were recruited in primary health care tuberculosis clinics in the Western Cape Province, South Africa, following full local ethical approval. Patients consented to be involved in the study. Full details of the patients in this cohort and the drug treatment regimen have been reported previously (12). Longitudinal samples were collected for 112 subjects. Details of how patient data were used are given in Fig. 1. One hundred patients studied were cured following treatment and 12 patients went on to relapse. Relapse patients were defined as those who were culture negative at 6 months but in whom tuberculosis disease was found to have recurred with the same *M. tuberculosis* strain as in the initial disease episode during the 2 year follow-up. One day 0 sputum was negative for 16S rRNA. This individual was excluded from the analysis since the patient was acid-fast bacillus (AFB) positive by standard microbiology (liquid culture and Ziehl-Neelsen staining) and would bias the results in the favor of the reported observations comparing relapse and cure.

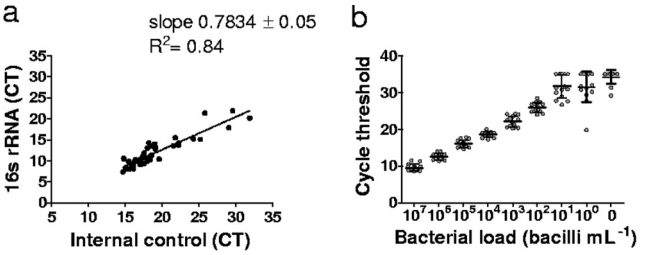
RNA preservation. Assay optimization control sputum samples, artificially spiked with serially diluted H37Rv, as detailed below, were treated with 5 ml of 5 M GTC (Promega) also containing 17 mM *N*-lauroylsarcosine sodium salt, 25 mM trisodium citrate (pH 7.0 with 1 M HCl), 1% Tween 80, and 0.7% β-mercaptoethanol to preserve the RNA.

Two patient sputum samples were collected in the clinic at each time point. The entirety of the first sample was used for routine bacteriology and the entirety of the second was transferred into 4 M GTC, 0.1 M Tris-HCl (pH 7.5), and 1% β-mercaptoethanol immediately after expectoration in order to preserve the *M. tuberculosis* RNA. Samples were transferred to the laboratory within 2 h and then frozen at −80°C until RNA extraction. Details of routine bacteriology processing for smear and liquid culture are outlined in the work of Hesseling et al. (12).

RNA extraction and preparation. Patient sputum samples in GTC were adjusted accordingly so that the RNA extraction was from a total volume of 0.5 to 1.5 ml of sputum. Following thawing, 50 ng of the internal control was spiked into each sputum sample and RNA extracted. Sputum containing RNA in GTC plus the internal control (IC) was centrifuged at 2,000 × *g* for 30 min at room temperature. The supernatant was discarded, the pellet was agitated, and 1 ml of RNApro (FastRNA Pro Blue kit; MP Biomedicals) was added. The manufacturer's instructions from the RNApro kit were then followed for the remainder of the extraction. Contaminating DNA was removed by treating with DNase I (TURBO DNA-free kit; Ambion) according to the manufacturer's instructions.

RT-qPCR. Expression of 16S-ROX and IC-Joe was measured using multiplex reverse transcriptase (RT)-quantitative PCR (qPCR) (see Table S1 in the supplemental material for detailed information on primer and probe sequences and Data S2 for information about assay specificity). All reagents were from the Quantitect Multiplex RT-PCR NR kit (Qiagen), except primers and dual-labeled probes, which were synthesized by Eurofins MWG Operon. For details about assay setup, see Data S3.

Nonlinear model fitting. Monoexponential and biexponential nonlinear models have been proposed to describe the decline in bacterial load over the first 2 months of treatment, with the biexponential model, reflecting two separate phases of decline, having been shown to best fit the data (4, 8, 25). To compare



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	Bacilli mL^{-1} sputum							
	10^8	10^7	10^6	10^5	10^4	10^3	10^2	$< 10^2$
Mean	6.6	9.6	12.6	16.0	18.5	22.1	25.9	>27.2
CT±SD	±0.9*†	±0.9	±0.9	±1.1	±0.7	±1.5	±1.3	

