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Rapid DNA extraction for specific detection and quantitation of *Mycobacterium tuberculosis* DNA in sputum specimens using taqman assays

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SUMMARY

Rapid tuberculosis (TB) detection is critical for disease control, and further quantitation of *Mycobacterium tuberculosis* (*Mtb*) in sputum is valuable for epidemiological and clinical studies. We evaluated a simple, robust and cost-efficient in-house DNA extraction and downstream taqman approach for detection and quantitation of *Mtb* genomes from sputum of newly-diagnosed TB patients and non-TB controls. DNA was extracted using guanidine isothiocyanate and silica-based spin columns in less than 2h, stored frozen, and taqman assays were used to detect *Mtb* with IS6110 and quantify it targeting RD1 and IS1081. The taqmans had a sensitivity > 95% in 108 culture-confirmed TB patients and specificity of 100% in 43 non-TB controls. Genome counts were correlated with the Mycobacterial Growth Indicator Tubes' (MGIT) time-to-detection values ($1/TTD \times 1000$; $\rho = 0.66$; $p < 0.001$) in 91 TB patients (33 excluded with MGIT contamination). This linear relationship was nearly identical between mycobacteria isolated from sputum and H37Rv *Mtb* grown in-vitro to its log phase. TB treatment between 3 and 7 days was associated with lower $1/TTD \times 1000$ values but not with genome counts. Together, our protocol provides rapid, specific, inexpensive and quantitative detection of *Mtb* DNA in fresh or stored sputa making it a robust tool for prompt TB diagnosis, and with potential use for clinical and epidemiologic studies.

Keywords

tuberculosis; real-time quantitative PCR; quantitation; diagnosis; sputum; PCR inhibitors

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Author contributions: Conceived and designed the experiments: BIR JBM SPF; Performed the experiments: DG, CM; Analyzed the data: BIR DG CM; Provided data/materials/field support: FMG JGC; Wrote the paper: BIR DG CM; Approved final version of the paper: all authors.

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1. Introduction

Tuberculosis is a major health problem worldwide, with the highest prevalence rates in developing countries.¹ Early TB diagnosis and treatment is important for patient outcome and prevent further spread.² Conventional methods such as direct smear lack sensitivity, and isolation of *Mycobacterium tuberculosis* (*Mtb*) in culture takes at least one week. A molecular approach for *Mtb* detection is highly appealing for TB diagnosis due to a short turnaround time (within 24h), particularly for patients with negative smears where positive results can influence the physician's decision to start treatment. Commercially-available assays for molecular detection of *Mtb* (e.g. COBAS Amplicor from Roche Diagnostic Systems, Branchburg, NJ and *MTB* direct from GenProbe, San Diego, CA) have the advantage of being standardized and reproducible, and exhibit high sensitivity and specificity in smear-positive samples. However, they have low sensitivity in smear-negative samples and high cost that limits their implementation in developing countries.²⁻⁴ In-house assays also have low sensitivity in smear-negative specimens and heterogeneity in their diagnostic accuracy but are more affordable.⁵ Another limitation of *Mtb* DNA detection in sputum is the presence of internal PCR inhibitors, with false-negative rates as high as 12%.^{6,7} Altogether, the numerous publications on molecular methods for prompt *Mtb* detection reflects the need for methods with improved performance, low cost and ease-of-use.³ Real-time PCR has been proposed for *Mtb* detection due to faster results and lower risk of contamination.⁸ The first commercial taqman assay has now been launched by Roche Diagnostics.⁹ A promising but expensive recent development is the Gene Xpert (Cepheid, Sunnyvale, CA), an automated system for *Mtb* DNA detection by PCR, with additional screening for rifampicin-resistance.¹⁰

Mtb quantitation is a valuable tool for clinical research aimed at evaluating new TB medications and for epidemiological studies assessing infectiousness of new TB patients.¹¹⁻¹⁵ The classical method for *Mtb* quantitation is by colony-forming units (CFU) but this approach is time-consuming and expensive.¹⁶ This approach has been replaced in most recent studies by time-to-detection (TTD) estimates of mycobacterial growth in automated culture systems (e.g. MGIT-960, Becton-Dickinson, Sparks, MD; BacT/ALERT MB, bioMerieux, Durham, NC) because it is simpler and exhibits less variability.^{16,17} However, microbial contamination of cultures is a well-known limitation of this technique.¹⁸

