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Quantification of multidrug-resistant *M. tuberculosis* bacilli in sputum during the first 8 weeks of treatment

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SUMMARY

SETTING: Mulago Hospital, Kampala, Uganda.

OBJECTIVE: To quantify *Mycobacterium tuberculosis* in sputum during the first 8 weeks of pulmonary multidrug-resistant TB (MDR-TB) treatment.

DESIGN: We enrolled consecutive adults with pulmonary MDR-TB treated according to national guidelines. We collected overnight sputum samples before treatment and weekly. Sputum samples were cultured on Middlebrook 7H11S agar to measure colony-forming units per mL (cfu/mL) and in MGIT™ 960™ media to measure time to detection (TTD). Linear mixed-effects regression was used to estimate the relational change in $\log_{10}$ cfu/mL and TTD.

RESULTS: Twelve adults (median age: 27 years) were enrolled. Half were women, and two-thirds were HIV-positive. At baseline, median $\log_{10}$ cfu/mL was 5.1, decreasing by 0.29 $\log_{10}$ cfu/mL/week. The median TTD was 116.5 h, increasing in TTD by 36.97 h/week. The weekly change was greater in the first 2 weeks (−1.04 $\log_{10}$ cfu/mL/week and 120.02 h/week) than in the remaining 6 weeks (−0.17 $\log_{10}$ cfu/mL/week and 26.11 h/week).

CONCLUSION: Serial quantitative culture measures indicate a slow, uneven rate of decline in sputum *M. tuberculosis* over 8 weeks of standardized pulmonary MDR-TB treatment.

RÉSUMÉ

Hôpital de Mulago, Kampala, Ouganda.

Quantifier *Mycobacterium tuberculosis* dans les échantillons de crachats pendant les huit premières semaines du traitement de la TB pulmonaire multirésistante (MDR-TB).

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Disclaimer: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the U.S. Centers for Disease Control and Prevention.

Nous avons inclus, de manière consécutive, des adultes atteints de MDR-TB pulmonaire traités conformément aux recommandations nationales. Nous avons recueilli les échantillons de crachats nocturnes avant l'instauration du traitement, ainsi qu'une fois par semaine. Les échantillons de crachats ont été mis en culture sur gélose Middlebrook 7H11S afin de mesurer les unités formant colonies par mL (cfu/mL) et en milieu MGIT™ 960™ pour mesurer le temps avant détection (TTD). Une régression linéaire à effets mixtes a été utilisée afin d'estimer le changement relatif en $\log_{10}$ cfu/mL et TTD.

Douze adultes (âge médian : 27 ans) ont été inclus. La moitié était des femmes et les deux tiers étaient infectés par le VIH. Lors de l'inclusion, la valeur $\log_{10}$ cfu/mL médiane était de 5,1, diminuant à 0,29 $\log_{10}$ cfu/mL/semaine. Le TTD médian était de 116,5 h, avec une augmentation du TTD de 36,97 h/semaine. Le changement hebdomadaire était plus important au cours des deux premières semaines (−1,04 $\log_{10}$ cfu/mL/semaine et 120,02 heures/semaine) qu'au cours des six semaines restantes (−0,17 $\log_{10}$ cfu/mL/semaine et 26,11 heures/semaine).

Les mesures quantitatives en série des cultures indiquent un taux de décroissance faible et irrégulier de *M. tuberculosis* dans les échantillons de crachats au cours de 8 semaines d'un traitement standardisé de la MDR-TB pulmonaire.

Keywords

anti-TB drug treatment; multidrug-resistant tuberculosis; sputum; *M. tuberculosis* bacilli; quantitative cultures

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The first randomized clinical trial in history—streptomycin for TB in 1947—showed that single-agent treatment led to acquired resistance and treatment failure.[1] Multidrug therapy cured patients and prevented acquired resistance.[2] This foundational principle of multidrug therapy made it hard to quantify the effects of individual new drugs because no one drug could be tested alone in humans. In 1980, Jindani et al. introduced the early bactericidal activity (EBA) study in which patients were treated with one drug for the first 2 days, and researchers quantified *Mycobacterium tuberculosis* colony-forming units (cfu) cultured from serial sputum specimens.[3] EBA studies were justified because the period of monotherapy was too short to lead to acquired resistance.[4] EBA studies were extended to 14 days without adverse effects, leading to more effective treatment and insights into pharmacodynamics.[4]

Until recently, EBA studies have not been used for multidrug-resistant TB (MDR-TB).[5–8] Consequently, the dynamics of EBA in MDR-TB treatment are less well understood. We investigated the extent to which quantitative culture methodologies can detect decreasing mycobacterial burden during the first 8 weeks of MDR-TB treatment, a standard duration for Phase-II clinical trials.