FIG. 2. (a to c) Optimization of the MBL assay. (a) Correlation between detection of the internal control (IC) gene and tuberculosis complex-specific 16S rRNA in 51 sputum samples, spiked with 10^7 *M. tuberculosis* bacteria and 50 ng IC. (b) Cycle threshold (C_T) range for 15 sputum samples spiked with serial dilutions of H37Rv. Cycle threshold values were normalized against IC as determined in panel a using the following equation: 16S rRNA C_T − [(IC C_T − 16.00) × 0.7834]. (c) Range of C_T values specified for each concentration of H37Rv (mean ± SD) according to the analysis shown in panel b. The internal control C_T was validated between 16.00 and 26.9. Symbols: ∗, value extrapolated from other data; †, unnormalized C_T values below 10.0 obtained in an RT-qPCR run were repeated as a 10-fold dilution.

the decline of the MBL assay-determined bacterial load in this study with the decline of bacterial load reported in other published studies, we used the same methods described in references 4 and 25 to fit and compare different nonlinear models. Nonlinear mixed-effects models were therefore used to analyze the decline in counts in the 111 patients. Models were fit using the nlme package in R (www.r-project.org). Mono-, bi-, and triexponential models were compared corresponding to one, two, or three phases of bacterial clearance, respectively. The full specification of the models used can be found in reference 25. A patient's first zero count was replaced with a value of 5 bacilli ml^{-1} sputum, with any subsequent zero counts excluded from the analysis using the approach described by Rustomjee et al. (25). Nonlinear models were compared using the likelihood ratio test and the Akaike information criterion (AIC). For calculation of bacterial decline transition times, see Data S4 in the supplemental material.

RESULTS

Determination of a standard curve for the molecular count. Preliminary experiments were performed to establish the effective range and reproducibility of the molecular bacterial load (MBL) assay. Studies of extraction efficiency revealed that a 50-ng starting quantity of the internal control (IC) resulted in an optimal cycle threshold (C_T) of 16.00 when detected in a multiplex RT-qPCR assay (data not shown). A total of 51 sputa were each spiked with 10^7 bacilli and 50 ng of IC. This was performed to ascertain whether variation in RNA extraction and different inhibitors in sputum samples affected the gene of interest (tuberculosis complex-specific 16S rRNA) and the internal control similarly. RNA was extracted from each and then the cycle threshold (C_T) was measured using RT-qPCR for 16S rRNA and IC. Using linear regression, the coefficient of determination, R^2, was determined to be 0.84 (Fig. 2a). Since the inhibition and RNA loss for the IC and gene of interest correlated closely, we were able to normalize the results obtained for the 16S with the IC result for RNA extracted from sputum. According to the relationship between the IC and 16S rRNA (Fig. 2a), the equation for normalization of 16S rRNA is given as follows: 16S rRNA C_T − [(IC C_T − 16.00) × 0.7834]. (A C_T of 16.00 represents the lowest possible inhibition/RNA loss as determined empirically and 0.7834 is the slope of the line (Fig. 2a).

Detection limit of the MBL assay was determined *in vitro* as 10^2 bacteria ml^{-1} sputum. Preliminary experiments were performed to establish the effective range and specificity of the MBL assay. The range in cycle threshold for 16S rRNA expression, determined as described above, was compared at each bacterial concentration (10^7 to 10^0) (Fig. 2b). The mean C_T value for each bacterial concentration was determined and a range set for each dilution as the mean C_T ± 1 standard deviation (SD) (Fig. 2c). Since the maximum spike tested was 10^7 bacilli, values for 10^8 bacilli ml^{-1} were extrapolated from the data obtained for 10^7 to 10^2 bacilli ml^{-1} sputum. Using the mean C_T ± 1 SD, 100% of 10^7 to 10^2 (86 of 86 samples) were within 1 $\log_{10}$, and 98% (84 of 86 samples) were within 0.5 $\log_{10}$ of the culture-defined bacterial count. Samples with $<10^2$ bacilli ml^{-1} were considered negative by this assay. Of 45 sputum samples spiked with $<10^2$ bacilli ml^{-1}, two were false positives (4.4%) (Fig. 2c). The internal control was validated empirically for cycle threshold values between 16 and 26.9. Where the internal control gave a C_T of >26.9, the sample was considered too inhibited to give a valid bacterial load measurement. Of 584 patient sputum samples included in this study, only 4 (0.7%) were too inhibited to be assigned a value using the MBL assay.