In a previous study we reported a method for efficient DNA extraction of few *Mtb* genomes from blood followed by downstream real-time quantitative PCR of the multi-copy insertion sequence IS6110.¹⁹ In the present study we report a faster and simpler DNA extraction protocol that is suitable for sputum and two new taqman assays for *Mtb* quantitation. Our enhanced protocol is specific for *Mtb* species, highly sensitive in smear-positive specimens, eliminates taq inhibitors that lead to false negatives, and is suitable for *Mtb* quantitation in sputum.

2. Methods

2.1. Participant enrollment

Participants were enrolled at pulmonary clinics in southern Texas (Hidalgo County Health Department) and northeastern Mexico (Secretaria de Salud de Tamaulipas, Matamoros). Inclusion criteria designated patients with suspected pulmonary TB based on a positive sputum smear, abnormal chest x-ray or clinical findings (TB suspects), or individuals in whom TB had been ruled out or was unlikely (TB-ruled out controls). Jail inmates, individuals < 18 years of age and patients who had received anti-TB treatment for more than 7 days were excluded. Enrolled and consented participants provided sociodemographic data.

Clinical (e.g. chest X-ray, treatment start date) and available microbiological findings (smear and mycobacterial culture) were extracted from medical records. Final participant classification was 'confirmed TB' when *Mtb* was isolated from culture (described below), and 'TB ruled out' when culture was negative, the patient's symptoms improved without TB treatment, and/or chest x-rays were normal. Participants with inconclusive classification (e.g. TB ruled out but abnormal chest x-ray) or clinical TB diagnosis (e.g. no isolation of *Mtb* but abnormal chest x-ray and/or response to TB treatment) were excluded from data analysis. The study was approved by the institutional review boards of the participating institutions in both countries and consents were signed by all participants.

2.2. Sputum processing and mycobacterial culture

Non-induced sputum was collected at the clinic during enrollment (on spot) and at the participant's home (first morning sputum) prior or within the first week of anti-TB treatment. Both specimens were subjected to mycobacterial culture (described below), but the specimen collected at the clinic was used for quantitative analysis if more than one ml was available. Otherwise, the second sputum was used. Sputum was transported within 24h to the research laboratory in Brownville, Texas, where volume was recorded and processed using standard laboratory techniques with slight modifications.²⁰ Namely, one volume of 4% NaOH (along with 1% of N-acetyl-L-cysteine) was added for digestion and decontamination, and after washing with PB buffer (0.03M Na₂HPO₄-0.03M KH₂PO₄) the pellet was resuspended in a 0.5x final volume of the original sputum. Five hundred microliters (equivalent to one ml of original sputum) was inoculated in Mycobacteria Growth Indicator Tube (MGIT, BD Diagnostics, Sparks, MD), and 100 microliters into Lowenstein Jensen Graft slant (LJ, Remel*, Lenexa, KS). The remaining volume was dispensed into 200 µl aliquots and frozen for subsequent DNA extraction and genome quantitation. Confirmation of *Mtb* species from positive cultures was assessed with Accuprobe testing in our research laboratory (Gen-Probe Inc., San Diego, CA) or by high-performance liquid chromatography of mycolic acids at the Texas State laboratory in Austin²¹ (data extracted from medical records when sputa was collected for routine TB assessment).

2.3. Mycobacterial strains

The reference *Mtb* H37Rv strain was grown in Difco™ Middlebrook 7H9 broth with glycerol (BD Diagnostic Systems, Sparks, MD) to an OD₆₀₀ of 0.5. Single-cell suspensions were prepared using a modified version of a previously reported protocol.^{22;23} Liquid cultures were vortexed in the presence of glass beads, sonicated to reduce mycobacterial clumps and submitted to a brief low-speed centrifugation to remove bacterial clumps. The upper bacterial suspension was used for quantitation. Seven species of atypical mycobacteria (*M. avium*, *M. abscessus*, *M. fortuitum*, *M. simiae*, *M. szulgai*, *M. marinum* and *M. kansasii*) that were isolated from patient specimens and speciated by high-performance liquid chromatography of mycolic acids and kindly provided by Ken Jost from the Texas Department of State and Health Services Laboratory in Austin.