METHODS

Study population

Patients aged ≥18 years with pulmonary MDR-TB were referred to Mulago Hospital, Kampala, Uganda. During March–August 2015, patients were recruited into a prospective study to determine the feasibility of quantitative cultures during 8 weeks of MDR-TB

treatment. According to national guidelines based on WHO recommendations, patients were treated with kanamycin (KM), levofloxacin (LVX), pyrazinamide (PZA), ethionamide (ETH), and cycloserine (CS) dosed by weight bands.

Participants had a productive cough, were sputum-positive for acid-fast bacilli (AFB), with resistance to rifampicin on Xpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA), and ready to start treatment based on the Xpert result.[9] An additional sputum specimen was collected for drug susceptibility testing (DST) using GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) and MGIT™ 960™ (BD, Franklin Lakes, NJ, USA) to first and second-line drugs.[10] Patients were excluded if they were deemed ineligible by the treating clinician for any reason; were HIV-positive with CD4 count <50 cells/mm^3; had any modification to standard dosing regimens; had extensively drug-resistant TB (MDR-TB plus resistance to an injectable agent and a fluoroquinolone); were incarcerated; or were unable to provide an adequate sputum specimen. Patients were followed for 8 weeks until the end of the intensive phase of treatment as in Phase-II clinical trials, not to the end of treatment.

Sputum collection and processing

Per routine, participants were admitted to hospital in Kampala. At baseline, patients began the first overnight, 16-h sputum specimen collection from 6:00 pm until 10:00 am the following morning, icing the container until pick up. The following morning (Day 1), directly observed treatment for MDR/rifampin-resistant TB (RR-TB) was started with LVX, KM, CS, ETH, and PZA, dosed by weight according to Ugandan national standards. Participants then collected overnight, 16-h sputum specimens weekly for 8 weeks. Specimens were stored at 4°C and processed within 24 h of collection. Sputum samples were homogenized with 1:10 proportion by volume of 0.1% dithiothreitol (final concentration: 0.01%) by vortex, and then rocked mechanically for 15–20 min.

Quantitative culture on Middlebrook 7H11S agar

Middlebrook 7H11 media was made selective by adding 5 μg/mL of amphotericin B, 25 μg/mL of carbenicillin, 100 units/mL of polymixin B, and 10 μg/mL of trimethoprim (final concentrations) during preparation. Five serial 10-fold dilutions of the homogenized sputum specimen were prepared in phosphate-buffered saline. We inoculated 100 μL of homogenized, undiluted sputum and all five dilutions from each sample on each segment of duplicate biplates. Culture plates were sealed with carbon dioxide-permeable tape, and incubated with carbon dioxide (5–10%) at 37°C. Plates were examined at Day 3 for contamination and, starting at Day 7, weekly thereafter for 6 weeks to count easily visible colonies. Once growth stopped and/or plates were incubated for 6 weeks, the dilution with colony counts ranging from 50 to 150 was chosen as most reliable. The average number of colonies was calculated for four bi-plate sides (those without growth of contaminants). Counts of cfu/mL of sputum were calculated as follows: cfu/mL = average number of colonies × 10 (100 μL inoculum per segment) × dilution factor.

MGIT 960 liquid culture and smear microscopy

After preparing quantitative culture dilutions, the remaining homogenized sputum was decontaminated with a final sodium hydroxide concentration of 1.5% for 15–20 min and

centrifuged in a refrigerated centrifuge at 3000 *g* for 15 min. Digested-decontaminated sputum sediment was re-suspended to 2 mL in phosphate-buffered saline. A 0.05 mL volume of re-suspended specimen was used for concentrated AFB smear microscopy. A 0.5 mL volume of re-suspended specimen was inoculated in MGIT prepared with PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) according to the manufacturer's directions. All positive MGIT cultures were identified and contamination was ruled out with AFB smear microscopy and blood agar plates. Instrument-generated time to detection (TTD) in days and hours was recorded for positive cultures.

Definitions and statistical analysis

Baseline demographic and clinical data were collected for each participant. Underweight was defined as a body mass index <18.5 kg/m^2. De-identified data were entered into a Microsoft® Access database (MicroSoft, Redmond, WA, USA) on a password-protected computer and analyzed using RStudio v1.2.5033 (RStudio, Boston, MA, USA). Univariate analyses were used to assess trend and distribution. After assessing distribution, we $\log_{10}$-transformed cfu/mL to normalize the distribution. $P < 0.05$ was considered statistically significant. Outliers greater than three times the standard deviation from the mean were truncated to three times the standard deviation. Linear mixed-effects regression models were used to quantify the change in $\log_{10}$ cfu/mL and TTD per week of MDR-TB treatment, with serial specimens treated as repeated measures with an autoregressive covariance structure. Bactericidal activity was estimated using the estimated parameters. Associations with contamination on 7H11S and MGIT 960 were investigated using Fisher Exact test.

