Serial image analysis of *Mycobacterium tuberculosis* colony growth reveals a persistent subpopulation in sputum during treatment of pulmonary TB

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**SUMMARY**

Faster elimination of drug tolerant 'persister' bacteria may shorten treatment of tuberculosis (TB) but no method exists to quantify persisters in clinical samples. We used automated image analysis to assess whether studying growth characteristics of individual *Mycobacterium tuberculosis* colonies from sputum on solid media during early TB treatment facilitates 'persister' phenotyping. As Time to Detection (TTD) in liquid culture inversely correlates with total bacterial load we also evaluated the relationship between individual colony growth parameters and TTD. Sputum from TB patients in Malawi was prepared for solid and liquid culture after 0, 2 and 4 weeks of treatment. Serial photography of agar plates was used to measure time to appearance (lag time) and radial growth rate for each colony. Mixed-effects modelling was used to analyse changing growth characteristics from serial samples. 20 patients had colony measurements recorded at $\geq$1 time-point. Overall lag time increased by 6.5 days between baseline and two weeks ($p = 0.0001$). Total colony count/ml showed typical biphasic elimination, but long lag time colonies ($>20$days) had slower, monophasic decline. TTD was associated with minimum lag time (time to appearance of first colony). Slower elimination of long lag time colonies suggests that these may represent a persister subpopulation of bacilli.

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**1. Introduction**

To meet global tuberculosis (TB) control targets, shorter duration treatment regimens are needed [1]. Recent attempts to shorten therapy for drug susceptible TB to less than 6 months in phase III clinical trials have resulted in high rates of treatment failure and relapse [2–4]. Such unfavourable outcomes are often attributed to the capacity of phenotypically drug-tolerant 'persister' bacilli to survive antibiotic exposure [5,6]. Development of techniques to monitor the specific activity of novel antimicrobial regimens against persister cells would significantly advance TB therapeutics research, and help select drug combinations with greater treatment shortening potential [7].

Bacilli in sputum have traditionally been quantified by counting colony forming units (CFUs) after incubation on solid media. Mathematical modelling of serial CFU counts during TB treatment has shown biphasic bacterial killing, supporting the hypothesis that different sub-populations of *Mycobacterium tuberculosis* are eliminated at different rates. However, total CFU counts do not selectively identify persister organisms. *In vitro* experiments have used...
days into therapy. Ethical approval was granted by the Research 
Abeth Central Teaching Hospital tuberculosis service, 0, 14 and 28 
home with an assisted early morning collection at the Queen Eliz-
obtained by combining one overnight collection at the patient's 
published protocol from our laboratory[15]) to a depth of ~4 mm. 

MAST Selectatabs 

threitol (Oxoid, SR0023A) and repeated light vortexing over the 

vidual 

M tuberculosis 

approach to provide lag-time and RGR measurements from indi-
results from liquid culture of the same specimens, to provide new 

persister organisms. We also compared these parameters to TTD 
TTD of the overall sample.

The study was conducted in Blantyre, Malawi as part of a large 
multicentre prospective cohort study named PanACEA Biomarkers 
Expansion Project (PanBioME). Patients aged 18 

multicentre prospective cohort study named PanACEA Biomarkers 

dilution was inoculated in duplicate, using a disposable loop, onto 

micropipette tip change between each dilution step [15]. Each 

automated image analysis for detailed study of individual Escher-
ichia coli colonies, measuring their time to appearance (lag-time) 
and radial growth rate (RGR) on culture plates under a range of 
conditions [8]. In these models, the primary adaptation to antibiotic 
stress was development of drug tolerance through a population 
shift in CFU lag-time distribution, without generation of resistance 
mutations [9]. Incorporation of similar methodology to sputum 
colony counting studies is required to test whether differential lag-
time or RGR measurements identify a persister TB phenotype from 
clinical samples. 