2.4 Mtb quantitation

Mtb quantitation was established by: i) microscopy using a Petroff-Hausser chamber, ii) colony forming units (CFU) on enriched Middlebrook 7H11 agar, iii) real-time quantitative PCR (qPCR) taqmans for the region of difference 1 (RD1)²⁴ or IS1081 element²⁵ (Table 1), and iv) time-to-detection (TTD) of bacterial growth in MGIT-960 cultures, where bacterial burden is inversely proportional to TTD (e.g. 1/TTD).¹⁷ To ensure that 1/TTD reflected *Mtb* burden, MGIT cultures with bacterial contamination (assessed by overnight incubation on blood agar at 37°C) were excluded from analysis. MGIT data is expressed as 1/TTD × 1000 (1/TTD×1000).

2.4. DNA extraction from sputum

DNA extraction from concentrated sputum was conducted as described previously with slight modifications.²⁶ Concentrated sputum (200 µl) was incubated with GITC lysis buffer and 500 µl of 0.1mm zirconia beads (Biospec, Inc., Bartlesville, OK), placed in a bead beater (Biospec, Inc., Bartlesville, OK) for 3 min at maximum intensity and boiled. After centrifugation the DNA was precipitated with isopropanol and purified through an EconoSpin™ All-in-1 Mini Spin Column (Epoch Biolabs, Inc, Sugar Land, TX). A reagent control (no sputum) was processed with each batch of patient samples to rule out reagent contamination during DNA extraction. DNA from mycobacterial isolates was extracted using the same protocol and quantified with Picogreen (Molecular Probes, Inc. Eugene, OR).

2.5. Quantitative real-time PCR (qPCR) for *Mtb*

The taqman assay for *Mtb* IS6110 was described previously.¹⁹ Identification of *Mtb* complex-specific DNA within the RD1 and IS1081 regions were identified using GenBank® (US National Library of Medicine, Bethesda, MD). Primers and probes were designed using Allele ID ® 6.0 for Windows (Premier Biosoft International, Palo Alto, CA) and the Basic Local Alignment Search Tool (BLAST, U.S. National Library of Medicine, Bethesda, MD) used to select regions specific for *Mtb* complex. Probes were labeled with a 6-Carboxyfluorescein dye (6-FAM) on the 5' end and a non-fluorescent quencher dye (Black Hole Quencher 1-BHQ1) on the 3' end (Table 1). Taqmans were run on a 7900HT sequence detector (Applied Biosystems, Foster City, CA) as described previously for 40 cycles¹⁹, but with the following conditions for RD1 and IS1081: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 4 mM MgCl₂, 200 µM dNTP mix, 200 nM each primer, 100 nM probe, 0.625 units of hot start Jumpstart *Taq* DNA polymerase (Sigma-Aldrich Corp., St. Louis, MO, USA) and 5 µl of sputum DNA sample. Each run included experimental samples, extraction reagent controls, a no-template negative control and a standard curve with known amounts of each gene target as described previously.¹⁹ The spectral data were analyzed with SDS 2.4 (Applied Biosystems). The number of *Mtb* genomes/ml of original sputum was extrapolated from the number of targets amplified in 5 µl of DNA. The presence of internal qPCR inhibitors was assessed by evaluating whether the amplification of 1000 targets of foreign DNA (*Planktothrix agardhii*) was compromised in the presence of DNA extracted from sputum (experimental well), when compared to a control with no sputum DNA (control well). *P. agardhii* DNA does not cross-react with human or *Mtb* complex DNA (initially assessed with BLAST and later confirmed with the first 50 sputum samples).²⁷

2.5 Data analysis

Data was analyzed with SPSS (v.19 SPSS Inc, Chicago, IL). Correlation coefficients were calculated using Spearman's rho and association between variables by the Wilcoxon rank-sum test. P-values ≤ 0.05 were considered significant.