Rate of bacterial decline during the first 3 days of treatment is rapid and dependent on bacterial load at day 0. To address whether the 16S rRNA species used in the MBL assay responds rapidly to changes in the live-bacillus number, we analyzed the decline of bacterial load measured with the MBL assay during the first 3 days of treatment. All 16S rRNA data were adjusted with the internal control as described above to normalize for variations in the efficiency of the reaction and RNA quality. Bacterial load could then be calculated in each sample according to the range given in Fig. 2c. The mean decline in bacterial load during the first 3 days was found to be 0.99 $\log_{10}$ (95% confidence interval [95% CI], 0.81 to 1.17) and therefore a 90% reduction in mean bacterial load during this time period (Fig. 3a to d), illustrating that 16S rRNA can respond rapidly to bacterial decline.

Fifty-four of 103 patients (52%) had a ≥1.0 $\log_{10}$ decline during the first 3 days of treatment (Fig. 4). Further analysis found that the rate of decline in bacterial load during the first 3 days of treatment correlated with the bacterial load at day 0 ($R^2 = 0.12$, $P = 0.0003$, linear regression) (Fig. 4). This analysis found that 93% of subjects with ≥10^7 bacilli ml^{-1} sputum (n = 15) had a ≥1.0 $\log_{10}$ decline in bacterial load after 3 days of treatment, whereas only 24% (n = 21) of samples presenting with ≤5×10^4 bacilli ml^{-1} sputum had a similar decline during the same phase.

Longitudinal decline in bacterial load as measured using the MBL assay was biphasic. In order to ascertain whether measuring bacterial decline in response to treatment by using the MBL assay is comparable to using solid culture, we analyzed the data using the same mathematical models previously established for serial colony-counting (SSCC) studies (25). The decline in MBL was found to be biphasic. A biexponential mixed-effects model was found to best fit the data when compared to either monoexponential or biexponential models ($P <$

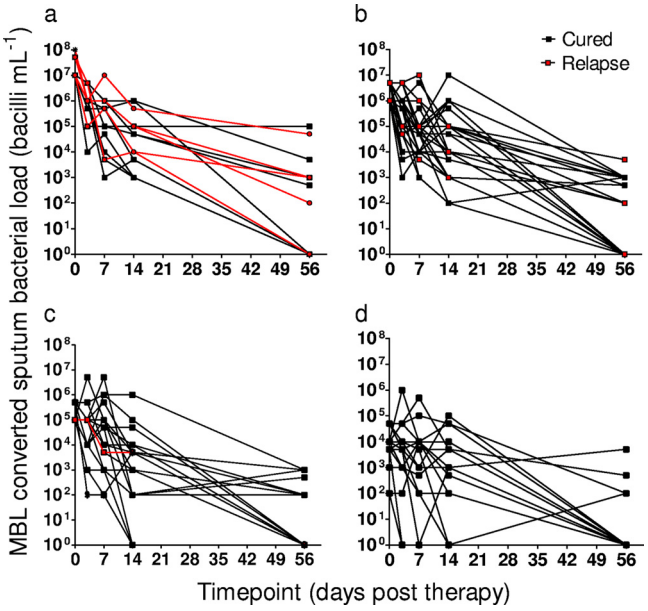
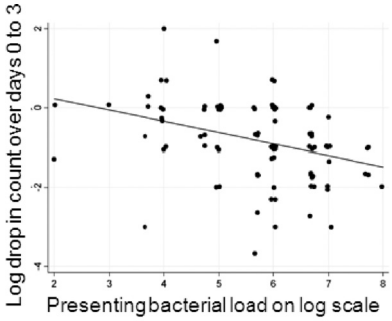


FIG. 3. (a to d) MBL assay-determined bacterial load in patient sputum samples collected longitudinally (days 0, 3, 7, 14, and 56) for 96 patients who had complete data sets for all of these time points and who were on chemotherapy (bacterial load results were determined using 16S rRNA and normalized against the IC; bacterial load values were assigned according to Fig. 2c). Day 0 bacterial loads (ml^{-1} sputum): 1×10^7 to 5×10^7 (a), 1×10^6 to 5×10^6 (b), 1×10^5 to 5×10^5 (c), and $\leq 5 \times 10^4$ (d).