Time to detection (TTD) in liquid culture is an alternative means of 
M tuberculosis quantification [10]. There is a known negative 
correlation between TTD and CFU counts, and recent studies have 
modelled treatment response on the basis of changes in TTD over 
time on therapy [11]. Whilst liquid culture offers several advant-
ages, including more successful revival of persisters than solid 
media, the precise relationship between TTD and CFU counts is 
incompletely understood and requires further study [12,13]. 

For the current study we adapted the in vitro colony imaging 
approach to provide lag-time and RGR measurements from indi-
vidual M tuberculosis CFUs recovered from serial sputum samples of 
adult pulmonary TB patients. We assessed whether heterogeneity 
in lag-time and RGR measurements revealed a sub-population of 
persister organisms. We also compared these parameters to TTD 
results from liquid culture of the same specimens, to provide new 
information on the relationship between individual CFU growth 
and TTD of the overall sample.

2. Methods 

2.1. Patient selection and sample collection 

The study was conducted in Blantyre, Malawi as part of a large 
multicentre prospective cohort study named PanACEA Biomarkers 
Expansion Project (PanBioME). Patients aged 18–65 years with 
smear positive pulmonary TB (grade 2–3+ by IUATLD criteria on 
Ziehl-Neelsen staining [14]) were recruited to PanBioME. Exclusion 
criteria included previous TB treatment in the last 5 years, or inability 
to complete the first two weeks of first-line treatment. Sputum was 
obtained by combining one overnight collection at the patient's 
home with an assisted early morning collection at the Queen Eliz-
abeth Central Teaching Hospital tuberculosis service, 0, 14 and 28 
days into therapy. Ethical approval was granted by the Research 
Ethics Committee, College of Medicine, University of Malawi.

2.2. Sample preparation and quantitative bacteriology 

Highly viscous sputum was liquefied by glass-bead vortexing 
with a volume of distilled water <15% of the sample volume. 
Two—four ml of un-decontaminated samples were then homoge-
nised by addition of an equal volume of working strength dithio-
threitol (Oxoid, SR0023A) and repeated light vortexing over the 
next 30 min. Ninety millimetre diameter plates were prepared with 
selective media (Middlebrook 7H11 oleic acid albumin agar with 
M AST Selectatabs™, and carbedazim, according to a previously 
published protocol from our laboratory [15]) to a depth of ~4 mm. 
Six serial ten-fold dilutions (near to 10⁶) of the processed 
sputum were prepared using the micropipetting method and 
phosphate buffered saline (PBS) diluent, with vortexing and 
micropipette tip change between each dilution step [15]. Each 
dilution was inoculated in duplicate, using a disposable loop, onto 
selective plates. Plates were then sealed using 12 mm Micropore™ 
tape, stored inside airtight polyethylene specimen bags and storage 
boxes and incubated at 37 °C in darkness, media-surface upside with 
tri-weekly shuffling. Colony counts were performed at 4 
weeks, and used to calculate CFU/ml counts for original sputum by 
back-calculation accounting for inoculum volume and dilution 
factor. A minimum of one colony per specimen was identified as 
containing acid-fast bacilli on Ziehl-Neelsen microscopy, including 
any colonies regarded as having ambiguous morphology. 

In parallel, aliquots of each sample was processed for liquid 
culture in BACTEC™ MGIT™ 960 Mycobacterial detection system 
(Becton Dickinson). One millilitre homogenised sputum was 
decontaminated with 1 ml N-acetyl-L-cysteine/NaOH 3% for 15 min 
before inoculation into BBL-MGIT™ 7 ml tubes prepared as per 
manufacturer instructions — 7 ml Middlebrook 7H9 broth base 
supplemented with oleic acid, albumin, dextrose, and catalase 
(BBL-MGIT™ OADC) and polymixin B, amphotericin B, nalidixic 
acid, trimethoprim (BBL-MGIT™ PANTA). Isolates which signalled 
positive were confirmed as M. tuberculosis if they demonstrated 
acid-fast bacilli with cording and MPT64Ag positivity on commercial 
identification kits (Becton Dickinson). TTD in hours was 
retrieved from BACTEC™ MGIT™ 960 software. Only TTD results 
from confirmed M. tuberculosis positive cultures were used in the 
analysis. Specimens which had not signalled positive by 8 weeks 
were reported as negative.