3. Results

3.1. Final TB classification and characteristics of study participants

Among the 174 participants enrolled (136 TB suspects and 38 non-TB controls), 24 were excluded due to an unclear diagnosis (n=1), clinical TB (n=16) or sputum volume below one ml (n=7; Figure 1). From the remaining 150 participants, pulmonary TB was culture-confirmed in 107 and ruled out in 43. Participant sociodemographics were similar to those described previously.²⁸ Namely, all were Hispanics in their mid-forties and 63% were male. Most (93%) were enrolled in Mexico, where TB diagnosis is based primarily on smear

results. Therefore, 99/107 (93%) of the *Mtb* culture confirmed TB cases had a positive sputum smear.

3.2. Performance of the DNA extraction and downstream taqman assays for *Mtb* detection in sputum

The first goal of this study was to assess the performance of our DNA extraction and qPCR amplification protocol to detect *Mtb* in sputum. To confirm that the RD1, IS1081 and IS6110 taqman assays were specific for *Mtb*, 42 pg of DNA (equivalent to 10e4 genomes) were extracted from in-vitro cultures of *Mtb*, *M. avium*, *M. abscessus*, *M. fortuitum*, *M. simiae*, *M. szulgai*, *M. marinum* and *M. kansasii* and subjected to qPCR. As expected, amplicons were only detected for *Mtb* DNA. The limit of detection of RD1 and IS1081 was one target, and of IS6110 was ten targets (data not shown). However, when these taqmans were tested with DNA extracted from sputa of participants in whom TB was ruled out, there was a low but detectable signal for RD1 in six specimens (6–30 targets/ml of sputa), for IS1081 in two specimens (12–13 targets/ml of sputa) and for IS6110 in 18 specimens (15–295 targets/ml of sputa). Since the clinical significance of these signals was unclear, we established cutoffs of 40 targets/ml of sputum (equivalent to detecting one target in 5 µl of DNA) for RD1 and IS1081, and 400 targets/ml of sputum (e.g. 10 targets/5 µl of DNA) for IS6110. With these cut-offs the sputum from the 108 TB patients was positive for RD1 in 103 (95.4%), for IS1081 in 104 (96.3%) and for IS6110 in 105 (97.2%; Table 2). The 43 participants without TB had negative taqman assays. Accordingly, the PPV was 100% for the three assays and the NPV was 89.6% for RD1, 91.5% for IS1081 and 93.5% for IS6110.

When smears were used as a reference, among the 100 smear-positive TB cases the sensitivity was 95%, 96% and 97% for RD1, IS1081 and IS6110, respectively. The five specimens with negative taqmans had no evidence of residual taq inhibitors that would explain this result (data not shown). Among the eight smear-negative, culture-confirmed TB cases, all qPCR assays were positive.

3.3. Quantitation of *Mtb* genomes using qPCR

The second goal of this study was to evaluate whether quantitation of *Mtb* genomes using qPCR is suitable to estimate *Mtb* burden in sputum within the first week of TB treatment. We designed taqmans that targeted sequences with a fixed number of copies per genome (RD1 one copy and IS1081 six copies). Since taqmans measure gene targets and not viable bacteria, we first determined the relationship between genome counts using RD1 versus techniques that measure mycobacteria by using *Mtb* grown in vitro. For this, single bacterial cell suspensions of the H37Rv strain in log phase were counted microscopically and serial 10-fold dilutions ranging from 600,000 to 60 *Mtb*/ml were prepared. Four quantitation methods were compared: microscopy (extrapolated counts from dilutions of the initial inoculum), CFUs, *Mtb* genomes with the RD1 taqman and 1/TTD×1000. Data from microscopy, qPCR and 1/TTD×1000 was available for all dilutions, but CFUs could only be counted for dilutions containing less than 300 colonies. Data for bacterial or genomes counts (microscopy, CFU and qPCR) were plotted against 1/TTD×1000 (Figure 2). The linear regression for all the methods had an R^2 above 0.98 and the slope of the three curves was similar. While the absolute values for genomes and microscopy were comparable, CFUs were 9-fold lower (-9.2 ± 4.5) than RD1 targets.