Ethics

The study protocol was approved by Makerere University School of Biomedical Sciences Research and Ethics Committee (Kampala, Uganda), the U.S. Centers for Disease Control and Prevention Institutional Review Boards (Atlanta, GA, USA), and the Uganda National Council for Science and Technology (Kampala, Uganda).

RESULTS

Twelve adults (median age: 27 years, interquartile range [IQR]: 24–36.5) were enrolled based on an Xpert result (10/12) of at least RR-TB or conventional phenotypic DST using MGIT 960 (11/12). Six participants were women, two-thirds were HIV-positive ($n = 8$), and 92% were underweight at treatment initiation ($n = 11$; Table 1). Ten participants were previously treated for TB; three of whom were receiving first-line anti-TB treatment when MDR-TB was diagnosed. Of eight participants with cavitation on chest radiograph, one had bilateral involvement. Of 11 participants with pulmonary infiltration or consolidation, two-thirds had bilateral involvement. All participants received LVX (500–750 mg/day), KM (500–750 mg/day), PZA (1,000–2,000 mg/day), CS (500–750 mg/day), and ETH (500–750 mg/day). The median time from initial diagnosis to start of MDR-TB treatment was 6.5 days (IQR: 3–8), 11/12 patients initiated MDR-TB treatment within 2 days after enrollment, one patient started 3 days after enrollment.

At baseline, nine participants had grade 3 sputum smears. Of the 108 total overnight sputum samples collected, 50 (46%) had unavailable colony counts due to contamination (n = 28; median: 2/patient; range: 0–8/patient) or no growth (n = 22; median: 1/patient; range: 0–5/patient) on Middlebrook 7H11S (Supplementary Table S1). In MGIT 960 cultures, 45 (42%) had unavailable TTD due to contamination (n = 33; median: 3/patient; range: 1–4/patient) or no growth (n = 12; median: 0/patient; range: 0–5/patient).

Change in the number of colony-forming units on Middlebrook 7H11S media

Overall, the median $\log_{10}$ cfu/mL at baseline was 5.1 (IQR: 4.0–6.4; n = 12). Figure 1 shows the weekly median $\log_{10}$cfu/mL results. Figure 2 shows individual participant response to therapy and the linear regression line. Ten participants (83%) had at least two non-zero data points and were included in the linear regression analysis of change in $\log_{10}$ cfu/mL. In the linear mixed-effects regression model, there was an overall decrease of 0.29 $\log_{10}$ cfu/mL per week (standard error [SE] 0.07; P = 0.0002) for 0–8 weeks (Table 2). Colony growth per week decreased more during the first 2 weeks (−1.04 $\log_{10}$ cfu/mL per week; SE 0.21; P = 0.0001) than during the final 6 weeks (−0.17 $\log_{10}$ cfu/mL per week; SE 0.10; n = 8; P = 0.09) during which the decrease was not statistically different from zero (Supplementary Table S1). There was no association between HIV infection and change in the number of cfu/ml or TTD.

Change in time to detection in MGIT 960

The median TTD at baseline was 116.5 h (IQR: 102.0–143.0) or 4.9 days (IQR: 4.2–6.0; n = 10; two patients were excluded due to contamination in baseline isolate). Figure 3 shows the median TTD during the first 8 weeks of MDR-TB treatment. Figure 4 shows the individual participant response to therapy and the linear regression line. Eleven participants (92%) had at least two data points included in the analysis of change in TTD. One outlier was truncated. Using a linear mixed-effects regression model, we found an overall increase in TTD of 40.59 h per week for 0–8 weeks (SE 5.79; P < 0.0001; Supplementary Table S1). TTD per week increased more during the first 2 weeks (120.02 h/week; SE 18.28; n = 10; P < 0.0001) than in the final 6 weeks (32.32 h/week; SE 8.91; n = 9; P = 0.001).

Contamination of agar plates and broth cultures

Of the 108 overnight sputum specimens, 28 (26%) were lost due to contamination in Middlebrook 7H11S cultures and 33 (31%) were lost due to contamination in MGIT 960 cultures (Supplementary Table S1). Contamination in Middlebrook 7H11S plates, but not MGIT 960, was statistically associated with individual participants (P = 0.002), women (P = 0.01), and negative AFB smear (P = 0.03).

