The relationship between Mycobacterium tuberculosis MGIT time to positivity and cfu in sputum samples demonstrates changing bacterial phenotypes potentially reflecting the impact of chemotherapy on critical sub-populations

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Objectives: The relationship between cfu and Mycobacterial Growth Indicator Tube (MGIT) time to positivity (TTP) is uncertain. We attempted to understand this relationship and create a mathematical model to relate these two methods of determining mycobacterial load.

Methods: Sequential bacteriological load data from clinical trials determined by MGIT and cfu were collected and mathematical models derived. All model fittings were conducted in the R statistical software environment (version 3.0.2), using the lm and nls functions.

Results: TTP showed a negative correlation with log10 cfu on all 14 days of the study. There was an increasing gradient of the regression line and y-intercept as treatment progressed. There was also a trend towards an increasing gradient with higher doses of rifampicin.

Conclusions: These data suggest that there is a population of mycobacterial cells that are more numerous when detected in liquid than on solid medium. Increasing doses of rifampicin differentially kill this group of organisms. These findings support the idea that increased doses of rifampicin are more effective.

Keywords: TTP, M. tuberculosis, TB

Introduction

If we are to improve the outcome of TB, it is essential that we develop shorter effective treatment regimens.1 Late relapse is thought to result from a population of dormant cells that are relatively resistant to chemotherapy.2 This has prompted extensive in vitro experiments with description of a range of dormancy models in vitro3,4 and in vivo.5 A number of recent publications have also postulated multiple populations of Mycobacterium tuberculosis cells in differing states within patients with pulmonary TB although there are few studies that link the different phenotypes. This concept has arisen from the findings of early-phase clinical trials that demonstrate that isoniazid has its greatest effect in the first 2 days.6,7 It is known that mycobacterial cells in artificial culture accumulate lipid bodies as they enter a stationary phase, an effect that is found in all species of the genus.8,9 Such cells may not grow on solid media unless resuscitated by the addition of resuscitation promotion factors (Rpf), first described in high-GC Gram-positive bacteria.10 A number of in vitro models have described these ‘dormant’ forms,3,4,11,12 Wayne and Hayes11 and Wayne12 described these cells as non-replicating-persistent (NRP) cells. The bacterial pheromones (Rfps) associated with activating non-plateable cells are expressed on the surface of M. tuberculosis in human granulomas.14 Importantly, it has also been recognized that, in human sputum samples, on average, up to 90% of the cells at
baseline cannot be cultured on solid medium without the addition of Rpf from spent medium.\textsuperscript{15}

Previous studies have demonstrated that liquid media are more sensitive than solid media for culture of mycobacteria from sputum and that liquid media reduce the number of subjects with negative cultures at 8 weeks.\textsuperscript{16} In this study we explore the relationship between matched liquid culture and colony count data from patients during the first 14 days of treatment. We also try to detect a population of organisms that cannot be cultured on solid medium but are capable of growth in liquid medium, and determine whether the proportion of these organisms remains constant during this initial phase and whether we are able to estimate the relationship between colony counts on solid medium and time to positivity (TTP).

**Methods**

**Patients and treatment dosage**

Clinical trial data were obtained from two patient datasets. The site’s local and national ethics boards approved these studies. The studies were conducted in compliance with ICH Good Clinical Practice Guidelines and the Declaration of Helsinki. All patients provided written informed consent to study participation.

We primarily focused on the HIGHRIF1 trial (www.clinicaltrials.gov, NCT01392911), a multiple ascending-dose study in which increasing doses of rifampicin, from 10 to 35 mg/kg, were evaluated. Adult patients were recruited at the University of Cape Town Lung Institute and Stellenbosch University. They were given rifampicin monotherapy alone for days 1–7, followed by the addition of isoniazid, pyrazinamide and ethambutol at standard doses for a further 7 days. Data were obtained from a total of 68 patients: 15 patients per arm and 8 in a control arm. Sputum was collected twice at baseline (pre-treatment) and then at days 1, 2, 3, 4, 5, 6, 7, 9 and 14 during treatment. At each visit, sputum was collected overnight (16:00 h until 08:00 h the following morning inclusive). This dataset was randomly split in two; the first half was used as a training dataset and the second as a test dataset.