16S rRNA bacterial load mL^{-1} sputum day 0	*Bacterial load Declined by ≥ 1.0 log_{10} by day 3*	*Bacterial load Declined by < 1.0 log_{10} by day 3*
$1\text{-}5x10^7$	14 (93%)	1 (7%)
$1\text{-}5x10^6$	26 (59%)	18 (41%)
$1\text{-}5x10^5$	9 (39%)	14 (61%)
$< 5x10^4$	5 (24%)	16 (76%)
Total	**54**	**49**

FIG. 4. Decline in bacterial load during the early phase of bacterial killing (day 0 to day 3) according to the presenting bacterial load.

0.0001) (Fig. 5). This model has also been found to be the best fit for SSCC data (4, 25). The triexponential model did not converge, most likely since it was too complex for the limited data.

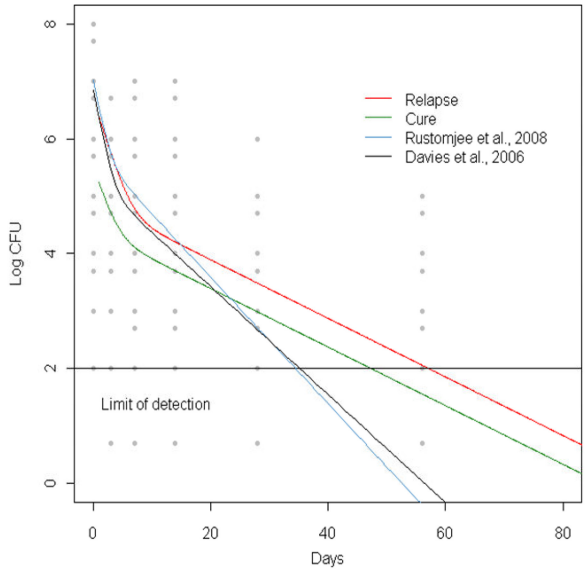
Molecular count as an early biomarker of treatment response. Further analysis using the nonlinear models found that a model that differed by relapse status (model 4) better fit the data than the pooled model (model 3) ($P = 0.0009$). The key difference was in the A parameter, reflecting the bacterial load at day 0 (cured A = 5.51, 95% CI, 5.28 to 5.74; relapse A = 6.70, 95% CI, 5.96 to 7.43; $P = 0.0002$) (Fig. 5). There was no significant improvement in model fit by allowing either gradient, α in the first phase or β in the second phase, to differ by relapse status. Using logistic regression there was a clear association between MBL-determined day 0 bacterial load and relapse status, with an increased odds of relapse of 3.62 (95% CI, 1.53 to 8.59) for every 1 $\log_{10}$ increase in day 0 bacterial load.

Culture conversion at day 56 has gained wide currency as a critical time point for treatment response and is the best currently available predictor of relapse (1). Previous studies have suggested that 16S rRNA has a long half-life in sputum following bacterial death that would interfere with the value of this measure as an endpoint (5). To address whether the MBL assay is comparable to liquid culture during the second phase (β) of bacterial elimination, the MBL assay data were compared to liquid culture results obtained at day 56. There were 94 subjects where bacterial load data, measured by the MBL assay and liquid culture, were available. Twenty-five of 26 samples at week 8 (96%) found to contain $\geq 10^3$ bacilli ml^{-1} sputum with the MBL assay were also culture positive (Table 1). Ten samples found positive using the MBL assay were negative by culture, and 24 samples were detected as culture positive but were negative by the MBL assay at this time point (Table 1).

DISCUSSION

The purpose of our work was to develop a rapid molecular assay to quantify *M. tuberculosis* that can substitute for culture-based methods and to validate it in a study of patient response to chemotherapy. Such a method would be useful in simplifying clinical trial methodology.