DISCUSSION

We quantified weekly bacterial density in sputum specimens from 12 adults with pulmonary MDR/RR-TB from the start of treatment to 8 weeks in Kampala, Uganda. Both cfu/mL on agar plates and TTD in MGIT 960 demonstrated significant trends in decreasing bacterial density overall (−0.29 $\log_{10}$ cfu/mL and 40.59 h per week, respectively), with a more rapid decline in density during the first 2 weeks (−1.04 $\log_{10}$ cfu/mL and 120.02 h per week,

respectively), and slower decline in density thereafter (−0.17 $\log_{10}$ cfu/mL and 32.32 h per week, respectively). There was a suggestion of a plateau from Week 2 to Week 4, resuming a downward slope afterward. Similar results were observed in a phase 2 trial of TMC207 (bedaquiline) against pulmonary MDR-TB among those receiving an optimized background regimen,[11] and in a study on ciprofloxacin in Tanzania among participants with TB who did not respond to a three-drug regimen.[12] Given the degree of variability, a large study is needed to understand this observation.

The median baseline quantitative culture on Middlebrook 7H11S in our study (5.1 $\log_{10}$ cfu/mL) was at least one log lower than what has been reported in many other EBA studies, including studies with treatment-naïve patients.[13,14] Compared with other studies that enrolled MDR-TB patients, the pretreatment median cfu/mL in our study was at least one-half log lower.[11] Our baseline $\log_{10}$ cfu/mL and TTD findings were similar to those of the moxifloxacin, pretomanid, and pyrazinamide study among pulmonary drug-susceptible TB patients, although their participants were treatment-naïve, and one arm included patients with drug-resistant TB.[15] The baseline TTD in MGIT 960 (116.5 h) was similar to findings in previous EBA studies.[13,16] Baseline bacillary load partly determines the ability to demonstrate a significant decline in cfu or increase in TTD, which are more difficult to demonstrate starting from lower bacterial density.[17,18] Nonetheless, although drug doses were lower than recommended for some of the drugs and patients, these results demonstrate the feasibility of quantitatively measuring the effect of chemotherapy over 8 weeks (and potentially longer) using serial sputum samples with either the gold standard cfu/mL on agar plates or the more practical method of TTD in MGIT 960. Our results give us an estimate of the rate of decline that may be useful in planning further studies, although the rate of change might be greater with higher drug doses.

In EBA studies, sputum specimens are not decontaminated before inoculation on Middlebrook 7H11 selective media because decontamination also kills mycobacteria, reducing the ability to grow countable colonies, while the selective agents prevent the growth of most oral flora.[19,20] Despite this, we lost fewer colony count results to contamination using Middlebrook 7H11S media (n = 28) compared to TTD in MGIT 960 media (n = 33).[21] However, we found that there were more unquantifiable cfu/mL results (n = 50) than TTD results (n = 45). However, for each study specimen there was one MGIT culture compared to the methodology for cfu/mL, which requires five serial dilutions, and each dilution is performed in quadruplicate. Culture-free molecular methods for quantifying viable mycobacteria in clinical specimens would obviate issues with contamination.

Our study had several limitations. The sample size was small. Nonetheless, certain findings were statistically significant, suggesting that the results were strong enough to be significant even with a small sample size. We lost about one-fourth and one-third of all cfu/mL and TTD endpoints to contamination, respectively. In subsequent studies, we had better results when we added carbendazim, an antifungal agent, to the 7H11S agar to suppress contaminants. Furthermore, not all mycobacteria from clinical specimens will grow in culture—the reason for not decontaminating specimens, as noted above, and for using at least two different media. Future studies could consider inoculating several MGIT cultures for TTD reproducibility and reducing risk of endpoint loss due to contamination. Another

important limitation of the present study was the MDR-TB regimen and the low drug doses received by the participants.[22] This analysis, along with recent MDR-TB clinical trial results, has spurred the WHO to recommend new MDR-TB regimens.[23] The lower dosing in our study could have caused the slow response or nonresponse to therapy among some of the participants. A similar lack of response has been seen in a dose-ranging EBA study among participants receiving a 9-mg/kg daily dose of isoniazid.[24] Finally, the full DST profile of our patients' isolates was not determined; however, WHO reports that quinolone resistance is uncommon among RR/MDR-TB patients in Uganda.[25]