3.4. Relationship between taqman assays and 1/TTD×1000 for *Mtb* quantitation in sputum

For *Mtb* quantitation in sputum, participants were excluded if MGIT cultures were contaminated (n=32; Figure 1) or TB had been ruled out (n=27). MGIT contamination would shorten the TTD resulting in unreliably higher *Mtb* estimates. Among the remaining 91 TB patients, the taqmans for IS1081 and RD1 in sputa were highly correlated ($\rho=0.95$),

but with a median 6.9-fold (11.7 IQR) higher copy number for *IS1081*. Given the similarities between both assays and the presence of a single copy/genome of RD1, additional genome quantitation was conducted with this assay. The correlation coefficient between RD1 and 1/TTD $\times$ 1000 in sputum specimens was 0.66 ($p < 0.001$; Figure 3A).

Given that TTD is affected by the metabolic status of *Mtb*, we evaluated whether the relationship between 1/TTD $\times$ 1000 and qPCR counts differed between mycobacteria isolated from patient sputum versus the H37Rv strain isolated from culture media during its log phase. We evaluated the subset of participants with sputum RD1 counts in the same range as that obtained for the reference strain in Figure 2 (1/TTD $\times$ 1000 between 1.5 and 9; $n=71$ patients). Figure 3B shows that the regression line for mycobacteria from sputum specimens had a similar slope and y-intercept to that of H37Rv. Even though the data from sputa had a wide dispersion ($R^2=0.41$), these results suggested that the overall metabolism of *Mtb* isolates from diverse patient sputum specimens is comparable to that from H37Rv.

3.5. Association between *Mtb* burden and host characteristics

Host characteristics known to be associated with higher bacterial burden among TB patients were compared to qPCR or 1/TTD $\times$ 1000 values (Table 3). Patients with positive smears had higher *Mtb* counts by both methods, but this association was only significant with 1/TTD $\times$ 1000 values. Self-reported productive cough was associated with higher bacterial burden by both methods ($p=0.001$). Presence of cavities showed a borderline association with higher genome counts, but not with 1/TTD $\times$ 1000. Treatment with anti-TB medication for 3–7 days (versus 0–3 days) was associated with reduced 1/TTD $\times$ 1000 values but not with qPCR ($p=0.98$). These findings provide further support for the validity of qPCR for *Mtb* quantitation in sputum at the time of diagnosis, and within the first week of TB treatment.

4. DISCUSSION

We describe a technically simple protocol that is specific for *Mtb* detection, with results available on the same day patient specimens are received and no evidence of internal taq inhibitors that lead to false-negative in complex specimens such as sputum. The protocol is inexpensive (estimated reagent cost less than \$5/taqman) and suitable for clinical laboratories worldwide with access to a bead-beater and qPCR equipment. We further show that with minor modifications to the routine sputum processing recommended for *Mtb* isolation, our protocol can be used for *Mtb* quantitation within the first week of TB treatment. This approach is appealing for clinical or epidemiological studies given that sputa can be stored frozen and batch tested at a convenient time, and quantitation is not affected by TB treatment or microbial contamination of *Mtb* cultures.

Sputum specimens pose challenges for microbial detection, including the presence of internal taq inhibitors and background with normal flora.^{6,7} We find that an aliquot of routinely digested and decontaminated sputum is suitable for our DNA extraction method, with effective removal of taq inhibitors and an estimated technician time of only 2 h which could be further reduced by automating the steps into existing platforms (e.i. QiaCube, Qiagen, Valencia, CA). Current in-house PCR methods can take 4 h of technician time during the estimated 6 to 24 hrs that can span between the initiation of sputum processing until results are available.²⁹ Minor modifications to our DNA extraction method also provides *Mtb* detection in formalin-fixed tissues (i.e. placenta, bone, lymph nodes), gastric aspirates, pleural fluids and cerebrospinal fluid (data not shown). The same principle for DNA extraction may also be suitable for detecting other bacterial or fungal pathogens from sputum.