Many molecular tests are compromised by variability created during nucleic acid extraction. To overcome these problems, we developed an improved internal control. A previous study has reported the use of a 101-bp fragment from the *phyB* gene in detecting inhibition during qPCRs. We demonstrated that extraction and inhibition effects are comparable between our 1,957-bp IC, but this was not case for the 101-bp fragment (our unpublished data) and *Mycobacterium tuberculosis* 16S rRNA when the 101-bp control was added prior to RNA extraction. Using our internal control, it was possible to normalize the RT-qPCR 16S rRNA data for robust detection of bacteria in spot sputum samples containing between 10^8 and 10^2 bacilli ml^{-1} sputum with up to 1,000-fold assay inhibition. This is based on detection of the internal control using RT-qPCR before and after spiking into the RNA extraction. A cycle threshold of 7.00 was observed prior to extraction, whereas the IC signal was not detected until at least cycle 16.00 after extraction. We found mRNA was not detected in samples with $<10^4$ bacilli ml^{-1} (5; our unpublished data), whereas the limit of bacterial detection using the MBL assay was 10^2 bacilli ml^{-1} and therefore a significant improvement over mRNA (10,



Model		Parameter estimates with 95% confidence intervals A	α	B	β	AIC[a]	Results of likelihood ratio test
1. Mono-exponential		5.11 (4.98, 5.25)	0.063 (0.058, 0.069)			1886	
2. Bi-exponential		5.62 (5.38, 5.86)	0.25 (0.16, 0.40)	4.52 (4.21, 4.84)	0.049 (0.042, 0.058)	1848	p<.0001[b]
3. Bi-exponential mixed effects (pooled)		5.65 (5.43, 5.88)	0.30 (0.25, 0.37)	4.41 (4.14, 4.68)	0.050 (0.045, 0.055)	1700	p<.0001[c]
4. Bi-exponential mixed effects	Cure	5.51 (5.28, 5.74)	0.33 (0.27, 0.39)	4.41 (4.14, 4.68)	0.051 (0.046, 0.056)	1690	p=0.0009[d]
	Relapse	6.70 (5.96, 7.43)		4.91 (4.07, 5.76)			
	Test[e]	p=0.0002		p=0.18			

FIG. 5. Mathematical modeling: MBL assay-determined bacterial decline for 111 patients using data from day 0 to day 56. The best-fit mixed-effect biphasic decay model is shown, and data from 2 SSCC studies, those of Rustomjee et al. (25) and Davies et al. (4), which previously found that the best fit was biphasic decay. Fit of nested sums of various exponential models. Parameters A and B are the intercepts for the phases of killing ($\log_{10}$ bacterial load ml^{-1}), and α and β are the corresponding rates of decrease in bacterial load ($\log_{10}$ bacterial load ml^{-1}) as derived from the model. [a], Akaike information criterion—a lower number indicates a model that fits the data better; [b], comparing models 1 and 2; [c], comparing models 2 and 3; [d], comparing models 3 and 4; [e], Wald test comparing parameter estimates between models for cured and relapse patients.

15). This is despite the observation that 16S rRNA may be downregulated as bacteria enter dormancy (28).

A challenge to any molecular method is whether it is able to detect changes in the numbers of organisms over short time frames. We have shown that the mean decline in bacterial load during the first 3 days was found to be 0.99 $\log_{10}$ (95% CI, 0.81 to 1.17), which suggests that the MBL assay does have the ability to rapidly detect changes in numbers of organisms.

We performed nonlinear modeling of MBL assay data in response to chemotherapy in order to assess how the assay compared to culture-based methods. The decline in the first phase of decay (α), 0.33 $\log_{10}$ per day, suggests that the 16S rRNA concentration fell rapidly in line with bacterial killing. Studies using solid agar have reported $\alpha = 0.52$ $\log_{10}$ per day (4, 25) when using the same biexponential mixed-effects model. Unfortunately, we did not have solid culture colony counts available for direct comparison.

