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We evaluated the use of the molecular bacterial load (MBL) assay, for measuring viable Mycobacterium tuberculosis in sputum, in comparison with solid agar and liquid culture. The MBL assay provides early information on the rate of decline in bacterial load and has technical advantages over culture in either form.

Assessment of Mycobacterium tuberculosis sputum bacterial load has routinely been performed using solid culture (1). Automated liquid culture has been proposed as an alternative (2), but all culture-based methods are hampered by contamination with other microorganisms (3), protracted time to obtain results (4), and populations of viable, nonculturable bacteria (5, 6). We recently described the molecular bacterial load (MBL) assay, based on 16S rRNA, observing that MBL declined biphascially in response to treatment (7). In the present study, we directly compared the MBL assay with solid and liquid culture on 148 sputum samples (collected overnight from 1600 h until 0800 h the following morning) from 20 patients enrolled and hospitalized at the EBA Unit, Tanzanian National Institute for Medical Research-Mbeya Medical Research Centre, Tanzania. Samples were collected pretreatment (2 overnight samples) and longitudinally (days 2, 4, 5, 7, 10, and 14) during standard WHO treatment for drug-sensitive tuberculosis. The OEBA (Observation of Early Bactericidal Activity) study was a capacity building project of the PanACEA consortium. Before patient enrolment, the study was approved by the site’s local ethics board (reference MRH/T.30/44/2) and national ethics board (reference NIMR/HQ/R.8a/Vol IX/1169) and the sponsor (University of Munich) ethics board. The study was conducted in compliance with the declaration of Helsinki. All patients provided written informed consent to study participation, including use of their samples for evaluation of novel molecular assays. The study was registered in the Pan African Clinical Trials Registry (pactr.org) under PACTR201209000394102.

Sputum was homogenized, and one half was decontaminated with N-acetyl-l-cysteine-sodium hydroxide (1% final NaOH concentration) prior to inoculation into mycobacterial growth indicator tubes (BBL MGIT; Becton, Dickinson and Company, MD, USA), according to the manufacturer’s instructions, in order to determine time to positivity (TTP) in the Bactec MGIT 960 (Becton, Dickinson and Company, MD, USA). The second portion was mixed with an equal volume of Sputasol (Oxoid, Limited, United Kingdom), and quadruplicate 100-µl volumes of 10-fold dilutions were inoculated onto 7H11 agar containing Mycobacteria Selectatab Kirchner supplement (Mast Group, Limited, United Kingdom). Nine hundred fifty microliters of sputum/Sputasol was preserved in guanidine thiocyanate containing 1% β-mercaptoethanol, and the MBL assay was performed as detailed in the work of Honeyborne et al. (7). MBL value was assigned based on 16S rRNA cycle threshold (CT) using the normalized 16S rRNA CT in the following equation: bacterial load (log_{10}) = (normalized 16S CT - 31.76)/-3.171 (7).

Artificial sputum (see Text S1 in the supplemental material) developed as a matrix for high assay standards was found to be a good substitute for pooled human sputum. High standards had mean 7.46 log_{10} bacilli (standard error of the mean [SEM], 0.08) and 7.49 log_{10} bacilli (SEM, 0.07) (P = 0.80) for pooled human sputum and artificial sputum, respectively (see Fig. S1 in the supplemental material). The low standard mean of 3.70 log_{10} bacilli (SEM, 0.13) for pooled human sputum was statistically different from that of artificial sputum (mean, 3.38 log_{10} bacilli; SEM, 0.13; P = 0.01) (see Fig. S1). However, the SEMs are identical for the two matrices, and therefore, the critical factor, reproducibility of extraction of an assigned bacterial number, was met.

Spearman rank correlations for 148 sputum samples revealed a high degree of correlation between the assays: log_{10} MBL compared to solid agar log_{10} CFU (r = +0.84; 95% confidence interval [CI], 0.78, 0.88), log_{10} MBL compared to solid agar log_{10} TTP (r = −0.81; 95% CI, −0.86, −0.74), and log_{10} TTP compared to solid agar log_{10} CFU (r = −0.78; 95% CI, −0.84, −0.71; for all, P < 0.0001) (see Fig. S2 and Text S2 in the supplemental material). Correlation between log_{10} CFU and log_{10} TTP for our study was comparable to that in a study of >2,000 sputum samples tested in South Africa (r = −0.72) (2), although TTP was not log-transformed in this study.