2.3. Digital imaging to assess colony lag time and RGR 

Media-side down plates were back-illuminated in a custom built 
light box by a low-heat LED panel light for photographing three 
times per week. A compact digital camera (Nikon™ Coolpix™ 
S6700 2.1 megapixel, settings: “MACRO”, “high quality, large 
image”) was fixed to the light box 20 cm from plate position. Plate 
edges were marked to allow standardised positioning in serial 
photographs and additional markings, visible in final images, 
permitted a post-hoc check for standardised positioning relative to 
the camera (Figure S1, available as online supplement).

The size and position of colonies in each digital image were 
measured using open source software (OpenCFU v3.9.0 beta) [16]. 
Combined colony observations from serial images of the same plate 
were used to extract growth curves for each unique colony.

Several colony growth models (including all those previously 
described in the literature [17,18]) were assessed for fit against 
observed growth data. A simple linear model of colony radius as a 
function of time for initial colony growth (i.e. after censoring 
plateau phase observations) showed better fit than a mono-
exponential, biexponential, or power-law function. Therefore, an 
individual linear model was fitted to the initial growth of each 
colonies using the ordinary least squares method. Colony RGR was 
defined as the slope co-efficient, and lag time as the x intercept, of 
this model (Figure S2, available as online supplement). 'Minimum 
lag time' was defined as the shortest colony lag time observed 
amongst colonies grown from a given sample.

Although every plate, at every dilution of each clinical sample 
was imaged, prior work on soil microbes [19], and optimization 
experiments on M tuberculosis laboratory strain H37Rv (Figure S3, 
available as online supplement) have shown that the concentra-
tion of bacteria inoculated onto a culture plate (plating density) 
influences the probability a CFU will form a colony, and its lag 
time in doing so. Therefore, analysis of individual colony growth kinetics 
from clinical samples in this study was restricted to the plate di-
lutions of each specimen which yielded a final count of 1–50 col-

ories per plate.

2.4. H37Rv control plates 

Two-hundred and sixty-nine M tuberculosis colonies grown from 
H37Rv laboratory strain were analysed in the same way as the 
clinical isolate colony growth for comparison.
2.5. Statistical analysis

The data structure for this study was hierarchical and nested, with serial measures of colonies, within plates, from repeated sampling of the same individuals at different time points. Therefore, statistical methods that accounted for non-independence of the observations (or their error terms) were employed, including use of summary measures and mixed-effects modelling [20,21]. In particular, linear mixed effects models were used to assess changes in colony growth kinetics from patient samples collected at different study visits. Correlations of TTD with solid media growth characteristics were assessed with $R^2$ from linear regression. All data management and statistical analysis was conducted in R Studio (Version 0.98.1102) [22].

3. Results

3.1. Patients, samples and quantitative bacteriology

Twenty-one patients were recruited; 20 patients had colony growth at one or more treatment timepoints. Positive samples were required for analysis of colony growth kinetics. 35 sputum specimens fulfilled these criteria; their time-points of collection, CFU counts and TTD data are summarised in Table 1.

3.2. Lag time and RGR of colonies recovered from clinical isolates vary over time

Approximately 16000 digital images of inoculated plates were captured across all specimens at all timepoints. However, based on criteria that only colonies from plate dilutions which yielded a captured across all specimens at all timepoints. However, based on criteria that only colonies from plate dilutions which yielded a CFU count of $1 \times 10^5$ CFU/ml at baseline, falling by $2.5 \log_{10}$ to $300$ CFU/ml by week 2. Over the same time period, median count for long-lag colonies fell by less than $1 \log_{10}$ CFU/ml, from $7.2 \times 10^4$ CFU/ml at baseline to $7500$ CFU/ml at two weeks. Short-lag colonies therefore accounted for the steep decline in CFU/ml in the rapid, early bactericidal phase of treatment, while long-lag colonies showed a constant rate of elimination over the first 4 weeks of therapy (Figure 2).