Thus, we believe that the MBL assay has considerable utility as a tool to understand the dynamics of treatment response. For example, we found that the rate of decline of MBL assay-determined bacterial load between days 0 and 3 was dependent on the bacterial load at day 0. If the bacteria were killed at a uniform rate, then the log decline should be the same regardless of the starting number. It may be that these differences are

TABLE 1. MBL assay-determined bacterial load compared to culture positivity at day 56 for 94 patients where both data were available[a]

16S rRNA bacterial load ml^{-1} sputum	No. (%) of samples		
	Total	Culture positive	Culture negative
1×10^5 to 5×10^5	1	1 (100)	0 (0)
1×10^4 to 5×10^4	2	2 (100)	0 (0)
1×10^3 to 5×10^3	23	22 (96)	1 (4)
1×10^2 to 5×10^2	21	12 (57)	9 (43)
0	47	24 (51)	23 (49)
Total	94	61	33

[a] The limit of detection for the MBL assay was determined to be 10^2 bacilli ml^{-1}. Therefore, assignment of 16S rRNA-determined bacterial load given as negative in the table represents any bacterial load that was $<10^2$ ml^{-1}.

attributable to the previously described heterogeneity of bacterial populations present in pretreatment sputum samples (16). The presence of the two phases of bacterial killing has previously been attributed to these heterogeneous populations of bacteria responding differently to the chemotherapy, with isoniazid killing rapidly dividing bacteria during the early treatment phase (α) and the other antibiotics acting on the nonreplicating population during the second phase (β) (16, 19). Higher bacterial load has previously been associated with the extent of cavitation (23, 24), and it might be expected that bacteria in the sputum expectorated by these patients would have a higher proportion of bacteria in a state of rapid division. Studies have also reported the presence of sputum bacteria with a persister phenotype (7). Both the proportion and the number of different bacteria in the lung may have an influence on how the patient responds to chemotherapy.

Bacterial load at day 0 was a predictor of later relapse, which mirrors liquid culture-based analysis in the same cohort (12). The number of relapses in this study was small (12 of 111 patients), and further work is required to confirm and strengthen this result.

When the MBL assay longitudinal data from 56 days of treatment were analyzed, we found that a biexponential mixed-effects model best described the data. Biphasic decay has been described by different authors using solid agar for quantification of bacterial load during treatment response (4, 25). The point on the fitted line where the decline transitions from the first phase (kill of rapidly dividing bacteria) to the second (kill of persister bacteria) can be calculated mathematically from the parameter estimates. This transition point occurs slightly later when using the MBL assay, at 3.99 and 6.49 days for cured and relapse, respectively. The transition points for the published SSCC data can be calculated from the printed parameter estimates, and these were 3.60 and 2.93 (4, 25). In these cohorts, relapse follow-up data were not available. The transition times are relatively comparable considering that the data came from different cohorts. Differences could be attributable to variations such as HIV status in the patient groups or could reflect the time taken for the 16S rRNA species to decay following bacterial cell death or the detection of nonculturable bacilli with the MBL assay.

For early bactericidal activity (EBA) studies and clinical trials modeling bacterial clearance, it may be possible to adopt the MBL assay in place of culture. However, the fact that both liquid culture and the MBL assay detected positive samples at day 56 when the other assay was negative suggests that the detection of culture-negative status at this time point may be improved by sample analysis with both assays. Culture-negative samples detected positive using the MBL assay at day 56 could be explained by viable bacilli that are refractive to culture media, as noted in a previous study when longitudinal sputum samples were tested for the presence of 16S rRNA during treatment (21). Several studies have also found increased recovery in bacilli by culture when resuscitation factors are added from exponential cultures (6, 22).

The advantages of the MBL assay reported here include results being obtained within 24 h of sputum expectoration regardless of bacterial load present, which contrasts to the long time delay required for low-burden samples to be tested by currently available culture-based methods. Also, <1% of samples were too inhibited when using the MBL assay, whereas contamination with other organisms invalidates 4.4 to 8.6% of liquid cultures depending on the method used (3). The specificity of our *M. tuberculosis* 16S primer and probe set to *M. tuberculosis* complex was determined empirically by testing a panel of 7 nontuberculosis bacteria and 5 other respiratory-relevant species (see Data S2 in the supplemental material). Our *in vitro* analysis calculated the false-positive rate of this assay to be 4.4%, which is in line with a median of 3.1% for culture false positives (interquartile range, 2.2% to 10.5%) (2).

In summary, we present a novel assay that is robust and reproducible for the quantification of tubercle bacilli in sputum samples. We believe that it will be of use in measuring treatment response in clinical trials.

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