We showed that taqman assays that target RD1 and IS1081 were suitable for *Mtb* quantitation in sputum from newly-diagnosed TB patients. The relationship between RD1 counts and established quantitation methods suggested that genome and microscopic counts are comparable, but CFUs are approximately one log lower than genomes. The latter may be due to *Mtb* clumping (despite preparation of single mycobacteria suspensions) or presence of dead bacteria (despite use of log-phase cultures). Even though RD1 and 1/TTD $\times$ 1000 were significantly correlated ($\rho=0.66$), the level of discrepancy between methods reflects the measurement of different aspects of the *Mtb*: genomes versus the metabolic state of bacteria in sputum.³⁰

We recognize potential study limitations. First, some of the TB-ruled out controls had low but detectable copies of *Mtb* genomes in sputum. Possible explanations include: First, some TB-ruled out participants may have had paucibacillary TB despite our thorough microbiological and clinical characterization. Second, there may be amplicon contamination during sputum collection at the TB clinics or in the laboratory despite the use of reagent controls. Amplicon contamination and the interpretation of low levels of *Mtb* DNA in specimens from non-TB controls are well-recognized obstacles for implementing PCR as a sole method for TB diagnosis.^{31;32} Another limitation of our study is that most TB specimens were smear-positive and therefore the estimates of qPCR sensitivity are premature for smear-negative specimens, where it is mostly needed. However, we were encouraged by the fact that the 8/8 smear-negative TB cases had a positive PCR. Thus, assessment of our assay in smear-negative specimens such as those from HIV-positive patients is justified.

Mtb quantitation with taqman assays can be useful for epidemiological and clinical studies.^{11–15} For example, the time-to-clearance of *Mtb* during TB treatment is associated with the effectiveness of TB treatment and risk of relapse, or the duration of infectiousness of a TB patient.^{11–13;15} A recent study suggested that time-to-clearance can be predicted based on a patient's bacillary load prior to treatment using TTD.¹¹ In a pilot study with 11 TB patients, we observed a correlation between time-to-clearance and RD1 qPCR of 0.054 (data not shown). Confirmation of such findings with a larger sample size would make the RD1 method useful to predict the length of time that will be required for isolation of newly-diagnosed TB patients, including those hospitalized in negative-pressure rooms.¹⁵

Mtb quantitation using qPCR can also be useful to assess the infectiousness of new TB cases, and estimates of infectivity index are largely based on smear results (e.g. four-fold weight if patient has a positive versus negative smear).¹⁴ In the present study *Mtb* burden among smear-positive cases varied by three orders of magnitude (10e4 to 10e7). Therefore, the RD1 or IS1081 taqman assays could be useful to refine infectivity index estimates of new TB patients.

Even though qPCR values are not readily affected by anti-TB treatment, we foresee a role for qPCR in clinical trials aimed at evaluating new TB treatment schedules. Specifically, qPCR can serve as a quality control for the baseline MGIT cultures (or other automated mycobacterial detection systems) where microbial contamination is a well-recognized problem.¹⁸ In our study we gradually increased the NaOH concentration to 4% for improved sputum decontamination before data was collected for analysis (data not shown). Despite this we were still forced to discard 20% of the MGIT cultures. In contrast, pre-treatment of sputa with 4% NaOH improved taqman sensitivity (data not shown).

In summary, we propose a simple protocol for detection and quantitation of *Mtb* in a complex specimen such as sputum. This approach is amenable to support TB diagnosis in reference laboratories throughout the world that have a capability for qPCR-based methods.

Validation of the use of *Mtb* quantitation by qPCR for epidemiological and clinical studies is warranted.

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Figure 1. Participant enrollment and exclusion criteria for TB diagnosis and quantitation studies
Among the 174 participants enrolled (136 TB suspects and 38 controls), 24 were excluded due to the various reasons shown in figure and explained in the Methods section. The quantitation study was conducted on the subset of 91 participants, after exclusion of those with contaminated MGIT-960 cultures (n=32) or TB ruled out (n=27; 4 with mycobacteria other than tuberculosis, MOTT). Inconclusive TB, smear and culture results unavailable or TB was diagnosed by physician but there was no documentation of TB treatment for at least 6 months; Clinical TB, no isolation of *Mtb* but abnormal chest x-ray and/or response to TB treatment.

Figure 2. Comparison of *Mtb* genomes from H37Rv versus other quantitation methods

The number of H37Rv mycobacteria was counted microscopically with a Petroff-Hauser chamber and serial 10-fold dilutions containing an estimated total between 10^3 and 10^5 cells (filled black triangle) were evaluated for bacillary burden using the taqman assay for RD1 (gray square), CFUs (X) and MGIT-960 $1/\text{TTD} \times 1000$. The mean from triplicate experiments is shown. The methods providing bacterial or genome counts (*Mtb* targets on Y axis) were plotted against $1/\text{TTD} \times 1000$. CFUs could only be counted for plates containing less than 300 colonies (dashed line). The regression lines were defined by: $y = 0.58x + 1.20$ (microscopy), $y = 0.66x + 0.64$ (PCR) and $y = 0.72x - 0.50$ (CFUs). The R-squared values for each counting method are provided.