A second dataset was used to test the observations and models that were created. The data were collected from the Mbeya site of the Oeba (Observation of Early Bactericidal Activity) Pan African Clinical Trials Registry (pactr.org) under PACTR201209000394102. A total of 23 eligible adults (males and females aged 18–65 years inclusive), with newly diagnosed, sputum smear-positive, drug-susceptible pulmonary TB were enrolled in the study at the Tanzanian National Institute for Medical Research—Mbeya Medical Research Centre. Individuals were hospitalized for up to 17 days at the Oeba unit and received standard isoniazid, rifampicin, pyrazinamide and ethambutol (HRZE) anti-TB treatment followed by standard treatment according to Tanzanian national guidelines. Sputum was collected twice at baseline (pre-treatment) and then at days 2, 4, 5, 7, 10, 14 (T2–T14) during treatment. At each visit, sputum was collected overnight (16:00 h until 08:00 h the following morning inclusive).

**Bacteriological methods**

Prospective sequential patient sputum samples were inoculated into the Mycobacterial Growth Indicator Tube (MGIT; BBL Becton Dickinson Microbiology Systems) and TTP was recorded. In parallel, cfu were enumerated using serial dilution on selective Middlebrook 7H10 medium as described previously.\textsuperscript{17}

**Statistical methods**

The relationship between TTP and log\(_{10}\) cfu was examined. Although some authors have previously used log-transformed TTP when analysing the relationship with log\(_{10}\) cfu,\textsuperscript{18} we found that it resulted in a poorer model fit and hence we chose to explore the relationship between the untransformed TTP and log\(_{10}\) cfu in this study.

All model fittings were conducted in the R statistical software environment (version 3.0.2). Linear regression analyses were carried out using the lm function, and \(R^2\) was used to assess goodness of fit by standard methodology. Other non-linear models were also fitted, using the nls function, and the Akaikes information criterion (AIC) was used to assess the quality of the statistical model. Model selection was then based on the smallest AIC value. Negative cultures were handled by setting cfu values to 1 and TTP measurements to 1008 h, which is the longest time MGIT will signal positive. Only timepoints where both TTP and cfu were measured were included in the analysis.

A conversion formula to translate TTP and cfu was created from half of the HIGHRIF1 dataset (the training set), based on the values from the Gompertz model (outlined below) for the changing gradients. This formula was then tested on the second half of the HIGHRIF1 dataset (the testing set).

**Results**

**Analysis of the combined dataset**

In this analysis we initially made the assumption that the dose of rifampicin did not affect the proportion of non-culturable cells detected. Bacterial load, as reflected in TTP (h) and cfu determined on solid medium, was analysed for each visit. TTP was plotted against log\(_{10}\) cfu, showing a negative correlation. This pattern was consistent on all 14 days of the study. These linear regression analyses are illustrated in Figure 1(a–j) and the numeric estimates for the absolute values of the gradients and the y-intercepts are tabulated in Table 1.

There was a trend for the gradient of the regression line and y-intercept to increase as treatment progressed. To demonstrate the changing relationship, all of the regression lines were plotted together on a single graph (Figure 2), where the shades/colours represent different days of treatment from, dark blue (baseline) to yellow (day 14). The steepness of the slope increases, particularly over the first few days (see Table 1). An analysis of covariance (ANCOVA) showed that this change in gradient was statistically significant, with a P value of 0.0002. This can be illustrated by the following example: if two samples had a cfu count of 10\(^2\) tested in MGIT, one at baseline and the other at day 14, they would take 150 and 280 h, respectively, to signal positive. If all of the bacterial cells were being detected this relationship would not change.

In order to find a parametric form to describe how the gradients and y-intercepts changed with each visit, we fitted a variety of functions to the changing profile: a simple straight-line model, a segmented straight-line model, a Gompertz function and other exponential models. The results of the two best-fitting functions, the simple straight-line model, \((m_1 t + c_1) \log(cfu) + (m_2 t + c_2)\), and the Gompertz function, \((i_1 e^{i_2 t}) \log(cfu) + (i_3 e^{i_4 t})\), are discussed, where \(t\) is the number of days on treatment.

The two models are illustrated in Figure 3, where the line shows the straight-line model and the curve shows the Gompertz model. Numeric estimates for the parameter values and AIC values for both models are tabulated in Table 2, where the parameters were estimated using half of the HIGHRIF1 data (the training dataset). Based on these
analyses, the Gompertz model was selected as the best model considered.

A similar pattern was observed in the OEBA dataset, with the gradient of the regression line and \( y \)-intercept increasing as treatment progressed (linear regression analyses are illustrated in Figure S1, available as Supplementary data at JAC Online). The increase in gradient, however, was not as steep as for the HIGHRIF1 data. A straight-line model and a Gompertz function were fitted for comparison with the HIGHRIF1 data. These models are illustrated in Figure 4, where the line shows the straight-line model and the curve shows the Gompertz model. Numeric estimates for the parameter values and AIC values for both models are tabulated in Table 3. Based on these analyses, the Gompertz model was selected as the best model.