Quantitative culture methods on Middlebrook 7H11S and MGIT can be used beyond the first 2 weeks of MDR-TB treatment to assess treatment effectiveness. High-quality cfu and TTD results are needed for comparators for novel regimens and biomarkers that are introduced for monitoring response to anti-TB therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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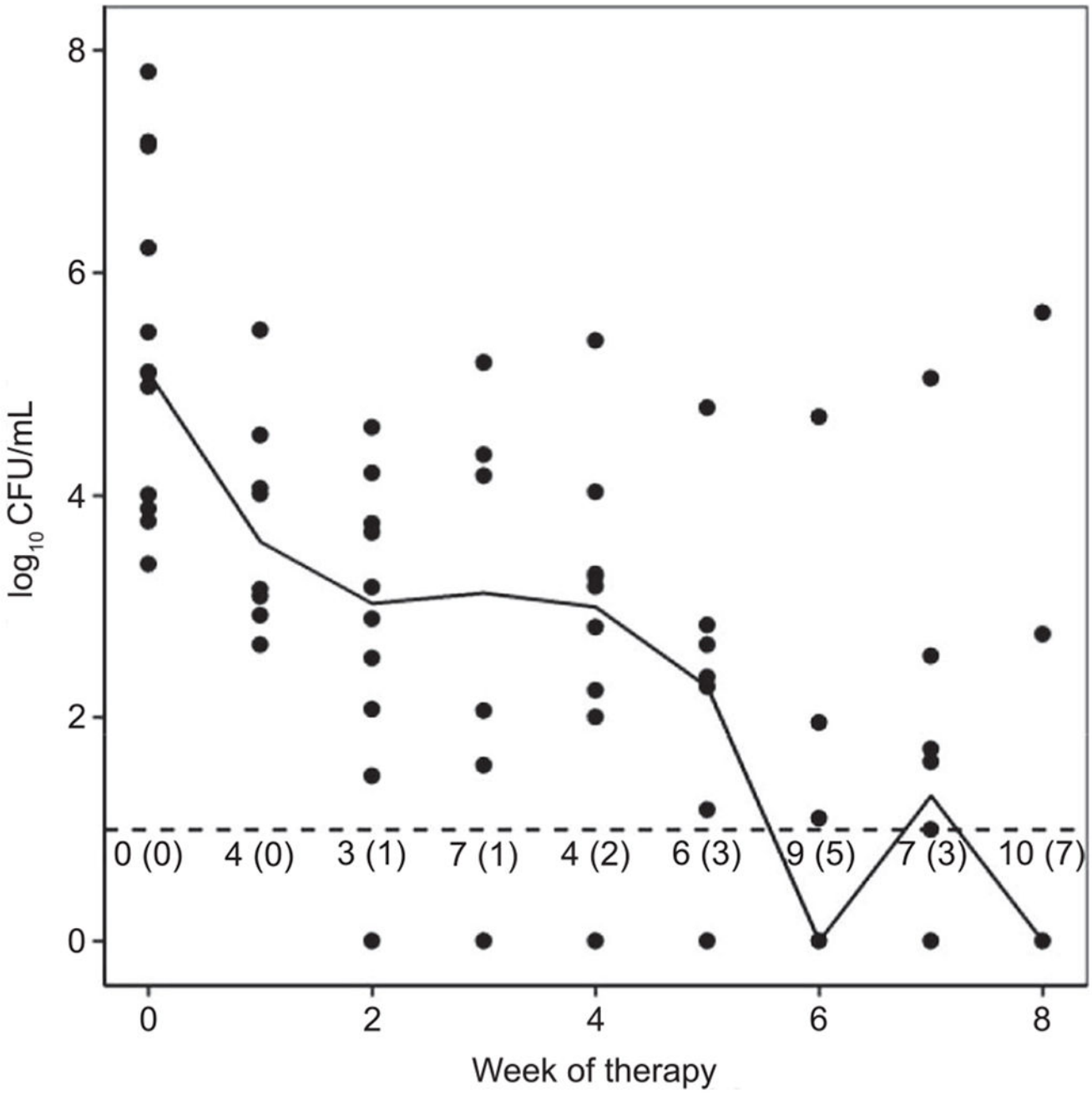

Figure 1.

Median $\log_{10}$ cfu/mL of participants (n = 12) during the first 8 weeks of multidrug-resistant TB treatment in Kampala, Uganda. The number of unquantifiable data points at each week are represented below the limit of detection (dotted line). The number in parentheses represents the number of *M. tuberculosis*-negative cultures. The remaining were contaminated. cfu = colony forming units.