Figure 3. Relationship between genomes and $1/TTD \times 1000$ values for *Mtb* from sputa and H37Rv
A. Correlation between *Mtb* genomes per ml of sputa by RD1 qPCR versus $1/TTD \times 1000$ among the 91 *Mtb*-confirmed cases ($\rho=0.66$; $p<0.0001$). **B.** Relationship between the RD1 qPCR values and $1/TTD \times 1000$ for either H37Rv (gray squares mean of triplicate experiments) or the subset of patient isolates that had $1/TTD \times 1000$ values in the same range as the reference strain ($1/TTD \times 1000$ between 1.5 and 9; open circles; $n=71$). The regression lines and corresponding equations for both data sets are shown.

Table 1

Primers and probes for Taqman assays

Gene name	Accession no.	Primers and probes
<i>M. tuberculosis</i> RD1 ^a	<u>BX842584.1</u>	11582 (+) GAGCCACTGGTGCCCATG
		116620 (-) TCAGATGAACAACGGATCCTTCC
		11636 (-) FAM-CGCCACCGACGAAGCCGAGACC-BHQ1
<i>M. tuberculosis</i> IS1081 ^a	<u>BX842582.1</u>	293 (+) GAGCTGAACGCGCACTGAC
		373 (-) CGACCAGGCGCTCCATCC
		315 (+) FAM-CGTGGTGGCGACCTGCTACCTGCT-BHQ1

^aLowest limit of quantification of each assay is 40 targets per mL of sputum.

Performance of qPCR assays versus microbiological findings among participants with a final diagnosis of either culture-confirmed pulmonary TB or TB ruled out^a

Table 2

PCR assays	TB patients (n=107)				TB-ruled out participants (n=43)			
	AFB smear		MTB culture		AFB smear		MTB Culture	
	Positive 99 (93)	Negative 8 (7)	Positive 107 (100)	Negative	Positive 2 (5)	Negative 41 (95)	Positive 43 (100)	Negative
RD1	Positive	94 (94.9)	8 (100)	102 (95.3)	0 (0)	0 (0)	0 (0)	0 (0)
	Negative	5 (5.1)	0 (0)	5 (4.7)	2 (100)	41 (100)	43 (100)	43 (100)
IS1081	Positive	94 (94.9)	8 (100)	102 (95.3)	0 (0)	0 (0)	0 (0)	0 (0)
	Negative	5 (5.1)	0 (0)	5 (4.7)	2 (100)	41 (100)	43 (100)	43 (100)
IS6110	Positive	96 (97)	8 (100)	104 (97.2)	0 (0)	0 (0)	0 (0)	0 (0)
	Negative	3 (3)	0 (0)	3 (2.8)	2 (100)	41 (100)	43 (100)	43 (100)

^aData expressed as n(column %); qPCR assays positive when ≥ 40 targets/ml of sputa for RD1 and IS1081, and ≥400 targets/ml sputa for IS6110; AFB, acid-fast bacilli; MTB, *M. tuberculosis*

Table 3

Association between quantitation of *Mtb* in sputum and host clinical characteristics associated with mycobacterial burden

Host characteristics	n	1/TTD × 1000		RD1: <i>Mtb</i> genomes/ml of sputum			
		Median	QRange	P value	Median	QRange	P value
AFB sputum smear							
Positive	83	6.06	3.12		131,357	334,709	0.112
Negative	8	4.70	1.57	0.046	3,436	70,648	
Productive cough							
Yes	77	6.25	3.12		147,626	340,424	0.001
No	14	4.25	1.42	0.001	1,058	5,575	
X-ray cavities							
Yes	31	6.25	1.61		255,935	396,092	0.052
No	52	5.51	4.52	0.51	30,202	248,715	
Days of TB treatment							
< 3 days	69	6.32	3.66		122,084	350,160	0.98
≥ 3 days	21	4.90	1.96	0.007	115,572	285,190	