Conversion formula
An equation relating \( \log_{10} \text{cfu} \) and MGIT TTP was developed based on the Gompertz model:

\[
\text{TTP} = \left( i_1 e^{t e^{1}} \right) \log(\text{cfu}) + \left( i_2 e^{t e^{2}} \right)
\]

\[
\log(\text{cfu}) = \frac{\text{TTP} - \left( i_2 e^{t e^{2}} \right)}{\left( i_1 e^{t e^{1}} \right)}
\]

where \( t \) denotes days on treatment.

Figure 1. Plots of MGIT TTP (h) against \( \log_{10} \text{cfu} \) for each timepoint in the PanACEA HIGHRIF1 study, from baseline to day 14 with regression lines fitted. The days are represented as follows: (a) day 0 (61 observations); (b) day 1 (60 observations); (c) day 2 (58 observations); (d) day 3 (55 observations); (e) day 4 (59 observations); (f) day 5 (59 observations); (g) day 6 (54 observations); (h) day 7 (53 observations); (i) day 9 (46 observations); and (j) day 14 (37 observations).
Using parameter estimates derived from the HIGHRIF1 training dataset, the following formula was constructed:

\[
\log(\text{cfu}) = \frac{TTP - (562.318 \times e^{-0.789e^{-0.195t}})}{-64.111 \times e^{-1.002e^{-0.218t}}}.
\]

In order to test this conversion formula, we validated it against the HIGHRIF1 testing dataset. The actual cfu values from the testing dataset and the predicted cfu counts using the conversion formula are plotted in Figure 5.

**Table 1.** Linear regression-fitted estimates with \(R^2\); note that all estimates were fitted with \(P\) values <0.001

<table>
<thead>
<tr>
<th>Absolute value of the gradient estimate</th>
<th>(y)-intercept estimate</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>19.33</td>
<td>224.42</td>
</tr>
<tr>
<td>Day 1</td>
<td>33.05</td>
<td>314.57</td>
</tr>
<tr>
<td>Day 2</td>
<td>35.61</td>
<td>337.97</td>
</tr>
<tr>
<td>Day 3</td>
<td>48.13</td>
<td>414.53</td>
</tr>
<tr>
<td>Day 4</td>
<td>44.81</td>
<td>399.51</td>
</tr>
<tr>
<td>Day 5</td>
<td>43.33</td>
<td>398.83</td>
</tr>
<tr>
<td>Day 6</td>
<td>51.93</td>
<td>443.23</td>
</tr>
<tr>
<td>Day 7</td>
<td>59.18</td>
<td>471.37</td>
</tr>
<tr>
<td>Day 9</td>
<td>55.30</td>
<td>468.75</td>
</tr>
<tr>
<td>Day 14</td>
<td>48.67</td>
<td>474.91</td>
</tr>
</tbody>
</table>

Bias and imprecision calculations for the conversion formula, which were expressed as median percentage error (MPE) and median absolute percentage error (MAPE) (for formulae, please see the Supplementary data), were calculated, with MPE ranging from -3.5% to 7.1% and MAPE from 6.9% to 17.1%. Table S1 tabulates MPE and MAPE for each visit.

**Differences in treatment arms**

We completed analyses on the gradient estimates for the whole HIGHRIF1 dataset over the first 7 days, analysing the treatment arm cohorts separately. Only the first 7 days were analysed because standard HRZE treatment was administered after this time. We used simple linear regression, as a straight-line model was found to fit best when only modelling the first 7 days of treatment.

In all cases, we found an increase in the magnitude of the gradient over time, i.e. the same general pattern as with the pooled data, but this differed between treatment arms. Results are illustrated in Figure 6 and the numeric estimates are tabulated in Table 4. The slope of the straight line between the gradient estimates increased with dose.

**Discussion**

The aim of this study was to relate TTP in MGIT to the number of cfu. By using sequential samples examined by both methodologies daily over 2 weeks, we were able to explore how the relationship between these two cell types changed over time and in Sub-populations of *M. tuberculosis*  

![Figure 2](https://academic.oup.com/jac/article-abstract/70/2/448/2911326/12345678)
response to treatment. We found that if two samples had the same cfu count, the sample taken at a later visit would take longer to signal positive in MGIT.