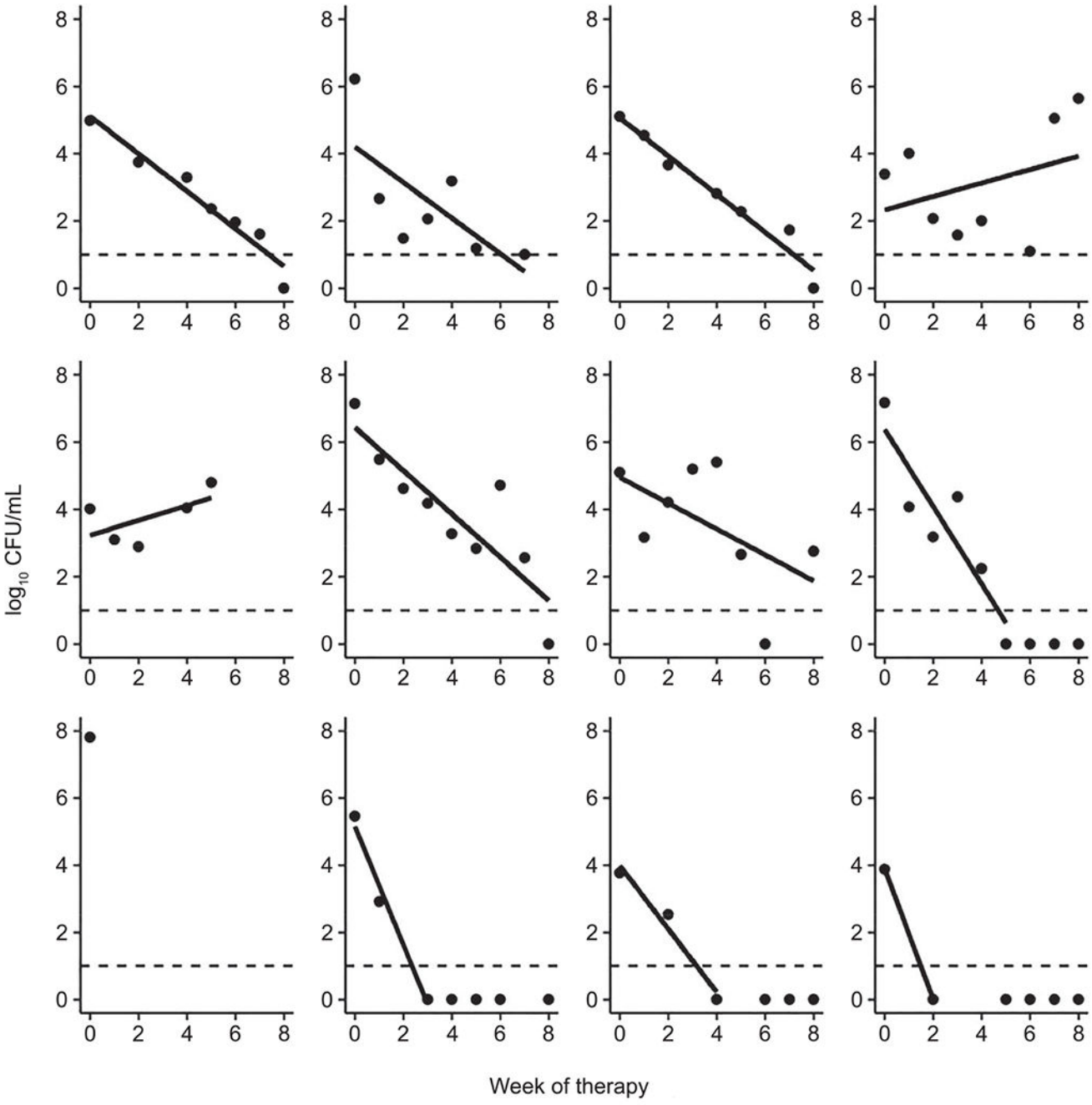

Figure 2.

Change in $\log_{10}$ cfu/mL during the first 8 weeks of multidrug-resistant TB treatment per participant ordered by date of enrollment in Kampala, Uganda. cfu = colony forming units.