3.3. Corrected colony counts show different bacterial elimination dynamics for long versus short lag time colonies

Observed colony numbers were corrected for inoculum volume and dilution factor to give CFU count per ml sputum. The median total bacterial load fell by $1.5 \log_{10}$ CFU/ml between baseline and week two, and by $0.5 \log_{10}$CFU/ml between week two and four, consistent with a typical biphasic elimination. However, a different elimination dynamic was seen for sub-populations of CFUs categorised by colony lag time as ‘short-lag’ (<20 days) or ‘long-lag’ (>20 days). Median count for short-lag colonies was $1.1 \times 10^5$ CFU/ml at baseline, falling by $2.5 \log_{10}$ to $300$ CFU/ml by week 2. Over the same time period, median count for long-lag colonies fell by less than $1 \log_{10}$ CFU/ml, from $7.2 \times 10^4$ CFU/ml at baseline to $7500$ CFU/ml at two weeks. Short-lag colonies therefore accounted for the steep decline in CFU/ml in the rapid, early bactericidal phase of treatment, while long-lag colonies showed a constant rate of elimination over the first 4 weeks of therapy (Figure 2).

3.4. TTD in MGIT correlates with minimum colony lag time and CFU count

TTD in MGIT of each sputum sample with confirmed $M. tuberculosis$ growth ($n = 29$) was compared to summary measures of solid media colony growth. As expected, samples with short TTD in MGIT had higher CFU count, with strong negative linear correlation between TTD and $\log_{10}$ CFU/ml ($R^2 = 0.41$, $p = 0.0002$). Minimum lag time for a discrete colony also correlated with TTD in liquid culture ($R^2 = 0.18$, $p = 0.02$). By contrast, mean lag time, minimum RGR, maximum RGR, and mean RGR all showed no correlation with TTD.

Table 1

| Summary of samples and colony growth by time of sample collection. |
|---------------------------------------------------------------|
|                                                              |
| **Baseline (Week 0)**  | **Week 2** | **Week 4** | **H37Rv colonies** |
| **Number of patients** | 21         | 13         | 12               | –                        |
| **Solid media results:**                                      |            |            |                  |
| Positive (CFU counted)                                        | 19         | 10         | 6                | –                        |
| Negative or contaminated                                     | 2          | 3          | 6                | –                        |
| **Liquid culture results:**                                  |            |            |                  |
| Positive                                                      | 17         | 7          | 5                | –                        |
| Negative                                                      | 2          | 3          | 1                | –                        |
| Contaminated                                                  | 2          | 3          | 6                | –                        |
| **Summary of colonies' growth kinetics:**                    |            |            |                  |
| Lag-time, median (IQR)                                       | 17.9 (13.6–24.1) | 25.9 (21.5–29.6) | 22.7 (19.4–26.5) | 20.0 (17.0–22.2) |
| Minimum lag time                                              | 2.1        | 4.3        | 11.6             | 5.7                      |
| RGR, median (IQR)                                             | 95 (59–129) | 92 (65–130) | 122 (93–157)     | 182 (134–226)             |
| Liquid culture TTD, median (IQR)                             | 117 (107–145) | 210 (147–308) | 270 (267–304)    | –                        |

CFU – colony forming unit; IQR – interquartile range; RGR – radial growth rate of colony during initial linear growth phase, in micrometres per day; lag time – time in days from plate inoculation to initial colony growth, defined by x intercept extrapolated from initial observed colony growth; TTD – time to detection by MGIT broth culture, in hours.
Minimum lag time on solid media and final colony count were strongly collinear, so their independent effects on TTD could not be reliably assessed in a multivariate analysis.