An important observation was that the magnitude of the regression line gradient increased in the first few days of treatment. This increase in magnitude of gradient then slowed for the remainder of the treatment. Although there was a negative relationship between cfu and TTP throughout the 14 days of treatment, the time it took for a sample with identical cfu to signal positive in MGIT changed during treatment, with higher TTP values later in the treatment period. This pattern was also shown with contour plots, which showed an upward trend in TTP over time for constant cfu values (data not shown). One explanation of this is that MGIT counts an extra sub-population of cells and this sub-population decreases in number during treatment, at a faster rate with higher doses of rifampicin. Alternative explanations include a change in lag phase or as a result of sub-lethal damage by rifampicin. The increase in gradient of the regression line relating TTP and cfu slowed after the initial few days of treatment, meaning that the disparity between cfu and total cells contributing to a positive signal in MGIT was decreasing. This would suggest that this extra cell population is being reduced rapidly by rifampicin. Alternatively, it may be due to mycobacterial cell damage changing their ability to grow on liquid or solid media. Although a post-antibiotic effect is an alternative explanation, it is likely to be too short to have such a large effect. An argument against a change in lag phase or post-antibiotic effect is that we found a similar pattern of increasing gradient during treatment emerging when we analysed data from the OEBA study, which used standard HRZE treatment. The gradient increased over time for these data, as in the HGHRIIF1 data, but here the increase was less rapid, with results similar to the control case for the HGHRIIF1 dataset.

When we analysed the treatment cohorts separately, we found that as the rifampicin dose increased the slope of the regression line gradient increased in the first few days of treatment, i.e. killing, ‘dormant’ cells. The increased clearance of these ‘dormant’ bacteria by rifampicin that is suggested by this study indicates that rifampicin may also be responsible for a large early
reduction in bacterial load. The data reported in this paper suggest that higher doses of rifampicin may help to eradicate many non-culturable cells in the early days of treatment and support treatment shortening.

We have been able to use these data to formulate a tool to translate TTP into cfu. Considering the variability in the colony counting methodology, the equation seems to predict cfu count well, with MAPE values between 6.9% and 17.1%. This shows acceptable predictive performance.\(^2\) In the future, this approach should be used to derive distinct formulae for patients treated by different therapeutic regimens. Once appropriately validated, these formulae would significantly reduce the cost of performing early-phase monotherapy and combination studies by removing the need for cfu counting on solid media.

In order to test whether our time-dependent formula converting TTP into cfu was an improvement on a simple straight-line relationship that does not change over time, we calculated least squares estimates of each predicted log\(_{10}\) cfu against the actual median value of the testing HIGHRIF1 dataset. We found that the straight-line relationship that did not change over time had larger least squares estimates than our time-dependent formula outlined earlier. We therefore conclude that a time-dependent conversion formula could be considered superior to a formula that does not take into account changes over time in the relationship between TTP and cfu.

When fitting regression lines to each visit to find the relationship between TTP and log\(_{10}\) cfu, the goodness of fit was measured

**Figure 5.** Log\(_{10}\) cfu values are plotted (grey circles) for all 68 patients in the HIGHRIF1 testing dataset during treatment. The asterisks show the median values of the testing dataset at each timepoint. The line shows the predicted decline in cfu using the TTP values from the testing dataset, predicted using the conversion formula.

**Figure 6.** Plots of the gradient estimates during the first 7 days of treatment in different treatment arms of the HIGHRIF1 study, identified by rifampicin dose: (a) control arm, (b) 20 mg, (c) 25 mg, (d) 30 mg and (e) 35 mg. Fitted straight lines are shown.
by $R^2$ with values ranging from 0.3 to 0.73 (Table 1). The high variability in accuracy of these counting methods, due to variability in the number of organisms in sputum samples on the same day and the tendency of organisms to clump, means that finding close-fitting relationships is often difficult, and this accounts for some of these low $R^2$ values. As we have shown, there are differences in the relationship between TTP and cfu when different therapies are administered. This means that the conversion formula derived for this combined HiGHRIF1 dataset may not be as accurate as if it had been derived separately for each cohort of patients receiving different treatments. With larger patient cohorts available, this method should be used to calculate distinct conversion formulae for different regimens.

In conclusion, we have demonstrated a population of mycobacterial cells that do not grow on solid medium. Increasing doses of rifampicin differentially kill this group of organisms. If it is true that this sub-population is representative of dormant cells, this could have important implications for treatment duration.

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**Supplementary data**

Figure S1, formulae and Table S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**

Sub-populations of *M. tuberculosis*