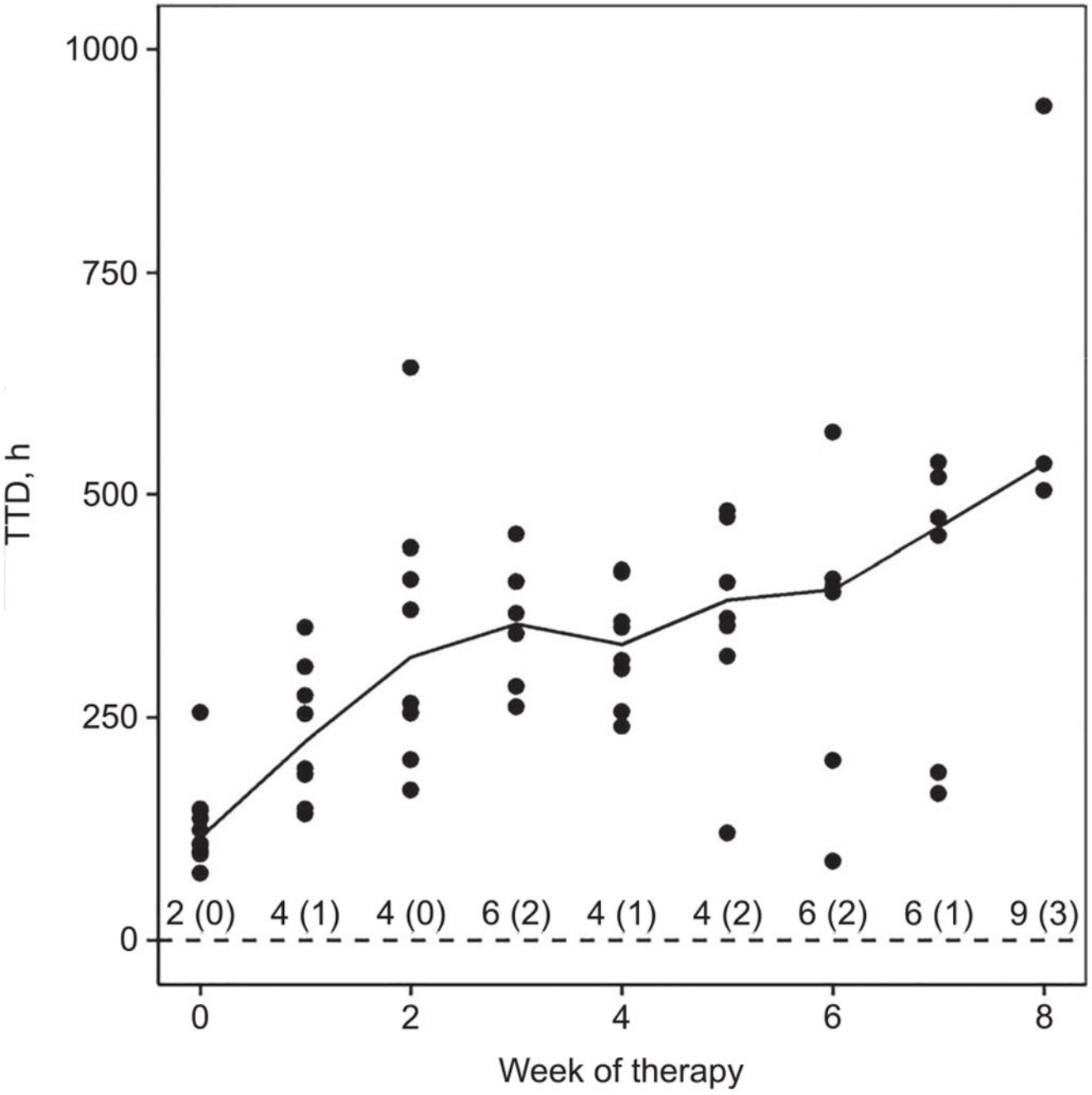

Figure 3.

TTD in hours of participants (n = 11) during the first 8 weeks of multidrug-resistant TB treatment. The number of unquantifiable data points at each week are represented just above the limit of detection (dotted line). The number in parentheses represents the number of *M. tuberculosis*-negative cultures. The remaining were contaminated. TTD = time to detection.