Direct comparison of solid culture and the MBL assay for individual patients revealed that the declines in bacterial load closely
matched during the first 14 days of therapy (Fig. 1). rRNA is downregulated in bacteria that are entering dormancy (8), and so one might expect that the MBL assay is an underestimate of bacterial load. However, the baseline median bacterial load was 0.43 log$_{10}$ (95% CI, 0.12, 0.73; $P = 0.008$) higher when measured using the MBL assay than when measured by the solid agar CFU method (paired $t$ test, $P = 0.008$). This may be explained by the selective antibiotics in the solid agar method killing a proportion of the bacteria present. We observed a similar effect for six clinical isolates (M. tuberculosis [n = 5] and Mycobacterium bovis [n = 1]) isolated from decontaminated sputum on Lowenstein-Jensen (LJ) slopes (Southern Group Laboratories, Limited, United Kingdom). Determination of bacterial number for these was done using the Miles-Misra method (9) on 7H10 agar with 10% oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton, Dickinson and Company, MD, USA) with and without the addition of antibiotics using bacteria subcultured from the LJ slope into 7H9 medium containing 0.2% Tween 80 and 10% ADC supplement (Becton, Dickinson and Company, MD, USA). Addition of antibiotics (Mycobacteria Selectatabs Kirchner; Mast Group, Limited, United Kingdom) reduced the bacterial count by 0.47 log$_{10}$ (95% CI, 0.23, 0.71; paired $t$ test, $P = 0.004$). This may explain the increased MBL readout observed here for patient sputum in comparison to that for solid culture despite comparable rates of decline.

A biphasic decline in bacterial load was observed for liquid and solid culture and the MBL assay with a node at day 3 (Fig. 2). Full details of statistical analysis are given in Text S2 in the supplemental material. The decline rate for days 0 to 3 for solid agar was $-0.278 \text{ log}_{10} \text{day}^{-1}$ (95% confidence interval, $-0.345, -0.212$), and that for MBL was $-0.307 \text{ log}_{10} \text{day}^{-1}$ ($-0.389, -0.226$). MBL decline was therefore slightly quicker over the first 3 days, although the confidence intervals overlap. The use of mRNA for the isocitrate lyase gene and the use of noncoding ribosomal promoter region mRNA as amplification targets for bacterial quantification have previously been found to respond comparably to culture during days 2 to 7 of therapy but not during the first 2 days of therapy (10). In contrast, MBL robustly matched solid and liquid culture over the early phase of treatment. Extended early bactericidal activities (EBAs) from days 3 to 14 were also comparable, with $-0.082 \text{ log}_{10}$ ($-0.119, -0.045$) and $-0.124 \text{ log}_{10}$ ($-0.170, -0.079$) for CFU on solid medium and MBL, respectively. These data match other EBA studies measuring treatment response with solid culture (11–16). The close comparability between MBL and culture contrasts with data for treatment monitoring using the GeneXpert MTB/RIF assay (Xpert; Cepheid, CA, USA). GeneXpert MTB/RIF did not respond to changes in bacterial load as rapidly as did culture (17). Within-subject variation of Xpert was high, at 56.7%. In contrast, MBL had a 9.6% variance between 2 baseline samples for 16 subjects (18), suggesting that MBL is more reproducible. To corroborate this, within-patient variabilities for solid (17.9%) and liquid (21.6%) culture in our study were compared to those in the study by Kayigire et al., with 16.5% and 22%, respectively (18). Prior to exclusion of outliers, solid culture had invalid results in 11% of samples (18 of 169), whereas MBL had only 1.2% of samples (2 of 169) unreadable.
In summary, our data show that the MBL assay is at least as good as culture for measuring EBA during standard tuberculosis therapy to day 14, with higher precision, fewer missing data, and a shorter time to result (24 h compared to weeks). This assay shows promise as a replacement for culture in future EBA trials testing new drugs.

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