4. Discussion

This study is the first to quantitatively describe individual \textit{M. tuberculosis} colony growth from clinical samples, and reports novel findings which suggest persister cells form colonies with longer lag times compared to bacilli which are rapidly eliminated by anti-tuberculosis treatment.

Pharmacodynamic modelling studies have previously reported biphasic elimination of sputum CFU counts over time on first-line TB treatment \cite{23}. This may represent differential killing of bacillary sub-populations with metabolically quiescent organisms exhibiting antibiotic tolerance and surviving longer, and it has been proposed that modelling the slower second phase of elimination is a potential surrogate for long-term clinical outcome \cite{11,24}. The current study is the first to measure phenotypic heterogeneity of \textit{M. tuberculosis} colonies obtained from sputum samples, and advances the sub-populations hypothesis by showing that distributions of colony growth kinetics vary non-randomly over the three time points assessed (baseline, week 2, week 4). In particular, counts of longer lag-time colonies (>20 days) decline more slowly than shorter lag-time colonies, and show linear decline when counts are aggregated across samples from different individuals.

One interpretation of these data is that a sub-population of \textit{M. tuberculosis} cells infecting the human host are stochastically generated with long lag-time and relative drug tolerance before antibiotic insult, and become the dominant phenotype through population restructuring after antibiotic exposure. Such a bet-hedging strategy is described for other bacteria, and has been designated type II persister formation \cite{25,26}. For example, when bacteria are grown \textit{in vitro} before inoculation onto solid media the distribution of resulting colony lag-times reflects the conditions of the original culture (e.g. nutrient restriction), and longer average lag times are associated with drug tolerance. By measuring individual colony growth for \textit{E. coli} strains, Levin-Reisman et al. correlated the magnitude of the resulting long lag-time ‘tail’ with the proportion of bacteria able to survive antibiotic exposure \cite{8}. Should the long lag-time colonies described in our analyses truly represent a metabolically quiescent sub-population of drug tolerant \textit{M. tuberculosis} cells, their existence might directly reveal the mechanism of biphasic bacillary clearance, and measuring their elimination rate may help predict the risk of eventual TB treatment failure or post-treatment relapse.

Alternative explanations of our findings are possible. Long lag-time colony count dynamics may not reflect population restructuring, but simply that bacilli damaged by antibiotics have longer lag times, or a post-antibiotic effect is being observed. However, prior reports that treatment outcomes can be predicted from 2 month sputum culture conversion rate \cite{27}, or the rate of bacillary

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Observed distributions of colonies’ lag times and growth rates change over time on treatment. A. Box plots showing lag times (top) and radial growth rates (bottom) of observed H37Rv plate colonies (n = 269), and observed colonies recovered from clinical isolates (n = 1352) at three time points into treatment (0, 2, and 4 weeks). Box shows median and interquartile range. B. Scatterplots of lag time and growth rate for colonies recovered from clinical isolates at the 3 treatment time points. Partitioning the colonies above and below the overall median lag time of 20 days, indicated by red circle and green triangle markers, shows that bacilli forming colonies with shorter lag time are the dominant sub-population at baseline, but are a minority after 2 or 4 weeks of treatment.}
\end{figure}
elimination from sputum [11], support the concept that type II persisters exist in sputum and that their phenotypic characteristics are likely to be relevant to clinical endpoints.

Serial sputum CFU counting is labour intensive, prone to experimental error, and shows systematic variation between laboratories. TTD in commercially available liquid culture systems is a convenient, readily standardised technique displaying moderate-strong correlation with colony counts, and is increasingly used as an alternative measurement. In addition, liquid culture has better sensitivity for quantifying colonies recovered from clinical specimens can be assessed its relationship with the growth kinetics of individual colonies. Our description of a correlation between TTD and minimum consumption by the entire batch culture

Not required.

Ethical approval: None.

Competing interests: None declared.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2016.03.001.
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