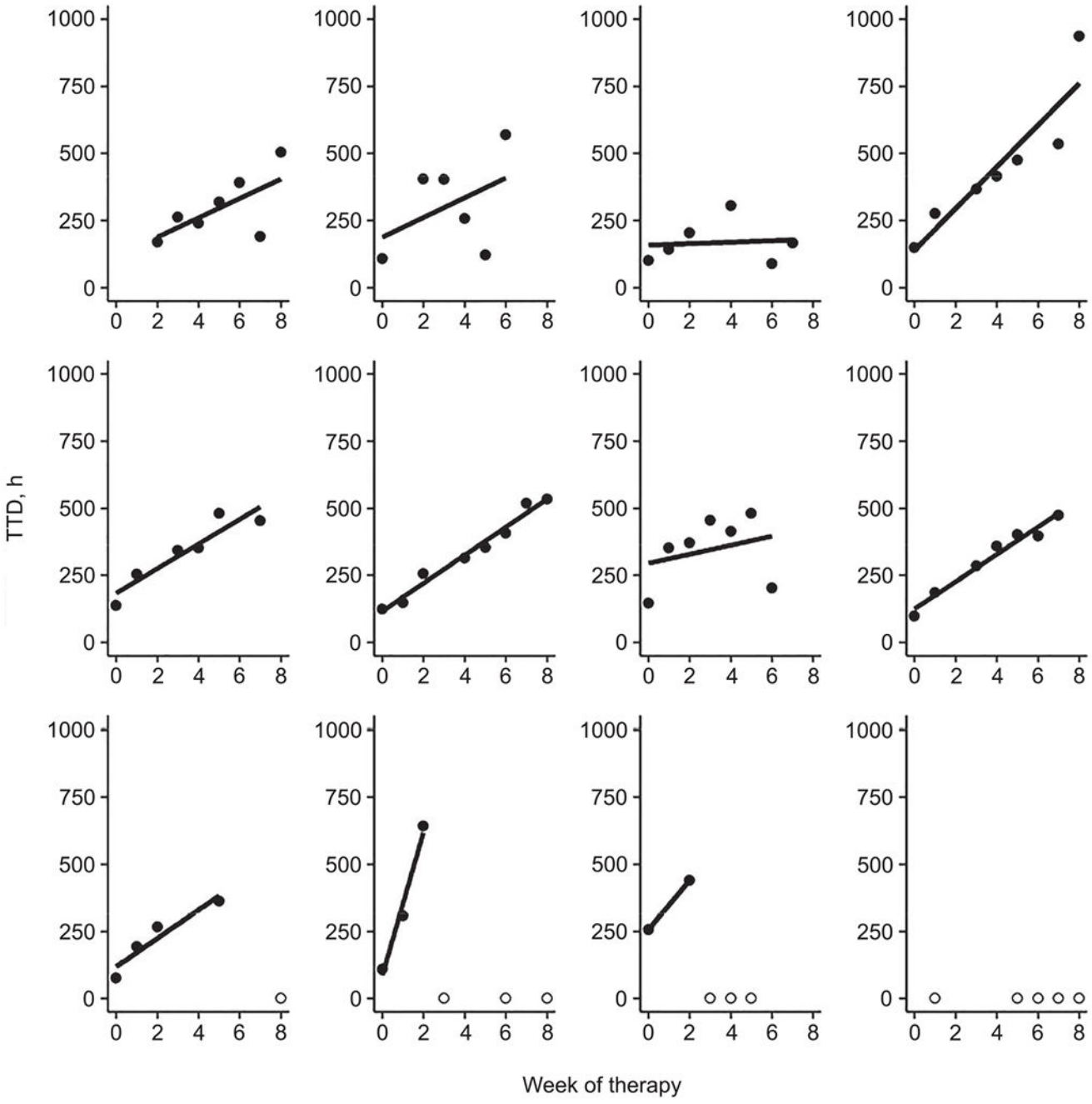

Figure 4.

Change in TTD of *M. tuberculosis* during the first 8 weeks of multidrug-resistant TB treatment per participant ordered by date of enrollment. Unfilled circles represent negative culture results. TTD = time to detection.

Table 1

Baseline characteristics of participants (n = 12) in a study of MDR-TB density in sputum during the first 8 weeks of treatment in Kampala, Uganda

Characteristics	*n* (%)
Female sex	6 (50)
Age, years, median [IQR]	27 [24–36.5]
HIV-positive	8 (67)
Underweight BMI at baseline	11 (92)
Any previous TB treatment	10 (83)
Still on TB treatment at time of MDR-TB diagnosis (n = 10)	3 (30)
Chest radiograph	
Any cavitation	8 (67)
Unilateral	7 (58)
Bilateral	1 (8)
Any infiltration or consolidation	11 (92)
Unilateral	3 (25)
Bilateral	8 (67)
Any other abnormalities	5 (42)
Enrollment laboratory results	
AFB smear grade	
1+	1 (8)
2+	2 (17)
3+	9 (75)
GenoType MTBDR*plus* results (n = 11)	
MDR-TB	4 (36)
RIF resistance only	6 (55)
INH resistance only	1 (9)
Phenotypic DST results (n = 11)	
MDR-TB	5 (46)
RIF resistance only	6 (55)
MDR-TB treatment	
Adequate dosage by weight	
Levofloxacin	10 (83)
Kanamycin	9 (75)
Cycloserine	12 (100)
Ethionamide	12 (100)
Pyrazinamide	12 (100)
Baseline quantitative culture results	
Baseline $\log_{10}$ cfu/mL, median [IQR]	5.1 [4.0–6.4]
Baseline TTD, h, median [IQR] (n = 10)	116.5 [102.0–143.0]

MDR-TB = multidrug resistant TB; IQR = interquartile range; BMI = body mass index; AFB = acid-fast bacilli; RIF = rifampicin; INH = isoniazid; DST = drug susceptibility testing; cfu = colony forming unit; TTD = time to detection.

Table 2

Bactericidal activity of standard MDR-TB treatment detected by change in $\log_{10}$ cfu/mL of *M. tuberculosis* in Middlebrook 7H11 selective media and TTD (h) in MGIT liquid media in a study of MDR-TB density in sputum during the first 8 weeks of treatment in Kampala, Uganda[*]

	Change in $\log_{10}$, cfu/mL		Change in TTD, h	
Study period, weeks	***n*** [*]	**Bactericidal activity/week (SE)**	***n*** [*]	**Bactericidal activity/week (SE)**
0–2	10	−1.04 (0.21)[†]	10	120.02 (18.28)[†]
2–8	8	−0.17 (0.10)	9	32.32 (8.91)[†]
0–8	10	−0.29 (0.07)[†]	11	40.97 (5.79)[†]

[*] Number of participants with at least two time points with quantifiable results and included in analysis.

[†] $P < 0.05$.

MDR-TB = multidrug-resistant TB; TTD = time to detection; MGIT = Mycobacteria Growth Indicator Tube; cfu = colony forming unit; SE = standard error.